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Novel insights in IBD pathogenesis:

T cell receptor N-glycosylation as a new molecular mechanism.

ANA MENDES DIAS
TESE DE DOUTORAMENTO APRESENTADA AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR DA UNIVERSIDADE DO PORTO EM BIOTECNOLOGIA MOLECULAR E CELULAR APLICADA ÀS CIÊNCIAS DA SAÚDE

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Novel insights in IBD pathogenesis: T cell receptor N-glycosylation as a new molecular mechanism.

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Aos meus Pais…

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Declaration

The author of this thesis declares that, in accordance with" nº 2, alínea a) do artigo 31º do Decreto-Lei n.º 115/2013 de 7 de Agosto", afforded a major contribution to the design and technical execution of the work, interpretation of the results and manuscript preparation resulting in the following accepted and submitted articles:

Scientific publications

- **Ana M. Dias**, Alexandra Correia, Márcia S. Pereira, Catarina R. Almeida, Inês Alves, Vanda Pinto, Telmo A. Catarino, Nuno Mendes, Magdalena Leander, M. Teresa Oliva-Teles, Luís Maia, Cristina Delerue-Matos, Naoyuki Taniguchi, Margarida Lima, Isabel Pedroto, Ricardo Marcos-Pinto, Paula Lago, Celso A. Reis, Manuel Vilanova, Salomé S. Pinho. (2018) Metabolic control of T cell immune response through glycans in Inflammatory Bowel Disease. *Proceedings of the National Academy of Sciences* 115 (20) E4651-E4660; doi:10.1073/pnas.1720409115.

- **Ana M. Dias*,** Joana Dourado *, Paula Lago, Joana Cabral, Ricardo Marcos-Pinto, Paulo Salgueiro, Catarina R. Almeida, Sandra Carvalho, Sónia Fonseca, Margarida Lima, Manuel Vilanova, Mário Dinis-Ribeiro, Celso A. Reis, Salomé S. Pinho (2014) Dysregulation of T cell receptor N-glycosylation: a molecular mechanism involved in ulcerative colitis. *Human Molecular Genetics* **23**, 2416-2427. **These authors contributed equally to this work.*

- **Invited review**

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- **Book chapter**

  Ana M. Dias, Catarina R. Almeida, Celso A. Reis, Salomé S. Pinho. (2015) Studying T cells N-glycosylation by Imaging Flow Citometry. *Methods in Molecular Biology*. pp 167-176.

Conference proceedings

A.M. Dias, A. Correia, M. Pereira, C.R. Almeida, I. Alves, M. Lima, R. Marcos-Pinto, C.A. Reis, M. Vilanova, P. Lago, S.S. Pinho (2017) DOP081 Glycosylation of T cells: a novel targeted-specific therapeutic strategy in IBD, Journal of Crohn's and Colitis, 11 S74-S75.

Patents Registration

Co-Inventor of the Patent, number 20131000040844. Provisional patent application in the National Institute of Industrial Property, Lisbon, Portugal. **Inventor:** Salomé S. Pinho.

Co-Inventor of the Patent, number 20151000007418. Provisional patent application in the National Institute of Industrial Property, Lisbon, Portugal. **Inventor:** Salomé S. Pinho.

Training in international institutions

2017- Visiting researcher in the laboratory of Prof. Mats Nilsson, at Molecular Diagnostics group, Science for Life Laboratory, Stockholm, Sweden and Prof. Carolina Wählby, Center for Image Analysis, Uppsala University, Sweden, from 1st September to 31st October 2017.
Research topic: Analysis of glyco-genes expression potentially involved in IBD by *in situ* RNA sequencing experiments using Ulcerative colitis biological samples.

2015- Visiting researcher in the laboratory of Prof. Vlatka Zoldos, at Division of Molecular Biology, Department of Biology, Faculty of Science University Zagreb, Croatia, from 20th September to 21st December 2015.
Research topic: DNA methylation and expression analysis of glyco-genes MGAT3 and MGAT5 using pyrosequencing method and quantitative PCR.

2014- Visiting researcher in Disease Glycomics Team of Prof. Naoyuki Taniguchi, RIKEN Advanced Science Institute, Wako, Japan, from 3rd to 28th February 2014.
Research topic: Evaluation of the glycosyltransferases enzymatic activity and preparation of fluorescent oligosaccharide acceptor substrate.

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“You raise me up to more than I can be”

By Brendan Graham

Resumo

A doença inflamatória intestinal (DII) é uma doença crónica do trato gastrointestinal que inclui a Colite ulcerosa (CU) e a doença de Crohn (DC). A etiopatogénese da DII ainda não está suficientemente elucidada, e a doença permanece incurável com a sua incidência a aumentar mundialmente. Não obstante, as estratégias terapêuticas atuais para DII são limitadas pela eficácia reduzida, custos elevados e / ou presença de efeitos tóxicos / colaterais. Uma das principais preocupações da gestão clínica dos pacientes com DII é a fraca caracterização dos mecanismos moleculares subjacentes à doença. Neste estudo demonstrámos que a desregulação da interação coordenada entre N-glicanos ramificados e a actividade das células T é um fator chave e um mecanismo molecular ainda não identificado subjacente à patogénese da DII. Com o reportar deste novo mecanismo, investigámos ainda se este seria um alvo terapêutico na DII. Os resultados obtidos usando células T *ex vivo,* isoladas de biópsias frescas de cólon e sangue periférico de doentes com CU, mostraram que a modulação da glicosilação destas células tem um impacto na supressão da resposta imunológica, nomeadamente na profileração e diferenciação das células T, supressão de citocinas pró-inflamatórias e sinalização pelo TCR. Adicionalmente, a regulação metabólica pela glicosilação resultou no controlo da severidade da doença e supressão da resposta adaptativa *in vivo*, em diferentes modelos de indução de colite em ratinhos que apresentam diferentes genótipos de síntese de N-glicanos ramificados.

Em suma, a presente tese de doutoramento contribuiu para identificar um novo mecanismo de doença em CU e propor uma nova terapia-alvo específica para CU, com potencial de evitar efeitos tóxicos desnecessários e terapias intensivas em DII. A eficácia terapêutica desta nova estratégia terapêutica em DII, aqui descrita, sustentou a avaliação da mesma em estudos clínicos que estão atualmente em curso.

Abstract

Inflammatory bowel disease (IBD) is a chronic disorder of the gastrointestinal tract that encompasses Ulcerative Colitis (UC) and Crohn´s disease (CD). The etiopathogenesis of IBD is far from being fully elucidated, and the disease remains incurable with incidence increasing worldwide. Furthermore, the current therapeutic strategies for IBD are limited by reduced effectiveness, high costs, and/or presence of toxic/side effects. One of the major concerns in the clinical management of IBD patients is the poor characterization of the underlying molecular mechanisms of the disease. In this study we demonstrated that the dysregulation of the coordinated interplay between N-glycan branching and T cell activity is a key factor and a yet uncovered molecular mechanism underlying IBD pathogenesis. The disclosure of this new mechanism set the ground to further investigate whether this altered mechanism can be therapeutically targeted in IBD. The results obtained on *ex vivo* T cells, isolated from fresh colonic biopsies and peripheral blood of UC patients, showed that modulation of glycosylation of these cells can have an impact in the suppression of the immune response, namely in T cell proliferation and differentiation, suppression of pro-inflammatory cytokines and TCR signaling. Additionally, the metabolic regulation by glycosylation also resulted in the control of disease severity and suppression of the adaptive immune response *in vivo,* in different colitis-induced mouse models with different branched glycosylated- encoded genotypes.

Overall, the present doctoral thesis contributed to identify a new disease mechanism in UC, further proposing a new targeted-specific therapy for UC with potential to avoid unnecessary toxic effects and step-up therapies in IBD. The therapeutic efficacy of this new therapeutic strategy in IBD, herein described, pave the way to test it in clinical studies that are currently ongoing.

Abbreviations

5ASA- 5-aminosalicylic acid

ADAb- anti-drug antibodies

ALDH- aldehyde dehydrogenase

ALG- asparagine linked glycosylation

AOM- azoxymethane

APC- antigen presenting cell

ASCAs- anti-Saccharomyces cerevisiae antibodies

Asn - asparagine

CA-CRC- colitis-associated colorectal cancer

CARD15- caspase recruitment domain-containing protein 15

CD3- cluster of differentiation 3

CD-Crohn's disease

CLRs-C-type lectin receptors

CNX - calnexin

COPII- Yeast cytosolic coat proteins

CRC-colorectal cancer

CRP- C-Reactive Protein

CRT - calreticulin

CTLA-4- cytotoxic T-lymphocyte–associated antigen 4

DAI-disease activity index

DC- Dendritic cell

DC- dendritic cell

DC-SIGN- dendritic cell- specific intercellular adhesion molecule-3 grabbing non- integrin

DNA- deoxyribonucleic acid

Dol-P- dolichol-phosphate

DPAGT1- Dolichyl-Phosphate N-Acetylglucosaminephosphotransferase 1

DSS- Dextran Sulfate Sodium

EAE- Experimental autoimmune encephalomyelitis

EDEM- ER Degradation Enhancing Alpha-Mannosidase Like Protein

ER- endoplasmic reticulum

ERAD- ER- associated degradation

EWAS- epigenome-wide association study

FUT2- α(1,2)- Fucosyltransferase

FUT8- α-1,6-Fucosyltransferase

GAGs- glycoaminoglycans

GATA3- GATA-binding protein 3

GII- glucosidase II

GlcNAc - N-acetylglucosamine

GlcNAcT-I (or GnT-I) - N-acetylglucosaminyltransferase I

GlcNAcT-II (or GnT-II) - N-acetylglucosaminyltransferase II

GlcNAcT-III (or GnT-III) - N-acetylglucosaminyltransferase III

GlcNAcT-IV (or GnT-IV) - N-acetylglucosaminyltransferase IV

GlcNAcT-V (or GnT-V) - N-acetylglucosaminyltransferase V

GlcNAcT-VI (or GnT-VI) - N-acetylglucosaminyltransferase VI

GWAS- genome-wide association study

IBD-Inflammatory bowel disease

IECs- intestinal epithelial cells

IFN-γ- interferon-gamma

IgG- immunoglobulin G

IL13R- interleukin

ILC- innate lymphoid cells

ITAMs- immunoreceptor tyrosine-based activation motifs

ITK- IL2 Inducible T-Cell Kinase

JAK- Janus tyrosine Kinase

KDa- Kilodaltons

LAT- Linker for Activation of T-Cells

Lck- lymphocyte-specific protein tyrosine kinase

LPLs- lamina propria T lymphocytes

LPS- lipopolysaccharides

MadCAM1- mucosal vascular addressin cell adhesion molecule 1

Man- mannose

MAN1A1/A2- Mannosidase Alpha Class 1A Member 1/2

MAN1B1- Mannosidase Alpha Class 1B Member 1

MAN2A1-α-mannosidase II

MAPK- Mitogen-Activated Protein Kinase

MGAT5- mannoside acetylglucosaminyltransferase 5 gene

MHC- major histocompatibility complex

miRNAs- MicroRNAs

MPDU1- Mannose-P-Dolichol Utilization Defect 1

mRNA- messenger RNA

MS- multiple sclerosis

NF-Kβ- factor nuclear kappa B

NK- Natural killer

NOD2- Nucleotide-binding oligomerization domain-containing protein 2

OST- oligosaccharyltransferase

pANCAs- perinuclear antineutrophil cytoplasmic antibodies

PD-1- programmed cell death protein-1

polyLacNAc - poly-N-acetyllactosamine

RA- rheumatoid arthritis

RNA- Ribonucleic acid

RORγT- RAR-related orphan receptor gamma

Ser - serine

SMACs- supramolecular activation clusters

SMAD7- SMAD Family Member 7

SMDs- small-molecules drug

ST6Gal-1- ST6 Beta-Galactoside Alpha-2,6-Sialyltransferase 1

T- bet- T-box transcription factor

TCR- T cell receptor

TGF-β- Transforming growth factor beta

Th1- T helper 1

Th17- T helper 17

Th2- T helper 2

Thr – threonine

TLR- toll-like receptor

TNBS- 2,4,6-trinitrobenzene sulfonic acid

TNF-Tumor necrosis factor

TRAF2- TNF receptor-associated factor 2

Treg - T regulatory

UC- Ulcerative colitis

UDP-GlcNAc

UGGT- UDP-Glc: glycoprotein glucosyltransferase

ZAP70- Zeta Chain of T-Cell Receptor Associated Protein Kinase 70

Table of Contents

I

General introduction

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic disorder of the gastrointestinal tract that comprises Ulcerative Colitis (UC) and Crohn´s disease (CD). The incidence of IBD is increasing worldwide, being estimated that over 1 million residents in the USA and 2.5 million in Europe are affected with these diseases (Kaplan, 2015),particularly in north European countries (Figure 1).

In Portugal, the prevalence of IBD has been increasing and the available data based on intestinal anti-inflammatory drugs consumption, estimated an increase from 86 patients per 100 000 persons in 2003 to 146 per 100000 in 2007(Azevedo, et al., 2010) with a similar increase in UC and CD. Interestingly, there is also a distinct distribution accordingly with Portuguese districts, in which, apart from large reference treatment centers like Porto (area of patients studied in this thesis) and Lisboa, districts like Castelo-Branco and Beja also come out in this pharmaco-epidemiological approach (Figure 2). Moreover, in terms of age and gender, in the Portuguese population, the prevalence of UC is higher between 40–64 years old and the prevalence of CD is higher between 17–39 years old. Overall, in Portugal, females had slightly higher prevalence than males.

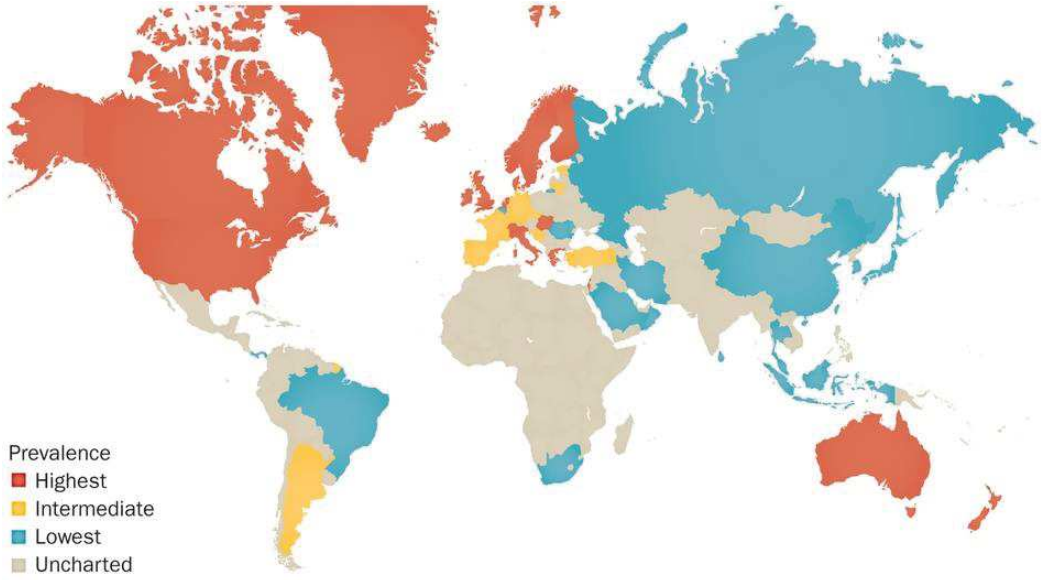

Figure 1. Global incidence of IBD. Reprinted by permission from Springer Nature: Nature Reviews Gastroenterology & Hepatology, (Kaplan, 2015), copyright (2015).

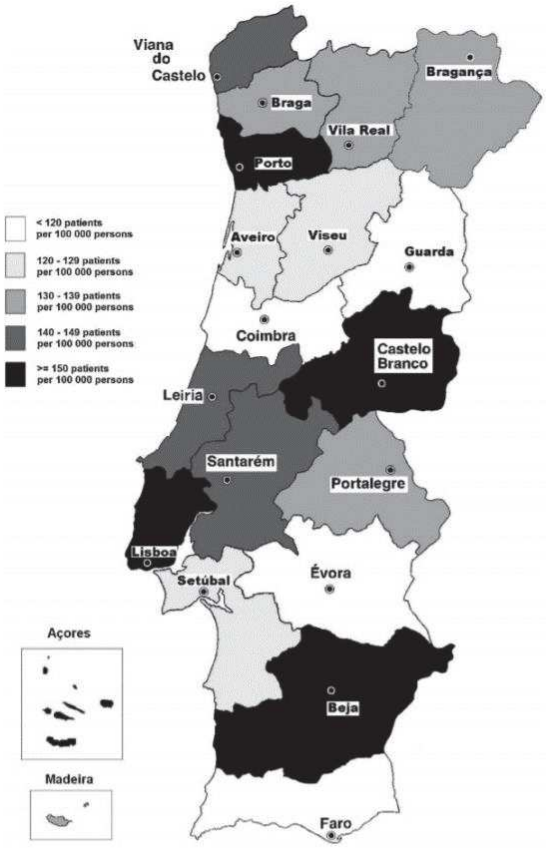

Figure 2. Prevalence of IBD, in the 20 districts of Portugal (patients per 100 000 persons), in 2007. Reprinted by permission from John Wiley & Sons, Inc: Pharmacoepidemiology & Drug Safety, (Azevedo, et al., 2010), copyright (2010).

The precise etiopathogenesis of IBD remains to be clarified. In fact, this disease does not arise as a consequence of an isolated/single cause, being a multifactorial disease that remains incurable. There is a synergitic effect of different risk factors involved in IBD pathogenesis that includes genetic predisposition, altered immune response, dysbiosis, environmental factors and lifestyle (diet, stress). There is an urgent need in the field to disclose the specific mechanisms underlying intestinal inflammation that can be therapeutically targeted as well as identify potential risk factors (markers of disease susceptibility) that could help to explain the complexity of IBD pathogenesis.

Interestingly, in less developed countries the incidence of IBD is lower and it is postulated that might be due to modulation of host immunity induced by helminths. It was observed in IBD murine models that the helminth, *Heligmosomoides polygyrus bakeri* prevents colitis, preventing antigen-specific gut T cell response by altering dendritic cell (DC) function (Blum, et al., 2012). This mechanism of suppression of inflammation that was found to occur through helmith´s glycans has been explored and recently, it was suggested that IL-4Rα signaling is the key pathway required for an effective suppression

of immune response (Matisz, et al., 2017) which has contributed to raise the interest for helminth's antigen cell-based therapy (Maizels, 2016), already tested in small clinic trials in UC (Summers, et al., 2005).

Genetics has a major contribution in defining disease susceptibility and since 2001, when the first gene, *NOD2* gene (Ogura, et al., 2001), was described to be associated with Crohn's disease, hundreds of risk loci were then identified to be associated with IBD, shared by both CD and UC, or only associated with CD or UC (Lees, et al., 2011).That knowledge is very helpful for the understanding of IBD etiopathogenesis. However, it does not completely explain IBD incidence worldwide. For instance, IBD in Asian populations is not associated with *NOD2*, *CARD15* or *IL23R* variants (Sood and Midha, 2007).

Moreover, the heritable component of CD and UC is supported by twin studies. Despite in Crohn's disease was found concordance rates in monozygotic twins (20-50%) and less in dizygotic twins (10%), in UC there is a weaker heritable component for monozygotic (16%) and dizygotic (4%) twins (Ananthakrishnan, 2015; Halme, et al., 2006; Orholm, et al., 2000).

During the last decades notable efforts emerged to unravel the pathogenesis of IBD. A recent clinical review summarizes it and in Figure 3 is illustrated the progression of disease stages.

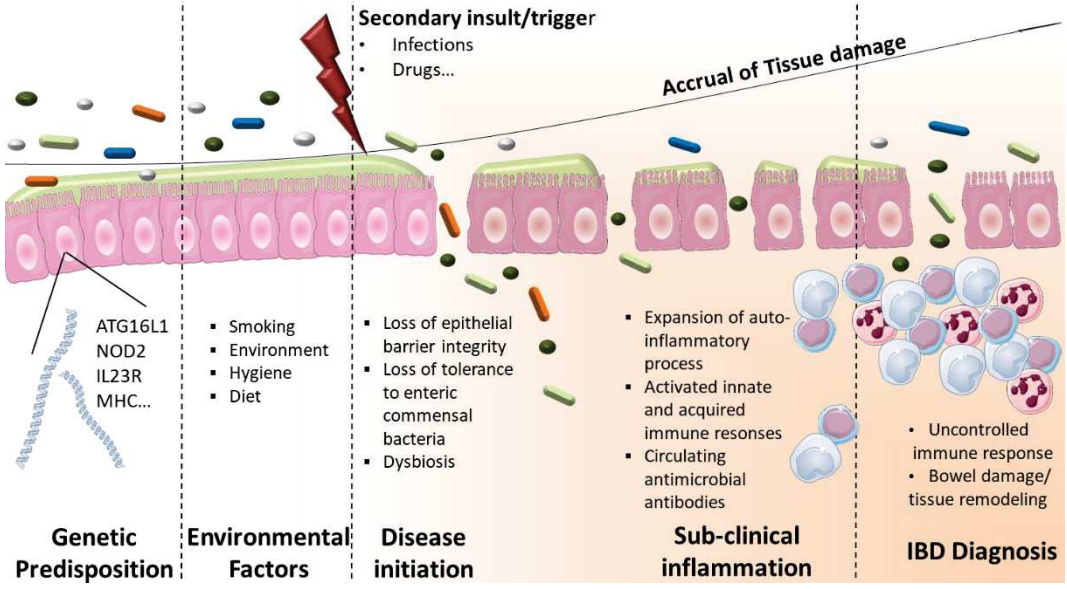

Figure 3. Proposed model of IBD pathogenesis and progression. Adapted from (Torres, et al., 2016).

The disease initiation triggered by genetic and environmental factors lead to loss of epithelial barrier integrity therefore compromising enteric commensal bacteria survival

and in consequence exposing inner mucosa layers to pathogenic microbiota. Consequently, it promotes an inflammatory process and activation of innate and adaptive immune responses. Once this inflammation is perpetuated and there is an uncontrolled immune response associated with tissue damage, the IBD diagnosis is stablished (Figure 3) (Torres, et al., 2016).

Apart from common pathways that link the pathogenesis of CD and UC, these two disorders are very distinct in terms of disease pattern. CD can affect any part of the gastrointestinal tract (from the mouth to the anus). The inflammation associated with CD is transmural, affecting all layers of the intestine and can extend into the deep layers of the intestinal wall. On the other hand, UC affects mainly the rectum and colon and the inflammation is confined to the mucosa occurring in an uninterrupted pattern (Baumgart and Sandborn, 2012; Ungaro, et al., 2017).

The work developed and presented in this doctoral thesis is mainly focused on UC. UC is classified into different categories depending on the location and extension of the disease. There are three main types of ulcerative colitis (represented in Figure 4). UC affects different parts of colon and rectum with different disease extension. **Proctitis** is confined to the rectum (the lower part of the colon that connects with the anus) and in about one third of the individuals, UC begins with ulcerative proctitis. **Left-Sided Colitis** causes continuous inflammation throughout the left side of the colon from the rectum to the area near the spleen. **Pancolitis** is when the disease and inflammation occurs throughout the entire colon (Ungaro, et al., 2017).

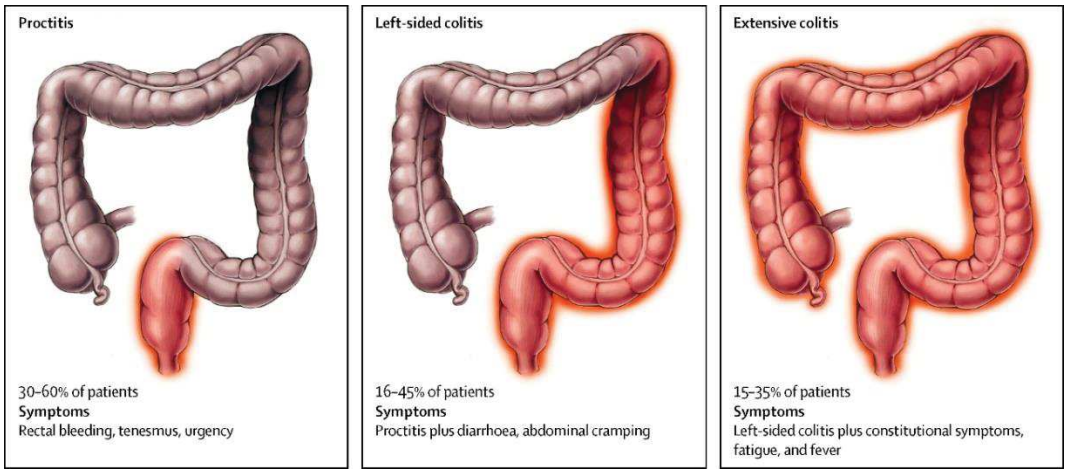

Figure 4. Ulcerative colitis is classified into different categories depending on the location and extension of the disease. Reprinted by permission from Elsevier: The Lancet Nature (Ungaro, et al., 2017), copyright (2017).

Diagnosis and clinical management of Ulcerative colitis

The diagnosis of UC takes into consideration the Mayo endoscopic score (detailed in Table 1). The final score combines the evaluation of clinical symptoms of disease (stool frequency, rectal bleeding); assessment of mucosa integrity and friability and physician's global assessment (Magro, et al., 2017).

Table 1. Mayo endoscopic score for Ulcerative colitis. Adapted from (Magro, et al., 2017).

Mayo index	0	1	2	3
Stool frequency	Normal	1-2/day > normal	3-4/day > normal	5/day > normal
Rectal bleeding	None	Streaks	Obvious	Mostly blood
Mucosa	Normal	Mild friability	Moderate friability	Spontaneous bleeding
Physician's global assessment	Normal	Mild	Moderate	Severe

The IBD clinical management also takes into consideration some biomarkers that are expected to help in the diagnosis and monitoring of IBD. By definition a good biomarker should be disease-specific; able to identify individuals at risk for the disease; able to detect disease activity; able to monitor the effects of treatment; and prognostically valuable for assessing disease relapse or recurrence. Therefore, it should present high sensitivity and specificity.

In the context of IBD, biomarkers are important to predict the occurrence of the disease, to distinguish IBD subtypes (CD or UC), to discriminate IBD from IBS and to predict the therapeutic response. However, there is no single "gold standard" test that can assess all these parameters. Among others listed in Table 2, there are biomarkers that can discriminate CD from UC, such as perinuclear anti-neutrophil cytoplasmic antibodies (**pANCAs**) and anti-*Saccharomyces cerevisiae* antibodies (**ASCAs**) and markers that can evaluate disease activity or inflammation in IBD, such as C-Reactive Protein (**CRP**) and **fecal calprotectin** (Viennois, et al., 2015). Nonetheless, efforts have been made to develop new tools to assess, for instance, circulating miRNAs or protein profiles, in order to identify high risk patients (Viennois, et al., 2015)

Table 2. Current IBD biomarkers. Adapted from (Viennois, et al., 2015).

Biomarker name	Distinguish CD vs UC (Yes/No)	Specificity for IBD (Yes/No)	Sensitivity (Low/High)	Predictive potential (Yes/No)
pANCAs ASCAs	Yes Yes	Yes Yes	Low Low	Yes Yes
CRP	No	No	High for CD Low for UC	Yes
Fecal Calprotectin	No	Yes	High	Yes
Fecal lactoferrin	No	Yes	High	Yes
S100A12	No	No	High	No
Lipocalin 2	No	No	High	No

The main goal of IBD clinical management is to achieve clinical remission and inactive disease with maintenance of no clinical symptoms (stool frequency ≤ 3/day with no bleeding) and mucosa healing detected by endoscopy (Harbord, et al., 2017; Magro, et al., 2017). In this regard, new molecular mechanisms need to be disclosed to design more target-specific therapies for IBD. The main achievement is to have a clinical management as closer as possible to a personalized medicine - “an emerging practice of medicine that uses an individual’s genetic profile to guide decisions made in regard to the prevention, diagnosis, and treatment of disease” (National Institutes of Health; http://www.genome.gov/glossary/).

Current IBD therapies and their known targets

IBD treatment is based in a step-up therapeutic strategy, both in CD and UC. Particularly in UC, the standard therapy is aminosalicylates for mild to moderate UC. Often, topical and systemic steroids can be used to treat UC flares, while immunosuppressants and biological drugs are used in moderate to severe disease. Colectomy is the extreme solution and it is needed in up to 15% of patients with UC (Ochsenkuhn and D'Haens, 2011) (Figure 5).

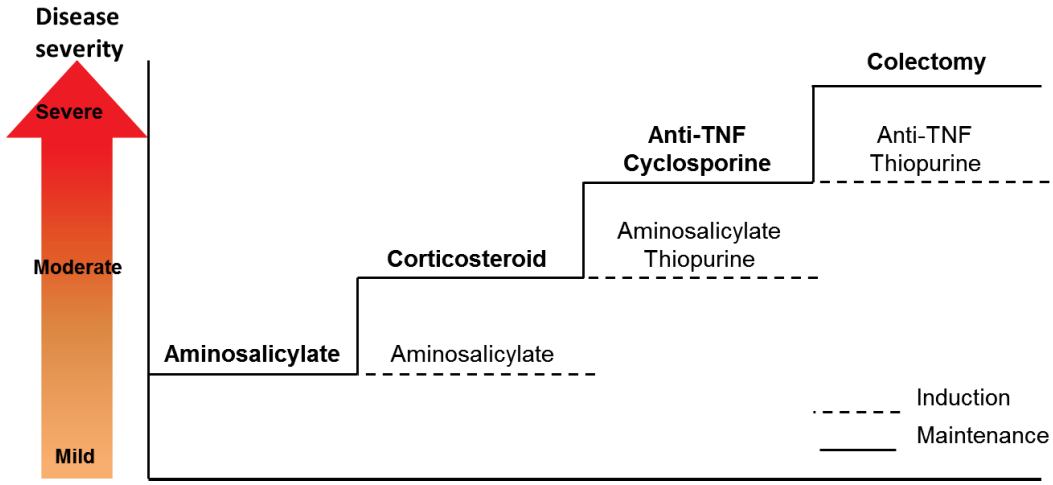

Figure 5. Common IBD step-up therapy following the course of disease severity. Adapted from (Marchioni Beery and Kane, 2014).

For decades, 5-aminosalicylic acid (5ASA) was the first line of therapy despite the fact that the underlying molecular mechanism has been suggested later in 2005. The action of 5ASA was found to be dependent on the increased expression of peroxisome proliferator-activated receptor-gamma (PPAR-gamma) (Rousseaux, et al., 2005).

In the last two decades, there was an exponential pharmaceutical development that gave rise to a new era of therapies, with the introduction of immunosuppressive (such as azathioprine) and biologic agents (such as tumor necrosis factor alpha (TNF- α) inhibitors) which markedly reduced the need to use corticosteroids. After that, many other different therapies have been investigated and are now available, including the α4β7 integrin blocker, vedolizumab; a number of new cytokine inhibitors (e.g. IL-6–IL-6R and IL-12–IL-23 blockers or apremilast); modulators of cytokine signaling events (for example, JAK inhibitors or SMAD7 blocker); inhibitors of transcription factors (e.g. GATA3 or RORγt), reviewed in (Neurath, 2017).

Moreover, new anti-adhesion and anti-T-cell-activation and migration strategies (e.g. β7 integrin, sphingosine 1-phosphate receptors and MAdCAM1 inhibitors, regulatory T-cell therapy and stem cells) are being evaluated in controlled clinical trials (Neurath, 2014).

Indeed, this therapeutic revolution contributed to increase the percentage of therapy success predominantly in the most severe cases. In both CD and UC, several anti-TNF-α either chimeric (e.g. infliximab), humanized (e.g. certolizumab pegol) or fully human (e.g. adalimumab and golimumab) antibodies display a high impact in controlling chronic intestinal inflammation. Briefly, anti-TNF-α therapies consist in antibodies that neutralize both soluble TNF-α and membrane-bound TNF-α (such as infliximab and adalimumab). Their effectiveness was found to be due to induction of T cell apoptosis *in vivo* (via TNF receptor 2 and $CD14^+$ macrophages) whereas agents that preferentially block only soluble TNF-α (for example, etanercept) had no therapeutic effect (Atreya, et al., 2011; Neurath, 2014; Van den Brande, et al., 2007).

In fact in IBD, there is an association with high production of both soluble and membrane-bound TNF-α which can be produced by several cell populations (immune and stroma) like macrophages, dendritic cells (DCs), effector T cells, adipocytes and fibroblasts. Indeed, TNF-α drives pleiotropic pro-inflammatory effect in several pathways. It can induce hypervascularization and angiogenesis; enhances pro-inflammatory cytokine production by macrophages and T cells; causes barrier alterations and promotes cell death of intestinal epithelial cells (IECs) and Paneth cells. Additionally, it promotes tissue damage through the production of matrix metalloproteinases (MMPs) by myofibroblasts. It also drives T cell resistance to apoptosis via the induction of TNF receptor-associated factor 2 (TRAF2) and the activation of nuclear factor-κB (NF-κB) (Neurath, 2014).

It is worth nothing that not all anti-cytokine therapies are efficient for the treatment of IBD. Some examples have demonstrated that neutralization of other cytokines, such as IFN-γ (with fontolizumab) (Reinisch, et al., 2010) or IL-17A (with secukinumab) (Hueber, et al., 2012) do not demonstrated therapeutic efficiency in Crohn's disease. Therefore future strategies in IBD treatment should target T cell subsets themselves or simultaneous targeting multiple cytokines, rather than targeting a single effector cytokine (Neurath, 2014).

However, immunogenicity is a well-known complication during treatment with biologic agents and involves the formation of anti-drug antibodies (ADAb). For anti-TNF-α drugs, ADAb are associated with alterations in anti-TNF-α levels, reduced efficacy, and side-effects reactions, being responsible for loss of response to biologics (Vincent, et al., 2013). Moreover, there are other drawbacks associated, namely infection related

complications, a potential risk of malignancy, the need for parenteral administration and high treatment related costs.

Despite that, and because the percentage of response to this type of therapy is still very significant in some cases, and, based on preclinical and clinical data, the European Medicines Agency (EMA) has allowed the introduction of biosimilars of anti-TNF to be marketed in rheumatoid arthritis, spondyloarthritis, UC and CD. Biosimilars appear to constitute efficient drugs but a less expensive option (Deiana, et al., 2017).

Recently, Olivera and colleagues discussed the new generation of small molecules (SMDs) based therapies (in late-stage clinical development) which present main advantages in comparison with monoclonal antibodies, being most of them under clinical investigation in UC patients, on phase II and III of clinical trials (Olivera, et al., 2017).

Indeed, SMDs have been showing promising results in other diseases with significant levels of efficacy and an acceptable safety. For instance, tofacitinib (JAK inhibitor) was found to be effective in phase III clinical trials, and Pfizer is seeking marketing authorization in UC (Olivera, et al., 2017).

In fact, the particularities of SMDs make them a good alternative over biologics as they can diffuse more easily through cell membranes due to their low molecular weight (<1kDa, usually <500Da). Moreover, they can be administered orally resisting to gastric degradation, and entering in to systemic circulation rapidly, displaying a short half-life (rapid drug elimination) in comparison to biologics (Leeson and Springthorpe, 2007; Olivera, et al., 2017; Veber, et al., 2002). Additionally, SMD's lack immunogenicity and they are less expensive than biologics so they represent a better relation of cost-effectiveness.

Despite all these progresses in IBD treatment, a considerable proportion of patients are still refractory to the treatment and overall half of the patients do not achieve sustained remission (Ochsenkuhn and D'Haens, 2011). Hence, there is an urgent unmet need in the clinic to develop new and optimized targeted-specific therapies. Moreover, with the introduction of this broad setting of new drugs, it is fundamental to identify reliable biomarkers able to predict and monitor therapeutic success, improving the individualized therapy in IBD.

Intestinal immune response in Ulcerative colitis

IBD is characterized by a perturbation on the intestinal homeostasis which is known to be critically dependent on complex interactions between the microbiota, the intestinal epithelium and the host immune system (Maloy and Powrie, 2011) . Upon injury of epithelial barrier function, innate and adaptive immune recognition precipitates a hyperimmune response in IBD (Figure 6) (Neurath, 2017), with distinct particularities, in UC and CD, as reviewed in (Baumgart and Sandborn, 2012; Ungaro, et al., 2017).

The gut microbial content is definitely a major player in IBD pathogenesis as alterations in microbiota composition and functions (dysbiosis) have been consistently associated with IBD (Ananthakrishnan, 2015; Hall, et al., 2017). In UC patients is commonly observed a decreased of biodiversity, with a lower proportion of Firmicutes and increased Gammaproteobacteria and Enterobacteriaceae (Frank, et al., 2007). However, it remains to be elucidated whether dysbiosis is cause or consequence of mucosal inflammation.

Together with colonic microflora composition, intestinal homeostasis is also dependent on the role of different gut cell types, from both epithelium and immune system.

The intestinal epithelium is mainly composed by **IECs** coated with mucus layer that prevent a direct contact of the commensal microbiota with IECs. This barrier comprises two layers, an outer layer of secreted mucins overlying a dense inner layer (glycocalyx) of membrane-anchored mucins that is inaccessible to most bacteria (Artis, 2008). In addition, as a biophysical barrier, mucus forms a matrix that allows the retention of high concentrations of antimicrobial molecules, such as defensins and secretory IgA, close to the epithelial surface. IBD is characterized by a reduction of goblet cells with consequent reduction of mucin secretion. In mice, the absence of MUC2 (principal mucin in intestine) leads to spontaneous colitis (Van der Sluis, et al., 2006).

On the other hand, IECs produce cytokines that can preclude the priming of T helper 1 (Th1)-cell responses, favoring thereby the induction of T regulatory (Treg)-cell and Th2-cell responses. On the contrary, upon sensing pathogenic invasion or damage, IECs secrete chemokines like IL-8 (CXCL8) that promote immune activation (Artis and Spits, 2015). Furthermore, IECs are involved in local antibody responses by producing transforming growth factor-β (TGF-β) and they also mediate the transport of secretory IgA into the mucus layer which complements the innate response by limiting the penetration of commensal bacteria across the epithelium (Maloy and Powrie, 2011).

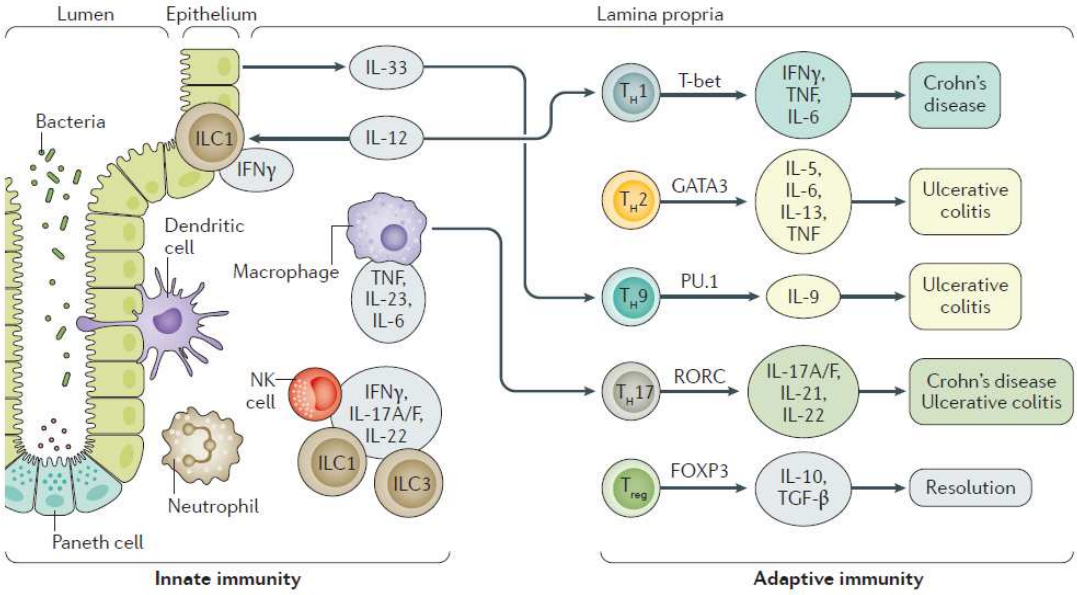

Figure 6. Immune response in UC and CD. Reprinted by permission from Springer Nature: Nature Reviews Gastroenterology & Hepatology,(Neurath, 2017), copyright (2017).

Another cellular component of bowel epithelium are **innate lymphoid cells** (ILCs) which constitute the most recently identified component of the innate immune system with an important role on the control of intestine homeostasis (Artis and Spits, 2015). Indeed, ILCs isolated from patients with active UC show increased gene expression of type 3 ILCs (ILC3s) cytokines (IL17A and IL22), transcription factors (RORC and AHR), and cytokine receptors (including IL23R) (Geremia, et al., 2011) . Interestingly, a recent report (Goto, et al., 2014) demonstrated that α-1,2 fucose expressed on the apical side of epithelial cells is controlled by ILC3s and luminal microbes, reviewed in (Goto, et al., 2016). In line with those findings, it was demonstrated that commensal bacteria, pathogenic bacteria and bacterial products (LPS) directly or indirectly stimulate (via gut dendritic cells) ILC3s to produce IL-22 promoting epithelial FUT2 expression and to induce α-1,2 fucosylation. Hence, epithelial α1,2-fucose protects against pathogenic bacteria and it is key in maintaining the commensal microbiota and thereby intestinal homeostasis.

Neutrophils are critical components of the innate immune system in protecting the host from pathogens through their uniquely capability to produce toxic molecules, like reactive oxygen species. In general, during intestinal inflammation, neutrophils present in the blood sense a chemoattractant gradient, generated through cytokines production by resident monocytes, and then translocate across the vascular endothelium infiltrating the intestinal lamina propria (Amulic, et al., 2012). In UC

patients is characteristic an increase of activated neutrophils at blood and biopsies in comparison to healthy controls (Hanai, et al., 2004). However, the role of neutrophils particularly in intestinal inflammation is still controversial whether beneficial or detrimental, reviewed in (Fournier and Parkos, 2012).

Antigen presenting cells (APCs) like **DCs** are crucial in mediating immune homeostasis by bridging innate and adaptive immunity to foreign or self antigens. Interestingly, intestinal DCs were shown to be distinct from those in blood and also between non-inflamed and inflamed human colon (Hart, et al., 2005). Intestinal DCs isolated from IBD patients present increased expression of Toll-like receptors (TLRs) namely, TLR2 and TLR4. DCs control microbial driven T-cell polarization in part through the ligation of TLRs (Kaisho and Akira, 2003). TLR4 is required for recognition of lipopolysaccharide from *Escherichia coli*, and TLR2 recognizes peptidoglycan and lipoteichoic acid from Gram-positive bacteria and lipoproteins from both Gram-positive and Gram-negative organisms (Chow, et al., 1999; Michelsen, et al., 2001; Morath, et al., 2002; Takeuchi, et al., 2000). Importantly, DCs express another type of receptor (a C-type lectin receptor), Dendritic cell specific intracellular adhesion molecule-3 (ICAM-3) grabbing nonintegrin (DC-SIGN) (that binds mainly high-mannose but also fucose moieties (Feinberg, et al., 2001; Svajger, et al., 2010)), being responsible to stablish the DCs' interactions with endothelial cells (via ICAM-2) (Geijtenbeek, et al., 2000a), with T cells (via ICAM-3) (Geijtenbeek, et al., 2000b) and with neutrophils (via Mac-1)(van Gisbergen, et al., 2005). In those circumstances, DCs play a key role on the initiation and perpetuation of inflammatory cascade in IBD.

Intestinal macrophages are also instrumental in gut homeostasis, owing to their plasticity and ability to change phenotype and function according to the neighboring environment. Most of the current knowledge about intestinal macrophages is predominantly based on mouse studies or on macrophages derived from peripheral blood monocytes from CD patients (Xue, et al., 2014) . Nonetheless, it is well established that bone marrow-derived monocytes are the precursors of tissue-resident intestinal macrophages. Hence, the production of interleukin (IL)-8 and transforming growth factor (TGF)-β promotes the recruitment of these monocytes (through blood circulation) into the intestinal mucosa (Smythies, et al., 2006). At least, in mice, there is a constant replacement of intestinal macrophages, eliminated by senescence or apoptosis, by newly recruited blood monocytes (Jenkins, et al., 2011).

Macrophages may be classified in distinct subsets according to produced cytokines or other functional features. Namely, pro-inflammatory (M1) and anti-inflammatory (M2) macrophages have been consequently regarded as mirrors of the polarization of T helper cell subsets (Mantovani, et al., 2004). In IBD, tissue-resident

macrophages are M2-type macrophages and $CD68^+$ macrophages which massively infiltrate the intestinal mucosa. Despite they can spread throughout the thick mucosa and submucosa, their composition and function is distinct in UC and CD (Kuhl, et al., 2015). Regarding UC, macrophages only reach mucosa level but in CD they can also infiltrate the muscular layer and the mesenteric fat (Kredel, et al., 2013; Mahida, et al., 1989).

Remarkably, reduction of macrophages in colon biopsies has been a unique histological change in successful cases of treatment with glucocorticoids or anti- TNF-α-antibodies (Caprioli, et al., 2013).Moreover, aldehyde dehydrogenase positive ($ALDH^+$) macrophages (meaning retinoic acid synthesis) are reduced in the intestinal mucosa of UC (both active and remission) but not of CD patients and there is also a clear involvement of macrophages on fibrosis development more evident in CD than UC (Magnusson, et al., 2016). Emerging evidences indicate that IL-33 plays a role in epithelial restoration, repair, and mucosal healing in UC and CD (Duan, et al., 2012). More recently, it was suggested that IL-33 can restore goblet cell numbers and induce macrophage switching from the M1 to the M2 phenotype, thereby controlling intestinal inflammation in IBD (Seo, et al., 2017).

In the late 1980's, the discovery that **Th cells** may differentiate into Th1 and Th2 cells subsets (Mosmann, et al., 1986) lead to the establishment of disease-specific cytokine patterns that distinct CD from UC. Those earlier evidences suggested that UC is a modified T-helper-2 (Th2) disease, while Crohn's disease is Th1 driven, the so-called Th1 /Th2 paradigm for CD *versus* UC (Strober and Fuss, 2011). Throughout the years, UC have been designated a "Th2-like" disease supported by the predominant expression of IL-13 and IL-4 in UC patients. Accordingly, IL-4 and IL-13 mRNA levels were found significantly increased in rectal biopsies from UC patients compared with healthy controls (Inoue, et al., 1999). Furthermore, IL-5 has been observed highly expressed in $CD4^+$ lamina propria cells of UC patients. On the other hand, CD has been designated Th1-driven due to the increased expression of IFN-γ in patients with this condition (Fuss, et al., 1996).

Over the past few years, the characterization of T cell differentiation, meaning T cell-subsets and their role in inflammation has been of paramount importance to understand their influence in chronic inflammatory diseases like IBD. The differentiation of naïve T cells, through the activation of separate signaling pathways, lead to differentiated Th cells, respectively designated as Th1, Th2 and Th17 or regulatory T cells (Treg), which suppress Th cells. This quartet of T cell subsets has an important role in orchestrating the adaptive immune response in IBD (Figure 7), as well summarized in (de Souza and Fiocchi, 2016). Importantly, the process of Th cell differentiation is mediated by STAT proteins which, although transiently activated, are gateways and the

first step on the road to commitment to a specific Th cell phenotype (O'Shea and Paul, 2010).

In 2014, a new population of $CD4^+$ Th cells, which produce IL-9 and express the transcription factor PU.1**, Th9 cells**, has been implicated in the development of UC. Th9 cells develop after Th0 cells encounter peptides presented on major histocompatibility complex II (MHC II) molecules in the presence of TGF- β and IL-4 cytokines. Through IL-9 production, these cells can impair tissue repair processes, increase intestinal permeability and may enhance pro-inflammatory Th cell responses. Additionally, IL-9 slightly increases tissue concentrations of TNF-α (Gerlach, et al., 2014).

Notably, constant progresses in the development of new therapies, for UC or CD, have relied on this better understanding of T cell differentiation and specific cytokines role (Figure 7).

