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Glycans as dual players of intestinal microbiome and
immune response – impact in Inflammatory Bowel
Disease immunopathogenesis

Maria Francisca Ribeiro Alves



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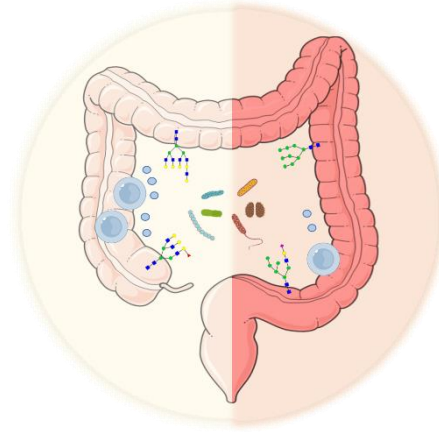
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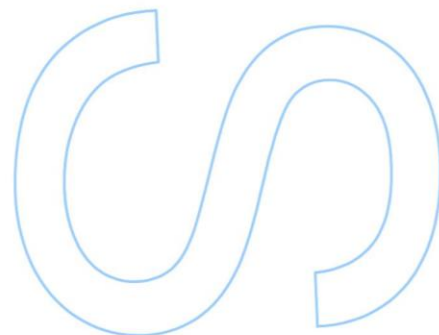
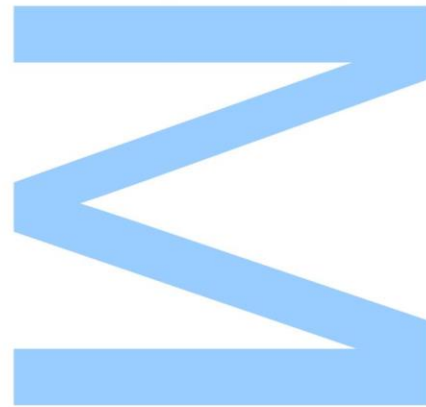
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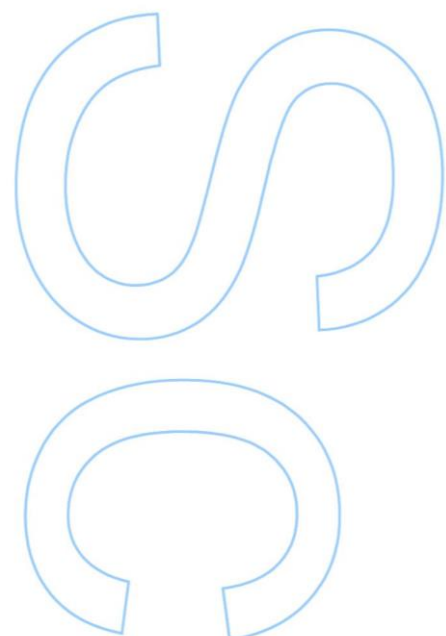
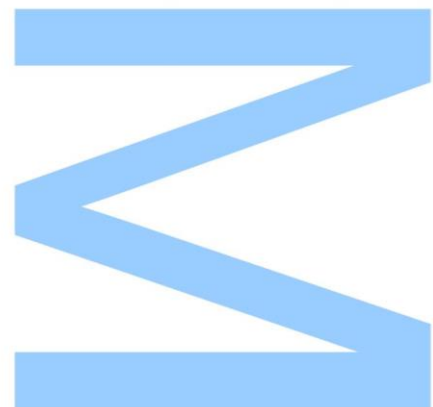




Todas as correções determinadas
pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Abstract

Inflammatory bowel disease (IBD) is a chronic immune-mediated disorder of the gastrointestinal tract that comprises Crohn's disease (CD) and ulcerative colitis (UC). The etiology of IBD remains unclear; nevertheless, evidence suggests that it results from an aberrant immune response to commensal microorganisms, triggered by environmental factors, in genetically susceptible individuals [1]. Glycosylation is a post-translational modification that occurs in the majority of proteins from mammalian cells, being essential in the regulation of many biological functions [2]. Indeed, glycans and the glycocalyx are a major interface between host and gut resident microbiota, serving as a source of energy and nutrients to commensal microbes [3]. Previous evidence from our group has demonstrated the relevance of changes in protein glycosylation in IBD immunopathogenesis [4-6]; yet, it is still unclear how glycans can modulate the microbiota composition and function and how this impacts gut immune responses associated with the disease. Therefore, in this thesis we aimed to: 1) unveil how an altered host glycocalyx impacts gut microbiota and the immune response associated with intestinal inflammation; 2) assess glycans supplementation as a strategy to create a homeostatic environment in the gut, putatively by modulating both the immune system and gut microbiota; 3) characterize the host glycosylation profile and immune landscape associated with human IBD pathogenesis.

Results from our group revealed that the absence of branched *N*-glycans in *Mgat5*-deficient (KO) mice imposes a dysbiotic microbiota composition, associated with an increased susceptibility to DSS-induced colitis. Also, within this thesis we observed that the glycosylation impairment observed in KO mice extends beyond branched *N*-glycans, since α 1,2-fucosylation was shown to be hindered in the colon of these mice. We also demonstrated that KO mice are depleted of a protective environment and in their capacity to sense microorganisms, as type 3 innate lymphoid cells (ILC3), ILC3-derived IL-22 and *Nod2* expression are decreased in the colon.

Understanding the impact of glycosylation in immune responses and considering that KO mice are partially depleted in bacteria capable to synthesize saccharides from the hexosamine pathway, as shown by our group (manuscript in preparation), we questioned whether supplementation of KO mice with GlycanC1 (a monosaccharide involved in this pathway), prior to DSS-induced colitis, could ameliorate disease severity in these mice. Interestingly, we found that GlycanC1-treated mice display improved disease courses comparing with non-treated mice. Indeed, we observed that, besides impacting α 1,2-fucosylation by promoting *Fut2* expression, GlycanC1 enhances ILC3

and ILC3-derived IL-22 levels in the colon, and rescues *Nod2* expression, ultimately promoting a homeostatic environment in the gut.

This evidence was then validated in human clinical samples, demonstrating the impact of host glycosylation in IBD immunopathogenesis in a clinical setting. We have observed that IBD patients exhibit an impaired mucosa glycoprofile, with the surface of colonic helper T (Th) and regulatory T (Treg) cells being deficient in glycans, particularly β 1,6-GlcNAc branched and high-mannose *N*-glycans, and α 2,6-sialic acid moieties. Resembling KO mice, the glycosylation impairment in the gut of IBD patients extends to fucosylation, as *FUT2* and *FUT8* appear to be downregulated and upregulated, respectively, in the inflamed colon. Importantly, ILC3 protective axis also seems to be hindered in patients, as they tend to be deprived of colonic ILC3 and Treg cells, while being enriched in Th17 cells.

Altogether, the findings presented in this thesis contribute to a deeper understanding of how altered mucosal glycoprofiles impact microbiota composition and immune responses associated with IBD pathogenesis, and provide novel insights regarding glycans supplementation as a potential prophylactic approach to be translated to IBD clinics.

Keywords: Inflammatory bowel disease, inflammation, immunity, glycosylation, *N*-glycans, ILC3, IL-22, glycans supplementation, prophylaxis, microbiome.

Resumo

A doença inflamatória intestinal (DII) é uma doença crónica imuno-mediada do trato gastrointestinal que inclui a doença de Crohn (CD) e a colite ulcerosa (UC). A etiologia da DII permanece pouco clara; no entanto, as atuais evidências sugerem que resulta de uma resposta imune alterada a microrganismos comensais, desencadeada por fatores ambientais, em indivíduos geneticamente suscetíveis [1]. A glicosilação é uma modificação pós-transducional que ocorre na maioria das proteínas de células de mamíferos, sendo essencial na regulação de muitas funções biológicas [2]. De facto, os glicanos e o glicocálice são uma importante interface entre o hospedeiro e a microbiota residente no intestino, servindo como fonte de energia e nutrientes para microrganismos comensais [3]. Estudos anteriores do grupo demonstraram a relevância das alterações na glicosilação proteica na imunopatogenicidade da DII [4-6]; no entanto, ainda não é claro de que forma podem os glicanos modular a composição e função da microbiota, e como isto influencia as respostas imunes intestinais associadas à doença. Por conseguinte, nesta tese, o nosso objetivo era: 1) desvendar como um glicocálice alterado no hospedeiro impacta a microbiota e a resposta imune associada à inflamação intestinal; 2) avaliar a suplementação de glicanos como estratégia para criar um ambiente homeostático no intestino, possivelmente modulando simultaneamente o sistema imune e a microbiota intestinal; 3) caracterizar o perfil de glicosilação do hospedeiro e a assinatura imunológica associada à patogénese da DII humana.

Os resultados do nosso grupo revelaram que a ausência de *N*-glicanos ramificados em ratinhos deficientes em *Mgat5* (KO) impõe uma composição disbiótica da microbiota, associada a uma maior suscetibilidade à colite induzida por DSS. Nesta tese observámos que a deficiência ao nível da glicosilação observada nos ratinhos KO se estende para além dos *N*-glicanos ramificados, uma vez que também a α 1,2-fucosilação está afetada no cólon destes. Para além disso, demonstrámos que os ratinhos KO são desprovidos de um ambiente protetor e da capacidade de perceber microrganismos, uma vez que as células linfóides inatas do tipo 3 (ILC3), os níveis de IL-22 derivada de ILC3 e a expressão de *Nod2* estão diminuídos no cólon.

Compreendendo o impacto da glicosilação nas respostas imunes e considerando que ratinhos KO têm uma diminuição ao nível de bactérias capazes de sintetizar sacarídeos da via da hexosamina, como demonstrado pelo nosso grupo (manuscrito em preparação), questionámos se a suplementação de ratinhos KO com GlycanC1 (um monossacarídeo envolvido nesta via), antes de induzir colite com DSS, poderia melhorar a severidade da doença nestes. De facto, descobrimos que os ratinhos tratados com GlycanC1 apresentam melhores cursos de doença em comparação com ratinhos não

tratados. Também observámos que, além de ter impacto na α 1,2-fucosilação ao promover a expressão de *Fut2*, o GlycanC1 aumenta os níveis de ILC3 e de IL-22 produzida pelas ILC3 no cólon e resgata a expressão de *Nod2*, promovendo em última instância um ambiente homeostático no intestino.

Esta evidência foi então validada em amostras clínicas humanas, demonstrando o impacto da glicosilação do hospedeiro na imunopatogenicidade da DII num ambiente clínico. Observámos que os doentes apresentam um perfil glicopatogénico ao nível da mucosa, com a superfície das células T auxiliares (Th) e células T reguladoras (Treg) exibindo um défice de glicanos, particularmente *N*-glicanos β 1,6-GlcNAc ramificados e ricos em manose, e unidades de α 2,6-ácido siálico. De forma similar aos ratinhos KO, as alterações na glicosilação no intestino de pacientes com DII estendem-se à fucosilação, uma vez que a expressão de *FUT2* e *FUT8* parece estar diminuída e aumentada, respetivamente, no cólon inflamado. É importante notar que o eixo protetor das ILC3 parece também estar comprometido nos pacientes, uma vez que estes apresentam uma tendência para uma diminuição de células ILC3 e Treg no cólon, concomitante com um enriquecimento em células Th17.

No conjunto, os resultados apresentados nesta tese contribuem para uma compreensão mais aprofundada de como a alteração dos glicoperfis da mucosa afeta a composição da microbiota e as respostas imunes associadas à patogénese da DII, fornecendo novos conhecimentos sobre a suplementação de glicanos como uma potencial abordagem profilática a ser traduzida para a clínica.

Palavras-chave: Doença Inflamatória Intestinal, inflamação, imunidade, glicosilação, *N*-glicanos, ILC3, IL-22, suplementação com glicanos, profilaxia, microbioma.

Index

Acknowledgments	V
Abstract.....	VI
Resumo.....	VIII
Figure Index.....	XII
Table Index	XV
Abbreviations	XVI
Chapter I - Introduction	1
1. Inflammatory Bowel Disease.....	3
1.1. Epidemiology	3
1.2. Etiology.....	5
1.3. Genetics	5
1.4. Symptoms and Diagnosis	7
1.5. Therapeutics.....	7
2. Immune system	10
2.1. Innate Immunity.....	10
2.2. Adaptive Immunity.....	12
2.3. Immunity in IBD	14
3. Microbiome	19
3.1. Dysbiosis in IBD	20
4. Glycobiology	22
4.1. Protein Glycosylation	22
4.2. Lectins	25
4.3. Glycosylation in IBD	26
Context and Main Goal	29
Specific Aims.....	29
Chapter II - Methods	31
Animals	33
Microbiota composition analysis.....	33
<i>In vivo</i> GlycanC1 supplementation and DSS-induced colitis.....	33
Isolation of murine lamina propria leukocytes	34
Isolation of leukocytes from murine mesenteric lymph nodes	34
Colonic tissue culture and cytokine quantification	35
Histology	35
Human samples	35

Isolation of human PBMCs	36
Human PBMCs defrosting	36
Isolation of lamina propria leukocytes from human fresh colonic biopsies.....	37
Flow cytometry.....	37
RNA extraction, cDNA synthesis and quantitative real-time PCR	39
Statistical Analysis	40
Chapter III - Results	41
I. Glycans as modulators of intestinal environment and their impact in homeostasis and disease.....	43
<i>Mgat5</i> -deficient mice exhibit a dysbiotic microbiota composition and higher susceptibility to develop severe colitis	43
KO mice display impaired <i>Fut2</i> expression and ILC3/IL-22 dynamics in the gut ..	45
GlycanC1 supplementation ameliorates disease severity in KO and WT mice.....	53
GlycanC1 supplementation improves ILC3/IL-22 dynamics in the colon of KO and WT mice	60
<i>Mgat5</i> deficiency seems to have a detrimental impact on <i>Nod2</i> expression, which is rescued by GlycanC1 supplementation	70
GlycanC1 impacts the transcription of genes involved in epithelial barrier integrity	74
II. Characterization of the host glycosylation profile and immune signature in IBD	76
Characterization of the glycoprofile associated with human IBD pathogenesis	76
<i>In situ</i> characterization of the immune landscape associated with human IBD pathogenesis	80
Characterization of the immune landscape at the periphery associated with human IBD pathogenesis	82
Chapter IV - Discussion	87
Conclusions and Future Perspectives	99
References	101
Appendix	117

Figure Index

Figure 1 Global incidence of IBD.....	4
Figure 2 Global prevalence of IBD in 2015.	4
Figure 3 Common step-up therapeutic approach used in IBD.	8
Figure 4 Helper ILCs.....	12
Figure 5 Defective mucosal barrier in IBD.	15
Figure 6 Intestinal anti-inflammatory environment promoted by SCFAs.....	20
Figure 7 Classes of <i>N</i> -glycans.	23
Figure 8 Synthesis of <i>N</i> -glycans.....	25
Figure 9 Lectins binding sites.....	26
Figure 10 KO mice are more susceptible to DSS-induced colitis.....	44
Figure 11 KO mice display an impaired glycoprofile in the colon, in homeostasis.. ...	45
Figure 12 KO mice display impaired ILC3 and IL-22 dynamics in the gut, in homeostasis.....	47
Figure 13 KO mice seem to display impaired IL-22 production in the colon, in homeostasis.....	48
Figure 14 KO mice display an impaired glycoprofile in the colon, upon inflammation.	49
Figure 15 KO mice display impaired ILC3 and IL-22 dynamics in the gut, upon inflammation.	51
Figure 16 KO mice display impaired IL-22 dynamics in the colon, upon inflammation.	52
Figure 17 GlycanC1-supplemented mice are protected against DSS-induced colitis.	54
Figure 18 GlycanC1 supplementation for 5 weeks does not seem to impact β 1,6-GlcNAc branched and high-mannose <i>N</i> -glycans, or α 2,6-sialic acid, in homeostasis..	55
Figure 19 GlycanC1 impacts epithelial glycosylation in WT mice, upon inflammation.	56
Figure 20 GlycanC1 upregulates <i>Fut2</i> in the colon of supplemented mice, in homeostasis and upon inflammation.	57
Figure 21 GlycanC1-supplemented WT mice have decreased levels of branched <i>N</i> -glycans in immune cells, upon inflammation.	59
Figure 22 GlycanC1 supplementation has a mild impact on the immune profile of KO mice, in homeostasis.	61
Figure 23 GlycanC1 downregulates <i>Il22ra2</i> in the colon of KO mice, in homeostasis.	62
Figure 24 GlycanC1 increases Treg-derived IL-10 in the colon of WT mice, in homeostasis.....	64

Figure 25 GlycanC1 tends to upregulate <i>Il22</i> in the colon of WT mice, in homeostasis.	65
Figure 26 GlycanC1 promotes ILC3 and ILC3-derived IL-22, GM-CSF and IL-17A, while decreasing Th17-derived IL-17A in the colon of KO mice, upon inflammation.	67
Figure 27 GlycanC1 increases ILC3 and Treg cells frequencies in the colon of WT mice, upon inflammation.	68
Figure 28 GlycanC1 increases IL-22 levels in the colon of WT mice, upon inflammation.	69
Figure 29 <i>Mgat5</i> deficiency seems to impact <i>Nod2</i> expression, which is then rescued by GlycanC1, in homeostasis.	71
Figure 30 GlycanC1 rescues <i>Nod2</i> expression in KO mice, upon inflammation.	73
Figure 31 GlycanC1 impacts barrier integrity through <i>Cldn1</i> or <i>Cldn2</i> , in homeostasis.	75
Figure 32 Inflamed colon tissues of IBD patients seem to display alterations in T cells' glycans expression.	77
Figure 33 Inflamed colon tissues of IBD patients seem to have impaired glycogenes expression.	78
Figure 34 Peripheral Th cells from IBD patients with active disease have increased levels of α 2,6-sialic acid.	79
Figure 35 The inflamed colon of IBD patients seems to present decreased frequencies of ILC3 and Treg cells, and an enrichment of Th17 cells.	81
Figure 36 Peripheral blood of IBD patients seems to be enriched in ILC3 and depleted in Th17 and Treg cells.	83
Figure 37 Peripheral blood of IBD patients is depleted in NK and NKT cells and is enriched in DCs.	85
Figure 38 Proposed model.	100

Supplementary Figure 1 Gating strategy used to define epithelial cells, ILC3, Th17, $\gamma\delta$ T and Treg cells in mouse samples.	117
Supplementary Figure 2 Gating strategy used to define epithelial cells, ILC3, Th17, Treg, NK, NKT and dendritic cells in human samples..	118
Supplementary Figure 3 Histograms of L-PHA staining in epithelial cells from mice colon.	119
Supplementary Figure 4 Histogram representation of cytokine staining of murine colonic immune cells.	119
Supplementary Figure 5 Characterization of the immune profile in the MLNs of WT and KO mice, in homeostasis.	120
Supplementary Figure 6 Characterization of the immune profile in the MLNs of WT and KO mice, upon inflammation.	121
Supplementary Figure 7 Characterization of the immune profile in the MLNs of KO and GlycanC1-treated KO mice, in homeostasis.	122
Supplementary Figure 8 Characterization of the immune profile in the MLNs of WT and GlycanC1-treated WT mice, in homeostasis.	123
Supplementary Figure 9 Characterization of the immune profile in the MLNs of KO and GlycanC1-treated KO mice, upon inflammation.	124
Supplementary Figure 10 Characterization of the immune profile in the MLNs of WT and GlycanC1-treated WT mice, upon inflammation.	125

Table Index

Table 1 Disease activity index used to evaluate mice disease severity.	34
Table 2 Characterization of the cohort of IBD patients involved in this study.	36
Table 3 Lectins and antibodies used in flow cytometry analyses.	38
Table 4 Mouse oligonucleotides used in quantitative real-time PCR.....	40
Table 5 TaqMan probes used in quantitative real-time PCR.	40

Abbreviations

5-ASAs	5-aminosalicilates
$\alpha\beta$	Alpha-beta
αMan-I	α 1,2-mannosidase I
$\gamma\delta$	Gamma-delta
ABC	Avidin–biotin–peroxidase
AhR	Aryl hydrocarbon receptor
AIEC	Adherent-invasive E.coli
AMPs	Antimicrobial peptides
APCs	Antigen-presenting cells
ASCA	Anti <i>Saccharomyces cerevisiae</i> antibodies
Asn	Asparagine
ATG16L1	Autophagy related 16-like 1
BfdA	Brefeldin A
CARD9	Caspase Recruitment Domain Family Member 9
CD	Crohn's disease
CLDN	Claudin
CHP/HSA	Centro Hospitalar do Porto-Hospital de Santo António
CRP	C-reactive protein
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTLs	C-type lectins
DAB	3,3'-Diaminobenzidine
DAI	Disease activity index
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICAM-3-grabbing nonintegrin
DII	Doença Inflamatória Intestinal
DMSO	Dimethyl sulfoxide
DoI-P	Dolichol phosphate
DSS	Dextran sulphate sodium
<i>E. coli</i>	<i>Escherichia coli</i>
ECM	Cell–extracellular matrix
ESR	Erythrocyte sedimentation rate
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FMO	Fluorescence minus one

FOXP3	Forkhead box P3
Fuc	Fucose
FUT	Fucosyltransferase
Gal	Galactose
GalNAc	<i>N</i> -acetylgalactosamine
GalNAc-T	GalNAc-transferase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBP	Glycan-binding proteins
GIT	Gastrointestinal tract
Glc	Glucose
GlcA	Glucuronic acid
GlcNAc	<i>N</i> -acetylglucosamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GNA	<i>Galanthus nivalis</i> agglutinin
GnT	<i>N</i> -acetylglucosaminyltransferase
GPR	G-protein coupled receptor
HBI	Harvey-Bradshaw Index
HBSS	Hanks' Balanced Salt Solution
HC	Healthy controls
IBD	Inflammatory bowel disease
ICI	Immune checkpoint inhibitors
IdoA	Iduronic acid
IECs	Intestinal epithelial cells
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IL-22BP	Interleukin-22 binding protein
<i>IL22ra2</i>	Interleukin 22 Receptor Subunit Alpha 2
ILCs	Innate lymphoid cells
IRGM	Immunity-related GTPase M protein
JAK-STAT	Janus-associated kinase–signal transducers and activators of transcription
JAM	Junctional adhesin molecules
KGF	Keratinocyte growth factor
KO	Knockout
L-PHA	<i>Phaseolus vulgaris</i> Leucoagglutinin
LPL	Lamina propria leukocytes

LTi	Lymphocytes tissue-inducer
Man	Mannose
MAP	Mitogen-activated protein
MFI	Median fluorescence intensity
<i>Mgat5</i>	α 1,6-mannosylglycoprotein 6- β -N-acetylglucosaminyltransferase
MHC	Major histocompatibility complex
min	Minutes
MLNs	Mesenteric lymph nodes
MUC2	Mucin 2
MR	Mannose-receptor
NF-κB	Nuclear factor κ B
NK	Natural killer
NKG2D	Natural killer group 2D
NKT	Natural killer T
NOD2	Nucleotide-binding oligomerization domain 2
Occludin	OCLD
OST	Oligosaccharyltransferase
p-ANCA	Perinuclear anti-neutrophil cytoplasmic antibodies
PBMCs	Peripheral blood mononuclear cells
PD-1	Programmed cell death protein 1
PMA	Phorbol 12-myristate 13-acetate
polyLacNAc	Poly-N-acetyllactosamine
PRR	Pattern recognition receptors
qPCR	Quantitative PCR
REG3	Regenerating Islet Derived Protein 3
RNA	Ribonucleic acid
RORγt	RAR-related orphan receptor gamma t
RPMI	Roswell Park Memorial Institute Medium
RT	Room temperature
SCFA	Short-chain fatty acid
SCFAR	SCFAs' receptor
SD	Standard deviation
Ser	Serine
SFB	Segmented filamentous bacteria
Sia	Sialic acids
SNA	<i>Sambucus nigra</i> agglutinin

SNPs	Single-nucleotide polymorphisms
SPF	Specific pathogen-free
T-bet	T-box expressed in T cells
Tc	Cytotoxic T
TCR	T cell receptor
TGF-β	Transforming growth factor- β
Th	Helper T
Thr	Threonine
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T
UC	Ulcerative colitis
WT	Wild-type
Xyl	Xylose

Chapter I

Introduction

1. Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is defined as a chronic, relapsing, immune-mediated disorder of the gastrointestinal tract (GIT) [4]. IBD comprises two disorders: ulcerative colitis (UC) and Crohn's disease (CD). Although UC and CD have common symptoms and similar disease courses, with flares that alternate with periods of remission, they differ in terms of localization and nature of the inflammatory changes [7]. UC inflammation may affect a variable extent of the rectum and the colon, in continuity, and is typically confined to the mucosa [5]. On the other hand, the inflammation in CD is discontinuous and can affect any part of the GIT, typically the terminal part of the ileum and the colon [8]. At the microscopic level, CD is transmural, since it may affect the entire bowel wall – mucosa, submucosa, muscularis propria and serosa/adventitia [9].

1.1. Epidemiology

IBD has evolved into a global disease in the 21st century [10], with its incidence, i.e., the rate of newly diagnosed cases, increasing worldwide. The highest incidences have been reported in Northern Europe, North America and the United Kingdom (Figure 1) [11]. Although the incidence of IBD plateaued in Western countries over the past decades, the incidence in newly industrialized countries in Asia, South America, and Middle East has emerged. Additionally, the prevalence of IBD is increasing in every continent, finding its higher values in North America, some Northern European countries, Australia and New Zealand (Figure 2) [12]. Considering the global scenario, in Portugal the prevalence of IBD is intermediate, increasing from 86 to 146 patients per 100 000 people from 2003 to 2007 [13].

IBD can affect individuals of any age, from infants to the elderly, but two peaks are described for the age of onset: a major one between 15-25 years old, and a second one between 40-69 years old [14].

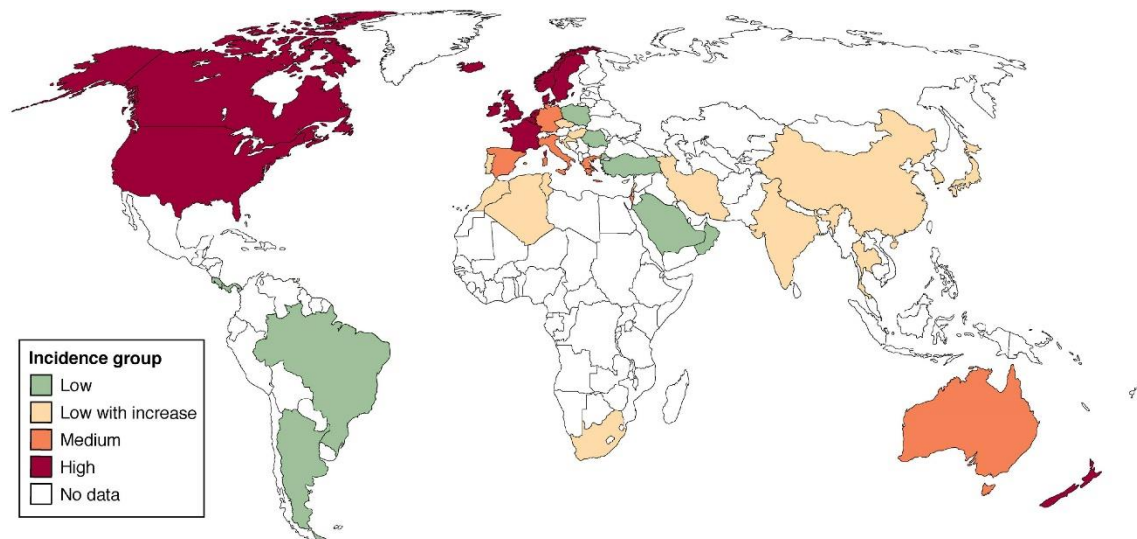


Figure 1| Global incidence of IBD. The incidence of IBD has emerged worldwide, particularly due to the increased incidence in newly industrialized countries in Asia, South America, and Middle East. On the other hand, the incidence of IBD in countries with the highest incidence rates, in Northern Europe, North America and the United Kingdom, has plateaued over the years [11].

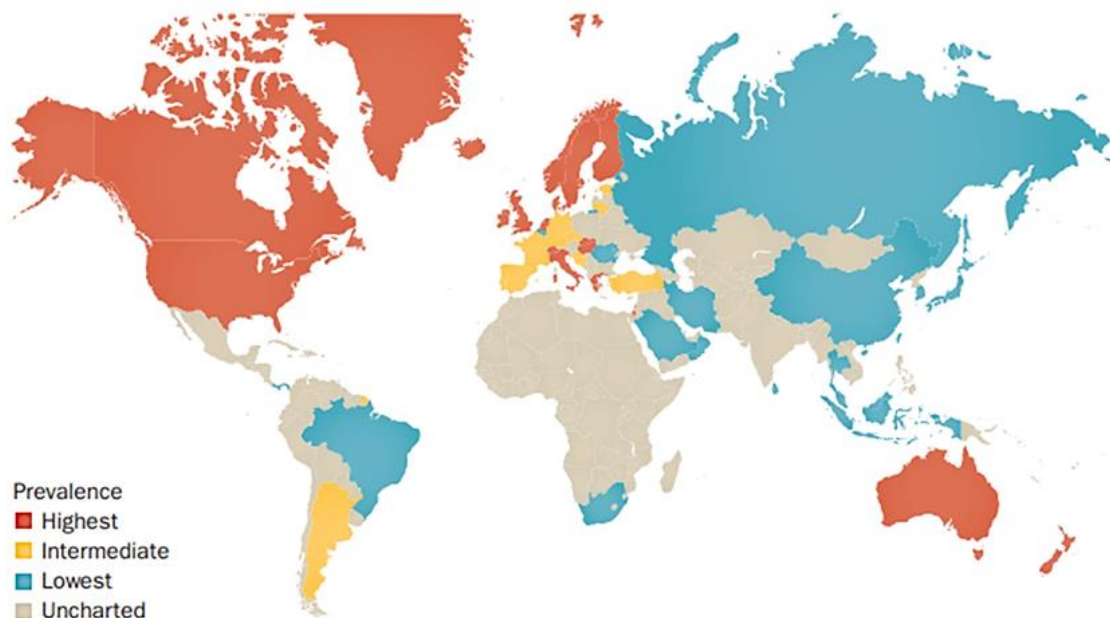


Figure 2| Global prevalence of IBD in 2015. The prevalence of IBD is increasing globally, with the highest prevalence occurring in North America, some Northern European countries, Australia and New Zealand [12].

1.2. Etiology

Although the precise etiology of this disease remains unknown, both forms of IBD seem to result from an inappropriate inflammatory response triggered by intestinal microbes in genetically susceptible individuals [1].

Environmental factors are closely associated with IBD, and among them, diet has a major contribution. This is evidenced by the rising incidence of IBD observed in newly industrialised countries due to prominent dietary changes, such as increased exposure to processed food, refined sugars, dairy, and decreased plant-based fibres consumption [12]. Smoking habits also affect IBD but differentially between CD and UC; smokers are at increased risk for CD, and for more severe manifestations of the pathology, whereas in UC this habit is associated with a protective effect [1]. In addition, childhood antibiotic exposure, stress, and the hygiene hypothesis are also related to the development of IBD [15].

1.3. Genetics

Several genetic variants have been closely linked to an increased susceptibility to develop IBD and, surprisingly, it is estimated that known variants correspond only to 20% of all genetic factors that increase the risk for this disease [1].

Genetic polymorphisms of innate immune receptors that sense microorganisms, namely nucleotide-binding oligomerization domain 2 (NOD2) and toll-like receptor 4 (TLR4), are major risk factors for the development of IBD, which emphasizes the compromised host immune response to microbiota highlighting this relationship as a key player in inciting the initiation and progression of inflammation [16].

NOD2 is an intracellular sensor of peptidoglycan and is expressed by immune cells, such as epithelial, endothelial, Paneth and dendritic cells, and macrophages. Activation of this protein triggers nuclear factor κ B (NF- κ B) and mitogen-activated protein (MAP) kinase signalling pathways, ending in the production of cytokines, such as interleukin (IL)-1 β , and antimicrobial peptides (AMPs) [1]. In carriers of genetic variants that impair NOD2 function there is a downregulation of the mechanisms responsible for minimizing the production of pro-inflammatory cytokines during chronic NOD2 stimulation, namely the production of IL-10 [17], resulting in increased inflammation of the gut. Heterozygosity and specially homozygosity for a polymorphism confer a great risk for CD [1]. *NOD2* variants are also associated with age of onset and disease location [6].

Caspase Recruitment Domain Family Member 9 (CARD9) is an adaptor molecule required for NOD2 [18] and TLR pathways, being therefore involved in innate immune

activation after the sensing of pathogenic microorganisms. *CARD9* variants are identified as susceptibility factors for IBD, with multiple risk alleles contributing to pathogenesis by predisposing to an increased expression of this gene, which leads to an hyperactivation of the immune response towards pathogens [19]. At adequate levels, *CARD9* has a notorious role in colitis, promoting recovery by inducing IL-22 production in a microbiota-dependent manner [20].

Other innate-immune-response genes have been associated with IBD, such as *autophagy related 16 like 1 (ATG16L1)* and *immunity-related GTPase M (IRGM)*, both involved in autophagy. Since the autophagy pathway contributes to T-cell tolerance, defective polymorphisms in these genes can increase CD patients' susceptibility to intestinal inflammation. Furthermore, T-cell tolerance is also associated with IL-10 signalling, and it has been shown that rare, recessive, loss-of-function mutations in *IL10RA* and *IL10RB* (components of the IL-10 receptor) result in CD [1].

IL-23 signalling has an important role in mediating inflammation, through the interaction of heterodimeric IL-23 (p19 and p40 subunits) with its heterodimeric receptor (IL-23R and IL-12RB1 subunits) that triggers the Janus-associated kinase–signal transducers and activators of transcription (JAK-STAT) signalling pathway. Genetic variants of *IL23R* have been associated with IBD and other immune diseases, correlating with the fact that IL-23 levels and IL-23-stimulated Th17-derived cytokines are elevated in the colonic mucosa of both CD and UC patients. On the other hand, some variants are protective, such as Arg381Gln, decreasing the probability to develop IBD. Other genes related to the IL-23 signalling pathway have been associated with both CD and UC, such as *JAK2*, *STAT3* and p40 [1].

The apical surface of intestinal epithelial cells (IECs) possesses glycans enriched in α 1,2-fucose, essential for protection against pathogenic bacteria and maintenance of commensal microbiota [21]. Some commensal bacteria, such as *B. thetaiotaomicron*, *B. fragilis* and segmented filamentous bacteria (SFB), and some pathogenic bacteria, for example *Salmonella typhimurium* and *Helicobacter bilis*, interact with type 3 innate lymphoid cells (ILC3), inducing them to produce IL-22. IL-22 then acts on IECs, promoting the expression of *fucosyltransferase (FUT)2* by these cells, and consequent α 1,2-fucosylation. Apart from this, ILC3 also produce lymphotoxin in a microbiota-independent manner that at baseline induces intestinal epithelial fucosylation [22, 23]. In accordance with the intimate relation between fucosylation, microbiota and immune system, and the fact that commensal bacteria and the host immune system are tightly associated with the development of IBD, *FUT2* nonsense polymorphisms are risk factors for this disease. In IBD patients, *FUT2* gene expression is generally decreased [23].

So far, around 240 genetic risk loci have been identified for IBD susceptibility [6]. Interestingly, loci associated with the risk to develop IBD may not be associated with the disease course, regarding relapses and best therapeutic approaches [24].

1.4. Symptoms and Diagnosis

According to disease location, CD can be subdivided in five categories: gastroduodenal, affecting the stomach and the duodenum; jejunoileitis, if inflammation occurs in the lower half of the small intestine; ileitis, if only the ileum is affected; ileocolitis, characterized by inflammation in the ileum and the colon; and, finally, Crohn's colitis, affecting only the colon [25, 26]. Similarly, UC can be subclassified according to disease extension: ulcerative proctitis, that affects only the rectum; proctosigmoiditis, where the rectum and the sigmoid colon are affected; left-sided colitis, in which inflammation occurs from the rectum to the splenic flexure; and extensive colitis, that affects the entire colon [27].

Despite the symptoms being very similar between UC and CD, which hinders the diagnosis of one form of IBD, it is more common in UC the presence of pain in the lower left abdomen, diarrhoea, weight loss and blood on rectal examination. In contrast, in CD is more frequent to identify pain in the lower right abdomen and swelling, which results in thickening of the bowel wall and malnutrition, in consequence of poor absorption [7].

To make a correct diagnosis, besides considering clinical features, physicians may evaluate endoscopic, histologic and radiological features [28, 29]. Blood and stool samples are used to complement the diagnostic work-up, allowing the identification of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), and calprotectin and lactoferrin, respectively, which are indicatives of inflammation [30, 31]. Some serological biomarkers are specific to one of the two IBD disorders, and these may be used to distinguish between them. For instance, perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA) point towards UC [32], while anti *Saccharomyces Cerevisiae* antibodies (ASCA) discriminate CD [33]. However, 15-20% of IBD patients do not have any of these markers [34]. Family history is also an important factor to consider, since there is a strong contribution from genetics to this disease, as described before.

1.5. Therapeutics

As a consequence of being a multifactorial and complex disease, IBD is extremely heterogenous among patients, which hampers the finding of an efficient treatment. Current therapeutic approaches focus on mitigating symptomatology in patients, as well as on inducing and maintaining remission.

As a guidance for therapy decision-making, disease severity can be determined by physicians using different scores. Mayo Score is the most popular and commonly used score for UC and relies on both clinical features, namely stool frequency, rectal bleeding and physician rating, and endoscopic appearance of mucosa, evaluating vascular pattern, friability, bleeding, and ulcerations. Each parameter is rated from 0 to 3, determining normal (0), mild (1), moderate (2) or severe (3) disease [35]. On the other hand, disease severity in CD can be assessed by Harvey-Bradshaw Index (HBI), a simple score that uses only clinical features. These include general well-being, abdominal pain, stool frequency - all three for the previous day and reported by the patient -, abdominal mass and presence or absence of complications, such as ulcers, new fistulas and abscesses [36].

Even though some patients only benefit from more potent drugs due to having severe forms of the disease, others can profit from a standard approach called “step-up approach” [37]. In this method, the treatment initiates with less effective and potentially less toxic strategies, such as aminosalicylates, progressing towards potentially more toxic treatments, namely corticosteroids, immunosuppressants and, finally, biologics, for patients that did not ameliorate with the previous approaches (Figure 3). In opposition, some studies have shown that the introduction of more potent and toxic treatments early in the disease course of a chronic illness is helpful to prevent disease progression - this is defined as “top-down approach” [38].

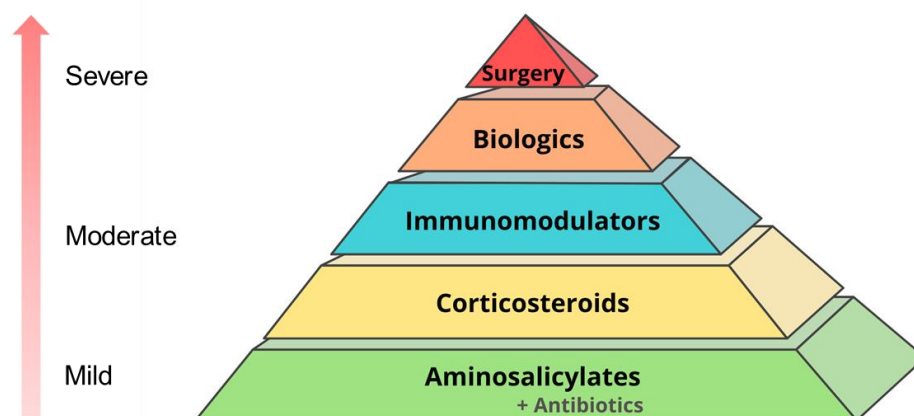


Figure 3| Common step-up therapeutic approach used in IBD. IBD treatment usually initiates with less toxic and effective compounds, which for UC patients is aminosalicylates, occasionally conjugated with antibiotics. According to disease severity, IBD patients may progress to more potent and toxic strategies: from corticosteroids to immunomodulators and biologics. For severer cases, surgery is performed. Adapted from [37].

5-aminosalicylates (5-ASAs), like mesalamine and sulfasalazine, are non-steroid anti-inflammatory drugs that are usually used as the first line of defence in UC. Although ineffective for the treatment of CD, 5-ASAs can efficiently reduce UC symptoms and help maintain remission in these patients. Anti-inflammatory drugs can inhibit the production of cytokines such as tumor necrosis factor-alpha (TNF- α) and interferon-gamma (IFN- γ), but, unfortunately, have vomiting, diarrhoea, nausea and others as side effects [7].

Antibiotics may influence disease's course by altering the composition of intestinal microbiota, which plays an important role in the pathogenesis and complications of IBD [39]. Available studies demonstrate that antibiotics may be used as a complement to other current treatments in IBD management [40].

Corticosteroids, such as budesonide and prednisone, are fast-acting anti-inflammatory steroids, being optimal to control flares [41]. They are involved in the stimulation of lipocortin, which inhibits phospholipase A2, preventing the synthesis of pro-inflammatory mediators prostaglandins and leukotrienes [42]. Corticosteroids are not recommended for long-term or maintenance use due to side effects, such as diabetes, osteoporosis and withdrawal symptoms caused by suppression of the adrenal glands [41, 43].

Immunosuppressants such as azathioprine, mercaptopurine and methotrexate regulate the immune system by triggering a Th2-mediated response that dampens Th1-mediated inflammation. This results in the production of anti-inflammatory cytokines, as IL-4, IL-5, IL-6 and IL-10, that inhibit the production of pro-inflammatory mediators, reducing the extent of inflammation [7]. Although immunosuppressant agents have significant side effects, such as increased risk of infection, weight gain, drowsiness, fluid retention, hypertension and vomiting, they are safer and better tolerated than long-term corticosteroid therapy [7, 41].

Biologics have been used as a promising therapy for IBD and other inflammatory diseases, since they act selectively, unlike other approaches such as corticosteroids. TNF- α is a pro-inflammatory cytokine that plays a major role in IBD-associated inflammation [7]. Therefore, anti-TNF- α , e.g., Adalimumab and Infliximab, are the most used biologics, being efficient in reducing symptoms and healing inflamed tissues. However, other biologic possibilities include integrin receptor antagonists, as Vedolizumab, and IL-12 and IL-23 antagonists, such as Ustekinumab [44].

Finally, in more severe cases in which the previous therapies were not able to control inflammation, there may be the need to perform surgery. In these situations, severe inflamed tissue portions are removed. It is estimated that around 20% of UC patients will require surgery, and up to 80% of CD patients will undergo operation during their lives [45].

2. Immune system

The immune system is a defence mechanism present in the human organism so as to protect against disease, eliciting a response not only against infectious microbes but also against non-infectious foreign substances, such as toxic or allergenic components, and products of damaged cells [46].

To effectively avoid damage, the immune system has to satisfy four main aspects: immunologic recognition, to identify the cause of a possible dysregulation; effective immune function, to contain invaders and, if possible, eliminate them; immune regulation, which translates its capacity to regulate itself, contributing to homeostasis; and immunologic memory, the ability to induce a stronger response to subsequent expositions to the same antigen [47].

The immune system can be divided in two categories, the innate and the adaptive immunity, which vary in the speed and specificity of reaction [48].

2.1. Innate Immunity

The innate immunity is essential to give an almost immediate unspecific response to microbes and injured cells [46]. As first line of defence against pathogens there are physical barriers. Focusing on the gut, which is the focal point in this work, these are extremely important since the digestive system is continuously exposed to antigens that enter by ingestion [48], and also due to the huge number of microorganisms that inhabit it.

The intestinal physical barrier is formed by epithelial cells and mucus. IECs are connected through junctional complexes, that include tight, adherens and gap junctions [49]. As components of tight junctions, claudins (CLDNs), occludin (OCLD) and junctional adhesion molecules (JAM) seal the space between IECs, controlling paracellular permeability [50-52] and hindering the passage of microorganisms from the lumen to the lamina propria. This is reinforced by the presence of two mucus layers: an inner layer, composed by the glycocalyx and anchored to the mucus-producing goblet cells of the epithelial membrane; and a loosely adherent outer layer that is composed by gel-forming mucins and bacteria [53, 54]. Besides physical barrier, the entrance of pathogens is hindered by a chemical barrier of AMPs, such as regenerating islet derived protein 3 (REG3) α [55], produced by secretory IECs [56].

2.1.1. Myeloid cells

Phagocytic cells, such as neutrophils and macrophages, have a crucial role in the ingestion and digestion of harmful particles and are major components of innate immunity, along with mast cells and blood proteins (such as the components of the complement system) [46].

Macrophages are the largest population of leukocytes in the intestine, which confers them the role of initiators of defence to maintain intestinal homeostasis. The majority of intestinal macrophages are located in the colon, which is directly associated with the vast number of bacteria present [57]. Originating from circulating monocytes that replenish the intestine and differentiate *in situ*, intestinal macrophages keep the integrity of the gut epithelial barrier, having several other essential functions, including phagocytosis, degradation of microorganisms and the production of mediators that promote epithelial cell renewal [58, 59].

Dendritic cells (DCs) are innate cells capable of identifying microbial ligands and responding quickly to pathogens, due to the presence of superficial and intracellular pattern recognition receptors (PRRs). Along with M cells and immunoglobulins (Igs), DCs are responsible for the continuous sampling of antigens present in the intestinal lumen, translocating them across epithelial, and then presenting to T cells in secondary lymphoid organs of the gut, such as mesenteric lymph nodes (MLNs), Peyer's patches, and isolated lymphoid follicles. In this sense, together with macrophages, DCs are designated as antigen-presenting cells (APCs). The described mechanism allows the induction of immune tolerance [1], crucial to avoid unnecessary inflammatory responses to food antigens and commensal bacteria, maintaining immune homeostasis in the intestine [60]. Antigen-presentation also allows the initiation of an adaptive immune response [61], if needed, in the presence of pathogenic microbes. For this reason, APCs can be considered a bridge between the innate and the adaptive system. Additionally, classical DCs are described to play a crucial role in promoting protective mucosal responses by serving as source of relevant cytokines, as IL-23 required for ILC3 activation [62].

2.1.2. Innate lymphoid cells

Innate lymphoid cells (ILCs) are a relatively small population in the gut that has been identified as decisive in the development of an immune response, which can have both protective or deleterious properties [63]. ILCs are a heterogeneous family of five subsets including natural killer (NK), lymphocytes tissue-inducer (LTi) cells, type 1 ILCs (ILC1), type 2 ILCs (ILC2) and type 3 ILCs (ILC3) [64]. NK have a cytotoxic profile due to their capacity to produce IFN- γ , perforin and granzymes, while LTi are responsible for

secondary lymphoid organogenesis through the production of lymphotoxin. ILC1, ILC2 and ILC3, known as ‘helper ILCs’, are associated with different contexts, with ILC1 being important during bacterial infections, ILC2 in helminth infections and allergic diseases, and ILC3 in chronic inflammation (Figure 4) [65, 66]. A few years ago, ILC3 have emerged as a critical subset in the regulation of intestinal homeostasis [67], through the production of IL-22, IL-17 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the physiological state [68].

ILC3 produce high amounts of IL-22 in response to IL-23 and IL-1 β pro-inflammatory cytokines and commensal bacteria. IL-22 plays an important role in maintaining intestinal barrier integrity, namely by stimulating the production of AMPs by epithelial cells, promoting mucosal healing and fostering the transcription of mucins [69, 70].

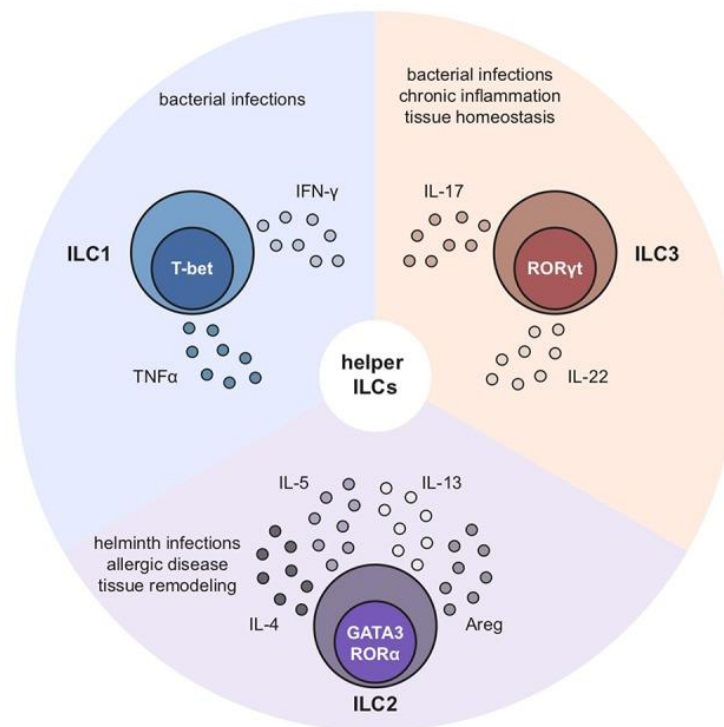


Figure 4| Helper ILCs. ILC1, ILC2 and ILC3 are known as ‘helper ILCs’, due to their involvement in host defence and tissue remodelling. T-bet⁺ ILC1 produce TNF- α and IFN- γ and have an important role against bacterial infections. GATA3⁺ROR α ⁺ ILC2 produce IL-4, IL-5, IL-13, being involved in helminth infections, allergic disease, and tissue remodelling. ROR γ t⁺ ILC3 mainly produce IL-17 and IL-22, having a fundamental role in bacterial infections, chronic inflammation, and tissue homeostasis [66].

2.2. Adaptive Immunity

The adaptive immune response is slower than innate immunity but is specific for distinct antigens and often for different portions of a single complex protein, polysaccharide, or other macromolecule, called epitopes. Moreover, it has memory,

which leads to faster and greater in magnitude responses in a repeated exposure to the same antigen [46].

The adaptive immune system is mediated by B and T lymphocytes. These cells are responsible for two different types of adaptive immunity: the humoral and the cellular immunity, respectively. Humoral immunity is mediated by antibodies, produced by B lymphocytes and present in the blood and mucosal secretions. This immunity is especially important to defend against microbes and their toxins located outside cells. On the contrary, cellular immunity promotes the destruction of microbes inside phagocytes and the killing of infected cells to eliminate reservoirs of infection [46].

2.2.1. T lymphocytes

T lymphocytes, also referred as T cells, originate from hematopoietic stem cells and mature in the thymus.

In the thymus, thymocytes, the precursors of T cells, acquire T cell receptors (TCRs), which are typically composed of two different polypeptide chains, nominated α and β , being therefore named as alpha-beta ($\alpha\beta$) T cells. In each of these chains there is a short cytoplasmic tail and a transmembrane region, followed by a constant region, and a top variable region, responsible for the recognition of the antigen. Constant and variable regions can be decorated with glycans, which modulates their properties. A minority of T cells have TCRs composed of γ and δ chains, being defined as gamma-delta ($\gamma\delta$) T cells [47].

During thymic development, thymocytes suffer a negative selection, where cells with self-reactive receptors are eliminated so as to avoid autoreactivity [71]. Moreover, thymocytes go through a positive selection, in which the ones bearing a functional $\alpha\beta$ TCR undergo differentiation into $CD4^+$ helper (Th) or $CD8^+$ cytotoxic (Tc) T cells, essentially [72]. Th cells are responsible for the production of cytokines that activate other immune cells, such as macrophages [73]. On the other hand, Tc cells are able to kill cells infected with virus or intracellular pathogens [74].

Natural killer T (NKT) cells is also a small subset of $\alpha\beta$ T cells that includes $CD4^+$, $CD4^-$, $CD8^+$ and $CD8^-$ cells, and that differs from conventional $\alpha\beta$ T cells in the very limited diversity of their TCR. NKT cells have this designation because they express NK1.1, a marker of NK cells [75].

Besides above-mentioned cells, a lineage-committed subset of forkhead box P3 (FOXP3)⁺ regulatory T (Treg) cells is also generated in the thymus. The development of these cells requires high-avidity interaction with self-peptides [76], leading to the generation of self-reactive cells that inhibit immune responses by producing suppressive IL-10, transforming growth factor- β (TGF- β) and IL-35 [46, 77].

Once matured, CD4⁺ and CD8⁺ T cells leave the thymus towards peripheral lymphoid organs as naive T cells, meaning they have not yet encountered their specific antigen. In peripheral lymphoid organs, $\alpha\beta$ T cells are activated through the interaction of the TCR with foreign proteins bound to major histocompatibility complex (MHC) molecules on APCs [73]. This leads to further antigen-mediated differentiation of CD4⁺ Th cells into effector subsets, depending on the cytokine milieu: Th1, Th2, Th17. These subtypes differ essentially in the cytokines they produce during an immune response [78]. In opposition to MHC restricted CD4⁺ and CD8⁺ $\alpha\beta$ T cells, $\gamma\delta$ T cells directly recognize non-peptidic molecules, independently of MHC-mediated antigen presentation [79]. Functionally, $\gamma\delta$ T cells have the capacity to rapidly respond to stimuli producing a variety of cytokines. In this sense, $\gamma\delta$ T cells are considered ‘unconventional’ T cells that act as a bridge between the innate and the adaptive immune system [80].

Interestingly, Th1, Th2 and Th17 share the same transcription factors as ILC1, ILC2 and ILC3, respectively, also producing common cytokines. T-box expressed in T cells (T-bet)⁺ Th1 and ILC1 produce IFN- γ and TNF- α , having an important role in phagocytosis and cellular immunity; GATA3^{hi} Th2 and ILC2 produce IL-5, IL-13 and IL-9, inducing eosinophil accumulation and IgE production, and inhibiting phagocyte function; while RAR-related orphan receptor gamma t (ROR γ t)⁺ Th17 and ILC3 produce IL-22, GM-CSF and IL-17 [81, 82].

Some activated T cells differentiate into memory T cells, which are responsible for long-term immunity since they are readily capable of differentiating into effector cells in a second exposure to their specific antigen [47].

2.3. Immunity in IBD

A compromised immune system can be a major trigger for the development of immune-mediated disorders but is also an outcome of these diseases. IBD patients have a defective mucosal barrier characterised by a thinner mucus layer, epithelial injury [83], flawed adhesion molecules [70] and decreased expression of AMPs [84]. In IBD, abnormalities in intercellular tight junctions occur due to decreased expression and redistribution of occludin, JAM and claudins, leading to increased paracellular permeability [85]. In particular, the expression of sealing claudins with the capacity to tighten the epithelium, such as CLDN-1, -3, -5 and -8, is altered in IBD [86]. The role of CLDN-2 is controversial, but several studies report it as a pore-forming claudin that decreases barrier function of CLDN1 and CLDN4, being upregulated in the colon of UC and CD patients, promoting barrier disruptions [87, 88].

The described fragility of the epithelium allows the entrance of intestinal microbiota from the lumen to lamina propria, triggering an inflammatory reaction [85]. Indeed, during active IBD there is an exacerbated infiltration of innate immune cells, such as neutrophils, macrophages and DCs, and adaptive immune cells, like B and T lymphocytes, into the lamina propria. Once inflammation is set, several immune cells produce different cytokines that amplify the immune response, namely TNF- α , IL-1 β , IFN- γ , IL-17 and IL-23 [1]. When the inflammatory event persists over time, the epithelium is injured, mainly due to the action of metalloproteinases that destroy the matrix [83]. This causes erosions and ulcerations, which ultimately increase exposure to intestinal microbiota, exacerbating local inflammation (Figure 5) [1].

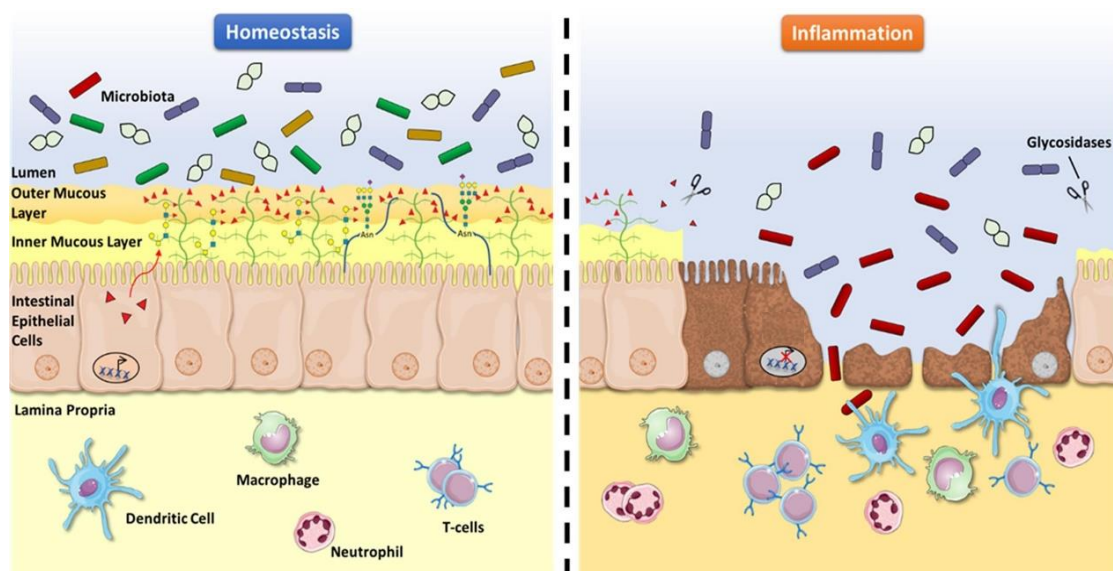


Figure 5| Defective mucosal barrier in IBD. The intestinal epithelium in IBD patients is characterized by a compromised integrity. This occurs as a consequence of a thinner mucus layer, epithelial injury and decreased production of AMPs, favouring the entrance of pathogenic bacteria (represented in red) that will establish an exacerbated pro-inflammatory response. Adapted from [21].

The predominant inflammatory response is slightly different between UC and CD. While in CD the major response involves Th1 cytokines, namely IFN- γ and TNF- α , in UC there is usually a Th2 response, with a high expression of IL-4, IL-5 and IL-13 [1, 68, 89]. Nevertheless, both UC and CD patients present a massive infiltration of Th17 cells in the gastrointestinal mucosa, together with increased levels of Th17-related cytokines, mainly IL-17A, IL-17F, IL-22 and IL-23 [90]. Th17 cells are intimately implicated in the pathogenesis of IBD and several other inflammatory diseases [91, 92].

Interestingly, it has been reported a dual role for IL-17 in IBD. Among the IL-17 family, IL-17A and IL-17F isoforms share 55% of homology [93] and seem to have opposite functions. In the dextran sulphate sodium (DSS)-induced colitis model, a

chemical murine model that resembles human UC [94], it has been reported that lack of IL-17F results in reduced colitis, translating the pathogenic role of this protein, while IL-17A deficiency promotes a more severe disease, showing a protective function of the later cytokine [95, 96]. Indeed, the immunotherapy with secukinumab (anti-IL-17A) has shown to be ineffective in IBD patients, which may imply an important regulatory role of this cytokine [97]. Notwithstanding, other studies in mice describe a pathogenic role for IL-17A in acute DSS-induced colitis, with its blockade ameliorating intestinal inflammation [98, 99]. In general, an equilibrated production of both isoforms is mandatory for an intestinal homeostasis in a healthy mucosa [100].

IL-22 is a cytokine commonly co-expressed with IL-17 by Th17 cells, although it can be produced by other innate and adaptive immune, such as $\gamma\delta$ T cells, NKT cells, LT α i cells and ILC3 [101, 102]. In fact, the latter are the main producers of IL-22 in the intestine [103]. Both pro-inflammatory and anti-inflammatory properties have been described for IL-22, depending on the environment [104]. IL-22 is important for gut homeostasis, contributing to tissue regeneration and wound healing through the promotion of epithelial cell proliferation and survival in the intestine. In DSS-induced colitis and other models of IBD, IL-22 has shown to be protective, with its blockade leading to a delayed recovery of the disease. This can be explained by its capacity to induce the production of IL-6 and IL-8 by colonic epithelial cells, mucins by goblet cells, AMPs, along with the mentioned ability to promote wound healing [102]. IL-22 and IL-17 are intimately correlated, being in part synergistic, and on the other hand balancing each other's functions. For instance, higher levels of IL-17 can exacerbate IL-22 pro-inflammatory properties, whereas a balanced co-expression of IL-17 and IL-22 can reduce the pathogenicity of IL-17. To avoid pathology, IL-22 and IL-17 production must be tightly controlled [102].

As co-producers of IL-22 and IL-17, ILC3 have also been described as essential in gut homeostasis. These cells contribute to intestinal immunity against pathogens and facilitate tissue repair and regeneration [67]. ILC3 have been detected in both UC and CD patients [105] having a decreased IL-22 production [106], which could also explain the enhanced epithelial injury in patients. However, other studies link ILC3 to the pathogenesis of IBD; although its involvement is still unclear, it may occur in consequence of ILC3 plasticity, i.e., during chronic inflammation and stimulation with IL-12 and IL-15, ILC3 are capable to acquire an ILC1-like phenotype, changing from ROR γ t to T-bet expression, and overexpressing IFN- γ [67, 68].

GM-CSF is a cytokine produced by ILC3, Th17 cells and $\gamma\delta$ T cells, that is associated with the proliferation and activation of immune cells during inflammation. Biological effects of GM-CSF in the human intestine are still unexplored, however, its

role in gut homeostasis is highlighted by several evidence: administration of GM-CSF to CD patients results in clinical improvements; higher levels of circulating anti-GM-CSF antibodies are associated with a worse CD prognosis; and GM-CSF^{-/-} mice are more susceptible to colitis [107]. More recently, it has been reported that microbiota-dependent IL-1 β production by intestinal macrophages activates ILC3 to secrete GM-CSF, which has a crucial homeostatic role by promoting local Treg cells' differentiation and tolerance [108].

$\gamma\delta$ T cells are a minor subset of T cells that, although rare in secondary lymphoid organs, are considerably located in the epithelial layer of mucosa lining [109]. Increased numbers of $\gamma\delta$ T cells have been identified in inflamed areas of the gut of both UC and CD patients [109], although its role in IBD is still controversial [110]. $\gamma\delta$ T cells are a source of IFN- γ [111], which could exacerbate inflammation. Notwithstanding, these cells are also able to produce IL-17, IL-22 and GM-CSF, and are known to be present in epithelial barrier tissues, being paramount for barrier surveillance, tissue repair and homeostasis [112]. Accordingly, a protective role from $\gamma\delta$ T cells has been suggested in various IBD animal models, namely in DSS-treatment in which mice deficient in $\gamma\delta$ T cells have a more severe disease and a delayed recovery after treatment [110, 113]. This could be explained by $\gamma\delta$ T cell production of keratinocyte growth factor (KGF), a potent IEC mitogen, in DSS-induced colitis [109].

Treg cells are fundamental for intestinal homeostasis [114]. The implication of these cells in IBD is evidenced using murine models of colitis such as T cell transfer model and the IL-10-knockout model. It has been reported that transfer of CD4⁺CD45RB^{high} (naive) T cells from healthy wild-type mice to T cells-knockout mice leads to colitis; on the other hand, transfer of CD4⁺CD45RB^{low} T cells, a population that includes CD25⁺Foxp3⁺ Treg cells, into immunodeficient recipients does not lead to intestinal inflammation. Along with findings showing that IL-10-knockout mice develop colitis spontaneously, this illustrates the importance of Treg cells in gut homeostasis [115]. Emerging evidence point to an impairment of Treg cells in IBD, being therefore involved in disease pathogenesis [116]. Indeed, some studies suggest that IBD patients have reduced levels of Treg cells both in the periphery and in colon, with these still being functional *in vitro* [117], while others identify increased levels of Treg cells in both inflamed and non-inflamed tissue of IBD patients, comparing with healthy individuals, yet with a compromised activity *in vitro* [115].

Differences in the humoral immunity have also been identified in IBD. Both UC and CD patients have an extended infiltration of B cells and plasmocytes [118]. In normal conditions, IgA is the major immunoglobulin in the intestine, however, in active IBD, IgG has a prominent role, being highly induced essentially against intestinal commensals

[100, 119], which illustrates the importance of microbes in the context of this disease. As mentioned earlier, both anti-commensal microbes antibodies and autoantibodies, such as ASCA-IgG and p-ANCA, have also been described in CD and UC, respectively [120, 121].

3. Microbiome

The human gut is home to a variety of microbes, including bacteria, fungi, virus, archaea and microbial eukaryotes. This complex community of microorganisms is defined as microbiota, and the combination of microbes with their genes, proteins and metabolites is called microbiome [122].

The microbiome operates as a secondary organ system with critical functions to the host. In most healthy human beings, the gut microbiome is dominated by bacteria in a relative abundance that varies throughout the GIT, reaching an upper level in the colon of 10^{11} or 10^{12} cells/g of the luminal content [83]. Besides possessing the greatest number of bacteria, the colon also contains the greatest diversity of species [17].

More than 1000 bacterial species inhabit the human GIT, with the most prevalent phyla being Firmicutes and Bacteroidetes [123]. Even between healthy individuals, the percentage of each of these two dominant phyla can be extremely variable, with the combined percentages being around 90%. In disease, larger percentages of other bacterial phyla are often seen, such as Proteobacteria, Verrucomicrobia, Actinobacteria and Fusobacteria [122].

Commensal bacteria and the host have coevolved towards a symbiotic relationship. The host provides a home for the gut microbiota and controls its homeostasis through mucosal immune cells. In turn, bacteria develop important functions that benefit the host, such as secretion of enzymes that digest substrates otherwise inaccessible to the host, and repression of detrimental microorganisms' overgrowth [83, 124].

Resident microbiota is also involved in the development and regulation of the immune system, namely by the production of short-chain fatty acids (SCFAs). Different bacterial families in the large intestine can produce SCFAs as end products of the fermentation of dietary fibres [125]. SCFAs include acetate, propionate and butyrate, and perform two major functions: serve as primary energy source for colonic epithelial cells, and induce the expansion of colonic Treg cells and the production of IL-10 by these (Figure 6) [83]. Bacteria can also influence intestinal Treg cells through a distinct pathway, independent of SCFAs, that involves the induction of TGF- β production by epithelial cells [125].

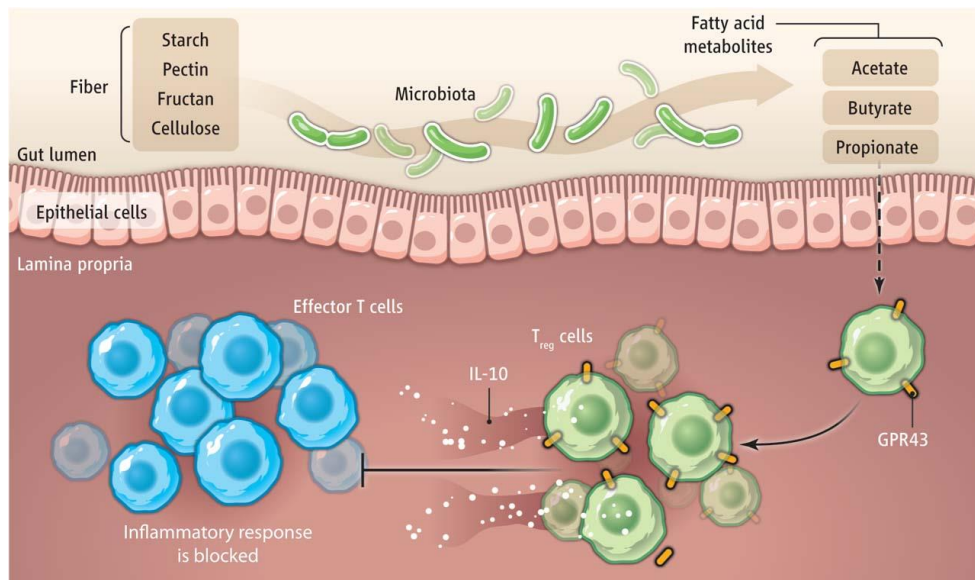


Figure 6| Intestinal anti-inflammatory environment promoted by SCFAs. Commensal microorganisms metabolize indigestible fibres to SCFAs. These are recognized by SCFAs' receptors on Treg cells, as G-protein coupled receptor (GPR)43, promoting their proliferation and IL-10 production [125].

3.1. Dysbiosis in IBD

In a healthy state, a symbiotic relationship is maintained between the host and the lumen-confined microbes. Most microorganisms that colonize human colon live in the outer loose mucus layer, whereas the inner mucus layer acts as a barrier to prevent bacteria from reaching epithelial cells. As mentioned above, there is a defective inner mucus layer in IBD, which leads to an intimate contact between bacteria and the epithelium, triggering severe inflammation [121]. Epithelial hyperpermeability and the presence of breaches in the mucosal barrier, also described in IBD, allow commensal microorganisms to migrate from the lumen to the lamina propria, which could overturn immune tolerance to hyperactivation [16, 126].

Besides the exacerbated immune response towards commensal bacteria, there is a compositional and functional imbalance of the microbiota associated with IBD pathogenesis, termed as dysbiosis [16]. It is not clear if microbiota dysbiosis comes as a cause or consequence of inflammation [127], although it seems likely that both hypotheses are true. Microbial dysbiosis is characterized by population, spatial, or number change in the human body [16]. In IBD, there is indeed a clear decrease in α diversity or species richness, which is a measure of the total number of species in a community [128]. Besides, there is also a decrease in the abundance of bacteria within each species, for instance within the dominant and protection-associated phylum Firmicutes [129]. These large changes in the microbiota composition of IBD patients can occur on time scales of weeks to months [122].

The composition of gut microbiota differs according to the physiological state of the host [121]. Several studies have been done comparing the microbiome of healthy individuals and patients with CD or UC, and variable results were documented. This is reasonable giving the fact that there is huge inter-individual variability even between the same group, with each person harbouring around 160 species and only 30-40 species being shared among individuals [16]. It appears that bacterial communities and not specific bacteria determine susceptibility towards complex diseases, a concept defined as 'the pathobiome' [130].

Compared to healthy individuals, IBD patients present decreased abundances of bacterial taxa within the phylum Firmicutes, and an increase in Proteobacteria and Bacteroidetes bacteria [21, 131]. Moreover, studies revealed that CD patients have decreased levels of the genera *Lactobacillus*, *Bifidobacterium*, and *Faecalibacterium*, which have shown to be protective against mucosal inflammation through various mechanisms, namely the stimulation of anti-inflammatory cytokines, such as IL-10, and the downregulation of inflammatory cytokines. Other genera such as *Clostridium* and *Bacteroides*, which are capable of inducing Treg cells and mitigate intestinal inflammation, are also decreased in IBD. As mentioned earlier, SCFAs have important roles in the gut homeostasis, and therefore it is of no surprise that a decrease in SCFAs-producing bacteria is related to disease. Indeed, *Faecalibacterium*, *Phascolarctobacterium* and *Roseburia* are reduced in CD, while *Leuconostocaceae* is depleted in UC [83].

High levels of mucosa-associated bacteria were reported in IBD patients, suggesting their correlation with gut inflammation. For example, *Escherichia coli* (*E.coli*) is abundant in tissue biopsies of CD and UC patients, and adherent-invasive *E.coli* (AIEC) in ileal CD lesions. In addition, a high amount of *Bacteroides fragilis* and *Enterococcus* strains with adherent and biofilm-forming abilities were found in the mucosa of patients with IBD. Therefore, mucosa-associated bacteria are correlated to gut inflammation [132].

4. Glycobiology

Living systems are composed by four types of organic molecules: proteins, nucleic acids, lipids and carbohydrates. Carbohydrates, also called saccharides or glycans, are the most abundant class, with nearly all organisms synthesizing and metabolizing them [133]. The study of the structure, biosynthesis, biology, and evolution of saccharides and of the proteins that recognize them is defined as glycobiology [134].

Monosaccharides (galactose (Gal), glucose (Glc), mannose (Man), fucose (Fuc), xylose (Xyl), *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), glucuronic acid (GlcA), iduronic acid (IdoA) and sialic acids (Sia)) can form free structures, but typically they are attached to lipid or protein molecules, in an enzymatic process defined as glycosylation [135]. This attachment is done in branched and linear patterns with α or β -glycosidic linkages (when the two carbons have the same or different stereochemistry, respectively), leading to the formation of a complete repertoire of glycoconjugates denominated glycome [22, 136]. Interestingly, glycans are not encoded in our genome but, in vertebrates, glycosylation is regulated by more than 700 genes, called glycogenes. Glycogenes code for several enzymes, such as glycosyltransferases and glycosidases, which are responsible for the addition and removal of sugars, respectively [22, 133].

Cell surfaces are essentially all covered by glycans that form a dense and complex coat termed glycocalyx [137]. Glycocalyx is involved in many biological important functions, including normal embryonic development, differentiation and growth, contact inhibition, cell–cell recognition, cell signalling, host–pathogen interaction during infection, host immune response, disease development, metastasis, intracellular trafficking and localization, rate of degradation and membrane rigidity, which points out the importance of glycosylation [133].

4.1. Protein Glycosylation

The addition of glycans to proteins, forming glycoproteins, is a post-translational modification that alters not only protein structure but also function. Glycans regulate protein stability and oligomerization, and serve as ligands for glycan-binding proteins (GBPs), including antibodies and lectins [22].

Depending on the glycan-protein linkage, two major classes of glycoproteins exist: *O*- and *N*-glycans.

O-glycosylation is catalysed mainly in the Golgi apparatus, where the synthesis of *O*-glycans begins by the action of polypeptide GalNAc-transferase (GalNAc-T). This enzyme adds a GalNAc to the oxygen atom of serine (Ser) or threonine (Thr) that are

not inserted in any conserved sequon, and, posteriorly, several sugar residues can be added progressively by specific enzymes, during the passage through Golgi, creating a great variety of glycans [22].

N-glycosylation occurs in the endoplasmic reticulum (ER) and the Golgi, and begins with the addition of a 14-sugar residues precursor to the nitrogen of an asparagine (Asn) on the polypeptide consensus sequence Asn-X-Ser/Thr, in which X denotes for any aminoacid except for proline. The glycoprotein formed suffers several modifications afterwards, leading to the formation of three classes of *N*-linked oligosaccharides: high-mannose, hybrid and complex (Figure 7) [133, 138, 139].

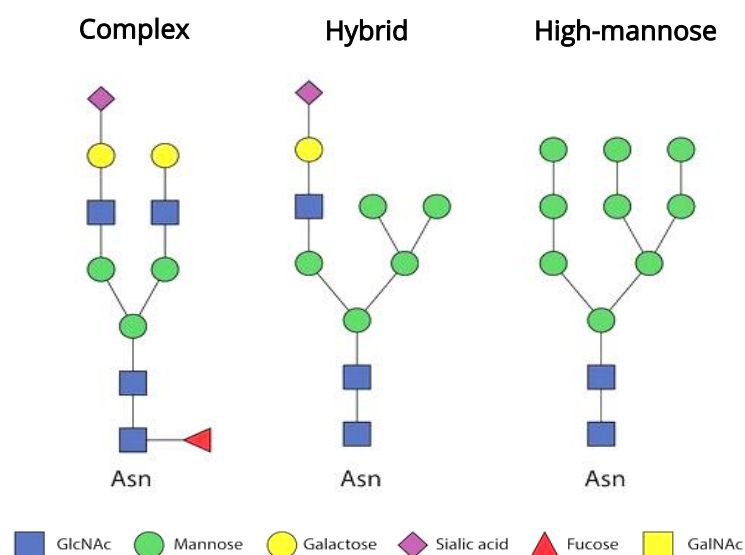


Figure 7] Classes of *N*-glycans. *N*-glycans share a common Man3GlcNAc2 core attached to an Asn-X-Ser/Thr sequence. According to their upper saccharide composition, they can be classified as complex, hybrid or high-mannose. Complex *N*-glycans are composed by different monosaccharides within their two antennae. Hybrid, on the other hand, are enriched in mannose in one antenna, and are similar to complex *N*-glycans in the other. High-mannose *N*-glycans are overall enriched in mannose. Adapted from [138].

4.1.1. *N*-Glycosylation

N-linked glycans formation is initiated by the construction of a precursor constituted by 3 Glc, 9 Man and 2 GlcNAc linked to the lipid carrier dolichol phosphate (Dol-P) in the ER. Later, in a co-translational manner, the precursor is transferred *en bloc* by oligosaccharyltransferase (OST) to an Asn on a Asn-X-Ser/Thr sequence [133]. Afterwards, the 3 Glc are removed by α -glucosidase, creating a high-mannose structure.

N-linked glycosylation is required for the proper folding of some eukaryotic proteins in the ER. Once the folding is assured, the glycoprotein is exported from the ER to the

Golgi apparatus. Firstly, in *cis*-Golgi, the glycoprotein is processed, with 3 Man being removed by α 1,2-mannosidase I (α Man-I). In *medial*-Golgi, biosynthesis of hybrid and complex *N*-glycans is initiated by *N*-acetylglucosaminyltransferase I (GnT-I). GnT I adds a GlcNAc in a β 1,2-linkage, forming the first antenna of the glycan, and giving rise to a hybrid structure [140]. GlcNAc derives from its precursor UDP-GlcNAc formed in the hexosamine pathway - a branch of glycolysis responsible for the production of this key substrate for protein glycosylation [141].

Subsequently, α 1,2-mannosidase II removes terminal α 1,3-Man and α 1,6-Man residues, in the majority of *N*-glycans, allowing GnT-II to add a GlcNAc in a β 1,2-linkage, forming a precursor to all biantennary, complex *N*-glycans. Branching can be initiated by other GnTs, which add GlcNAc to glycans in diverse linkages. For instance, GnT-V, encoded by *α 1,6-mannosylglycoprotein 6- β -N-acetylglucosaminyltransferase (MGAT5)* gene, forms β 1,6-GlcNAc branched *N*-glycans [140].

Maturation of complex *N*-glycans takes place in *trans*-Golgi, with the addition of sugars to the core, elongation of branching GlcNAc residues and capping of elongated branches. Frequently, antennae of complex and hybrid *N*-glycans are elongated with poly-*N*-acetylglucosamine (polyLacNAc), which is a tandem repeat of GlcNAc and Gal. On the other hand, capping usually involves the addition of Sia, Fuc, Gal, GlcNAc and sulfate to the branches, in an α -linkage, opposing to the β -linkage of polyLacNAc branches, thus facilitating recognition by lectins and antibodies (Figure 8) [140, 142].