Altogether, these cellular and molecular discoveries set the ground for the development of new and successful therapeutic approaches essential to improve the global IBD burden. For instance, Tofacitinib, an oral Janus kinase inhibitor, was the first JAK multi-cytokine blocker reported for the treatment of IBD (Sandborn, et al., 2012). And very recently the final results of clinical trial (OCTAVE) with tofacitinib, in moderately to severely active UC patients, demonstrated its efficacy, by induction and maintenance of both remission and mucosal healing (Sandborn, et al., 2017), two critical aspects to avoid IBD flares and sustained clinical remission.

Protein Glycosylation

Since more than 25 years ago, the field of glycobiology had a tremendous growth confirming that glycans are fundamental players in biological processes. The new era of glycomics, in parallel with genetics and proteomics, brought new perspectives in biomedical research, from cancer research to infection and other diseases (Pinho and Reis, 2015; Rudd, et al., 2001).

Interestingly, the magnitude of glycome repertoire - the spectrum of all glycans structures- is estimated to be 10-10^4 times bigger than the proteome and far more complex than the genome and proteome (Cummings, 2009; Cummings and Pierce, 2014). From the four fundamental biomolecules in cells (nucleic acids, proteins, lipids and glycans), glycans are by far the ones that bring more diversity. If "*DNA is made up of four nucleotides (G, A, T and C), so there are theoretically 4,096 possible ways to build a string of six elements, or a 6-mer. Proteins have more building blocks (20 amino acids) and can potentially assemble into 64 million different 6-mers. But 6-mer carbohydrates can adopt 193 billion possible configurations*" (Peter Seeberger).Therefore, the diversity of glycan structures can provide an additional level of information content in a diversity of biological systems (Moremen, et al., 2012).

By definition, glycosylation consists in the covalent attachment of a carbohydrate to proteins and lipids producing different families of glycoconjugates (Pinho and Reis, 2015; Stanley, et al., 2015) (Figure 7).

Glycoprotein is a glycoconjugate in which a protein carries one or more glycans linked to a polypeptide backbone, usually via nitrogen (in *N*-glycans) or oxygen (in *O*-glycans) linkages. *N*-linked glycans are attached to asparagine (Asn) residue of proteins in the consensus peptide sequence Asn- X- Ser/Thr, where X is any amino acid except proline. *O*-glycans, particularly found on secreted or membrane bound mucins, consist of O-linked glycan attached to serine (Ser) or threonine (Thr) residue which can be further extended resulting in different types of *O*-glycans structures (Moremen, et al., 2012; Pinho and Reis, 2015).

Nonetheless, as represented in figure 7, there are other classes of glycoconjugates like, glycosaminoglycans (GAGs) which are O-linked glycans, constituted by linear co-polymers of acidic disaccharide repeating units. Glycoproteins carrying one or more GAG chains are called proteoglycans and Hyaluronic acid is a GAG primarily found as a free sugar chain.

Glycosphingolipids are ceramide – linked glycans which are major components of the outer leaflet of the cell plasma membrane. Other glycoproteins can also be found in

the outer leaflet of the plasma membrane linked to a phosphatidylinositol (GPI) termed GPI-anchored proteins (Moremen, et al., 2012; Pinho and Reis, 2015).

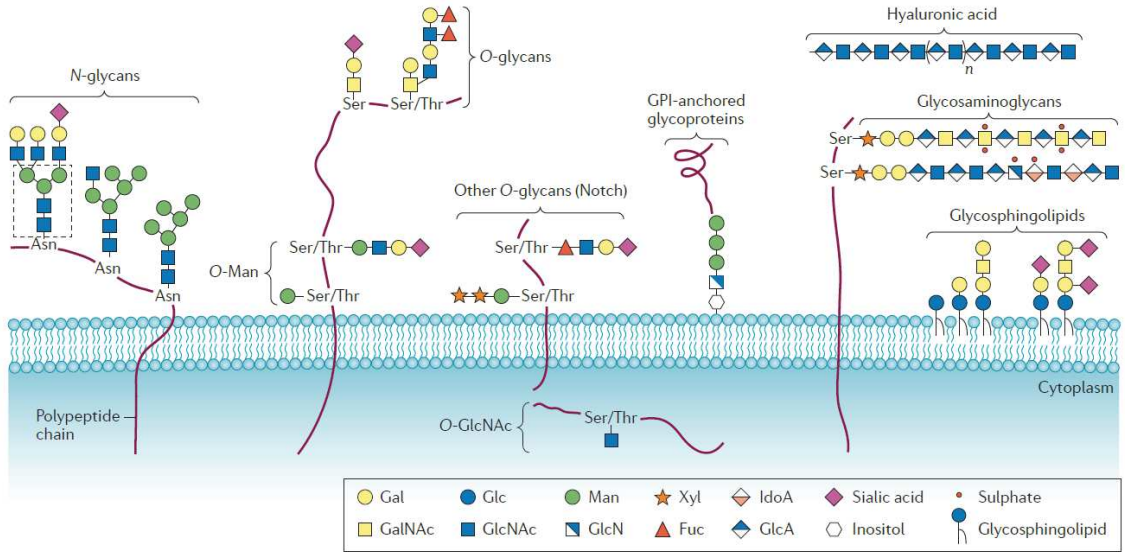

Figure 7 – Major classes of glycoconjugates in mammalian cells. Adapted from (Pinho and Reis, 2015).

Protein glycosylation can confer many advantages in biological systems due to the diversity, complexity, hydrophilicity, and structural mobility of cell-surface glycans (Gagneux and Varki, 1999).

Importantly, this process is not template driven and is subject to multiple sequential and competitive enzymatic pathways (Drickamer and Taylor, 1998; Esko and Selleck, 2002). Moreover, it is also not predictable by gene expression patterns *per se* once glycans composition is dynamically altered in response to small variations in the extracellular environment and intracellular events.

N-glycosylation

Glycosylation is one of the most abundant and complex forms of protein posttranslational modifications in which approximately 90% of glycoproteins are N-glycosylated. It is a highly regulated process catalyzed, by a portfolio of specific enzymes (glycosyltransferases) that mediate the addition of carbohydrate structures (glycans) to proteins and lipids in the endoplasmic reticulum (ER) /Golgi secretory pathway. Notably, N-glycosylation is firmly stablished to be species-, cell- and tissue-specific process (Varki, 2006).

All eukaryotic ***N*-glycans** share a common core sequence, Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1–4GlcNAcβ1–Asn-X-Ser/Thr being classified in three major types (Stanley, et al., 2015): (1) **oligomannose**, in which only Man residues extend the core; (2) **complex**, in which "antennae" initiated by GlcNAc extend the core; and (3) **hybrid**, in which Man extends the Manα1-6 arm of the core and one or two GlcNAcs extend the Manα1-3 arm (detailed structures in figure 7) (Pinho and Reis, 2015).

The **N-glycans biosynthesis in eukaryotic cells** is mainly divided in two phases. The first phase (Figure 8), at the ER membrane, in which a lipid-like polyisoprenoid molecule termed dolichol-phosphate (Dol-P), hold/mediate the assembly of a precursor oligosaccharide structure that is then transferred to the secretory and membrane proteins. In the second phase, the N-glycans processing, in the lumen of the ER and Golgi, is orchestrated by a diverse repertoire of glycosidases and glycosyltransferases (which main substrates are nucleotide sugars and dolichol-sugars) (Figure 8). This phase is dependent on the expression of glycosylation genes in each cell type in which the glycoprotein is processed, as well as on the physiological state of the respective cell which can affect the localization and activity of glycosylation enzymes and transporters (Stanley, et al., 2015).

The **assembly of precursor glycan in ER** involves a GlcNAc-1-phosphotransferase (ALG7, DPAGT1 in mammals) which transfers GlcNAc-1-P from UDP-GlcNAc to Dol-P forming the Dol-P-P-GlcNAc. Dol-P-P-GlcNAc is extended with two GlcNAc and five Man residues, from Dol-P-Man and Dol-P-Glc, respectively, originating the Dol-P-P-$GlcNAc_2Man_5$ before it is "flipped" across the ER membrane to the luminal side. Subsequently, other four Man residues are added from Dol-P-Man and three Glc residues from Dol-P-Glc. Dol-P-Man and Dol-P-Glc (used by mammalian MPDU1) are also made on the cytoplasmic side of the ER membrane and "flipped" onto the luminal side where occurs the synthesis of the mature N-glycan precursor **$Glc_3Man_9GlcNAc_2$-P-P-Dol** (detailed structure in Figure 8). Lastly, this 14-sugar chain is transferred by an oligosaccharyltransferase (OST) to Asn residue in the consensus Asn-X-Ser/Thr site in protein regions that have translocated across the ER membrane (Stanley, et al., 2015).

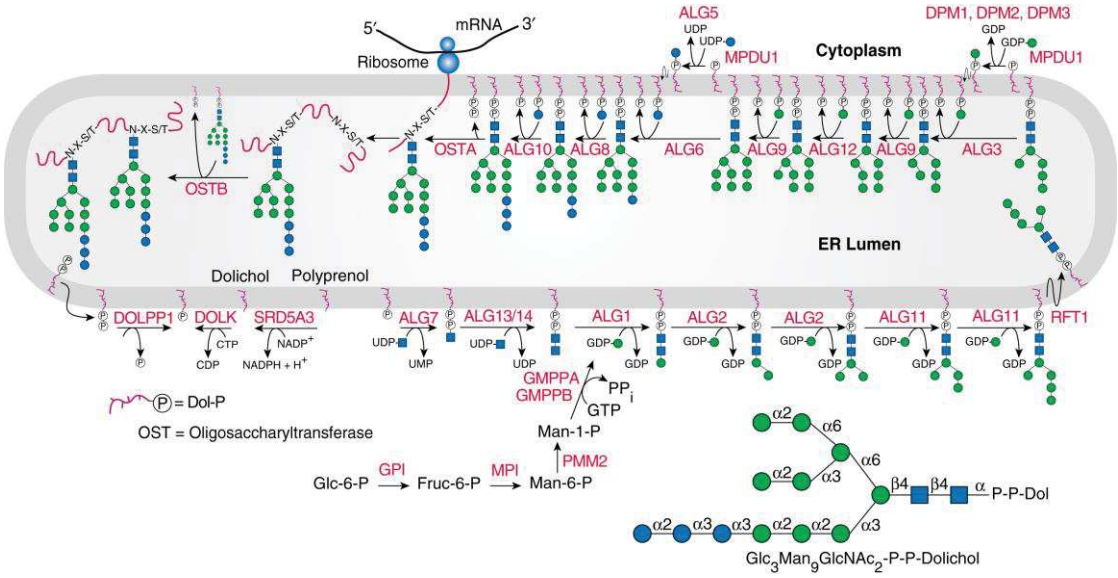

Figure 8. Schematic representation of the N-linked oligosaccharide assembly. Reprinted by permission from The Consortium of Glycobiology Editors, La Jolla, California: (Stanley, et al., 2015), copyright (2017).

The N-glycans processing occurs upon covalent attachment of the mature N-glycan percursor ($Glc_3Man_9GlcNAc_2$) to a protein. Then the process of **trimming by glucosidases (I and II) takes place in the lumen of the ER**, in which α-glucosidase I remove on the terminal α1–2Glc and α-glucosidase II sequentially removes the two inner α1–3Glc residues. This process originates the monoglucosylated intermediate Glc1Man9GlcNAc2 (G1M9 structure), the key ligand to initiate chaperone-assisted proofreading of protein folding by the calnexin (CNX) / calreticulin (CRT) quality control cycle. These lectins determine whether the newly made membrane and secreted proteins continue to the Golgi or are degraded (Caramelo and Parodi, 2015).

Additionally to CNX/CRT, the quality control mechanism of protein folding involves three other components, ERp57 (a lectin-associated oxidoreductase acting on monoglucosylated glycoproteins), UGGT (a glucosyltransferase that creates monoglucosylated epitopes in protein-linked glycans) and a glucosidase (GII) that removes the glucose units added by UGGT (Parodi, et al., 2015). Cycles of deglucosylation by GII and reglucosylation by UGGT continue until the glycoprotein folds properly. If properly folded, these glycoproteins are packaged into COPII-coated vesicles and transferred to the Golgi. In case they remain unfolded, those proteins are recognized by UGGT which adds the single α1–3Glc to the glycan (removed by GII), thus recreating the identical N-glycan (G1M9) first recognized by CNX/CRT and contributing for ER retention time (Parodi, et al., 2015).

Glycoproteins that fail to properly fold are eventually driven to proteasomal degradation in the cytosol following the ER-associated degradation (ERAD) pathway, in which the extent of N-glycan demannosylation by ER mannosidases play a relevant role in the identification of irreparably misfolded glycoproteins (Parodi, et al., 2015).

Normally, before leave the ER the last event is mediated by ER α-mannosidase I (MAN1B1) which removes the terminal α1-2Man from the central arm of $Man_9GlcNAc_2$ to produce a $Man_8GlcNAc_2$ isomer.

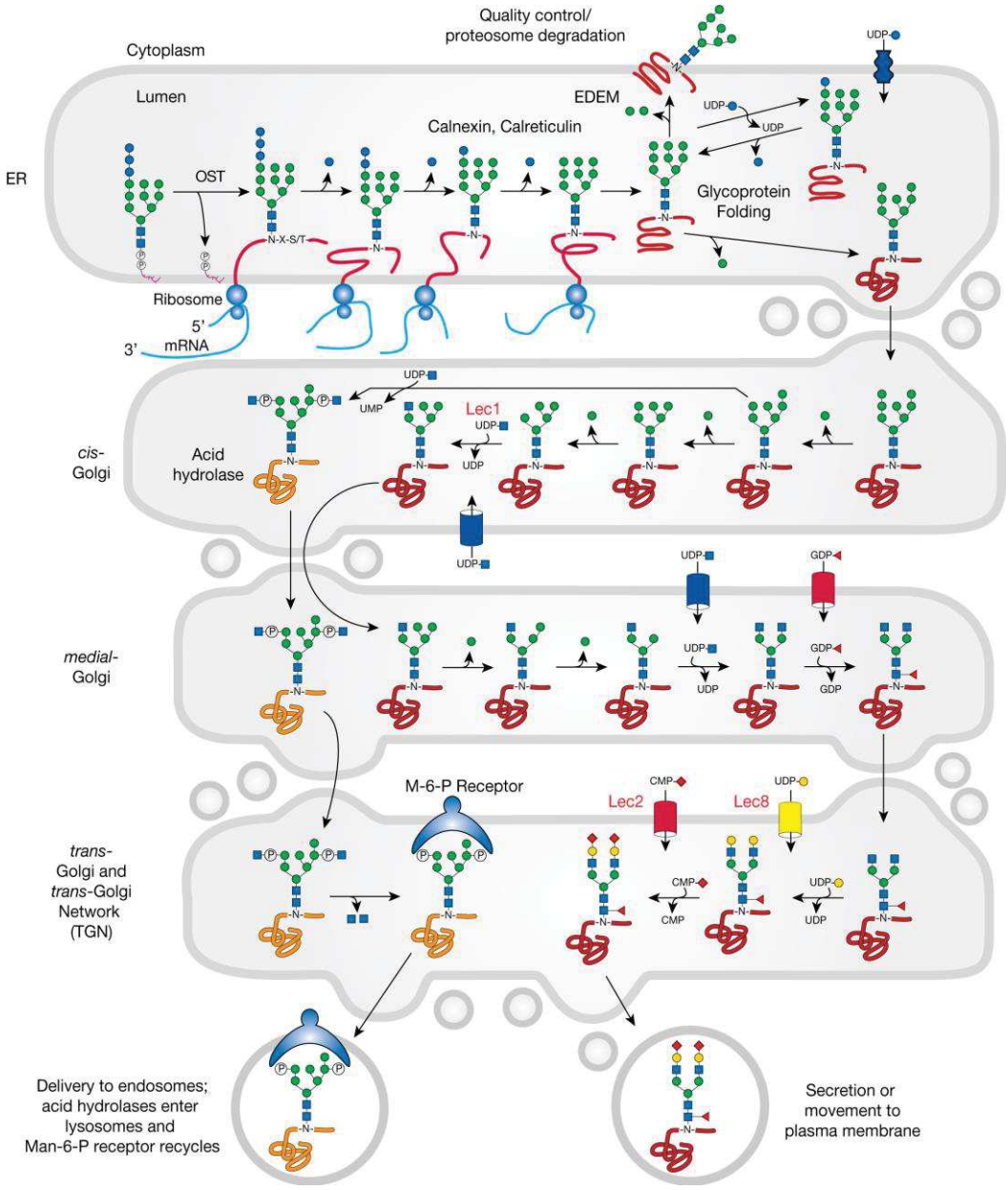

Figure 10. Processing and maturation of N-glycans in ER/Golgi secretory pathway. Reprinted by permission of the *Annual Review of Biochemistry*, from Kornfeld R, Kornfeld S. 1985. *Annu Rev Biochem* **54:** 631–634, adapted in (Stanley, et al., 2015), copyright (2017).

Late processing: Biosynthesis of hybrid and complex N-glycans

The majority of glycoproteins exiting the ER to the Golgi carry N-glycans with either eight or nine Man residues.

Trimming of α1-2Man residues continues with the action of α1-2 mannosidases IA and IB (MAN1A1, MAN1A2) in the *cis*-Golgi to give rise to **$Man_5GlcNAc_2$**, a key intermediate in the pathway to hybrid and complex N-glycans (Figure 10).

The action of an N-acetylglucosaminyltransferase called GlcNAc-TI (*MGAT1*) on $Man_5GlcNAc_2$ in the *medial*-Golgi initiates the first branch of an N-glycan (deletion of *MGAT1* gene blocks $Man_5GlcNAc_2$ to be further processed preventing synthesis of complex and hybrid N-glycans) (Stanley, et al., 2015). Subsequently, the majority of N-glycans are trimmed by α-mannosidase II enzymes MAN2A1 or MAN2A2 in the *medial*-Golgi, which remove the terminal α1-3Man and α1-6Man residues form $GlcNAcMan_3GlcNAc_2$. Following that acts another N-acetylglucosaminyltransferase, GlcNAc-TII (*MGAT2*) to add a second GlcNAc to the C-2 of the α1-6Man in the N-glycan core originating the precursor for all biantennary, complex N-glycans (Stanley, et al., 2015).

Hybrid N-glycans (small oligomannose N-glycans found in invertebrates and plants) can be formed if the $GlcNAcMan_5GlcNAc_2$ glycan (produced by *MGAT1*) do not suffer action of α-mannosidase II or due to incomplete action of α-mannosidase II, resulting in hybrids synthesis, $GlcNAcMan_4GlcNAc_2$ (Stanley, et al., 2015).

In the *medial*- Golgi, complex N-glycan has two antennae or branches initiated by the addition of two GlcNAc residues. Additional branches can be initiated at C-4 of the core α1-3Man (by GlcNAc-TIV; *MGAT4A*, *MGAT4B*) and C-6 of the core α1-6Man by GlcNAc-TV (*MGAT5*) to originate tri- and tetra-antennary N-glycans. Similar reaction is catalyzed by *MGAT5B* or GlcNAc-TIX but preferentially on O-mannose glycans in brain (Taniguchi and Kizuka, 2015) .

GlcNAc-TVI (*MGAT6*) catalyzes the formation of the most highly branched penta-antennary complex-type N-glycan initiated at C-4 of the core α1-6Man by *MGAT6*. It is present in various chicken (*Gallus gallus*) tissues and fish however, both *MGAT6* gene and its enzymatic activity have not been detected in mammalian tissues (Brockhausen, et al., 1989; Sakamoto, et al., 2000).

Complex and hybrid N-glycans may also carry a “bisecting” GlcNAc residue that is attached to the β-Man of the core by GlcNAc-TIII (*MGAT3*). A bisecting GlcNAc on a biantennary N-glycan can be present in all of the more highly branched N-glycans.

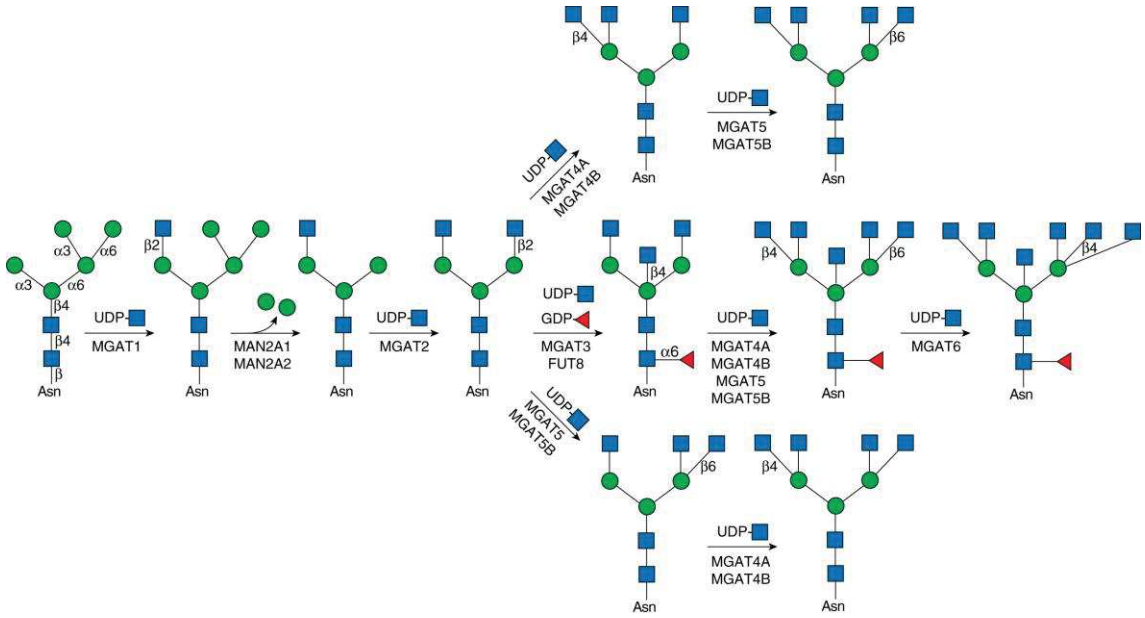

Figure 10. Branching and core modification of complex N-glycans. Reprinted by permission from The Consortium of Glycobiology Editors, La Jolla, California: (Stanley, et al., 2015), copyright (2017).

Glycan maturation in the Golgi

During the terminal glycosylation, at *trans*-Golgi, the repertoire of hybrid and branched N-glycans can be further extended giving rise to higher sugar diversity at several levels including branch number, composition, length, capping arrangements and core modifications. For example, in vertebrate N-glycans, a major core modification is the addition of α1-6Fuc to the Asn-linked GlcNAc in the N-glycan core (Figure 10). Importantly, the action of α1-6fucosyltransferase (FUT8) requires the prior action of *MGAT1* (Stanley, et al., 2015).

Moreover, the majority of complex and hybrid N-glycans have extended branches resulting from the addition of Gal to the initiating GlcNAc to produce the ubiquitous building block Galβ1-4GlcNAc which is a type-2 *N*-acetyllactosamine (LacNAc) sequence. The sequential addition of LacNAc disaccharides, in tandem repeats, originate biosynthesis of poly-*N*-acetyllactosamines (poly-LacNAc) which are key ligands of lectins (as galectins) and antibodies (Stanley, et al., 2015).

Role of glycans in the regulatory circuits of the immune response

Glyco-immunology is an emergent area in health and life sciences with increasing amount of evidences demonstrating how the immune system is tightly controlled by cellular glycosylation. In fact, almost all of the key molecules involved in the innate and adaptive immune response are glycoproteins (Johnson, et al., 2013; van Vliet, et al., 2008; Wolfert and Boons, 2013). Alterations in protein glycosylation both at the cell surface and on secreted glycoproteins can, positively and negatively dictate the immune response.

The complex interplay between glycans and glycan-binding proteins (such as galectins, siglecs and other C-type lectin receptors (CLRs)) are recognized as crucial factors in the immune system. Additionally, other processes like antigen presentation, immune signaling cascades, innate recognition of microbial products and T cell response are also regulated in a glycan-dependent manner (Johnson, et al., 2013).

Selectins are surfaces localized members of CLR family which are carbohydrate-binding molecules that bind to fucosylated and sialylated glycoprotein ligands and are found on endothelial cells, leukocytes and platelets (E-, L- and P-selectin, respectively) (Ley, 2003). During an inflammatory response, the release of chemokines stimulates the endothelial cells to express selectins at their surface which are responsible for controlling leukocytes recruitment and homing to sites of inflammation and injury. An update of leukocyte adhesion cascade is reviewed in (Ley, et al., 2007). Interestingly, this particular feature leads to the successful use of anti-selectin antibodies in preclinical models as a new targets for inhibiting tissue-specific inflammation (Everts, et al., 2002; Jubeli, et al., 2012). However the challenge is inhibit specific subsets of leukocytes that lead to inflammation without affecting trafficking and function of other unaffected leukocytes avoiding systemic immunodeficiency (Luster, et al., 2005).

Siglecs have a key role in self- versus non-self-discrimination. Several pathogenic microorganisms, including several strains of *E. coli,* have evolved the capacity to synthesize or capture sialic acids from their hosts and incorporate these into their own glycoconjugates using it as a mechanism of immune escape. Interestingly, it is also widely assumed that negatively charged sialic acids could reduce pathogen interactions with the host by electrostatic repulsion, and/or by inhibiting the alternative pathway of complement activation (Crocker, et al., 2007).

Another important siglec is the cell surface CD22 glycoprotein, a key B-cell co-receptor, which recognizes α(2,6)-linked sialylated glycans suppressing B-cell receptor (BCR) signaling (Poe and Tedder, 2012) and further preventing autoimmunity.

Accordingly, CD22-deficient mice exhibit hyperimmune responses *in vitro* and *in vivo* (Collins, et al., 2006). In contrast, ST6Gal1–deficient mice, which cannot make CD22 ligands, exhibit hypoimmune responses (Hennet, et al., 1998).

Galectins, are a family of soluble conserved carbohydrate-binding proteins, that preferentially bind N-acetyllactosamine sequences (Galβ(1,4)GlcNAc) of O- and N-linked glycoproteins. They can form galectin–glycan structures termed lattices (van Kooyk and Rabinovich, 2008), that contribute to restrict the interactions between receptors and co-receptors on immune cells regulating intracellular signaling pathways, apoptosis, proliferation and migration (Liu and Rabinovich, 2010). After their first description in 1975 (Teichberg, et al., 1975), galectins were later associated with modulation of the immune response in 1983 where an eletrolectin could prevent the onset of experimental autoimmune myasthenia gravis in rabbits (Levi, et al., 1983).

Galectins family have three distinct classes based on their global structure, protopypical (e.g. galectin 1), tandem-repeat (e.g. galectin 8) and chimeric (e.g. galectin 3). Galectins are highly expressed in leucocytes namely in activated T cells, Treg cells, macrophages and DCs. They can also play distinct functions in modulating either negatively or positively the immune system. Galectin 1 and 3 are known to suppress inflammation and T cell response (Chung, et al., 2000; Demetriou, et al., 2001; Toscano, et al., 2007). Galectin 1 is described to negatively regulate Th1 and Th17 effector cells by inducing cell death (Toscano, et al., 2007). Galectin 3 has been described to limit TCR clustering due to lattice formation in MGAT5-expressing cells controlling thereby the threshold of T cell activation (Chen, et al., 2009b; Demetriou, et al., 2001). Similarly, galectin 2 also exhibits a suppressive effect by inducing apoptosis of lamina propria T lymphocytes attenuating acute and chronic colitis in mouse (Paclik, et al., 2008). In contrast, galectin 8 and galectin 4 induce T cell immune response. When binding to T cells, galectin 8 promotes T-cell proliferation, possibly through unique interactions with CD45 (Tribulatti, et al., 2009). Galectin 4 mediates $CD4^+$ T cells stimulation (by IL-6 production) leading to exacerbation of T cell- mediated chronic colitis (Hokama, et al., 2004).

A good example of how glycosylation affects the adaptive immune system is shown by immunoglobulins (large Y-shaped glycoproteins) that are produced by B cells and plasma cells. Their glycosylation profile critically determines their biological function, namely recognition of microbial antigens. IgG is the most abundant class of antibody in human plasma (accounting to approximately 75% of serum immunoglobulins) representing the major antibody isotype mediating immunity against pathogens (Aschermann, et al., 2010). The IgG conserved functional (Fc) domain contains a single, highly conserved, glycosylation site (Asn 297) that carries complex N-glycans. This N-

linked glycans can directly affect IgG antibody function. IgG agalactosylation, meaning loss of terminal galactose residues, has been observed in the sera of patients with autoimmune disorders like rheumatoid arthritis (RA) and IBD (Dube, et al., 1990; Parekh, et al., 1985; Shinzaki, et al., 2008). Interestingly, RA patients, in remission stages of disease, present an increase of α-2,6 sialylated (by ST6Gal-1 enzyme) IgG in circulation similarly with healthy controls. Moreover, high-dose intravenous immunoglobulin (IVIg) has been used as an effective treatment to control autoimmunity and later it was discovered that the anti-inflammatory portion of IVIg is exactly the α-2,6 sialylated N-glycans on the Fc domain (Kaneko, et al., 2006).

On the other hand, changes in the glycans repertoire can also fine tune host immune response by bi-directionally regulate microbiota content and function. Accumulating evidences have shown that gut microbiota and intestinal homeostasis are mediated by glycans influx (either from diet or mucosal secretion). Particularly in the intestinal mucosa, glycocalix (dense and complex coat of glycans) represents an extra physical and biological barrier. Glycoproteins on intestinal brush border membrane suffer a dynamic remodeling of its glycan content that shapes the intestinal homeostasis. The carbohydrate moieties (glycocalyx) at epithelial cells (ECs) surface, suffers a turning over of approximately every 6 to 12 hours in human jejunum (Moran, et al., 2011). This remodeling is crucial to maintain not only the protection barrier but also as a continuous source of nutrients that sustain commensal microbiome. For instance, mice experiments revealed a protective role of *B4GALT1* expression, which encodes for β-1,4-galactosyltransferase I, that mediates the addition of galactose moeieties to glycoproteins. Higher galactosylation of N-glycans on mucus proteins has been associated with increased ratio of Firmicutes/Bacteroidetes in healthy conditions, conferring protection against TNF-induced systemic inflammation and DSS-induced colitis (Vanhooren, et al., 2013). By contrast, the sialic acid composition of the intestinal glycocalyx has been also associated with dysbiotic conditions. The overgrowth of *E. coli* that occurs in colitis was demonstrated to be dependent on the sialic acid release from the host´s glycans after sialidase activity (Huang, et al., 2015). The dependence of *E. coli* on sialidases secreted by *Bacteroides* spp. (abundant in intestinal inflammation) contribute to the overgrowth of *E. coli* and thereby to dysbiosis in patients with colitis (Gophna, et al., 2006).

Moreover, several microorganisms (including meningococci, *Trypanosoma* and *Helicobacter)* can synthesize terminal glycan structures that are similar to those found in mammalian cells in a process of “molecular mimicry”. This “glycan escape” strategy contributes to hide from host immune system and thereby triggering pro-inflammatory responses (van Die and Cummings, 2010; van Kooyk and Rabinovich, 2008).

Another example of glycosylation impact in innate immune response was demonstrated by an elegant study, where mice deficient in a Golgi enzyme, α-Mannosidase II (αM-II; *MAN2A1*) that partially abolishes the complex N-glycans production, lead to the exposure of unusual hybrid glycans epitopes (commonly expressed at pathogen surfaces) on erythroid lineage, contributing to the abnormal recognition by innate immune system and triggering an excessive co-stimulation of the immune response (Green, et al., 2007).

Emergent evidences pointing out for a crucial role of glycans in mediating tolerogenicity of DCs. It was shown that sialylation of antigens can induce antigen-specific immune tolerance which promotes a dual-tolerogenic function of DCs in inducing Treg cells and concomitantly, suppressing the IFN-γ- producting T cells. Hence, it can be a potential strategy to dampen excessive T-cell pathologies (Perdicchio, et al., 2016).

Protein glycosylation can also influence the adaptive immune response and consequently T cell priming (Wolfert and Boons, 2013). On this process there are key glycoproteins, namely the MHC class I and II proteins which form a complex with glycosylated peptides to be recognized by T cells. Hence, T helper cell (by MHC II) and cytotoxic T cell (by MHC I) responses are highly driven in a glycan dependent manner (Neefjes, et al., 2011).

More recently, protein O-GlcNAcylation, which consist in the addition of O-GlcNAc moieties to serine or threonine residues of nuclear and cytoplasmic proteins by the O-GlcNAc transferase (OGT) through UDP-GlcNAc (Hart, et al., 2007), has been demonstrated to be implicated in the metabolic processes involved in T cell self-renewal, differentiation and proliferation (Swamy, et al., 2016). This work highlights that access to nutrients which enables all cellular functions is more highly regulated than previously imagined. Current data showed that T cells activation accompanies increased nutrients (glucose and glutamine) uptake inducing increase of UDP-GlcNAc which in turn can enhance OGT activity and glycosylation of multiple proteins such as c-Myc. The glycosylated form of c-Myc is an important factor that regulates a feedback mechanism controlling the nutrients uptake and UDP-GlcNAc production thereby regulating O-GlcNAcylation which is a key factor in the regulation of T cells and their precursors. The loss of OGT enzyme blocked T cell progenitor renewal, malignant transformation and peripheral T cell clonal expansion (Swamy, et al., 2016) .

Additionally, complex O-glycans have also been demonstrate to be key in protein modification from which T cells depend to interact with selectins and in consequence, they are indispensable in regulating the trafficking of T cells, namely the distribution and homing of both naive and activated T cells *in vivo* (Hobbs and Nolz, 2017). Evidences support also that the capacity for memory T cells to rapidly traffic into a site of infection is

critical for protective immunity and is highly dependent on *de novo* synthesis of core 2 O-glycans. Interestingly, it is postulated that stimulation of core 2 O-glycans on tumor-specific T cells can be a potential combinatory therapy to enhance T cell trafficking and ultimately improve cancer immunotherapy (Hobbs and Nolz, 2017).

Overall, glycans are key players in the regulatory circuits of both innate and adaptive immune response. However, it remains unclear whether glycans changes are cause or consequence of the inflammatory cascade. Therefore, the short-term goal in glyco-immunology field is the unceasingly effort on decoding and selectively modulate cellular glycomes which certainly will provide exciting opportunities to control innate and adaptive immune responses (Rillahan and Paulson, 2011; Smith and Cummings, 2013).

T cell receptor

TCR structure and activity

The T cell receptor (TCR) is the primordial receptor at the surface of T lymphocytes. It is a glycoprotein that interacts with other glycoproteins like MHC I and II in response to an immunological stimulus. MHC I interacts with TCRs on $CD8^+$ T cells, whereas MHC II is recognize by $CD4^+$ T cells (Johnson, et al., 2013; Rossy, et al., 2012).

The stoichiometry of the TCR/CD3 complex has not yet been clearly established but is generally accepted to consist of a αβTCR heterodimer, two CD3ϵ chains, one CD3γ, one CD3δ chain and a ζ homodimer (Brownlie and Zamoyska, 2013; Rudd, et al., 1999).

According to the literature, TCR α, β subunits have at least 7 N-glycan addition sites and alterations on the glycoprofile can interfere with its flexibility, movement and interactions with surface molecules. TCR-CD3 complex has a total of 12 N-glycan addition sites (Kuball, et al., 2009; Rudd, et al., 1999).

Activation of T cells is a key element in adaptive immune response and requires the coordination of complex signal transduction networks. The process of T cell activation begins at the immunological synapse formed at the interface of the TCR and APC (Figure 11). The synapse has a classic bull's-eye pattern and it is organized into supramolecular activation clusters (SMACs), with the central SMAC (cSMAC) rich in TCRs. Then, surrounding this layer there is the peripherial SMAC (pSMAC) that is enriched in signaling co-factors and adhesion complexes. The distal SMAC (dSMAC) comprises CD45 glycoprotein among others (Rossy, et al., 2012).

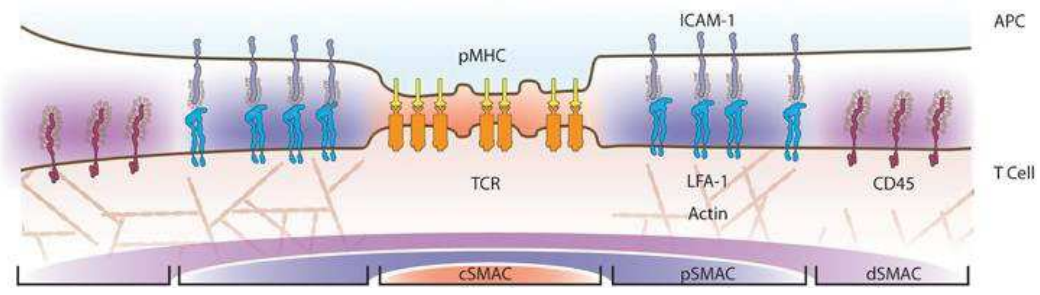

Figure 11. The immunological synapse formed at the interface of the TCR and APC and organization of distinct supramolecular activation clusters (SMACs). Adapted from (Rossy, et al., 2012).

This bull's-eye pattern has been described in Th cells, cytotoxic T cells, Treg cells, B cells and natural Killer (NK) cells. However the SMAC organization is not necessarily required for T cell signaling because for instance at the interface between DCs and Th2 cells there are multiple focal structures instead (Rossy, et al., 2012). Interestingly, the pattern of immunological synapses in self-reactive T cells clonally derived from patients with multiple sclerosis (MS) and type 1 diabetes showed a strong TCR phosphorylation and signaling activity but cSMAC was not formed in self-reactive T cells (Schubert, et al., 2012).

The TCR has no intrinsic enzymatic activity thus it depends on the kinase activity of the Src family kinase (SFKs), particularly Lck which initiates the signaling by binding to the cytoplasmic domains of the TCR co-receptors CD4 and CD8 (Brownlie and Zamoyska, 2013).

Summarizing the cascade of events occurring upon activation of TCR signaling pathway (Figure 12), the TCR signal transduction is initiated by the recognition of cognate peptide-MHC molecules. As already mentioned, Lck is the first to be recruited to the TCR-CD3 complex, which phosphorylates immunoreceptor tyrosine- based activation motifs (ITAMs) in the CD3γ chain, CD3δ chain, CD3ϵ chains and the ζ-chains. Phosphorylation of the ITAMs enables the recruitment of ZAP70 (ζ- chain associated protein kinase), that is phosphorylated by LcK and activated. Activated ZAP70 further phosphorylates four key tyrosine residues on linker for activation of T cells (LAT), which recruits numerous signaling molecules to form a multiprotein complex, termed the LAT signalosome. This complex includes phospholipase Cγ1 (PCγ1), growth factor receptor-bound protein 2 (GRB2), GRB2- related adaptor protein GADS, SLP76 (SH2 domain-containing leukocyte protein), adhesion- and degranulation-promoting adaptor protein (ADAP), interleukin-2-inducible T cell kinase (ITK), NCK1 and VAV1. Then, LAT signalosome propagates signal to three major signaling pathways: the calcium (Ca^{2+}), the mitogen-activated protein kinase (MAPK) and the nuclear factor- κB (NF- κB) signaling pathways, leading to the mobilization of transcription factors that are crucial for gene expression and for T cell growth and differentiation. Signals initiated from the TCR also result in actin reorganization and the activation of integrins by inside-out signaling (Brownlie and Zamoyska, 2013).

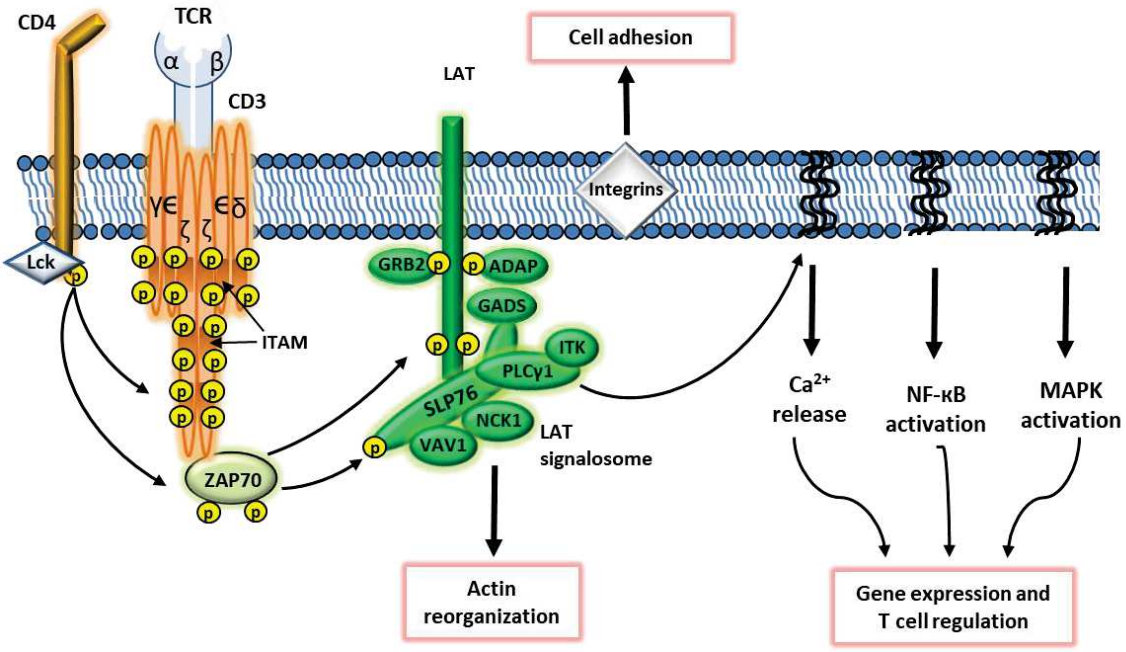

Figure 12. T cell receptor signaling cascade. Adapted from (Brownlie and Zamoyska, 2013).

In fact, from all these elements in TCR signaling, LAT is considered to be a gatekeeper since it ensures signal propagation in a regulated manner. Indeed, cells deficient in LAT, such as Jurkat cell mutant JCAM2.5, cannot propagate TCR signals (Finco, et al., 1998). Moreover, LAT-deficient mice do not have mature T cells in spleen and lymph nodes, and the thymocytes of these mice were exclusively $CD4^{-}CD8^{-}$ meaning that *LAT*$^{-/-}$ thymocytes fail to develop beyond the double negative (DN) stage. Thus, LAT also have a critical role on T cell development (Zhang, et al., 1999).

However, there is a considerable number of evidences that point toward the existence of LAT-independent signaling pathways (Brownlie and Zamoyska, 2013). Surprisingly, it was observed that LAT-deficient T cells still proliferate and produce cytokines. Therefore, the simplistic idea of TCR signaling propagation in a linear manner through the LAT signalosome are still under investigation as well as spatiotemporal concepts of how LAT reaches the TCR (reviewed in (Brownlie and Zamoyska, 2013)).

Overall, a critical understanding of the regulation of these signaling cascades is essential as these mechanisms regulate the thresholds of T cell activation, controlling antigen sensitivity, which is particularly important in naïve T cells that respond only to foreign-peptide-MHC-complexes to maintain immune tolerance (Adachi and Davis, 2011).

Notably, during TCR signal transduction, glycans play a key role in stabilizing individual molecules in the complexes at the immunological synapse and by protecting them from the action of proteases during T cell engagement (for class II APCs this

process may take several hours). Additionally, glycans can restrict nonspecific protein-protein interactions like aggregation of TCRs on the membrane helping to orient the interactions of the proteins in the central clusters (Rudd, et al., 1999).

N-glycosylation and T-lymphocytes

The proper function of T-lymphocytes function is highly dependent on their surface receptors which in turn are highly mediated by glycosylation.

One of the major negative regulators of T-cell responses is the cytotoxic T-lymphocyte protein 4 (**CTLA-4**) which function is dependent on its retention at T cell surface. Indeed after TCR activation, endocytosis rates are increased and Src-family kinases and phosphatidylinositol 3-kinase/Erk stimulate hexosamine flux in the Golgi to generate β1,6 GlcNAc branched N-glycans which in turn glycosylate CTLA-4 enhancing its surface retention and thereby suppresses T cell activation promoting immune tolerance. On the contrary, β1,6GlcNAc branched N-glycans reduction on T cells induces CTLA-4 endocytosis contributing to exacerbation of T cell response which can lead to autoimmunity (Dennis, et al., 2009).

Programmed cell death protein-1 (**PD-1**) is another inhibitory receptor of T cells that leads to inhibition of T lymphocyte proliferation, cytokine production, cytolytic activity and suppression of immune response (Freeman, et al., 2000). Recently, it was demonstrated that core fucosylation (which refers to fucose attached to the innermost N-acetylglucosamine of N-linked glycans, catalyzed by α1-6 fucosyltransferase (FUT8)) on N-linked oligosaccharides is required for cell-surface expression of PD-1 on T cells. Blocking this post-translational modification results in anti-tumor immune responses mediated by T cells, being a new attractive target for enhancing anti-tumor immunity in future clinical settings (Okada, et al., 2017).

The T cell activity is also influenced by complexes of TCR and **CD45** which are also critically dependent on glycosylation. Particularly, galectin-3 is a key mediator of the CD45 and the TCR signaling complex organization which via their glycans close proximity originate a lattice formation. Consequently, CD45 phosphatase activity induces downregulation of T-cell signaling, preventing T-cell activation (Wolfert and Boons, 2013).

The **CD28** is another T cell surface glycoprotein important as secondary signaling molecule of T cell activation. Interestingly, nearly 50% of the molecular mass

of CD28 is constituted by N-glycans (Aruffo and Seed, 1987). Previous studies reported that N-glycosylation of human CD28 can negatively regulate CD28-mediated T cell adhesion and costimulation, namely the interaction between CD28/CD80. Different approaches like mutation of all potential CD28 N-linked glycosylation sites as well as treatment of Jurkat cells with inhibitors of N-glycosylation resulted in a defective CD28 glycosylation and enhancement of the binding to CD80 expressed on APCs (Ma, et al., 2004).

CD25 receptor surface retention by branching N-glycans is important to control T differentiation and immune tolerance. Recently, it was demonstrated that by reducing UDP-GlcNAc and branching it induces a decrease of CD25 surface retention and IL-2 signaling and promotes T_H17 over iTreg differentiation (Araujo, et al., 2017).

Notably, all these evidences reinforce that glycosylation plays a key role in T cell activation regulating not only TCR but also many of its key partners (co-stimulatory receptors and multiple receptor-ligand interactions between T cell and an APC). Hence, glycans alterations directly or indirectly dictate the overall T cell function (Johnson, et al., 2013).

Dysregulation of TCR N- glycosylation in autoimmunity

Accumulating evidences have been demonstrating that β1,6GlcNAc branched N-glycans structures regulate T cell activation (Demetriou, et al., 2001).This type of structures are catalyzed by the β1,6 N-acetylglucosaminyltransferase V (GnT-V) which is encoded by MGAT5 gene. MGAT5 gene transcription is positively regulated by Ras-Raf-Ets (Chen, et al., 1998; Ko, et al., 1999), a pathway commonly activated in cancer cells as well as on activated T cells.

In homeostasis, when an antigen presenting cell binds a specific TCR through the MHC II-peptide complex, the triggered TCR signaling activation has been shown to regulate multiple Golgi N-glycan branching-processing enzymes at the mRNA level, including MGAT5 (Chen, et al., 2009a). Increasing expression of MGAT5 lead to the enhancement of GnT-V activity and increase β1,6GlcNAc branched N-glycans structures on TCR which precludes the TCR clustering and signaling, leading to T cell growth arrest and, consequently, to a controlled immune response (Chen, et al., 2009a). The dysregulation of this key regulatory mechanism of T cell glycosylation with branched N-glycans results in T-cell hyperactivity and susceptibility to immune-mediated diseases. Mice deficient in MGAT5 and lacking GnT-V function (no synthesis of β1,6GlcNAc

branched N-glycans structures), display a significantly increased TCR clustering, leading to a decreased threshold of T-cell activation and increased Th1 differentiation resulting in a hyperimmune response and increased susceptibility to autoimmunity (Demetriou, et al., 2001; Morgan, et al., 2004). This increased sensitivity is also due to the lack of lattice formation between TCR complex and the endogenous polylactosamine-binding lectin, galectin 3 (Demetriou, et al., 2001).