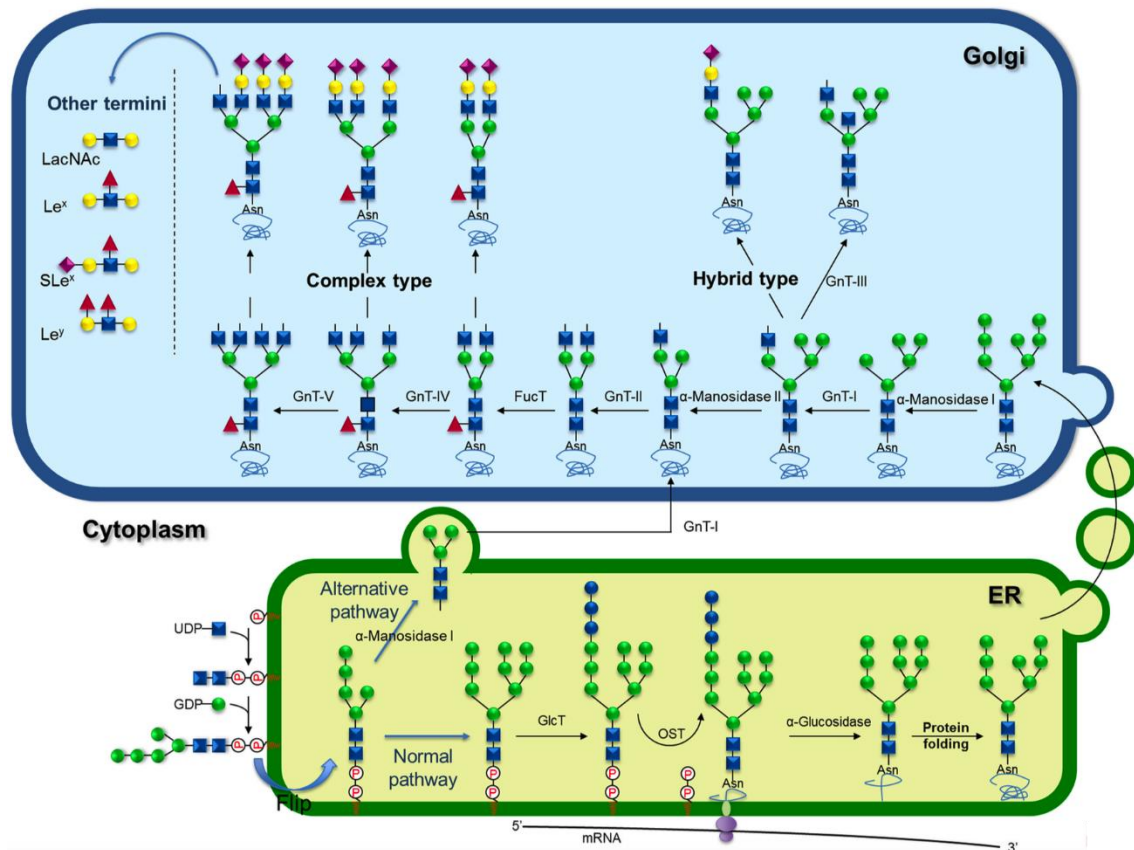


Figure 8| Synthesis of N-glycans. The synthesis of N-glycans begins with the OST-mediated *en bloc* addition of a 14-sugar glycan precursor to an Asn, in the ER. After protein folding, the glycoprotein is transferred to Golgi, where by the action of glycosidases and glycosyltransferases three groups of N-glycans will be formed: high-mannose, hybrid or complex glycans. Adapted from [142].

4.2. Lectins

Lectins are proteins that recognize and bind carbohydrates in glycolipids or glycoproteins, or even to free-floating glycans, including monosaccharides [22, 133]. Lectins can be divided into classes based on their amino acid sequences and biochemical properties [143]. C-type lectins (CTLs) are the most abundant family of GBPs and depend on calcium for their function. In general, CTLs are large, exist in either secreted or bound forms, and can be subdivided into selectins, collectins and endocytic lectins. S-type lectins, also known as galectins, are generally small, non-glycosylated, soluble calcium-independent GBPs that appear both extra and intracellularly [144]. Other less common lectins can be distinguished, such as L-, P- and I-type lectins.

The interaction of lectins with particular carbohydrates is very specific, similar to the interaction between antigen and antibody, or substrate and enzyme [22]. This allows the usage of lectins to detect the present of specific *N*-linked carbohydrate structures, namely *Phaseolus vulgaris* Leucoagglutinin (L-PHA) for β 1,6-GlcNAc branches of tri- and tetra-antennary oligosaccharides, and *Sambucus nigra* agglutinin (SNA) for α 2,6-sialic acid attached to terminal galactose [145-147]. For high-mannose *N*-glycans detection, *Galanthus nivalis* agglutinin (GNA) is commonly used, binding specifically to α 1,3-linked terminal mannoses (Figure 9) [148, 149], as well as dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) which recognizes *N*-linked high-mannose and fucose-containing glycans [150].

Endogenous lectins are involved in numerous biological processes such as cell–cell self-recognition, cell–extracellular matrix (ECM) interactions, cell growth, differentiation, signalling, adhesion and migration, apoptosis, immunomodulation and inflammation, host–pathogen interactions, glycoprotein folding, and homeostasis [133].

4.3. Glycosylation in IBD

Alterations of the glycosylation pattern have been linked to various diseases, including IBD [121]. The mucus layer covering the GIT consists of large, glycosylated mucin proteins that can be either transmembrane or secretory. Transmembrane mucins are involved in cell-surface signalling and sensing, while secretory mucins have the capacity to bind water molecules through their glycans, forming a gel that serves to lubricate and protect the GIT. Mucin 2 (MUC2) is the main secretory mucin in the colon, and it is highly *O*-glycosylated, which confers protection from digestive proteases. It has been reported that patients with active UC have reduced mucus carbohydrate content, comparing with healthy controls and patients with quiescent disease. Also, it was observed that the strongest alterations in MUC2 *O*-glycosylation occur in patients with the most severe phenotypes. Changes in intracellular *O*-glycosylation also appear in IBD patients, namely a reduction in intracellular *O*-GlcNAcylation in the intestinal epithelia. Notwithstanding, in remission, the glycan pattern reverts to normal, which shows that decreases in glycosylation in IBD are related to inflammation [151]. The simplified *O*-

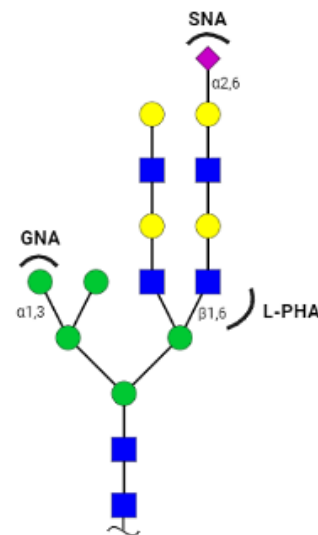


Figure 9| Lectins binding sites.

Lectins bind specifically to different carbohydrates. SNA binds to terminal α 2,6-sialic acid, GNA to terminal α 1,3-mannose, and L-PHA to β 1,6-GlcNAc branched *N*-glycans. Adapted from [4, 139, 145].

glycome associated with IBD, characterized by increased levels of the truncated O-glycans [22], reduces the carbohydrate content present which may increase the contact of bacteria with colonic epithelium, triggering inflammation. On the other hand, the reverse could also be true, with a defective inner mucus layer being a consequence of inflammation [121].

Alterations regarding N-glycosylation are also described in the context of IBD. Indeed, a decrease in hybrid and high-mannose structures, a decrease in fucosylation and galactosylation, and an increase in sialylation were observed in plasma proteins from IBD patients [152].

Sites of glycosylation in IgG fragment crystallizable (Fc) domain determine if a pro-inflammatory or an anti-inflammatory response is established. To initiate an anti-inflammatory activity, the Fc region of IgG must be galactosylated. This process is decreased in IBD, which possibly contributes to the pathogenesis of the disease. Interestingly, the extent of IgG galactosylation could be used as a novel diagnostic and prognostic marker for UC and CD, with higher specificity and sensitivity than ASCA, a current serological marker for CD. The degree of GalNAc in the O-linked oligosaccharides of IgA also serves as a way to diagnose CD, since IgA N-acetylgalactosylation is lower in CD than in UC and controls [121].

Glycans are intimately related to T cells by regulating thymocyte selection, T cell development, activation, signalling, differentiation and proliferation. Therefore, a dysregulation in glycosylation of these cells may lead to an undesirable response [2]. Major forces were made towards T cells glycosylation research, unveiling that in IBD there is a decrease in branched N-glycans (via *MGAT5* downregulation) and an increase in core fucosylation (due to enhanced *FUT8* expression), which increases T-cell immune response [21, 137].

4.3.1. The impact of *MGAT5* alterations in immune responses

As earlier mentioned, GnT-V is a glycosyltransferase encoded by the *MGAT5* gene that catalyses the synthesis of β 1,6-GlcNAc branched N-glycans structures. It has been demonstrated that lamina propria T cells from UC patients display decreased branched N-glycans in TCR, due to reduced *MGAT5* expression. This glycosylation defect hinders the linkage of specific GBPs such as Galectin-3 to elongated chains of the branched N-glycans, a crucial interaction to preclude the TCR clustering and thus control the threshold of T cell activation [21]. Besides increasing T cell activation, deficiency of branched N-glycans also increases Th1 differentiation, with more pro-inflammatory cytokines being produced. Ultimately, an hyperimmune response is established, which

is in accordance with the evidence that the variability in branched *N*-glycans expression on T cells correlates with disease severity [5].

Low transcription levels of *MGAT5* in colonic and circulating T cells from patients with UC may occur due to *MGAT5* variants. *MGAT5* single-nucleotide polymorphisms (SNPs) not only interfere with T cells glycosylation but also with IgG glycome composition, specifically decreasing monogalactosylation of Fc region of IgG1, 2 and 3, leading to a pro-inflammatory effector function of IgG. *MGAT5* genetic variants are of clinical relevance in UC, determining the disease outcome and patients' stratification [6].

Recurrent evidence that shed light on the importance of glycans in several diseases, particularly in IBD, led to the evaluation of branched *N*-glycans as biomarkers in UC [153]. It has been reported that, around time of diagnosis, levels of branched *N*-glycans can predict non-response to conventional therapy with 75% specificity, immediately suggesting that patients will benefit from other therapeutic strategies. Besides, it was also disclosed that high levels of branched *N*-glycans predict 78% of UC patients who will display a favourable disease course; again, facilitating clinician's decision of therapeutics [154].

Context and Main Goal

IBD results from complex interactions between genes, immune response, environmental factors, and microbiota, although the underlying mechanisms are still unclear, hampering the finding of an effective and targeted treatment to this disease. Glycosylation plays a pivotal role in IBD, as glycans are essential determinants in the regulation of immune responses. Despite this evidence, how host glycans modulate the microbiota composition and function and how this impacts the regulation of immune response associated with IBD development is still unclear. Hence, the main goal of this thesis was to unravel the unexplored role of glycans as potential shapers of gut microbiota and immune response in IBD, paving the way for novel therapeutic interventions.

Specific Aims

To tackle our main goal, we aimed to:

- 1) unveil how an altered host glycocalyx modulates gut microbiota and how this impacts the immune response associated with intestinal inflammation.
- 2) assess glycans supplementation as a strategy to create a homeostatic immune environment in the gut: impact in microbiota modulation?
- 3) characterize the host glycosylation profile and immune landscape associated with human IBD pathogenesis.

Chapter II

Methods

Animals

C57BL/6J (WT) mice and *Mgat5*-deficient C57BL/6J (KO) mice were housed in i3S animal facility under specific pathogen-free (SPF) conditions and fed with standard chow diet (2014S, Teklad). To reduce experimental variability, all murine experiments were performed with female animals. Mice were euthanized by CO₂ inhalation using a specific chamber and death was confirmed by cervical dislocation. All procedures were approved by the ethical committee for animal experimentation of i3S following the Portuguese regulations and FELASA guidelines.

Microbiota composition analysis

Stools from WT and KO mice were collected and snap frozen in liquid nitrogen until analysis. Microbiota composition was evaluated by 16S rDNA gene sequencing using Illumina MiSeq platform, as described in detail in [20].

In vivo GlycanC1 supplementation and DSS-induced colitis

WT and KO mice with 5 weeks were treated with GlycanC1 (WT+GlycanC1; KO+GlycanC1), a monosaccharide from the hexosamine pathway, in drinking water *ad libitum* for 5 weeks. Control groups mice received regular unsupplemented water.

Colitis was induced with 2% (wt/vol) dextran sulfate sodium (DSS; MP Biomedicals) *ad libitum* in drinking water for 7 days. Mice were let to recover for 4 days with tap water. Body weight, stool consistency and blood in the stools were examined daily to monitor disease course. These parameters were used to calculate the disease activity index (DAI), used to classify disease severity (Table 1).

Mice were euthanized either before or after colitis induction. Colon and mesenteric lymph nodes (MLNs) were collected in Hanks' Balanced Salt Solution (HBSS; Sigma), for further processing. All mouse procedures were approved by the i3S ethics committee for animal experimentation under Portuguese regulations.

Table 1| Disease activity index used to evaluate mice disease severity. Disease severity was monitored daily by rating from 0 to 4 the weight loss, consistency of faeces and the presence of blood. The presence of blood was assessed by evaluating the cage and using a swab in the anus of the animal.

DAI score	Weight Loss	Consistency of faeces	Blood
0	Normal	WF	Any blood in the swab
1	1-5%	WF/P	Vestiges in the swab + Blood or no blood in the cage
2	6-10%	P	Blood in the swab + Blood or no blood in the cage
3	11-18%	P/L	Blood in the swab + High blood quantity in the cage
4	> 18%	L	Blood in anus at naked eye + Blood in the cage

WF - well formed; **P** - pasty; **L** – liquid.

Isolation of murine lamina propria leukocytes

To isolate lamina propria leukocytes (LPL), colons were flushed with phosphate buffer saline (PBS, pH=7.4), opened longitudinally and cut into small pieces. Then, the tissue was digested with 0.9 mg/mL of collagenase IV (Sigma) in RPMI (RPMI-1640 GlutaMAX; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL Penicillin/Streptomycin (Pen/Strep; Gibco), 1 mM CaCl₂ and 1 mM MgCl₂, during 45 minutes (min) with agitation at 37 °C. Cell suspensions were then filtered in 70 µm cell strainers (SPL Life Sciences) and washed with RPMI. Following, cells were submitted to a Lymphoprep (Alere Technologies) gradient density centrifugation at 900 g, without acceleration or brake, for 30 min, at room temperature (RT). Immune cells were collected in the interphase and epithelial cells in the pellet. After washing once with PBS and centrifuging at 300 g for 10 min in 4 °C, cells were resuspended in PBS with 1% FBS (FACS Buffer), displayed in a 96-well plate (Applied Biosystems® by Life Technologies), and proceeded for flow cytometry staining.

Isolation of leukocytes from murine mesenteric lymph nodes

Mouse MLNs were collected and gently teased between glass slides, washed with cold PBS, followed by a centrifugation at 300 g, for 10 min, at 4 °C. Then, cells from MLNs were resuspended (1x10⁶ cells/well) in PBS and passed through a 70 µm filter to obtain a single cell suspension that was then used for flow cytometry staining.

Colonic tissue culture and cytokine quantification

The distal part of the colon was cultured in RPMI supplemented with 1% Pen/Strep and 0.1% Gentamicin (Gibco) for 24 h. The supernatant of colon explants was collected, centrifuged at 10000 g to remove cell debris and stored at -80 °C until analysis. The tissue was weighed in order to normalize the cytokine levels.

IL-22 and IL-10 were quantified by Enzyme-Linked Immunosorbent Assay (ELISA), using specific kits from R&D Systems®, according to the manufacturer's instructions. After stopping the reaction with 2 N sulfuric acid (H₂SO₄), the absorbance was measured at 450 nm and 570 nm using the Biotek MQX200 uQuant Microplate Reader. Cytokine concentration was determined using a standard curve and the results were normalized to the weight of the explant (pgmL⁻¹ /g of tissue).

Histology

Samples of colons from WT, WT+GlycanC1, KO and KO+GlycanC1 mice were fixed in 4% paraformaldehyde and 3 µm sections were used for haematoxylin and eosin (H&E) staining (n=3 per group) and for lectin histochemistry with L-PHA (n=3 per group).

For lectin, paraffin samples were deparaffinized and hydrated. Endogenous peroxidase was blocked with 3% H₂O₂ (Fisher Scientific) in methanol (Fisher Scientific). Before staining, unspecific proteins were blocked with Carbo-free Blocking Solution (Vector Laboratories). Sections were incubated for 1 h with biotinylated L-PHA diluted in PBS at 1:100 (Vector Laboratories). For detection, the avidin–biotin–peroxidase (ABC) complex from the Vectastain ABC Kit (Vector Laboratories) was used in 1:100 for 30 min, followed by 3,3'-Diaminobenzidine (DAB) substrate from DAB Quanto kit (Thermo Scientific). Sections were counterstained with haematoxylin, dehydrated and mounted.

Human samples

The IBD patients' cohort used in this study was established in collaboration with the Gastroenterology Department of Centro Hospitalar do Porto-Hospital de Santo António (CHP/HSA) and comprised 15 IBD patients, 12 with CD and 3 with UC. The median age of patients was 43 years old (interquartile range = 18). From the cohort, 6 fresh biopsies from inflamed mucosa and 12 blood samples were collected. Detailed clinical information was provided by CHP/HSA (Table 2). As controls, 3 biopsies from healthy individuals (healthy controls – HC; submitted to planned colonoscopy, without

IBD or cancer; non-inflamed mucosa) and blood samples from 6 healthy individuals were gathered.

All participants gave informed consent about all clinical procedures and research protocols were approved by the ethics committee of CHP/HSA.

Table 2| Characterization of the cohort of IBD patients involved in this study. Data regarding disease categorization (UC or CD), gender, disease status (activity or remission) and use of biologics (naive vs non-naive) was gathered for future integrated analysis.

		<i>N</i>
Disease	UC	3
	CD	12
Gender	F	67%
	M	33%
Disease status	Activity	53%
	Remission	47%
Biologics	naive	33%
	non-naive	67%

Isolation of human PBMCs

Peripheral blood mononuclear cells (PBMCs) from whole blood of HC and IBD patients were isolated by a density gradient centrifugation using Lymphoprep™. Briefly, 2 volumes of blood were added on top of 1 volume of Lymphoprep™ and centrifuged for 30 min at 900 g without acceleration or brake, at RT. PBMCs were aspirated from the interphase between plasma and Lymphoprep, washed with PBS, and centrifuged for 10 min at 300 g, RT. The pellet was resuspended with 90% FBS and 10% dimethyl sulfoxide (DMSO, PanReac AppliChem), and placed in a Corning CoolCell™ freezing container (to freeze slowly) at -80 °C.

Human PBMCs defrosting

Frozen PBMCs were quickly thawed and washed with PBS with FACS Buffer. Following, PBMCs were centrifuged for 8 min at 300 g, RT, and resuspended in RPMI medium supplemented with 10% FBS and 1% Pen/Strep. Then, cells recovered in culture at 37 °C in 5% CO₂ for 2 hours (h). After this period, PBMCs were collected and resuspended in supplemented RPMI, for further flow cytometry staining.

Isolation of lamina propria leukocytes from human fresh colonic biopsies

After mechanical dissociation in HBSS with 1% Pen/Strep and 0.1% Gentamycin, human fresh colonic biopsies were digested with 0.9 mg/mL of collagenase IV in RPMI supplemented with 10% FBS, 100 U/mL Pen/Strep, 1 mM CaCl₂ and 1 mM MgCl₂, for 45 min with agitation, at 37 °C. Cell suspensions were then filtered in 70 µm cell strainers and washed with PBS. Following, cells were centrifuged for 10 min at 300 g and 4 °C, and the pellet was resuspended in FACS buffer and ready to proceed for flow cytometry staining.

Flow cytometry

Human and murine cells were plated in a round-bottom 96-well plate and stained with a fixable viability dye (FVD) for 30 min at 4 °C. Cells were kept in the dark until analysis. Afterwards, they were washed with FACS Buffer and centrifuged at 2000 rpm for 1 min, which was always repeated between staining steps. Lectin staining was performed for 15 min at 4 °C. Lectins used were *Phaseolus vulgaris* Leucoagglutinin (L-PHA), *Galanthus nivalis* agglutinin (GNA) and *Sambucus Nigra* agglutinin (SNA), that specifically recognise β 1,6-GlcNAc branched *N*-glycans, terminal α 1,3-linked mannose, and α 2,6-sialic acid preferably linked to *N*-glycans, respectively. Later, cells were incubated with surface antibodies, streptavidin, or secondary antibodies for 30 min at 4 °C. Wells that did not require intracellular staining were fixed with formaldehyde 2% (diluted in PBS) for 10 min at RT, centrifuged, resuspended in FACS Buffer and stored at 4°C until analysis. For intracellular staining, cells were previously cultured with Phorbol 12-myristate 13-acetate (PMA; 20 ng/ml), Ionomycin (200 ng/ml) and Brefeldin A (BfdA; 10 ng/ml) in RPMI-1640 with 10% FBS and 1% Pen-Strep, for 4 h at 37 °C, before starting the staining procedure. Cells were collected after stimulation and then stained as described above, followed by fixation with Fix-Perm (30 min at RT) and permeabilization with Perm Buffer (20 min at RT), using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience™). After permeabilization, cells were incubated with intracellular antibodies diluted in Perm Buffer for 30 min at RT. Finally, these cells were washed with Perm Buffer and resuspended in FACS Buffer.

Cell analysis was performed on a FACSCanto II (BD Biosciences, US) and collected with BD FACSDiva™ v8.0.3 Software (BD Biosciences, US). For proper definition of cell populations, fluorescence minus one (FMO) stainings were performed. Data were analysed using FlowJo software (Tree Star, USA). Gating strategies are

presented in Supplementary Figures 1 and 2. Populations were analysed according to their frequency and the median fluorescence intensity (MFI; Supplementary Figures 3 and 4). All antibodies and lectins used are compiled in Table 3.

Table 3| Lectins and antibodies used in flow cytometry analyses.

<i>Species</i>	<i>Antibodies/ Lectins</i>	<i>Conjugated Fluorochrome</i>	<i>Dilution</i>	<i>Clone</i>	<i>Company</i>
Human/Mouse	FVD	APC-Cy7	1:2000	-	eBioscience
Mouse	CD45	APC	1:400	30-F11	eBioscience
		PE-Cy7	1:400	30-F11	eBioscience
Mouse	CD3	AmCyan	1:100	145-2C11	Biolegend
Mouse	CD19	AmCyan	1:300	1D3	eBioscience
Mouse	CD11c	AmCyan	1:100	N418	eBioscience
Mouse	CD11b	AmCyan	1:400	M1/70	eBioscience
Mouse	Thy1.2	PB	1:300	53-2.1	eBioscience
Mouse	TCR $\gamma\delta$	PB	1:100	GL3	eBioscience
Human/ Mouse	ROR γ t	Pe-Cy7	1:100	AFKJS-9	eBioscience
Mouse	IL-17	PE	1:100	17B7	eBioscience
Mouse	IL-22	PerCP-Cy5.5	1:100	1H8PWSR	eBioscience
Mouse	GM-CSF	FITC	1:100	MP1-22E9	Biolegend
Mouse	CD4	Pacific Blue	1:300	RM4-5	eBioscience
Mouse	CD25	PerCP-Cy5	1:400	PC61.5	eBioscience
Mouse	Foxp3	APC	1:200	FJK-16s	eBioscience
Mouse	IL-10	PE	1:100	554467	BD Pharmingen
Human/Mouse	L-PHA	FITC	1:1000	-	Vector Laboratories
Human/Mouse	SNA	APC	1:1000	-	Vector Laboratories
Human/Mouse	GNA	Biotinylated	1:1000	-	Vector Laboratories
Mouse	mDectin-2-hFc	PE	1:200	-	Kindly provided by Bernd Lepenies
Human/Mouse	Streptavidin	PE	1:200	-	Biolegend
		PE-Cy7	1:200	-	eBioscience
Human	CD45	Brilliant Violet 510	1:100	HI30	Biolegend
Human	CD3	PE	1:100	OKT3	Biolegend
		PB	1:100	OKT3	Biolegend
Human	NKp44	PerCP-Cy5.5	1:100	P44-8	Biolegend

Human	CD4	eFluor450	1:200	RPA-T4	eBioscience
Human	CD25	PerCP-Cy5.5	1:100	BC96	BioLegend
Human	CD56	PE	1:100	HCD56	BioLegend
Human	CD11c	APC	1:100	BU15	eBioscience
Human	CD206	PE-Cy7	1:100	15--2	BioLegend
Human	CD209 (DC-SIGN) - IgG	-	1:100	Polyclonal	Bio-Rad
-	Secondary antibody: swine anti-rabbit IgG	FITC	1:75	-	Dako
Human	FOXP3	PE	1:100	PCH101	eBioscience
Human	RORyt	APC	1:100	AFKJS-9	eBioscience
Human	IL-17A	FITC	1:75	BL168	BioLegend
Human	IL-22	PE-Cy7	1:100	2G12A41	BioLegend
Human	GM-CSF	PB	1:100	BVD2-21C11	BioLegend

APC: Allophycocyanin; Cy: Cyanine; FITC: Fluorescein Isothiocyanate; PB: Pacific Blue; PE: Phycoerythrin; PerCP: Peridinin Chlorophyll Protein.

RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA from mouse and human colonic samples was extracted using RNAqueous-Micro Kit (Invitrogen) according to the manufacturer's protocol. Total RNA was quantified using the Nanodrop system, and 500 ng of RNA were retrotranscribed to cDNA using Superscript IV Reverse Transcriptase (Invitrogen), according to the instructions of the kit.

The quantitative real-time PCR (qRT-PCR) was performed in 96-well reaction plates. cDNA was amplified using LightCycler® 480 SYBR Green I Master (Roche) using specific oligonucleotides (Table 4). Alternatively, cDNA was amplified using Taqman Universal PCR Master Mix (Applied Biosystems) and Taqman probes (Table 5). Amplification data were acquired with 7500 Fast Real-Time PCR System (Applied Biosystems). *Glyceraldehyde 3-phosphate dehydrogenase (Gapdh)* or *18S* were used as housekeeping genes. Relative quantification (RQ) values for gene expression were calculated based on the ΔC_t method, as follows: $2^{-(\text{Target gene mRNA expression} - \text{housekeeping gene mRNA expression})}$.

Table 4| Mouse oligonucleotides used in quantitative real-time PCR.

Primers	Forward Sequence	Reverse Sequence
<i>Fut2</i>	AGGCGGTTCAAATGTCCTCA	CGTTGTGCATGGATTGAGGG
<i>Il22</i>	TCCAGCAGCCATACATCGTC	CCTCGGAACAGTTTCTCCCC
<i>Il22ra2</i>	TCAGCAGCAAAGACAGAAGAAAC	GTGTCTCCAGCCCAACTCTCA
<i>Il23</i>	AGCGGGACATATGAATCTACTAAGAGA	GTCCTAGTAGGGAGGTGTGAAGTT
<i>Nod2</i>	CTGTCCAACAATGGCATC	GTTCCCTCGAAGCCAAACCT
<i>Card9</i>	CTCACTGCCTCAGGATCTGG	CCCTGTCTGCCAGTACACCT
<i>Reg3g</i>	TCAGGTGCAAGGTGAAGTTG	GGCCACTGTTACCACTGCTT
<i>Gpr43</i>	AATTCCTGGTGTGCTTTGG	ACCAGACCAACTTCTGGGTG
<i>Ocln</i>	GCTGTGATGTGTGTTGAGCT	GACGGTCTACCTGGAGGAAC
<i>Cldn1</i>	GGCTTCTCTGGGATGGATCG	CCCCAGCAGGATGCCAATTA
<i>Cldn2</i>	TTTTGGGGCTGTTAGGCACA	AGAATCCTGGCAGAACACGG
<i>Cldn4</i>	ACGTCATCCGCGACTTCTAC	TTGTCGTTGCTACGAGGTGG
<i>Gapdh</i>	GAAGGTCGGTGTGAACGGAT	CTCGCTCCTGGAAGATGGTG

Table 5| TaqMan probes used in quantitative real-time PCR.

Species	TaqMan assays	Reference	Company
Mouse	<i>Mgat5b</i>	Mm01252571	Applied Biosystems
Human	<i>MGAT5</i>	Hs00159136	Applied Biosystems
Human	<i>FUT2</i>	Hs00382834	Applied Biosystems
Human	<i>FUT8</i>	Hs00189535	Applied Biosystems
Human	<i>CD209</i>	Hs01588349	Applied Biosystems
Human	<i>CD3E</i>	Hs0162241	Applied Biosystems
Human/Mouse	<i>18S</i>	Hs.PT.39a.22214856.g	Integrated DNA Technologies

Statistical Analysis

All statistical analyses were executed using GraphPad Prism 7 Software. The presence of outliers was evaluated using ROUT method (Q=1%). Normality was assessed by performing D'Agostino-Pearson and Shapiro-Wilk normality tests. For statistical analyses unpaired t-test or Mann-Whitney test were used, according to normality. For DAI analysis, two-way ANOVA was used. Characterization of immune populations in mice colon involved pools of two animals and thus the total sample size was considered for analysis.

Data obtained were expressed as mean $\pm$ standard deviation (SD). Statistical significance was considered at $p \leq 0.05$ and statistically significant values were represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

Chapter III

Results

I. Glycans as modulators of intestinal environment and their impact in homeostasis and disease

***Mgat5*-deficient mice exhibit a dysbiotic microbiota composition and higher susceptibility to develop severe colitis**

Previous reports from our group have disclosed that mucosal T lymphocytes from UC patients harbour a deficiency in β 1,6-GlcNAc branched *N*-glycans in the TCR due to reduced *MGAT5* expression, leading to T cell hyperactivation [5], and that genetic polymorphisms of *MGAT5* are linked with disease severity in UC [6]. Additionally, the group has shown that *Mgat5*-deficient (KO) mice are associated with early-onset of DSS-induced colitis and severe forms of the disease [4].

To better understand the factors underlying the increased susceptibility displayed by *Mgat5*-KO mice, the gut microbiota composition was assessed by 16S rDNA sequencing. It was observed that mice lacking branched *N*-glycans display a clear dysbiotic microbiota composition at baseline when compared with *Mgat5*-wild-type (WT) mice (Figure 10A). Particularly, KO mice have a decreased abundance of several bacteria belonging to the Firmicutes phylum, which is highly associated with gut protection [155]. Nevertheless, some Firmicutes bacteria also increase in KO mice together with bacteria from the phyla Tenericutes, Bacteroidetes and Actinobacteria (Figure 10A). In this sense, we hypothesised that glycans may act as major shapers of intestinal counterparts, playing a dual role as regulators of microbiota composition and of host immune response. To unveil how glycosylation influences intestinal immune responses, we analyzed the immune profile of lamina propria leukocytes of WT and KO mice at baseline (homeostasis) and after DSS-induced colitis. Colitis was induced by administering DSS 2% in the drinking water for 7 days. Afterwards, mice were let to recover until day 11, receiving water with no treatment (Figure 10B). During this period, disease severity was monitored daily and scored according to the disease activity index (DAI), considering weight loss, stool consistency and the presence of blood. As expected, KO mice developed a more severe disease than WT, with a higher peak of disease, although no differences were seen regarding the onset or recovery (Figure 10C). Accordingly, histological analysis of the colon following the complete procedure (day 11) showed that KO mice display more extensive inflammatory infiltrates in the lamina propria than WT, accompanied with abnormal glandular morphologies (Figure 10D).

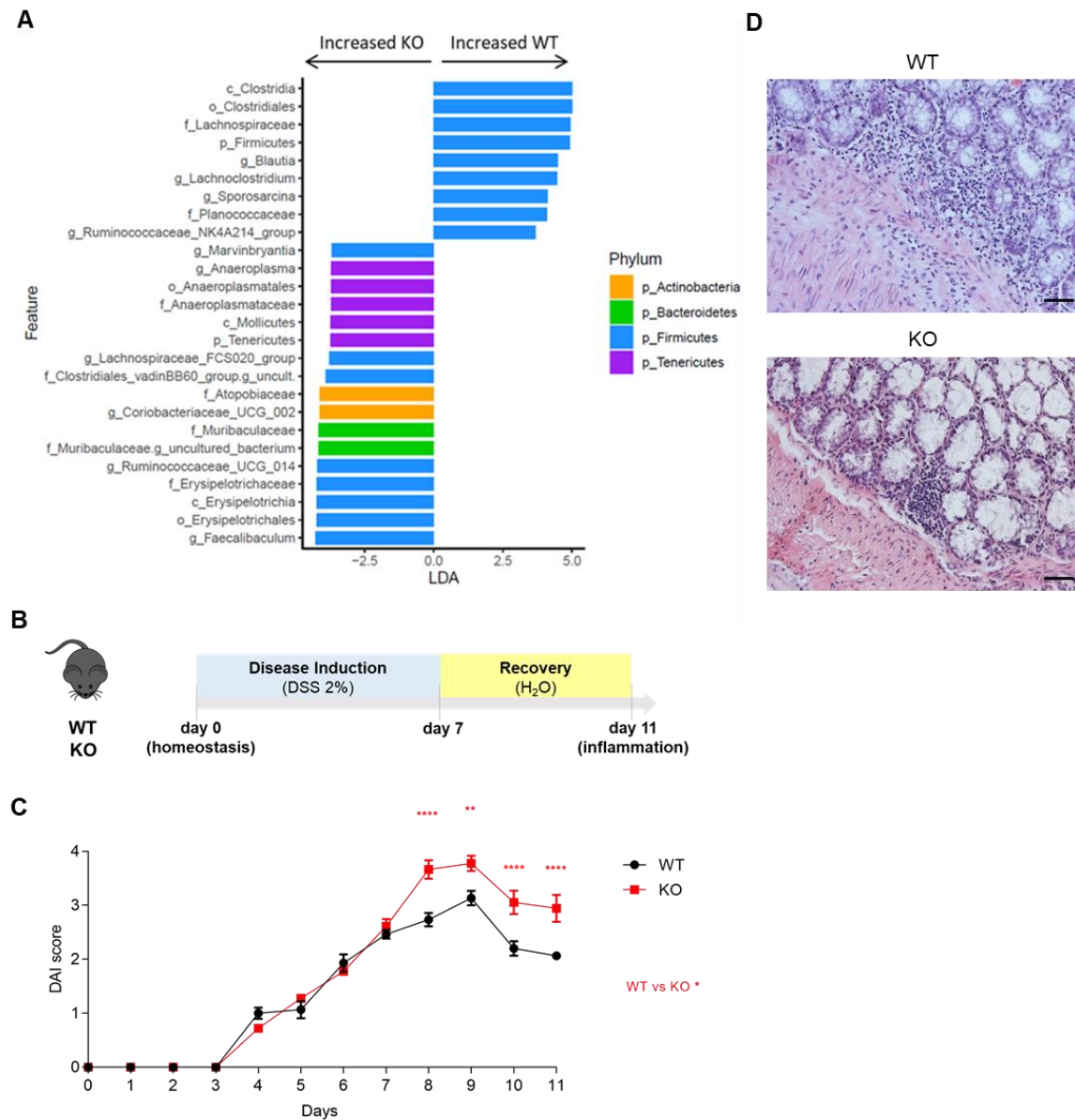


Figure 10| KO mice are more susceptible to DSS-induced colitis. (A) Linear discriminant analysis (LDA) of the microbiota composition in WT and KO mice. Phylum, class, order, family and genus are represented as p, c, o, f and g, respectively. **(B)** C57BL/6 WT and KO mice were induced to colitis using dextran sulfate sodium (DSS) 2% *ad libitum* in drinking water for 7 days. Mice were let to recover for 4 days with tap water. **(C)** Disease progression was assessed by scoring the disease activity index (DAI) throughout the experiment. Data is presented as mean ± standard deviation (SD). Statistically significant values are: **p ≤ 0.01; ****p ≤ 0.0001. **(D)** Histological analysis of haematoxylin & eosin staining of mice after colitis induction (day 11). Scale bar = 50 μm.

KO mice display impaired *Fut2* expression and ILC3/IL-22 dynamics in the gut

In homeostasis (before colitis induction)

To unveil the basal differences between the two genotypes, we performed a characterization of the glycocalyx in the colon of WT and KO mice at homeostatic conditions. The absence of branched *N*-glycans in the epithelium of KO was confirmed by negligible detection of L-PHA binding (Figure 11A). Interestingly, we found that the expression of *Mgat5b*, a homologous gene of *Mgat5* (also called *Mgat5a*) that codes for GnT-Vb enzyme [140], is tendentially increased on overall colonic tissue in KO mice (Figure 11B), probably as a compensatory mechanism to increase branched *N*-glycans, as previously described by us [4]. No alterations were observed between the two genotypes for epithelial α 2,6-sialylation, inferred by SNA binding (Figure 11A).

Since epithelial fucosylation by *Fut2* is well-described to be associated with gut homeostasis [124], we evaluated the expression of its encoding gene on total colonic tissue. Importantly, we found that, in homeostasis, KO mice show a tendency for decreased *Fut2* expression (Figure 11B). Despite not reaching statistical significance, this may be in line with reduced gut protection.

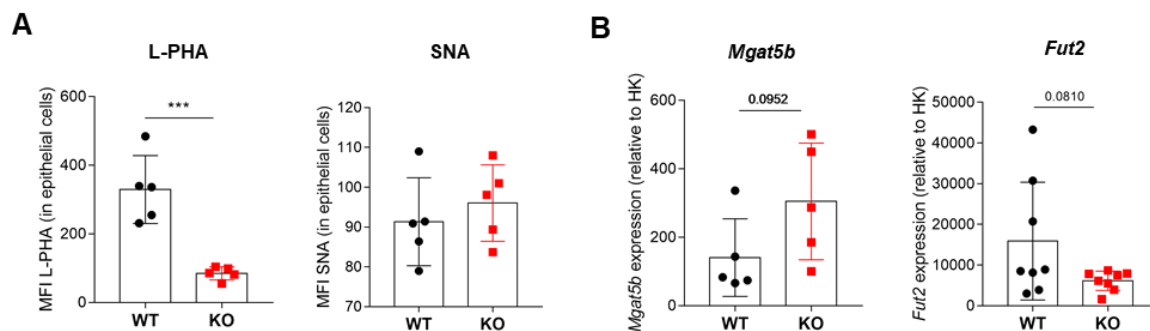


Figure 11| KO mice display an impaired glycoprofile in the colon, in homeostasis. (A) MFI of L-PHA and SNA in epithelial cells of the colon of WT and KO mice. **(B)** *Mgat5b* and *Fut2* mRNA expression levels normalized to *18S* and *Gapdh* expression, respectively, in the colon of WT and KO mice. Each dot represents one individual biological sample, presented as mean ± SD. Statistically significant values are: *** $p \leq 0.001$.

To evaluate how these changes in gut glycocalyx impact the immune signature residing in KO mice, which can be contributing to the increased colitis severity found, we analysed the immune profile in the colon of WT and KO mice by flow cytometry, focusing on cellular populations that are known to play a pivotal role in the gut immune response.

As mentioned in Introduction, ILC3 are tightly associated with intestinal homeostasis, very much owed to their capacity to moderately produce IL-22, GM-CSF, and IL-17A [68]. We observed that, in homeostasis, KO mice display defective ILC3 levels in the colon. Although no differences were seen concerning ILC3 production of IL-

17A, we observed a significant decrease in their capacity to produce IL-22, a cytokine highly associated with gut protection (Figure 12A).