The production of these polylactosamine extensions in glycoproteins of T cells is central to the control of the immune response (Togayachi, et al., 2007). Accordingly, β3GnT2- deficient mice show a T cell hypersensitivity due to the reduction of polylactosamine on the N-glycan similarly to the observations in MGAT5 deficient mice (Demetriou, et al., 2001; Togayachi, et al., 2007) .

On the other hand, the metabolic supplementation of mouse models of EAE and type I non-obese diabetic mice and T cells with GlcNAc resulted in the enhancement of GlcNAc branching on T cells by GnT-V activity, which increased the threshold for T cell activation, suppressing T cell growth and inhibiting Th1 differentiation concomitantly with a decreased disease severity (Grigorian, et al., 2011; Grigorian, et al., 2007). These results demonstrate that GnT-V-mediated glycosylation plays a key role in the regulation of T cell activity and signaling in immune-mediated disorders.

The mechanism of GlcNAc uptake is still debatable but to date it was observed that exogenous GlcNAc added to mammalian cells appeared to be taken up by pinocytosis and was then converted to UDP-GlcNAc (Grigorian, et al., 2007). A recent study focused on the impact of GlcNAc supplementation on mouse physiology and metabolism showed that oral GlcNAc supplementation is mainly converted into body-mass and fat content meaning weight gain and lipid storage in adult mice. Moreover, no significant alterations were observed in other parameters like calorie-intake, total activity, energy expenditure and gut microbiome (Ryczko, et al., 2016). However, it remains unclear whether O-linked GlcNAc is also altered when cells are exposed to exogenous GlcNAc. Nonetheless, Araujo and colleagues observed that the effects of GlcNAc on T cell differentiation were reversed by directly blocking Golgi branching activity, confirming that the effects of UDP-GlcNAc enhancement were predominantly through branching rather than other pathways (Araujo, et al., 2017).

Recent evidences on mice suggested that composition of glycans, rather than their structure, determines their role (Mkhikian, et al., 2016). In mice lacking specific MGAT enzymes, disruption of branching pathway produce LacNAc with fewer branches glycans due to reduction of UDP-GlcNAc consumption at *medial* Golgi. Alternatively this unused GlcNAc is driven forward to *trans* Golgi where the local β3GnTs enzymes are postulated to be able to produce bioequivalent poly-LacNAc structures. Accordingly, the

authors proposed that loss of LacNAc branches is balanced by increased production of linear LacNAc polymers, a Golgi self-correcting ability that sustain cell surface LacNAc density and thereby the galectin-glycoprotein lattice controlling the risk of autoimmunity (Mkhikian, et al., 2016).

In the last decade, Demetriou and colleagues have been exploring this mechanism of T cells regulation by N-glycosylation and how N-glycan biosynthesis can rescue branching deficiency in MS disease model. In this regard, it has been proposed that *MGAT5* is a gene that appears to be associated with severity and susceptibility to MS (Brynedal, et al., 2010; Li, et al., 2013), including in a GWAS (Brynedal, et al., 2010). Furthermore, it was proposed that the association between environmental factors (sunlight/vitamin D_3 and metabolism) combined with multiple genetic variants (IL17RA, IL2RA, MGAT1 and CTLA-4) converge to dysregulate Golgi N-glycosylation and regulate MS disease development and severity (Mkhikian, et al., 2011).

Another type of N-glycans structures with implications in T cell-mediated inflammation is core fucose catalyzed by α1-6 fucosyltransferase (FUT8). Recently, Fujii and colleagues demonstrated that FUT8 dysregulation is associated with IBD pathogenesis (Fujii, et al., 2016). In mouse models of induced colitis, the authors described that T cells display an increased expression of core fucosylation when compared with mice without colitis. Accordingly, the *Fut8*$^{-/-}$ mice developed a less severe colitis than *Fut8*$^{+/+}$ mice, and T cells from *Fut8*$^{-/-}$ mice produced lower levels of T-helper 1 and 2 cytokines. Accordingly, colonic samples from both CD and UC also revealed that inflamed mucosa exhibited higher levels of core fucosylation comparing to non-inflamed mucosa and healthy colon (Fujii, et al., 2016).

Altogether, these emerging evidences on the impact of glycosylation in immune-mediated diseases have been drawing the attention of many researchers and became also one of the main purposes of this doctoral thesis in the IBD field.

Experimental murine models of colitis

In vivo models are generally used as good alternatives to test new hypothesis as they constitute more complex systems than cell lines, being frequently used as proof-of-concept studies for new drug development. In IBD, there are suitable animal models available that closely resembles human IBD.

In contrast with genetically engineered mouse models of IBD or adoptive T-cell transfer and spontaneous models of IBD, the chemically induced mouse models develop a much faster onset of inflammation and the involved procedures are relatively straightforward, being extensively used in the last decades.

Chemically induced mouse models of colitis

- **Dextran sulfate sodium model**

In this doctoral thesis, we have used one of the most widely used mouse models of colitis, the dextran sulfate sodium (DSS). This model was stablished since almost 30 years ago, by Okayasu and colleagues, and it is the model that most closely resembles human UC in which mice develop similar symptoms like bloody stools, diarrhea and weight loss (Okayasu, et al., 1990). Although the exact mechanism of its colitogenicity remains to be elucidated, it is believed that DSS induces intestinal inflammation by damaging the intestinal epithelial cells allowing the dissemination of pro-inflammatory intestinal contents (e.g. bacteria and their products) into underlying tissue (Chassaing, et al., 2014; Wirtz, et al., 2017).

The main advantages of this model is that induction of colitis is very simple (oral administration in drinking water), rapid (*acute DSS colitis*: 8–14 days; *chronic DSS colitis:* 52–56 days) and reproducible. It is possible to implement acute, chronic and relapsing models of intestinal inflammation by simply modifying the concentration of DSS and its frequency of administration (Wirtz, et al., 2017).

Throughout the years, different studies demonstrated particularities of this model that need to be taken into account dependent on the final goal of the study namely strain differences (e.g. C57BL/6 mice are more susceptible to DSS-induced colitis than BALB/c mice); gender differences (e.g. male mice tend to be more susceptible than female mice) and age of induction is also a crucial aspect (e.g. mice must have 6 to 8 weeks at the beginning of induction). Moreover, the colitogenic potential of DSS is critically dependent on its molecular weight, ideally 36 to 50 KDa (Wirtz, et al., 2007; Wirtz, et al., 2017). Additionally, depending on the study purpose, a certain mouse strain can reveal other

critical aspects, for instance, a certain strain with a genotype never tested with DSS model can demonstrate outcomes different due to gender or age. In this regard, pilot studies are always required to initial test and to help in further protocols refinements despite the existence of detailed ones (Wirtz, et al., 2017).

Interestingly, the DSS model can also be integrated in the type of mouse models used to investigate the narrow relationship between chronic colitis and neoplasia development. The model AOM/DSS was proposed in 2003 by Takuji Tanaka and colleagues and consists on the combination of repeated cycles of DSS with the genotoxic agent azoxymethane (AOM). AOM is administered by an intraperitoneal injection following one week of oral DSS (in drinking water) and together these agents induce colonic epithelial cell damage and mutagenesis. Shortly, in 10 weeks, this model develops tumors (Tanaka, et al., 2003). Importantly, this model shows histopathological features of human colitis-associated colorectal cancer (CA-CRC) like distant-located tumors and invasive adenocarcinomas (De Robertis, et al., 2011). In addition to this chemically induced model, genetically modified mouse models, like TRUC mice(Garrett, et al., 2009), IL-10 KO mice (Berg, et al., 1996) and TCR-α KO mice (Dianda, et al., 1997), have been used to shed light on the understanding of CA-CRC.

- **Trinitrobenzene Sulfonic Acid model**

The hapten reagent 2,4,6-trinitro-benzene sulfonic acid (TNBS) was introduced in 1989, by Morris et al. (Morris, et al., 1989), as a model of chemically induction of colitis by rectal administration. Ethanol is used as a vehicle as it is needed for disruption of intestinal barrier enabling the interaction of TNBS with colon tissue proteins (Ikeda, et al., 2008; Neurath, et al., 1995). After the first week upon intracolonic administration, signs of severe disease characterized by bloody diarrhea and loss of body weight are observed (Antoniou, et al., 2016).

TNBS –induced colitis is a suitable model to assess therapeutic studies that closely resembles features of human CD like transmural inflammation with increase infiltration of macrophages, neutrophils and lymphocytes as well as colonic patch hypertrophy. This model is also known to develop an acute Th1 inflammation (Elson, et al., 1995). Chronic TNBS colitis in BALB/c mice is characterized by persistent lamina propria fibrosis, a well-known feature of CD (Antoniou, et al., 2016).

- **Oxazolone model**

In 1998, oxazolone was described by Boirivant M. and colleagues, as a new method of experimental colitis (Boirivant, et al., 1998). As TNBS, oxazolone is also a hapten reagent that needs to be dissolved in ethanol and be administered topically to induce colitis. Of note, both methods are distinct in the type of T cell responses induced. Comparing with TNBS, oxazolone mouse model develops colitis restricted to the distal half of the colon. Moreover, oxazolone induces the release of TGF-β which plays an important role in the control of inflammation. This model typically induces a Th2- immune response, and tissue damage is characterized by an increased production of interleukin, IL-4 and IL-5 (Boirivant, et al., 1998).

Importantly, mouse strain susceptibility is an issue to be considered. Accordingly, C57BL/6 mouse strain is more resistant to oxazolone colitis and may require a subcutaneous sensitization step before intrarectal administration. In this case, it resembles histologically UC features with a mixed neutrophil/lymphocyte infiltration limited to the superficial layer. More recently, it was accepted as the ideal model to study the type-2- and type-9-related immune responses during intestinal inflammation (Gerlach, et al., 2014).On the other hand, in BALB/c mouse strain, chronic colitis can be induced by repetitive administration of oxazolone which can be useful to study specific characteristics on the progression of inflammatory response to a chronic stage (Wirtz, et al., 2017).

Other IBD models

- **Adoptive T cell Transfer model**

Firstly described by in 1993 by Morrissey et al. and Powrie et al., adoptive T cell transfer model consists in the adoptive transfer of a subset of $CD4^+$ T cells to syngeneic SCID or Rag-knockout mice, results in the development of a chronic, progressive colitis and wasting disease, usually 5 to 10 weeks after treatment (Morrissey, et al., 1993; Powrie, et al., 1993). As other models, it develops common colitis symptoms such as chronic, progressive disease with diarrhea and weight loss, heavily inflamed colon- occasionally transmural damage and loss of mucus from goblet cells.

This model has been extensively used for studying the function of effector cells and regulatory cells in IBD pathogenesis as well as testing new IBD drug candidates (Coombes, et al., 2005; Powrie, et al., 1994; Uhlig and Powrie, 2009). It has contributed to understand the mechanisms that control intestinal inflammation being, for instance,

widely used to identify mechanisms through which $CD4^+CD25^+$ Treg cells suppress intestinal inflammation *in vivo*. The initial findings demonstrate that transfer naive $CD45RB^{high}$ T cells (which cannot be induced to generate Treg cells) to lymphopenic mice induces colitis but if naïve T cells were transferred with mature $CD45RB^{low}$ T cells (already contains Treg cells) prevents the development of intestinal inflammation (Kiesler, et al., 2015). The role of Th1 and Th17 effector T –cell responses in this model has been challenging to define but current data support that transfer colitis is largely mediated by Th1 cells producing IFN-γ. However, there are some evidences showing an atypical IFN-γ origin indirectly from a Th17 differentiation pathway depending on IL-23 rather than directly from a Th1 differentiation (Kiesler, et al., 2015).

Overall, cell transfer models are crucial to understand colitis immune regulation but it cannot be assumed that a model that depends on cell expansion in a lymphopenic host completely mimics human IBD. However, comparing with other chronic colitis models, adoptive transfer colitis present several practical advantages to pharmacological testing (e.g., the synchronized onset of disease, no generation of anti-drug antibodies and commercial availability of mice).

- **IL-10 Knockout model**

IL-10 is a key immunosuppressive cytokine that drives the maintenance of gut homeostasis and in 1993, it was identified that IL-10- deficient mice develop chronic enterocolitis. This murine model develops spontaneous inflammation of the colon characterized by the presence of an inflammatory infiltrate made up of lymphocytes, macrophages and neutrophils (Kuhn, et al., 1993). Currently, it is known that genetic polymorphisms of IL-10 confer risk to both UC and CD (Franke, et al., 2008; Franke, et al., 2010).

Throughout the years, several studies using this model shed light on how IL-10, alone or in combination with other molecular alterations, is a key factor on colitis development. Recently, the ability to respond to TLR stimulation through regulation by PTEN phosphatase was shown to be important in IL-10-deficient mice' susceptibility to develop colitis. It was shown that mice lacking both IL-10 and PTEN develop early onset disease and increase severity and could form colonic tumors (Im, et al., 2014). Moreover, PTEN/ IL-10-deficiency seems to influence colitis pathogenesis through alterations in gut microbiome due to a predominance of Bacteroides organisms (Im, et al., 2014). Furthermore, IL-10R murine studies indicate that IL-10R-dependent signals suppress pro-inflammatory macrophages function as well as enhance tolerogenic

macrophages properties, both at the peripheral level and in the intestine. This study defined innate immune IL-10R signaling as a key factor regulating mucosal immune homeostasis in mice and humans (Shouval, et al., 2014).

Taken together, this model not only has been essential to understand intestinal immune response mediated by IL-10 signaling pathways but it can also represent an important tool to further explored target drug development to a subset of patients once that particularly early-onset IBD patients harboring mutations in *IL-10R* genes (Shouval, et al., 2014).

Apart from the different aforementioned animal models, other models have emerged to study human gastrointestinal disorders like IBD, such as the *in vitro* organoid models (Dedhia, et al., 2016). Despite they are hard to implement, they have been optimized and became more frequently used because we can gain insights on the cell composition and organization once their cellular complexity and physiology is similar to that of native organ systems. Therefore, they represent an extraordinary technical breakthrough being an important tool for basic biology and clinical applications (Dedhia, et al., 2016; Noben, et al., 2017a). Emergent evidences focused on studying stem cells from IBD patients, demonstrate distinct expressed genes in organoids from patients with UC in comparison to controls pointing out to a genetic imprinting which is recapitulated *in vitro* (Dotti, et al., 2017; Noben, et al., 2017b).

Additionally, linking those models with the topic of this doctoral thesis, it is worth to mention that all these *in vivo* models of IBD have also shed light on the importance of glycosylation alterations, both N- and O- glycosylation, on colitis development. In fact, several studies demonstrate that glycosylation-based modifications in mice have an association with intestinal inflammation presenting different phenotypes, some promoting colitis protection, others increasing colitis susceptibility or even spontaneously developed colitis as summarized in Table 3.

Table 3. Examples of glycosylation alterations associated with intestinal inflammation.

Type of alterations	Colitis association	Intestinal feature	References
Core 3 $^{-/-}$	Susceptible to DSS	Mucins were more susceptible to proteolysis; Loss mucus barrier	(An, et al., 2007; Bergstrom, et al., 2017)
Core 1 $^{-/-}$	Spontaneously develop colitis	Mucins were more susceptible to proteolysis; Loss mucus barrier	(Bergstrom, et al., 2017)
FX $^{-/-}$ (fucosylation deficiency)	Develop colitis	Altered the composition of the fecal microbiota, epithelial barrier dysfunction, altered epithelial proliferation	(Wang, et al., 2017)
MUC 2 $^{-/-}$	Spontaneously develop colitis	Associated with epithelial barrier dysfunction	(Van der Sluis, et al., 2006)
FUT 8 $^{+/+}$	Develop more severe colitis than FUT 8 $^{-/-}$	Increase TCR signaling and production of inflammatory cytokines	(Goto, et al., 2014)

In summary, the use of well-established murine models of colitis and the continuous development of novel *in vivo* models of colitis are a crucial tool to better understand intestinal inflammation and IBD pathogenesis. Together with the progress on routine methods for analysis of the degree of intestinal inflammation such as *in vivo* imaging of inflammation (Becker, et al., 2005), it can promote a new body of knowledge regarding the abnormalities that lead to the genesis of intestinal inflammation.

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Main aims

IBD is a multifactorial disorder and although progress has been made in IBD research, its precise etiopathogenesis is far from being fully elucidated. Therefore it is of paramount importance to identify and characterize the underlying molecular mechanisms of IBD pathogenesis in order to improve the development of novel biomarkers that may help the determination of prognosis and also improve patients' stratification for appropriate treatment. Accumulating evidences have been shown that GnT-V-mediated glycosylation on T cells has a critical role in the regulation of some immune-mediated disorders, such as multiple sclerosis (Demetriou, et al., 2001; Grigorian and Demetriou, 2011). Since this question was never addressed in IBD, the main goal of this project is to assess for the first time whether the dysregulation of this coordinated interplay (T cell function/branched N-glycans) is a novel underlying molecular mechanism in IBD with potential clinical and therapeutic applications.

The general aim of the present thesis is to disclose how glycans regulate the immune response in IBD, emphasizing the role of N-glycosylation of T cells in IBD pathogenesis and the potential of specific glycans, such as N-acetylglucosamine (GlcNAc) as an immunomodulatory strategy in IBD.

Specific aims

1. **To uncover the underlying molecular mechanism of T cells´ branching N-glycosylation in UC.**

A growing body of evidence has demonstrated that in homeostasis and self-tolerance, the T cell activation (TCR signaling) induces up-regulation of the MGAT5 gene which in turns leads to GnT-V-mediated glycosylation of the TCR**.** The N-glycan branching on T cells, catalyzed by GnT-V glycosyltransferase, promotes the formation of multivalent galectin binding which negatively regulates T cell growth by precluding TCR clustering, and increasing the TCR activation thresholds, ultimately modulating the immune response.

We aimed to assess whether T cell regulation by GnT-V-mediated glycosylation is a new underlying molecular mechanism in UC.

In chapter II, we investigated the relationship between the levels of expression of branched N-glycan structures in the intestinal lymphocytic infiltrate in relationship with clinicopathological parameters of UC patients. We also analyzed the MGAT5 gene expression and GnT-V enzymatic activity comparing UC patients with different disease severity *versus* controls.

2. **To evaluate the relationship between the glycosylation of T cells and regulation of the immune response underlying UC to understand the impact of glycophenotype modulation in intestinal inflammation.**

Despite the recent advances in IBD therapeutic resources, a high proportion of patients remain refractory to conventional treatment, and approximately half of the patients with UC do not achieve sustained remission. Furthermore, issues related to side effects and failure in therapy response highlight the need to identify more effective and targeted-specific therapies. Following the disclosure of the molecular mechanism based on dysregulation of TCR N-glycosylation (in chapter II), we hypothesized that modulating N-glycosylation of T cells, by enhancing the hexosamine pathway, can control immune response in UC.

In chapter III, we investigated the biological effects and the potential therapeutic efficacy of GlcNAc in T cells function and activity. We used an *ex vivo* approach to assess the effects of supplementation with GlcNAc in the regulation of T cell response, testing T lymphocytes isolated from blood and biopsies of UC patients. The clinical efficacy of GlcNAc was further tested in pre-clinical models of colitis using the DSS-induced mouse model. Moreover, we assessed whether deficiency in branched N-glycosylation (in *MGAT5* null or heterozygous mice) has an impact in disease onset and severity. We also conducted *in vivo* studies to assess the therapeutic efficacy of GlcNAc in severe forms of colitis testing different routes of administration.

II

Dysregulation of T cell receptor N-glycosylation: a molecular mechanism involved in ulcerative colitis.

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Dysregulation of T cell receptor *N*-glycosylation: a molecular mechanism involved in ulcerative colitis

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The incidence of inflammatory bowel disease is increasing worldwide and the underlying molecular mechanisms are far from being fully elucidated. Herein, we evaluated the role of *N*-glycosylation dysregulation in T cells as a key mechanism in the ulcerative colitis (UC) pathogenesis. The evaluation of the branched *N*-glycosylation levels and profile of intestinal T cell receptor (TCR) were assessed in colonic biopsies from UC patients and healthy controls. Expression alterations of the glycosyltransferase gene *MGAT5* were also evaluated. We demonstrated that UC patients exhibit a dysregulation of TCR branched *N*-glycosylation on lamina propria T lymphocytes. Patients with severe UC showed the most pronounced defect on *N*-glycan branching in T cells. Moreover, UC patients showed a significant reduction of *MGAT5* gene transcription in T lymphocytes. In this study, we disclose for the first time that a deficiency in branched *N*-glycosylation on TCR due to a reduced *MGAT5* gene expression is a new molecular mechanism underlying UC pathogenesis, being a potential novel biomarker with promising clinical and therapeutic applications.

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic immune-mediated disorder of the gastrointestinal tract that includes ulcerative colitis (UC) and Crohn's disease (CD) (1). The incidence of IBD is increasing worldwide and the disease remains incurable. IBD places a heavy burden on populations reducing quality of life and incurring substantial medical and societal costs (2). Although progress has been made in understanding the disease, the etiopathogenesis of IBD is far from being fully elucidated. Accumulating evidence suggests that IBD results from an inappropriate inflammatory response in a genetically susceptible host (1,3), although the underlying molecular mechanisms remain elusive.

UC is a chronic inflammatory condition causing continuous mucosal inflammation of the colon affecting the rectum and a variable extent of the colon in continuity, which is characterized by a relapsing and remitting course. The inflammation in UC is typically confined to the mucosa (1,2), and the course of the disease is characterized by flares that alternate with periods of remission. Severity of flares and their response to treatment vary and are hard to predict. In addition, prognosis of patients with UC is difficult to determine (2). In line with this, it is of paramount importance to further identify and characterize the underlying molecular mechanisms of UC pathogenesis in order to improve the development of novel biomarkers that may help the determination of prognosis and also improve the patients' stratification for appropriate treatment (4).

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Table 1. Relationship between L-PHA expression in intestinal lymphocytic infiltrate and the inflammatory activity

L-PHA expression (%) in intestinal lymphocytic infiltrate Clinical features	Number of cases ($n = 64$)	Low	High	P-value
Inflammatory activity				<0.0001
Control (no disease)	13	0	13 (100%)	
Inactive disease (Mayo 0)	16	1 (6.25%)	15 (93.75%)	
Active				
Mayo 1	13	9 (69.23%)	4 (30.77%)	
Mayo 2	14	13 (92.86%)	1 (7.14%)	
Mayo 3	8	8 (100%)	0	

Glycosylation is a complex post-translational mechanism characterized by the addition of carbohydrate structures (glycans) to proteins and lipids in the endoplasmic reticulum/Golgi secretory pathway, by specific enzymes (glycosyltransferases) (5). The immune system is tightly controlled by cellular glycosylation as almost all of the key molecules involved in innate and adaptive immune responses are glycoproteins (6–8). The *N*-acetylglucosaminyltransferase V (GnT-V) is a glycosyltransferase encoded by the human *MGAT5* gene that catalyses the synthesis of β1,6 GlcNAc branched *N*-glycans structures, which are known to play pivotal roles in many glycoproteins in cancer (9,10), and particularly in T cell function (11). This *N*-glycan branch commonly includes polylactosamine chains (*N*-acetyllactosamine disaccharide repeats) that are ligands for various lectins of the galectin family (12). Mice deficient in the *MGAT5* gene have been shown to develop autoimmune diseases, increased delayed-type hypersensitivity responses and an enhanced susceptibility to experimental autoimmune encephalomyelitis (EAE, a mouse model of multiple sclerosis) (13–15). These mice lacking GnT-V function (no synthesis of β1,6GlcNAc branched *N*-glycans structures) display a significantly increased T cell receptor (TCR) clustering, leading to a decreased threshold of T cell activation, and increased T_H1 differentiation resulting in hyperimmune response and increased susceptibility to autoimmunity (16,17). Overall, these reports support that T cell activity and signaling is tightly regulated by GnT-V-mediated glycosylation. In fact, in homeostasis and self-tolerance, T cell activation (TCR signaling) induces up-regulation of *MGAT5* gene which in turns leads to GnT-V-mediated glycosylation of the TCR (18). This phenotype promotes growth arrest of T cells by at least two mechanisms: early, by raising T cell activation thresholds via limiting TCR clustering at the immune synapse and the consequent hyperimmune response, and later by increasing surface retention of growth inhibitory receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4) (18).

The abovementioned evidences in other immune-mediated disorders set the ground to the present study that aims to address for the first time whether the dysregulation of this critical interplay between *N*-glycan branching and T cell activity is a major contributory factor and a yet uncovered mechanism underlying UC. In this study, we first report that dysregulation of the GnT-V-mediated glycosylation of the TCR on lamina propria T cells is a new molecular mechanism underlying UC pathogenesis. We further showed that UC patients exhibit a deficiency in T lymphocytes *MGAT5* gene transcription comparing with normal controls, which underlies the observed dysregulation of T cell glycosylation in UC.

RESULTS

Active UC cases exhibit a decreased expression of branched *N*-glycans in intestinal lymphocytic infiltrates

We first evaluated whether there were alterations in the expression of β1,6 GlcNAc branched *N*-glycans structures in intestinal lamina propria lymphocytic infiltrates positive for CD3 expression in a well-characterized series of colonic biopsies ($n = 64$) from UC patients comparing with normal controls. By using L-PHA lectin histochemistry, we observed that active UC cases had significantly lower expression ($P < 0.0001$) of branched *N*-glycans in intestinal lamina propria cell infiltrates $CD3^+$, when compared with normal controls (Table 1, Fig. 1A and B). UC patients with inactive disease (Mayo subscore 0), which have a controlled T cell response, showed increased expression of lymphocytic branched *N*-glycans expression (Table 1) comparing with active UC (Mayo subscore ≥1), but a tendency to have less lymphocytic *N*-glycans expression levels than normal controls. In the controls, the lamina propria lymphocytes (LPLs) $CD3^+$ exhibit high expression of L-PHA lectin (Table 1, Fig. 1A and B). No differences were observed comparing the levels of L-PHA expression with age, gender and disease extension.

These results showed significant alterations of β1,6 GlcNAc branched *N*-glycans expression in the intestinal lymphocytic infiltrate of UC cases comparing with normal controls. The variability in branched *N*-glycans expression on T cells among active and inactive UC is in accordance with disease severity. All of the severe UC cases (Mayo subscore 3) showed the lowest expression (<25%) of lymphocytic branched *N*-glycans. Moreover, we have observed that, for the same patient and during the course of disease, the percentage of expression of lymphocytic branched *N*-glycans varies accordingly with disease severity. These results were next explored in-depth at the molecular level.

Levels of TCR and CD3 expression in UC patients and normal controls: phenotypic characterization of T cells subsets and activation state

The results showed increasing levels of TCRβ and CD3 protein expression from normal controls; inactive UC (Mayo subscore 0); to active UC patients (Mayo subscore ≥1). The severe UC patients (Mayo subscore 3) had the higher levels of both TCRβ and CD3 proteins of the analyzed samples groups (Fig. 2A). The TCRβ and CD3 protein expression levels correlated with the clinical criterions; Mayo endoscopic subscoring and the histological standards for these setting of patients.

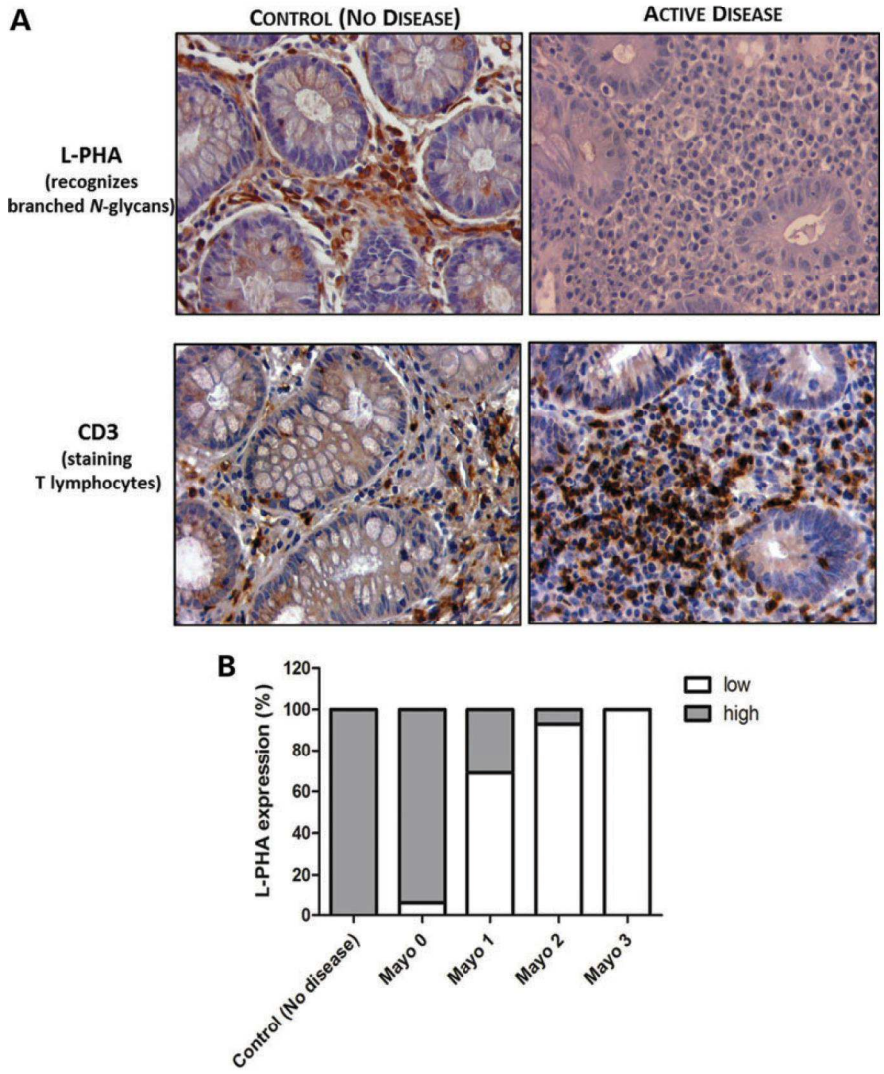

Figure 1. Relationship between the levels of lymphocytic branched *N*-glycans expression and the inflammatory activity. (**A**) A total number of 64 colonic biopsies were analyzed for L-PHA expression in the intestinal lamina propria lymphocytic infiltrate positive to CD3 expression (**B**). There was a significant association ($P < 0.0001$) (Table 1) between the levels of lymphocytic branched N-glycans and the inflammatory activity. UC cases with active disease (Mayo subscore 1,2,3) exhibit significantly lower levels ($\leq$25%) of L-PHA lymphocytic expression compared with normal controls. (A) Illustrates that normal controls showed a significant increased reactivity of L-PHA (>75%) in intestinal lymphocytic infiltrates $CD3^+$ (left figures). On the contrary, right figures represent a case of active UC (Mayo subscore 3) showing a significant decrease of L-PHA reactivity in the lamina propria T lymphocytes (<25%) displaying heavy CD3 cell infiltrates. Amplification ×400. (The evaluation of the relationship between the expression of lymphocytic β1,6 GlcNAc branched structures and the inflammatory activity considering the four percentual categories (<25%; 25–50%; 50–75% and >75%) is statistically significant (CI:95%) and the table is provided in Supplementary Material, Table S1. The regrouping in two categories (Low and High) did not change the P value and did not influence statistically the results.)

In addition, the isolated T cells obtained from the fresh colonic biopsies were analyzed by imaging flow cytometry analysis (Fig. 2C). The results showed that the expression of TCR from isolated cells were higher in active UC patients comparing with normal controls (Fig. 2C), validating the results obtained by western blot (Fig. 2A and B).

The phenotypic characterization of the T cells subsets showed that the intestinal T cell lymphocytic infiltrate from UC patients is predominantly represented by $CD4^+$ T cells, whereas in the normal individuals the intestinal T cells population is predominantly $CD8^+$ (Fig. 2D and E). Curiously, intestinal T cells from patients with UC had higher levels of TCR-alpha/beta expression when compared with intestinal T cells from controls (Fig. 2C and D), this being more evident in $CD4^+$ T cells than in $CD8^+$ T cells (data not shown). In addition, both normal and UC intestinal T cells express activation-related markers, HLA-DR and CD45RO (Fig. 2D and E). However, intestinal T cells from UC patients exhibit a higher level of expression of these activation-related markers compared with controls, as evaluated by the median fluorescence intensity (MFI) of the antigen expression (Fig. 2E).

TCRs from UC patients exhibit a decreased modification with β1,6 GlcNAc branched *N*-glycans structures

In order to evaluate the levels of β1,6 GlcNAc branched *N*-glycans expression specifically on the TCR of LPLs from UC patients and controls, we performed different experimental

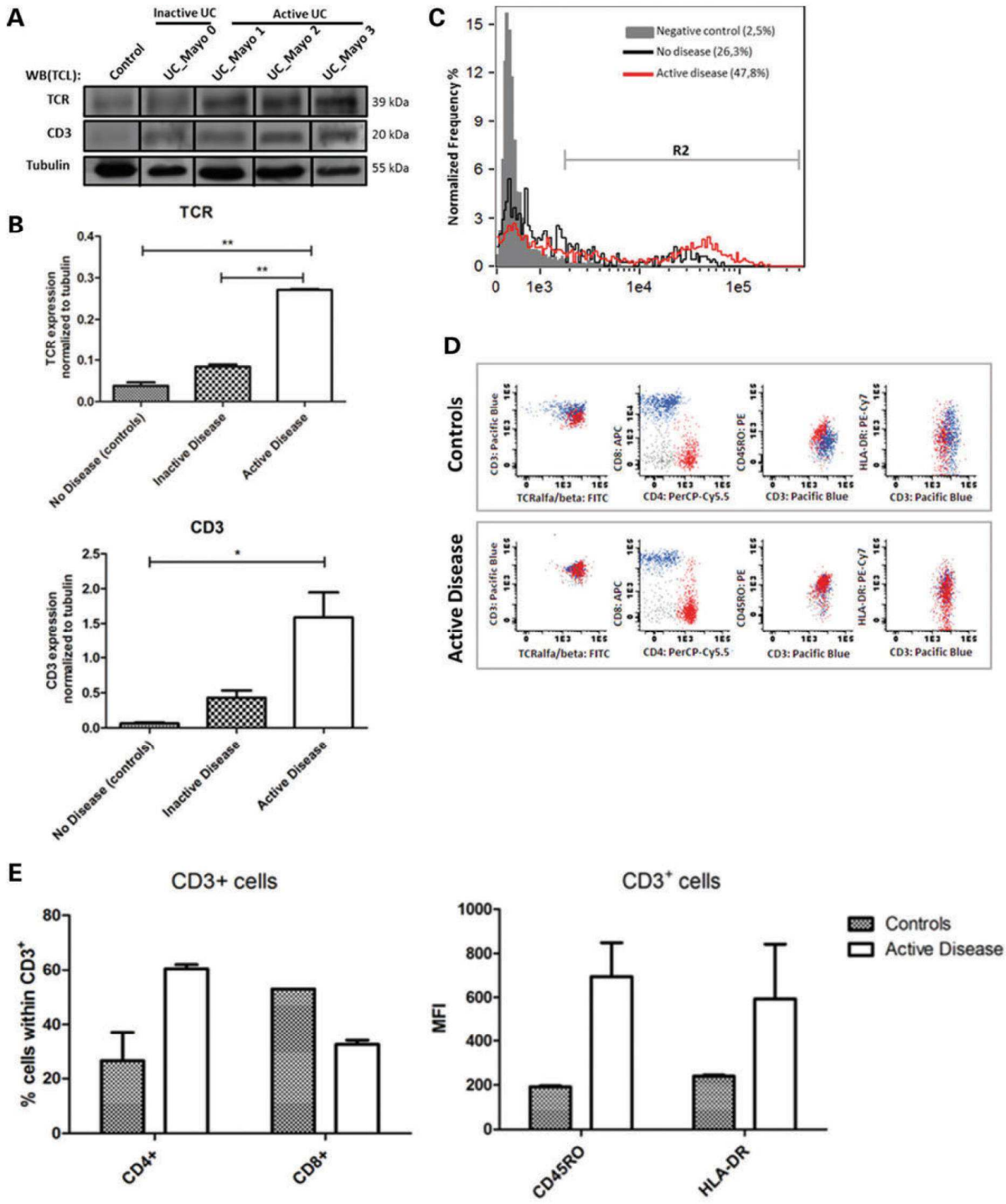

Figure 2. TCR and CD3 expression levels. (**A**) The results showed that active UC patients had significantly higher levels of both TCRβ and CD3 expression compared with normal controls. UC patients with severe disease (Mayo subscore 3) showed the highest levels of TCRβ and CD3 protein expression. Inactive UC patients (Mayo endoscopic subscore 0) exhibited an increased TCRβ and CD3 expression levels compared with normal controls. (**B**) Bar graphs, quantification of TCRβ and CD3 densities normalized to tubulin. Results are described as mean ± SEM of three independent experiments (Student's *t*-test: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$). In order to confirm the differential expression levels of TCR between normal controls and active UC patients, LPLs obtained from the fresh colonic biopsies were analyzed for this marker by imaging flow cytometry (**C**). The percentage of cells expressing TCRαβ in gate R2 is indicated in brackets. The results showed that isolated LPLs from active UC patients express higher TCRαβ expression levels compared with normal controls. Negative control refers to unstained cells (**C**). Lanes in (**A**) were on the different gels (black lines). Characterization of the T cells subsets and activation state. (**D**) Flow cytometry dot-plots illustrating the $CD4^+$ (red) and $CD8^+$ (blue) T cells populations and expression of CD45RO and HLA-DR on intestinal T cells from normal individuals ($n = 2$ biological replicates) and UC patients ($n = 2$ biological replicates). (**E**) Bar graphs, showing that $CD3^+$ T cells are predominantly represented by $CD4^+$ cells in UC patients and by $CD8^+$ cells in normal individuals (left graph). The activation state of $CD3^+$ T cells was evaluated by the MFI due to antigen staining of the activation-related markers, HLA-DR and CD45RO. Intestinal T cells from UC patients exhibit a higher level of expression of these activation-related markers (right graph).

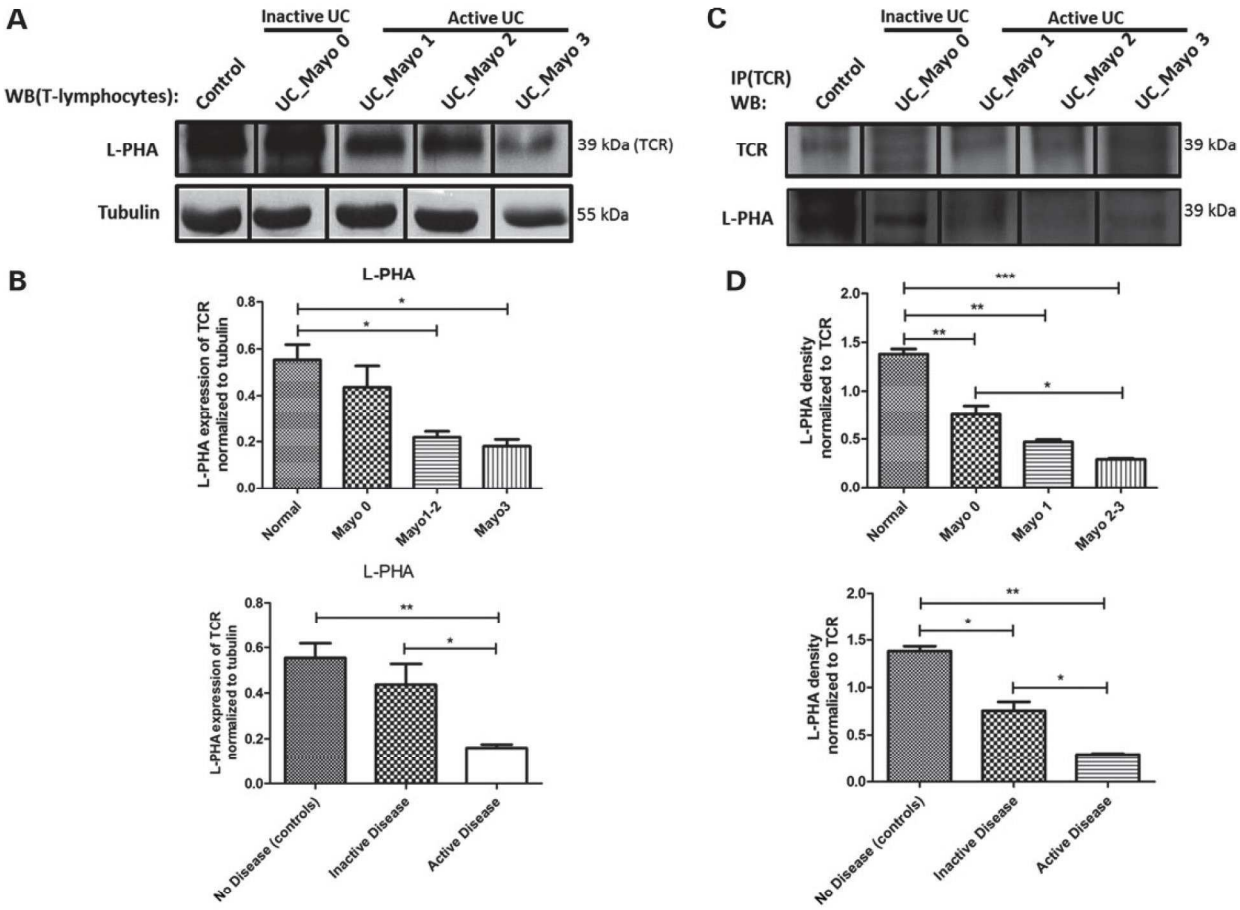

Figure 3. Alteration of intestinal TCR *N*-glycosylation in UC patients. (**A**) Protein lysates from the isolated LPLs were subjected to L-PHA lectin blot in order to evaluate the expression levels of β1,6 GlcNAc branched *N*-glycans on the TCR (39 kDa). The results showed a decreased TCR branched glycosylation in UC patients compared with normal controls. Patients with severe disease (Mayo subscore 3) had the lowest branched *N*-glycosylation modification of TCR. (**B**) *Bar graphs*, quantification of L-PHA densities on TCR band normalized to tubulin. Results are described as mean + SEM of three independent experiments (Student's *t*-test: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$). (**C**) Immunoprecipiation of TCR followed by β1,6 GlcNAc branched *N*-glycans recognition. As observed in (A) and (B), the intestinal TCR from UC patients (with inactive and active disease) suffered a decreased modification with branched *N*-glycans compared with normal controls. UC patients with severe disease (Mayo subscore 3) showed the lowest levels of TCR branched *N*-glycosylation. (**D**) *Bar graphs*, amounts of branched *N*-glycan structures were determined from the ratios of densities of L-PHA reactivity normalized to TCR. Results are described as mean ± SEM of three independent experiments (Student's *t*-test: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.005$). Lanes in (**A**) and (**C**) were on the different gels (black lines).

approaches that mutually validate each other. On the one hand, we isolated the LPLs from a subset of fresh biopsies collected from UC patients and controls which were analyzed by the L-PHA blot. We demonstrated that the TCR was differently glycosylated with branched *N*-glycans compared with UC patients and normal controls (Fig. 3A and B). UC patients with active disease (Mayo subscore ≥ 1) showed a significant decrease in the levels of TCR modification with branched *N*-glycans when compared with controls. Patients with severe disease (Mayo subscore 3) displayed the lowest levels of TCR glycosylation. Moreover, the levels of branched *N*-glycans modification on the TCR surface decreased concomitantly with disease severity (from Mayo subscore 0 to 3) (Fig. 3A and B), as also observed by histochemistry (Table 1 and Fig. 1). The levels of bisecting GlcNAc structures (catalyzed by GnT-III) on the TCR did not vary significantly among UC patients and controls (Supplementary Material, Fig. S1). These results on the isolated LPLs were further validated, by performing TCR immunoprecipitation from the total protein lysate (TCL) of the UC and controls biopsies, followed by β1,6 GlcNAc branched *N*-glycans recognition (L-PHA blot) (Fig. 3C and D). The results demonstrated that UC patients (both with inactive and active disease) showed significant decreased levels of TCR glycosylation with branched *N*-glycan structures compared with normal controls. Again, patients with severe disease (Mayo subscore 3) showed the lowest levels of β1,6 GlcNAc branched *N*-glycans on TCR. These observations obtained at the molecular level by two different technical approaches, pinpoint the existence of a dysregulation of intestinal TCR *N*-glycosylation mediated by GnT-V in UC patients compared with controls, which appear to be associated with disease severity.

Furthermore, we also confirm this dysregulation by imaging flow cytometry that combines features of both conventional flow cytometry and fluorescence microscopy and thus allows quantification of imaging parameters, including membrane intensity of different probes (19). Interestingly, we observed that isolated LPLs from active UC patients showed a decreased fluorescence intensity of L-PHA on the membrane of the TCRα/β

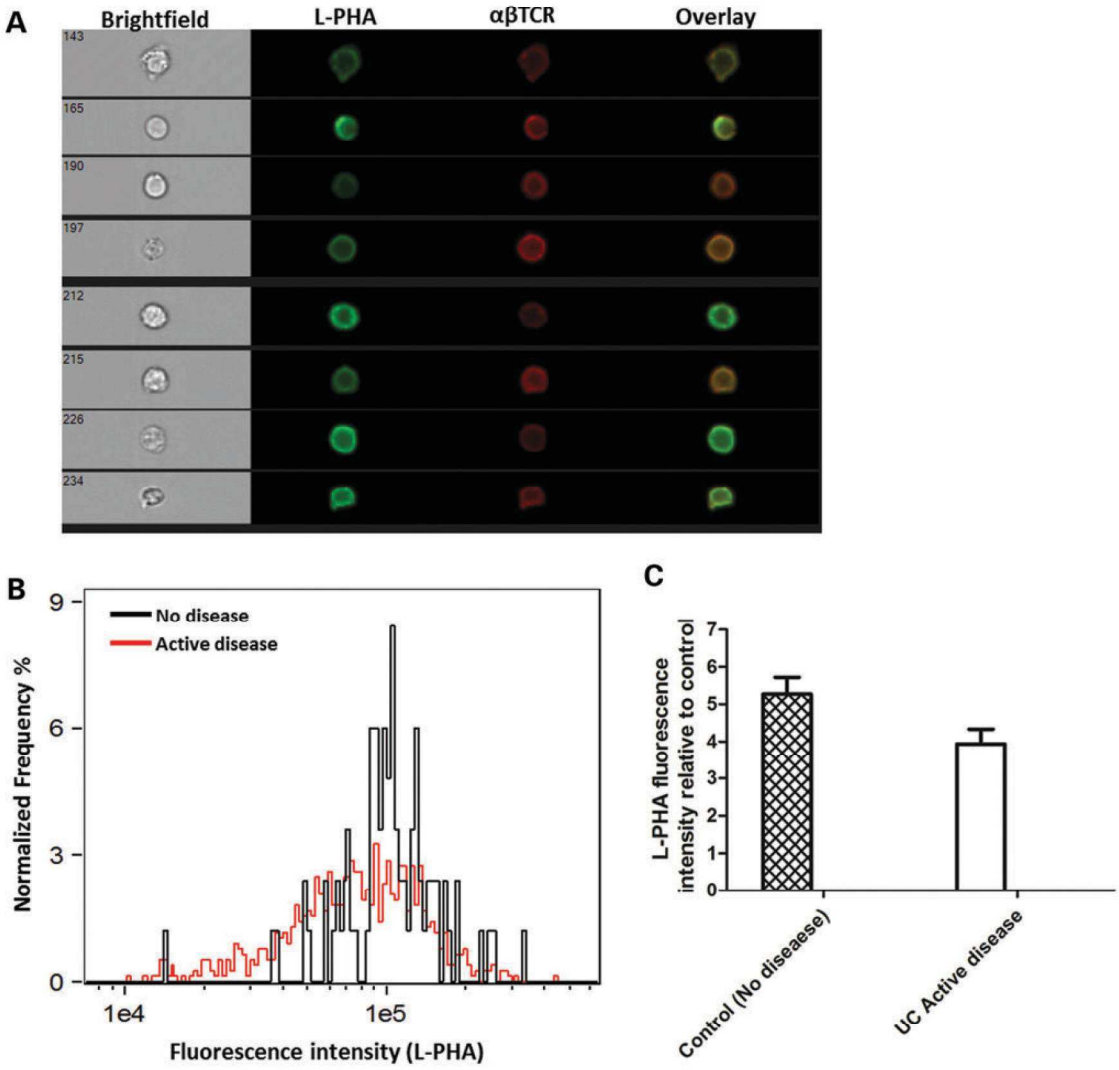

Figure 4. Imaging flow cytometry analysis of L-PHA membrane distribution in TCR positive cells. Intestinal LPLs were surface stained for TCRαβ and branched *N*-glycans (L-PHA) and analyzed on an ImageStream^X. (**A**) Examples of brightfield, L-PHA (green), TCRαβ (red) and overlaid images showing co-localization between TCRαβ and L-PHA. (**B**) Histogram representing the intensity of L-PHA staining on the TCRαβ$^+$ cells. (**C**) The intensity of L-PHA staining on the cell membrane of TCRαβ$^+$ cells was measured by imaging flow cytometry, and normalized relatively to cells stained only with streptavidin-FITC (negative control). Data show the average of two independent experiments. Error bars represent the mean ± SEM. The results indicate that UC patients with active disease (Mayo subscore ≥1) exhibit decreased levels of L-PHA staining at the cell membrane of TCRαβ$^+$ cells comparing with normal controls.

positive cells than the one detected in normal controls (Fig. 4). These bioimaging observations corroborate the above results and further pointing toward a deficiency in TCR branched *N*-glycans in UC patients.

MGAT5 gene expression alterations in T cells from UC patients

It has been demonstrated that TCR signaling regulates multiple Golgi *N*-glycan branching-processing enzymes at the mRNA level, such as *MGAT5* (18). Therefore, and in order to assess if the deficiency in TCR *N*-glycan branching, catalyzed by GnT-V, could be due to genetic alterations of *MGAT5* glycogene, we evaluated the mRNA transcription levels of *MGAT5* in a representative sub-series of intestinal LPLs isolated from active UC patients and normal controls (Fig. 5A and B). Our results consistently showed that LPLs from UC patients with active disease significantly express altered mRNA levels of *MGAT5* compared with normal controls (Fig. 5). The *MGAT5* mRNA analysis performed both independently (Fig. 5A) or using RNA pooled from different individuals (Fig. 5B) showed that LPLs from active UC patients had significantly lower levels of *MGAT5* transcription than normal controls. No significant alterations were observed on *MGAT3* transcription (Supplementary Material, Fig. S1). This is the first evidence suggesting the identification of a possible genetic alteration in the *MGAT5* gene from LPLs, which appears to underlie the deficiency of TCR *N*-glycosylation associated with UC pathogenesis, an issue that is being further studied in larger cohort populations.

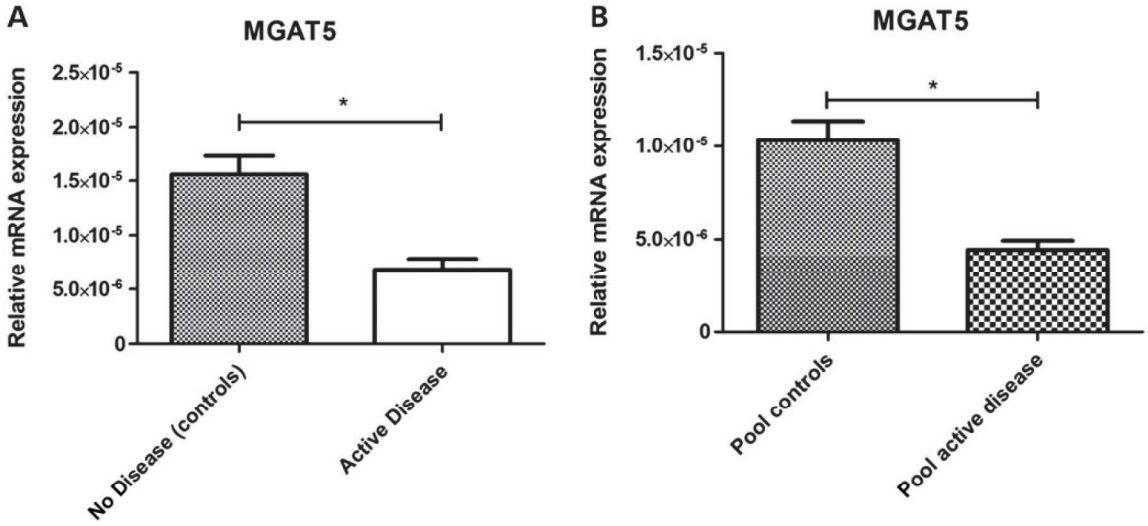

Figure 5. Deficiency of *MGAT5* gene expression in LPLs from UC patients. (**A**) qRT-PCR analysis for mRNA expression of *MGAT5* from intestinal LPLs from normal controls and active UC patients (Mayo subscore 1,2,3). Results in (A) are an average of three independent experiments, performed in triplicate, using RNA from three independent normal controls and three independent active UC patients. (**B**) Represents the average of two independent experiments using RNA of intestinal LPLs pooled from four different normal controls and four different UC patients, performed in triplicate. There is a significant decrease of *MGAT5* mRNA expression from intestinal LPLs of UC patients (with active disease) compared with normal controls. The mRNA expression levels are expressed as mean ± SEM (Student's *t*-test: $^{*}P \leq 0.01$).

DISCUSSION

In the present study, we have disclosed a novel molecular mechanism contributing for UC pathogenesis. We demonstrated that UC patients have dysregulation of intestinal TCR *N*-glycosylation, which has been associated with T cell hyperactivity and hyperimmune response (16,17). We further provide the first evidence supporting that UC patients exhibit a transcriptional alteration of the *MGAT5* gene in intestinal LPLs that appear to be the causative mechanism for the deficiency on TCR *N*-glycosylation of these patients, which associates with, and may possibly determine disease severity or susceptibility.

A growing body of evidence has demonstrated that in homeostasis and self tolerance, the T cell activation (TCR signaling) induces up-regulation of the *MGAT5* gene which in turns leads to GnT-V-mediated glycosylation of the TCR (18). The *N*-glycan branching on T cells, catalyzed by GnT-V glycosyltransferase, promotes the formation of multivalent galectin binding which negatively regulates T cell growth by precluding TCR clustering, and increasing the TCR activation thresholds, ultimately modulating the immune response (Fig. 6). Interestingly, recent evidences support that environmental and genetic dysregulation of *N*-glycosylation are involved in the molecular mechanism of human multiple sclerosis pathogenesis (15,20).

In UC we consistently observed, both by immunohistochemistry and at molecular level, that UC patients exhibit a defective *N*-glycan branching on intestinal TCR compared with normal controls (Fig. 6). Interestingly, patients with severe forms of endoscopic activity (Mayo endoscopic subscore 3) showed the lowest levels of TCR *N*-glycan branching compared with patients with mild, moderate or inactive disease. This variability in the levels of TCR *N*-glycan branching among UC patients was demonstrated to accompany disease severity, which at the end may determine different susceptibilities to the different forms of UC. These data are in accordance with previous observations in models of EAE (21). The authors reported that among inbred mouse strains, *N*-glycan GlcNAc branching in T cells was highly variable (different hypomorphic forms) and inversely correlated with EAE susceptibility (21).

In addition, we observed that levels of TCR glycosylation are associated with different T cell subsets (predominantly represented by $CD4^+$ T cells in UC patients and by $CD8^+$ T cells in normal individuals) and with different levels of T cell activation (higher surface expression of HLA-DR and CD45RO on T cells from UC patients, when compared with controls). Lower levels of TCR glycosylation (in UC patients) were found in the intestinal T cell subsets that were predominantly represented by $CD4^+$ T cells in an activated state. These differences on TCR branched glycosylation associated with different T cells subsets/activation (Fig. 2D and E) are in accordance with previous observations showing that $CD4^+$ and $CD8^+$ T cells have been shown to have different profiles of *N*-linked glycans (22,23). Differential effects of glycosylation on specific T cell lineages have been reported previously. For instance, sialyltransferase ST3 Gal-I-deficient mice lacked $CD8^+$, but not $CD4^+$ T lymphocytes (24). Moreover, and in line with our observations, the disruption of *N*-glycosylation by *MGAT5* ablation has been shown to lower T lymphocyte activation thresholds and cause autoimmunity (17). Altogether, our results further support that the differential glycosylation of $CD4^+$ versus $CD8^+$ T cells could cause differential activation thresholds and/or mechanisms (23). In addition, these glycosylation differences in the T cells subsets compared UC patients and controls may have major impact on $CD4^+$ versus $CD8^+$ T cell differentiation and function. This issue should be further examined in the future.

To further explore the possible causative mechanism of the dysregulation of the TCR branched *N*-glycosylation in UC patients, we evaluated the mRNA transcription levels of the

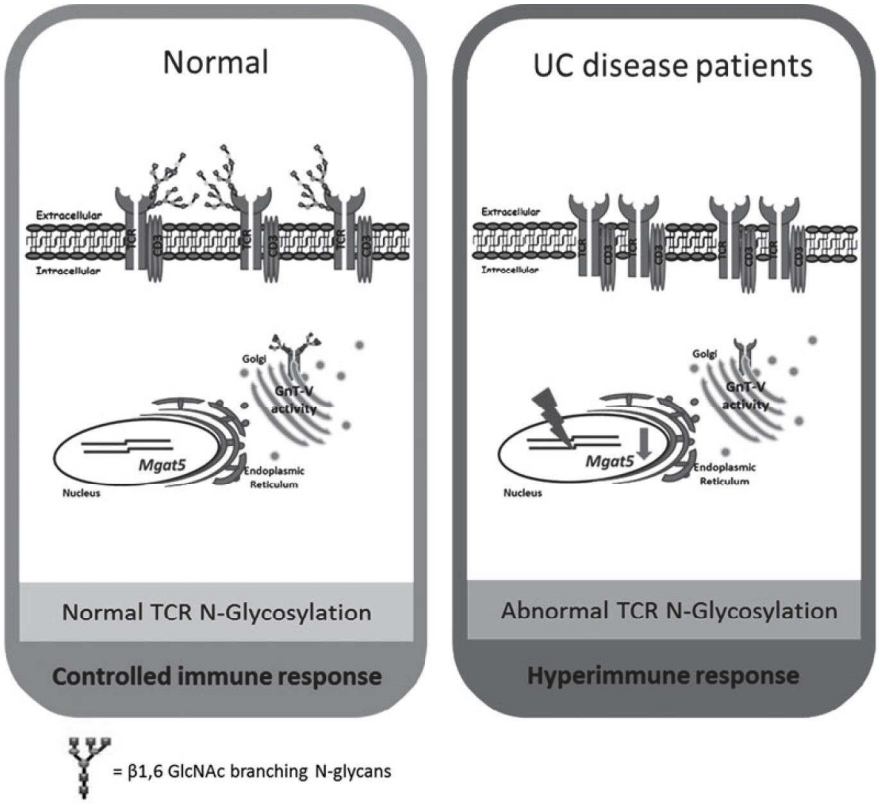

Figure 6. A model for the dysregulation of TCR *N*-glycosylation in UC. In intestinal homeostasis and under normal conditions, the T cell activation (TCR signaling) induces up-regulation of *MGAT5* gene which in turns leads to GnT-V-mediated glycosylation of the TCR. The increased β1,6GlcNAc branching synthesis on TCR, catalyzed by GnT-V, negatively regulates T cell response promoting growth arrest of T cells by raising T cell activation thresholds via limiting TCR clustering at the immune synapse. This GnT-V-mediated branched glycosylation of the TCR is associated with a controlled immune response. In UC patients, we propose the existence of a dysregulation of the intestinal TCR *N*-glycosylation catalyzed by GnT-V. Alterations on the transcription levels of *MGAT5* glycogene of the intestinal T cells appear to underlie the deficiency of GnT-V-mediated glycosylation on the TCR. This dysregulation of TCR branched *N*-glycosylation contribute to decrease the threshold of T-cell activation leading to a hyperimmune response, which is a feature of UC patients.

MGAT5 gene (that encodes GnT-V enzyme). Interestingly, we consistently observed a reduced *MGAT5* mRNA transcription levels in LPLs from UC patients with active disease compared with controls. These results support a genetic alteration of the *MGAT5* gene from intestinal T lymphocytes of UC patients, being the underlying event that promotes a dysregulated immune response through a disturbance in protein branched *N*-glycosylation catalyzed by GnT-V on intestinal TCR (Fig. 6). Interestingly, and in line with our results, it has been proposed that *MGAT5* is a gene that determines severity and susceptibility to multiple sclerosis (25,26). Our observations open new avenues for further exploring *MGAT5* as a potential susceptibility gene in IBD pathogenesis. Nevertheless, whether this dysregulation of *MGAT5*/GnT-V-mediated glycosylation on TCR is a cause or consequence of the Ulcerative Colitis disease is a premature conclusion that deserves to be carefully addressed.

In addition, the identification of a specific molecular mechanism underlying UC pathogenesis constitutes an opportunity to improve the target-specific therapy of UC patients. In fact, it was showed that metabolic supplementation of mice or T cells with UDP-GlcNAc enhances GlcNAc branching of T cells catalyzed by GnT-V which increases the threshold for T cell activation, suppresses T cell growth and inhibits Th1 differentiation leading to a controlled immune response and a decreased disease clinical severity in EAE and type I non-obese diabetic mice (27,28). Interestingly enough, a pilot study of oral GlcNAc in pediatric treatment-resistant IBD reveals the potential of GlcNAc as a therapeutic agent. In that pilot study, 8 out of 12 children with severe IBD went into clinical remission with evidence of histological improvement (29). Our results further contribute to explain the therapeutic effect of GlcNAc observed in pediatric IBD that was at that time unknown. The identification of an underlying disease mechanism prone to be targeted by specific therapy is a major unmet need for the management of IBD in general, enlarging the therapeutic options and improving the success of the therapy. In this regard, we are conducting further studies and controlled trials in order to test the therapeutic efficacy of this inexpensive and non-toxic agent in IBD.

In conclusion, our study demonstrates that dysregulation of branched *N*-glycosylation on TCR is a key mechanism in UC pathogenesis. Moreover, we identified that *MGAT5* gene expression alterations underlie the observed defect on TCR branched *N*-glycosylation. This deficiency in T cells *N*-glycosylation is positively associated with disease severity. Taken together, the disclosure of this new molecular mechanism in UC disease opens new windows of opportunity to further explore the potential applicability of this mechanism in predicting disease course

Table 2. Patients' characteristics

	Patients' characteristics	
	Controls	UC patients
n	13	17
Gender (M/F)	7/6	8/9
Age, range (years)	30–89	25–62
Age, mean (years)	68	45

and/or susceptibility. In addition, our observations further suggest the potential of metabolic supplementation with GlcNAc as a promising therapeutic tool that directly targets an underlying mechanism.

MATERIALS AND METHODS

Patient selection and colonic biopsies collection

The present study includes 30 patients from which 13 were normal controls and 17 were IBD patients diagnosed with UC that underwent scheduled colonoscopy (between 1991 and 2013) at the Gastroenterology Department of Centro Hospitalar do Porto-Hospital de Santo António (CHP/HSA), Porto, Portugal. The patients were randomly enrolled in the study prospectively from a cohort of outpatients of IBD clinics. All the 17 patients were studied: retrospectively by analyzing formalin-fixed paraffin-embedded (FFPE) samples at different stages of disease course/activity (obtained from the Pathology archive of CHP/HSA); and prospectively, where collected fresh colonic biopsies (8–20 biopsies per UC patient) were analyzed for different purposes: histology/morphology; isolation LPLs; protein and RNA extraction. From these 17 UC patients, we have analyzed 51 colonic biopsies (FFPE samples) that were collected at different times of the disease course. The fresh biopsies ($n = 8–20$ per UC patient) were representative of macroscopically active/inactive disease topography, as defined by Mayo endoscopic score (UC samples). Eligibility criteria for inclusion in this study were no history of human immunodeficiency virus. Age and gender were not exclusion factors (Table 2). Patients with UC were excluded if they had evidence of dysplasia or malignancy on colonoscopy with biopsy or positive to *Clostridium difficile* or infectious agents in stool assay in active UC cases. Cytomegalovirus (CMV) infection was also excluded using histopathology combined with immunohistochemistry against CMV antigens.

Endoscopic assessment of the severity of UC was determined using the Mayo endoscopic subscore for UC as follows: normal or inactive disease—subscore 0; mild disease—subscore 1 (erythema, decreased vascular pattern, mild friability); moderate disease—subscore 2 (marked erythema, lack of vascular patterns, friability, erosions); severe disease—subscore 3 (spontaneous bleeding, ulceration) (30,31).

Normal controls ($n = 13$) were randomly enrolled in the study (prospectively) and were represented by individuals that attend the gastroenterology department of CHP-HSA for a planned colonoscopy. The control group includes individuals without detectable colorectal lesions or previous history of IBD, colorectal hereditary syndromes or cancer (adenocarcinomas). In the patients of the control group, biopsies (8–20 biopsies per control used for the different purposes) were performed in mucosa without endoscopic abnormalities.

All specimens were subjected to histological examination and classification. All participants gave informed consent about all clinical procedures and research protocols were approved by the ethics committee of CHP/HSA, Portugal (233/12(179-DEFI/177-CES).

Overall, we have analyzed 64 FFPE colonic biopsy samples. In addition, from a subset of UC patients and controls, we have also analyzed fresh colonic biopsies at different stages of disease course which were collected prospectively from 2011 to 2013.

Tissue histochemistry

A total of 64 FFPE colonic biopsies from normal controls ($n = 13$) and UC patients ($n = 51$) (16 Mayo subscore 0; 13 Mayo subscore 1; 14 Mayo subscore 2; 8 Mayo subscore 3) were analyzed by histochemistry in order to evaluate the expression of CD3 and the β1,6GlcNAc branched *N*-glycan structures.

Identification of intestinal T lymphocytes at lamina propria was performed by CD3 immunohistochemistry. Heat-induced antigen retrieval was performed and endogenous peroxidase activity was blocked. Slides were washed and incubated with swine normal serum (Dako) before incubation with rabbit IgG anti-human CD3 monoclonal antibody (clone EP449E, Thermo Scientific, diluted at 1:50) overnight, at 4°C. The slides were washed and incubated with biotinylated swine anti-rabbit secondary antibody (Dako).

For evaluation of the expression of β1,6 GlcNAc branched structures, sections were incubated 1 h with biotinylated *Phaseolus Vulgaris Leucoagglutinin* (L-PHA) lectin that specifically recognizes the β1,6GlcNAc branched *N*-glycan structures (Vector Laboratories) diluted at 1:150.

The avidin–biotin–peroxidase complex detection method was the Vectastain ABC Kit (Vector Laboratories). The chromogen used was 3,30-diaminobenzidine (DAB). Sections were counterstained with hematoxylin, dehydrated and mounted. Controls were incubated with PBS instead of the primary antibody or lectin. As positive control, sections of thymus (for CD3 expression) and colon carcinoma (for L-PHA staining) were used.

The percentage of expression of the β1,6 GlcNAc branched structures (L-PHA reactivity) in the intestinal lymphocytic infiltrate (T cells at lamina propria) positive to CD3 were evaluated by three independent observers (J.C.; S.S.P.; C.A.R.) and scored as follows: less than 25%; 25–50%; 50–75% and more than 75% of intestinal T lymphocytes at lamina propria stained. For statistical analysis, the percentage of expression of L-PHA lectin reactivity was regrouped into two percentual categories ($\leq$25%: low expression; and $>$25% to $\geq$75%: high expression) in order to increase the number of cases in each category and in this manner improve the statistical power of the tests.

The statistical relationship among variables was analyzed in StatView (SAS Institute) using tables of frequencies and their significance tested by the Chi-square (χ^2). *P*-values less than 0.05 were considered a significant association.

Isolation of LPLs

LPLs were isolated from fresh colonic biopsies, following an adapted protocol (32). In order to isolate the LPLs, Percoll (Sigma) density gradients were used (1.05–1.09 g/ml). Lymphocytes were washed twice and cell yields were determined using a hemacytometer and cell viability was determined by trypan blue exclusion. After LPLs isolation, the levels of CD3 and/or TCR expression were evaluated by flow cytometry and/or imaging flow cytometry analysis.

Flow cytometry

To perform the characterization of the T cells subsets and activation state, isolated LPLs from normal individuals ($n = 2$ biological replicates) and UC patients ($n = 2$ biological replicates) were resuspended in 1000 μl of FACS Buffer (PBS1x containing 0.1% sodium azide and 2% BSA) following 15 min of incubation at room temperature with the appropriate volume of monoclonal antibodies specific for CD45 (clone GA90, IgG2a mouse; Cytognos SL, Salamanca, Spain), CD3 (clone SK7, IgG1 mouse; Becton Dickinson Biosciences, California, USA—BDB), TCR-alpha/beta (clone T10B9.1A-31, IgM mouse; BD Pharmingen, California, USA), CD4 (clone SK3, IgG2a mouse; BDB), CD8 (clone SK1, IgG1 mouse; BDB), CD45RO (clone UCHL-1BDB, IgG2a mouse; BDB) and HLA-DR (clone Immu-357, IgG1 mouse; Immunotech, Marseille, France—IOT), conjugated with orange cytognos 550 (OC550), pacific blue (PB), fluorescein isothiocyanate (FITC), peridinin-chlorophyll-protein complex Cy5.5 tandem (PerCP-Cy5.5), allophycocyanin (APC), phycoerythrin (PE) and phycoerythrin-Cy7 tandem (PE-Cy7), respectively. Lastly, cells were fixed with 4% formaldehyde. Data acquisition was performed in a FACS Canto v.2 flow cytometer (BDB), using the FACSDiva software (BDB). Instrument alignment and standardization were done according to the recommendations of the Euroflow consortium. Data on a minimum of 2×10^5 events were acquired for each staining. The data were analyzed using the Infinicyt™ software (Cytognos SL). Lymphocytes were first identified and gated based on their bright CD45 expression and light scatter characteristics, and T cells were gated based on the expression of surface CD3. Afterwards, the major T-cell populations were identified and quantified, based on the type of TCR receptor (alpha/beta and gamma-delta), and CD4/CD8 molecules ($CD4^+$, $CD8^+$ and $CD4^-CD8^-$ T cells). Finally, T cells were characterized for the expression of the activation-related markers, CD45RO and HLA-DR. Results were expressed as percentage (%) of cells that stained positively for each analyzed antigen, as well as the MFI due to antigen staining.

Imaging flow cytometry

Isolated LPLs were centrifuged and the pellets resuspended in FACS Buffer. For TCR staining, cells were incubated with anti-TCRα/β mAb (clone BW242/412 mouse IgG2b) conjugated with R-phycoerythrin (PE) (Miltenyi Biotech) diluted in FACS Buffer, on ice for 30 min, in the dark. For L-PHA staining, cells were then incubated with L-PHA/FITC (4 μg/ml) (Vector Laboratories) and as negative control cells were incubated with Streptavidin-conjugated fluorescein isothiocyanate (FITC) (1 μg/ml) (Caltag Laboratories). Afterwards cells were washed, fixed with 4% formaldehyde and analyzed by imaging flow cytometry.

Images of isolated cells were acquired on a 6-Channel ImageStream^X Imaging Flow Cytometer (Amnis, EMD Millipore) at the Bioimaging Center for Biomaterials and Regenerative Therapies (b.IMAGE, INEB, Porto, Portugal). Analysis was performed with IDEAS 5.0 (Amnis, EMD Millipore). Upon compensation, co-localization of the TCRα/β protein with L-PHA was determined with co-localization wizard. The intensity of L-PHA staining within the TCRα/β positive events was also determined. The experiment was reproduced two times using cells from two different biological replicas.

Western-blot and immunoprecipitation

Evaluation of the total protein expression levels of TCR and CD3 was performed using 40 μg of TCL obtained from the whole fresh biopsy digestion. The samples were subjected to 12% SDS–PAGE electrophoresis and membranes were blocked before incubation with primary antibodies against TCRβ (mouse monoclonal antibody anti-human, Santa Cruz Biotechnology) (dilution 1:100) and CD3 (rat anti-human CD3ε mAb, Cell Signaling Technologies) (dilution 1:1000). The respective secondary antibodies used were goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) and donkey anti-rat IgG-HRP (Jackson ImmunoResearch). For loading control analysis, mouse IgG anti-tubulin (Sigma) was used. The target proteins were visualized using ECL reagent (GE Healthcare, Life Sciences).

For TCR immunoprecipitation (IP), equal amounts of TCL obtained from the whole biopsy (500 μg) were precleared with protein G-sepharose beads (GE Healthcare, Life Sciences) and the supernatant was incubated overnight with rabbit anti-human TCRβ polyclonal antibody (Santa Cruz Biotechnology). The immune complexes were released by boiling and subjected to 12% SDS–PAGE. Membranes were blocked and probed with the primary anti-human TCRβ polyclonal antibody (dilution 1:100), and revealed with secondary antibody goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology). For the β1,6 GlcNAc branched structures analysis on TCR, membranes were probed with biotinylated L-PHA or E-PHA (10 μg/ml) lectin. Immunoreactive bands were then visualized using the Vectorstain ABC kit and detection was performed by an ECL reagent. Quantitative analyses were performed by densitometric scanning of bands.

For L-PHA or E-PHA lectin blot analysis, 20 μg of LPLs protein lysate was used. Membranes were blocked before incubation with L-PHA or E-PHA lectin and bands were then visualized using the Vectorstain ABC kit. The detection was performed by an ECL reagent. For loading control, anti-tubulin mAb was used.

The experiments were reproduced at least three times. For all data comparisons, the Student's *t*-test was used (two tailed, unequal variance). Results were considered statistically significant when $P < 0.05$.

Real-time PCR

Total RNA from isolated LPLs was extracted with Tri-Reagent (Sigma) or RNAqueous- Micro Kit (Applied Biosystems)

according to the manufacturer's protocol. The quantitative real-time PCR (qRT-PCR) was performed using TaqMan Gene Expression Assays (Applied Biosystems). T lymphocytes total RNA were reversed transcribed to single-stranded cDNA using random hexamer primers and Superscript II Reverse Transcriptase (Invitrogen). qRT-PCR was carried out in triplicates using RNA source from three biological replicates (three normal individuals and three UC patients) and RNA pooled from four different normal controls and four different UC patients with active disease, for the target genes *MGAT5* (Taqman probe: hs.00159136_m1, Applied Biosystems), *MGAT3* (Taqman probe: hs.02379589_s1, using RNA source from 2 biological replicates: two normal individuals and two UC patients) and for the appropriated lymphocytes endogenous control *18S* (Hs.PT.39a.22214856.g, Integrated DNA Technologies) and qRT-PCR reactions were performed on the ABI Prism 7000 Sequence Detection System. Data were analyzed by the comparative $2(-\Delta\Delta CT)$ method (33). For all data comparisons, the Student's *t*-test was used (two-tailed, unequal variance). Results were considered statistically significant when $P < 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

Conflict of Interest statement. None declared.

FUNDING

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III

Metabolic control of T cell immune response through glycans in Inflammatory Bowel Disease

Metabolic control of T cell immune response through glycans in inflammatory bowel disease

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Mucosal T lymphocytes from patients with ulcerative colitis (UC) were previously shown to display a deficiency in branched N-glycosylation associated with disease severity. However, whether this glycosylation pathway shapes the course of the T cell response constituting a targeted-specific mechanism in UC remains largely unknown. In this study, we demonstrated that metabolic supplementation of ex vivo mucosal T cells from patients with active UC with *N*-acetylglucosamine (GlcNAc) resulted in enhancement of branched N-glycosylation in the T cell receptor (TCR), leading to suppression of T cell growth, inhibition of the T helper 1 (Th1)/Th17 immune response, and controlled T cell activity. We further demonstrated that mouse models displaying a deficiency in the branched N-glycosylation pathway (*MGAT5*$^{-/-}$, *MGAT5*$^{+/-}$) exhibited increased susceptibility to severe forms of colitis and early-onset disease. Importantly, the treatment of these mice with GlcNAc reduced disease severity and suppressed disease progression due to a controlled T cell-mediated immune response at the intestinal mucosa. In conclusion, our human ex vivo and preclinical results demonstrate the targeted-specific immunomodulatory properties of this simple glycan, proposing a therapeutic approach for patients with UC.

T lymphocytes | T cell receptor | adaptive immune response | branched N-glycosylation | intestinal inflammation

Inflammatory bowel diseases (IBDs), encompassing Crohn's disease and ulcerative colitis (UC), are chronic, relapsing, and life-long inflammatory disorders of the gastrointestinal tract affecting mainly young populations. The incidence of IBD is increasing worldwide, and the disease remains incurable, placing a heavy burden on populations by reducing patients' quality of life and increasing disability (1). The current therapeutic strategies for IBD are limited by reduced effectiveness, high costs, and/or side effects. This scenario highlights the urgent need in the clinic of identifying novel molecular markers capable of being selectively targeted with new and optimized therapies. Future progress in IBD monitoring and therapy mostly depends on the identification of key mechanism(s) mediating intestinal inflammation that could be therapeutically targeted.

The immune system is tightly regulated by glycosylation, through the addition of carbohydrate structures (glycans) to key molecules (proteins) involved in innate and adaptive immune responses (2). The *N*-acetylglucosaminyltransferase V (GnT-V) is a glycosyltransferase encoded by the human *MGAT5* gene that catalyzes the synthesis of β1,6-*N*-acetylglucosamine (GlcNAc) branched N-glycans, which are known to play pivotal roles in many glycoproteins in cancer (3–6) and also in T cell activity and function (7, 8). In homeostasis and self-tolerance, T cell activation [via T cell receptor (TCR) signaling] induces up-regulation of the *MGAT5* gene, which, in turn, leads to GnT-V–mediated glycosylation of the TCR (9). Consequently, it can promote growth arrest of T cells early, by raising T cell activation thresholds via limiting TCR clustering at the immune synapse (and restricting TCR signaling), and, later, by increasing surface retention of growth inhibitory receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4) (9).

In fact, mice deficient in the *MGAT5* gene display an increased susceptibility to autoimmune diseases (7, 10, 11). These mice lacking GnT-V function (no synthesis of β1,6-GlcNAc branched N-glycan structures) display an increased TCR clustering and increased T helper 1 (Th1) differentiation that result in a hyperimmune response in mouse models of multiple sclerosis (7, 10). Interestingly, in mouse models of experimental autoimmune encephalomyelitis (EAE) and type I diabetes, it was shown that supplementation with GlcNAc induces increased N-glycan branching, through increasing the hexosamine pathway, that was associated with inhibition of T cell growth and differentiation (12, 13), leading to delayed disease progression. Moreover, N-glycan branching was also found to regulate T cell development (14). Recently, it was demonstrated that

Significance

Our findings demonstrate that metabolic supplementation of mucosal T cells, isolated from patients with active ulcerative colitis (UC), with *N*-acetylglucosamine (GlcNAc) leads to the enhancement of branched N-glycosylation on the T cell receptor, which was associated with the control of T cell activation and function. These results were validated in "glycoengineered" mouse models with severe colitis. Overall, our results open new avenues for a targeted-specific therapy in inflammatory bowel disease (IBD). The therapeutic use of GlcNAc (either alone or in combination with other antiinflammatory therapies) represents a simple immunomodulatory strategy in IBD, with absence of side effects, low costs, and the possibility of being used as a simple rescue therapy to avoid unnecessary toxic effects and step-up therapies in IBD.

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branching N-glycans potentiate the differentiation of induced regulatory (iTreg) T cells over Th17 differentiation (15). Importantly, and in the setting of human immune-mediated disorders, we have recently found that patients with UC exhibit a deficiency in branched glycosylation (catalyzed by GnT-V) in mucosal T cells that was associated with disease severity. Patients with UC who have severe disease showed the most pronounced defect on branched N-glycans in intestinal T cells, together with a significant reduction of *MGAT5* gene transcription in these cells (16).

In the present study, and building upon our previous findings in patients with UC (16), we have evaluated the impact of glycosylation, particularly the branched N-glycosylation pathway, in the regulation of the T cell-mediated immune response in patients with UC. We further explored whether this mechanism could be therapeutically targeted in vivo through a simple glycan-based strategy. Our results showed that metabolic supplementation of mucosal T cells, isolated from patients with active UC, with GlcNAc led to the enhancement of branched N-glycosylation on the TCR, controlling T cell activation and function. Preclinical data further demonstrated that GlcNAc treatment of *MGAT5* null or heterozygous mice developing severe forms of induced colitis significantly controlled disease severity and progression due to suppression of the intestinal T cell-mediated immune response, with good clinical effects when GlcNAc was topically administered by enemas. Altogether, this study highlights the potential of glycans as novel immunomodulatory agents in IBD, warranting validation in human clinical trials.

Results

Ex Vivo GlcNAc Supplementation Increased Branched N-Glycosylation of T Cells from Patients with Active UC. We have previously demonstrated that patients with UC display reduced branched N-glycosylation on mucosal T cells (16). To assess the ability of glycans as repairers of the above-mentioned mechanistic defect, we herein promoted, ex vivo, the hexosamine biosynthetic pathway (*SI Appendix*, Fig. S1*A*) in purified intestinal T cells by metabolic supplementation with GlcNAc. Previous studies showed that supplementation with GlcNAc increases the availability of the substrate (UDP-GlcNAc) to Golgi enzymes such as GnT-V, enhancing β1,6-GlcNAc branching N-glycans, particularly in T cells (13). To test this hypothesis, T cells ($CD3^+$) were isolated ex vivo from both the intestinal lamina propria of fresh colonic biopsies and peripheral blood of patients with UC who have active disease and were supplemented with increasing doses of GlcNAc. Different GlcNAc concentrations (40 mM, 80 mM, and 100 mM) were tested, and 40 mM did not reveal major alterations compared with nontreated T cells (*SI Appendix*, Fig. S1*B*). The expression of β1,6-GlcNAc branched N-glycans on colonic T cells was evaluated by flow cytometry using *Phaseolus vulgaris* leukoagglutinating (L-PHA) lectin. We observed a dose-dependent increase of branched N-glycans on intestinal T cells upon GlcNAc supplementation across different patients (Fig. 1*A*). This increased modification with branched N-glycans was also observed in T cells isolated from peripheral blood mononuclear cells of patients with active UC displaying FSC^{high} and SSC^{high} light-scattering parameters, characteristic of activated T lymphocytes (Fig. 1*B*). The increased expression of β1,6-GlcNAc branched N-glycans was detected both on $CD4^+$ and $CD8^+$ T cells (*SI Appendix*, Fig. S1 *C* and *D*). No effects of GlcNAc treatment in the proportion of $CD4^+$ and $CD8^+$ T cell subsets in the cultures were observed (*SI Appendix*, Fig. S1 *E* and *F*), supporting that GlcNAc supplementation leads to a specific modification with branched glycans on T cells in a dose-dependent manner. Importantly, the enhancement of branched N-glycans was only observed in T cells from patients with active UC (Fig. 1*C* and *SI Appendix*, Fig. S1*G*). T cells from healthy controls and from patients with inactive disease did not show alterations in the levels of branched glycans upon treatment with increasing concentrations of GlcNAc (Fig. 1*C* and *SI Appendix*, Fig. S2), possibly due to the higher baseline branching comparing with patients with active UC. These results were further confirmed by other technical approaches. Increased expression of β1,6-GlcNAc branched N-glycans on a band the same size as the TCR β-chain (TCRβ) after GlcNAc supplementation was also detected by L-PHA blotting (Fig. 1*C*) and by TCR immunoprecipitation using lysates of lamina propria T lymphocytes (LPLs) purified from patients with UC (Fig. 1*D*). Interestingly, this increased branching of N-glycans after GlcNAc supplementation was found to occur in intestinal T cells from patients with UC with different Mayo subscores (with Mayo endoscopic subscores 1, 2, and 3) with a trend association with disease severity, as depicted in *SI Appendix*, Fig. S1*G*. The internalization of externally given GlcNAc was already demonstrated in cell lines (17). The specific effects of GlcNAc in enhancing branched glycosylation on T cells from patients with active UC was further demonstrated by the reversed effects on L-PHA mean fluorescence intensity when T cells were treated with the N-glycan branching inhibitors kifunensine (KF) and swainsonine (SW) (*SI Appendix*, Fig. S3) in T cells from biopsies and blood of patients with active UC. Moreover, the specific effects of GlcNAc in the enhancement of branched glycosylation in T cells was further validated by supplementation of T cells from patients with active UC with other glycan types such as D-mannose, which revealed no impact in branched N-glycan expression (*SI Appendix*, Fig. S3). To further validate these observations, we also performed imaging flow cytometry showing that $TCR\alpha/\beta^+$ cells display an increase of fluorescence intensity due to staining with L-PHA on the cell membrane. This increase was observed in T cells displaying blast-like morphology (Fig. 1 *E* and *E1*). Taken together, these results demonstrate that treating ex vivo T cells from patients with active UC with GlcNAc promotes the hexosamine biosynthetic pathway enhancing β1,6-GlcNAc branched N-glycans on the TCR, and thus restoring the deficiency on branched N-glycans previously shown in mucosal T cells from patients with UC (16). Next, we have determined the specificity of this enhancement of β1,6-GlcNAc N-glycan branching by analyzing the correspondent GnT-V enzymatic activity. Interestingly, and in line with our previous observations on *MGAT5* gene transcription (16), T cells from patients with active UC displayed reduced GnT-V enzymatic activity compared with healthy controls (*SI Appendix*, Fig. S4). Our results showed that this reduced GnT-V enzymatic activity of T cells could be significantly recovered after metabolic supplementation with GlcNAc (Fig. 2*A*), which further supports the effects of GlcNAc in the enhancement of N-glycan branching mediated by GnT-V. In the N-glycosylation branching pathway, the β1,6-GlcNAc branched N-glycan catalyzed by GnT-V can be further extended with polylactosamine structures (ligands for galectins), which, in turn, can be terminally sialylated (*SI Appendix*, Fig. S1*A*). Our results showed that GlcNAc supplementation of ex vivo activated T cells led to increased expression of β1,6-GlcNAc branched N-glycans (as detected by L-PHA lectin) (Figs. 1*E* and 2 *B* and *B1*) with a trend of increased extension with polylactosamine structures, as indicated by staining with the *Lycopersicon esculentum* agglutinin (LEL) (Fig. 2 *B* and *B1*). Additionally, we determined whether there was a terminal addition of α2,6-linked sialic acid, recognized by binding of *Sambucus nigra* agglutinin (SNA), and/or α2,3-sialic acid, recognized by *Maackia amurensis* agglutinin (MAL-II). The results demonstrated a trend of increase in α2,6-linked sialic acid, and no consistent alterations in α2,3-sialic acid linkages were detected (Fig. 2 *B* and *B1*). Overall, our results support that ex vivo supplementation of T cells from patients with UC with GlcNAc has the potential to enhance the branched N-glycosylation on T cells, remodeling the T cell glycoprofile, which is ultimately expected to have an impact on the regulation of the immune response.

Shaping the T Cell-Mediated Immune Response in UC Through Increased Branching N-Glycans. After demonstrating the ability of GlcNAc supplementation to repair the deficiency of branched N-glycans on ex vivo T cells, we next evaluated its impact on the modulation of T cell responsiveness. The metabolic supplementation with GlcNAc of ex vivo activated T cells from naive patients (without therapy) resulted in significant suppression of their proliferative response to anti-CD3/CD28 mAb stimulation (Fig. 3 *A* and *A1*). In addition, and importantly, the increased N-glycan branching on T cells resulted in a significant reduction in the production of the proinflammatory cytokines TNF-α, IFN-γ, and IL-17A, which are associated with UC pathogenesis (18),

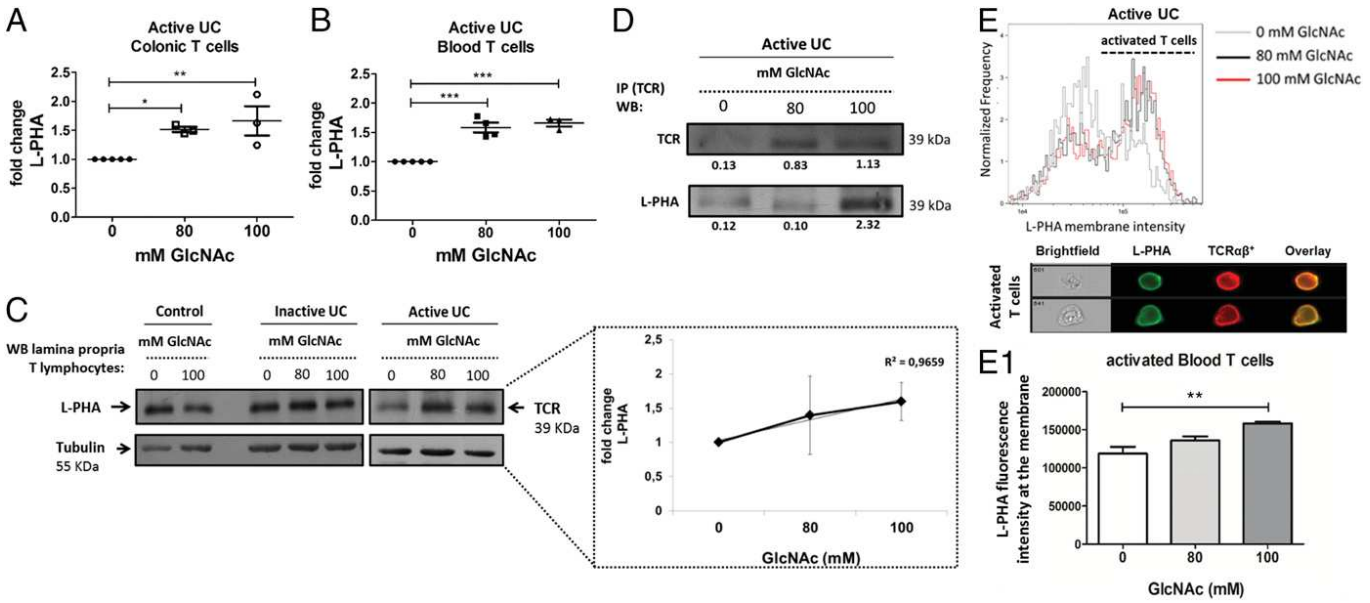

Fig. 1. Ex vivo GlcNAc supplementation of T cells from patients with UC resulted in increased branching N-glycans. (*A* and *B*) $CD3^+$ T cells from patients with active UC were cultured with different concentrations (millimolar) of GlcNAc, and the fold change of mean fluorescence intensity due to L-PHA staining was determined by flow cytometry. The scatter plots illustrate the mean ± SEM of five biological replicates. One-way ANOVA using the Newman–Keuls multiple comparison posttest: $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$. (*C*) Protein lysates of purified $CD3^+$ T cells under GlcNAc treatment were subjected to L-PHA lectin blotting to evaluate the expression levels of β1,6-GlcNAc branched N-glycans on a protein band corresponding to the migration profile of the TCRβ. WB, Western blot. (*Inset*) Linear regression using mean values per treatment condition. (*D*) Immunoprecipitation (IP) of TCR followed by β1,6-GlcNAc branched N-glycan recognition with L-PHA. The density of bands is indicated below each band. (*E*) Imaging flow cytometry analysis (on an ImageStreamX) of L-PHA membrane distribution on TCR^+ cells after GlcNAc supplementation in T cells isolated from blood of patients with active UC. Representative images of activated T cells display blast-like morphology showing colocalization (overlaid images) of TCRαβ and L-PHA staining on the cell membrane. (*E1*) Bars depict the mean ± SEM of L-PHA staining intensity on gated $TCR\alpha\beta^+$ $L\text{-}PHA^{high}$ cells from three independent experiments. One-way ANOVA using Dunnett's multiple comparison posttest: $**P \leq 0.01$. In all experiments, results are normalized to the corresponding untreated condition (0 mM).

in a GlcNAc dose-dependent manner (Fig. 3*B*), supporting its effect on the control of Th1/Th17-type immune responses. The effects of GlcNAc on the suppression of proinflammatory cytokine production were found to be independent of cell death and/or decreased T cell proliferation. After normalization of cytokine concentrations to the respective T cell proliferation index (index of division), we still observed a reduction in the production of proinflammatory cytokines (*SI Appendix*, Fig. S5*A*). Moreover, the percentage of IFN-γ– and TNF-α–expressing cells among total $CD4^+$ T cells, evaluated by intracellular flow cytometry, was decreased upon GlcNAc treatment (Fig. 3*B1*). The impact of GlcNAc supplementation on other cytokines, such as TGF-β and IL-10, is not significant (*SI Appendix*, Fig. S5 *B* and *C*). The specific effects of GlcNAc in suppressing proinflammatory cytokine production is further confirmed by inhibitors (KF and SW), which blocked the regulatory impact of branched N-glycans in T cell function (*SI Appendix*, Fig. S6). Next, we have evaluated the expression of the transcription factors (T-bet, RORγt, Foxp3, and Gata3) in T cells ($CD3^+$) under ex vivo GlcNAc treatment. The results showed that GlcNAc supplementation was associated with a reduction in the expression of T-bet and RORγt, which corroborates the negative impact on the Th1/Th17-type response (Fig. 3 *C* and *C1*). The expression of Foxp3, a transcription factor associated with regulatory T cells as well as with human activated T cells (18), was also found to be reduced upon GlcNAc treatment. To gain insight into the molecular basis of this modulation of the T cell response through metabolic supplementation with GlcNAc, we analyzed its impact on the TCR signaling pathway. We observed that GlcNAc supplementation led to an inhibition of the phosphorylation of ZAP70 and LAT, thereby hindering T cell activity by controlling the TCR signaling pathway (Fig. 3*D*). Additionally, we evaluated whether GlcNAc supplementation had an impact on T cell apoptosis. Our results showed that T cells from patients with active UC treated with GlcNAc displayed an increased susceptibility to apoptosis compared with nontreated T lymphocytes as soon as 3 h after stimulation (*SI Appendix*, Fig. S7*A*). This effect was not seen in T cells from controls and patients with inactive UC, which demonstrated no differences, or even a trend to decrease apoptosis, respectively, at the same time points upon GlcNAc supplementation. This argues against a putative effect of hyperosmolarity in the observed increase of apoptosis/cell death in GlcNAc-supplemented T cells from patients with active UC and in controls (16) (*SI Appendix*, Fig. S7*A*). Treatment with the branched N-glycan inhibitors (KF and SW) did not reverse the effects of GlcNAc in apoptosis (*SI Appendix*, Fig. S7*B*). At 72 h, a significant decrease in branched glycosylation levels was observed with KF and SW (*SI Appendix*, Fig. S3); however, no differences in the apoptosis were detected (*SI Appendix*, Fig. S7*B*). The percentage of cell death was unchanged or even higher with SW and KF than with treatment with GlcNAc only (*SI Appendix*, Fig. S7*B1*). Taken together, these data collectively demonstrate that ex vivo GlcNAc supplementation of T cells from patients with active UC enhances the branched N-glycans and resulted in a significant suppression of T cell proliferation and TCR signaling, as well as controlled Th1/Th17-type immune responses.