Th17 and $\gamma\delta$ T cells are also important producers of IL-17 and IL-22 in the gut, which can be protective or pathogenic depending on their levels. In homeostasis, KO mice have lower frequencies of Th17 cells, as well as decreased levels of Th17-derived IL-22, with no differences regarding IL-17A (Figure 12B). Concerning $\gamma\delta$ T cells, despite being also decreased in frequency in the colon of KO mice, these cells show an increased production of the pro-inflammatory cytokine IL-17A, with no alterations found on IL-22 (Figure 12C).

Our immune characterization also encompassed Treg cells, due to their importance in controlling inflammation. Although no statistically significant differences were seen for intracellular IL-10 on these cells by flow cytometry analysis, we observed that the frequency of Treg cells is decreased in KO mice (Figure 12Di), which could underlie the exacerbated inflammation in these mice. In fact, cytokine quantification in colonic explants of KO mice has shown decreased levels of IL-10 when compared to WT mice (Figure 12Dii). The immune profile was also evaluated on the mesenteric lymph nodes (MLNs), since these are in closed proximity to the colon and are major pools of immune cells. Nevertheless, no major alterations were seen between the two genotypes in MLNs at baseline (Supplementary Figure 5).

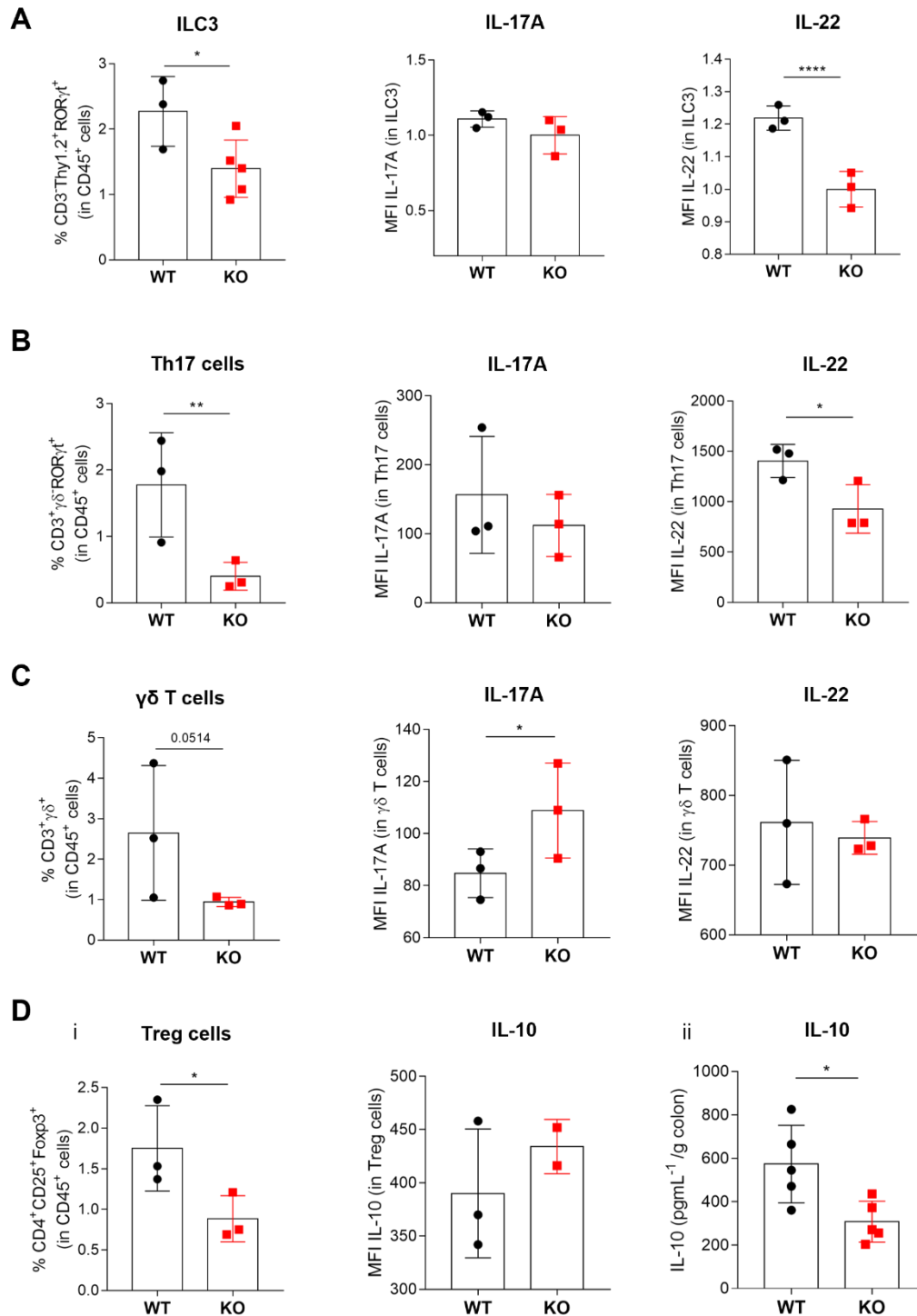


Figure 12| KO mice display impaired ILC3 and IL-22 dynamics in the gut, in homeostasis. Frequencies of **(A)** ILC3, **(B)** Th17 and **(C)** γδ T cells, and MFI of IL-17A and IL-22 inside each population (normalized to KO mice in (A)). **(D)** (i) Frequency of Treg cells and MFI of IL-10 inside these cells. (ii) Concentration of IL-10 (pgmL⁻¹/g colon) in the supernatants of 24 h colonic explants cultures. Each dot represents a pool of two mice, except in (Dii) in which each dot represents one individual biological sample. Data is presented as mean ± SD. Statistically significant values are: * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$.

To further compare the immune environment in WT and KO mice, we evaluated by quantitative PCR (qPCR) the expression of several genes encoding for relevant participants on gut homeostasis. Firstly, we assessed the expression of *Il22* and *interleukin 22 receptor subunit alpha 2* (*Il22ra2*), the genes that encode for IL-22 and IL-22 binding protein (IL-22BP), a soluble inhibitory IL-22 receptor expressed by DCs and T cells [156, 157]. We observed that the expression of these genes is not altered in the colon of KO mice (Figure 13A). However, and in line with the previously shown decreased intracellular protein levels of IL-22 in ILC3 and Th17, we observed that supernatants from KO colon explants have lower amounts of IL-22, although it did not reach statistical significance (Figure 13B). These results suggest that although there are no major differences between WT and KO mice at transcription levels, KO mice display an impaired production of IL-22.

Since IL-23 induces the production of IL-22 by ILC3 [124] and IL-17A by Th17 [158], therefore being able to participate in both protective and pro-inflammatory axes, and GM-CSF (coded by *Csf2*) is an ILC3-derived cytokine involved in homeostasis, we have also analysed the expression of respective genes, but no differences were found within the two groups (Figure 13A).

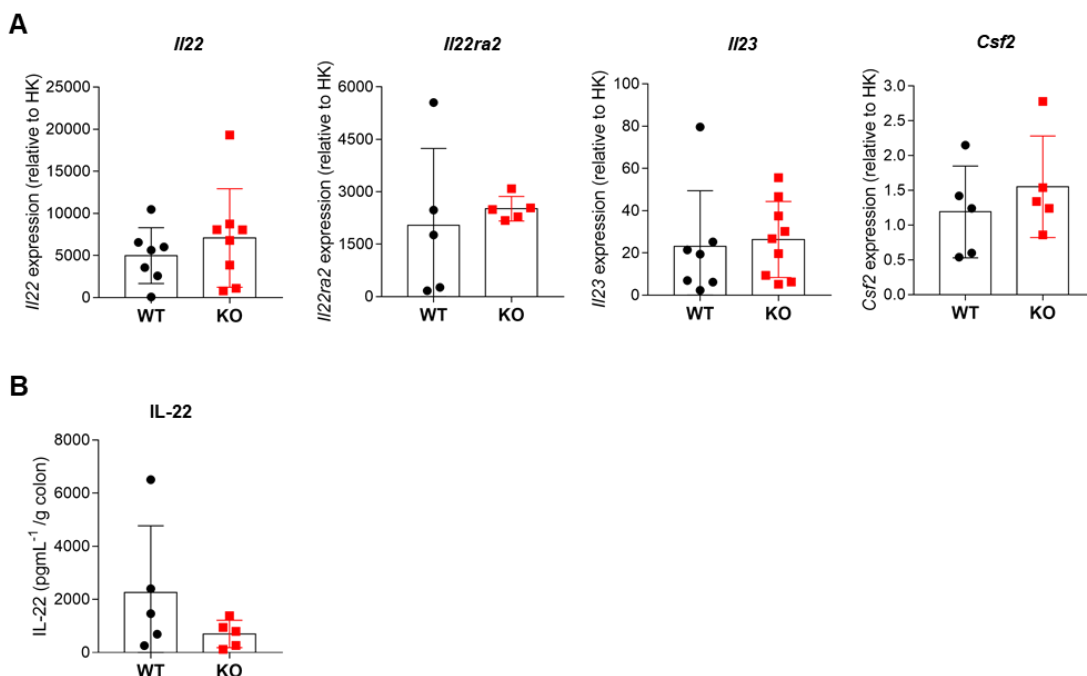


Figure 13| KO mice seem to display impaired IL-22 production in the colon, in homeostasis. (A) *Il22*, *Il22ra2*, *Il23* and *Csf2* mRNA expression levels normalized to *Gapdh* expression in the colon of WT and KO mice. **(B)** Concentration of IL-22 (pgmL⁻¹/g colon) in the supernatants of 24 h colonic explants cultures. Each dot represents one individual biological sample, presented as mean $\pm$ SD. No statistical significance was reached (significant *p*-value ≤ 0.05).

After induction of inflammation

The glycoimmune profile in the colon of WT and KO mice was also assessed upon an inflammatory trigger induced by DSS.

Firstly, we have studied the epithelial glycoprofile by staining CD45⁺ cells with L-PHA, SNA and a Dectin-2-hFc fusion protein, the latter binding to high-mannose glycans [159]. As expected, we observed a lack of branched *N*-glycans in KO mice, however, contrary to what was evidenced before the DSS, upon inflammation the levels of α 2,6-sialic acid are increased in these mice (Figure 14A). This may be detrimental for the host since, as an important shaper of the microbiome [160], an increased availability of sialic acid may promote the overgrowth of deleterious bacteria that utilise it as a carbon source [161, 162]. No differences were seen concerning epithelial Dectin-2-binding motifs (Figure 14A), nor on *Fut2* expression in total colonic tissue (Figure 14B).

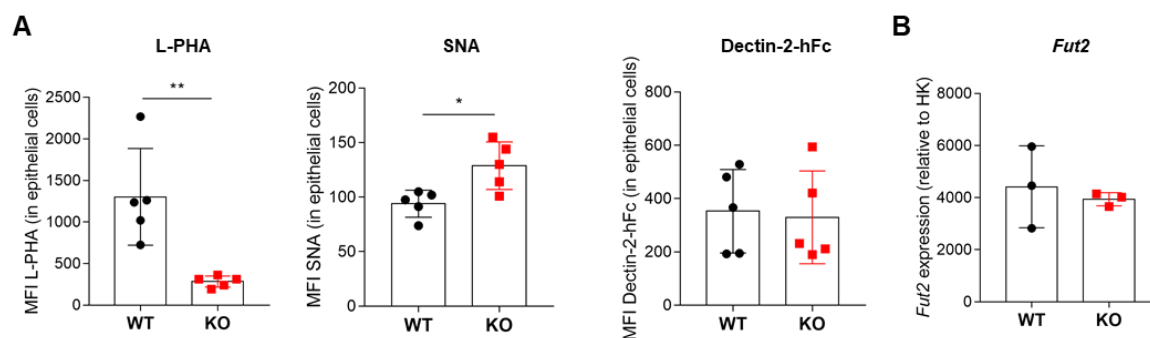


Figure 14| KO mice display an impaired glycoprofile in the colon, upon inflammation. (A) MFI of L-PHA, SNA and Dectin-2-hFc in epithelial cells of the colon of WT and KO mice. **(B)** *Fut2* mRNA expression levels normalized to *Gapdh* expression in the colon of WT and KO mice. Each dot represents one individual biological sample, presented as mean $\pm$ SD. Statistically significant values are: * $p \leq 0.05$; ** $p \leq 0.01$.

Characterization of the immune profile in colonic lamina propria after disease induction revealed that ILC3 levels and IL-22 production by these cells remain compromised in KO mice upon inflammation. Once more, ILC3-production of IL-17A is no different between the two groups (Figure 15A). Nevertheless, ILCs-derived IL-17A is augmented in the MLNs of KO mice (Supplementary Figure 6).

In accordance with the development of a more severe disease and the presence of more extensive inflammatory infiltration at the lamina propria (Figure 10C,D), KO mice reveal increased frequencies of both colonic Th17 and $\gamma\delta$ T cells, and IL-17A production by these, comparing with WT mice (Figure 15B,C). Moreover, MLNs from KO mice show decreased frequencies of Th17 cells (Supplementary Figure 6), which may be explained by a possible recruitment of these cells to the colon. Contrary to Th17, colonic $\gamma\delta$ T cells

also display decreased IL-22 production (Figure 15C), which may be detrimental for gut homeostasis.

We also observed that the lamina propria of KO mice tends to have increased frequencies of Treg cells, probably as a compensatory mechanism to control inflammation, despite these displaying the same intracellular levels of IL-10 as Treg cells from WT mice (Figure 15Di). Yet, cytokine quantification has shown that supernatants from KO colon explants tend to have lower levels of IL-10 (Figure 15Dii), hindering its anti-inflammatory power. In MLNs, Treg cells from KO mice produce more IL-10 than those in WT, which may also occur as an attempt to control exacerbated inflammation within these mice (Supplementary Figure 6).

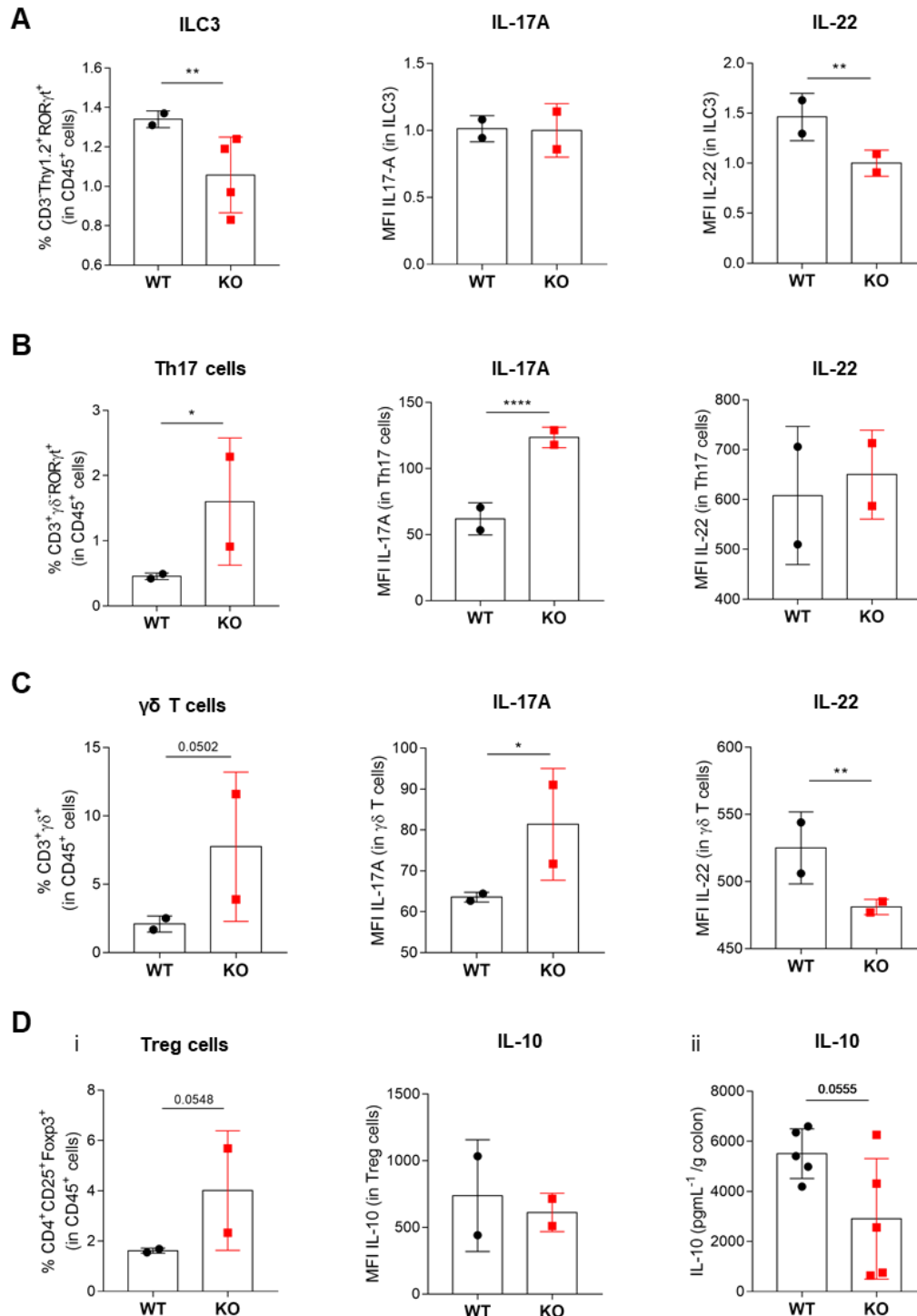


Figure 15| KO mice display impaired ILC3 and IL-22 dynamics in the gut, upon inflammation. Frequencies of **(A)** ILC3, **(B)** Th17 and **(C)** γδ T cells, and MFI of IL-17A and IL-22 inside each population (normalized to KO mice in (A)). **(D)** (i) Frequency of Treg cells and MFI of IL-10 inside these cells. (ii) Concentration of IL-10 (pgmL⁻¹/g colon) in the supernatants of 24 h colonic explants cultures. Each dot represents a pool of two mice, except in (Dii), in which each dot represents one individual biological sample. Data is presented as mean ± SD. Statistically significant values are: * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$.

To further characterize the defective IL-22 production in KO mice, we checked *Il22* expression on colonic tissue and, unexpectedly, observed that, after the inflammatory trigger, the expression of *Il22* is increased in these mice (Figure 16A). However, this may occur as a compensatory mechanism for the concomitant increased expression of the gene encoding for IL-22BP (Figure 16A), which impairs the availability of IL-22 to exert its protective function. Indeed, IL-22 levels on supernatants from KO mice colonic explants are significantly decreased (Figure 16B).

Along with flow cytometry analysis showing increased Th17-derived IL-17, *Il23* expression tends to increase in the KO group (Figure 16A), opening the possibility that in these mice IL-23 is participating in the IL-23/Th17 axis, promoting the production of IL-17A by Th17 and exacerbating inflammation.

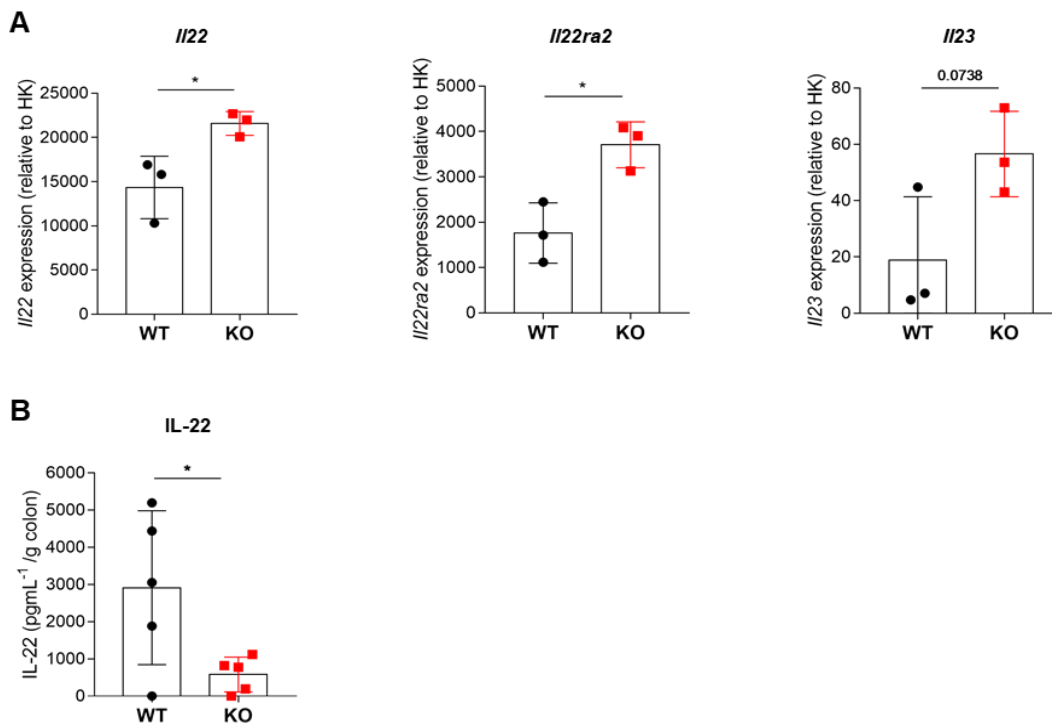


Figure 16| KO mice display impaired IL-22 dynamics in the colon, upon inflammation. (A) *Il22*, *Il22ra2*, and *Il23* mRNA expression levels normalized to *Gapdh* expression in the colon of WT and KO mice. **(B)** Concentration of IL-22 (pgmL⁻¹ /g colon) in the supernatants of 24 h colonic explants cultures. Each dot represents one individual biological sample, presented as mean $\pm$ SD. Statistically significant values are: * $p \leq 0.05$.

Overall, these results highlight that a deficiency in the enzyme responsible for the synthesis of β 1,6-GlcNAc branched *N*-glycans imposes a dysbiotic microbiota composition and a compromised immunological environment in the gut, with emphasis on the impairment of ILC3 and IL-22 dynamics, which was evident before and after the DSS treatment.

GlycanC1 supplementation ameliorates disease severity in KO and WT mice

As mentioned above, a dysbiotic microbiota composition is found in KO mice at baseline (Figure 10A). Since the outcome of the genetic difference between WT and KO mice is the absence of β 1,6-GlcNAc branched *N*-glycans within the latter, and host and dietary glycans have a crucial role in shaping the composition and physiology of the microbiota and in regulating immune responses [163], it was raised the hypothesis that the deficiency in branched *N*-glycans was at the basis of dysbiosis and immune dysregulation. Moreover, metabolic analysis previously done by our group have shown that KO mice are particularly depleted in bacteria capable to synthesise saccharides from the hexosamine pathway (manuscript in preparation). In this sense, we wanted to explore whether the supplementation of KO mice with a monosaccharide from this pathway that is redundantly used in branched *N*-glycosylation could overcome their baseline deficiency and, ultimately, serve as a strategy to restore beneficial microbiota and create a homeostatic immune environment in the gut. This monosaccharide was designated as GlycanC1, and it was added in the drinking water for 5 weeks, a period sufficient to modulate the microbiota, not only to KO (KO+GlycanC1) but also to WT (WT+GlycanC1) mice, to additionally answer if GlycanC1 has the potential to be beneficial to mice without glycan deficiency at baseline. Later, we induced colitis with DSS 2% in drinking water for 7 days, allowing recovery until day 11 (Figure 17A), as previously done.

We found that GlycanC1 supplementation has a protective effect against DSS-induced colitis in both WT and KO mice, with mice treated with GlycanC1 displaying an improved disease course when compared to non-supplemented groups, and WT+GlycanC1 mice being the most protected group (Figure 17B). Colon size measurement has underlined the protection against disease conferred by GlycanC1 to WT mice, with these mice showing a decreased colon shortening usually associated with DSS-induced inflammation (Figure 17C). Histological images demonstrated that GlycanC1 is able to decrease the lymphocytic infiltrate and improve the glandular architecture in the colon of supplemented mice (Figure 17D).

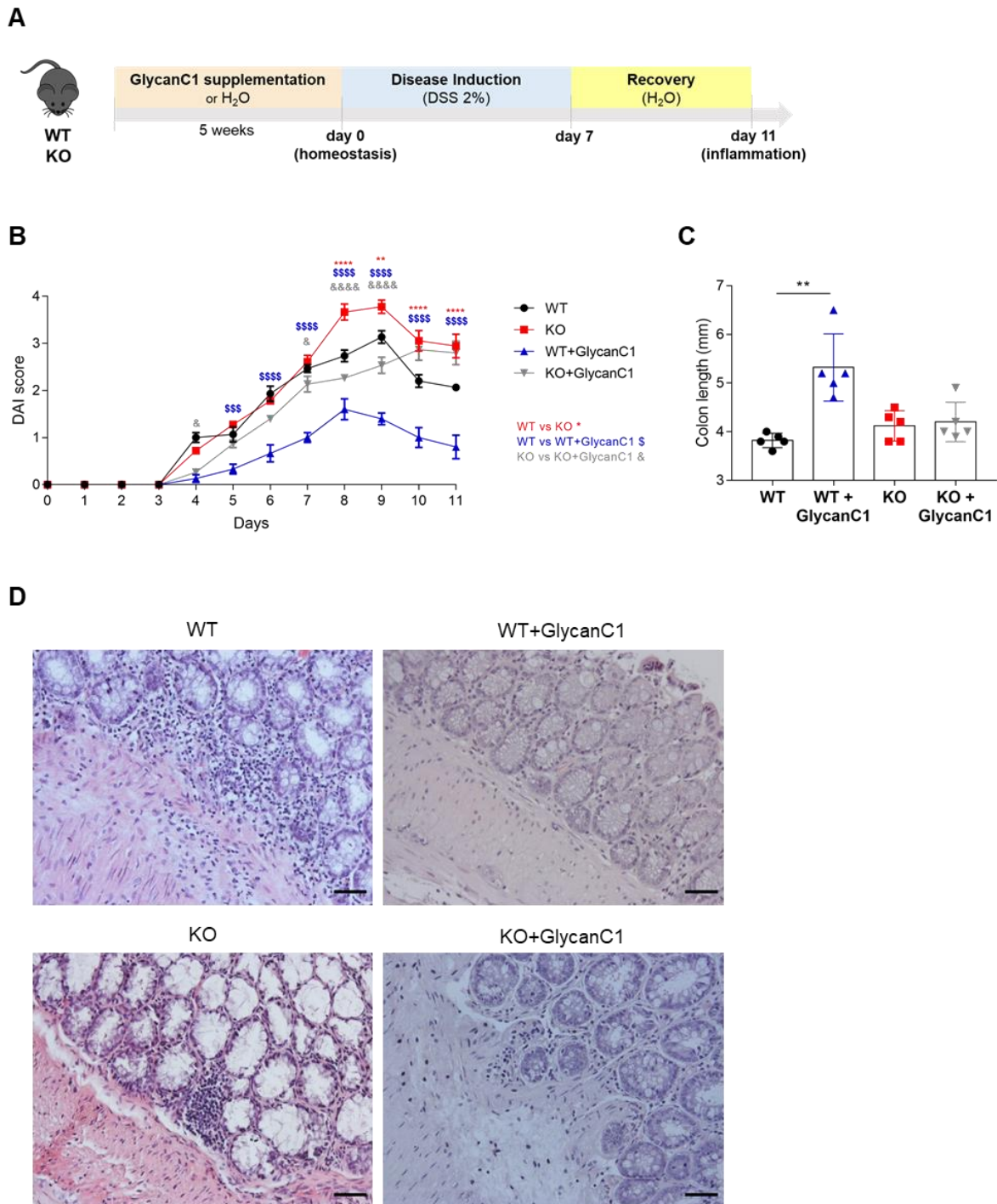


Figure 17| GlycanC1-supplemented mice are protected against DSS-induced colitis. (A) WT and KO mice were supplemented with GlycanC1 for 5 weeks. Unsupplemented mice were used as controls. Mice were induced to colitis using DSS 2% *ad libitum* in drinking water for 7 days, and then let to recover for 4 days with tap water. (B) Disease progression was assessed by scoring the DAI throughout the experiment. (C) Colon length was measured at day 11, after excision. Each dot represents one biological sample. Data is presented as mean $\pm$ SD. Statistically significant values are: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. (D) Histological analysis of haematoxylin & eosin staining of mice after colitis induction (day 11). Scale bar = 50 μ m.

GlycanC1 promotes colonic *Fut2* expression

To unveil whether GlycanC1 treatment for 5 weeks alters the host glycome, we evaluated the epithelial glycosylation in the colon of supplemented WT and KO mice, both in homeostasis and under inflammation.

At day 0 (baseline), staining with L-PHA, GNA and SNA showed that 5 weeks' administration of GlycanC1 does not significantly alter the levels of expression of branched and high-mannose *N*-glycans, or α 2,6-sialic acid, respectively, on epithelial cells of WT and KO mice (Figure 18A). Overall colonic *Mgat5b* expression is not significantly altered upon GlycanC1-treatment (Figure 18B).

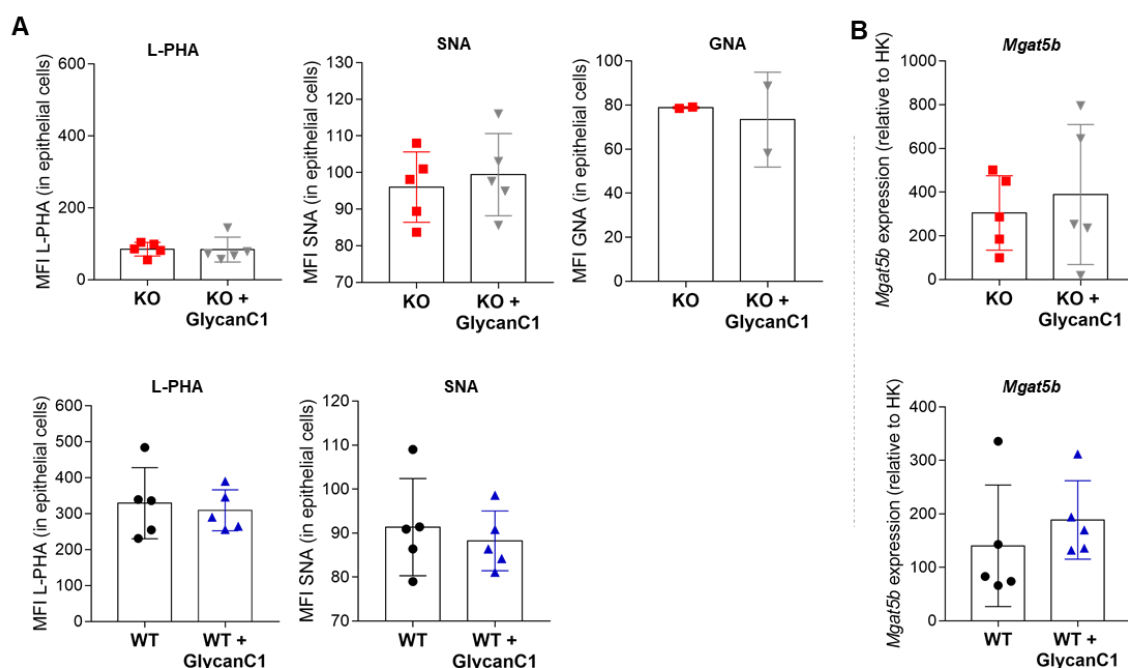


Figure 18| GlycanC1 supplementation for 5 weeks does not seem to impact β 1,6-GlcNAc branched and high-mannose *N*-glycans, or α 2,6-sialic acid, in homeostasis. (A) MFI of L-PHA, SNA and GNA in epithelial cells of the colon of KO and KO+GlycanC1 mice or WT and WT+GlycanC1 mice. **(B)** *Mgat5b* mRNA expression levels normalized to 18S expression in the colon of KO and KO+GlycanC1 mice or WT and WT+GlycanC1 mice. Each dot represents one individual biological sample, presented as mean $\pm$ SD. No statistical significance was reached (significant *p*-value $\leq$ 0.05).

At day 11 (upon inflammation), KO+GlycanC1 mice remain with negligible levels of branched *N*-glycans and display a tendency to decrease α 2,6-sialylation on epithelial cells, while WT+GlycanC1 mice present a decrease in branched *N*-glycans, together with a tendency for decreased high-mannose glycans (assessed with Dectin-2) (Figure 19A), which could suggest a putative glycans' consumption by microorganisms. In addition, L-PHA lectin staining of the colon at day 11 reinforces that GlycanC1 is apparently being

consumed by microbiota (Figure 19B), an issue that needs to be further confirmed and validated.

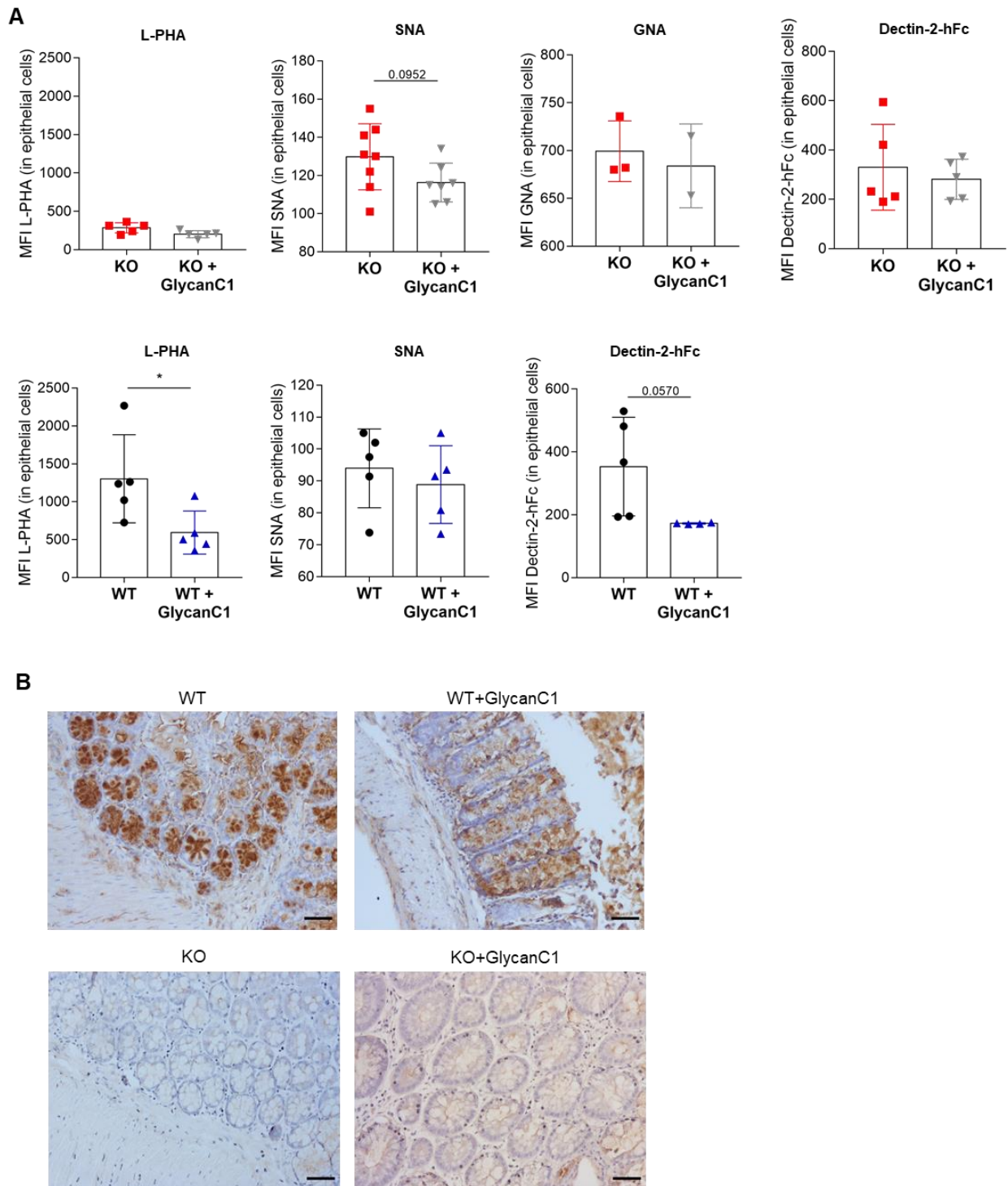


Figure 19| GlycanC1 impacts epithelial glycosylation in WT mice, upon inflammation. (A) MFI of L-PHA, SNA, GNA and Dectin-2-hFc in epithelial cells of the colon of KO and KO+GlycanC1 or WT and WT+GlycanC1 mice. Each dot represents one individual biological sample, presented as mean $\pm$ SD. Statistically significant values are: * $p \leq 0.05$. **(B)** Lectin histochemistry staining with L-PHA in colon samples of WT, WT+GlycanC1, KO and KO+GlycanC1 mice, at day 11. Scale bar = 50 μ m.

Since we have previously observed that *Fut2* expression tends to be impaired in KO mice and knowing that on epithelial cells it has an important role in protecting the host against pathogenic bacteria [124], we assessed whether GlycanC1 is able to induce α 1,2-fucosylation in overall colonic tissue. Importantly, we saw that, in homeostasis, GlycanC1 significantly rescues *Fut2* expression in KO mice to levels previously observed in WT mice (Figure 20A). During inflammation recovery, at day 11, both KO+GlycanC1 and WT+GlycanC1 mice tend to have increased *Fut2* expression relative to respective non-supplemented mice (Figure 20B). We hypothesise that GlycanC1 promotes α 1,2-fucosylation on epithelial cells, thus exerting a protective role in the gut.

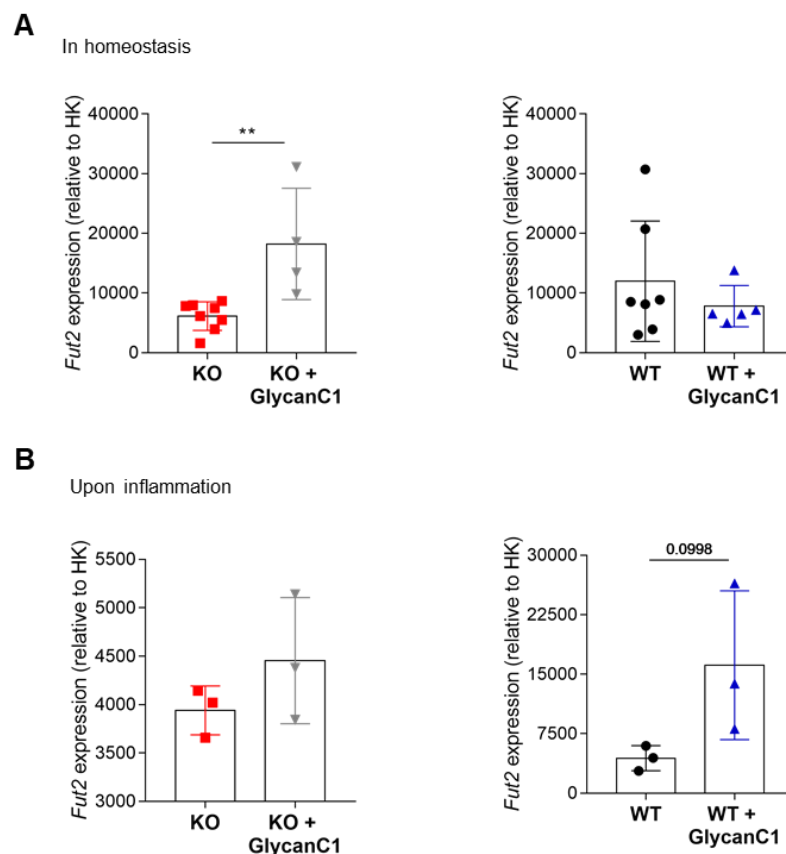


Figure 20| GlycanC1 upregulates *Fut2* in the colon of supplemented mice, in homeostasis and upon inflammation. *Fut2* mRNA expression levels normalized to *Gapdh* expression in the colon of KO and KO+GlycanC1 or WT and WT+GlycanC1 mice, **(A)** in homeostasis and **(B)** upon inflammation. Each dot represents one individual biological sample, presented as mean $\pm$ SD. Statistically significant values are: ** $p \leq 0.01$.