Treatment with GlcNAc Reduces Disease Severity and Ameliorates Clinical Signs of Disease in Mice with Colitis. To determine whether dysregulation of branched N-glycans on TCR occurs in different experimental mouse models of colitis, we have evaluated two different chemically induced colitis mouse models, the dextran sodium sulfate (DSS)-induced and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced models (19), in C57BL/6 wild-type mice. In both models, colitis was successfully induced, and we have observed a similar impact on the dysregulation of branched N-glycans in the TCR (*SI Appendix*, Fig. S8 *A* and *B*), which is in accordance with our previous observations on human patients with UC (16). The DSS-induced colitis model was selected as the ideal one to proceed with, since the rectal induction of disease in the TNBS model would interfere with the enema administration of GlcNAc. After disease

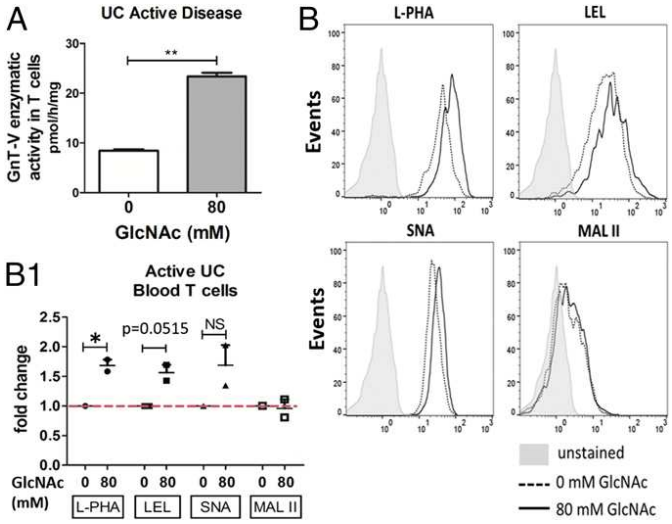

Fig. 2. Remodeling of the glycosylation phenotype upon metabolic supplementation with GlcNAc. (*A*) Impact of GlcNAc supplementation on GnT-V activity was determined using a pool of lysates from treated vs. nontreated peripheral blood T cells, in three biological replicates of patients with active UC, from two independent technical experiments. Student's *t* test: $^{**}P \leq 0.01$. (*B*) Flow cytometry evaluation of glycophenotype of T cells upon GlcNAc supplementation. (*B1*) Scatter plot: fold change of MFI due to staining with each lectin on T cells, in two biological replicates with different stages of disease severity (Mayo endoscopic scores 1 and 2), from two independent experiments. Results are normalized to the untreated condition, which was taken as 1. Student's *t* test: $^{*}P \leq 0.05$. NS, not significant.

onset using 2% DSS, GlcNAc was administered through two different routes: orally, by supplementing the drinking water with 0.25 mg/mL GlcNAc, and/or rectally, using 0.5 mg/mL GlcNAc enemas. With this approach, we assessed the therapeutic effects of GlcNAc on disease activity and on the control of intestinal inflammation. The experiment design is summarized in Fig. 4*A*. Our results showed that mice with colitis and treated with GlcNAc exhibited lower body weight loss (Fig. 4*B*) and significant improvements in disease activity index (DAI) (Fig. 4*C*) in comparison to mice with colitis not treated with GlcNAc (DSS control). Importantly, whenever animals received GlcNAc topically by enema administration (either alone or in combination with oral GlcNAc), the body weight changes were lower in comparison to animals receiving only oral GlcNAc. These results suggest that GlcNAc enemas may have promising topical effects on the control of disease severity (Fig. 4 *B* and *C*, *Insets*). Accordingly, macroscopic observation of the colon showed that mice with colitis displayed visible colonic edema (swelling of the bowel wall) that recovered upon GlcNAc treatment (*SI Appendix*, Fig. S8*C*). In addition, mice with colitis displayed extensive lymphocytic infiltrates (Fig. 4*D*, arrowhead) presenting with $CD3^+$ cells (Fig. 5*A*) in the intestinal lamina propria, together with notable alterations of the glandular morphology. Furthermore, these animals also showed a reduced mucus layer (produced by glycoproteins at the glycocalyx), a natural barrier that confers protection from microbiota, preventing disease aggressiveness and progression (20). When animals with colitis were treated with GlcNAc, there was a decrease in the lymphocytic infiltrate and an improvement of the glandular architecture (Fig. 4*D*), which is compatible with disease remission (21). To further investigate the relationship between this overall improvement of disease severity through GlcNAc treatment and the levels of β1,6-GlcNAc branched N-glycans on LPLs, we performed L-PHA and CD3 histochemistry in the formalin-fixed, paraffin-embedded (FFPE) colonic specimens from the different groups of animals (Fig. 5*A*). The results showed that induction of colitis was accompanied by a notable intestinal lymphocytic infiltrate in the lamina propria, including CD3-expressing cells that expressed low levels of branched N-glycans. When mice were treated with GlcNAc, an increased expression of β1,6-branched glycans in the inflammatory infiltrate was observed (Fig. 5*A*, arrowheads). Importantly, the administration of GlcNAc also resulted in increased mucus lining, with augmented expression of branched glycans in glycoproteins in the superficial mucus layer, which is compatible with disease remission. Afterward, we investigated whether the TCR was particularly targeted by the observed enhancement of branched N-glycans in vivo. The results showed that GlcNAc treatment resulted in increased branching glycosylation on intestinal T cells, particularly in the TCR (Fig. 5 *B* and *B1*). To further address the mechanistic basis of the clinical efficacy of GlcNAc, we have evaluated the impact on T cell activity and signaling. Interestingly, LPLs from GlcNAc-treated mice displayed a decrease in the phosphorylation levels of ZAP70 and LAT that was more pronounced in mice treated with GlcNAc enemas (Fig. 5*C*). This result supports control of T cell activity through GlcNAc treatment, with effective results in topical/enema administration. Next, the effects of GlcNAc administration on the expression levels of T-bet, the transcription factor associated with proinflammatory Th1 cell polarization, were assessed in situ in the intestinal lamina propria. We have observed abundant cells expressing T-bet in lymphocytic infiltrates in mice with colitis that were markedly reduced in GlcNAc-treated mice (Fig. 5*D*, *Insets*). Furthermore, mice treated with GlcNAc revealed a significant reduction of IFN-γ production and a trend in the suppression of IL-17A secretion, further supporting that the enhancement of branched N-glycans by GlcNAc treatment controls Th1/Th17-type immune responses in vivo (Fig. 5*E*). Taken together, our in vivo results demonstrate a therapeutic effect of GlcNAc in a colitis-induced mouse model, revealing the immunomodulatory properties of this agent in the control of intestinal inflammation and, consequently, in the control of disease severity and progression.

Deficiency in Branched Glycosylation in *MGAT5* Null or Heterozygous Mice Is Associated with Early-Onset Disease and Increased Severity of Colitis that Is Suppressed by GlcNAc Treatment. To gain insights into the targeted-specific therapeutic effects of GlcNAc, we used *MGAT5* null or heterozygous mice that display a deficiency in branched N-glycosylation, mimicking the mechanistic defect described in humans (16). The impact on DSS-induced colitis onset and severity was assessed in *MGAT5* heterozygous ($^{+/-}$, partial deficiency on branched N-glycosylation) and *MGAT5* knockout ($^{-/-}$, absence of branched N-glycans) mice. Those genotypes represent intermediate N-glycans and the loss of one branch (β1,6-branching) of N-glycans, which may mimic mild/moderate versus severe deficiency on branched glycosylation. Our results showed that after DSS induction, *MGAT5* null or heterozygous mice exhibited increased susceptibility to early-onset disease and to severe forms of colitis. At day 2 after DSS induction, more that 50% of $MGAT5^{-/-}$ mice developed both mild and moderate forms of colitis compared with WT mice, which only developed clinical signs of colitis at day 6 postinduction (Fig. 6*A*). Moreover, at the end of DSS induction period (day 7), and based on the DAI, we observed increased susceptibility to severe forms of colitis in *MGAT5* null or heterozygous mice (Fig. 6*A*). As depicted in the pie chart in Fig. 6*A*, on day 7, more than 50% of $MGAT5^{-/-}$ mice exhibited severe forms of disease (scores ≥3) and 29% of $MGAT5^{+/-}$ mice developed severe colitis. In contrast, WT mice presented only mild/moderate forms of the disease. These results reinforce that *MGAT5*-mediated branched glycosylation has a strong impact on UC disease onset and progression.

Afterward, we tested the effect of GlcNAc treatment on the control of disease severity in mice with the different *MGAT5* genotypes. As shown in Fig. 6 *B* and *B1*, $MGAT5^{+/-}$ mice with colitis and treated with GlcNAc presented lower body weight loss and lower DAI compared with nontreated controls. With regard to body weight changes and DAI scores, the same tendency was observed in $MGAT5^{-/-}$ mice treated with GlcNAc (Fig. 6 *C* and *C1*). Due to their higher susceptibility to colitis, nontreated $MGAT5^{-/-}$ animals were euthanized on day 10 since they reached the established humane end points. $MGAT5^{-/-}$ mice, besides developing a much more aggressive disease phenotype,

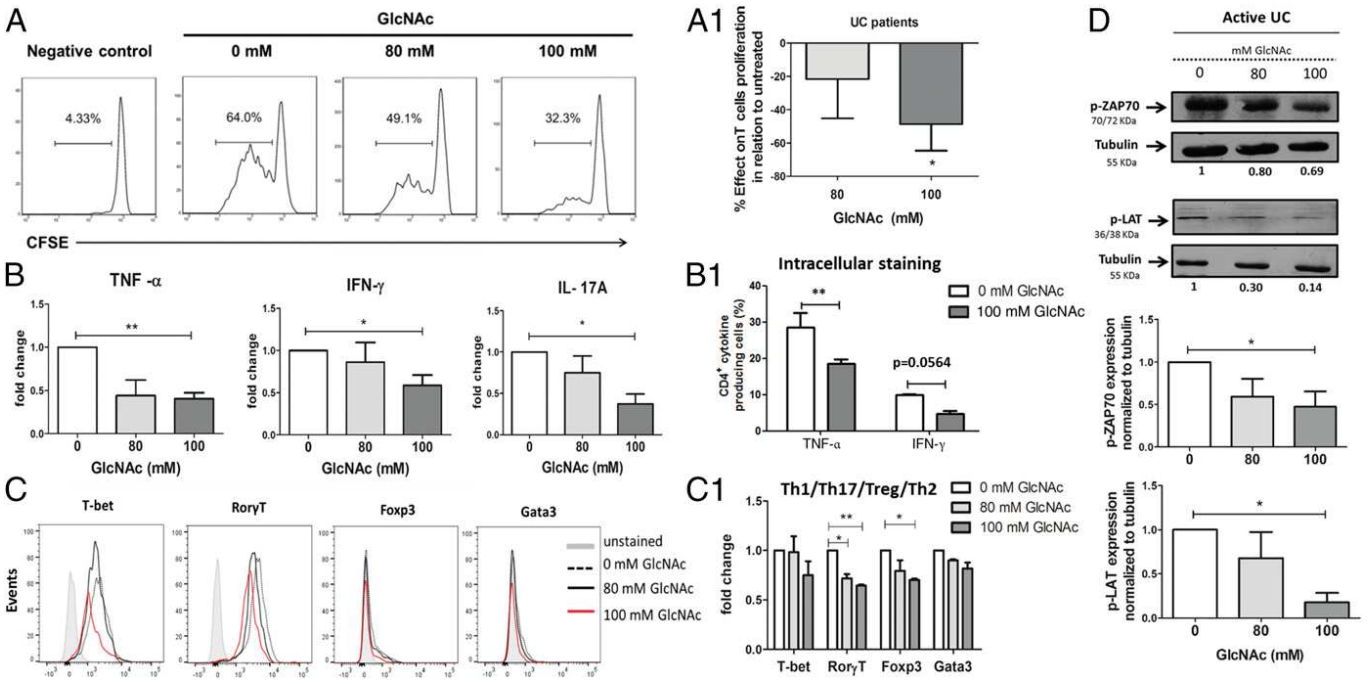

Fig. 3. Control of T cell-mediated immune response through enhancing branching N-glycosylation. (*A*) Purified $CD3^+$ T cells from fresh biopsies of naive patients with active UC were labeled with CFSE and cultured with GlcNAc treatment. The gated cells in the histograms correspond to the percentage of live cells. (*A1*) Bar plot: the mean percentage of effect ± SEM due to GlcNAc supplementation on T cell proliferation in comparison to untreated cells. Results include four biological replicates. Student's *t* test: $*P \leq 0.05$. (*B*) Cytokine profile assessed by flow cytometry in the supernatants from ex vivo T cell cultures under GlcNAc supplementation. Bar plots: mean fold change ± SEM of cytokine concentrations (picograms per milliliter) in six biological replicates. Student's *t* test: $*P \leq 0.05$; $**P \leq 0.01$. (*B1*) Evaluation of the percentage of IFN-γ– and TNF-α–producing $CD4^+$ T cells treated vs. nontreated with GlcNAc. Bar plots: mean ± SEM percentage of $CD4^+$cytokine-producing cells in three biological replicates from two independent experiments. Two-way ANOVA with Bonferroni postcorrection: $**P \leq 0.01$. (*C*) Expression of the transcription factors (TFs) in $CD4^+CD45^+$ T cells isolated from patients with UC and analyzed by flow cytometry. Histogram overlays correspond to the expression of the indicated TFs observed upon GlcNAc supplementation (gray-shadowed histograms depict the respective unstained control). (*C1*) Bar plots: mean fold change in TF mean fluorescence intensity ± SEM in two biological replicates, from two independent experiments. Two-way ANOVA with Bonferroni postcorrection: $*P \leq 0.05$; $**P \leq 0.01$. (*D*) Western blot analysis of TCR signaling, p-ZAP70, and p-LAT assessed in T cell lysates from cultures supplemented with GlcNAc. Bar plots: mean ± SEM fold change of p-ZAP70 and p-LAT densities normalized to tubulin in five biological replicates, from three independent experiments. Student's *t* test: $*P \leq 0.05$. In all experiments, results are normalized to the corresponding untreated condition (0 mM), which was taken as 1.

were also less responsive to GlcNAc, which makes GlcNAc therapy in these mice more challenging. Nevertheless, the results in $MGAT5^{+/-}$ and $MGAT5^{-/-}$ mice (Fig. 6 *B–D*) showed that even in these mice, treatment with GlcNAc improved clinical scores compared with the scores of DSS mice. This beneficial effect was pronounced whenever GlcNAc was administered topically by enema (single or in combination with oral administration), as evidenced by lower body weight loss and lower colitis scores compared with DSS mice. Interestingly, when GlcNAc treatment was initiated earlier in disease course of $MGAT5^{-/-}$ mice, at day 5, when animals started to develop severe colitis, we observed a decrease of the colitis scores compared with nontreated diseased animals (DSS), which supports the benefits of treating these susceptible animals earlier with GlcNAc, as demonstrated in Fig. 6*D*.

GlcNAc Treatment of MGAT5 Null or Heterozygous Mice Attenuates Disease Progression by Controlling Th1/Th17-Type Immune Responses. *MGAT5* null and heterozygous mice showed disorganization of the glandular architecture and an increase of inflammatory infiltrates in the colonic mucosa after DSS-induced colitis that was improved overall upon GlcNAc treatment (Fig. 7*A*). Notably, the evaluation of β1,6-GlcNAc branched N-glycan levels on $CD3^+$ LPLs confirmed that the induction of colitis was accompanied by decreased expression of branched N-glycans in LPLs from $MGAT5^{+/-}$ mice and by the absence of branched N-glycans in $MGAT5^{-/-}$ mice with induced colitis (DSS) (Fig. 7*A*). Interestingly, when mice of both *MGAT5* genotypes were treated with GlcNAc, a recovery of β1,6-branched glycan expression was observed in the intestinal inflammatory infiltrate (Fig. 7*A*, arrowhead) and in the superficial mucus layer, which is compatible with mucosal healing. The positive detection of L-PHA staining in *MGAT5* null mice upon GlcNAc supplementation was unexpected, as these mice lack the GnT-V enzyme. This positive detection was confirmed at the protein level by L-PHA blot (Fig. 7*B* and *SI Appendix*, Fig. S9*A*). In fact, the reactivity of L-PHA in $MGAT5^{-/-}$ mice is the lowest, compared with heterozygous and WT mice, but it is still positive. These observations may be in line with redundant effects of other GnTs at the Golgi (22–24) that, within an activated hexosamine pathway, may compensate for the absence of *MGAT5* by producing the β1,6-GlcNAc branched glycans, although with a much lower yield of synthesis, as we have observed. In an attempt to explore the potential compensatory synthesis of β1,6-GlcNAc branched N-glycans in *MGAT5* null mice, interestingly, we have observed that the *MGAT5b* gene [a homologous gene of MGAT5a that codifies the GnT-IX or GnT-Vb enzyme (25)] is apparently up-regulated in colonic T cells from *MGAT5* null mice treated with GlcNAc compared with control mice (nontreated mice) (*SI Appendix*, Fig. S9*B*). *MGAT5* null mice with DSS-induced colitis do not express *MGAT5b*. This preliminary evidence suggests GnT-IX/Vb as a potential candidate that might compensate for the synthesis of β1,6-GlcNAc branched glycans in *MGAT5* null mice. This issue needs further investigation. We then assessed the enhancement of branched N-glycans specifically on T cells after GlcNAc treatment. *MGAT5* null or heterozygous mice treated with GlcNAc showed an enhanced expression of branched N-glycans in the TCR compared with nontreated diseased animals (DSS) (Fig. 7 *C* and *D*). This effect was highlighted when animals were treated topically with GlcNAc enemas (Fig. 7 *C* and *D*). To explore the mechanistic effects of GlcNAc treatment in the T cell-mediated immune response, the

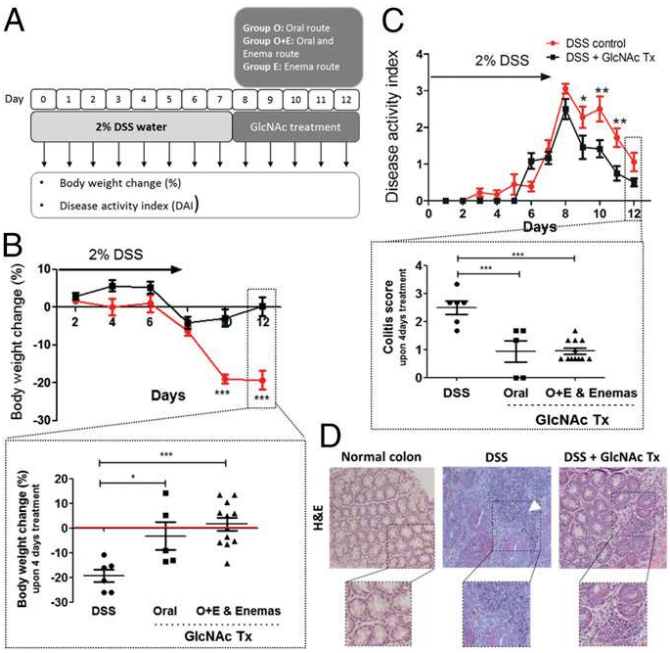

Fig. 4. Colitis-induced mouse model treated with GlcNAc displays significant control of disease severity and recovery from clinical signs. (*A*) Schematic representation of the in vivo study performed on C57BL/6 mice. (*B* and *C*) Body weight changes and DAI. (*B*) Effects of GlcNAc on body weight change (%) comparing DSS control and GlcNAc-treated animals. Graphs depict the mean ± SEM. Two-way ANOVA with Bonferroni postcorrection: $***P \leq 0.001$. (*Inset*) Discrimination of the effects of GlcNAc on body weight change (%) using different routes of administration. Scatter plots include the mean ± SEM. One-way ANOVA with Bonferroni postcorrection: $*P \leq 0.05$; $***P \leq 0.001$. (*C*) DAI comparing mice with colitis that were untreated with those that were treated with GlcNAc. Plots depict the mean ± SEM. Two-way ANOVA with Bonferroni postcorrection: $*P \leq 0.05$; $**P \leq 0.01$. (*Inset*) Discrimination of the effect of GlcNAc on colitis scores using different routes of administration. Plots depict the mean ± SEM. One-way ANOVA with Bonferroni post correction: $***P \leq 0.001$. (*D*) Representative histological images (H&E) of colonic samples from mice [normal colon, DSS (DSS-induced colitis), and GlcNAc treatment (Tx) (DSS + GlcNAc Enema Tx)]. (Magnification: 40×.)

impact on TCR signaling was evaluated in *MGAT5* null and heterozygous mice. An overall decrease of ZAP70 phosphorylation, indicative of reduced TCR signaling, was detected in colonic T cells from GlcNAc-treated mice that was evident when GlcNAc was administered topically (Fig. 7*E*). This topical effect was particularly observed in $MGAT5^{-/-}$ mice, where oral treatment did not affect TCR signaling. The more marked effect achieved through GlcNAc enema administration suggests that this molecule may be more efficiently taken up by cells in this way, likely by increasing its local concentration, thus facilitating its entry into the hexosamine pathway and usage by glycosyltransferases other than GnT-V (*SI Appendix*, Fig. S9*A*) that may redundantly catalyze the branched N-glycans. These redundant effects need further investigation. Additionally, the evaluation of the Th1 proinflammatory response revealed that GlcNAc treatment in both genotypes was associated with reduced proportions of cells expressing T-bet in lymphocytic infiltrates compared with control mice with colitis (Fig. 7*F*). Notably, similar to our observations of GlcNAc treatment in T cells from patients with UC, colonic explants from *MGAT5* null or heterozygous mice treated with GlcNAc indicate a trend for IFN-γ suppression, but with a more pronounced effect in reducing IL-17A. These results further support the impact of GlcNAc treatment and, consequently, the enhancement of branched N-glycans in controlling Th1/Th17-type immune responses also in the IBD in vivo model (Fig. 7*G*). Regarding the impact of GlcNAc on regulatory T cells, no apparent difference in the numbers of FoxP3-expressing cells was observed at the intestinal lamina propria comparing non-treated versus GlcNAc-treated mice of $MGAT5^{wt}$ and $MGAT5^{-/-}$ genotypes (*SI Appendix*, Fig. S9*C*). Nevertheless, further studies are needed to better characterize the regulatory effects of GlcNAc treatment in the different components of the immune response, such as in macrophages (*SI Appendix*, Fig. S9 *D* and *E*), as proposed by previous reports (26), and other T cell populations. Taken together, these data support the targeted-specific effects of GlcNAc that were able to repair the deficiency in branched glycosylation on T cells associated with *MGAT5* deficiency, thus controlling progression of colitis.

Discussion

IBD is characterized by a substantial heterogeneity concerning disease onset, course, response to therapy, and progression to complications (e.g., hospitalization, need for surgery, cancer) (1). Moreover, and despite recent advances in IBD therapeutic resources, a high proportion of patients remain refractory to conventional treatment, and approximately half of the patients with UC do not achieve

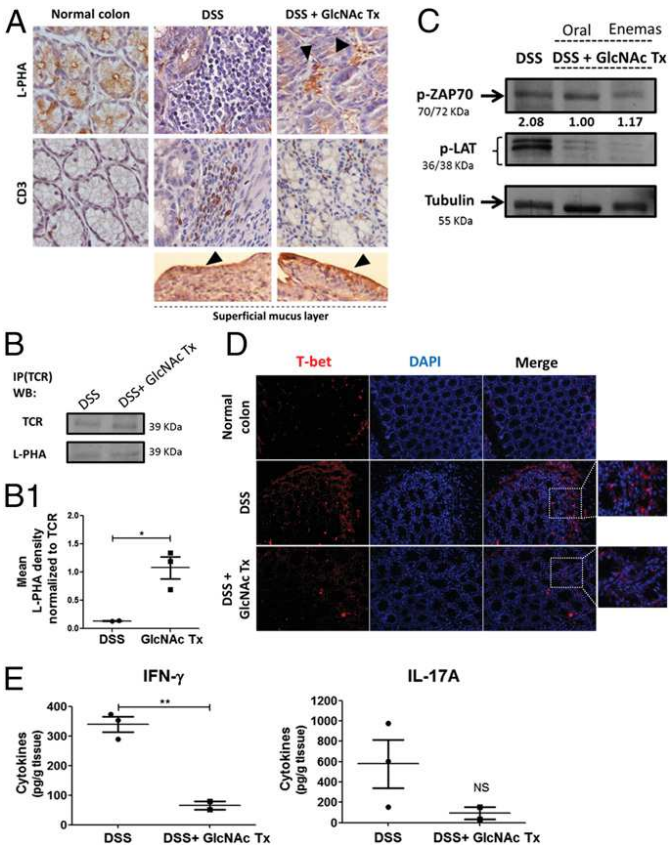

Fig. 5. Colitis-induced mouse model treated with GlcNAc showed increased branched N-glycosylation associated with suppression of T cell function. (*A*) L-PHA histochemistry and CD3 immunohistochemistry. L-PHA lectin reactivity showed an increased expression of β1,6-branched structures in the intestinal inflammatory infiltrate (positive to CD3) as well as an increase in mucus lining in mice treated with GlcNAc enemas (arrowheads). (Magnification: 63×.) (*B*) Immunoprecipitation (IP) of TCR followed by β1,6-GlcNAc branched N-glycan recognition in mouse colon, DSS (DSS-induced colitis) vs. DSS + GlcNAc treatment (Tx). WB, Western blot. (*B1*) Scatter plot: ratio of densities of L-PHA reactivity normalized to that of TCR depicted as the mean ± SEM comparing DSS ($n = 2$) mice with DSS + GlcNAc Tx ($n = 3$) mice. Student's *t* test: $*P \leq 0.05$. (*C*) TCR signaling by Western blot analysis of the phosphorylation levels of ZAP70 and LAT in LPLs. Values of pZAP70 densities normalized to tubulin are indicated. (*D*) Immunofluorescence of T-bet in colonic sections of DSS vs. DSS + GlcNAc Tx. (*Insets*) T-bet–expressing cells at intestinal inflammatory infiltrate are highlighted. (Magnification: 20×.) (*E*) Concentration of IFN-γ and IL-17A in the supernatants of 24-h colonic explant cultures from DSS and DSS + GlcNAc Tx $MGAT5^{wt}$ ($n = 5$) mice by ELISA. Plots depict the mean ± SEM of two to three animals per group. Student's *t* test: $**P \leq 0.01$. NS, not significant.

sustained remission (27). In addition, issues related to side effects and failure in therapy response highlight the need for more effective and targeted-specific drugs (28). We have recently demonstrated that patients with UC exhibit a deficiency in branched glycosylation on intestinal T cells due to a transcriptional reduction of the *MGAT5* gene that accompanied disease severity (16).

Herein, we uncovered a prominent role for the branched glycosylation pathway in IBD pathogenesis, by shaping the course of the T cell response. This pathway is thus an attractive target for novel therapies. Indeed, we have shown here that checkpoints of T cell immune response in UC could be modulated by metabolic supplementation with the simple sugar GlcNAc. We showed that GlcNAc therapy concomitantly increased branched N-glycosylation on T cells and down-regulated T cell proinflammatory responses both ex vivo and in vivo. In line with these observations, it was previously reported that lack of β1,6-GlcNAc branched N-glycans, by targeted deletion of the locus encoding GnT-V, results in enhanced TCR signaling and increased susceptibility to multiple sclerosis (7, 10). Moreover, and in accordance with our results, the increase of N-glycan branching through GlcNAc salvage into the hexosamine pathway was associated with a decreased threshold in T cell activation and more stable CTLA-4 surface expression, which resulted in the inhibition of adoptively transferred EAE (7, 13).

The evidence presented here suggests that GlcNAc supplementation of T cells isolated from patients with active UC resulted in remodeling of the glycophenotype of T lymphocytes through a marked increase of β1,6-GlcNAc branched N-glycans and an increase of polylactosamine structures, the ligand for galectins (29), that can then be terminally sialylated, predominantly with α2,6-sialic acid residues. This glycan reprogramming on T cells was shown to translate into key immunomodulatory effects in UC. Importantly, the enhancement of branched N-glycans on T cells induced by GlcNAc supplementation led to the suppression of T cell proliferation; inhibition of T cell signaling; reduced production of the proinflammatory cytokines TNF-α, INF-γ, and IL1-7A; and controlled Th1- and Th17-type responses. Both Th responses have been associated with IBD pathogenesis (30). Furthermore, these results are in accordance with a very recent report using mouse T cell cultures, which showed the ability of GlcNAc to promote iTreg over Th17 differentiation (15).

The glycosylation of T cells can also have an impact on the susceptibility to cell death (31). Accordingly, treatment of T cells with GlcNAc induced an increased susceptibility to apoptosis, which is at the core of different regulatory processes controlling T cell activation and expansion, thus avoiding exacerbated inflammation (32). This effect of GlcNAc on apoptosis was dose-dependent and limited to T cells from patients with active UC (*SI Appendix*, Fig. S7*B*). In agreement, previous reports have shown that extension with polylactosamine structures, which are ligands for galectins (such as galectin 1), was associated with proapoptotic effects of $CD4^+$ T cells (31, 33). Nevertheless, and given that treatment with KF and SW did not reverse the apoptotic effects induced by GlcNAc in the cells of patients with active UC, it cannot be excluded that factors other than branching glycans can also contribute to GlcNAc-mediated regulation of in vitro T cell apoptosis. Importantly, besides the TCR, the enhancement of branched N-glycosylation can also modify other receptors like the coreceptors CD4 and CD8, as well as the growth inhibitory receptor CTLA-4 (7, 10, 22). Moreover, CD45 and CD25 are also potential targets of branched glycosylation modification that can further contribute to the regulation of the T cell-mediated immune response through branching N-glycans (15, 34). Immunomodulation through GlcNAc-mediated enhancement of branched glycosylation, as described here, is a promising therapeutic approach to restore T cell homeostasis in IBD (*SI Appendix*, Fig. S10). Indeed, metabolic regulation of T cell function has been highlighted by recent research (35) and may be manipulated to reduce T cell-mediated inflammation (15, 36).

The preclinical data reported here provide the proof of concept supporting such a therapeutic approach in IBD. Deficiency of the *MGAT5* gene was associated with higher susceptibility to severe

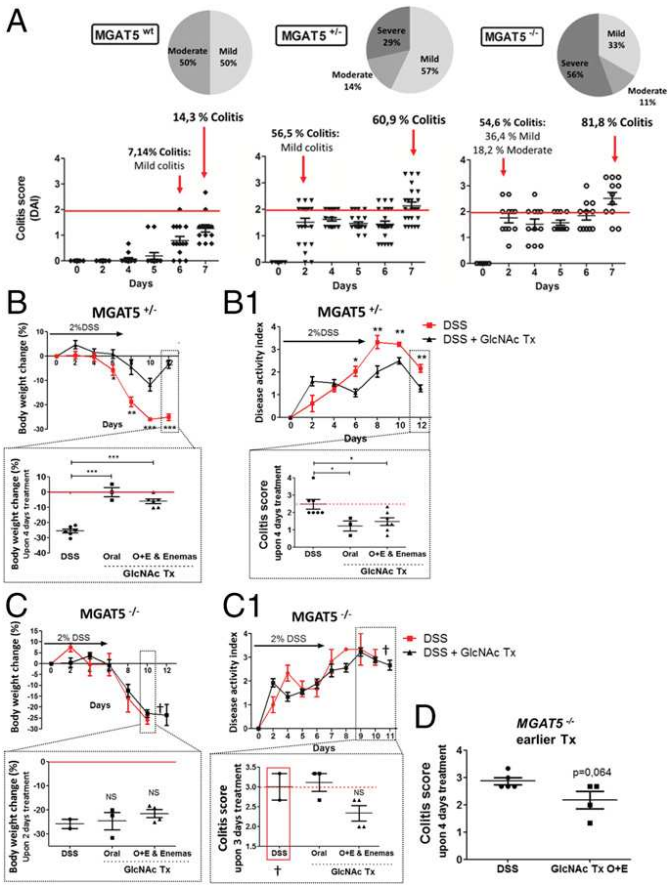

Fig. 6. *MGAT5* null or heterozygous mice develop early-onset colitis and an increase in disease severity that is suppressed by GlcNAc treatment. (*A*) Evaluation of colitis onset and disease severity in *MGAT5* null or heterozygous mice: C57BL/6 WT (n = 14), $MGAT5^{+/-}$ (n = 23), and $MGAT5^{-/-}$ (n = 11) mice. Active disease was defined when animals showed a DAI of ≥2, and three stages of severity were defined: mild (≥2 and <2.5), moderate (≥2.5 and <3), and severe (≥3). Average results of body weight change (*B* and *C*) and DAI (*B1* and *C1*) of $MGAT5^{+/-}$ (n = 23) and $MGAT5^{-/-}$ (n = 9) mice, respectively, randomly distributed in controls and GlcNAc treatment groups are shown. DSS-induced colitis (DSS) vs. DSS treated with GlcNAc treatment (DSS + GlcNAc Tx). Animals showing severe signs of disease were euthanized (†). (*B* and *B1*, *Insets*) Discrimination of the efficiency of GlcNAc treatment (colitis scores) with different routes of administration upon 4 d of treatment. Graphs correspond to the mean ± SEM of 17 animals (three to seven animals per route of administration). Student's t test (*B* and *B1*) and one-way ANOVA with Bonferroni postcorrection (*B* and *B1*, *Insets*): $*P \leq 0.05$*; $**P \leq 0.01$; $***P \leq 0.001$. Body weight changes of $MGAT5^{-/-}$ mice treated through different routes vs. nontreated upon 2 d of treatment (*C*) and DAI scores of $MGAT5^{-/-}$ mice treated (n = 7) vs. nontreated (n = 2) (*C1*) are shown. (C and *C1*, *Insets*) Discrimination of the efficiency of GlcNAc treatment (colitis scores) with different routes of administration upon 3 d of treatment. (*D*) Evaluation of the impact of early oral route (O) + enema route (E) GlcNAc treatment (starting on the second day of disease onset: 5–6 d after DSS induction) on the colitis scores (DAI of animals per group) of $MGAT5^{-/-}$ mice, comparing DSS (n = 5) with GlcNAc treated mice (n = 4).

forms of colitis and early-onset disease. These data highlight the prominent role of branched N-glycosylation in the pathogenesis of IBD, and are in accordance with previous studies on multiple sclerosis (11). GlcNAc supplementation improved clinical scores and was associated with a better disease course in mice developing the most severe disease phenotype ($MGAT5^{-/-}$). These immunosuppressive effects catalyzed by GlcNAc were observed by both oral and enema administration routes, with promising effects when mice received GlcNAc topically via enemas. This topical effect of GlcNAc is in line with the ability of GlcNAc to be more efficiently taken up by the intestinal mucosa, thereby entering directly into the

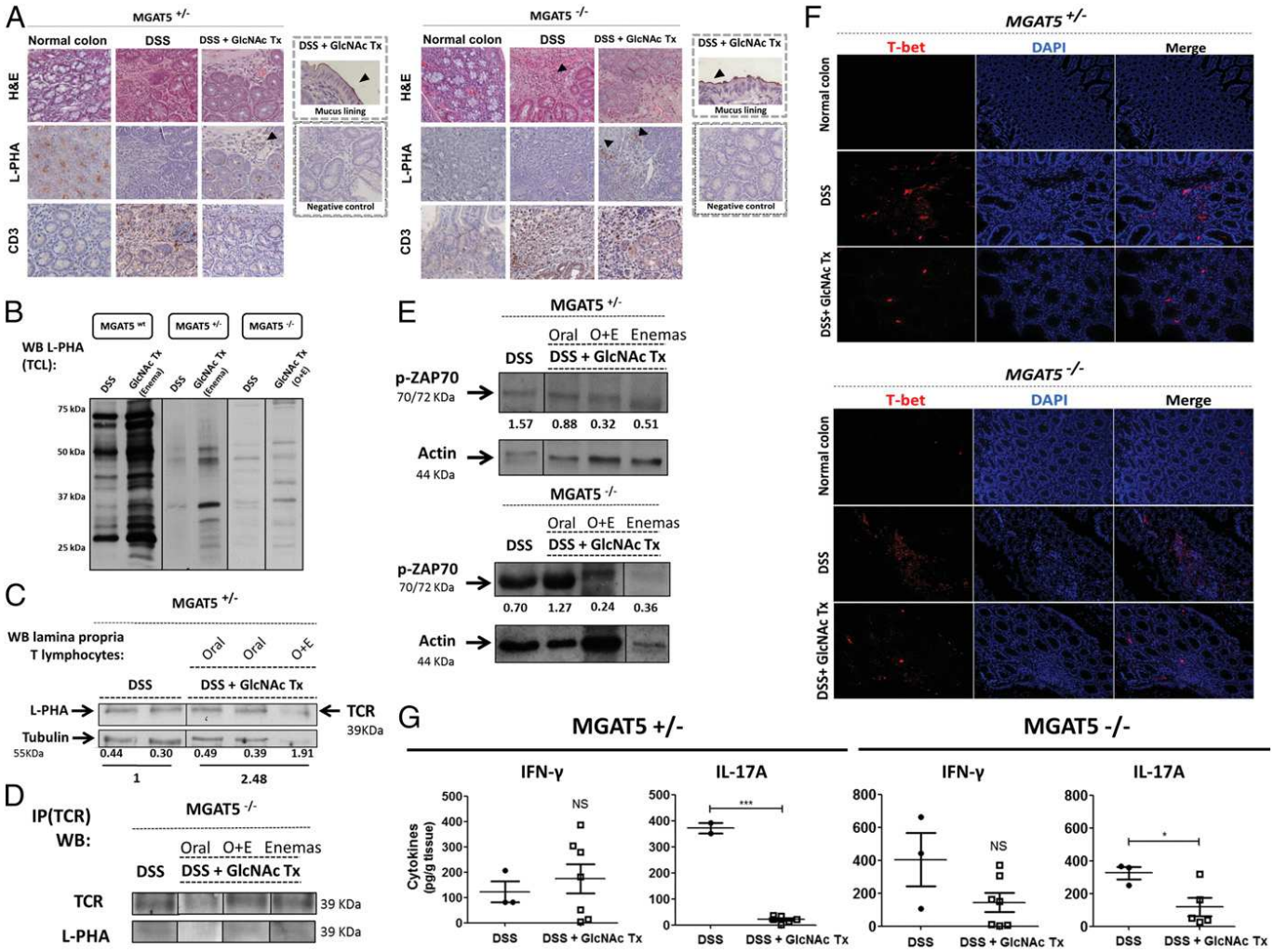

Fig. 7. GlcNAc treatment of *MGAT5* null or heterozygous mice attenuates disease progression by controlling Th1/Th17-type immune responses. (*A*) Representative histological images (H&E) of colonic sections from *MGAT5*$^{+/-}$ and *MGAT5*$^{-/-}$ [normal colon, DSS-induced colitis (DSS), and GlcNAc treatment (DSS + GlcNAc Tx)] (Magnification: 20×.) DSS mice displayed visible signs of lymphocytic infiltrate in the intestinal lamina propria (arrowheads). L-PHA histochemistry and CD3 immunohistochemistry of mouse colon from the different groups. (Magnification: 20×.) (*B*) Evaluation of branching N-glycans on colonic total cell lysates from *MGAT5*^{wt}, *MGAT5*$^{+/-}$, and *MGAT5*$^{-/-}$ mice comparing DSS control with GlcNAc Tx enema by Western blot (WB). TCL, total cell lysate. (*C*) Protein lysates from LPLs isolated from the colon of *MGAT5*$^{+/-}$ mice with DSS (colitis) or treated (DSS + GlcNAc Tx) mice were subjected to L-PHA lectin blotting to evaluate the expression of β1,6-GlcNAc branched N-glycans on the TCR (39 kDa). L-PHA density normalized to tubulin is indicated for each case, and fold change differences of DSS vs. DSS + GlcNAc Tx are highlighted. (*D*) Immunoprecipitation (IP) of the TCR in total cell lysates from *MGAT5*$^{-/-}$ mouse colon followed by β1,6-GlcNAc branched N-glycan recognition. DSS vs. DSS + GlcNAc Tx with different routes of administration. (*E*) Analysis of the phosphorylation levels of ZAP70 in LPL lysates from colon of *MGAT5*$^{+/-}$ and *MGAT5*$^{-/-}$ mice. Values of pZAP70 normalized to actin in *MGAT5*$^{+/-}$ and *MGAT5*$^{-/-}$ mice are indicated. (*F*) Immunofluorescence of T-bet in *MGAT5*$^{+/-}$ and *MGAT5*$^{-/-}$ mice comparing normal colon, DSS, and DSS + GlcNAc Tx mice. (Magnification: 20×.) (*G*) Concentration of IFN-γ and IL-17A in the supernatants of 24-h colonic explant cultures from DSS and DSS + GlcNAc Tx *MGAT5* heterozygous ($n = 10$) and null ($n = 10$) mice by ELISA. Plots depict the mean ± SEM of two to seven animals per group. Student's *t* test: $^{*}P \leq 0.05$; $^{***}P \leq 0.001$. NS, not significant.

hexosamine pathway to increase branched glycosylation. Intriguingly, in *MGAT5*$^{-/-}$, with the absence of *MGAT5*/GnT-V, expression of the β1,6-GlcNAc branched N-glycans detected by L-PHA lectin histochemistry and blotting was positive and slightly increased with GlcNAc treatment. This unexpected result may be in accordance with the fact that several glycosyltransferase-deficient mice exhibit no or only mild phenotypes due to redundancy or compensation of glycan functions. In fact, several family members of glycosyltransferases are known to be functionally redundant (37, 38). This potential redundant effect observed in *MGAT5*$^{-/-}$ mice treated with GlcNAc might be due to the fact that other Golgi glycosyltransferases within an activated hexosamine pathway triggered by UDP-GlcNAc may compensate for the lack of this specific glycan structure, thereby guaranteeing immune homeostasis. One of the candidate enzymes is the homologous GnT-Vb that may compensate for the synthesis of the branched glycans; however, further studies are needed to clarify this issue. Similar examples of the redundancy of glycosyltransferases were described, such as for FUT8-deficient mice (39). Nevertheless, this redundant effect observed upon GlcNAc supplementation needs to be further explored.

The clinical effects of GlcNAc were further evidenced by the decreased frequency of T-bet–expressing cells in colonic mucosa of treated mice, together with reduced release of the proinflammatory cytokine IL-17A of respective colonic explants.

GlcNAc is a naturally occurring amino sugar for which no adverse effects were reported in humans (40). It is available as a dietary supplement, and oral GlcNAc administration showed no toxicity in rats (41). Interestingly, 17 y ago, oral GlcNAc was described to promote intestinal lining through mucus production in children with severe treatment-resistant IBD (21). In that study, eight of the 12 children studied went into clinical remission, which could have resulted from the immunomodulatory effects of

GlcNAc proposed here. Taken together, the combined evidence from both ex vivo and preclinical data provides proof of concept for the therapeutic use of GlcNAc (either alone or in combination with other antiinflammatory therapies) as a simple immunomodulatory strategy in IBD. Assessment of this strategy in clinical studies is currently ongoing. Some of the most relevant properties associated with GlcNAc treatment are the absence of side effects, low cost, and possibility of being used as a simple rescue therapy to avoid unnecessary toxic effects and step-up therapies in IBD.

Materials and Methods

Patient Selection and Colonic Biopsy Collection. Fresh colonic biopsies were obtained from 75 patients (three patients in remission) diagnosed with UC and normal controls ($n = 3$) who underwent a scheduled colonoscopy (2014–2017) at the Gastroenterology Department of Centro Hospitalar do Porto–Hospital de Santo António (CHP-HSA), Porto, Portugal. All participants gave informed consent, and procedures were approved by the Ethics Committee of CHP/HSA [233/12(179-DEFI/177-CES)].

Isolation of $CD3^+$ T Cells from Fresh Colonic Biopsies and Blood of Patients with Active UC: Ex Vivo Culture of T Cells. After mechanical dissociation of colonic biopsies and blood density gradient centrifugation using Lymphoprep, $CD3^+$ T cells (from biopsies and blood) were magnetically sorted using an EasySep Human T Cell Enrichment Kit (STEMCELL Technologies) following the manufacturer's instructions. $CD3^+$ T cells were cultured for 72 h with anti-CD3 mAb (clone OKT3) and soluble anti-CD28 mAb (clone CD28.2) (eBioscience). T cell cultures were supplemented with GlcNAc (Sigma and Wellesley Therapeutics, Inc.).

Imaging Flow Cytometry. Imaging flow cytometry analysis was performed as previously described (42).

Flow Cytometry. $CD3^+$ T cells were stained with CD4 and CD8 (BD Biosciences) and fluorescein isothiocyanate (FITC)-conjugated L-PHA (Vector Laboratories), as well as with cell surface markers (CD4, CD45), intracellular antigens (T-bet, RORγt, Foxp3, and Gata3), and cytokine intracellular staining (TNF-α, IFN-γ). Various antibodies used for staining are described in *SI Appendix*, Table S1.

Proliferation Assay. $CD3^+$ T cells were purified from colonic biopsies and labeled with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) using a CellTrace CFSE Cell Proliferation Kit (Invitrogen), as described by Oliveira et al. (43).

Cytokine Production. Supernatants from colonic T cell cultures were analyzed by flow cytometry using the BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences) following the manufacturer's instructions. Human TGF-β1 quantification was performed using ELISA kits (R&D Systems) according to the manufacturer's instructions. The supernatants from mouse colonic explant cultures were concentrated using Amicon Ultra-2 mL Centrifugal Filters (Merck Millipore), according to manufacturer's instructions. The levels of IFN-γ and IL-17A (anti-mouse, Ready-SET-Go! kits; eBioscience) and TNF-α and IL-6 (anti-mouse; Biolegend) were quantified by ELISA, according to the manufacturers' instructions.

Western Blot and TCR Signaling. TCR signaling and L-PHA lectin blot analysis (44), using T cell protein lysates (extracted with radioimmunoprecipitation buffer), were performed as described by Dias et al. (16). Incubation of phospho–Zap-70 [Tyr319/Syk (Tyr352)] rabbit mAb and anti–phospho-LAT (Tyr191) rabbit mAb (Cell Signaling Technologies) was performed. Goat anti-rabbit IgG-HRP mAb was used as a secondary antibody, and rabbit IgG antiactin (Santa Cruz Biotechnology) or mouse IgG antitubulin (Sigma) was used as a loading control.

Immunoprecipitation. TCR immunoprecipitation, using total cell lysates obtained from mouse colons or from ex vivo human T cell cultures, was performed as previously described (16).

Glycophenotype. T cells were incubated with biotinylated L-PHA, biotinylated LEL, biotinylated SNA, or biotinylated MAL-II (Vector Labs). Lectins were revealed with FITC-conjugated streptavidin.

Apoptosis Assays. Apoptotic cells were identified by flow cytometry, using an FITC Annexin V Apoptosis Detection Kit I (BD Biosciences), following the manufacturer's instructions.

Enzymatic Reaction and HPLC Analysis. The GnT-V enzymatic activity analyses in T cells from patients with UC and controls were performed as previously described by Takamatsu et al. (45).

DSS- and TNBS-Induced Colitis and in Vivo GlcNAc Treatment. Colitis was induced with DSS in C57BL/6, MGAT5 wild-type, heterozygous, and knockout mice (kindly provided by Michael Pierce, University of Georgia, Athens, GA) (19). The TNBS model was also performed using C57BL/6 mice (19). After disease onset, DSS mice were treated with GlcNAc (Sigma and Wellesley Therapeutics, Inc.) (12). LPLs were isolated from mouse colon as previously described (16).

Tissue Immunohistochemistry and Immunofluorescence. FFPE colonic tissue slides were used for H&E staining and for immunohistochemistry with L-PHA and anti-CD3 mAb as well as Foxp3 and F4/80, as described, respectively, by Dias et al. (16) and Teixeira et al. (46). For T-bet immunofluorescence, mouse IgG_1 T-bet–specific mAb (clone 4B10; Santa Cruz Biotechnology), goat anti-mouse Alexa 594 secondary antibody (Invitrogen), and DAPI staining were used.

Real-Time PCR. Total RNA from isolated LPLs was extracted and the quantitative real-time PCR (qRT-PCR) was performed using TaqMan Gene Expression Assays (Applied Biosystems), as previously described (16). qRT-PCR was carried out in triplicates for the target gene MGAT5b (Taqman probe: Mm01252571_m1) and the endogenous control 18S (Hs99999901_s1) (Applied Biosystems) (16).

Statistics. Statistical significance was assessed by one-way or two-way ANOVA using a Bonferroni's, Dunnett's, or Newman–Keuls multiple comparison post-test and, where appropriate, by an unpaired Student's *t* test (two-tailed) using GraphPad Prism 5. *P* values of <0.05 were considered statistically significant.

More details can be found in *SI Appendix, SI Materials and Methods*.

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IV

Glycans as immunomodulators in inflammation: Inflammatory Bowel Disease as a model

Glycans as immunomodulators in inflammation: Inflammatory Bowel Disease as a model.

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Abstract

The diversity of glycans expression within a cell or an organism is enormous and the amount of relevant biological information that each glycan structure encodes is far from being clarified. The importance of glycans in health and life sciences is highlighted by their multiple functional implications in cellular and molecular biology processes with impact in homeostasis and in diseases, such as cancer and immune-mediated disorders. Glycans are major participants in the intricate regulatory circuits that govern immune response. Changes in the glycans repertoire occur during the transition from normal to inflamed conditions and this aberrant expression of glycans contribute to the excessive immune-stimulation that characterizes inflammatory diseases, such as Inflammatory Bowel Disease (IBD). This review summarizes the most recent evidences underlying how glycans integrate the regulatory networks of immune response with a particular focus on IBD pathogenesis. The translational value of glycans to the clinical setting is discussed, addressing its potential applications as biomarkers and as immunomodulatory agents.

1. Introduction

Inflammation is a complex biological process that occurs as a normal host response to tissue damage inflicted by infections or other stimuli. An inadequate resolution of inflammation and/or an unchecked inflammatory reaction can evoke chronic inflammation, predisposing the host to various diseases, including chronic immune-mediated disorders, such as Inflammatory Bowel Disease (IBD), a focus of this review, and cancer.

A compelling body of evidences has been revealing that glycans (carbohydrates or sugar chains) integrate into the canonical circuits that govern inflammation as demonstrated by the fact that alterations in the cellular glycosylation repertoire have remarkable effects in the development and progression of immune-mediated disorders (Marth and Grewal, 2008; Rudd, et al., 2001), such as IBD.

The intestinal mucosa is considered a major physical and biological barrier that supports the body´s immune system. The extensive and diversified microbiota content of the gut appears to positively contribute to the host´s immune system. The glycans composition of the gut is considered a key factor that guides the establishment of the microbial community (Koropatkin, et al., 2012), being also instrumental in shaping the course of immune response both in homeostasis and in diseases such as in IBD.

This review summarizes the importance of glycosylation as a fundamental player in inflammation using IBD as a disease model system. The recent evidences on the impact of glycans, with a focus on N-linked glycans, in the regulatory circuits that govern inflammation is discussed, highlighting their impact on T cell-mediated immune response in the context of IBD pathogenesis. The implications of glycans as a source of promising clinical biomarkers and targets for new therapeutic strategies in immune-mediated diseases are introduced and discussed.

2. Inflammatory Bowel Disease pathogenesis: glycans as the missing factor?

Inflammatory bowel disease (IBD) is a global disease with incidence increasing worldwide. It is estimated to affect over 1,5 million people in USA and 2 million people in Europe (Burisch, et al., 2013). It comprises two major bowel disorders, Crohn's disease (CD) and Ulcerative Colitis (UC), that arise in a multifactorial context in which a chronic immune-mediated inflammatory process takes place triggered by genetic factors, environmental conditions and alterations in the composition of the gut microbiota (Figure 1). Nevertheless, this is far from being a perfectly matched puzzle on IBD pathogenesis

since it remains unclear the precise factors underlying the excessive co-stimulation of the immune system.

Importantly, the advent of genome-wide association studies (GWAS) studies imposed a tremendous progress in the **genetic** discovery of IBD susceptibility (Lees, et al., 2011). Although genetics only explains 7,5% of UC and 13,6% of CD (Jostins, et al., 2012), the identification of UC and CD specific loci was a major contribution to identify causal genes, related signaling pathways and to understand shared pathways between IBD and other autoimmune disorders(Lees, et al., 2011).

A meta-analysis of GWAS together with an ImmunoCHIP has identified genetic variants in 163 loci that explain the susceptibility of patients to develop CD and UC in almost all the loci (Jostins, et al., 2012). These loci are enriched in genes related with immune deficiencies, cytokine production (specifically interferon gamma (INF-γ), tumor necrosis factor alpha (TNF-α), interleukin (IL)-12, and IL-10 signaling), lymphocyte activation, response to molecules of bacterial origin and JAK-STAT signaling pathway. The impairment of immune system and the predisposition for host-microbe interactions (genetic variants in nucleotide-binding oligomerization domain-containing protein 2 (NOD2), caspase recruitment domain-containing protein (CARD) 9 and IL10) are considered the main triggers for IBD pathogenesis, however this huge number of loci are far to explain the total disease variance in IBD. In fact, the risk loci to develop IBD may not explain the disease-course over time, concerning disease relapsing, need of surgical intervention and also therapeutic outcomes. A recent study showed that the loci associated with CD prognosis are independent of the loci that contribute to disease susceptibility (Lee, et al., 2017b). The *NOD2/CARD15* (Yarur, et al., 2011) has been shown to predict the clinical course. Other studies showed that genetic variants in IBD risk loci, as in Major histocompatibility complexes (*MHC*) (Kolho, et al., 2016) and multidrug resistance protein 1 (*MDR1*) (Potocnik, et al., 2004) genes were associated with bad prognosis as well as with the non-response to therapy. Moreover, a GWAS in UC identified 46 single-nucleotide polymorphisms (SNPs) associated with refractory treatment that required colectomy (a marker of severe disease) (Haritunians, et al., 2010).

Nevertheless, all of these genetic data require further functional studies to validate its clinical impact.

Multidisciplinary efforts have been made to identify **environmental** factors associated with IBD (Ananthakrishnan, 2015).Evidences suggest that IBD incidence is particularly rising in the newly industrialized countries (Ng, et al., 2017) which provides strong evidences supporting the effect of the environment for both CD and UC. Smoking has been described to increase the risk of CD, conferring protection in UC. Diet, lifestyle, high standards of hygiene and use of antibiotics and other medications, have been

postulated to have an impact in disease susceptibility and pathogenesis, as reviewed in (Ananthakrishnan, 2015).

The gut **microbiome** content is definitely a major player in IBD pathogenesis as alterations in microbiota composition and functions (dysbiosis) have been consistently associated with IBD (Ananthakrishnan, 2015; Hall, et al., 2017).Generally, in IBD, there is a depletion of bacteria belonging to Firmicutes phylum (such as *Faecalibacterium prausnitzii*), particularly in CD patients (Hansen, et al., 2012) and an increase in bacteria belonging to the Proteobacteria phylum (such as *Escherichia coli*) (Marchesi, et al., 2016). The association of the Bacteroidetes phylum with IBD is not consensual, however, evidences suggests that there is a possible spatial reorganization of the Bacteroides species in patients with IBD, being *Bacteroides fragilis* an important species able to distinguish IBD patients from healthy controls (Swidsinski, et al., 2005).

Regarding the fungal microbiota content, little is known about its influence in IBD pathogenesis. However, evidences suggest that mice lacking important genes involved in fungi sensing, such as *Dectin-1* or *CARD9*, have an increased load of fungal microbiota being thus more susceptible to colitis (Iliev, et al., 2012; Sokol, et al., 2013). Recently, the analysis of the faecal microbiota of 235 IBD patients *versus* 38 healthy subjects demonstrated that a distinct fungal microbiota dysbiosis occur in IBD being characterized by alterations in their biodiversity and composition (Sokol, et al., 2017).

The contribution of **glycans** to intestinal microbiome composition and functions as well as to dysbiotic conditions is far from being fully elucidated. In fact, the colon mucosa is heavily glycosylated and different parts of the intestine display a specific glycosylation signature. Changes in mucins glycosylation have been reported to occur over the entire healthy colon (Arike and Hansson, 2016). Advanced glycoproteomics approaches, demonstrated that the cellular glycosylation profile vary among the different parts of the intestine when comparing IBD patients and controls (Stavenhagen, et al., 2015). This differential glycans composition at the level of colonic mucosa might influence the microbiota composition and function due to the fact that individual microorganisms prefer different glycans for degradation. This selective consumption of nutrients (glycans) can influence which microbial groups proliferate and persist in the gastrointestinal tract. For instance, mice experiments revealed a protective role of *B4GALT1* expression, which encodes for β-1,4-galactosyltransferase I, that mediates the addition of galactose moeieties to glycoproteins. Higher galactosylation of N-glycans on mucus proteins were associated with increased ratio of Firmicutes/Bacteroidetes in healthy conditions, conferring protection against TNF-induced systemic inflammation and DSS-induced colitis (Vanhooren, et al., 2013).

Glycans can also act at the interface of microbiota and immune response. As example, glycans in helminth act as a conserved molecular pattern that instructs dendritic cells (DCs) functions to drive T helper (Th)2-polarized responses (Smits, et al., 2010). Curiously, in less developed countries in which the incidence of IBD is rare, it is postulated that helminths might have a protective effect by modulating the host immune response. Using IBD murine models, it was demonstrated that the helminth, *Heligmosomoides polygyrus bakeri* prevents colitis by inhibiting antigen-specific gut T cell response through alterations of dendritic cells function (Blum, et al., 2012). This mechanism of suppression of inflammation through helmith´s glycans has been explored and recently, it was suggested that IL-4Rα signaling is the key pathway required for an effective suppression of immune response (Matisz, et al., 2017), which has been raising the interest for helminth antigen cell-based therapy (Maizels, 2016), already tested in small clinic trials in UC (Summers, et al., 2005).

Taken together, IBD is a multifactorial disease, which consequently imposes a major challenge in the clinical and therapeutic management of IBD patients. There is an urgent need in the clinics to identify and characterize novel molecular mechanisms underlying disease pathogenesis in order to improve the development of novel clinical biomarkers and new targeted-specific therapeutic strategies in IBD.

In the last decade novel insights on the impact of the glycome (biological repertoire of glycans structures in an organism) in the regulation of immune response (Demetriou, et al., 2001; Grigorian, et al., 2011; Mkhikian, et al., 2016; Rabinovich and Toscano, 2009; Toscano, et al., 2007; Zhou, et al., 2014) have been contributing to push the glyco-immunology field to the “prime-time” of immunity with relevant clinical implications in immune-mediated disorders such as IBD (Dias, et al., 2014) (Figure 1).

3. Glycosylation as a major biological barrier of the gut. Impact in host-interaction and immune response.

Glycosylation is a well-orchestrated post-translational process catalyzed by a diverse repertoire of glycosyltransferases (enzymes that catalyze the transfer of a sugar chain from a nucleotide sugar donor to a specific substrate) and glycosidases (enzymes that catalyze the hydrolysis of glycosidic bonds in glycan structures). The genes encoding the portfolio of those enzymes contribute to more than 1% of the total human genome which results in a huge diversity of glycosylation modifications in which the same protein or lipid can exhibit different glycoforms and consequently different biological functions (Gagneux, et al., 2015).

In fact, all cells are covered with a dense and complex coat of glycans called glycocalyx that, particularly in the intestinal mucosa represents an important physical and biological interface. In fact, the glycans composition of the intestinal glycocalyx creates a meshwork of different types of sugar chains that project to the lumen acting as molecular sensors with a vast potential for information display. Glycoproteins on intestinal brush border membrane suffer a dynamic remodeling of its glycan content that shapes the intestinal homeostasis. The carbohydrate moieties (glycocalyx) at epithelial cells (ECs) surface, suffers a turning over of approximately every 6 to 12 hours in human jejunum (Moran, et al., 2011).

One of the major components of the intestinal glycocalyx is the oligomeric mucus gel-forming glycoprotein Mucin 2 (MUC2) which is the main intestinal mucin produced by goblet cells. Curiously, in a cancer context, expression of MUC2 is associated with intestinal metaplasia being the most prominent mucin in mucinous type gastric carcinomas (Reis, et al., 2000).

Mucin's glycans serve as a nutritional source for bacteria. Recent reports have shown that in UC the alterations on MUC2 glycosylation are associated with inflammation (Larsson, et al., 2011; Larsson, et al., 2009). Moreover, core 3–derived O-glycans seems to be pivotal in the intestinal barrier function as C3GnT-deficient mice revealed a reduction of MUC2 glycoprotein content and a consequent impairment of mucosal integrity making these mice more prone to develop colitis and colorectal adenocarcinoma (An, et al., 2007).

Changes in cellular glycosylation occur during the transition from normal to inflamed conditions. This altered expression of glycans in inflammation has major implications in the regulation of both innate and adaptive immune responses (Johnson, et al., 2013; van Kooyk and Rabinovich, 2008; Wolfert and Boons, 2013).

Regarding **innate immunity**, glycans are key mediators of immune response due to its interaction with a particular family of receptors called C-type lectin receptors (CLRs) that bind to carbohydrates in a calcium-dependent manner. CLRs, such as dendritic cell specific intracellular adhesion molecule-3 (ICAM-3) grabbing nonintegrin (DC-SIGN) (that bind mainly high-mannose but also fucose moieties) (Feinberg, et al., 2001; Svajger, et al., 2010)), sialic acid-binding immunoglobulin-type lectins (siglecs) and macrophage mannose receptor (MMR), are expressed in various innate immune cells including DCs and macrophages. These CLRs are involved in glycan-mediated pathogen recognition and internalization of antigen for loading on MHC class I and II molecules inducing T cell response (Rabinovich and Croci, 2012; Singh, et al., 2009; van Gisbergen, et al., 2005; van Kooyk and Geijtenbeek, 2003).

In the process of loss of tolerogenicity associated with the development of autoimmunity and allergies (Buckner, 2010), an interesting report demonstrated that

sialylation of antigens is able to induce antigen-specific tolerogenic immune response through recognition by DCs and consequent induction of T regulatory (Treg) cells and suppression of IFN-γ production (Perdicchio, et al., 2016).

The sialic acid composition of the intestinal glycocalyx has been also associated with dysbiotic conditions. The sialic acid catabolism was reported to mediate intestinal inflammation concomitantly with the expansion of *E. coli* during colitis. The overgrowth of *E. coli* that occurs in colitis was demonstrated to be dependent on the sialic acid release from the host´s glycans after sialidase activity (Huang, et al., 2015). The dependence of *E. coli* on sialidases secreted by *Bacteroides* spp. (abundant in intestinal inflammation) contribute to the overgrowth of *E. coli* and thereby to dysbiosis in patients with colitis (Gophna, et al., 2006). In line with this, it was proposed that sialidase inhibitors can constitute a glycan-mediated strategy to decrease *E. coli* outgrowth preventing dysbiosis and thereby controlling the severity of colitis (Huang, et al., 2015).

In fact, the glycan influx to intestine either from diet or mucosal secretion, directly influence the adhesion, composition and the metabolic activity of microbiota with consequences in intestinal immune response.

Additionally, proteins glycosylation can also influence the **adaptive immune response**. In this regard, the MHC class I and II (MHC I and II) can display glycosylated peptides that are recognized by T cells, supporting the impact of glycans in the modulation of T helper cell (by MHC II) and cytotoxic T cell (by MHC I) responses (Neefjes, et al., 2011).

Protein O-GlcNAcylation, consisting in the addition of O-GlcNAc moieties to serine or threonine residues of nuclear and cytoplasmic proteins by the O-GlcNAc transferase (OGT) through UDP-GlcNAc (Hart, et al., 2007), has been revealed to be implicated in the metabolic processes involved in T cell self-renewal, differentiation and proliferation (Swamy, et al., 2016).

Interestingly, a recent study demonstrated that expression of α-1,2 fucose on the apical side of intestinal epithelial cells (IECs) protects against pathogenic bacteria being essential in the maintenance of the commensal microbiota (Goto, et al., 2014) (Figure 2). This process of fucosylation was found to be mediated by group 3 innate lymphoid cells (ILC3s). It was demonstrated that commensal bacteria, pathogenic bacteria and bacterial products (lipopolysaccharide (LPS)) stimulate (via gut DCs) ILC3s to produce IL-22 which in turn induces α-1,2 fucosylation of IECs. Accordingly, inactivating polymorphisms of fucosyltransferase (FUT) 2 (non-secretor status) were associated with CD (Franke, et al., 2010; McGovern, et al., 2010).

Another important type of fucosylation is core fucose which refers to fucose attached to the innermost N-acetylglucosamine of N-linked glycans, catalyzed by α1-6 fucosyltransferase (FUT8). A dysregulation of FUT8 was described to be associated with

IBD pathogenesis (Fujii, et al., 2016). In mouse models of induced colitis, T cells were found to display an increased expression of core fucosylation when compared with mice without colitis. Accordingly, the $Fut8^{-/-}$ mice developed a less severe colitis than $Fut8^{+/+}$ mice, and T cells from $Fut8^{-/-}$ mice produced lower levels of Th1 and Th2 cytokines. Accordingly, colonic samples from both CD and UC also revealed that inflamed mucosa exhibited higher levels of core fucosylation comparing to non-inflamed mucosa and healthy colon (Fujii, et al., 2016).

The complex branching N-linked glycan have been also described to impact in immune response, particularly the β1,6 GlcNAc branched complex N-glycans, catalyzed by N-acetylglucosaminiltransferase V (GnT-V). In homeostasis and self-tolerance, the T cell activation (T cell receptor (TCR) signaling) was described to induce up-regulation of MGAT5 gene (encoding GnT-V glycosyltransferase) leading to GnT-V-mediated branched glycosylation of the TCR. This phenotype promotes growth arrest of T cells by at least two mechanisms: early, by raising T cell activation thresholds via limiting TCR clustering at the immune synapse and controlling hyperimmune response, and later by increasing surface retention of growth inhibitory receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4)(Chen, et al., 2009a; Demetriou, et al., 2001). The dysregulation of this mechanism between GnT-V-mediated glycosylation and T cells function results in T-cell hyperactivity and susceptibility to multiple sclerosis (Demetriou, et al., 2001). Recently, it was also demonstrated that UC patients exhibit a deficiency of branched N-glycans on intestinal T cells associated with UC pathogenesis (discussed in detail in section below) (Dias, et al., 2014). Accordingly, mice deficient in *MGAT5* and lacking GnT-V function (no synthesis of β1,6 GlcNAc branched N-glycans structures), display a significantly increased TCR clustering, leading to a decreased threshold of T-cell activation which result in a hyperimmune response and increased susceptibility to autoimmunity (Demetriou, et al., 2001; Morgan, et al., 2004).

Besides the TCR, the enhancement of branched N-glycosylation can also modify other receptors like the co-receptors CD4 and CD8 as well as the growth inhibitory receptor CTLA-4 (Demetriou, et al., 2001; Lau, et al., 2007; Morgan, et al., 2004) . Moreover, CD45 and CD25 are also potential targets of branched glycosylation modifications with impact in the regulation of T cell-mediated immune response (Araujo, et al., 2017; Chen, et al., 2007).

The branched *N*-glycans can be further modified and elongated with poly-*N*-acetyllactosamine (repeats of Galβ1,4GlcNAcβ1,3) that can be capped with sialic acid and fucose. This poly-*N*-acetyllactosamine structure is the preferred ligand for galectins, a family of soluble conserved carbohydrate-binding proteins, forming galectin–glycan structures termed “lattices” (van Kooyk and Rabinovich, 2008). This molecular complex contributes to restrict the interactions between receptors and co-receptors on

immune cells regulating intracellular signaling pathways, apoptosis, proliferation and migration (Liu and Rabinovich, 2010). Galectins can be found in activated T and B cells, being significantly upregulated in activated macrophages and regulatory T cells (Treg) (Liu and Rabinovich, 2005; Rabinovich, et al., 2007). These C-type lectins have been implicated in a wide range of key biological processes including: regulation of host-pathogen interactions; innate and adaptive immune responses; acute and chronic inflammation, and immune tolerance (Sundblad, et al., 2017).

Interestingly, galectins family can play an opposite role in immune response as they can act as negative or positive regulators of T cell immune response. Galectin 1 and 3 are known to suppress inflammation and T cell response (Chung, et al., 2000; Demetriou, et al., 2001; Toscano, et al., 2007). Galectin 1 is described to negatively regulate Th1 and Th17 effector cells by inducing cell death (Toscano, et al., 2007). Galectin 3 has been described to limit TCR clustering due to lattice formation in MGAT5-expressing cells controlling thereby the threshold of T cell activation (Chen, et al., 2009b; Demetriou, et al., 2001). Similarly, Galectin 2 also exhibits a suppressive effect by inducing apoptosis of lamina propria T lymphocytes attenuating acute and chronic mouse colitis (Paclik, et al., 2008). In contrast, galectin-8 and galectin-4 act in opposite way. When binding to T cells, galectin-8 promotes T-cell proliferation, possibly through unique interactions with CD45 (Tribulatti, et al., 2009).Galectin-4 mediates $CD4^+$ T cells stimulation (by IL-6 production) leading to exacerbation of T cell- mediated chronic colitis (Hokama, et al., 2004).

Glycans can also function as a “sweet escape” strategy used by many prokaryotic and eukaryotic pathogens (including meningococci, *Trypanosoma* and *Helicobacter*). These microorganisms can synthesize terminal glycan structures that are similar to those found in mammalian cells in a process of “molecular mimicry”. This “glycan escape” strategy contributes to hide from host immune system and thereby triggering pro-inflammatory responses (van Die and Cummings, 2010; van Kooyk and Rabinovich, 2008).

Taken together, the integration of glycans in the regulatory networks that govern both innate and adaptive immune response, translating these glycan-circuits in clinical implications is an emerging topic in immunology. In fact, glycans act as a pivotal molecular interface in intestinal mucosa that bidirectionally regulate the cycle of transmitted signals from microbiota via glycans-mediated pathogen recognition to IECs and to immune cells, as well as the cycle of transmitted signals from glycans-dependent regulation of immune cells to IECs that are further converted into signals that regulate the composition of gut microbiota (Figure2) (Goto, et al., 2016).

4. Glycosylation alterations in Inflammatory Bowel Disease: a mechanism involved in pathogenesis.

A compelling body of evidences have been pointing toward the importance of complex branched N-glycans catalyzed by GnT-V in controlling T cell activity and functions in different immune-mediated disorders as demonstrated in mouse models of Multiple sclerosis (MS) and Type I diabetes (Demetriou, et al., 2001; Grigorian, et al., 2007; Lau, et al., 2007). IBD is characterized by a massive infiltration of inflammatory cells in intestinal mucosa but the mechanisms underlying this excessive stimulation of the immune system with loss of immune tolerance remains largely unknown and thereby the disease is still incurable.

Glycans have been shown to have an instrumental role in IBD pathogenesis. Particularly, the GnT-V-mediated branched glycosylation of intestinal T cells was found to play a role in UC pathogenesis. Patients with UC exhibit, at the level of intestinal mucosa, a deficiency in the expression of branched N-glycans on the TCR that was associated with disease severity. This decreased expression of branched glycans in UC patients comparing with healthy controls was demonstrated to be due to a reduced transcription of *MGAT5* glycogene in intestinal T lymphocytes (Dias, et al., 2014).

Nevertheless, whether this dysregulation of *MGAT5*/GnT-V-mediated glycosylation on T cells is a cause or consequence of intestinal inflammation in UC remains unclear. Evidences suggest that GnT-V-mediated β1,6GlcNAc N-glycans are implicated in the development of Th2 over Th1 responses (Morgan, et al., 2004). Recently, it was demonstrated that branching N-glycans potentiate the differentiation of induced Treg cells over Th17 differentiation (Araujo, et al., 2017).

Moreover, in other models of immune-mediated disorders such as MS (that can constitute models for IBD understanding) an association between environmental factors (sunlight/vitamin D_3 and metabolism) combined with multiple genetic variants (*IL17RA, IL2RA, MGAT1* and *CTLA-4*) was described to dysregulate Golgi N-glycosylation with impact in disease development and severity (Mkhikian, et al., 2011). Accordingly, GWAS studies revealed that *MGAT5* polymorphisms cooperatively with *MGAT1* and interleukin-2 and 7 receptor variants were associated with susceptibility to multiple sclerosis (Brynedal, et al., 2010; Li, et al., 2013). Preliminary evidences point towards a promising association between *MGAT5* genetic variants and IBD severity (Salomé S. Pinho *et al*, unpublished) which support the prominent role of *MGAT5* glycogene and other related genes involved in the branched glycosylation pathway for determining genetic susceptibility to immune-mediated disorders.

Additionally, DNA methylation has been demonstrated to have a physiological role on IBD development, mediating genetic risk in IBD (Howell, et al., 2017; Kraiczy, et al., 2016; Ventham, et al., 2016). The DNA methylation and EWAS (Epigenome-wide association studies) have been providing novel insights in complex diseases (Callaway, 2014) and will certainly contribute to clarify how epigenetic alterations of key glycogenes in a tissue/cell-specific manner are implicated in IBD pathogenesis.

In summary, and taking into account the multifactorial nature of autoimmune disorders such as IBD, MS and type I diabetes, it is very likely that the integration, in a common pathway, of genetic factors (including glycogenes), environmental triggers and specific glycans modifications (both in microbiota, epithelial cells and in immune cells) will contribute to gain novel insights into the mechanisms underlying the global loss of immune tolerance that characterizes autoimmune disorders (Figure 1).

The rapid advance of cutting edge (glyco)technological approaches that are decoding the human glycome (Holst, et al., 2016; Rillahan and Paulson, 2011; Smith and Cummings, 2013) will be an asset to disclose how glycans impact in immunity and autoimmunity, paving the way for the development of optimized (glyco)biomarkers and novel glycan-based therapeutic approaches.

5. Glycans as immunomodulatory agents in the control of T cell-mediated immune response in IBD.

The current IBD therapeutic strategies are limited by reduced effectiveness, high costs, and/or presence of toxic/side effects (Plevy and Targan, 2011), highlighting the need of novel and targeted-specific therapeutic strategies.

Interestingly, the metabolic supplementation of mice models of Experimental autoimmune encephalomyelitis (EAE) and type I non-obese diabetic mice and T cells with UDP-GlcNAc resulted in the enhancement of branching N-glycans on T cells by GnT-V activity, which increased the threshold for T cell activation, suppressing T cell growth and inhibiting Th1 differentiation which led to a controlled immune response (Grigorian, et al., 2011; Grigorian, et al., 2007). Interestingly, 17 years ago, oral N-Acetylglucosamine (GlcNAc) was described to promote intestinal lining through mucus production in children with severe treatment-resistant IBD in which 8 out of 12 children studied went into clinical remission (Salvatore, et al., 2000). Evidences from our group, both *ex vivo* (in human intestinal T cells from UC patients) and *in vivo* (in mouse models of colitis) have been demonstrating that GlcNAc supplementation is actively acting as an immunomodulatory agent by leading to suppression of T cell growth, inhibition of Th1/Th17 immune response

and by inhibiting TCR signaling (Dias, et al., 2017) (Ana M. Dias and Salomé S. Pinho, In revision), which set the ground to test the therapeutic effects of GlcNAc in IBD clinical trial (ongoing).

Accordingly, in MS, an ongoing pilot study using low-dose oral GlcNAc (3 g/day) revealed increased serum GlcNAc levels and branching N-glycans in T cells (Araujo, et al., 2017).

The immunomodulatory effects of glycans (such as the complex branched N-glycans), place them at the interface of inflammation and cancer, where they can exert dual roles in these pathological conditions, acting simultaneously as a "benefit" in the control of immune-mediated disorders through its immunosuppressive functions (Dias, et al., 2014), but also as a "threat" in cancer by contributing to malignant transformation and cancer aggressiveness (Carvalho, et al., 2016a; Carvalho, et al., 2016b; Pinho, et al., 2012a; Pinho, et al., 2012b; Pinho and Reis, 2015).

In the context of IBD, and taking into consideration that this disease is one of the three highest risk factors for colorectal cancer (CRC) after familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer syndrome (HNPCC) (Eaden, et al., 2001; Lutgens, et al., 2015), and that CRC accounts for 1/6 of ulcerative colitis-related deaths (Jess, et al., 2006), it is definitely worth explored how glycans impact in colitis-associated colorectal cancer.

6. GlycoMedicine in IBD: a near future?

The complex mechanisms underlying the pathogenesis of IBD explain the substantial heterogeneity of the disease with respect to disease onset, course, response to therapies and progression to complications (Cosnes, et al., 2011). Consequently, there is a major need in the field to identify novel molecular markers able to help on the identification of patients who are most likely to develop aggressive/complicated disease and carefully select them for appropriate therapy. This tailored approach will certainly improve the long-time course of the disease and the success of the therapeutic results (D'Haens, 2010).

Recent advances in IBD research have been contributing to the identification of genetic, serological, fecal and biopsy-based markers with relevant implications in the clinical decision. How glycans can integrate the clinical-algorithm of IBD by constituting reliable prognostic biomarkers as well as providing a source for the development of new therapeutic strategies are introduced and discussed in the following sections.

6.1. Glycans as a promising prognostic biomarker

6.1.1. *Genetic glycobiomarkers*

As mentioned above, glycosylation constitutes a key player in immunity. The contribution of glycans-encoding genes to IBD pathogenesis is far from being fully characterized. In fact, the emerging role of glycoconjugates in IBD pathogenesis gave rise to the creation of an online database called Glycosylation and Gut Associated Immune Tolerance (GlycoGAIT-https://apps.connexios.com/glycogait/), in which the profile of the glycome can be analyzed under different inflammatory conditions (Oommen, et al., 2016).

Genetic alterations in glycogenes have been associated with multiple primary immunodeficiency diseases (Lyons, et al., 2015). Accordingly, mouse genetics in which selected glycogenes are mutated revealed a remarkable impact in T cell development and thymus positive and negative selection (Zhou, et al., 2014). Interestingly, transgenic mice with overexpression of α1,2-fucosyltransferase (FUT1) spontaneously develop colitis, by influencing T-cell development and altering glycosylation of mucosal barrier (Brown, et al., 2004). Moreover, a GWAS of the human Immunoglobulin G (IgG) N-glycome was performed and 16 genes involved in IgG glycosylation were identified, in which 4 out of 16 encode glycosyltransferases (*ST6GAL1*, *B4GALT1*, *FUT8*, and *MGAT3*) and 5 genes were associated with IBD (*IKZF1, LAMB1, MGAT3, IL6ST* and *BACH2*) (Lauc, et al., 2013). Genetic variants associated with loss-of-function of *FUT2*, also demonstrated to increase susceptibility to CD by altering host-microbial interactions. Specifically, the non-secretor status of those patients, display an altered microbiome at both composition and functional levels potentially explaining the CD risk (McGovern, et al., 2010; Tong, et al., 2014). Additionally, *FUT3* polymorphisms were also associated with higher susceptibility of Southeast China individuals to UC (Hu, et al., 2016). Recent evidences also point toward the identification of genetic variants in the intronic region of *MGAT5* gene that are associated with UC severity and response to therapy (Márcia S. Pereira and Salomé S. Pinho, unpublished data).

Furthermore, genetic alterations in immuno-genes, associated with IBD, can indirectly lead to alterations on glycogenes, with a synergistic impact in the dysregulation of the immune system. The very early onset (VEO) – IBD can be caused by loss-of-function mutations of IL10 and IL10R. A point mutation in the intronic region of IL10R1 (only stimulated by IL10), observed in three VEO-IBD patients, impaired the mRNA splicing with consequences in the N-linked glycosylation, and thus with the anti-inflammatory signal mediated by IL10 (Murugan, et al., 2014). Interestingly, IL-22/IL-22RA1 signaling in intestinal organoids was shown to up-regulate glycosylation genes such as *FUT2*, *FUT8*,

SEC1 and *B4GALT1*, in which IL-22RA1/FUT2 axis was demonstrated to be involved in the maintenance of healthy microbiota (Pham, et al., 2014).

Taken together, glycogenes constitute important genetic determinants with functional impact in IBD pathogenesis and prognosis.

6.1.2. *Tissue specific glycobiomarkers*

The histological information obtained from colonic biopsies is routinely performed in the clinical practice helping IBD diagnosis (Canavese, et al., 2015), serving also to monitor mucosal inflammation and healing. Taking into consideration that a colonic biopsy is a snapshot of the inflammatory state of the patient, relevant *in situ* information can be further explored from biopsies. On this regard, the *in situ* glycosylation-signature, at the cellular and tissue-specific level is able to provide important molecular information that can be translated into clinical and prognostic value. Recent evidences have been showing that changes in the glycans signature of the intestinal lamina propria can be associated with the therapeutic outcome of the patients. At the time of diagnosis, low levels of branched N-glycans on intestinal inflammatory infiltrate are able to predict 75% of the patients that not respond to standard therapy, thus needing to step-up to biologics. The predictive capacity of this glycobiomarker was showed to be independent and improved when used together with C-reactive protein (CRP). Importantly, the best performance (best predictive capacity) of the glycobiomarker was observed in patients displaying a severe endoscopic Mayo subscore (Mayo 3) at diagnosis (Pereira, et al., 2017). These results are in accordance with previous evidences showing that, low levels of branching N-glycans on intestinal T lymphocytes are associated with severe disease phenotypes (Dias, et al., 2014).

Alterations in the O-glycans profile of the MUC2 from colonic biopsies are also associated with UC disease activity and severity (Larsson, et al., 2011). Active UC patients display an increase of short O-glycans (SialylTn (STn) – glycan 513) and a decrease of more complex O-glycans (glycan 1104) (Itzkowitz, et al., 1996; Larsson, et al., 2011).

Taken together, the determination of the glycosignature *in situ* provides a plethora of important biological information that can be translated in relevant molecular parameters with clinical and therapeutic applications.

6.1.3. *Glycans as non-invasive biomarkers*

Non-invasive biomarkers are attracting tools for monitoring disease activity and for predicting therapy response. Glycosylated proteins have been already demonstrated to be useful in the clinical decision (Lakatos, et al., 2011). For instance, fecal Lactoferrin is an N-glycosylated protein secreted by neutrophil-specific granules that act as selective anti-

microbial, and is able to distinguish active IBD from inactive IBD (Lakatos, et al., 2011; Masoodi, et al., 2009; Siqueiros-Cendon, et al., 2014). In IBD, specific antibodies are produced by the host immune system against specific bacterial glycans, such as the case of anti-glycans antibodies, including anti-Saccharomyces cerevisiae antibody (ASCA) that is used as a clinical marker in CD to differentiate the diagnosis from UC (Lakatos, et al., 2011; Lichtenstein and McGovern, 2016). Studies using GlycoChip microarrays showed the importance of the detection of serum anti-glycan antibodies for CD diagnosis and stratification (Dotan, et al., 2006; Seow, et al., 2009).

Another important non-invasive glycobiomarker that has been described to be associated with IBD is the glycosylation profile of serum IgG. In fact, IgG exhibits in the constant domain a single N-glycosylated site that can comprises hundreds of different glycoforms, generally resulting from the combination of the presence or absence of fucose, galactose, sialic acid and bisecting GlcNAc (Kiyoshi, et al., 2017). The analysis of IgG glycome comparing IBD patients and controls showed that IBD patients display a distinct pattern of IgG glycosylation that is characterized by a decrease in galactosylation in both UC and CD, decreased sialylation and increased bisecting GlcNAc in IgG glycans from CD patients (Trbojevic Akmacic, et al., 2015). The combination of agalactosyl IgG with ASCA further showed higher specificity in IBD diagnosis (Shinzaki, et al., 2013). Moreover, the analysis of IgG glycoprofile of 75 UC patients (Miyahara, et al., 2013) demonstrated that high sialylation in IgG was associated with higher disease activity. A ratio between the presence of bisecting GlcNAc in monogalactosylated structures with the absence of bisecting GlcNAc in these structures were associated with a poor prognosis of UC patients (Trbojevic Akmacic, et al., 2015).

In fact, alterations in the glycosylation of IgG are able to switch the phenotype of IgG from anti-inflammatory to pro-inflammatory. Loss of sialylation and low levels of galactosylation were associated with pro-inflammatory properties of IgG. Sialylation of fragment crystallizable (Fc) region reduces the affinity of IgG to activating Fc gamma receptors (FcγR, notably FcγRIII/IV, that are present in monocytes and macrophages) and also increase the expression of inhibiting FcγRIIB, promoting the anti-inflammatory action of IgG (Kaneko, et al., 2006). In addition, agalactosyl IgG was shown to enhance antibody-dependent phagocytosis (Nakajima, et al., 2011).

The determination of the glycosylation signature of human biological fluids, such as serum constitutes a non-invasive tool with promising prognostic value that is worth explored.

6.2. Glycans as an emerging therapeutic strategy in IBD

In the last years, an explosion of new targeted therapies arose to overcome the ineffectiveness of the standard therapy. The continuously discloser of the mechanisms behind IBD, gave rise to several therapies that target many proteins involved in immune response, such as TNF blockers, anti-adhesion molecules, cytokines inhibitors, JAK inhibitors, among others (Coskun, et al., 2017; Danese, et al., 2015). However, and given the heterogeneity of IBD in terms of disease course, progression to complication and therapy response, there are still a significant proportion of patients that remain refractory to therapy.

Glycosylation has been demonstrated to be an important step to consider in the design of effective therapeutic monoclonal antibodies (mAb) (Gomollon, 2014; Sha, et al., 2016). The quality of an antibody relies not only in the primary structure, charge and purity but also in the type, quantity and position of glycosylation. The Fc glycosylation influences the clearance rate, pharmacokinetic, pharmacodynamic and the binding to Fc receptors (Sola and Griebenow, 2010). Moreover, antigen-binding fragment (Fab) glycosylation also influences the binding to antigens and glycan binding proteins (Plomp, et al., 2016). Therefore, the efficacy of a therapeutic mAb is critically dependent on its glycosylation pattern. For example, a comparative study of biosimilars and biologics of anti-TNF that took into consideration its glycans profile support the relevance of glycans in determining efficacy and stability of the mAb (Lee, et al., 2017a).

The pharmaceutical industry has been using different hosts to produce mAb in order to promote glyco-heterogeneity of the antibodies. As described above, the altered pattern of glycosylation observed in the IgG of IBD patients influence their capacity to suppress inflammation. Accordingly, intravenous immunoglobulin (IVIG) administration containing a pool of serum IgG´s from thousands of healthy individuals are used as an infusion therapy to modulate the immune response in a variety of chronic inflammatory and autoimmune diseases (Schwab and Nimmerjahn, 2013). The de-glycosylated Fc from IVIG was shown to have no anti-inflammatory activity, since the terminal sialic acid of Fc revealed to play a key role in the immuno-modulatory effects of IgG (Kaneko, et al., 2006). IVIG, dependent on sialylated IgG glycovariants was demonstrated to result in Treg cell expansion, suppression of T cell activity by selective engagement of the type II Fc receptor SIGN-R1 (human DC-SIGN) (Fiebiger, et al., 2015). Moreover, IVIG therapy in mice with colitis was shown to reduce the intestinal inflammation by induction of IL-33 (Fiebiger, et al., 2015), supposedly through promotion of Treg cell expansion, since this regulation was already described (Schiering, et al., 2014). Additionally, sialylated IgG reduce antibody-dependent cell-mediated cytotoxicity (ADCC) by decreasing IgG affinity to FcγR, suggesting that sialic

acid in Fc promotes a conformational change in IgG. The administration of IVIG has been demonstrated to be beneficial in IBD treatment (Horton, et al., 2017). Despite being tolerated by the patients and reduce disease activity in refractory IBD patients, these therapy is not chosen as an option due to high cost comparing with standard therapy, however the cost is much less than biologics therapy (Rogosnitzky, et al., 2012).

The therapeutic benefits of glycans in the regulation of immune response are evident, as described above. Interestingly, glycomacropeptide (GMP) (Sawin, et al., 2015), which is released during cheese making, has been shown to exert immunomodulatory activities in some disorders such as IBD. This macropeptide is extensively O-glycosylated, and enriched with *N*-acetylneuraminic acid (sialic acid), galactose and N-acetylgalactosamine, and its oral administration in colitis-induced mice showed an improvement on the disease severity due to its anti-inflammatory effects (Lopez-Posadas, et al., 2010). Moreover, this glycomacropeptide also control gut microbiota (Sawin, et al., 2015), which can potentially act as a prebiotic agent in IBD.

In addition, the metabolic supplementation with glycans that are salvage into the hexosamine pathway, can also represent also a promising immunomodulatory therapeutic strategy for IBD (Dias, et al., 2017).

Overall, the specific effects of glycans in the control of immune response as well as in the regulation of microbiota content and functions highlight the clinical and therapeutic effects of glycans in controlling inflammation, particularly in IBD.

7. Concluding Remarks

Glycosylation is now considered an important “building block” of life. Glycans encode a huge diversity of different biological functions within a cell or an organism with remarkable implications both in homeostasis and in disease. Particularly, glycans are essential regulatory elements that fine tune the immune response. During the transition from normal to inflamed conditions, changes in the glycans repertoire occur and the aberrant expression of glycans integrates into the regulatory circuits underlying immune stimulation or immune inhibition.

In intestinal mucosa, the abundance and diversity of glycan structures that compose the glycocalix reveal its prominent role at the interface of host-pathogen interaction in which glycans can bi-directionally regulate microbiota content and function and the host immune response. This holistic implication of glycans in the gut homeostasis and intestinal immunity, place them as fundamental molecules with major implications in the pathogenesis of bowel diseases such as IBD or gastrointestinal cancer.

The detailed identification and characterization of the impact of aberrant expression of specific glycans will bring to light new mechanisms of intestinal inflammation and consequently new biomarkers of disease and novel targeted-specific therapeutic strategies, able to be included in the clinical and therapeutic algorithm of IBD patients.

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Figure legends

Figure 1

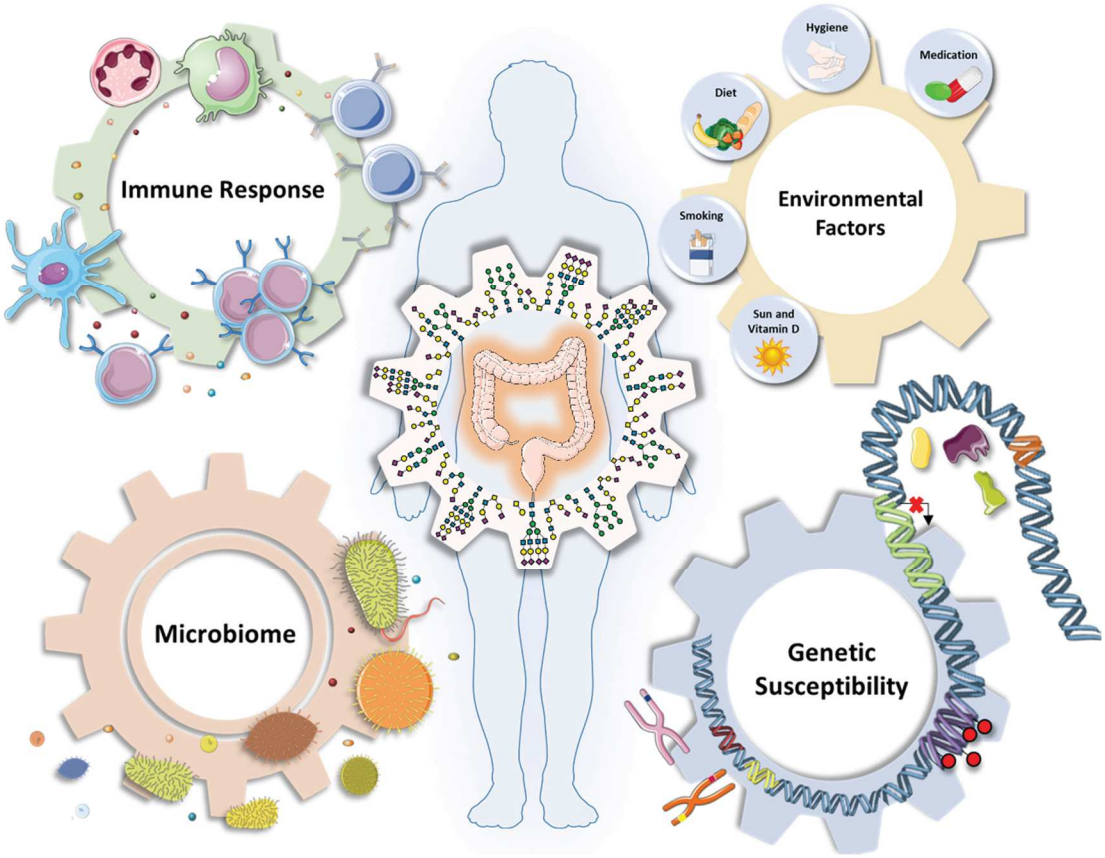

Figure 1. Glycans as a missing factor in IBD pathogenesis. IBD is a multifactorial disorder in which the combination of genetic factors, environmental conditions, alterations in the microbiome composition and functions as well as a dysregulated immune response are known to contribute to this chronic disease of the gastrointestinal tract. However this is not a perfectly matched puzzle and the precise pathogenesis of IBD remains to be fully understood and therefore the disease is still incurable. A compelling body of evidences suggests that glycosylation constitute a fundamental factor in IBD pathogenesis as glycans act at the interface of the four well-known risk factors of IBD. In fact, glycans and their binding partners are implicated in the regulation of both innate and adaptive immune responses that occur in IBD. The glycome composition of each individual can also be influenced by environmental factors such as diet, or medication. Many glycogenes displaying aberrant promoter methylation and/or deficient *cis*-regulation have been described to be associated with IBD-susceptibility locus. Therefore the host`s glycome alterations is a central component in IBD pathogenesis imposing new perspectives in IBD clinical management and targeted-specific therapies.

Figure 2

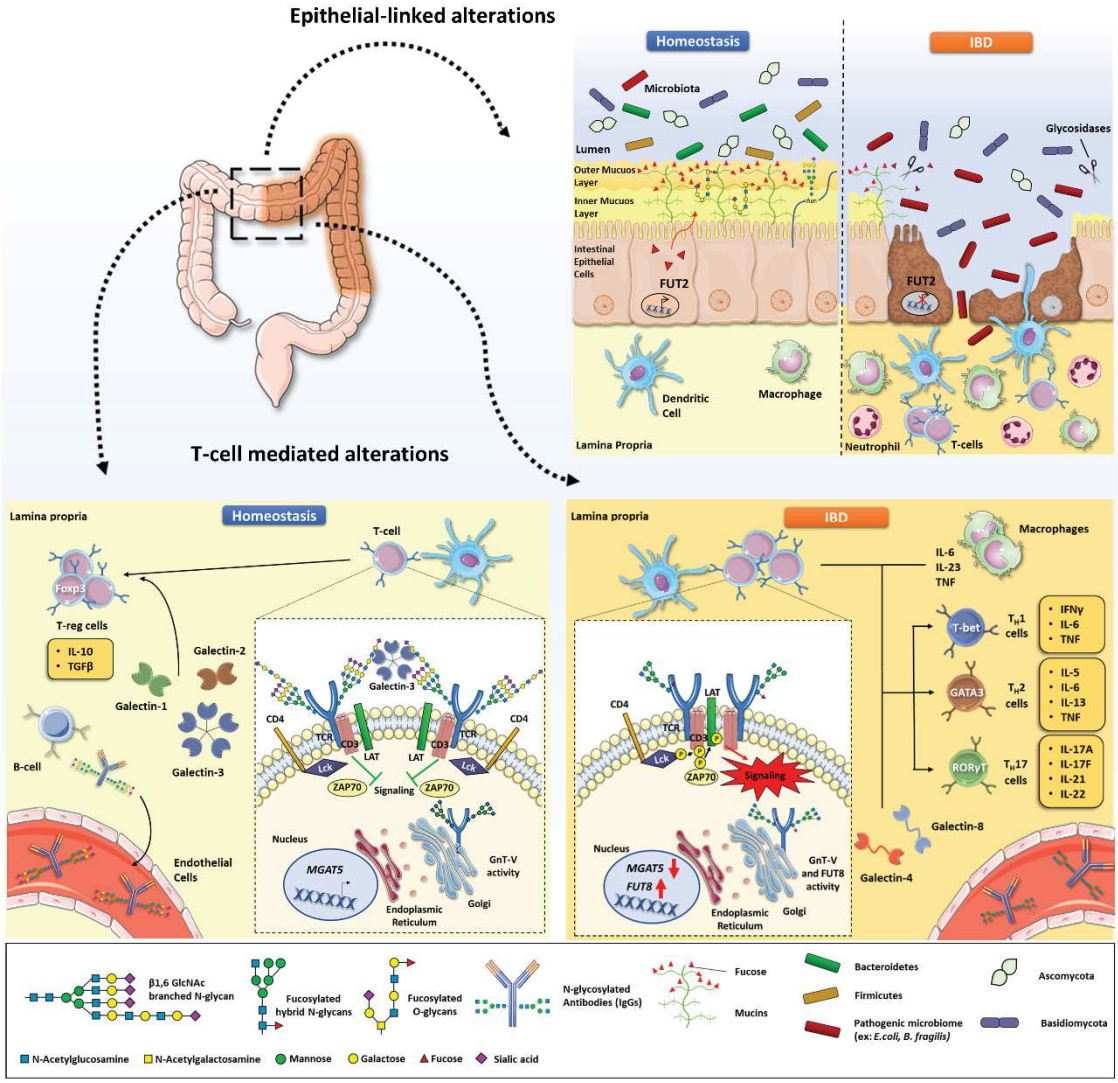

Figure 2. Impact of glycosylation in IBD pathogenesis. During the transition from normal to inflamed gut mucosa changes in the glycans repertoire occur. The aberrant expression of specific glycans that accompany intestinal inflammation impacts not only in the host´s immune response but also in the microbiota content and functions. Glycans composition namely in mucins are crucial to maintain the protective function of the intestinal barrier against pathogenic microbiota. Particularly, the α-1,2 fucosylation catalyzed by FUT2 in epithelial cells is crucial in maintaining commensal microbiota protecting against pathogenic microorganisms. The loss of mucosal integrity results in pathogens invasion concomitantly with a cascade of inflammatory events mediated by dendritic cells, macrophages, neutrophils and later by T cells. These pathogenic microorganisms can took advantages from host´s glycans, through glycosidades activity as strategies to “escape” recognition by host´s immune system. This selective process gives rise to dysbiosis that in

IBD is characterized by a decreased in microbiota diversity (less Firmicutes and more Proteobacteria) and a higher Fungi ratio of Basidiomycota/ Ascomycota.