As GlycanC1 can be redundantly used by different enzymes involved in branched *N*-glycosylation, we also tested if this carbohydrate was able to increase branched *N*-glycans on immune cells. Similar to what was found for the epithelial compartment, no alterations on L-PHA staining in immune cells were observed between KO and KO+GlycanC1 mice, prior and after disease induction (Figure 21A,B). Regarding branched *N*-glycosylation on immune cells of WT+GlycanC1 mice, no differences were seen in homeostasis, comparing with WT (Figure 21A). Nevertheless, upon inflammation (day 11), there is a decreased expression of L-PHA (Figure 21B), similar to what we observed for non-immune (epithelial) cells.

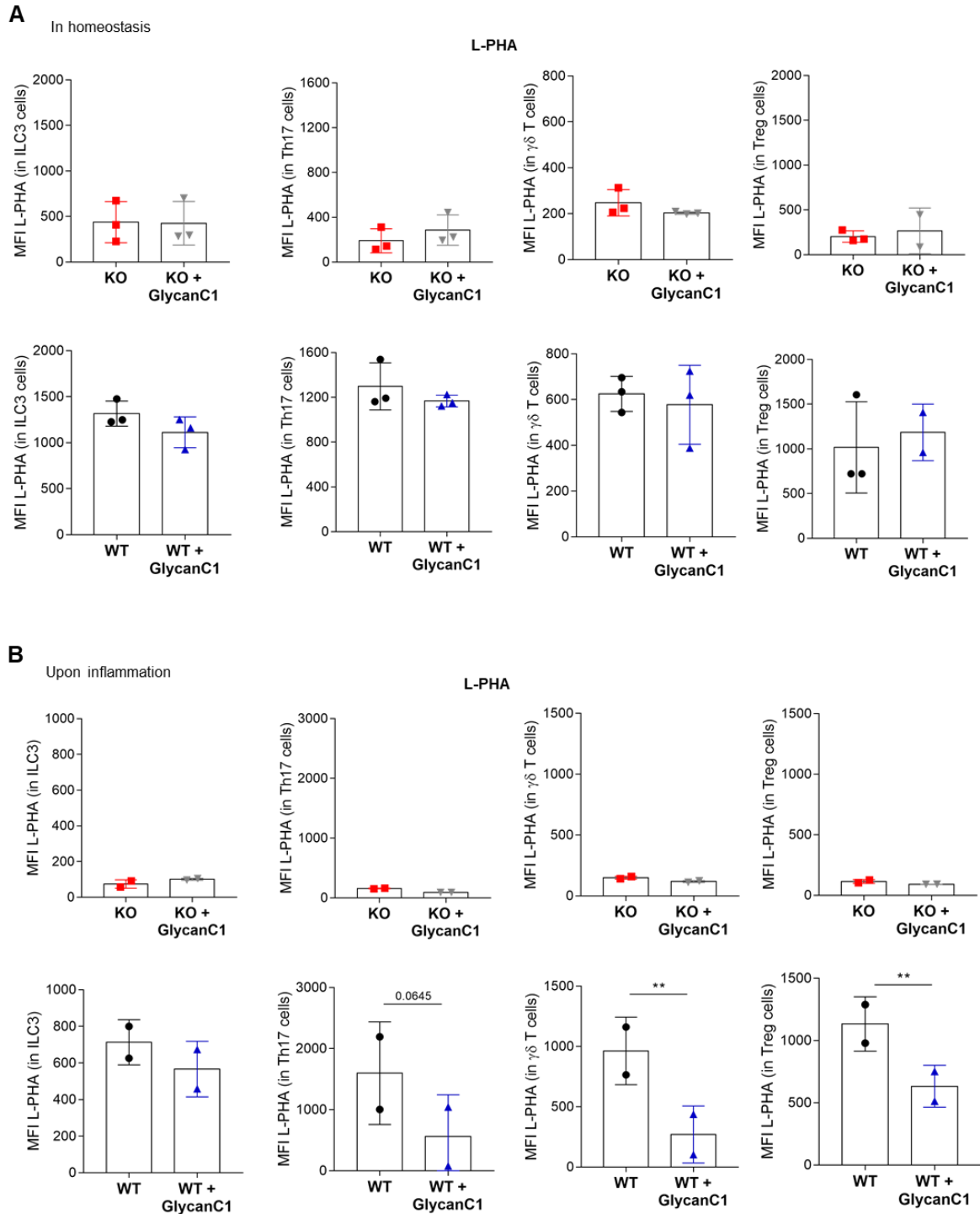


Figure 21| GlycanC1-supplemented WT mice have decreased levels of branched *N*-glycans in immune cells, upon inflammation. MFI of L-PHA in ILC3, Th17, $\gamma\delta$ T, and Treg cells in the colon of KO and KO+GlycanC1 mice, or WT and WT+GlycanC1 mice, **(A)** in homeostasis and **(B)** upon inflammation. Each dot represents a pool of two mice, presented as mean $\pm$ SD. Statistically significant values are: ** $p \leq 0.01$.

GlycanC1 supplementation improves ILC3/IL-22 dynamics in the colon of KO and WT mice

In homeostasis (before colitis induction)

To understand the potential ability of GlycanC1 to modulate the immune system towards the protective response observed upon an inflammatory stimulus, we characterized the immune profile of GlycanC1-treated WT and KO mice, prior DSS treatment.

Regarding the effect of GlycanC1 on KO mice, we observed that there is a slight tendency to restore ILC3 levels in the colon of KO mice, although without statistical significance. Moreover, GlycanC1 supplementation tendentially reduces IL-17A production by these cells, which may contribute to reduce inflammation. No differences on ILC3-derived IL-22 were observed due to GlycanC1 (Figure 22A).

At basal level, KO+GlycanC1 mice tend to have increased frequencies of Th17 cells in comparison with KO mice, although not reaching statistical significance (Figure 22B). Despite there were no differences regarding cytokine production by these cells in the colon (Figure 22B), Th17 cells in the MLNs of KO+GlycanC1 mice show a significant decrease of IL-17A (Supplementary Figure 7). KO+GlycanC1 mice also display higher frequencies of $\gamma\delta$ T cells in the colon, although with less IL-22-production (Figure 22C). It is described that $\gamma\delta$ T cells may exert a protective role in inflammation through the production of cytokines other than IL-22, namely IL-10 and TGF- β , which are involved in epithelial regeneration and wound healing [164]. Thus, it is plausible that $\gamma\delta$ T cells may be protective by producing these cytokines, which requires further evaluation in future experiments.

Concerning Treg cells, we have found that in KO+GlycanC1 mice these have decreased amounts of intracellular IL-10 when compared to KO mice (Figure 22D). The higher levels in KO mice may be possibly explained as a compensatory mechanism to balance pro-inflammatory responses. Even so, although there is a trend for higher frequencies of Treg cells in KO+GlycanC1 mice and thus a more anti-inflammatory profile (Figure 22D), due to the low number of samples this needs to be fully addressed in the future.

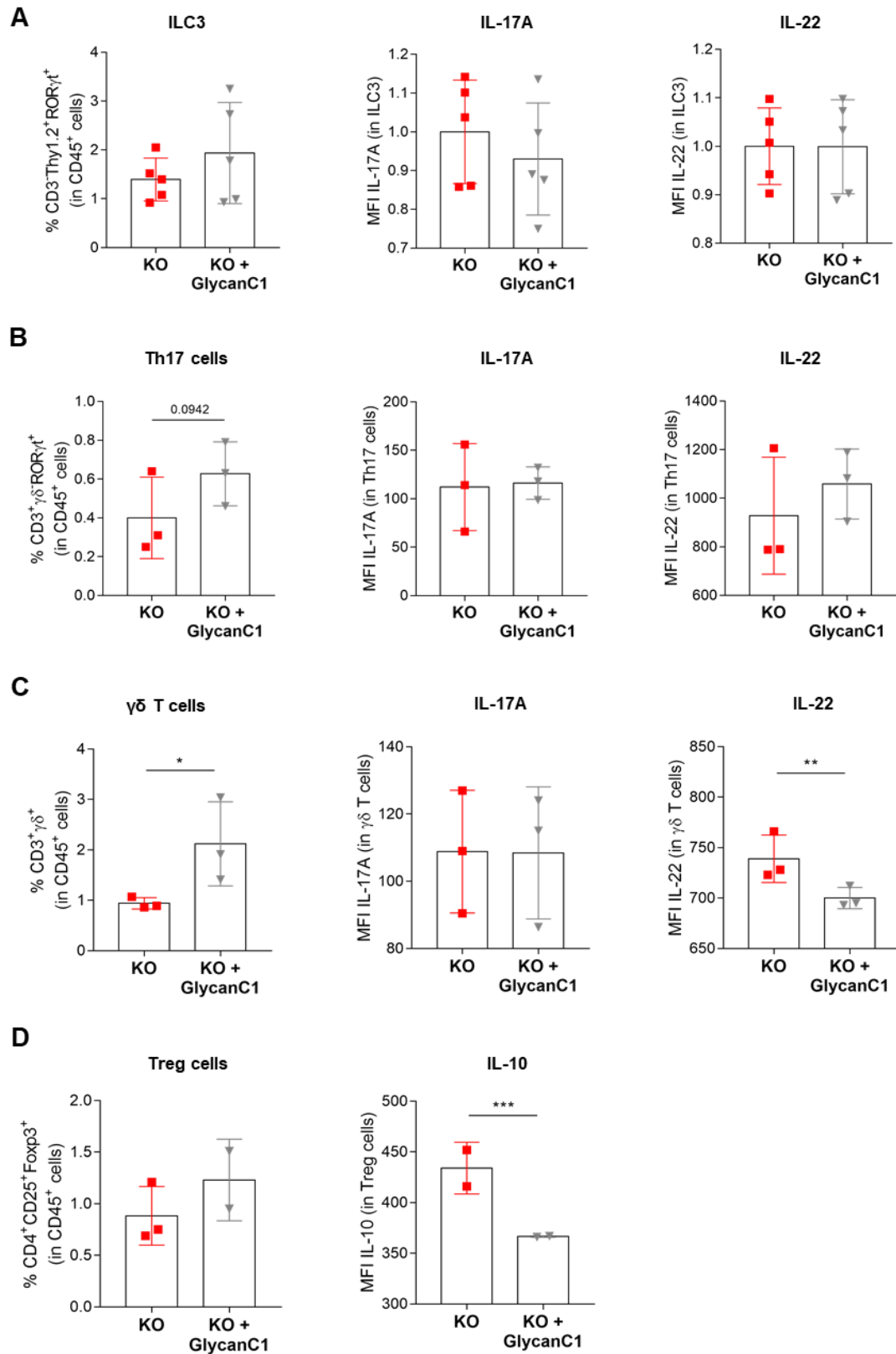


Figure 22| GlycanC1 supplementation has a mild impact on the immune profile of KO mice, in homeostasis. Frequencies of **(A)** ILC3, **(B)** Th17 and **(C)** γδ T cells, and MFI of IL-17A and IL-22 inside each population (normalized to KO mice in (A)). **(D)** Frequency of Treg cells and MFI of IL-10 inside these cells. Each dot represents a pool of two mice, presented as mean ± SD. Statistically significant values are: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Besides not observing significant differences on the production of IL-22 by the analysed cell populations through flow cytometry, with exception of $\gamma\delta$ T cells, qPCR analysis also showed that KO and KO+GlycanC1 mice have similar *Il22* transcription levels in the colon. Yet, KO+GlycanC1 mice have a significant decrease in the expression of *Il22ra2* (Figure 23A), which may imply an increased IL-22 availability. In line with this, the levels of IL-22 in supernatants from colon explants of KO+GlycanC1 mice tend to be increased in comparison with supernatants from KO mice, but no statistical differences were found (Figure 23B).

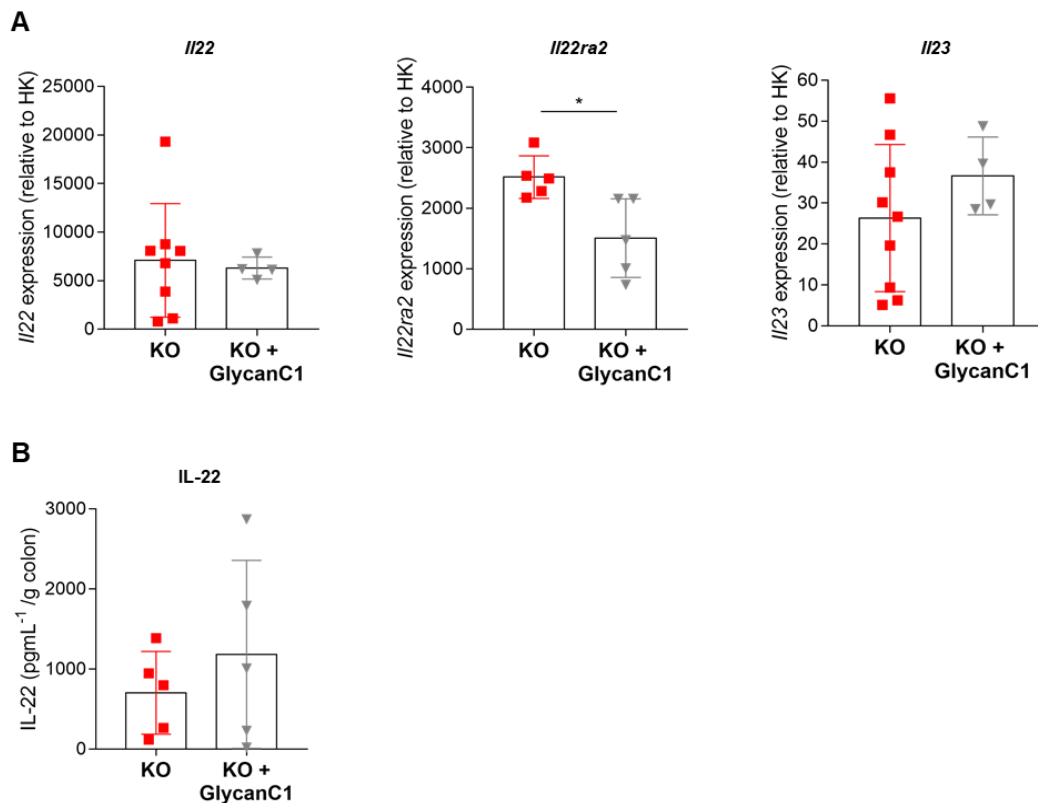


Figure 23| GlycanC1 downregulates *Il22ra2* in the colon of KO mice, in homeostasis. (A) *Il22*, *Il22ra2* and *Il23* mRNA expression levels normalized to *Gapdh* expression in the colon of KO and KO+GlycanC1 mice. **(B)** Concentration of IL-22 (pgmL⁻¹/g colon) in the supernatants of 24 h colonic explants cultures. Each dot represents one individual biological sample, presented as mean $\pm$ SD. Statistically significant values are: * $p \leq 0.05$.

Regarding GlycanC1 supplementation in WT mice, no major alterations were found in homeostasis, with the exception of increased IL-10 production by Treg cells (Figure 24D). Nevertheless, some tendencies were observed after supplementation, such as a decrease in Th17 cells frequencies (Figure 24B), as well as an increase in *IL22* transcription (Figure 25A). Additionally, in the MLNs WT+GlycanC1 mice have decreased levels of IL-17A produced by ILCs and increased IL-22 production by Th17 cells (Supplementary Figure 8). These minimal immune alterations may be explained by the fact that WT mice have no baseline impairments conditioned by the deficiency in branched *N*-glycans to be surpassed, especially under no inflammatory trigger.

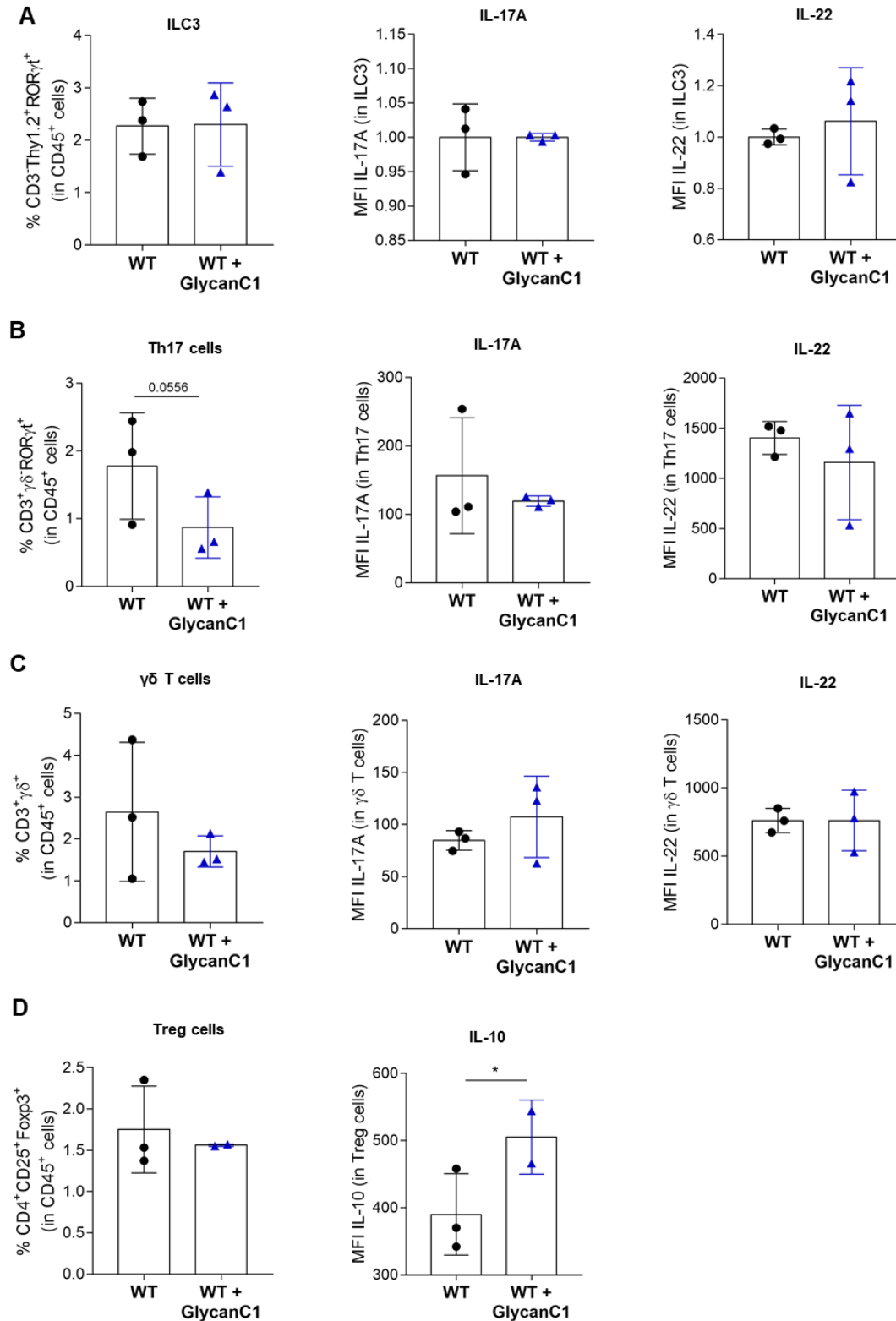


Figure 24| GlycanC1 increases Treg-derived IL-10 in the colon of WT mice, in homeostasis. Frequencies of **(A)** ILC3, **(B)** Th17 and **(C)** γδ T cells, and MFI of IL-17A and IL-22 inside each population (normalized to WT mice in (A)). **(D)** Frequency of Treg cells and MFI of IL-10 inside these cells. Each dot represents a pool of two mice, presented as mean ± SD. Statistically significant values are: **p* ≤ 0.05.

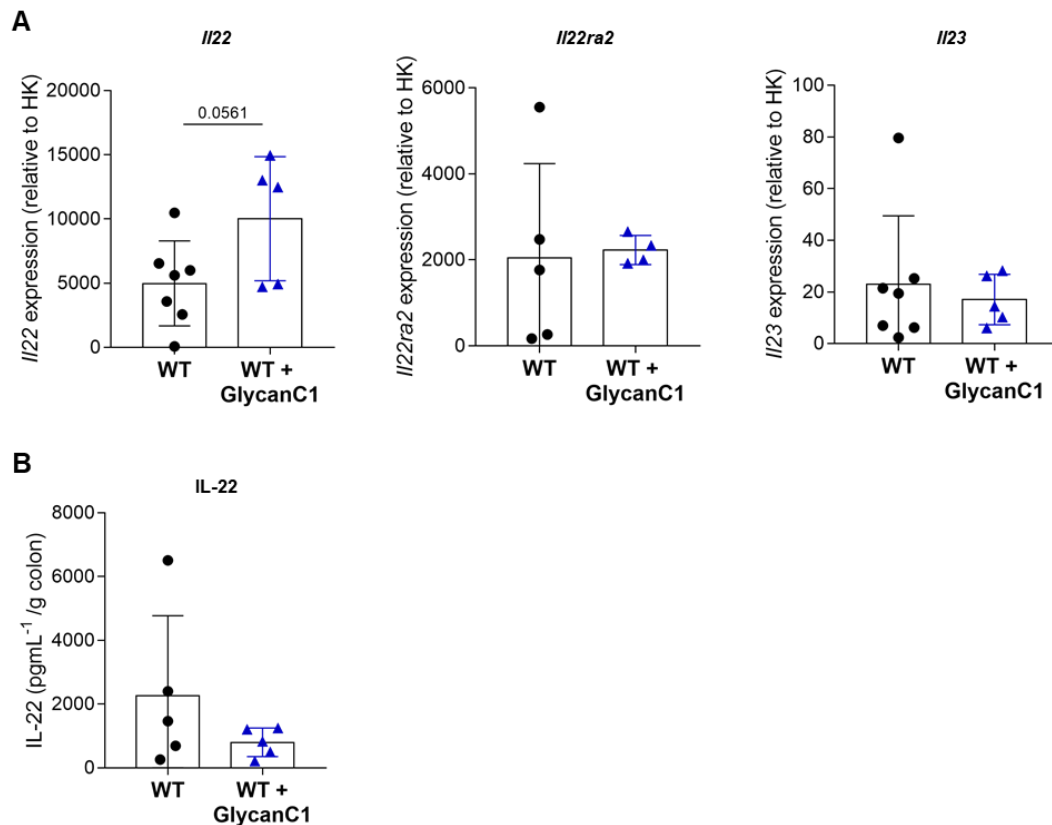


Figure 25| GlycanC1 tends to upregulate *IL22* in the colon of WT mice, in homeostasis. (A) *IL22*, *IL22ra2* and *IL23* mRNA expression levels normalized to *Gapdh* expression in the colon of WT and WT+GlycanC1 mice. **(B)** Concentration of IL-22 (pgmL⁻¹/g colon) in the supernatants of 24 h colonic explants cultures. Each dot represents one individual biological sample, presented as mean $\pm$ SD. Statistically significant *p*-value ≤ 0.05 .

After induction of inflammation

We have previously described the immune alterations imposed by glycans supplementation that may create an intestinal environment more prone to respond to an inflammatory insult. Additionally, we wanted to understand whether this immunomodulatory effect of GlycanC1 is maintained upon inflammation induced with the DSS treatment. Therefore, a characterization of the immune profile similar to the one performed in homeostasis (day 0) was applied in the presence of inflammation (day 11).

We have noted that, at day 11, i.e., during disease, but in a phase in which mice are starting to recover from inflammation, both KO+GlycanC1 and WT+GlycanC1 mice have a significant increase in ILC3 frequencies in the colon, relatively to respective controls (Figure 26A, 27A). While supplemented WT mice only tend to increase ILC3-derived IL-22-production in comparison with non-supplemented WT (Figure 27A), supplemented KO mice display a significant increase of IL-17A, IL-22 and GM-CSF produced by ILC3 (Figure 26A). Since ILC3-derived IL-22 is a promoter of epithelial *Fut2*

expression [124], these results support the hypothesis that increased *Fut2* expression observed earlier in supplemented WT and KO mice (Figure 20) is occurring in the epithelium. Although IL-17 may exert pro-inflammatory properties, that occurs specially if produced by Th17 cells and under an overall impaired context. In fact, IL-17 also plays a pivotal role in gut barrier [165], being important to balance IL-22 as it can engage a pathogenic role in the absence of IL-17 and if produced in excessive amounts. Indeed, clinical trials have already evidenced that IL-17 depletion in some CD patients is detrimental for intestinal function, exacerbating disease [97]. In the MLNs of WT+GlycanC1 mice, ILCs produce less IL-17A and IL-22 (Supplementary Figure 10), which raises the hypothesis that ILCs with the highest capacity to produce those cytokines (ILC3) are being recruited to the gut.

At this timepoint, the frequencies of Th17 and $\gamma\delta$ T cells in KO+GlycanC1 mice tend to decrease (Figure 26B,C), with the latter tendency being also observed in WT+GlycanC1 mice (Figure 27C). This sustains the decrease in lymphocytic inflammatory infiltrates observed in colonic histological images of GlycanC1-treated mice (Figure 17D). Moreover, Th17 cells from KO+GlycanC1 mice produce significantly less pro-inflammatory IL-17A (Figure 26B), which may be beneficial to control inflammation. Regarding MLNs, $\gamma\delta$ T cells of WT+GlycanC1 mice produce more IL-22, in comparison with WT (Supplementary Figure 10).

Interestingly, GlycanC1-treated KO mice tend to have decreased frequencies of Treg cells in the colon (Figure 26D), restabilising it to levels slightly above those observed in WT. The same profile was observed for these cells in the MLNs (Supplementary Figure 9). On the other hand, GlycanC1 induces an increase of Treg cells in the gut of supplemented WT mice (Figure 27D), reinforcing that even in a glycan-competent environment, supplementation with glycans may exert a beneficial effect on gut function.

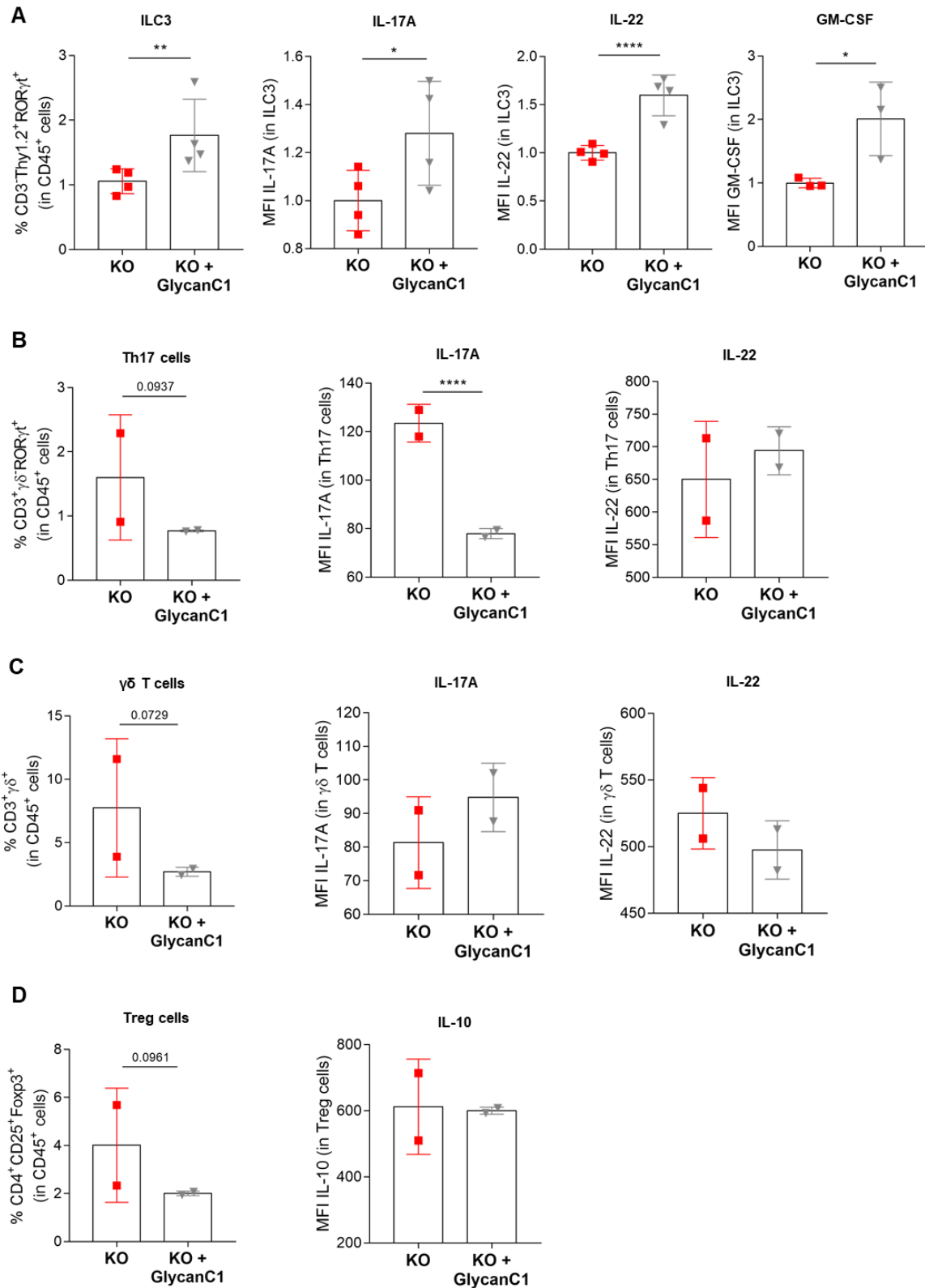


Figure 26| GlycanC1 promotes ILC3 and ILC3-derived IL-22, GM-CSF and IL-17A, while decreasing Th17-derived IL-17A in the colon of KO mice, upon inflammation. Frequencies of **(A)** ILC3, **(B)** Th17 and **(C)** γδ T cells, and MFI of IL-17A and IL-22 inside each population. **(A)** also contains the MFI of GM-CSF inside ILC3 (normalized to KO mice). **(D)** Frequency of Treg cells and MFI of IL-10 inside these cells. Each dot represents a pool of two mice, presented as mean ± SD. Statistically significant values are: * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$.

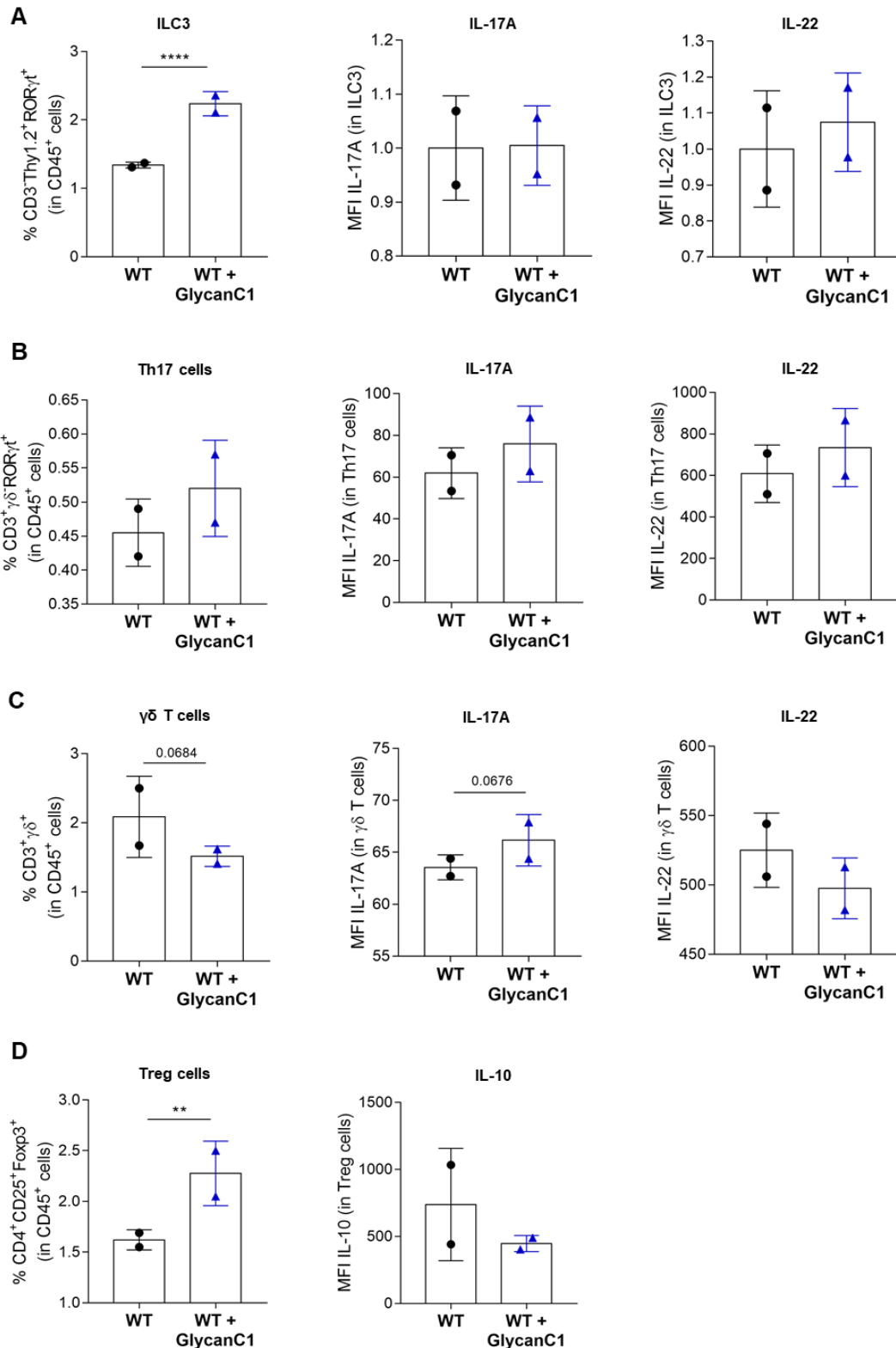


Figure 27| GlycanC1 increases ILC3 and Treg cells frequencies in the colon of WT mice, upon inflammation. Frequencies of **(A)** ILC3, **(B)** Th17 and **(C)** γ δ T cells, and MFI of IL-17A and IL-22 inside each population (normalized to WT mice in (A)). **(D)** Frequency of Treg cells and MFI of IL-10 inside these cells. Each dot represents a pool of two mice, presented as mean $\pm$ SD. Statistically significant values are: ** $p \leq 0.01$; **** $p \leq 0.0001$.

To complement the analysis, the transcriptional levels of *Il22*, *Il22ra2* and *Il23* were evaluated by qPCR, but no major alterations were found (Figure 28Ai,Bi). Nevertheless, the levels of IL-22 in supernatants from WT mice supplemented with GlycanC1 are significantly increased in comparison with unsupplemented mice (Figure 28Bii), which is in accordance with the highly protective effect found upon DSS-induced colitis.

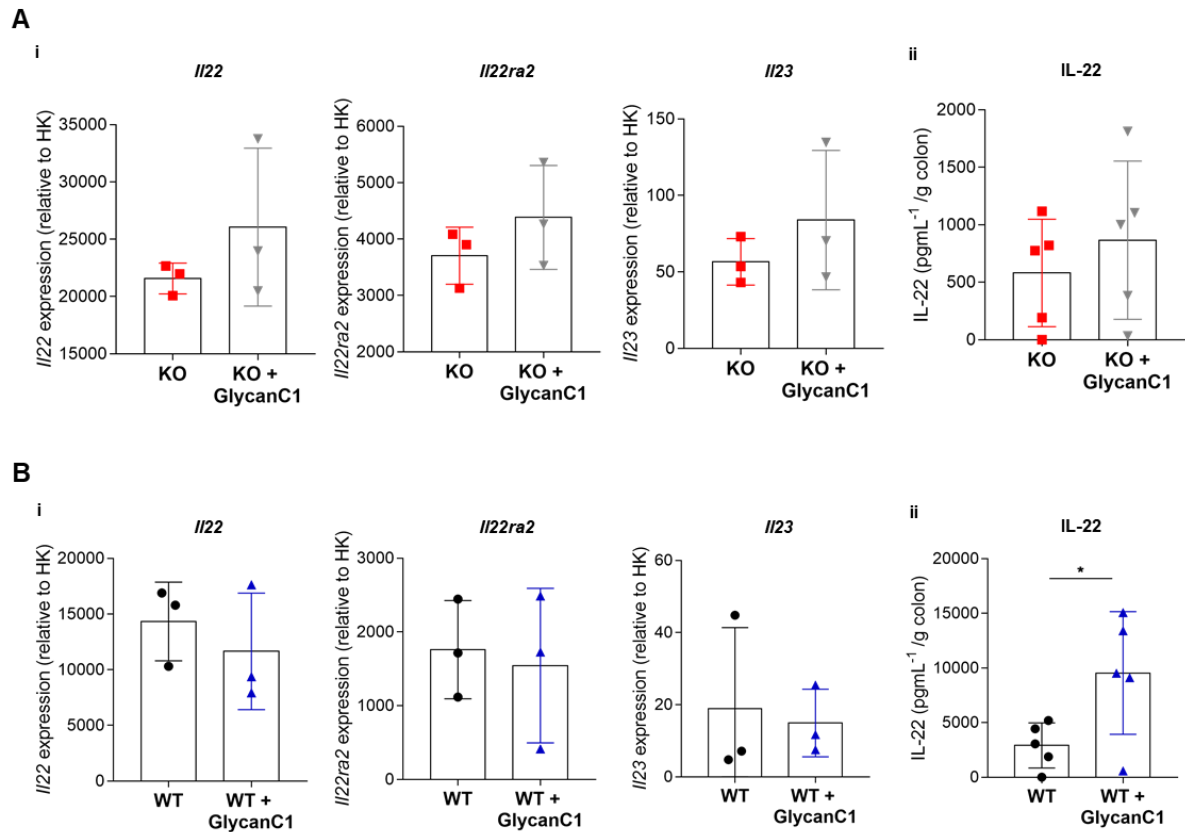


Figure 28| GlycanC1 increases IL-22 levels in the colon of WT mice, upon inflammation. *Il22*, *Il22ra2* and *Il23* mRNA expression levels normalized to *Gapdh* expression in the colon of **(A)** (i) KO and KO+GlycanC1 mice, or **(B)** (i) WT and WT+GlycanC1 mice. (A, B) (ii) Concentration of IL-22 (pgmL⁻¹/g colon) in the supernatants of 24 h colonic explants cultures. Each dot represents one individual biological sample, presented as mean ± SD. Statistically significant values are: **p* ≤ 0.05.

These findings have demonstrated that GlycanC1 displays immunomodulatory properties, seen both prior and after inflammation. In particular, GlycanC1 positively impacts ILC3, which seem to be the cellular population having the most pivotal role in gut protection upon glycans supplementation, particularly through IL-22 production.