At the level of lamina propria, glycosylation alterations are known to regulate T cell-mediated immune response. In homeostasis, the expression of β1,6 GlcNAc branched N-glycans, catalyzed by GnT-V is crucial to control T cell receptor (TCR) function and signaling. On this process, specific carbohydrate recognition proteins (C-type lectins) such as galectins (Galectin 3) recognizes the elongated chain (polylactosamine) of the branched N-glycans forming a "lattice" that precludes the TCR clustering and in this way control the threshold of T cell activation. Other galectins, such as Galectin 1 and Galectin 2, are also important in controlling T cell-mediated immune response towards a T regulatory response. Importantly, in homeostatic conditions, the profile of IgG glycosylation released in the serum has been described to exhibit predominantly galactosylated and terminal sialylated N-glycans in the Fc portion.

In IBD, specific alterations on the expression of glycans, particularly the upregulation of α1-6 fucosyltransferase (FUT8) with overexpression of core-fucose and the downregulation of MGAT5 gene with decreased expression of GnT-V-mediated branched N-glycans negatively impact in T cell response. This altered expression of N-glycans (branched and core-fucosylated) lead to T cell hyperactivation and signaling of intestinal T lymphocytes, being also associated with a T cell differentiation towards Th1 and Th17 immune responses. In addition, the expression of the C-type lectins, galectins 4 and 8 further stimulate T cell proliferation in colitis. Moreover, in IBD the expression profile of serum IgG is different from homeostasis, being predominantly characterized by agalatosylation.

Altogether, the glycosignature that accompany IBD pathogenesis provides a plethora of opportunities to understand the mechanisms underlying intestinal inflammation which will consequently improve the development of novel disease biomarkers and targets for new therapeutic strategies.

V

General discussion

In the last couple of years significant progresses in the understanding of IBD occurred. Namely the development of new therapeutic targets, the disclosure of new molecular mechanisms (Neurath, 2017), numerous clinical trials (Olivera, et al., 2017), and even the development and approval of biosimilars (FDA approved CT-P13 and ABP 501 as biosimilars for infliximab and adalimumab, respectively) (Ha and Kornbluth, 2018). However, as the incidence of IBD has been continuously increasing, it is now considered a global disease. The most updated review point out that IBD incidence has raised in the newly industrialized countries (Ng, et al., 2017), with 70,000 new cases diagnosed each year, and with approximately 80,000 children suffering from CD or UC (Colombel and Mahadevan, 2017).

In fact IBD places a heavy burden in young populations incurring in substantial healthcare and societal costs, impeding career aspirations with impact in patients' quality of life (Kaplan, 2015).

Taking into consideration the complexity of IBD pathogenesis, it is of paramount importance to identify risk factors as well as relevant environmental influences since "low risk" populations are witnessing an increasing of IBD incidence. Therefore, renewing efforts in unravelling the natural history of IBD is crucial to develop new avenues for therapeutic intervention and improvement of patient's outcomes. This doctoral thesis have contributed to improve IBD clinical management as this PhD project culminates with new insights on a new molecular mechanism, proposing an alternative target-specific therapy for IBD based in glycans.

Chapter II- Dysregulation of T cell receptor N-glycosylation: a molecular mechanism involved in ulcerative colitis.

Accumulating evidences have shown that GnT-V-mediated glycosylation plays a key role in regulating T cell activity in immune-mediated disorders (Demetriou, et al., 2001; Grigorian, et al., 2007; Lau, et al., 2007). TCR is a glycoprotein with at least 7 N-glycan sites on α and β subunits (Rudd, et al., 1999) and in homeostasis and self-tolerance, T cell activation (TCR signaling) induces up-regulation of *MGAT5* gene which in turns leads to GnT-V-mediated glycosylation of the TCR (Chen, et al., 2009). The branching N-glycans on TCR promote growth arrest of T cells by at least two

mechanisms: early, by raising T cell activation thresholds via limiting TCR clustering at the immune synapse and the consequent hyperimmune response, and later by increasing surface retention of growth inhibitory receptors such as CTLA-4 (Chen, et al., 2009). Together these evidences from other immune-mediated disorders set the ground to this project which intends to address whether the dysregulation of this critical interplay between N-glycan branching and T cell activity is a major contributory factor and a yet uncovered mechanism underlying UC.

Herein, we have demonstrated that GnT-V-mediated glycosylation of the TCR on lamina propria T cells is a new molecular mechanism underlying UC pathogenesis. We found a significant correlation between low expression levels of branched N-glycans in intestinal lymphocytic infiltrates and disease severity. Then, and taking into consideration that TCR signaling regulates multiple Golgi N-glycan branching-processing enzymes at the mRNA level (Chen, et al., 2009), such as *MGAT5*, we showed that the mRNA expression of glycogene *MGAT5* is reduced in intestinal lamina propria lymphocytes (LPLs) isolated from UC patients. No significant alterations were found on *MGAT3* glycogene (Dias, et al., 2014). The competition between branching N-glycans (by GnT-V) and bisecting (by GnT- III) known in cancer context (Pinho, et al., 2013) appears to be not observed at least in UC, however further studies are needed. Moreover, we further validate that this dysregulation of GnT-V-mediated branched N-glycans in T cells from UC patients also occurs at the level of GnT-V enzymatic activity (Ana M. Dias et al., under revision, in chapter III in this thesis).

Nevertheless, whether this dysregulation of MGAT5/GnT-V-mediated glycosylation on TCR is a cause or consequence of UC remains to be elucidated. It has been proposed that *MGAT5* is a gene that determines severity and susceptibility to MS (Brynedal, et al., 2010; Li, et al., 2013), including in a GWAS (Brynedal, et al., 2010). Future studies are warranted to clarify whether dysregulation *MGAT5* expression represents a common alterations determining autoimmune susceptibility together with other susceptibility loci raised with GWAS (Lees, et al., 2011).

These findings described in chapter II give rise to many other questions: what are the underlying mechanisms that could explain this *MGAT5* mRNA dysregulation? Epigenetics?; Does this relationship between deficiency of branched N-glycans with disease severity can help to identify patients' likely to develop severe forms of UC? ; Do T cells from normal adjacent mucosa display this deficiency in branched N-glycans? ; Can this deficiency in branched N-glycans in T cells be repaired representing thereby a new opportunity for IBD treatment?

Despite, DNA methylation demonstrates to have a physiological role on the IBD development being associated with genetic risk in IBD (Kraiczy, et al., 2016; Ventham, et al., 2016), so far, no major alterations on DNA methylation were found and thereby it does not represent the major cause of *MGAT5* gene downregulation in IBD (Ana M. Dias and Salomé S. Pinho, unpublished data) (results from training visit and collaboration with Prof. Vlatka Zoldos group in Zagreb, Croatia). In fact, DNA methylation and EWAS (Epigenome-wide association studies) are providing new insights in complex diseases (Callaway, 2014) and soon they may help to understand epigenetic alterations in different cell-types, particularly at mucosa level, on the different gut cell types and also explore how it may regulate or is regulated by post-translational modifications.

Interestingly, Demetriou and colleagues reported a direct environmental impact on genetic risk in MS. They demonstrated that the association between environmental factors (sunlight/vitamin D_3 and metabolism) combined with multiple genetic variants (*IL17RA*, *IL2RA*, *MGAT1* and *CTLA-4*) converge to dysregulate Golgi N-glycosylation and regulate disease development and severity (Mkhikian, et al., 2011).

On the other hand, N-glycan branching pathway includes at least 30 different genes, representing a major harbour of genetic variants which together with additional risk factors is likely to be an important hit on the discloser of autoimmune disorders, like MS, IBD and type I diabetes. Further studies are warranted.

We cannot discard the possibility that this *MGAT5* mRNA dysregulation can be mediated through a disease-dependent immune modulatory process. Critically, it is also not clear yet if this dysregulation is confined to T cells from the inflamed colon mucosa of UC patients or not.

Hence, two possibilities/ hypothesis arise: Is it confined to the inflamed colonic mucosa due to the inflammation? Or, Is it systemic meaning a general alteration of *MGAT5* gene (in blood, inflamed tissue and normal adjacent mucosa) that might be derive from genetic plus environmental causes?

In the near future, those questions may be easily elucidated with development of innovative glyco-approaches (Holst, et al., 2016; Rillahan and Paulson, 2011; Smith and Cummings, 2013) and others approaches like *in situ* RNA sequencing (Ke, et al., 2013) which I had the opportunity to perform and initiate the optimizations to apply it in UC samples, during my training visit in collaboration with Prof. Mats Nilsson and Prof. Carolina Wählby (Sweden).

Moreover, it remains an open question whether this mechanism is also present in CD patients and we need other cohorts to further validate the association with UC and CD. In fact, this study was conducted in a restricted geographic population from Porto (Portugal) (Dias, et al., 2014), and another cohort (either national or international) needs

to be used to validate the association with UC pathogenesis. As in the case of *NOD2* gene (specific marker of CD but not representative of all geographic regions in the world), dysregulation of *MGAT5* gene expression can possibly be restricted to a geographic area and associated only to UC. Nonetheless, all these findings/observations can be considered a step forward on the understanding of IBD pathogenesis, representing a major contribution for IBD clinical management, namely by improving the development of novel biomarkers that may help the determination of prognosis and also improve the patients' stratification for appropriate treatment.

Undoubtedly, the disclosure of this new molecular mechanism in UC pathogenesis opens also new windows of opportunity to improve the target-specific therapy of UC patients. The findings in chapter II represented the basis of the research developed in chapter III.

Chapter III- Metabolic control of T cell immune response through glycans in Inflammatory Bowel Disease.

A compelling body of evidences have been pointing toward the importance of complex branched N-glycans catalyzed by GnT-V in controlling T cell activity and functions in immune-mediated disorders as demonstrated in mouse models of MS and Type I diabetes (Demetriou, et al., 2001; Grigorian, et al., 2007; Lau, et al., 2007). Moreover, from these models of IBD understanding, it was shown that metabolic supplementation of mice and T cells with UDP-GlcNAc enhances GlcNAc branching of T cell catalyzed by GnT-V which increases the threshold for T cell activation, suppresses T cell growth and inhibits Th1 differentiation leading to a controlled immune response and a decreased disease clinical severity in EAE and type I non-obese diabetic mice (Grigorian, et al., 2011; Grigorian, et al., 2007). Interestingly, 18 years ago, oral GlcNAc was described to promote intestinal lining through mucus production in children with severe treatment-resistant IBD. In this study, 8 out of the 12 children studied went into clinical remission (Salvatore, et al., 2000). However the specific therapeutic effect of GlcNAc was not understood at the time.

Therefore, in line with our findings shown in chapter II (Dias, et al., 2014), we investigated whether the mechanism described could be therapeutically targeted in IBD. Therefore, we conducted *ex vivo* and *in vivo* studies in order to evaluate the impact of GlcNAc supplementation in the regulation of T cell-mediated immune response. We

evaluated the effect of the supplementation with N-glycans in the adaptive immune response in purified mucosal T cells (obtained from fresh colonic biopsies) from UC patients with active disease.

Our results on *ex vivo* T cells cultures revealed that the supplementation with N-glycans is able to enhance the glycosylation of T cells, repairing the previously described deficiency on branched N-glycans in T lymphocytes (Dias, et al., 2014). The enhancement of N-glycosylation in T cells was validated by several techniques and by an innovative approach designated Imaging flow cytometry (which combines features of both conventional flow cytometry and fluorescence microscopy) that was specifically adapted to analyze the β1,6 GlcNAc branched N-glycans, on the membrane of T cells from IBD patients ((Dias, et al., 2016) in Appendix 3).

Moreover, we observed that increasing doses of GlcNAc resulted in a significant reduction of T cell proliferation, suppression of Th1 and Th17 response through decreasing the expression of the transcription factors, T-bet and RORγt and the respective cytokines production, TNF-α, INF-γ and IL17A. Downstream TCR signaling was also suppressed as observed by the reduction in phosphorylation levels of ZAP70 and LAT (Dias, et al., 2017).

Besides the TCR, the enhancement of branched N-glycosylation can also modify other receptors like the co-receptors CD4 and CD8 as well as the growth inhibitory receptor CTLA-4 (Demetriou, et al., 2001; Lau, et al., 2007; Morgan, et al., 2004) . Moreover, CD45 and CD25 are also potential targets of branched glycosylation modification that can further contribute to the regulation of T cell-mediated immune response through branching N-glycans (Araujo, et al., 2017; Chen, et al., 2007).

In our *in vivo* approach, we took advantage of transgenic mice with different glycosylation profiles and we induced colitis to study the impact of glycans in the control of disease severity and disease progression. Interestingly, our *in vivo* data reveal that mice with colitis treated with GlcNAc exhibited a suppression of disease severity and a delay in disease progression as demonstrated by low disease activity index (DAI) and suppression of Th1 immune response in the gut (Dias, et al., 2017).

Importantly, it would be also important to further explore the therapeutic effect of GlcNAc in other systems (like organoids or explants cultures) where the consequences of the uptake by other cells like intestinal epithelial cells would be investigated. Taking into account that GlcNAc treatment improves mucosal lining, it seems that GlcNAc helps in maintaining mucins layer on the epithelial barrier. Hence, it is worth exploring on GlcNAc treatment context, like this possible crosstalk between epithelial and immune cells (T cells and others) which deserves to be deeply investigated.

Furthermore, it would be interesting to explore other mouse models in order to validate that the immunomodulatory effect by GlcNAc treatment is specifically mediated by T cells. The T cells transfer mouse models or Cre-*loxP* mouse models (conditional inactivation of a target gene only in a selected cell population) (Kiesler, et al., 2015; Sharma and Zhu, 2014) would be two possible strategies to assess this important question.

On the other hand, the development of new strategies, other than the enemas tested in this study, to improve the local delivery of GlcNAc at the site of inflammation by implementing, for instance, the GlcNAc incorporation in "customized" nanoparticules with targeted effects, is an interesting strategy that is worth explored. Through this targeted approach we might direct the GlcNAc to those mucosal T cells, with TCR displaying lower branching, which would preferentially uptake the GlcNAc nanoparticles and presumably inducing a quicker and more stable immunomodulatory effect. Indeed, nanotechnology delivery systems of drugs to IBD treatment have been explored and tested in IBD patients. The main advantage is that we can decrease the number of administrations, get the desired pharmacologic effects with smaller doses and therefore allowing more stable and prolonged remissions (Viscido, et al., 2014).

Furthermore, and despite the several advantages of GlcNAc (natural compound, nontoxic, less expensive) for treating IBD patients, whether a single versus a combined administration of GlcNAc is complete effective remains to be explored. The use of GlcNAc as adjuvant/combination (COMBO) therapy with other existent IBD drugs has being now tested in the group. In this regard, we have very preliminary data indicating that a COMBO strategy with GlcNAc and other standard IBD therapy appear to be even more effective than the single therapy in reducing inflammation, through the control of the release of pro-inflammatory cytokines namely TNF-α and IL-6.

Certainly, the continuous improvements in the identification of new molecular mechanisms and consequently identification of new therapies are fundamental to develop, in the near future, a personalized therapy in IBD.

Overall, in chapter III, we demonstrate that enhancing the glycosylation of T cells resulted in a significant suppression of T cell mediated-immune response associated with the control of intestinal inflammation and suppression of disease severity and progression.

Importantly the association of pre-malignant disease like IBD and cancer deserves attention and future studies must address the overall impact of this specific glycans on the narrow/vulnerable line between *MGAT5* expression "benefit" in IBD (Dias, et al., 2014) and *MGAT5* expression "threat" to colorectal cancer (CRC) or other gastrointestinal related carcinomas (Carvalho, et al., 2016; Pinho, et al., 2012a; Pinho, et al., 2012b; Pinho and Reis, 2015).

Remarkably, in addition to the examples of glycosylation-based models of IBD listed in Table 3 (chapter I), this work propose now *MGAT5* null mice as a model of IBD susceptibility.

Chapter IV- Glycans as immunomodulators in inflammation: Inflammatory Bowel Disease as a model.

Lastly, the work presented in chapter II and chapter III was included in an updated review (chapter IV) summarizing the most updated evidences on the impact of glycans as key players in maintaining the equilibrium between homeostasis and exacerbation of immune responses.

In chapter IV, we discuss the pivotal role of glycans in mediating immune response, particularly in IBD, proposing glycans as the missing factor in IBD pathogenesis.

Evidences derived from the studies performed during this doctoral thesis and from other authors, namely Prof. Miyoshi's group, clearly demonstrate that, in IBD patients, there are two main glycosylation alterations in T cells; the upregulation of α1-6 fucosyltransferase (*FUT8*) (Fujii, et al., 2016) and the downregulation of branched N-glycans (by reduction of *MGAT5* gene and glycosyltranferase GnT-V) (Dias, et al., 2014) in IBD. Both alterations lead to a T cell hyperactivation (increasing of TCR signaling and pro-inflammatory cytokines production) on intestinal T lymphocytes from IBD patients, representing promising specific targets for new IBD therapies.

In chapter IV is highlighted the dynamic process, mediated by glycans, between transmitted signals of microbiota via pathogen recognition (by the innate immune system) to intestinal epithelial cells (IECs) and vice versa, immune cells transmitted signals to IECs and translate that into signals which regulate composition of gut microbiota. An elegant study demonstrated a clear example of this dynamic process (Goto, et al., 2014). At epithelial level, α-1,2 fucosylation by FUT2 in intestinal epithelial cells is crucial in maintaining commensal microbiota and protect against pathogenic microbiota. The α-1,2 fucose expression on ECs is controlled by ILC3s immune cells and luminal microbes. Basically, it was demonstrated that commensal bacteria, pathogenic bacteria and bacterial products (LPS) stimulate directly or indirectly (via gut DCs) ILC3s to produce IL-22 to promote epithelial FUT2 and to induce α-1,2 fucosylation on epithelial cells (Goto, et al., 2014).

In chapter IV is also described how the development of glyco-based approaches namely genetic glycobiomarkers, tissue specific glycobiomarkers and certain glycosylated proteins (as non-invasive biomarkers) have been essential in enhancing clinical decision making of IBD in the last years.

In conclusion, a compelling body of evidence pinpoint that glycans are on the road as attractive targets with a tremendous potential to help in improving the understanding of mechanisms underlying loss of immune tolerance that will catalyze the development of target-specific IBD therapies and new clinical biomarkers.

Concluding remarks

The main goal of the present doctoral thesis was to contribute to the disclosure of a new molecular mechanism in IBD pathogenesis, namely in UC and to purpose a new molecular target to improve IBD clinical management promoting the development of targeted-specific therapies. To achieve this main goal, we set specific aims from which resulted the following main conclusions.

1. Taking in consideration that in other autoimmune disorders T cell functions are modulated by N-glycosylation of the T cell receptor (TCR), we have explored whether a dysregulation of GnT-V-mediated branch glycosylation in T cells influences IBD pathogenesis.

In this doctoral thesis, we have shown that GnT-V-mediated glycosylation of the TCR on lamina propria T cells is a new molecular mechanism underlying UC pathogenesis. We found a correlation between disease severity and decreased expression of branched N-glycans in intestinal lymphocytic infiltrates. Furthermore, we found that the glycogene *MGAT5* mRNA expression is reduced in intestinal lamina propria lymphocytes (LPLs) isolated from UC patients with active disease compared with controls. These results support a genetic alteration of the *MGAT5* gene from intestinal T lymphocytes of UC patients, being the underlying event that promotes a dysregulated immune response through a disturbance in protein branched N-glycosylation catalyzed by GnT-V on intestinal TCR.

2. In line with the urgent need for target-specific therapies in IBD and following the disclosure of a dysregulation on TCR N-glycosylation as a new important factor in UC pathogenesis, we explored whether this deficiency can be repaired and whether it has an impact on the control of immune response in UC.

In this doctoral thesis, we found that metabolic supplementation with GlcNAc (precursor of hexosamine pathway) in *ex vivo* T cells, was able to enhance TCR glycosylation, repairing the previous identified deficiency on branched glycans in T lymphocytes. More important, we found that it induces a suppression of T cell function mainly via suppression of Th1 and Th17 responses. Additionally, our *in vivo* studies

provided the proof-of-concept of GlcNAc as a new immunomodulatory agent for IBD treatment. We demonstrated for the first time that deficiency in branched glycosylation in *MGAT5* null or heterozygous mice is associated with early onset disease and increased severity of colitis. Moreover, the GlcNAc treatment of *MGAT5* mice with severe colitis, particularly via enemas, exhibited a suppression of disease severity and a delay in disease progression as demonstrated by low disease activity index (DAI) and suppression of Th1 and Th17 immune response.

Overall, the present study contributed to ,***"Bridge Basic Research and Clinical Practice"*** improving IBD clinical management and paving the way for the development of novel target-specific therapies that ultimately will culminate in a better IBD patient quality of life, especially in young populations that are commonly affected.

Future perspectives

This multidisciplinary and translational work provided novel insights on the pivotal role of glycans in T cell-mediated immune response in IBD pathogenesis with impact in IBD treatment. The findings herein described, raised many other pertinent questions with clinical relevance (some of them ongoing in the group):

- The correlation of the deficiency of branched N-glycans in lamina propria T cells with UC disease severity can be further explored as a potential predictive biomarker to stratify patients accordingly with disease severity.

- The deep understanding of the mechanism(s) behind decreased expression of MGAT5 mRNA in T cells from UC patients may help to screen some possible genetic susceptibility factor in UC.

- With the advance of new cutting edge techniques in glycoproteomics remaining questions can be accurately assessed soon, such as: Do T cells from normal adjacent mucosa from UC have also this deficiency in branched N-glycans? Or is it similar to healthy controls? On this regard, we have initiated collaboration with Prof. Mats Nilsson and Prof. Carolina Wählby, and I have been conducting the first experiments to perform RNA *in situ* sequencing and investigate MGAT5 expression in normal adjacent mucosa and respective inflamed mucosa from UC patients.

- The herein described glycosylation dependent molecular mechanism and proposed therapy need to be tested in other UC cohorts. It would be also interesting to evaluate whether it is involved also in CD.

In summary, this doctoral thesis contributed to identify a mechanism dysregulated in autoimmune disorders like IBD which result in the development of a targeted-specific strategy of treatment that can be applied not only in IBD but it can also be tested in other autoimmune disorders. The results from this doctoral thesis gave a main contribution to support the development of an IBD clinical trial which is now ongoing (where I will be involved as study coordinator).

Overall, in the near future, GlcNAc therapeutic strategy might be an alternative IBD therapy, single or in combination. Most patients refractory to the current therapies might benefit from this less expensive, natural and non-toxic alternative.

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Appendixes

Appendix I

Dysregulation of T cell receptor N-glycosylation: a molecular mechanism involved in ulcerative colitis

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Supplementary Figures of Chapter II

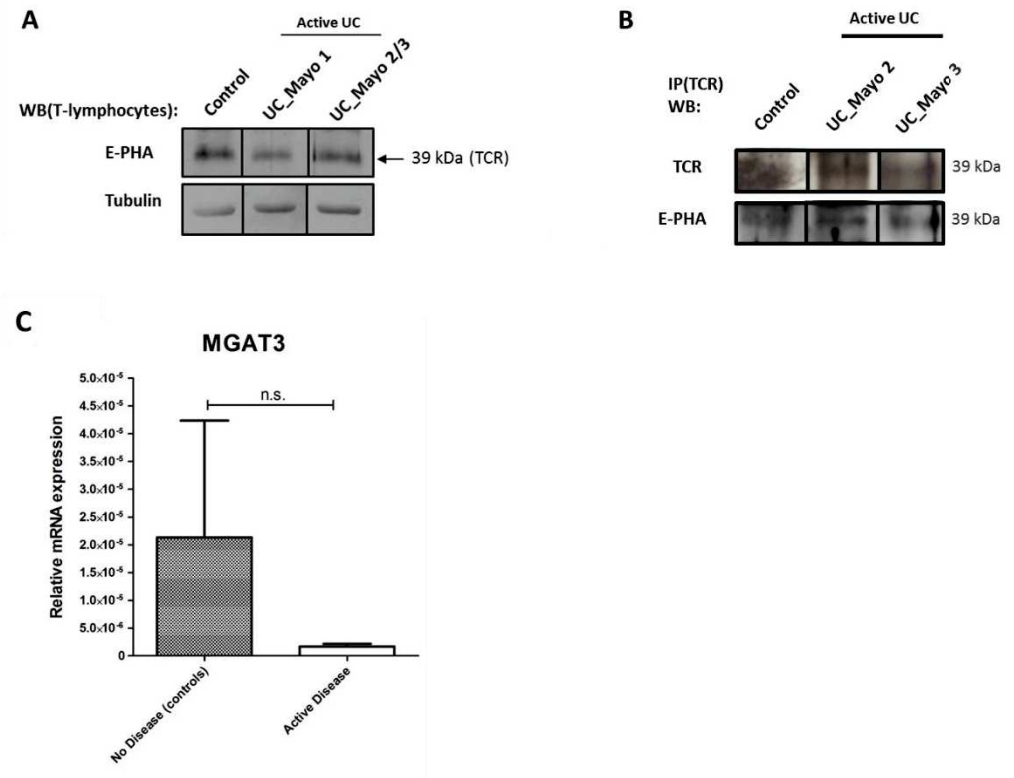
Supplementary Table S1: Relationship between L-PHA expression in intestinal lymphocytic infiltrate and the inflammatory activity.

Supplementary Fig.1: Levels of bisecting GlcNAc *N*-glycans expression and *MGAT3* gene expression in LPLs from UC patients and normal controls.

Supplementary Table S1 - Relationship between L-PHA expression in intestinal lymphocytic infiltrate and the inflammatory activity.

Clinical Features	Number of cases (n=64)	L-PHA expression (%) in intestinal lymphocytic infiltrate				
		< 25	25-50	50-75	> 75	*P value*
Inflammatory activity						**< 0,0001**
Control (No disease)	13	0	2 (15,39%)	10 (76,92%)	1 (7,69%)	
Inactive Disease (Mayo 0)	16	1 (6,25%)	3 (18,75%)	8 (50%)	4 (25%)	
Active						
Mayo 1	13	9 (69,23%)	3 (23,08%)	0	1 (7,69%)	
Mayo 2	14	13 (92,86%)	1 (7,14%)	0	0	
Mayo 3	8	8 (100%)	0	0	0	

Supplementary Fig.1



Supplementary Fig.1. Levels of bisecting GlcNAc *N*-glycans expression and *MGAT3* gene expression in LPLs from UC patients and normal controls. (**A**) Protein lysates from the isolated LPLs were subjected to E-PHA lectin blot in order to evaluate the expression levels of bisecting GlcNAc N-glycans on the TCR (39kDa). (**B**) Immunoprecipiation of TCR followed by bisecting GlcNAc structures recognition (E-PHA reactivity). The results in A and B demonstrate no significant alterations of the TCR glycosylation with bisecting GlcNAc structures comparing UC patients and normal controls. (**C**) qRT-PCR analysis for mRNA expression of *MGAT3* from intestinal LPLs from normal controls and active UC patients (Mayo subscore 2) showing no significant alterations. Results in (C) are an average of two independent experiments, performed in triplicate, using RNA from 2 independent normal controls and 2 independent active UC patients. The mRNA expression levels are expressed as mean ± SEM (Student's t-test: *P* = 0.45). Lanes in **A** and **B** were on the different gels (black lines).

Appendix II

Metabolic control of T cell immune response through glycans in Inflammatory Bowel Disease

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Supporting information of Chapter III

1- **SI Material and Methods**

2- **Supplementary Figures and Tables**

Supplementary Fig.1: Enhancing *ex vivo* the hexosamine biosynthetic pathway in T cells by metabolic supplementation with N-acetylglucosamine (GlcNAc).

Supplementary Fig.2: Upon GlcNAc treatment, N-glycosylation of T cells from controls and UC inactive disease remains unaltered.

Supplementary Fig.3: Enhancement of branched glycosylation on T cells from active UC patients is abolished by specific inhibitors of branching N-glycans synthesis.

Supplementary Fig.4: T cells from active UC patients display reduced GnT-V enzymatic activity comparing with healthy controls.

Supplementary Fig.5: Secreted cytokines in supernatants of *ex vivo* T cells from active UC patients.

Supplementary Fig.6: Inhibition of branching N-glycans synthesis abrogates the regulation of pro-inflammatory cytokines production.

Supplementary Fig.7: Impact of *ex vivo* GlcNAc treatment in cell apoptosis/death of T cells from control versus active UC patients.

Supplementary Fig.8: Evaluation of branched N-glycosylation on TCR of colonic T cells from DSS and TNBS-induced colitis mouse models.

Supplementary Fig.9: GlcNAc administration effects in different genotypes of MGAT5.

Supplementary Fig.10: Shaping the T cell-mediated immune response in IBD through metabolic enhancement of branched N-glycosylation. An opportunity for new therapeutic strategies.

Table S1: The table summarizes the list of antibodies used for staining by flow cytometry experiments.

1- SI Material and Methods

Patient's selection and colonic biopsies collection

The present study includes fresh colonic biopsies obtained from 75 patients (including 3 patients in remission stage) diagnosed with UC that underwent scheduled colonoscopy (between 2014 and 2017) at the Gastroenterology Department of Centro Hospitalar do Porto- Hospital de Santo António (CHP-HSA), Porto, Portugal. Fresh colonic biopsies (5-10 biopsies per patient) were representative of macroscopically active disease topography, as defined by Mayo endoscopic score (41) and intestinal $CD3^+$ T cells were purified. Blood was also collected for T cell isolation at the time of colonoscopy.

The eligibility criteria for inclusion in this study were UC patients with inaugural disease (naïve, without therapy) or with standard 5ASA therapy with no history of human immunodeficiency virus infection or cancer.

Normal controls (n=3) are represented by individuals that attend the gastroenterology department of CHP-HSA for a planned colonoscopy (no history of IBD or cancer).

All specimens were subjected to histological examination and classification. All participants gave informed consent about all clinical procedures and research protocols were approved by the ethics committee of CHP/HSA, Portugal (233/12(179- DEFI/177-CES).

Isolation of $CD3^+$ T cells from fresh colonic biopsies and blood of active UC patients. *Ex vivo* culture of T cells.

Colonic biopsies from controls, inactive and active UC patients were mechanically dissociated to prepare single cell suspensions using the Hanks' Balanced Salt solution Modified medium, without calcium chloride and magnesium sulfate (HBSS) (Sigma) with Penicilin/Streptomycin and Gentamicin. Peripheral Blood Mononuclear Cells (PBMCs) were obtained by density gradient centrifugation using Lymphoprep.

$CD3^+$ T cells (from biopsies and blood) were magnetically sorted by using the EasySep™ Human T Cell Enrichment Kit (STEMCELL) following the manufacturer's instructions. $CD3^+$ T cells (2×10^4) were cultured for 72h in 96-well round bottom plates with plate-bound anti-CD3 mAb (clone OKT3) (0,5µg/ml) and soluble anti-CD28 (clone CD28.2) mAb (0,5µg/ml) (eBioscience). *N*-acetylglucosamine (GlcNAc) (Sigma and Wellesley Therapeutics Inc) was added to the T cells cultures (0, 40, 80, 100mM). Kifunensine (10µm) and Swainsonine (500nM) (both from Sigma) were used as inhibitors of N-glycosylation,

specifically inhibits the enzymes mannosidase I (α-MAN I) and mannosidase II (α-MAN II), respectively (Supplementary Fig.1a). D-Mannose (80, 100 mM) (Sigma).

Imaging Flow Cytometry

Imaging flow cytometry analysis was performed as previously described (42) to assess co-localization of the TCRα/β$^+$ with L-PHA on T cells cultured for 72h under T cell-stimulation and different concentrations of GlcNAc. Data acquisition was performed in ImageStreamX (Amnis, Millipore). Data analysis was performed using IDEAS 5.0 software (Amnis, EMD Millipore), to determine the percentage of TCR$^+$ cells, mean bright detail similarity and mean fluorescence intensity of L-PHA at the cell membrane by creating a specific mask to evaluate fluorescence at the cell membrane.

Flow cytometry

CD3$^+$ T cells were resuspended in PBS containing 10mM sodium azide and 2%BSA and incubated for 30 min at 4°C with specific conjugated antibodies (detailed Table S1) and for β1,6-GlcNAc branched N-glycans detection, fluorescein isothiocyanate (FITC)-conjugated L-PHA (Vector Lab) (2μg/ml). Results were expressed as percentage (%) of cells that stained positively for CD4 and CD8, as well as the MFI due to L-PHA staining within each T cell population.

For cell surface and intracellular antigens used are indicated in TableS1. Surface-stained cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer's instructions. Fluorescence minus one stainings and isotype controls (eBioscience) were included for each assessed transcription factor.

For cytokine intracellular staining, CD3$^+$ T cells, cultured as described above for 72 h with anti-CD3 and anti-CD28, were washed with fresh medium and further incubated for 3 h at 37 °C in complete RPMI medium containing 500 ng/mL of ionomycin, 50 ng/mL PMA and 10 μg/mL of Brefeldin A (all from Sigma). Cells were stained with APC-eFluor® 780 Fixable viability dye before being surface stained with PerCP Cy 5.5 anti-human CD4 (clone RPA-T4) (Biolegend). Cells were then fixed with 2% formaldehyde, washed, permeabilized with 0.5% saponin (Sigma) and pre-incubated with 2% mouse serum before intracellular staining with human antibodies for TNF-α and IFN-γ (details in Table S1) or respective isotype controls (all from eBioscience).

In all flow cytometry experiments, dead cells were excluded with Fixable Viability Dye APC-eFluor® 780-conjugated (eBioscience).

Data acquisition was performed on a FACSCanto™ II system (BD Biosciences, San Jose, CA) using the FACSDiva™ software (BD) and compensated and analysed in FlowJo version 10.4. (Tree Star, Inc., Ashland, OR). Doublets were excluded from the analysis based on FSC-A versus FSC-H parameters. Fluorescence minus one gating was used to define the gates for cytokine-producing cells. Isotype controls were used to evaluate unspecific staining.

Proliferation assay

For T cell proliferation assays, $CD3^+$ cells were purified from colonic biopsies of naïve patients (with inaugural disease) and 5-(and-6)- carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled using the CellTrace CFSE Cell Proliferation Kit (Molecular Probes, Invitrogen), as previously described (43), prior to culture. Dead cells were excluded based on propidium iodide incorporation. Acquisition was performed in an EPICS XL cytometer (Beckman-Coulter Corporation) and data were analyzed (always gating in live cells) using FlowJo software.

Cytokine production

Supernatants from colonic T cell cultures (treated and untreated) were analyzed by flow cytometry using the BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit (BD) following the manufacturer's instructions. Sample acquisition was performed in BD FACS Canto™ II flow cytometer (Becton Dickinson) and data were analyzed using the Flow Cytometric Analysis Program (FCAP) Array™ software. Human TGF-β1 was quantified in the concentrated culture supernatants by using the ELISA kits (R&D systems), according to manufacturer's instructions.

The supernatants from mouse colonic explant cultures were concentrated 15 fold by using Amicon® Ultra-2 mL Centrifugal Filters (Merck Millipore, Billerica, MA), according to manufacturer's instructions. The levels of the cytokines IFN-γ and IL-17A were quantified in the concentrated culture supernatants by using the respective anti-mouse Ready-Set-Go!® ELISA kits (eBioscience), according to manufacturer's instructions. The levels of the cytokines TNF-α and IL-6 were quantified in the concentrated culture supernatants by using the respective anti-mouse (Biolegend), according to manufacturer's instructions.

Western-blot and TCR signaling

TCR signaling was evaluated by assessing the phosphorylation levels of LAT and ZAP70. After 72h of *ex vivo* T cell culture in absence or presence of GlcNAc lysates were extracted using RIPA buffer. Twenty µg of protein lysates were separated by 12% SDS–PAGE electrophoresis and transferred onto nitrocellulose membranes (GE Healthcare, Life Sciences, UK). Membranes were blocked before incubation phospho-Zap-70 (Tyr319/Syk (Tyr352) rabbit mAb (1:500) and anti-phospho-LAT (Tyr191) rabbit mAb (1:500) (both from Cell Signaling Technologies, USA). Goat anti-rabbit IgG-HRP mAb (Santa Cruz Biotechnology, USA) was used as secondary antibody.

For L-PHA lectin blot analysis (44), 20µg of T cell protein lysates separated by 12% SDS-PAGE electrophoresis was performed as in previous methods(16) . For loading control analysis, mouse IgG anti-tubulin (Sigma) or rabbit IgG anti-actin (Santa Cruz Biotechnology,USA) was used. The target proteins were visualized using ECL reagent (GE Healthcare, Life Sciences). Positive reaction was observed in a band the same size as TCRβ (mouse monoclonal antibody anti-human, Santa Cruz Biotechnology, USA) (39kDa, accordingly with manufacturer antibody details).

Immunoprecipitation

For T cell receptor (TCR) immunoprecipitation (IP), equal amounts of total cell lysates (TCL) obtained from mouse colons or from *ex vivo* human T cell cultures (in absence or presence of GlcNAc) were treated as previously described(16). Positive reaction was observed in a band the same size as rabbit anti-human TCR β (39kDa) polyclonal antibody (Santa Cruz Biotechnology).

Glycophenotype

T cells were incubated with biotinylated L-PHA (2µg/ml), biotinylated LEL (recognizes poly-lactosamine structures) (1 µg/ml), biotinylated SNA (recognizes α2,6 sialic acid) (5µg/ml) or biotinylated MALII (recognizes α2,3 sialic acid) (10µg/ml) (Vector Labs, USA). Lectins were revealed with (FITC)-conjugated streptavidin. FITC-conjugated streptavidin alone was used as control. Propidium iodide (PI) was used to exclude dead cells. Data acquisition was performed in a FACS Canto^{TM} II flow cytometer. Data were analyzed using FlowJo software.

Apoptosis assays

Apoptotic cells were identified by flow cytometry using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences), following the manufacturer's instructions. Only cells staining positive with FITC-Annexin V and negative with propidium iodide (indicative of early apoptosis) were considered. Data acquisition was performed in a FACS Canto™ II flow cytometer. Data were analyzed using FlowJo software.

Enzymatic reaction and HPLC analysis

Blood and colonic T cells of UC patients and controls were prepared as described above to evaluate the enzymatic activity of GnT-V, using a previously described methodology (45).

DSS and TNBS-induced colitis and *in vivo* GlcNAc treatment

Intestinal inflammation was induced in both male and female C57BL/6 mice, MGAT5 wildtype (WT), heterozygous (*MGAT5*$^{+/-}$), knockout mice (*MGAT5*$^{-/-}$) (kindly provided by Prof. Michael Pierce, CCRC, Georgia University, Athens, USA) , (6 to 8 weeks old), at IPATIMUP/i3S's animal facility by treating *ad libitum* with 2% dextran sodium sulfate (DSS) (36,000–50,000 Da; MP Biomedicals) in the drinking water, as previously described (19). The 2,4,6-trinitrobenzene sulfonic acid (TNBS) model was also performed using male C57BL/6 mice (6 to 8 weeks old), following previously described protocols(19) and including the following groups (animals randomly distributed): Control Group_water (H_2O, via rectal), Control Group_ethanol (50% ethanol, via rectal) and Group TNBS (3% TNBS in 50% ethanol, via rectal).

All procedures involving animals and their care were conducted in conformity with institutional guidelines in compliance with national and International laws and policies on the protection of animals used for scientific purposes (Directive 2010/63/EU, Guide for the Care and Use of Laboratory Animal. Eight edition, 2011). All the procedures were approved by local and national committees.

Both colitis models showed a dysregulation of branched N-glycosylation on T cells in mice with colitis (Supplementary Fig. 8a-b). In this study we have chosen the DSS model of colitis because it does not interfere with the enema treatment strategy the as TNBS model, that imply a rectal induction, would originate biased results.

The therapeutic effects of GlcNAc administration were evaluated by treating the DSS mice with GlcNAc (Sigma and Wellesley Therapeutics Inc) after disease onset, either orally by supplementing the drinking water at 0.25 mg/ml (as previously described (12)) and/or rectally, by GlcNAc enemas at 0.5 mg/ml (using a catheter). Treatment was performed daily for 7 consecutive days (19).Animals were randomly distributed per group of treatment. Oral consumption was verified by measuring the amount of drinking water left over each treatment.

Mice were clinically evaluated daily and over the next 7 days upon GlcNAc treatment and scored in a blinded fashion as follows: body weight change (values of body weight are expressed as percentage of body weight on day 0) ; disease activity index (DAI), the mean of the following parameters: % weight; stool consistency and blood stool (46).

The evaluation of colitis onset and disease severity in mice with different *MGAT5* genotypes: C57BL/6 wild-type mice (n=14); *MGAT5*$^{+/-}$ (n=23) and *MGAT5*$^{-/-}$(n=11) was based on DAI score per animal per day. Active disease was defined when animals showed DAI ≥ 2 and three stages of severity were defined: mild (≥2 and < 2,5), moderate (≥2,5 and <3) and severe (≥3).

In both *in vivo* models (TNBS and DSS), lamina propria T lymphocytes (LPLs) were isolated from mice colon samples following previously described methods (16).

Tissue immunohistochemistry and immunofluorescence

Formalin-fixed paraffin-embedded (FFPE) colonic tissue slides prepared from the different group of animals were used for H&E staining to evaluate histopathological alterations, and for immunohistochemistry with L-PHA and anti-CD3 mAb, following a previously described protocol(16). The evaluation of the mucus layer was focused on glycoproteins and glycans modifications in proteins (such as TCR) not removed by FFPE processing(47). Immunohistochemistry to evaluate Foxp3 and F4/80 expression was evaluated as described in (48).

T-bet immunofluorescence, in colon sections, was performed using heat-induced antigen retrieval with EDTA (Sigma), blocking with goat normal serum (Dako, diluted) before incubation with mouse IgG_1 T-bet-specific mAb (clone 4B10, Santa Cruz, 1:50) overnight, at 4ºC. Then slides were incubated with goat anti-mouse Alexa 594 secondary antibody (Invitrogen, 1:250) following nuclear staining with DAPI (1:100). Immunofluorescent images were obtained using a Zeiss Imager.Z1 AxioCam MRm (Carl Zeiss).

Real -time PCR

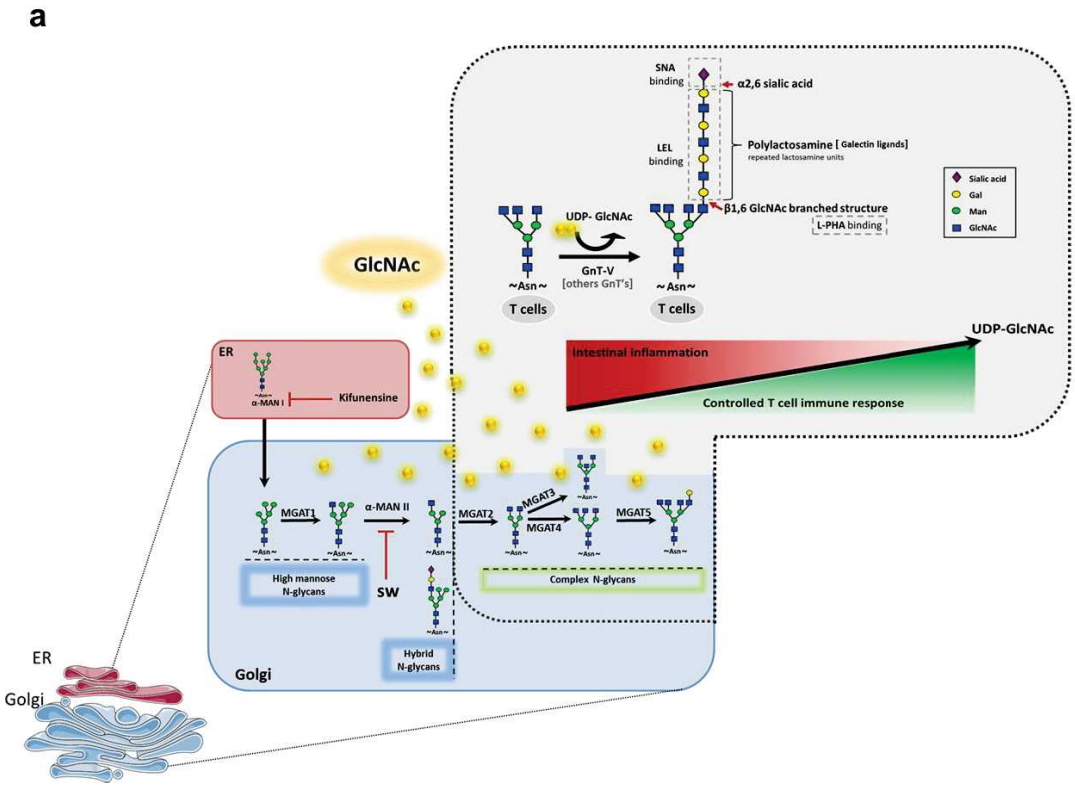
Total RNA from isolated LPLs was extracted and the quantitative real-time PCR (qRT-PCR) was performed using TaqMan Gene Expression Assays (Applied Biosystems) as previously described (16). qRT-PCR was carried out in triplicates using RNA source from intestinal LPLs from MGAT5 null mice controls (n=2) versus GlcNAc treated mice after DSS (n=2), for the target gene MGAT5b (Taqman probe: Mm01252571_m1, Applied Biosystems) and for the appropriated lymphocytes endogenous control 18S (Hs99999901_s1, Applied Biosystems).

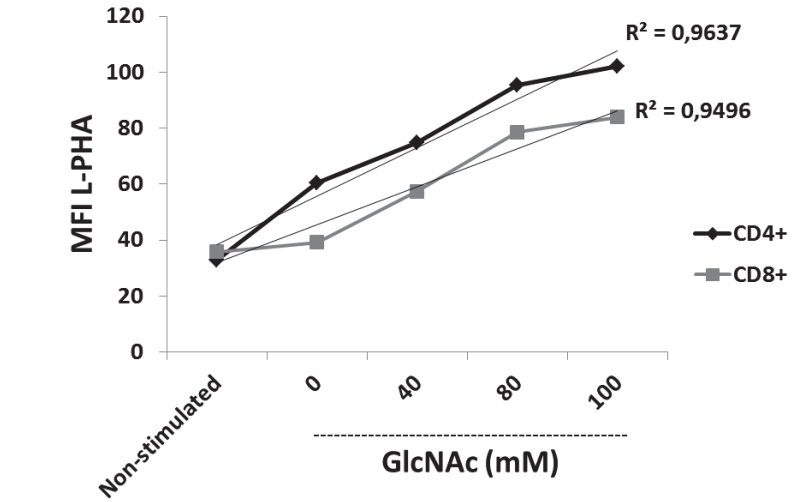
Statistical analysis

Statistical significance was assessed by one-way or two-way analysis of variance (ANOVA) using Bonferroni's, Dunnett's or Newman–Keuls multiple comparison post-tests and, where appropriate, by unpaired Student's t-test (two-tailed) using GraphPad Prism 5. *P* values of <0.05 were considered statistically significant.

In the *in vivo* studies, we have estimated the number of animals per genotype n >20, α= 0,05 and power >0,07. Statistical outliers were identified and excluded from the analysis, such as the animals that were euthanized when they reached the established humane endpoints.