***Mgat5* deficiency seems to have a detrimental impact on *Nod2* expression, which is rescued by GlycanC1 supplementation**

In homeostasis (before colitis induction)

Herein, we have disclosed that the paucity of ILC3 and ILC3-derived IL-22 production in KO mice is rescued by GlycanC1 supplementation, therefore, we wanted to further unlock if other molecular components associated with the ILC3/IL-22 axis are impaired in these mice and, subsequently, can be rescued upon supplementation. As GlycanC1 also positively impacts WT mice, namely through ILC3 and IL-22, the analysis was extended to WT+GlycanC1 mice.

In this sense, we evaluated the expression of *Nod2*, an intracellular PRR expressed by most cells in the gut [166], that senses the microbiome and can act upstream of ILC3 [167, 168]. In homeostasis, we noticed for the first time that KO mice have an almost statistically significant decrease in the expression of *Nod2*. Remarkably, we observed that GlycanC1 significantly rescues the transcriptional levels of *Nod2* in supplemented KO mice, once again pinpointing its role on ILC3 modulation (Figure 29A).

Considering that *CARD9* is a susceptibility gene for IBD [169] that, as an adaptor protein, can be associated with *Nod2* and TLR signalling [170], we assessed its expression. In agreement with studies describing that excessive *CARD9* transcription occurs in IBD patients and is associated with an hyperimmune response [19], we found that KO mice have a slight increase in *Card9* expression when compared to WT mice in homeostasis. GlycanC1 supplementation does not impact the expression of this gene (Figure 29B).

Reg3 γ is a CTL, AMP produced by intestinal epithelial cells in response to ILC3-derived IL-22 and TLR signalling [171]. In homeostasis, WT and KO mice present similar expression of *Reg3g* (Figure 29C), probably targeted by IL-22 in WT and by the sensing of pathogenic bacteria through TLR in KO mice, since we know that the latter are dysbiotic and present decreased IL-22 production. Notably, GlycanC1 treatment tends to increase the production of this AMP, both in WT and KO mice (Figure 29C), most likely through IL-22. This is supported by the fact that WT+GlycanC1 mice present higher *Il22* expression, and that KO+GlycanC1 mice, although not presenting higher production of IL-22 by the analysed cells, present a significantly decreased transcription of the gene encoding for IL-22BP, thus predisposing a higher availability of IL-22, as previously described, which may foster Reg3 γ production.

Finally, we have evaluated the expression of *Gpr43*, a receptor of microbiome-derived SCFAs present on colonic ILC3 and Treg cells, and important for respective IL-22 and IL-10 production [125, 172]. No major differences were found at baseline between the two genotypes and glycans-treated and non-treated mice (Figure 29D).

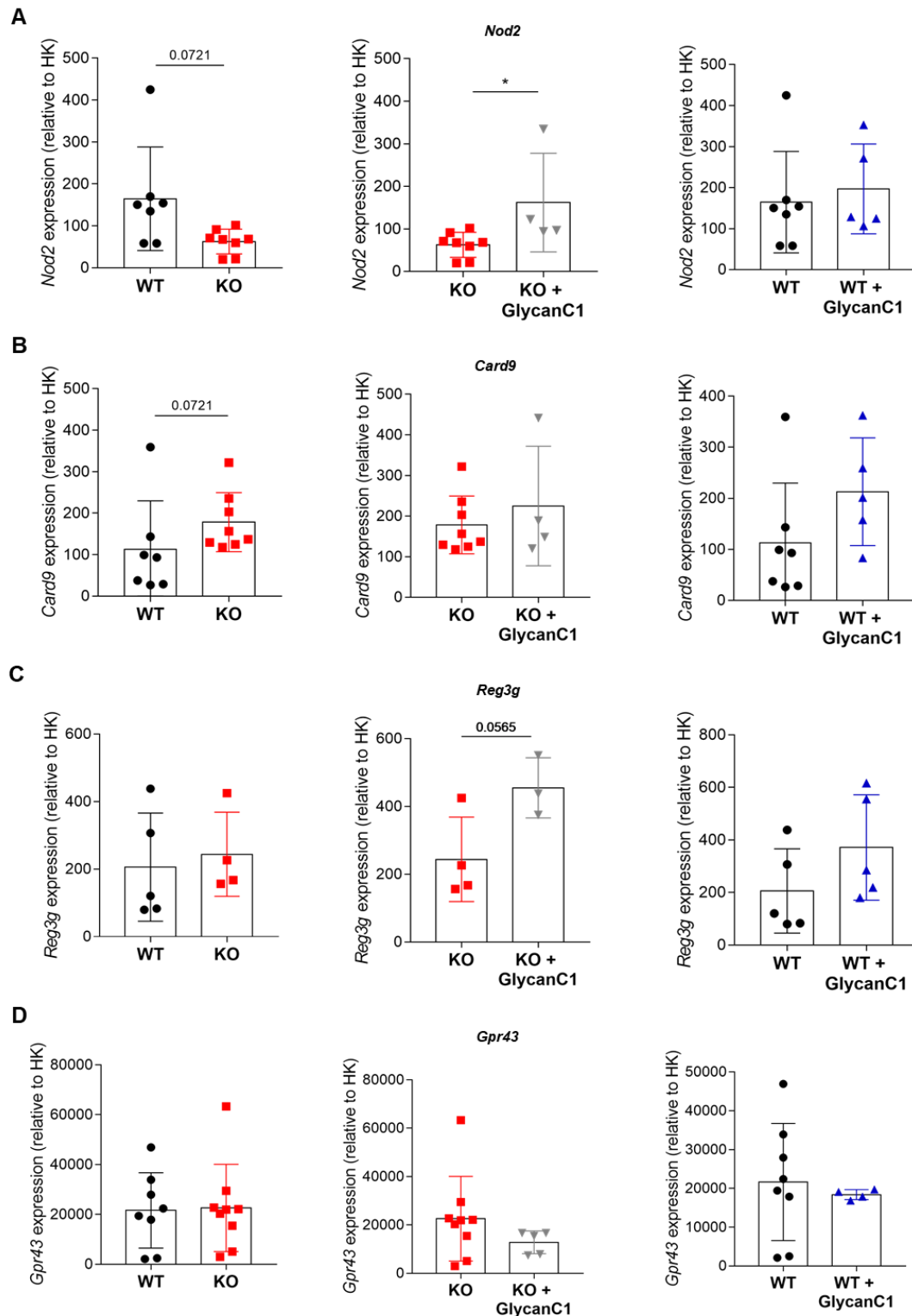


Figure 29| *Mgat5* deficiency seems to impact *Nod2* expression, which is then rescued by GlycanC1, in homeostasis. (A) *Nod2*, (B) *Card9*, (C) *Reg3g* and (D) *Gpr43* mRNA expression levels normalized to *Gapdh* expression in mice colon. Each dot represents one individual biological sample, presented as mean $\pm$ SD. Statistically significant values are: * $p \leq 0.05$.

After induction of inflammation

Similar to what was described for baseline, upon inflammation we observed that KO+GlycanC1 mice present a significant increased expression of *Nod2*, comparing with KO mice, meaning that the protective action conferred by GlycanC1 is sustained during inflammation. At this timepoint, a similar tendency was observed in WT+GlycanC1 mice, in comparison with WT mice (Figure 30A). Regarding *Card9*, no differences were noticed (Figure 30B).

Although without reaching statistical significance, we observed that KO mice have a decreased expression of *Reg3g* (Figure 30C), hampering the inhibition of bacterial translocation and antibacterial defence [55]. Similar to what we disclosed for baseline, herein supplemented WT and KO mice also display a tendency to increase *Reg3g* expression. Once more, it may occur in consequence of an increased enhancement of IL-22 production by ILC3. This suggests that GlycanC1 allows a tighter regulation of the microbiota composition, once again spotlighting a role for the microbiome in this interplay.

Regarding *Gpr43*, KO mice present a significant higher expression of this gene than WT mice (Figure 30D). We may hypothesise that this represents an attempt to enhance the sensing of environmental SCFAs and, in consequence, produce more IL-22 to overcome inflammation and the increased expression of the gene encoding IL-22BP. Supplementation with GlycanC1 imposes no major alterations on the *Gpr43* transcriptional levels in both WT and KO mice (Figure 30D).

These results illustrate that the protective role provided by GlycanC1 is associated with its capacity to increase the expression of microbiota-sensing molecules, as *Nod2* and *Reg3g*, allowing a greater perception of environmental cues and a more protective immune response.

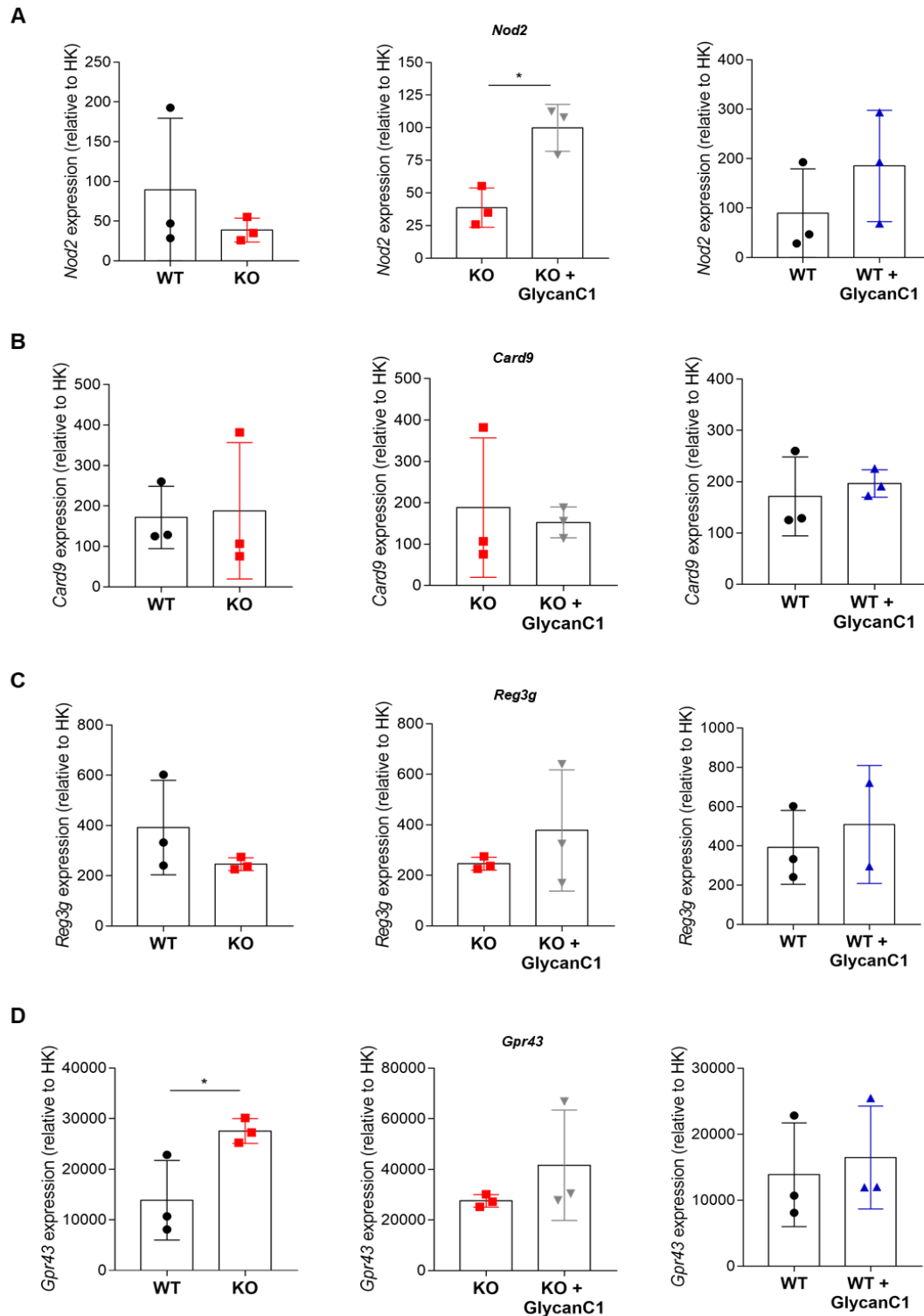


Figure 30| GlycanC1 rescues *Nod2* expression in KO mice, upon inflammation. (A) *Nod2*, (B) *Card9*, (C) *Reg3g* and (D) *Gpr43* mRNA expression levels normalized to *Gapdh* expression in mice colon. Each dot represents one individual biological sample, presented as mean $\pm$ SD. Statistically significant values are: $*p \leq 0.05$.

GlycanC1 impacts the transcription of genes involved in epithelial barrier integrity

Since the epithelial barrier dynamics can be influenced by both the microbiome and the immune populations, which are impaired in KO mice, we assessed the integrity of the epithelium in WT and KO mice by evaluating the transcriptional levels of genes encoding for claudins, such as *Cldn1*, *Cldn2* and *Cldn4*, and occludin. In homeostasis, no differences were seen for the mentioned genes (Figure 31A-D).

To evaluate whether the protective role of GlycanC1 on WT and KO mice is associated with an increased epithelium integrity, aside from immune modulation, we evaluated the expression of claudins and occludin in GlycanC1-treated groups at baseline. We have found that supplemented KO mice tend to have an increased expression of *Cldn1* (Figure 31A), a well-established sealing-claudin that reinforces barrier integrity [173]. On the other hand, GlycanC1 supplementation in WT significantly increases *Cldn2* expression (Figure 31B). Although the role of *Cldn-2* is still controversial, with evidence that it is a pore-forming claudin [174], it has been described that it promotes barrier integrity and induces immune tolerance, protecting mice against DSS-induced colitis [175]. This evaluation was not performed after DSS-induced colitis, since it is expected that upon inflammation the epithelial barrier components become altered independently of GlycanC1 supplementation, and, therefore, it would impose a confounding factor.

In conclusion, we observed that aside from modulating the immune compartment, GlycanC1 impacts the epithelial barrier integrity, playing a pivotal role in gut protection.

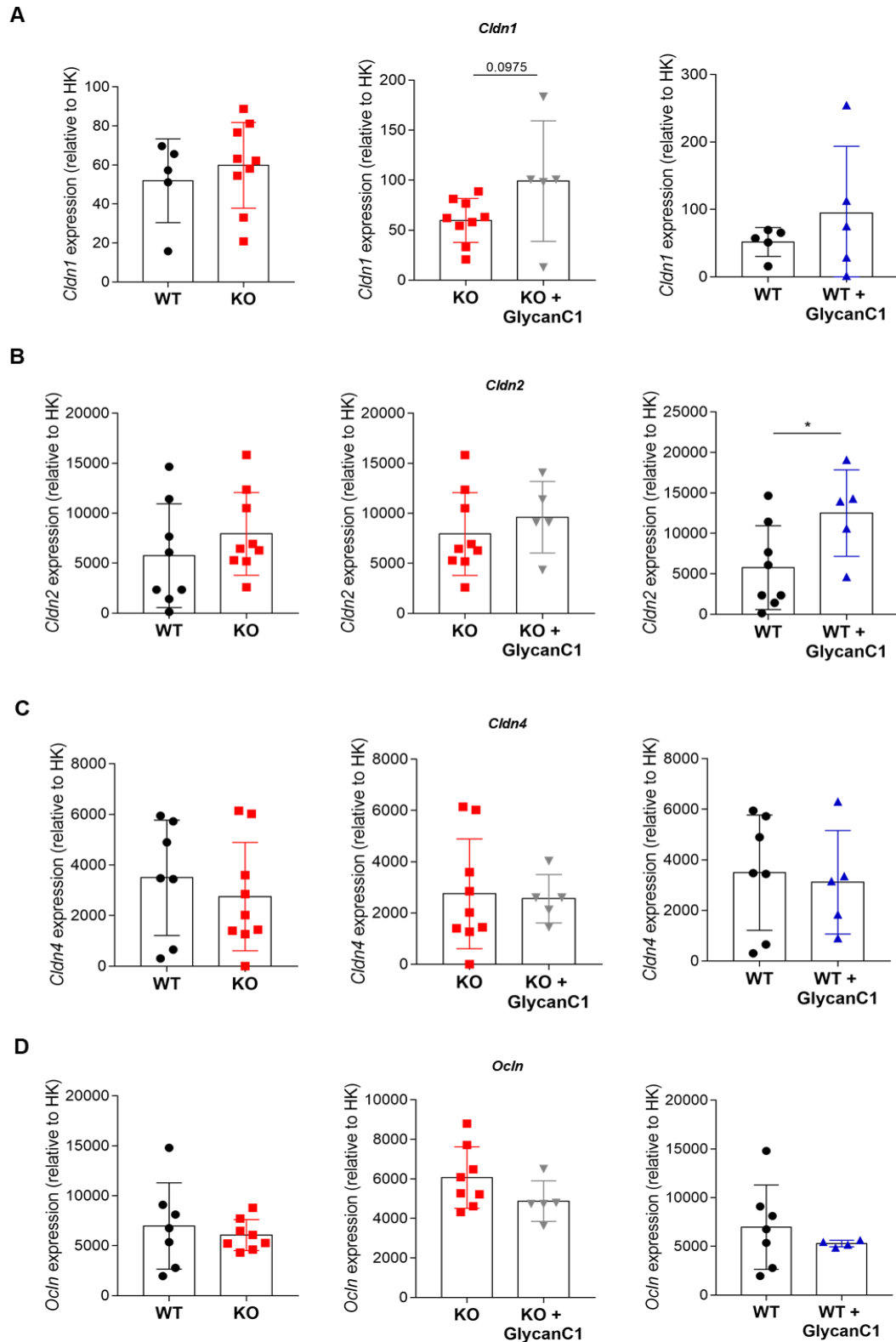


Figure 31| GlycanC1 impacts barrier integrity through *Cldn1* or *Cldn2*, in homeostasis. (A) *Cldn1*, (B) *Cldn2*, (C) *Cldn4* and (D) *OcIn* mRNA expression levels normalized to *Gapdh* expression in mice colon. Each dot represents one individual biological sample, presented as mean $\pm$ SD. Statistically significant values are: * $p \leq 0.05$.

II. Characterization of the host glycosylation profile and immune signature in IBD

Characterization of the glycoprofile associated with human IBD pathogenesis

Our murine experiments data have demonstrated how glycosylation and immune impairments contribute to intestinal inflammation. To translate these findings to a clinical human IBD setting, we characterized the host glycosylation profile and immune landscape in individuals diagnosed with CD or UC.

The glycoprofile of intestinal epithelial and immune cells from IBD patients remains largely unexplored. To unlock a putative glycosignature on these cells, we resort to flow cytometry and qPCR analysis, evaluating the expression of several lectins and glycogenes in inflamed colon biopsies from IBD patients (identified as CD or UC) and non-inflamed colon mucosa from healthy controls (HC). Our preliminary results showed no major differences in the epithelial glycosylation profile between inflamed and non-inflamed colon mucosa (Figure 32A) which can be explained by the reduced sample size. However, we observed that in inflamed biopsies Th and Treg cells tend to be deficient in glycans, particularly β 1,6-branched and high-mannose *N*-glycans, and α 2,6-sialic acid (Figure 32B,C), despite the variability of expression in controls.

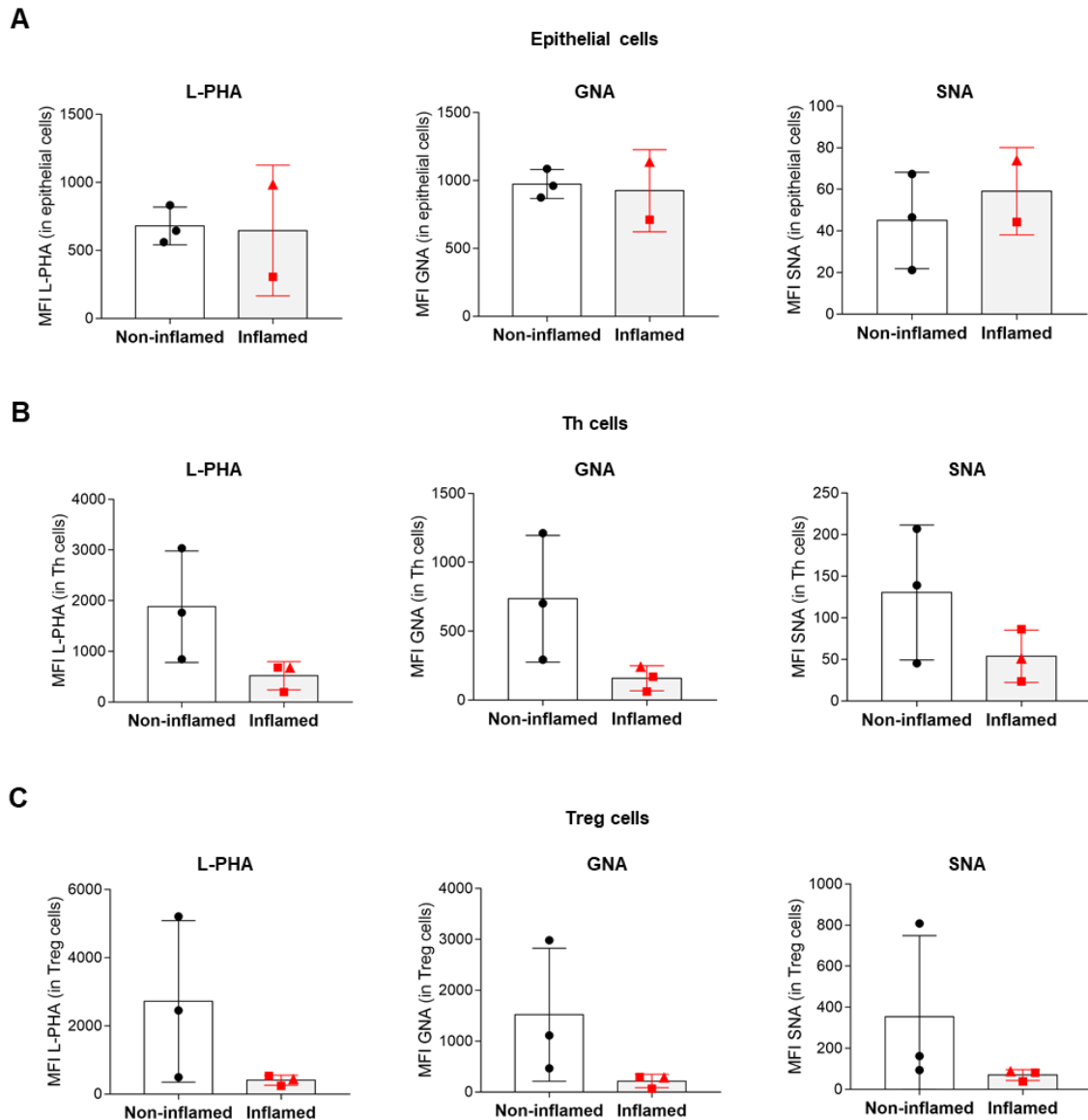


Figure 32| Inflamed colon tissues of IBD patients seem to display alterations in T cells' glycans expression. MFI of L-PHA, GNA and SNA in **(A)** epithelial, **(B)** Th and **(C)** Treg cells in non-inflamed or inflamed colon tissues. Each dot represents one individual biological sample, presented as mean $\pm$ SD. $\square$: CD; $\triangle$: UC. Statistical significance was not assessed in (A) due to sample size. No statistical significance was found (significant p -value ≤ 0.05).

To further infer about the presence of branched *N*-glycans, we also evaluated the expression of *MGAT5* in overall biopsies tissues. Supporting previous reports from the group showing that UC patients exhibit impaired β 1,6-GlcNAc branched *N*-glycosylation on lamina propria T lymphocytes [5], our results suggested that *MGAT5* expression is decreased in inflamed colons (Figure 33A).

Fucosylation has different implications according to the linkage involved and the site it occurs. For example, an α 1,2-fucose linked by FUT2 to the terminal β -D-galactose

residues in intestinal epithelial cells is associated with gut homeostasis as a mechanism of host–microbiota symbiosis [124]. On the other hand, a fucose residue attached by FUT8 to the innermost GlcNAc of *N*-glycans (core fucosylation) on TCR is associated with hyperactivation of T cells, while on co-inhibitory receptors (cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein1 (PD-1)) promotes immune tolerance [2]. Interestingly, despite the high deviation and small sample size of the non-inflamed group, we observed that the expression of *FUT2* tends to be decreased in inflamed biopsies (Figure 33B). Opposingly, the expression of *FUT8* seems to be increased in inflamed colons (Figure 33C). We hypothesise that this overexpression of *FUT8* is occurring in the TCR, which needs to be confirmed in future experiments.

Besides glycans and glycosyltransferases, we also evaluated DC-SIGN (CD209) as a CTL that senses glycans, particularly by binding to high-mannose and fucose-containing glycans [176]. We observed that the expression of *CD209* is tendentially decreased in inflamed colons of IBD patients (Figure 33D). As DC-SIGN is essentially expressed on immature DCs [177], this may suggest that under inflammation the gut is enriched in mature and activated DCs, as expected.

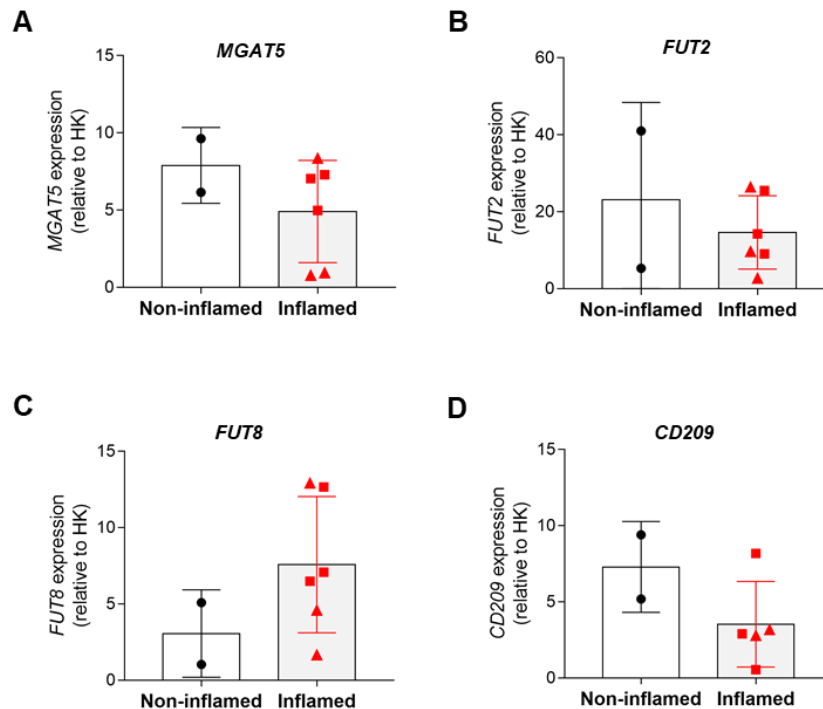


Figure 33| Inflamed colon tissues of IBD patients seem to have impaired glycosyltransferases expression. (A) *MGAT5*, (B) *FUT2*, (C) *FUT8* and (D) *CD209* mRNA expression levels normalized to 18S expression in non-inflamed or inflamed human colon tissues. Each dot represents one individual biological sample, presented as mean ± SD. □: CD; △: UC. Statistical significance was not assessed due to sample size.

The glycoprofile of peripheral immune cells was also assessed by flow cytometry. Here, besides stratifying IBD patients as CD and UC patients, the different disease state, i.e., activity or remission, was accounted in the analysis, despite no major distinctive signatures were found so far between the two groups.

To this point, no main differences were observed on L-PHA and GNA binding in peripheral Th and Treg cells (Figure 34A,B). Interestingly, although no major changes were found on SNA binding in Th cells from clustered IBD samples, a significant increase in $\alpha 2,6$ -sialic acid is observed in Th cells from patients with active disease when compared with HC (Figure 34A). This increase in $\alpha 2,6$ -sialic acid in active IBD impairs galectin binding [178], which if occurring in TCR may lead to T cell hyperactivation [179], in line with the physiopathology. No differences were found for SNA binding on peripheral Treg cells (Figure 34B).

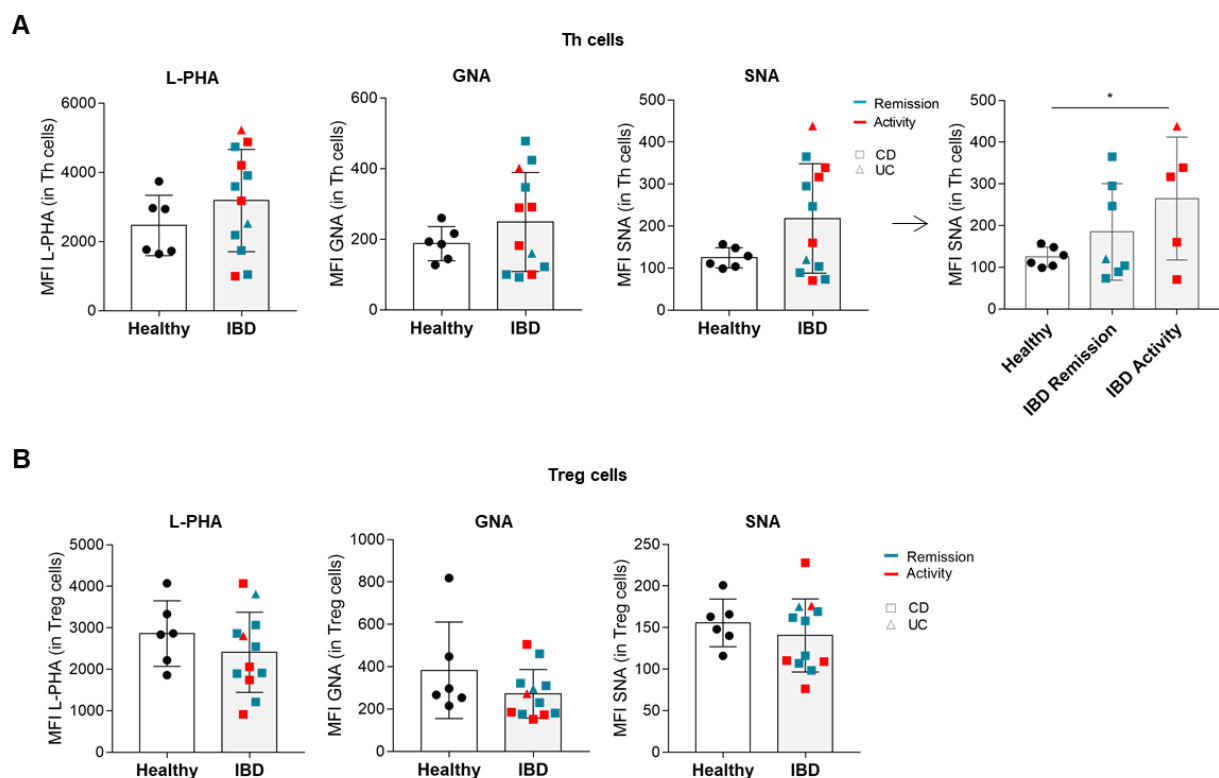


Figure 34| Peripheral Th cells from IBD patients with active disease have increased levels of $\alpha 2,6$ -sialic acid. MFI of L-PHA, GNA and SNA in **(A)** Th and **(B)** Treg cells in PBMCs from healthy individuals or IBD patients. Each dot represents one individual biological sample, presented as mean $\pm$ SD. Blue: remission; red: activity; $\square$: CD; Δ : UC. Statistically significant values are: * $p \leq 0.05$.

Overall, these results illustrate an altered glycoprofile in IBD that may have the ability to shape immune responses.

***In situ* characterization of the immune landscape associated with human IBD pathogenesis**

To understand how an altered glycoprofile is associated with the immune landscape in the context of human IBD pathogenesis, we analysed the immune profile in inflamed colon biopsies from IBD patients, comparing with non-inflamed colon biopsies.

As earlier mentioned, among innate immune cells, human ILC3 have an important role in maintaining intestinal tolerance and homeostasis [180] and can be subdivided into NKp44⁺ and NKp44⁻ subsets [181]. Even though both subsets produce GM-CSF and IL-17A [182, 183], the production of IL-22 is restricted to NKp44⁺ILC3 [184]. As IL-22 promotes epithelial homeostasis and suppresses inflammation [185], NKp44⁺ILC3 are described as tissue protective in the human gut [181]. Interestingly, although still preliminary due to the number of samples, our results showed that this population tends to be decreased in the inflamed colon of IBD patients (Figure 35A). Concerning the capacity of NKp44⁺ILC3 to produce IL-22, GM-CSF and IL-17A, only the latter shows a tendency to increase in inflamed biopsies (Figure 35A), which despite the protective role commonly associated with these cells may contribute to exacerbated inflammation.

T cells are major drivers of inflammation in IBD [186]. As expected, intestinal T cells seem to be infiltrated in the inflamed colon mucosa (Figure 35B), which is supported by a tendency to increase the expression of CD3 (Figure 35C). While CD and UC are associated with local Th1 and Th2 responses, respectively [86], in both disorders Th17 cells massively penetrate the inflamed intestine [90]. We observed that Th17 cells follow a tendency to increase in inflamed biopsies similarly to total T cells (Figure 35D). Moreover, Th17 production of IL-17A, IL-22 and GM-CSF tends to be decreased in inflamed biopsies (Figure 35D), although it is important to consider that these are preliminary results that display high variability and, therefore, need to be further assessed with an increased sample size.

Given the importance of Treg cells in maintaining homeostasis by controlling immune responses [187], we also assessed the frequency of these in biopsy samples. Importantly, we found that the inflamed colon of IBD patients seems to be depleted in protective Treg cells (Figure 35E).

In conclusion, these results suggest that upon inflammation, the colonic mucosa of IBD patients is depleted in protective NKp44⁺ILC3 and Treg cells, and is enriched in Th17 cells, contributing to the disruption of the intestinal equilibrium, and promoting a pro-inflammatory environment in IBD.

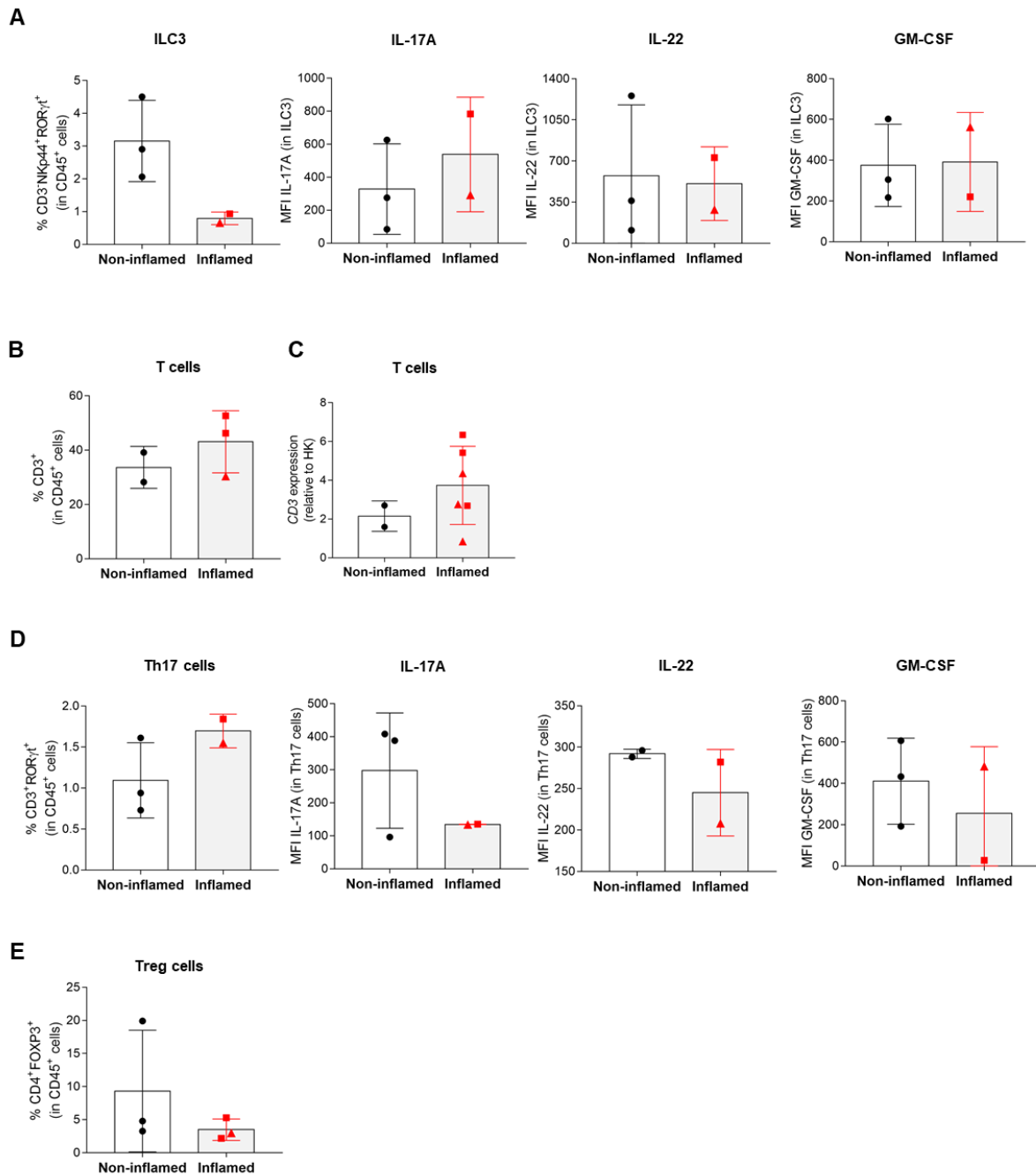


Figure 35| The inflamed colon of IBD patients seems to present decreased frequencies of ILC3 and Treg cells, and an enrichment of Th17 cells. Frequencies of **(A)** ILC3, **(B)** T, **(D)** Th17 and **(E)** Treg cells in non-inflamed or inflamed human colons. (A) and (D) contain the MFI of IL-17A, IL-22 and GM-CSF inside ILC3 and Th17 cells, respectively. **(C)** CD3 mRNA expression levels normalized to 18S expression in non-inflamed or inflamed human colon tissues. Each dot represents one individual biological sample, presented as mean $\pm$ SD. □: CD; △: UC. Due to sample size, statistical significance was assessed only in (E), although no statistical significance was reached (significant p -value ≤ 0.05).

Characterization of the immune landscape at the periphery associated with human IBD pathogenesis

To perform a more extensive characterization of the immune landscape, we also evaluated the immune profile at the periphery, in the blood of IBD patients and HC.

In the blood, ILC3 appear in very low frequencies [184]. Because of this, and since the NKp44⁺ population identified by flow cytometry was residual and therefore not sufficient to correctly infer the real profile of these cells, we tackled ILC3 by the negative expression of CD3 and positive expression of ROR γ t. Interestingly, we found that IBD patients tend to have more peripheral ILC3 than HC (Figure 36A), supporting the previous results that suggested that ILC3 are not being recruited to the inflamed colon (Figure 35A) and thus display higher levels in circulation. Additionally, we observed that ILC3-derived IL-22 is increased in the blood of IBD patients, suggesting that ILC3 with a higher capacity to produce IL-22 may be particularly those not being promptly directed to the gut. Although this hypothesis was not evidenced in colon biopsies analysis, it needs to be validated with sample increment. We also observed that peripheral ILC3 from IBD patients produce significantly less GM-CSF (Figure 36A). Despite no significant alterations were found regarding IL-17A production by peripheral ILC3 from clustered IBD samples, there is a significant decrease on IL-17A levels by ILC3 from patients with active disease (Figure 36A). This goes in line with the hypothesis that ILC3 more prone to produce IL-17A are increased *in situ* (Figure 35A).

Although without reaching statistical significance, IBD patients tend to have decreased frequencies of total T and Th17 cells in the blood (Figure 36B,C), which is in accordance with the increased lymphocytic inflammatory infiltrate in the gut (Figure 35B-D). Even though no differences were seen regarding Th17-derived IL-17A and GM-CSF, we observed a significant increase in IL-22 (Figure 36C). With this, we hypothesise that Th17 cells capable to produce more IL-22 and, therefore, balance IL-17A production to avoid exacerbated inflammation, are decreased in the colon of IBD patients. So far, that tendency seems to be evidenced in colon biopsies (Figure 35D) but, as mentioned above, that remains to be further confirmed in a higher sample size.

We previously observed a decrease in Treg cells' frequency in inflamed mucosa (Figure 35E); in the periphery, no significant alterations were found for Treg cells despite following the same tendency for decrease (Figure 36D), as reported by other studies [188, 189]. Therefore, these results suggest that IBD patients have an overall decline in the proportion of anti-inflammatory cellular counterparts.

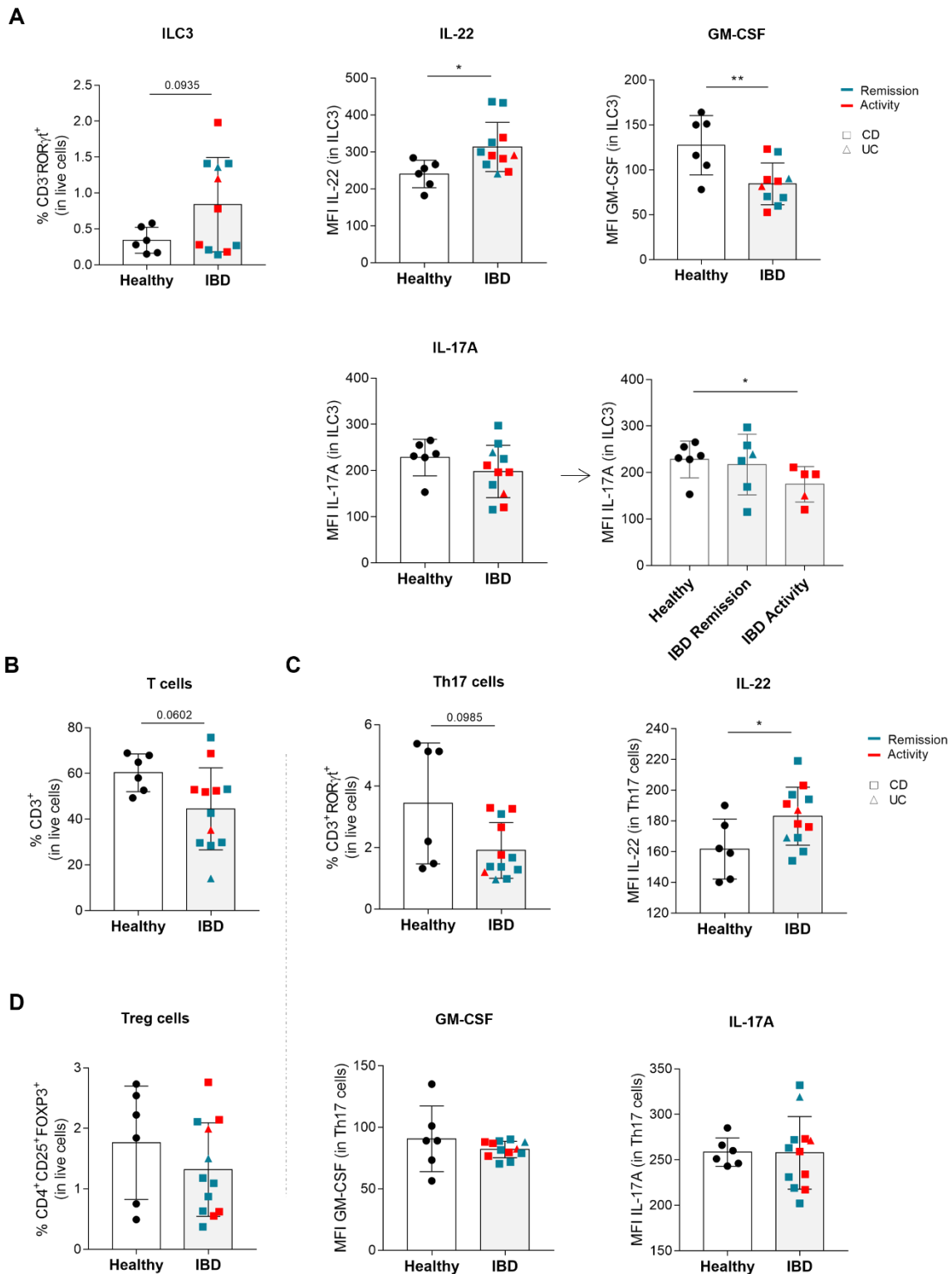


Figure 36| Peripheral blood of IBD patients seems to be enriched in ILC3 and depleted in Th17 and Treg cells. Frequencies of (A) ILC3, (B) T, (C) Th17 and (D) Treg cells in PBMCs from healthy individuals or IBD patients. (A) and (C) contain the MFI of IL-22, GM-CSF and IL-17A inside ILC3 and Th17 cells, respectively. Each dot represents one individual biological sample, presented as mean $\pm$ SD. Blue: remission; red: activity; $\square$: CD; $\triangle$: UC. Statistically significant values are: * $p \leq 0.05$, ** $p \leq 0.01$.

Our peripheral characterization was extended to other immune cell populations known to take part in inflammation in IBD. Our results showed that the frequencies of circulating NK and NKT cells, well-known producers of pro-inflammatory cytokines such as IFN- γ and TNF- α [190, 191], are significantly decreased in IBD patients comparing with HC (Figure 37A,B). These differences suggest an enrichment of these cells in the gut, where inflammation is occurring, as seen by other studies [192, 193]. This needs to be validated in the future in mucosal tissue samples.

DCs are antigen-presenting cells that play a fundamental role in initiating immune responses [194]. We found that DCs are significantly increased in the blood of IBD patients (Figure 37C), possibly as a consequence of an increased recirculation from the gut to secondary lymphoid organs. DCs are equipped with glycan-binding receptors that enable the recognition and response to pathogens, namely mannose-receptor (MR; CD206) and DC-SIGN (CD209). In this sense, we assessed the expression of these receptors on DCs by the mean fluorescence intensity (MFI). While we found no differences in the MFI of MR between DCs from IBD patients and HC, circulating DCs from patients have a significant decrease of DC-SIGN (Figure 37C). This is particularly relevant since it is described that the expression of DC-SIGN is augmented in immature DCs and downregulated on mature and activated DCs, that migrate from inflammation sites to lymphoid tissues such as the lymph nodes [177]. This reinforces that the identified circulating DCs of IBD patients are activated DCs probably coming from the gut and homing to lymphoid tissues.

Overall, these results demonstrate that IBD patients present several alterations regarding the glycosylation and immune profiles that may contribute to its pathogenesis.

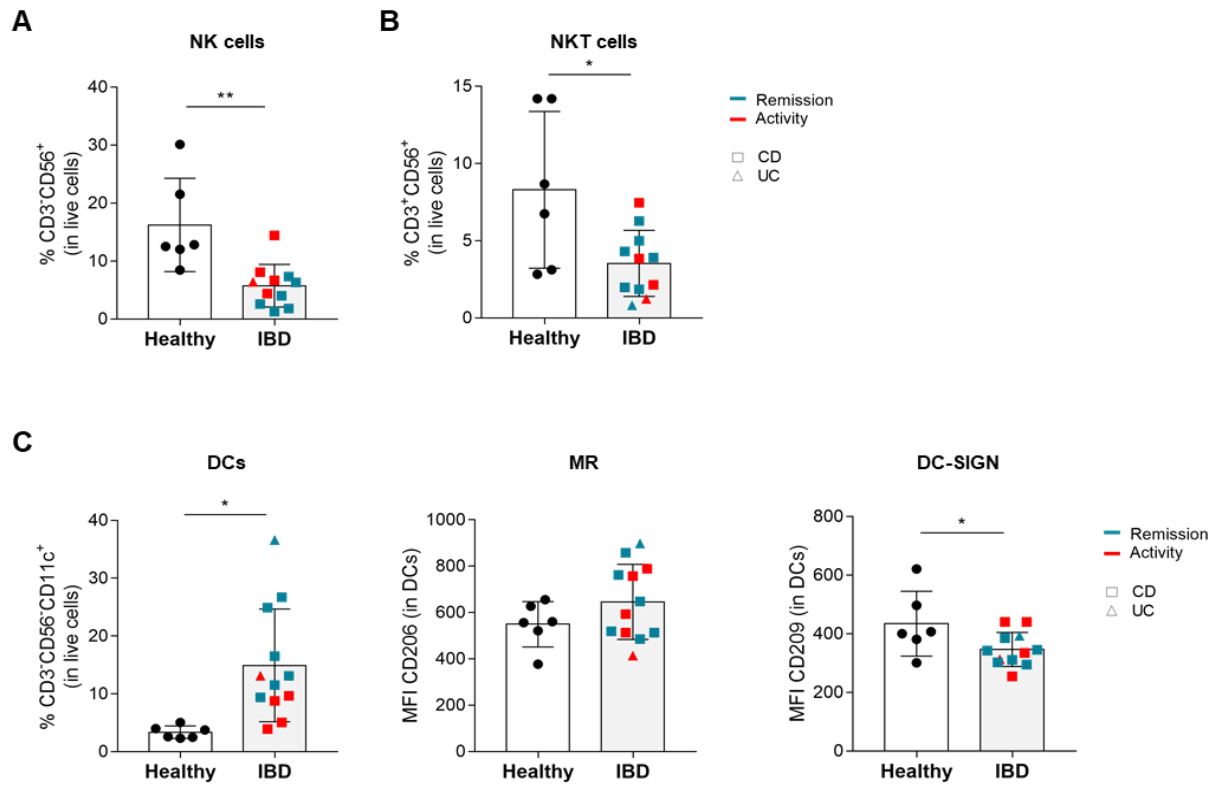


Figure 37| Peripheral blood of IBD patients is depleted in NK and NKT cells and is enriched in DCs.

Frequencies of **(A)** NK and **(B)** NKT cells in PBMCs from healthy individuals or IBD patients. **(C)** Frequency of DCs and MFI of MR and DC-SIGN inside this population, in the blood of healthy individuals or IBD patients. Each dot represents one individual biological sample, presented as mean $\pm$ SD. Blue: remission; red: activity; $\square$: CD; $\triangle$: UC. Statistically significant values are: * $p \leq 0.05$, ** $p \leq 0.01$.

Chapter IV

Discussion

IBD is a chronic immune-mediated disorder of the gastrointestinal tract that, due to frequent relapsing and the absence of an efficient treatment, accentually aggravates patients' quality of life. Although it is known that aberrant immune responses in this disease arise from complex interactions between genetics, the microbiome and the immune system, the underlying mechanisms are still unclear. Therefore, an improved understanding of the mechanisms involved in IBD pathogenesis is mandatory to tackle its clinics.

Glycosylation is a post-translational modification that occurs in the majority of proteins from mammalian cells [195]. In IECs, glycans are intimately associated with the symbiosis between host and commensals, serving as energy sources and interaction sites to microorganisms [3]. In the immune system components, glycans are involved in many biological functions such as pathogen recognition, immune tolerance, as well as T cells activity, differentiation, and proliferation [2]. Our group has previously evidenced the impact of glycosylation in IBD pathogenesis, finding that T cells from the colonic mucosa of UC patients display impaired GnT-V-mediated β 1,6-branched *N*-glycans in TCR (due to reduced *MGAT5* expression), which leads to a decreased threshold for T cell activation and, therefore, an hyperactivation of T cells [5]. Moreover, the group has shown that polymorphisms in the glycogene *MGAT5* are associated with UC severity and the need for biologics [154]. In this sense, our first aim was to unveil how an altered host glycoprofile modulates gut microbiota and the immune response associated with intestinal inflammation.

Herein, we demonstrated that the deficiency in branched *N*-glycans harboured by *Mgat5*-KO mice imposes a dysbiotic microbiota composition, with a decrease in bacteria from the Firmicutes phylum (which is described to display a protective function in intestinal barrier and to induce Treg cells [155, 196]). This disruption contributes to the increased susceptibility of KO mice to develop severe colitis, presenting more extensive inflammatory infiltrates and abnormal glandular morphologies in the colon. Nevertheless, we have found that, besides dysbiosis, the exacerbated inflammation in KO mice lies in an abnormal immune profile present in the colon. Particularly, our results have shown that KO mice have decreased levels of ILC3 and ILC3-derived IL-22, both prior and after colitis-induction. In fact, IL-22 production by other populations (Th17 and $\gamma\delta$ T cells) is also impaired. Interestingly, *Il22* expression is either unmodified or increased in the colon of KO mice, in homeostasis and upon inflammation (day 11); however, not only intra but also extracellular protein levels of this cytokine are diminished. As glycosylation is an important post-translation process that generally increases proteins' stability [197], and IL-22 has three *N*-glycosylation consensus sites that can be modified with *N*-glycans

[198], we question whether the deficiency in branched *N*-glycans is directly impacting IL-22 stability in KO mice.

Prior to colitis, we have also found decreased frequencies of the typically pro-inflammatory population of Th17 cells in the colon of KO mice. In fact, we observed a reduction in all analysed immune populations in this timepoint, in comparison with WT mice. We hypothesise that this occurs due to an enrichment of myeloid cells in the gut as a result of their crucial role in gut defence, as KO mice are dysbiotic even before an inflammatory trigger. This requires further analysis to validate our hypothesis. As a consequence of the depletion of Treg cells in homeostasis, colonic explants of KO mice have decreased levels of IL-10 when compared with WT mice, hampering an anti-inflammatory response necessary to control the exacerbated inflammation in these mice. Nevertheless, under inflammation, KO mice are enriched in lymphocytic infiltrates of Th17 and $\gamma\delta$ T cells, with both producing more IL-17A and the latter producing less IL-22, which contributes to a more pro-inflammatory environment due to unbalanced amounts of IL-17 and IL-22. To worsen this environment, IL-10 remains depleted in the colon of KO mice.

In this thesis, we have described for the first time that, both in homeostasis and upon inflammation, KO mice have an impaired expression of *Nod2*, a susceptibility gene for human CD [199, 200], although not reaching statistical significance. *Nod2* is a sensor of the microorganisms, as it binds to small peptides derived from the peptidoglycan [201], and is present in most gut immune and epithelial cells. Upon a stimulus, *Nod2* activation on DCs leads to the production of IL-1 β and IL-23 [202], which stimulates the production of IL-22 by ILC3 [68]. Besides the fact that *Nod2* enhances ILC3-derived IL-22, ILC3-derived IL-22 promotes the expression of *Nod2* [68]. Therefore, there seems to be a positive feedback loop between IL-22 and *Nod2* that in KO mice is impaired, hindering microbiome sensing, which is in accordance with the dysbiotic phenotype of these mice.

Aside from enhancing *Nod2*, it has been described that ILC3-derived IL-22 promotes the addition of α 1,2-fucose to epithelial cells by *Fut2*, which, as mentioned, is intimately associated with gut protection [124]. Supporting our results showing decreased IL-22 levels in KO mice, we have demonstrated that in homeostasis these mice also have a downregulation of *Fut2* in the colon, potentially in the epithelium, which could be further analysed. Aside from fucosylation and the absence of branched *N*-glycans, the abnormal glycosylation profile was also seen regarding sialylation, but this time upon inflammation, as after colitis-induction KO mice display increased α 2,6-sialic acid on the epithelium. As this carbohydrate is preferentially used by several pathogenic microorganisms, the increased availability of sialic acid may foster the overgrowth of deleterious bacteria [161, 162]. Therefore, these compromised immune and glycome

profiles may be intimately associated with the reported dysbiosis and inflammation in KO mice, although it is still unknown what is cause and what is consequence.

The relevance of dietary supplementation is nowadays undeniable in the context of IBD amelioration. Indeed, studies have shown that implementing fibres, polyphenols, and fatty acids to IBD patients can improve their quality of life and incite clinical remission [203]. Moreover, clinical trials have revealed that probiotics and prebiotics are capable of preventing relapse and of ameliorating IBD activity [204], although additional trials need to be performed. Previously, our group has observed that dysbiotic KO mice are depleted in bacteria capable to synthesize carbohydrates further used in the hexosamine pathway (manuscript in preparation). In this sense, our second aim was to assess whether metabolic supplementation of the hexosamine pathway with GlycanC1 could overcome the basal privation consequence of branched *N*-glycans scarcity and attenuate colitis severity in KO mice, envisioning clinical applications. Besides supplementing KO mice, GlycanC1 was also administered to WT mice to infer whether it could be beneficial to animals with no baseline privation, which we could then transpose to IBD patients with no genetic *MGAT5* or branched *N*-glycans susceptibility. GlycanC1 was administered for five weeks, since we knew that dysbiosis was playing a pivotal role in this interplay and our group has already observed that this period is sufficient to modulate the microbiota (manuscript in preparation). Interestingly, we saw for the first time that oral administration of GlycanC1 prior to colitis induction ameliorates the course of the disease, as well as it improves colonic glandular architecture and inflammatory infiltrates in mice from the two genotypes, opening the way for its usage as a prophylactic strategy in clinics. Dissecting what was being modulated by GlycanC1 that could justify the protective phenotype observed in supplemented mice, we found that GlycanC1 interferes with glycosylation. Indeed, GlycanC1 has shown to be able to restore *Fut2* expression in the colonic tissue of supplemented KO mice to values even higher than those seen in WT mice, prior to colitis induction. Additionally, at day 11, supplemented KO and WT mice also displayed that tendency, which may contribute to gut homeostasis and protection against pathogens.

Although no other major glycosylation differences were observed prior to colitis induction, after the DSS treatment KO+GlycanC1 mice tend to have lower levels of α 2,6-sialic acid in the epithelium, hampering the overgrowth of pathogenic bacteria, while WT+GlycanC1 mice have decreased levels of epithelial high-mannose *N*-glycans. This latter evidence could lead us thinking that branched *N*-glycosylation was being promoted by GlycanC1 (and therefore, there would be a decrease in high-mannose glycans) but, in fact, a significant decrease in branched *N*-glycans was detected in the epithelium of supplemented WT mice. Aside from epithelial cells, all analysed immune populations

seemed to be depleted in β 1,6-GlcNAc branched *N*-glycans in these mice, comparing with control WT. Since GlycanC1 supplementation was stopped at the initiation of DSS treatment, we hypothesise that GlycanC1 modulates the microbiome in a way that promotes the growth and colonization of bacteria that use GlycanC1 as a nutrient source; by the time GlycanC1 is not supplied to the system, these microorganisms may metabolize other carbohydrates from the hexosamine pathway, depleting the availability of these for branched *N*-glycosylation in the host. Nevertheless, this needs to be further explored.

Previous research findings revealed that the microbiota composition is capable of modulating innate and adaptive immunity [205]. Digestion of dietary fibres by several bacterial families leads to the production of SCFAs: acetate, butyrate, and propionate. Studies have shown that IBD patients present decreased mucosal and faecal levels of SCFAs [206], which may also occur in KO mice as they are dysbiotic. In this thesis, we observed that GlycanC1 leads to several immune modifications that can be explained by a modulation of the microbiota in a way that promotes the overgrowth of bacteria capable of producing SCFAs, positively impacting gut homeostasis, which should be investigated in the future. In line with findings demonstrating that butyrate increases the expression of *Nod2*, particularly in IECs [207], our results have shown that, prior and after inflammation, *Nod2* is upregulated in the overall colonic tissue of supplemented KO mice, which have a baseline impairment in its expression. Additionally, supplemented WT mice also display that tendency at day 11. Nonetheless, the upregulation of *Nod2* can also be explained in light of IL-22, as it seems to be increased in the colon of supplemented mice and, as mentioned, also promotes the production of *Nod2* [68]. In future experiments, it would be interesting to explore in which cells *Nod2* is being promoted, as we hypothesise that not only it may be occurring in IECs but also in myeloid cells as DCs.

Importantly, in this study we observed that GlycanC1 increases the levels of colonic ILC3 and ILC3-derived IL-22. Supporting the theory that GlycanC1 modulates the metabolites in the colon of supplemented mice, studies have shown that propionate and acetate lead to ILC3 proliferation and its production of IL-22 [172]. Herein, we have analysed whether *Gpr43*, one of the majors SCFAs' receptor (SCFAR) in colonic ILC3 [208], was overexpressed upon supplementation, finding no transcriptional differences between GlycanC1-treated and -nontreated groups. However, an improved sensing of SCFAs can occur through increased expression of other receptors, as *Gpr41*, *Gpr109a* and *Olf78* [209], that remain to be analysed, or simply through increased colonic levels of SCFAs. Aside from SCFAs, ILC3 can directly sense metabolites derived from microorganisms through its surface aryl hydrocarbon receptor (AhR), that binds to indole derivatives. These interactions also promote the maintenance of ILC3 and secretion of

IL-22 by these cells, promoting the integrity of intestinal mucosa [210]. Therefore, it becomes evident that the microbiome must be playing a pivotal role in gut dynamics upon glycans supplementation, being mandatory to perform metabolite analysis in the future.

Aside the aforementioned, other important beneficial immune modifications occur upon GlycanC1 supplementation. In homeostasis, GlycanC1 significantly reduces the expression of *Il22ra2* in KO mice, predisposing to a greater availability of homeostatic IL-22, while decreasing the frequency of Th17 cells and increasing IL-10 levels in the colon of WT mice. Once more, the increase in IL-10 can be explained in light of higher levels of SCFAs, or increased microbiota-dependent production of IL-23 and IL-1 β in the colon [68, 125]. Interestingly, under inflammation, GlycanC1 modulation towards protection was more evident than in homeostasis, demonstrating that although at baseline glycans supplementation ameliorates the environment in the gut, the major protective impact conferred by GlycanC1 occurs upon an inflammatory insult as the DSS, allowing an improved response to colitis. Particularly, at day 11, besides increased levels of ILC3 and ILC3-derived IL-22, GlycanC1-treated KO mice display increased ILC3-derived GM-CSF, which has been reported to occur under the stimulation of IL-23 and/or IL-1 β in a microbiota-dependent manner [68]. With regard to WT mice, GlycanC1 increases the abundance of IL-22 and Treg cells frequency in the colon, the latter described to occur in response to higher levels of SCFAs perceived by Gpr43 on the surface of Treg cells [125].

A defective mucus layer and barrier integrity are identified in IBD in part due to reduced expression of AMPs [84], necessary to hinder the entrance of pathogens to colon mucosa. Reg3 γ is an AMP, CTL that displays bactericidal activity against Gram-positive bacteria [211]. In this work, no differences were seen on the expression of *Reg3g* between WT and KO mice; nevertheless, *Reg3g* tends to be upregulated in the colonic tissue of GlycanC1-treated mice, both prior and after colitis induction. This can be explained by different aspects. Firstly, Reg3 γ can be enhanced in consequence of the increased *Nod2* expression, as it is described that the activation of Nod2 signalling promotes the secretion of AMPs [68]. Secondly, studies have shown that both ILC3-derived IL-22 and butyrate (which we have not yet explored) promote the induction of AMPs by epithelial cells and macrophages, respectively [124, 212]. Importantly, this enhancement of *Reg3g* caused by GlycanC1 must contribute positively to the restoration of a healthy microbiome, as it has been described that the overexpression of *Reg3g* in mice leads to the enhancement of beneficial bacteria *Lactobacilli* in the gut [213].

Finally, our results showed that GlycanC1 impacts the epithelium integrity of mice of the two genotypes by increasing *Cldn1* expression in KO mice, although not reaching

statistical significance, and *Cldn2* expression in WT mice, in homeostasis. While *Cldn-1* is typically described as a sealing claudin that increases barrier integrity [173], the role of *Cldn-2* is still controversial, being described either as pore-forming and promoter of barrier integrity [174, 175]. Although the effects of SCFAs in *Cldns* expression need to be consolidated, the literature shows that butyrate is capable to upregulate *Cldn1* [214], once more spotlighting a putative microbiome modulation accounting for an overproduction of SCFAs. Conversely, the upregulation of *Cldn2* can be explained by the increased colonic expression of IL-22 in WT+GlycanC1 mice, as this has been pointed in literature [215].

Notably, contrary to colon analysis, our study of the MLNs revealed that only mild peripheral changes occur due to GlycanC1 supplementation, evidencing that the effect of this compound is essentially local.

Initially, we were hypothesising that GlycanC1 supplementation would increase the levels of branched *N*-glycans in KO mice, promptly modulating the microbiome and repairing the exacerbated inflammation in these mice. Although inflammation was indeed controlled, at day 0 the increase in branched *N*-glycans was not observed, therefore, it would be interesting to replicate this experiment extending the administration of GlycanC1 to more than 5 weeks to check whether *N*-glycosylation is promoted. Surprisingly, at day 11, WT+GlycanC1 mice presented decreased expression of branched *N*-glycans, which we hypothesise it occurred in consequence of a microbiota modulation in a way that when deprived of GlycanC1 (day 0), microorganisms used other carbohydrates from the hexosamine pathway, decreasing the availability of these for host glycosylation. If our hypothesis is confirmed, the decline in branched *N*-glycans may be avoided with a continuous and sustained administration of GlycanC1 during the treatment with DSS. Our main goal is to transpose this assay to the clinics, envisioning patients orally taking GlycanC1 as a prophylactic approach to attenuate disease severity in periods of flares. If its capacity to modulate the microbiome is verified, GlycanC1 could be used as a prebiotic, which are in spotlight as adjuvants in IBD prevention and treatment, conferring protective effects to the host [216]. Therefore, the rationale of a continuous administration of GlycanC1 is even more interesting, as it is not expected that patients would stop the supplementation when needing it the most during crisis. Moreover, to mimic human cyclic active and remission stages, it would be of interest to perform a chronic DSS-induced colitis treatment in spite of an acute model. Finally, although polymorphisms in *MGAT5* impose an increased susceptibility to colitis development [6], patients are not fully knockout for *MGAT5*. Therefore, heterozygous *Mgat5*^{+/-} mice could be a model more similar to humans, reflecting clearer patients' disease phenotype. In this case, we can hypothesise that supplemented *Mgat5*^{+/-} mice

would display an intermediate disease severity comparing with GlycanC1-treated WT and KO mice, which remains to be further evaluated in future experiments.

To translate the findings gathered in murine experimental models to a clinical setting and clarify how glycosylation and immune alterations can contribute to IBD pathogenesis, we characterized the host glycosylation and immune profiles of patients with CD and UC.

Our preliminary results demonstrated several abnormalities in the glycosylation profile present *in situ* and in the periphery, which may certainly be detrimental for IBD. Although no glycosylation alterations were observed in epithelial cells, immune Th and Treg cells from the inflamed colon of IBD patients appear to be deficient in glycans, particularly β 1,6-GlcNAc branched and high-mannose *N*-glycans, and α 2,6-sialic acid. Supporting the observed decrease in β 1,6-branched *N*-glycans, *MGAT5* expression is also tendentially impaired in the colon of IBD patients, in line with the previous reports from our group [5]. This is very relevant as, upon activation, T cells from healthy individuals upregulate *MGAT5*, increasing tetra-antennary *N*-glycans in the TCR that are typically elongated with polyLacNAc [140]. Demetriou and colleagues demonstrated that this is necessary for a high affinity binding to galectin-3, forming a lattice that reduces TCR clustering and, therefore, increases the threshold for T cell activation [217, 218]. Additionally, the binding of galectins to branched *N*-glycans in CD25 and CTLA-4, a marker of Treg cells and a T cell receptor that represses immune responses, respectively, avoids their endocytosis and promotes immune tolerance [2]. Therefore, although we haven't explored which proteins were harmed, we predict that a compromised branched *N*-glycosylation is contributing to an inflamed environment.

Our results in the second subchapter of the results section have also suggested that colonic fucosylation is hindered in IBD. We have shown that *FUT2* tends to be downregulated in patients' inflamed colon, as reported in the literature [219], which supports a dysbiotic environment as epithelial α 1,2-fucose is an important nutrient source and adhesion site to commensal bacteria, while also protecting against infections with pathogens [124]. Indeed, the literature shows that non-functional mutations of *FUT2* in IBD patients increases their susceptibility to disease due to intestinal microbiota modulation [220-222]. Regarding α 1,6-fucosylation by *FUT8* (core fucosylation), in the TCR it is required for Th cell activation as it ameliorates the interaction between the TCR and MHC-II [223]. In this sense, it promotes the production of inflammatory cytokines and the induction of colitis in mice [224]. In line with reports showing that in IBD core fucosylation is increased in T cells TCR [224], our results showed that the inflamed colon of IBD patients tends to have a higher expression of *FUT8*. This way, core fucosylation blockade can be pointed as a potential therapeutic approach to treat IBD.

Modifications of the glycoprofile in IBD patients were not as evidenced in the periphery as in the colon, which may be explained by the fact that the dynamic nature of these modifications is influenced by the inflammatory environment, highly perceived *in situ*. Indeed, it has been demonstrated that pro-inflammatory cytokines modulate cells' glycosylation by regulating the expression of glycosyltransferases [225]. Nevertheless, we have found that circulating Th cells from patients with active disease have increased levels of terminal α 2,6-sialic acid. In this work we were unable to dissect which proteins were upper sialylated, but this is particularly relevant as α 2,6-sialic acid is described to impair galectins-binding to branched *N*-glycans [178]. Due to the above mentioned, despite the proteins affect, as TCR, CD25 or CTLA-4, this enhancement may be associated with disease pathogenesis. The fact that this was only observed in patients with active disease and not in those in remission demonstrates that important glycosylation shifts occur between disease stages.

In this thesis, our preliminary results also demonstrated that IBD patients develop an *in situ* inflammatory environment that appears to result from a differential cellular recruitment from the periphery to the colon. We have seen that, under inflammation, the colon of IBD patients appears to be depleted in protective populations, NKp44⁺ILC3 and Treg cells, while being enriched in typically pro-inflammatory cells, as Th17, as reported by other studies [226, 227]. An opposite profile was found in the blood of IBD patients, in which ILC3 and Th17 cells were tendentially increased and decreased, respectively.

More extensive analysis showed that peripheral ILC3 from IBD patients have higher intracellular levels of IL-22 and lower of IL-17A than peripheral ILC3 from HC. From this, we could infer that ILC3 more prone to produce IL-22 are those particularly impaired to be recruited to the colon, hindering its protective *in situ* effect, while those with the highest capacity to produce IL-17A are enriched in the colon of patients with active disease, exacerbating inflammation. Although this was not evident in colon biopsies analysis, it needs to be validated with an increased set of samples. Our results also showed that peripheral ILC3 from IBD patients produce less GM-CSF. The role of this cytokine *in situ* is still controversial, existing some evidence indicating that ILC3-derived GM-CSF recruits pro-inflammatory macrophages to the gut, exacerbating colitis inflammation [228], while others pinpoint that GM-CSF promotes the differentiation of Treg cells and reinforces tolerance [70]. Therefore, it will be paramount to confirm in an increased subset of biopsies whether the peripheral decrease of GM-CSF is not associated with an alteration of its levels in the inflamed colon, as our preliminary results suggested.

In the gut, NK and NKT cells play a dual role. While these cells participate in physiological processes, for example, by producing IFN- γ during intestinal bacterial

infections, amplifying the anti-bacterial immune response, they can also be associated with the pathogenesis of IBD. In fact, a recent clinical trial has shown that inhibiting natural killer group 2D (NKG2D), a receptor present in NK cells that binds to stress-related molecules, induces clinical remission in a subset of CD patients [229]. Additionally, it has been demonstrated that NKT cells from the lamina propria of UC patients produce IL-13, damaging epithelial cells, which if inhibited leads to clinical improvement of UC symptoms [230]. The fact that our results showed that peripheral NK and NKT cells are significantly decreased in IBD suggests that these are being recruited to the colon of IBD patients, as reported by other groups [193, 231], contributing to the exacerbated inflammation.

As antigen-presenting cells playing a pivotal role in initiating immune responses when in presence of pro-inflammatory stimuli [194], we could expect that DCs would be increased in the gut of IBD patients and, consecutively, decreased in the periphery. Yet, circulating DCs were found to be significantly increased in these patients. A possible explanation is that there may be a massive recirculation of DCs that have previously been in the gut, uptaking and presenting antigens, to the secondary lymphoid organs, where they will present antigens to naive T cells and recruit them to the site of inflammation. Indeed, it has been shown that the blood of CD patients has increased amounts of DCs displaying both $\beta 7$ and C-C motif chemokine receptor 7 (CCR7), gut- and lymph node-homing markers, respectively, which reinforces this hypothesis [232]. Supporting this idea that the identified circulating DCs in IBD patients are activated DCs coming from the gut and homing to lymphoid tissues is that DC-SIGN, which is described to be augmented in immature DCs and downregulated on mature and activated DCs that migrate from inflammation sites to lymphoid tissues [177], is significantly decreased in peripheral DCs from IBD patients. Additionally, *CD209* (that codes for DC-SIGN) tends to be downregulated in the inflamed colon of IBD patients, suggesting that DCs in the inflamed colon are as well essentially matured and activated. These hypotheses need to be further validated in colonic biopsies of an extended cohort.

The microbiota is an important mediator of inflammation, with its imbalance leading to the activation of immune responses [205]. Recently, Wang and colleagues have evidenced that modulation of the gut microbiome is a potential therapeutic approach in IBD, as the reestablishment of the microbiota composition through faecal microbiota transplantation from healthy individuals successfully treated immune checkpoint inhibitors (ICI)-associated colitis [233]. Due the intimate association of microorganisms with glycans, as energy sources and interaction sites to commensals and pathogens, glycosylation can modulate microbiota. As our results showed that glycosylation and the immune profiles are altered in IBD, similar to what was observed in dysbiotic KO mice,

in future experiments it would be paramount to characterize the microbiome in patients to unravel distinctive glycosylation-microbiota-immunity signatures in IBD.

Conclusions and Future Perspectives

In this master thesis, we have demonstrated the relevance of glycans in the modulation of the microbiota and the immune environment associated with disease severity.

Within this study it was shown that the deficiency in branched *N*-glycans imposes a dysbiotic microbiota composition in KO mice that may contribute to an increased susceptibility to colitis. It has also come to light that both IBD patients and colitis-susceptible mice have altered glycoprofiles, with emphasis in α 1,2-fucosylation, branched *N*-glycosylation, and sialylation. Concomitantly, the immune profile is impaired, particularly in the protective ILC3/IL-22 axis, hampering gut homeostasis. This can lead us to assume that these components are major contributors of gut pathogenesis that deserve a special focus in further experiments. Importantly, in this work it has been deciphered for the first time that GlycanC1 supplementation is efficient in protecting both KO and WT mice against colitis development, namely by regulating the epithelial barrier integrity in the colon, and restoring ILC3 and IL-22 levels, important for gut homeostasis and protection. Moreover, this project revealed that GlycanC1 ameliorates microbiota sensing by enhancing the expression of *Nod2* that was shown to be impaired in the colon of *Mgat5*-KO mice. Therefore, these findings pave the way for novel therapeutic interventions using glycans supplementation. The proposed model is demonstrated in Figure 38.

In the future, it is essential to validate the presented analyses in an extended human cohort and to evaluate the microbiota composition in patients, in order to establish a correlation between glycosylation, microbiome and immunity that could be used as a biomarker in the clinics, either in prognosis and therapy decision-making, or as an axis to look up to in future research regarding IBD therapeutics. Furthermore, the contribution of GlycanC1 as a putative prebiotic to be employed in IBD clinics should be explored, due to its beneficial properties for intestinal regulation.

Concluding, the data presented in this thesis provides novel background in glyco and immune alterations associated with IBD pathogenesis and highlights pioneering developments in the finding of a prophylactic approach using glycans supplementation to be applied in IBD clinics.

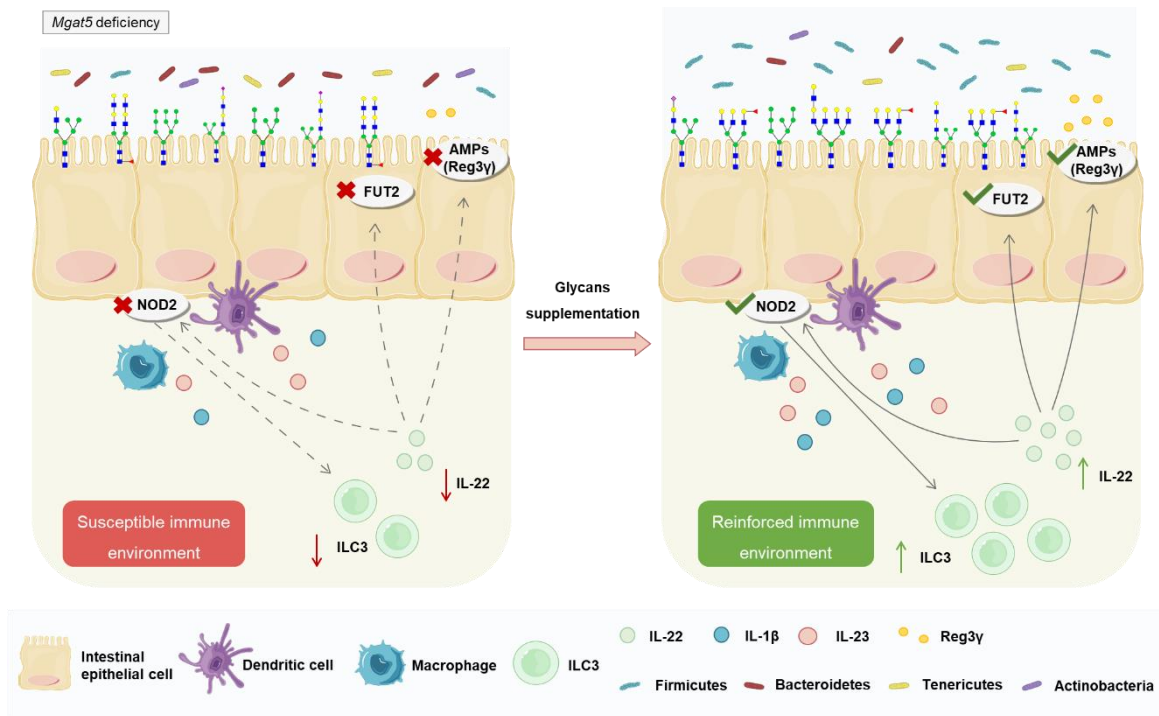


Figure 38| Proposed model. *Mgat5* deficiency is associated with increased susceptibility to colitis correlated with a dysbiotic microbiota composition and an impairment in counterplayers involved in gut homeostasis and protection. The deficiency in branched *N*-glycans is concomitant with a downregulation of the microbial sensing gene *Nod2*, which is associated with the maintenance of ILC3 pool and the production of ILC3-derived IL-22. This process is described in the literature to occur through IL-1β and IL-23 produced by innate cells as DCs and macrophages, yet it remains to be explored in the context of this work. In turn, deficiency in IL-22 drives an altered epithelial fucosylation by the downregulation of *Fut2*, and possibly hinders the production of antimicrobial peptides as Reg3γ, essential to positively regulate the microbiota. Glycans supplementation is able to revert the aforementioned impairments, exerting a beneficial impact in the colonic niche towards a more protective environment against an inflammatory insult. This may occur through a modulation of the microbiome, which represents an emerging topic of research in the context of IBD that should be further investigated.

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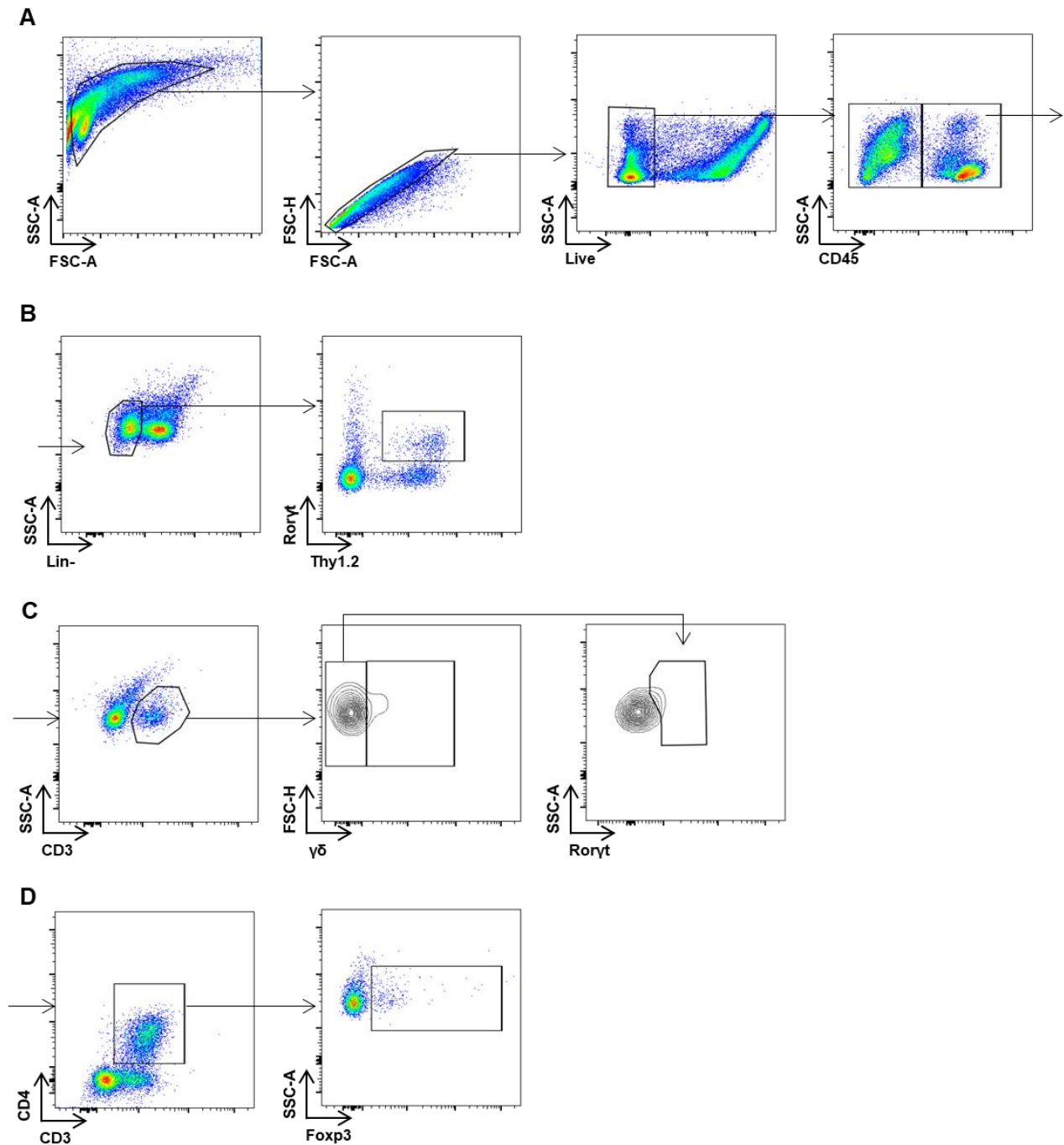
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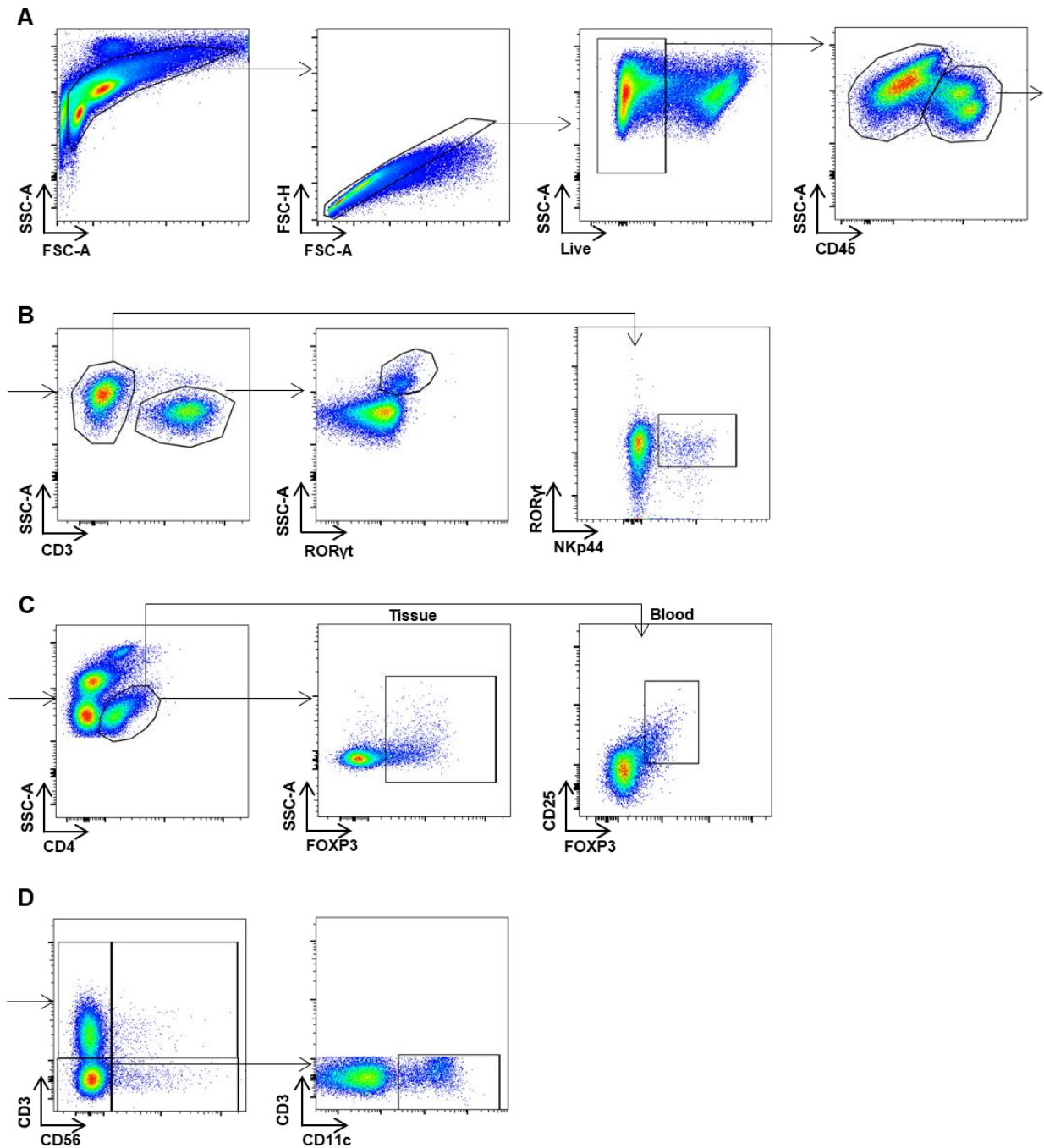
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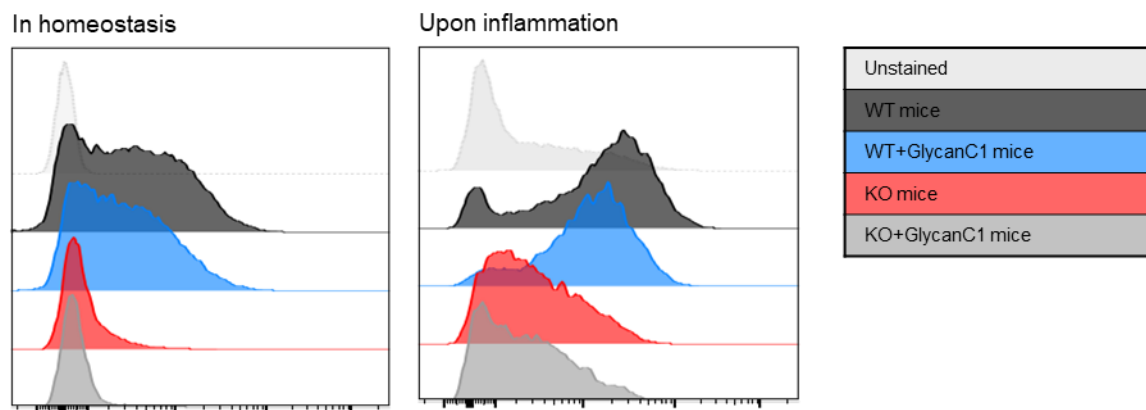
Appendix



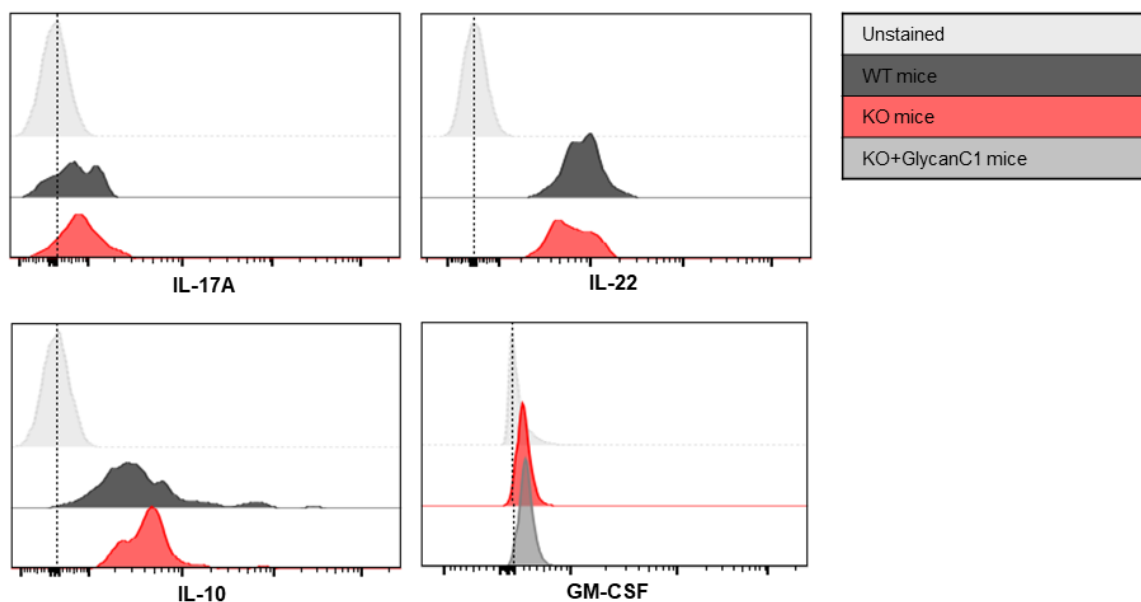
Supplementary Figure 1| Gating strategy used to define epithelial cells, ILC3, Th17, $\gamma\delta$ T and Treg cells in mouse samples. (A) Cells were selected by side scatter area (SSC-A) and forward scatter area (FSC-A) profile, and doublets were excluded based on forward scatter height (FSC-H) and forward scatter area (FSC-A). Live cells were gated as negative for FVD. Epithelial cells were gated as CD45⁻. Hematopoietic cells were gated by CD45 expression, and further diverged in (B), (C) and (D). **(B)** ILC3 were gated with Lin⁻ (negative for CD3, CD19, CD11c and CD11b) and Thy1.2⁺Rorγt⁺. **(C)** Th17 cells were identified as CD45⁺CD3⁺ $\gamma\delta$ ⁺Rorγt⁺ cells, while $\gamma\delta$ T cells were identified as CD45⁺CD3⁺ $\gamma\delta$ ⁺. **(D)** Treg cells were identified as CD45⁺CD3⁺CD4⁺Foxp3⁺.



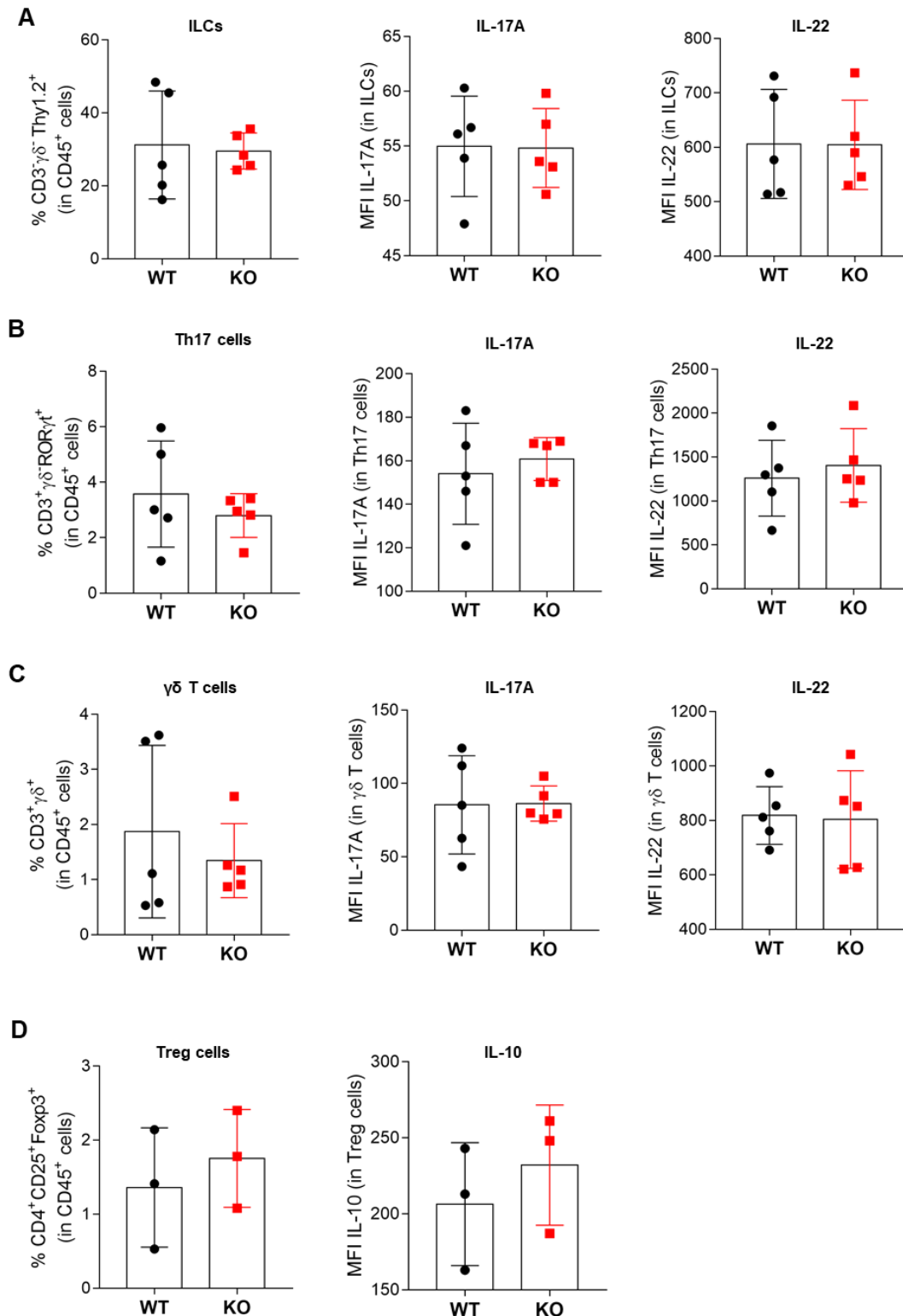
Supplementary Figure 2| Gating strategy used to define epithelial cells, ILC3, Th17, Treg, NK, NKT and dendritic cells in human samples. (A) Cells were selected by side scatter area (SSC-A) and forward scatter area (FSC-A) profile, and doublets were excluded based on forward scatter height (FSC-H) and forward scatter area (FSC-A). Live cells were gated as negative for FVD. In biopsies, epithelial cells were gated as CD45⁻. Hematopoietic cells were gated by CD45 expression, and further diverged in (B), (C) and (D). **(B)** Th17 cells were identified as CD45⁺CD3⁺RORγt⁺ cells, while ILC3 were identified as CD45⁺CD3⁺NKp44⁺RORγt⁺. **(C)** Treg cells were identified as CD45⁺CD4⁺FOXP3⁺ (tissue) or CD45⁺CD4⁺CD25⁺FOXP3⁺ (blood). **(D)** NK cells were identified as CD45⁺CD3⁻CD56⁺, NKT cells as CD45⁺CD3⁺CD56⁺ and DCs as CD45⁺CD3⁻CD56⁻CD11c⁺.



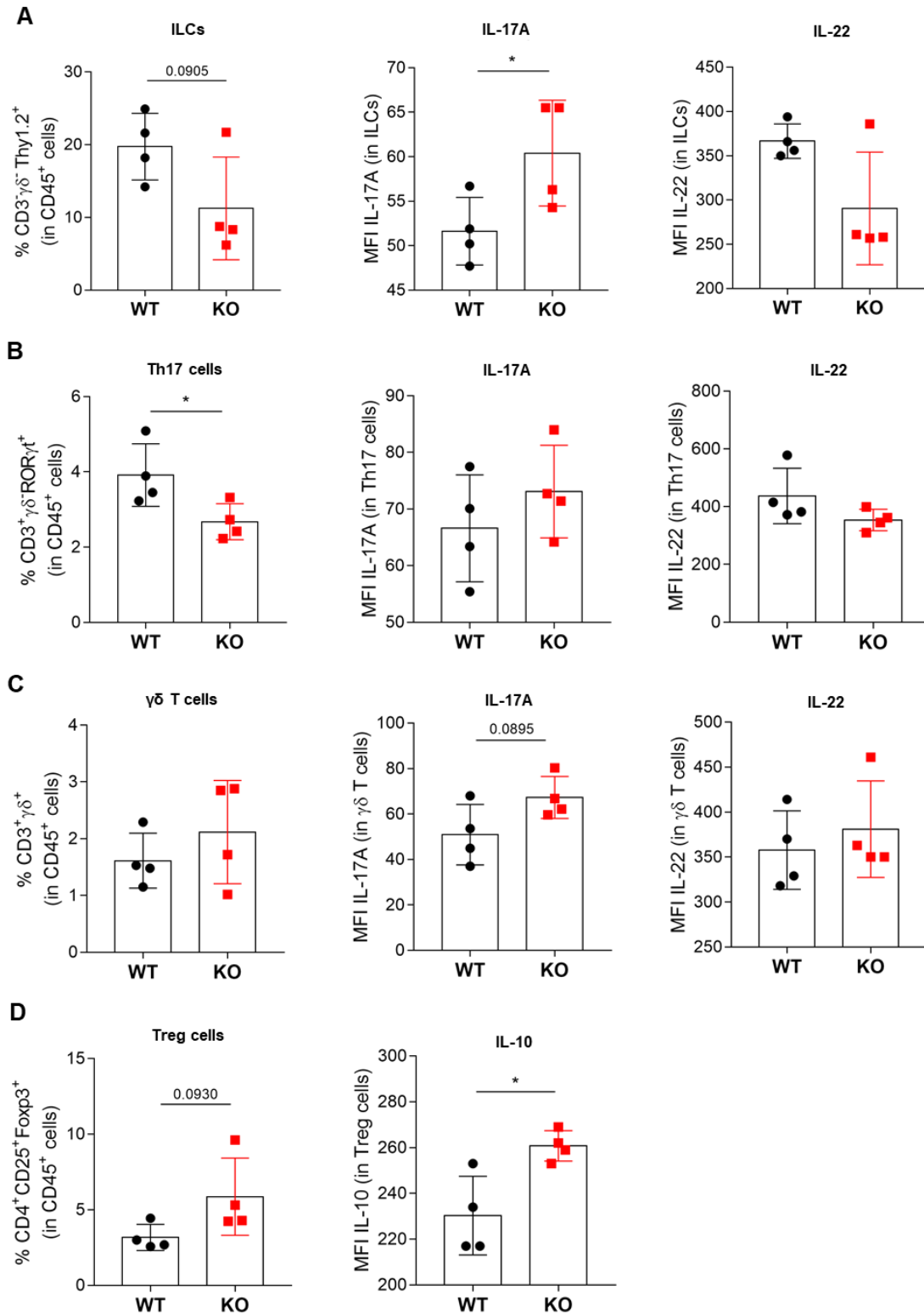
Supplementary Figure 3| Histograms of L-PHA staining in epithelial cells from mice colon. MFI of L-PHA in epithelial cells in the colon of WT, WT+GlycanC1, KO and KO+GlycanC1 mice, in homeostasis and upon inflammation, analysed by flow cytometry. Light gray-shadowed histograms depict the unstained controls. KO and KO+GlycanC1 mice present negligible levels of L-PHA binding. Histograms are representative of L-PHA expression in analysed immune cells.



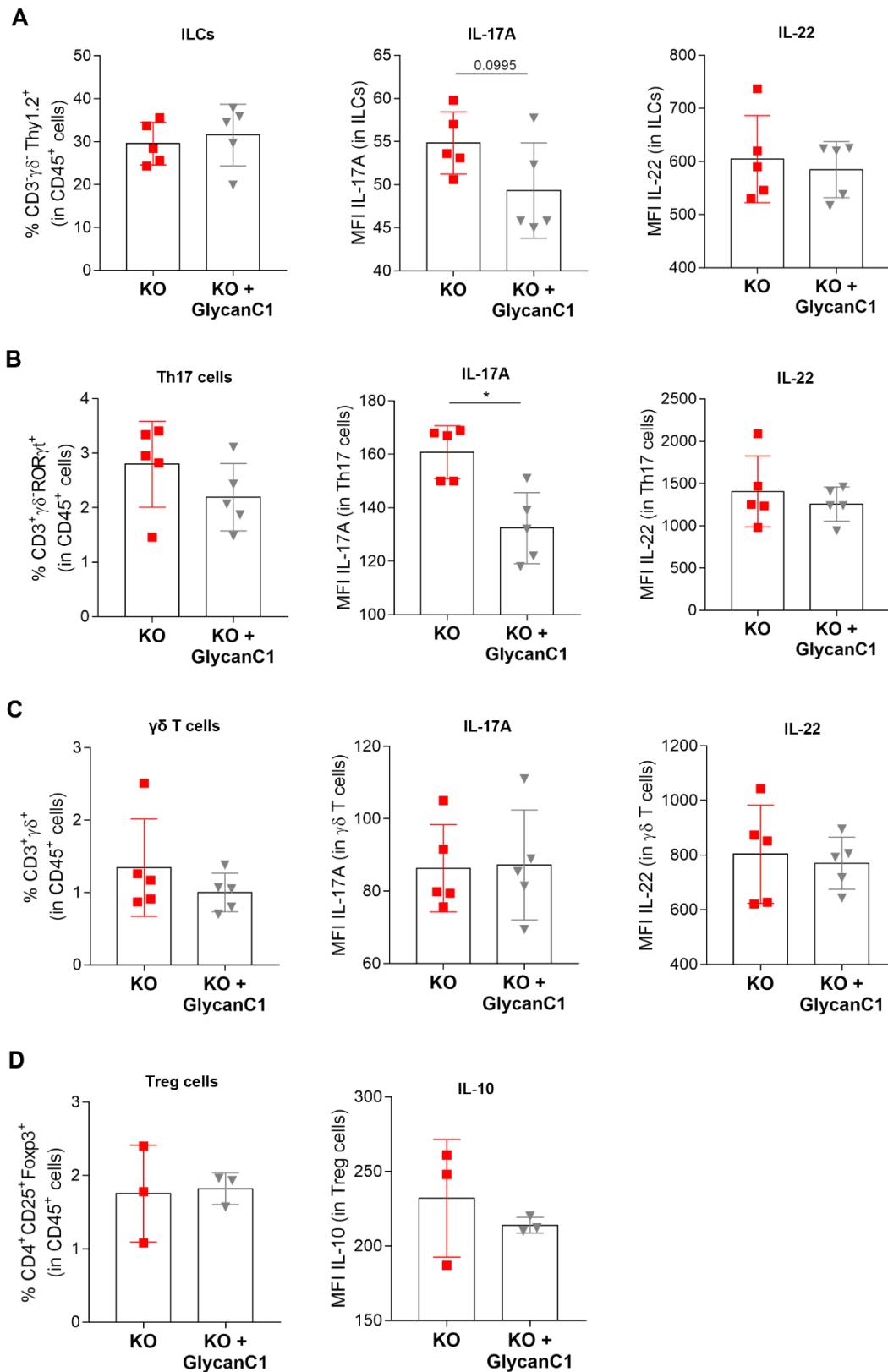
Supplementary Figure 4| Histogram representation of cytokine staining of murine colonic immune cells. MFI of IL-17A, IL-22, IL-10 and GM-CSF in the colon of WT and KO, or KO and KO+GlycanC1 mice are shown as a representation of the positive staining compared to unstained control (light gray-shadowed histograms).



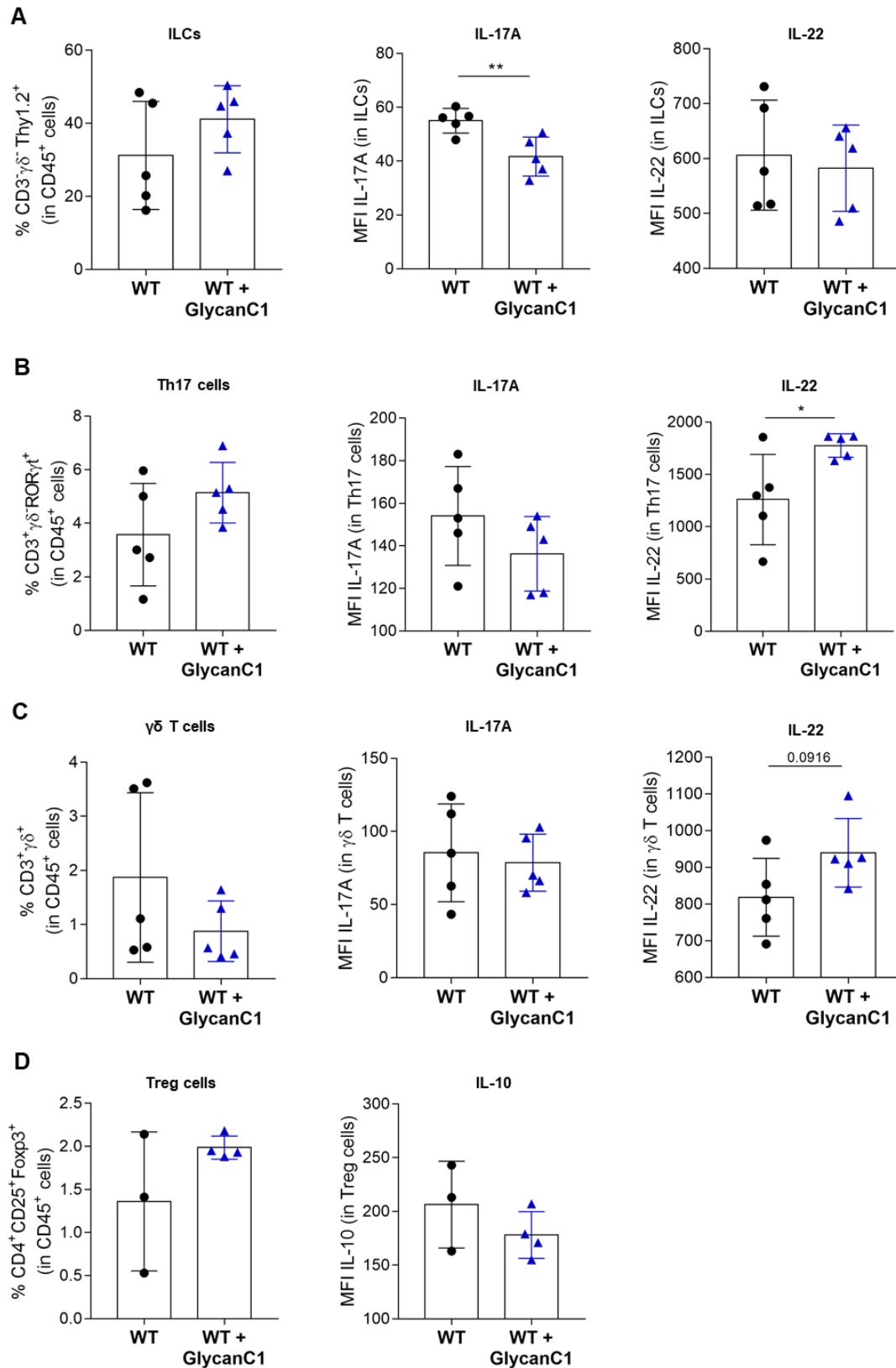
Supplementary Figure 5| Characterization of the immune profile in the MLNs of WT and KO mice, in homeostasis. Frequencies of **(A)** ILCs, **(B)** Th17 and **(C)** γδ T cells, and MFI of IL-17A and IL-22 inside each population. **(D)** Frequency of Treg cells and MFI of IL-10 inside these cells. Each dot represents one individual biological sample, presented as mean ± SD. No statistical significance was reached (significant *p*-value ≤ 0.05).



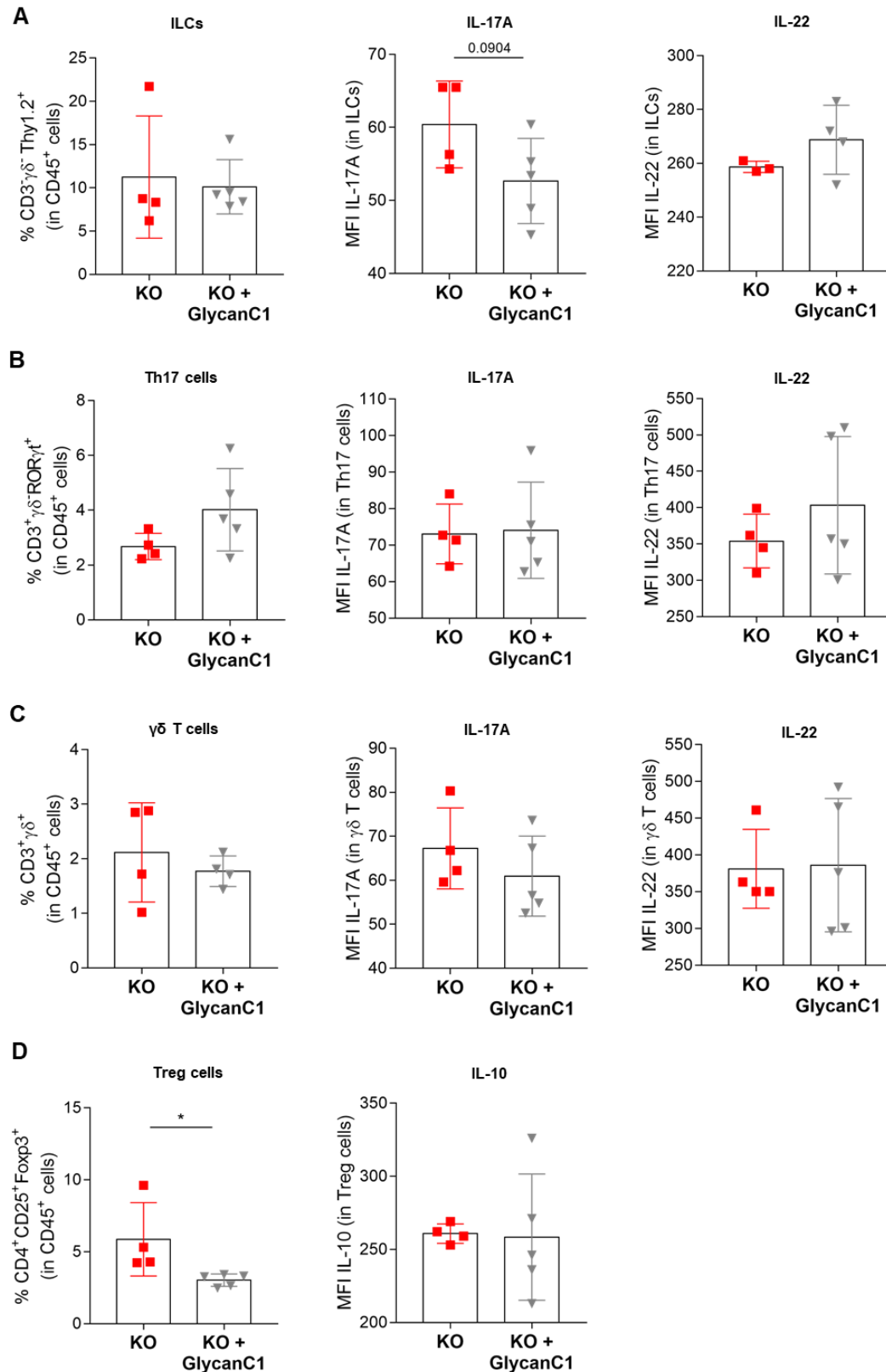
Supplementary Figure 6| Characterization of the immune profile in the MLNs of WT and KO mice, upon inflammation. Frequencies of **(A)** ILCs, **(B)** Th17 and **(C)** γδ T cells, and MFI of IL-17A and IL-22 inside each population. **(D)** Frequency of Treg cells and MFI of IL-10 inside these cells. Each dot represents one individual biological sample, presented as mean ± SD. Statistically significant values are: * $p \leq 0.05$.



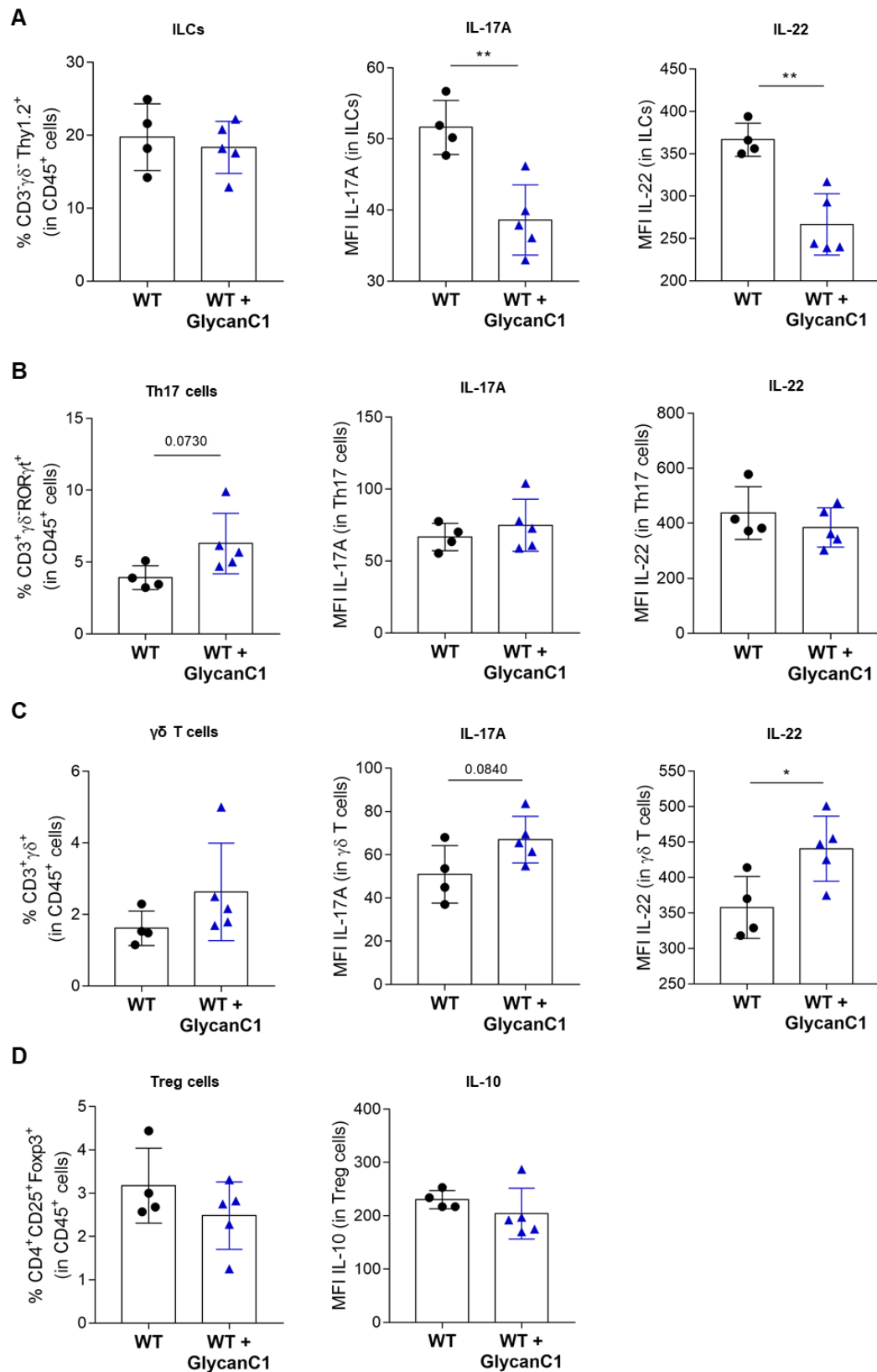
Supplementary Figure 7 | Characterization of the immune profile in the MLNs of KO and GlycanC1-treated KO mice, in homeostasis. Frequencies of **(A)** ILCs, **(B)** Th17 and **(C)** γδ T cells, and MFI of IL-17A and IL-22 inside each population. **(D)** Frequency of Treg cells and MFI of IL-10 inside these cells. Each dot represents one individual biological sample, presented as mean ± SD. Statistically significant values are: * $p \leq 0.05$.



Supplementary Figure 8J Characterization of the immune profile in the MLNs of WT and GlycanC1-treated WT mice, in homeostasis. Frequencies of (A) ILCs, (B) Th17 and (C) γδ T cells, and MFI of IL-17A and IL-22 inside each population. (D) Frequency of Treg cells and MFI of IL-10 inside these cells. Each dot represents one individual biological sample, presented as mean ± SD. Statistically significant values are: * $p \leq 0.05$, ** $p \leq 0.01$.



Supplementary Figure 9| Characterization of the immune profile in the MLNs of KO and GlycanC1-treated KO mice, upon inflammation. Frequencies of **(A)** ILCs, **(B)** Th17 and **(C)** γδ T cells, and MFI of IL-17A and IL-22 inside each population. **(D)** Frequency of Treg cells and MFI of IL-10 inside these cells. Each dot represents one individual biological sample, presented as mean ± SD. Statistically significant values are: * $p \leq 0.05$.



Supplementary Figure 10| Characterization of the immune profile in the MLNs of WT and GlycanC1-treated WT mice, upon inflammation. Frequencies of **(A)** ILCs, **(B)** Th17 and **(C)** γδ T cells, and MFI of IL-17A and IL-22 inside each population. **(D)** Frequency of Treg cells and MFI of IL-10 inside these cells. Each dot represents one individual biological sample, presented as mean ± SD. Statistically significant values are: * $p \leq 0.05$, ** $p \leq 0.01$.