Supplementary Fig.1





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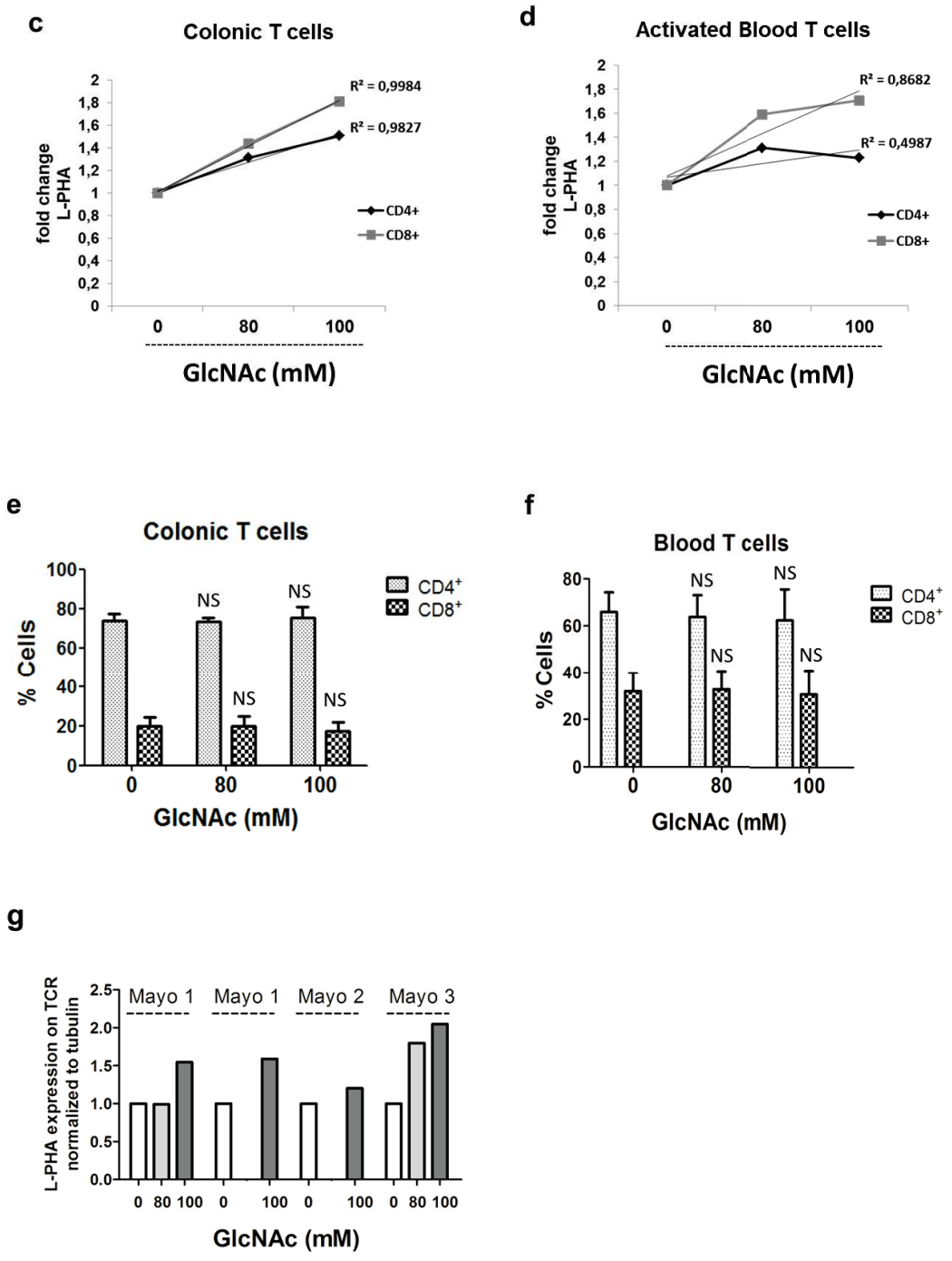
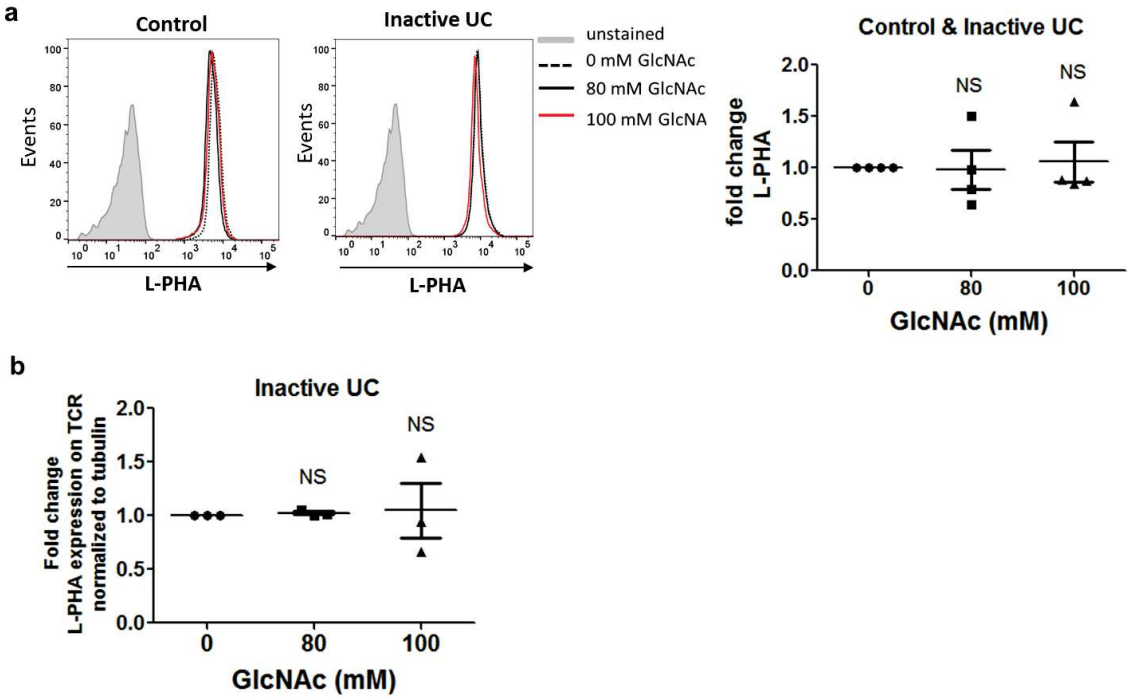


Supplementary Fig. 1. Enhancing *ex vivo* the hexosamine biosynthetic pathway in T cells by metabolic supplementation with N-acetylglucosamine (GlcNAc). (**a**) Schematic representation of T cells glycophenotype upon GlcNAc supplementation. In the canonical pathway, the GnT-V glycosyltransferase catalyzes the addition of β1,6-GlcNAc branched N-glycans (as detected by L-PHA lectin) on T cells that are further extended with polylactosamine structures (detected by plant lectin *Lycopersicon esculentum* agglutinin

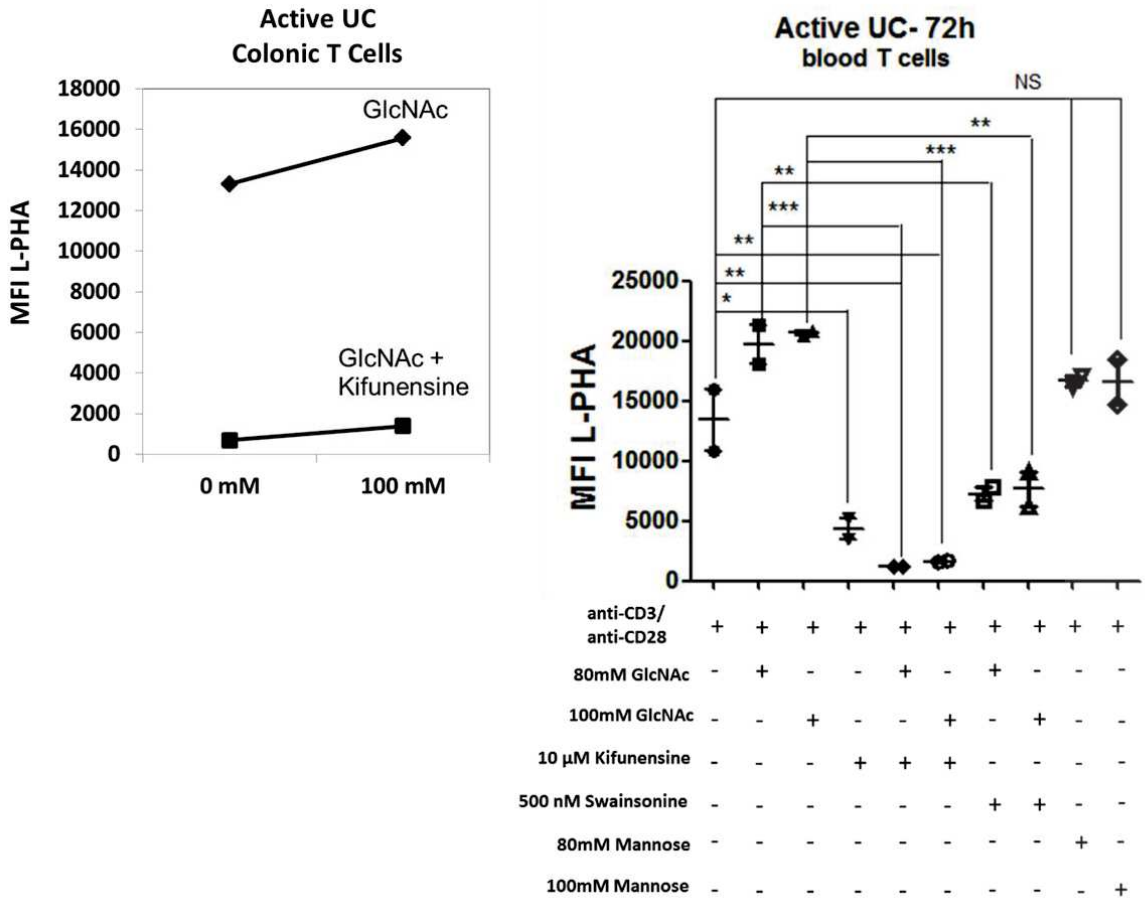
(LEL)). Additionally, our results demonstrate a trend increase in α2,6- linked sialic acid recognized by binding of *Sambucus nigra* agglutinin (SNA) and no significant alteration in α2,3- sialic acid residues. This glycosylation signature in T cells was shown to regulate the adaptive immune response. (**b**) $CD3^+$ T cells, magnetically sorted from blood of active UC patient were cultured for 72h under anti-CD3/CD28 mAb stimulation. Different concentrations (mM) of GlcNAc were added to cultures, as indicated. Fold change of L-PHA staining was determined by flow cytometry. (**c** and **d**) In $CD4^+$ and $CD8^+$ T cell subsets, N-glycosylation was determined by co-staining with L-PHA (lectin that recognize β1,6-branched N-glycans) and evaluated by flow cytometry. Fold change of L-PHA staining of $CD4^+$ and $CD8^+$ T cells sorted from intestinal biopsies or from blood of UC patients were *ex vivo* cultured for 72h under anti-CD3/CD28 mAb stimulation, in the presence of GlcNAc at indicated concentrations. (**e** and **f**) GlcNAc treatment does not affect the proportion of the T cell subsets. Results are presented as mean ± SEM of two to four independent experiments. (**g**) Fold change of L-PHA expression on a protein band corresponding to TCRβ migration profile normalized to tubulin. Results represent 4 biological replicates, using T cells from patients at different stages of disease severity (Mayo 1 (n=2), Mayo 2 (n=1) and Mayo 3 (n=1)). Results are normalized to untreated that was taken as 1.

Supplementary Fig.2



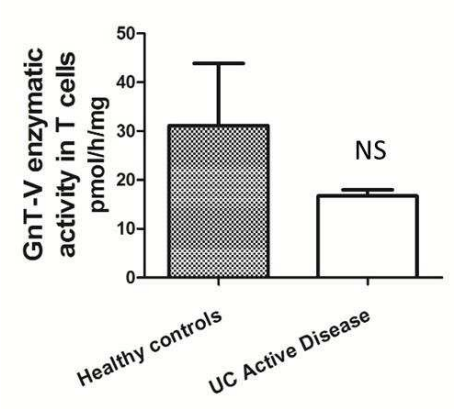
Supplementary Fig. 2. Upon GlcNAc treatment, N-glycosylation of T cells from controls and UC inactive disease remains unaltered. (**a**) T cell N-glycosylation was determined by staining with L-PHA (lectin that recognize β1,6-branched N-glycans) and evaluated by flow cytometry. Fold change of L-PHA staining on *ex vivo* T cells from UC activated blood T cells after GlcNAc supplementation. Results of healthy controls (n=3) and UC inactive disease (n=2) are presented as fold change of MFI values ± SEM. (**b**) Protein lysates from the *ex vivo* colonic T cells from inactive UC patients (n=3), after supplementation with GlcNAc, were subjected to L-PHA lectin blot in order to evaluate the expression levels of β1,6-GlcNAc branched N-glycans on a protein band with the size of the TCRβ. Quantification of L-PHA densities on TCRβ band normalized to tubulin, presented as fold change relative to the 0 mM Glc condition. No significant alterations were observed in the expression of β1,6-GlcNAc branched N-glycans in T cells from inactive UC patients.

Supplementary Fig.3



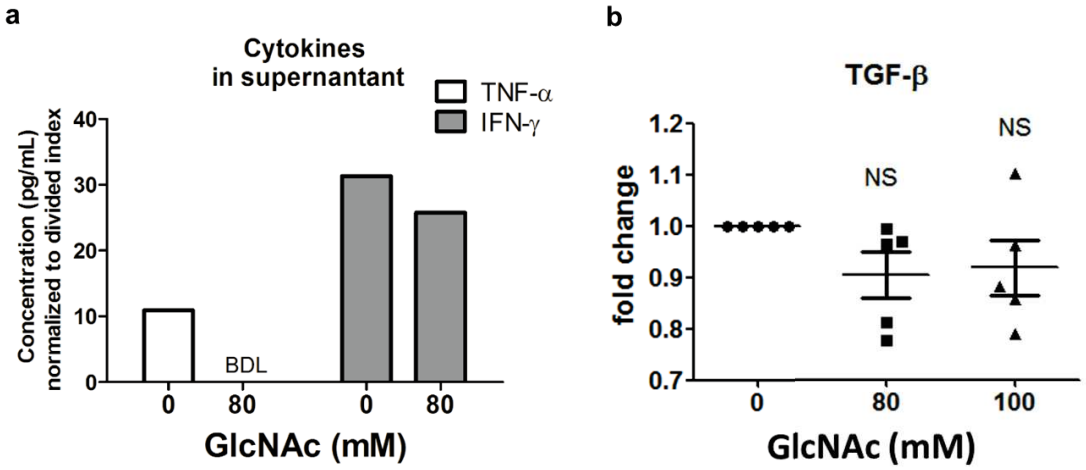
Supplementary Fig. 3. Enhancement of branched glycosylation on T cells from active UC patients is abolished by specific inhibitors of branching N-glycans synthesis. Graphs represent the effect of the treatment of T cells isolated from biopsies (n=1, left) and blood (n=2, right) of active UC patients with GlcNAc and the inhibitors of branching N-glycans synthesis, Kifunensine and Swainsonine as well as a different sugar, D-mannose.

Supplementary Fig. 4



Supplementary Fig. 4. T cells from active UC patients display reduced GnT-V enzymatic activity comparing with healthy controls. (**a**) Evaluation of the enzymatic activity of GnT-V assessed in a pool of lysates from colonic T cells obtained from different healthy individuals (n=2) and different active UC patients (n=2) and analyzed in two independent technical replicates.

Supplementary Fig. 5

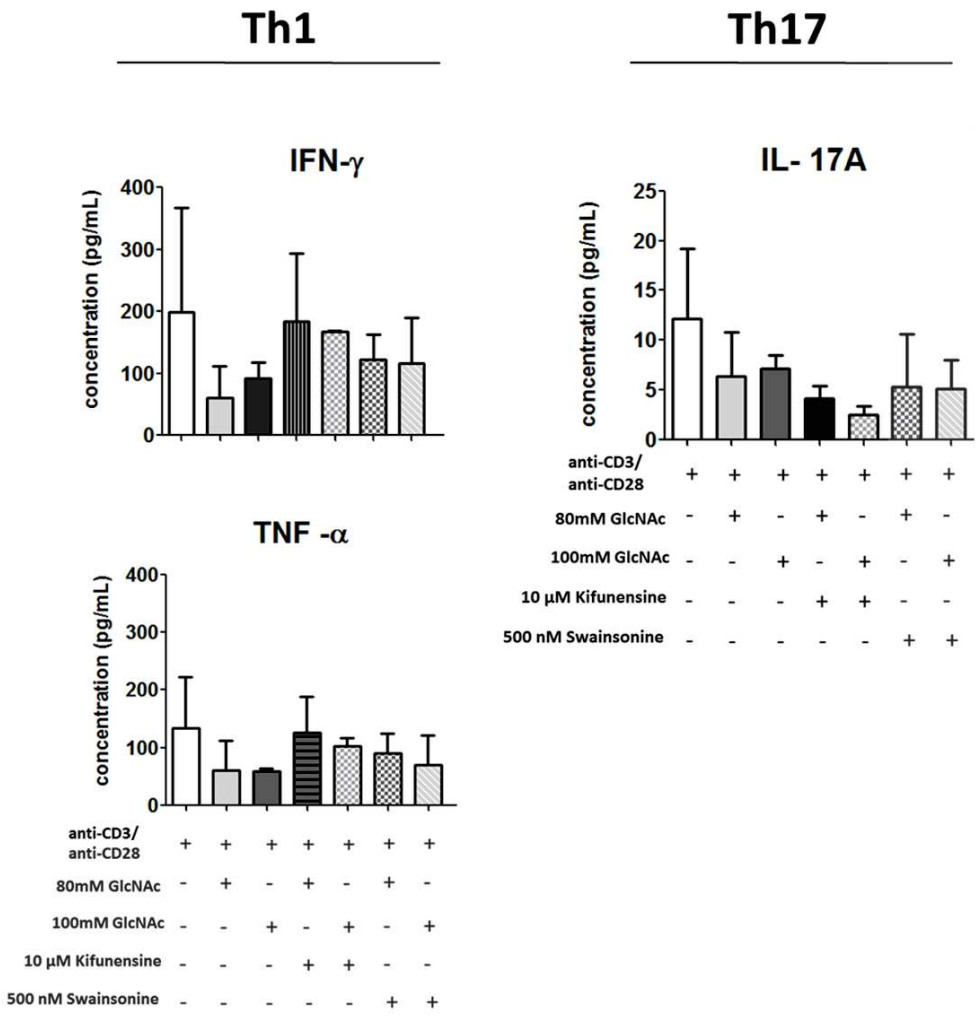


c

	IL-10 secreted by Active UC colonic T cells (pg/ml)		
	0 mM	**80 mM GlcNAc**	**100 mM GlcNAc**
Patient 1	15,24	11,1	4,87
Patient 2	0,28	0	0
Patient 3	7,8	4,57	3,02
Patient 4	0	0	0
Patient 5	0,38	0	0
Patient 6	1,5	0,61	0,35

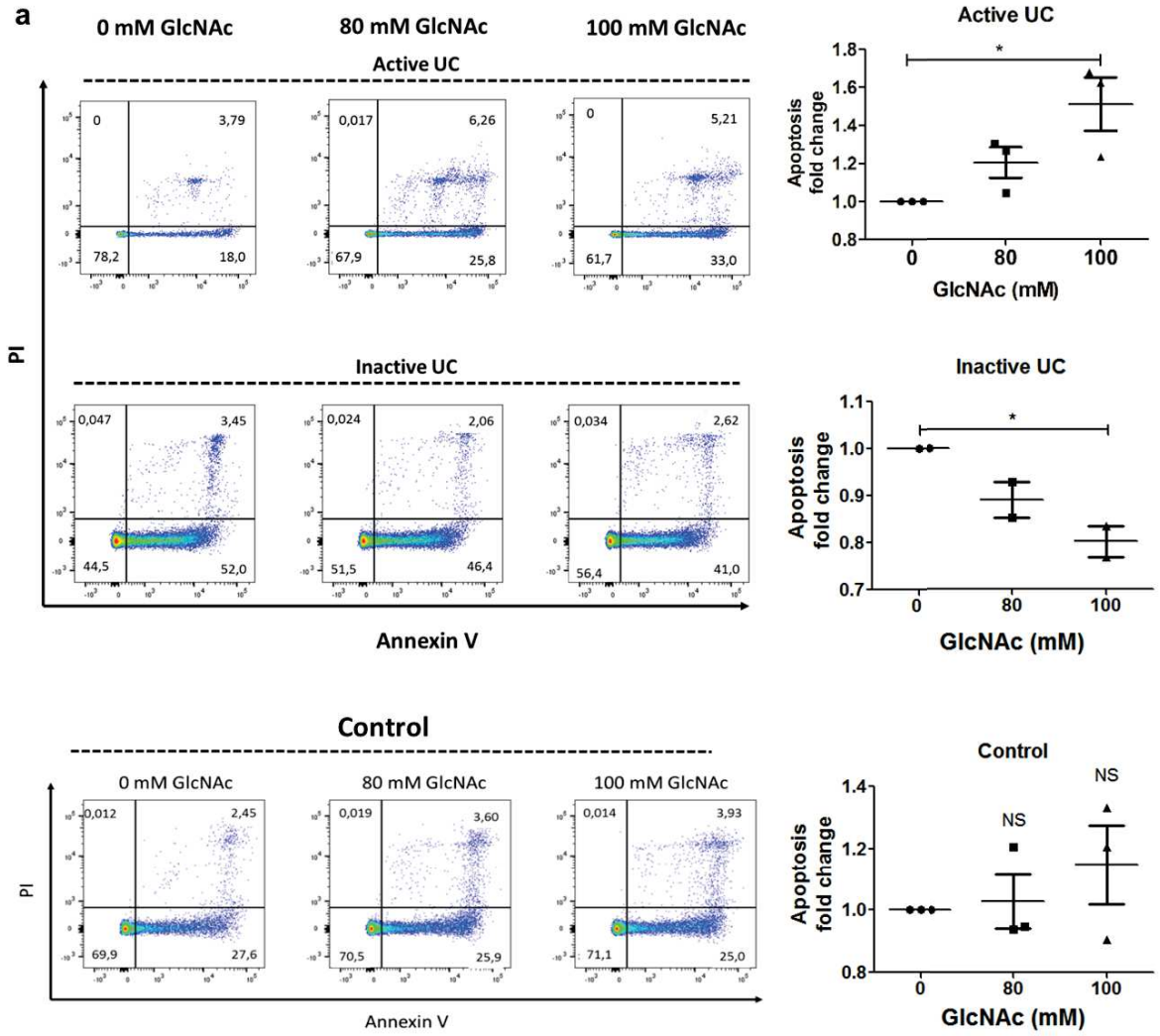
Supplementary Fig. 5. Secreted cytokines in supernatants of *ex vivo* T cells from active UC patients. (**a**) Concentration of pro-inflammatory cytokines detected in the supernatants from *ex vivo* cultures of mucosal T cells, normalized to respective division index (obtained from CFSE analysis) of T cells in the respective conditions (0 and 80 mM GlcNAc); BDL, means below detection limit. The effects of GlcNAc in the suppression of pro-inflammatory cytokines were found to be independent of cell death and/or decreased T cell proliferation. (**b**) Quantification of TGF-β in supernatants from *ex vivo* T cell cultures under GlcNAc supplementation. Scatter plots including mean fold change and ± SEM error bars of 5 biological replicates (biopsies: Mayo 2 (n=3); Mayo 3 (n=2). NS, not statistically significant. (**c**) The expression of IL-10 assessed by flow cytometry in supernatants from *ex vivo* T cell cultures under GlcNAc supplementation. Results include raw data of six biological replicates (Mayo 2 (n=3), Mayo 3 (n=3).

Supplementary Fig.6



Supplementary Fig. 6. Inhibition of branching N-glycans synthesis abrogates the regulation of pro-inflammatory cytokines production. Graphs represent the effect of *ex vivo* treatment of T cells isolated from blood (Mayo 3, n=1) of active UC patient with GlcNAc and the inhibitors of branching N-glycans synthesis, Kifunensine and Swainsonine revealing no regulatory effects in T cell function, with no apparent impact in Th1- (TNF-α, IFN-γ) and Th17-associated (IL-17A) cytokine production.

Supplementary Fig.7



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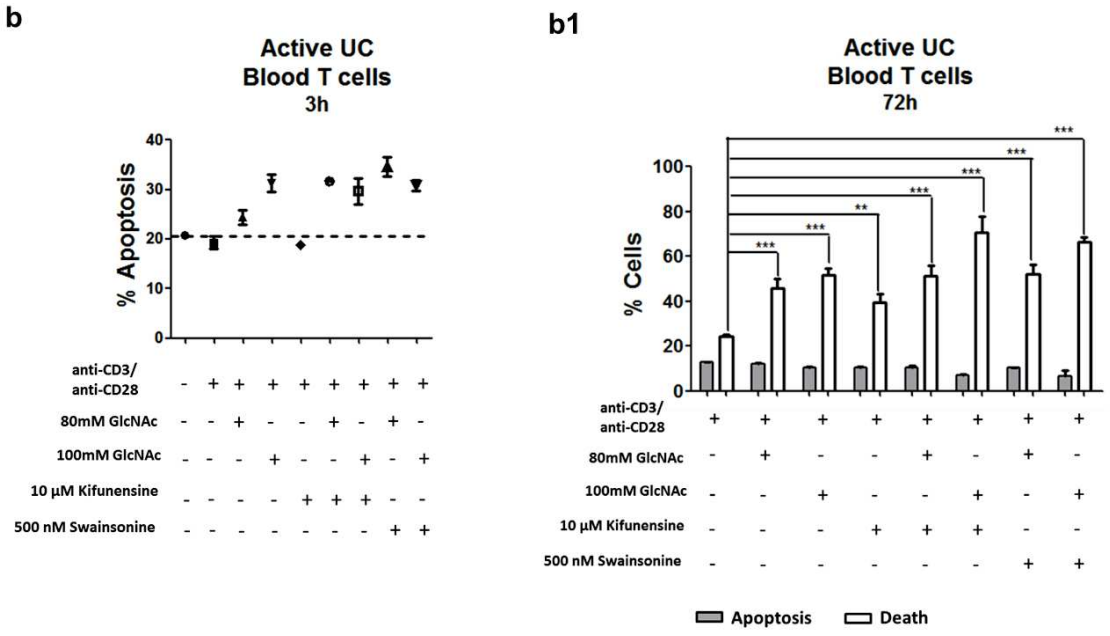
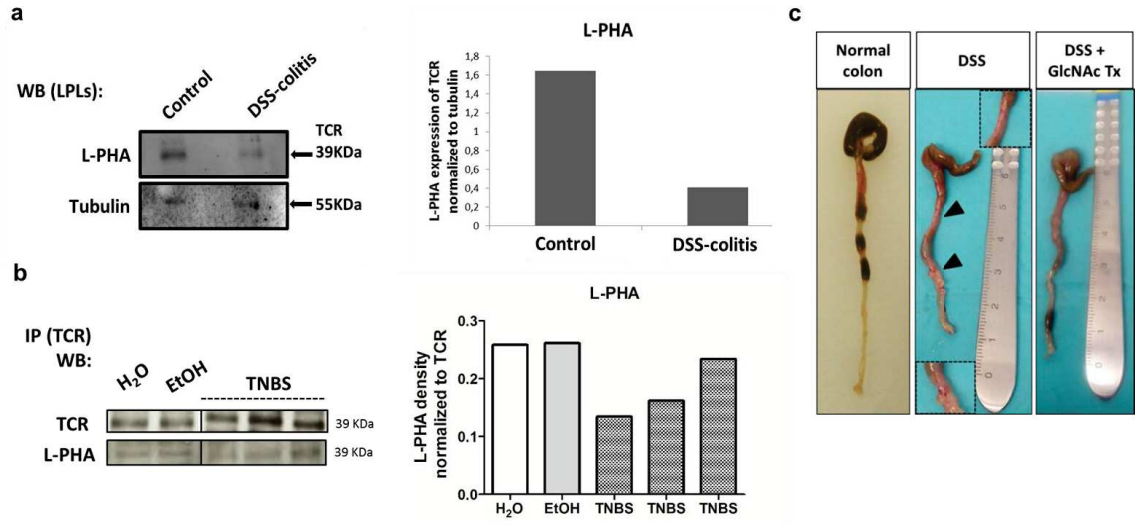


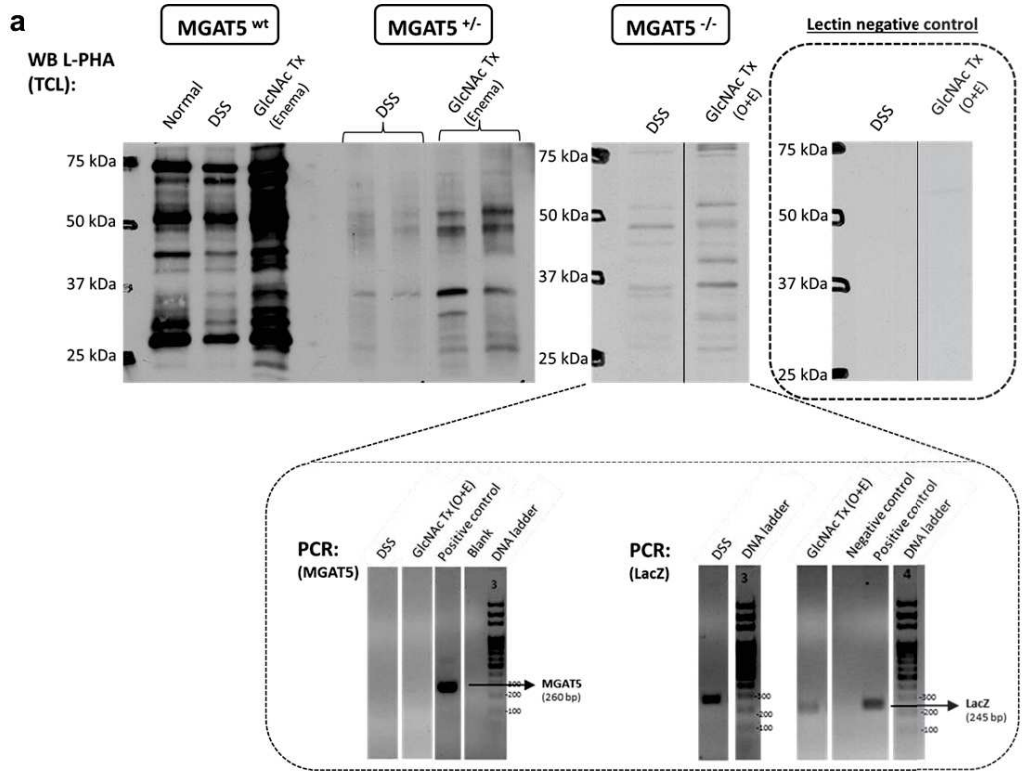
Supplementary Fig. 7. Impact of *ex vivo* GlcNAc treatment in cell apoptosis/death of T cells from control versus active UC patients. (**a**) Flow cytometry evaluation of T cell apoptosis by Annexin V/PI staining upon GlcNAc supplementation. The three dot plots correspond to a representative example of blood T cells from one active, one inactive UC patient and one heathy control. Numbers inside dot-plots correspond to the percentage of cells within each quadrant. Fold change in early apoptosis (Annexin V^+PI^-) after 3h of culture with and without GlcNAc, as indicated. Graphs correspond to mean fold change ± SEM of early apoptotic cells from biological replicates (Active: 3 biological replicates, Mayo 2 (n=2) and Mayo 3 (n=1); Inactive: 2 biological replicates; Control: 3 biological replicates). Results are normalized to the corresponding untreated condition that was taken as 1. Each value corresponds to the mean of two independent technical replicates. One-way ANOVA using Bonferroni's multiple comparison post-test: * $P \leq 0.05$. (**b**) Scatter plots correspond to percentages ± SEM of apoptotic cells at 3 hours in colonic T cells cultures from active UC patients, under different treatments and concentrations, as indicated. (**b1**) Percentage of apoptotic cells (Annexin V^+PI^-) and dead cells (Annexin V^+PI^+) in 72 hours cultures of blood T cells from active UC patients, under different treatments and concentrations, as indicated. Two-way ANOVA using Bonferroni's multiple comparison post-test: ** $P \leq 0.01$;***$P \leq 0.001$.

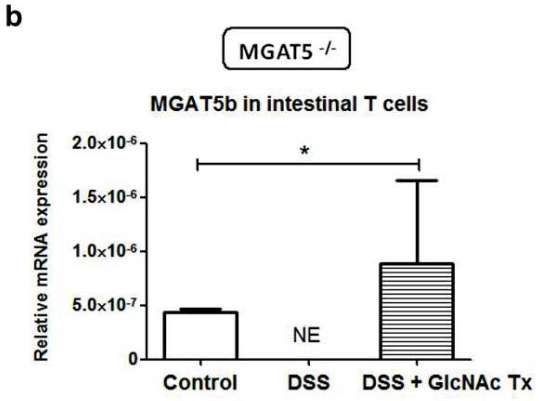
Supplementary Fig. 8



Supplementary Fig. 8. Evaluation of branched N-glycosylation on TCR of colonic T cells from DSS and TNBS-induced colitis mouse models. (**a**) Protein lysates from the isolated colonic T cells from control mice (drinking water) and DSS-colitis mice (DSS in drinking water) were subjected to L-PHA lectin blot to evaluate the expression levels of β1,6-GlcNAc branched N-glycans on the TCRβ (39 kDa). Bar graph, quantification of L-PHA densities on TCR band normalized to tubulin. (**b**) Immunoprecipitation of TCR from total cell lysates of mouse colon followed by β1,6-GlcNAc branched N-glycans recognition with L-PHA as indicated. Bar in the graph correspond to the amounts of branched N-glycan structures, determined from the ratios of densities of L-PHA reactivity normalized to TCR, comparing control groups, water (H_2O, via rectum) and ethanol (50% ethanol, via rectum) with TNBS- induced colitis group (3% TNBS in 50% ethanol, via rectum). (**c**) Representative macroscopic images of the colon and cecum of *MGAT5* WT mice at the end of the experimental period: DSS control (DSS-induced colitis) and GlcNAc treatment (Tx) (DSS+ GlcNAc Tx, enemas), comparing with a normal colon. Mice under DSS treatment showed a visible colonic edema (swelling of the bowel wall) (arrowheads, further magnified in the inserts) comparing to normal mice or mice treated with GlcNAc (enemas route) after DSS-induced colitis.

Supplementary Fig. 9





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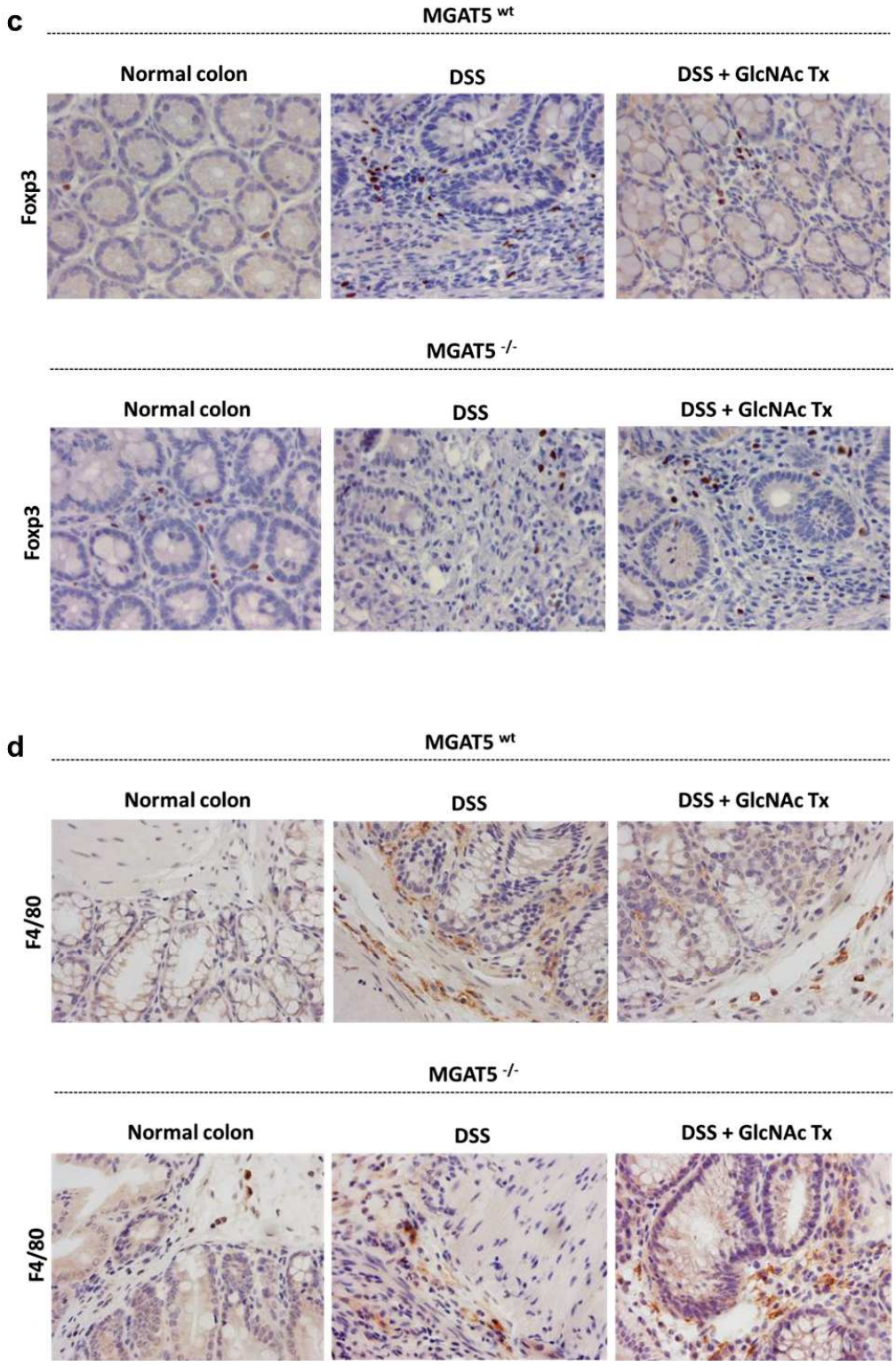

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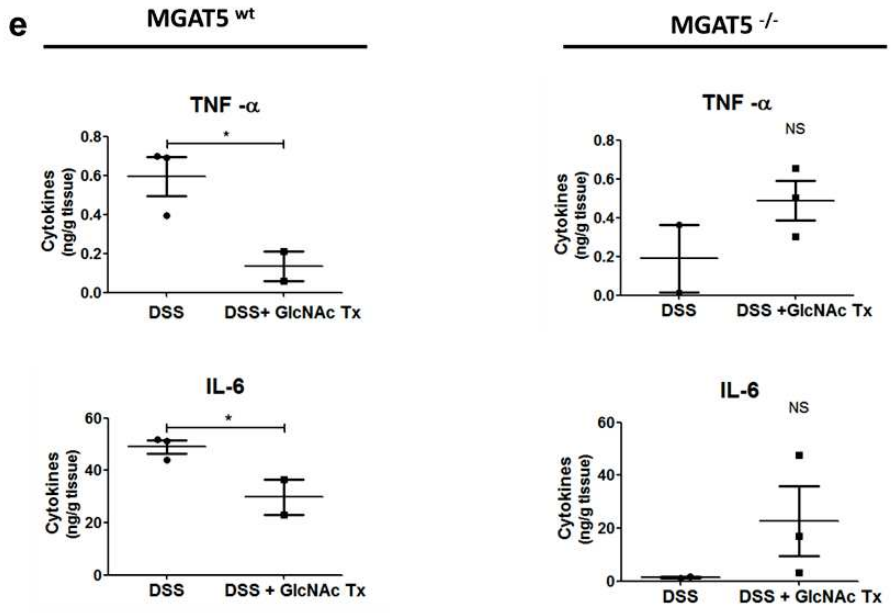
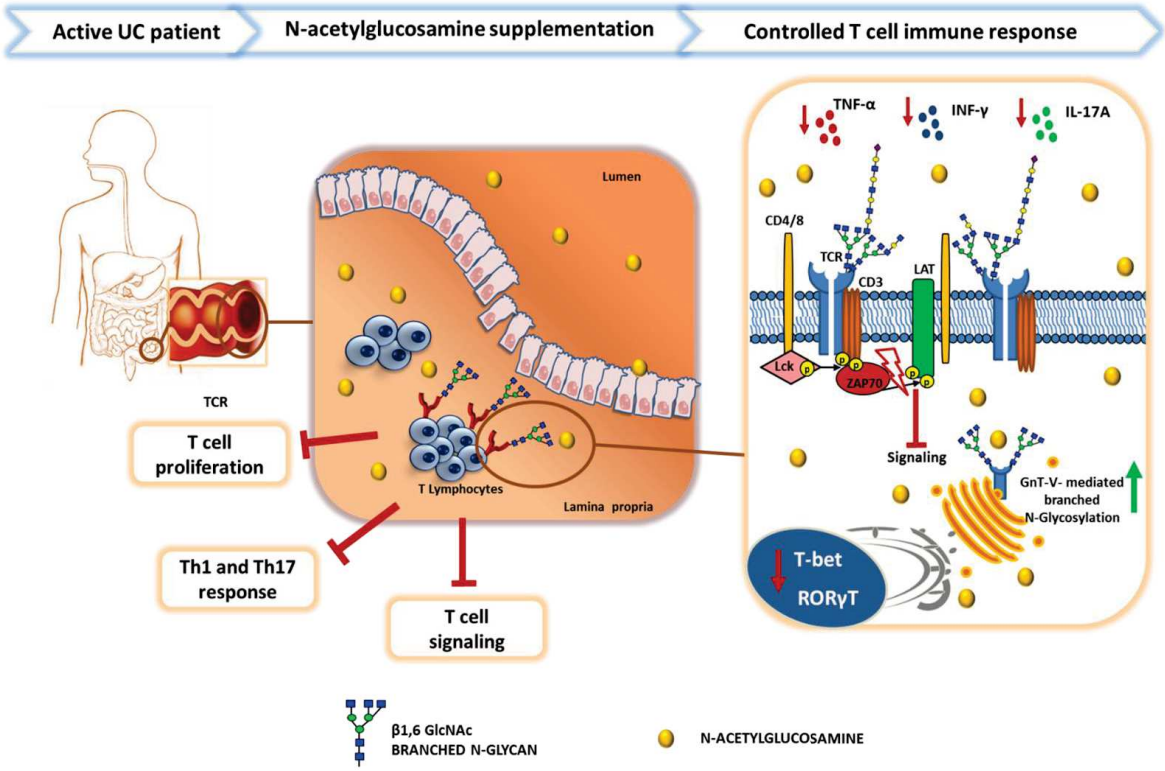


Supplementary Fig. 9. GlcNAc administration effects in different genotypes of MGAT5. (**a**) GlcNAc administration via enema reveal an enhancement of branched N-glycosylation on total cell lysates from mice with different MGAT5 genotypes. Evaluation of branching N-glycans on colonic total cell lysates from *MGAT5* ^{wt} mice, *MGAT5* $^{+/-}$ and *MGAT5* $^{-/-}$, comparing DSS control (DSS-induced colitis) with GlcNAc treatment Enema (GlcNAc Tx) by Western blot. Insert represents the *MGAT5* $^{-/-}$ genotyping (gels of PCR products of genes *MGAT5* and Lac Z) from the same animals represented in the *MGAT5* $^{-/-}$ L-PHA staining and lectin negative control. (**b**) Intestinal T cells from *MGAT5* null mice showed an increased expression of the homologous *MGAT5b* gene upon GlcNAc treatment. qRT-PCR analysis for mRNA expression of MGAT5b from intestinal LPLs from MGAT5 null mice controls (n=2) *versus* GlcNAc treated mice after DSS (n=2), performed in triplicate. The mRNA expression levels are expressed as mean ± SEM, (Student's t-test: $*P \leq 0.05$). NE, not expressed. (**c**) T reg cells expression at the intestinal lamina propria of MGAT5 ^{wt} *versus* MGAT5 $^{-/-}$ mice. The impact on T reg cells was evaluated by the expression of Foxp3 by immunohistochemistry in the colon of MGAT5^{wt} mice and MGAT5 $^{-/-}$, comparing normal colon, DSS-induced colitis (DSS) and treated mice with GlcNAc (DSS + GlcNAc Tx). Represented colon sections of treated mice in both genotypes correspond to GlcNAc Oral+ Enema, x40 original magnification. (**d-e**) The control of inflammation upon GlcNAc treatment is associated with differences in *MGAT5* wildtype and null mice regarding macrophages recruitment and function. (**d)** The impact of GlcNAc treatment on macrophages was evaluated by the expression of F4/80

by immunohistochemistry in the colon of MGAT5^{wt} mice and MGAT5 $^{-/-}$, comparing normal colon, DSS-induced colitis (DSS) and treated mice with GlcNAc (DSS + GlcNAc Tx). Represented colon sections of treated mice in both genotypes correspond to GlcNAc Oral+ Enema, x40 original magnification. (**e**) Concentration of TNF-α and IL-6 in the supernatants of 24h colonic explant cultures from DSS and DSS + GlcNAc Tx *MGAT5* wildtype (n=5) and null (n=5) mice by ELISA. Cytokine levels are expressed as ng/g of dry colon explant weight. Results correspond to mean ± SEM of 2 to 3 animals per group. Student's t-test: * P ≤ 0.05. NS, not statistically significant.

Supplementary Fig.10



Supplementary Fig.10. Shaping the T cell-mediated immune response in IBD through metabolic enhancement of branched N-glycosylation. An opportunity for new therapeutic strategies. UC patients with active disease are characterized by a massive infiltration of lamina propria T lymphocytes (LPLs) that we previously demonstrated to display a deficiency in branched N-glycosylation catalyzed by GnT-V(16). In this study we demonstrated that the simple glycan N-acetylglucosamine (GlcNAc) is able to repair the abovementioned deficiency having an important impact on the control of T cell-mediated immune response. The *ex vivo* supplementation of GlcNAc in T cells (purified from colonic mucosa of active UC patients) induces suppression of T cell proliferation, increased susceptibility to T cell apoptosis, inhibition of Th1/Th17-type inflammatory response, and suppression of T cell signaling and activation. Specifically, we found that GlcNAc supplementation resulted in T cell surface glycans remodeling characterized by an enhancement of the expression of branched N-glycans on the TCR that can be further extended with polylactosamine residues (ligand for galectins) and known to prevent TCR clustering and activation. These poly-LacNAc structures can be terminal sialylated. This modulation of the glycophenotype of T cells was shown to be translated in important regulatory effects on the adaptive immune response by

hampering T cell function and activation, suppressing the release of potent pro-inflammatory cytokines known to promote the disease.

Taken together, metabolic enhancement of branched glycosylation on T cells, revealed to have immunomodulatory effects in UC with clinical relevance, paving the way to further explore the clinical applicability of this novel immunomodulatory agent in UC patients.

Table S1.

Fluorochrome	Antibody name	Source	Clone	Specificity
PerCP	CD4	BD Bioscience	clone SK3	anti-human
PE	CD8	BD Bioscience	clone SK1	anti-human
eF450	CD4	eBioscience	clone RPA-T4	anti-human
BV510	CD45	eBioscience	clone HI30	anti-human
Alexa Fluor488	Gata3	eBioscience	clone,TWAJ	anti-human
APC	RorγT	eBioscience	clone AFKJS-9	anti-human/mouse
PerCP-Cyanine 5.5	T-bet	eBioscience	clone eBio4B10	anti-human/mouse
PE	FoxP3	eBioscience	clone PCH101	anti-human/mouse
PerCP Cy 5.5	CD4	Biolegend	clone RPA-T4	anti-human
AF488	TNF-α	eBioscience	clone MAb11	anti-human
APC	IFN-γ	eBioscience	clone 4S.B3	anti-human
APC-eFluor® 780	Fixable Viability Dye	eBioscience		

The table summarize es the list of antibodies used for staining by flow cytometry experiments

Appendix III

Studying T cells N-glycosylation by Imaging Flow Citometry

Ana M. Dias, Catarina R. Almeida, Celso A. Reis, Salomé S. Pinho

Book chapter with detailed protocol applied in the work included in Chapter II and Chapter III

Chapter 11

Studying T Cells *N*-Glycosylation by Imaging Flow Cytometry

Ana M. Dias, Catarina R. Almeida, Celso A. Reis, and Salomé S. Pinho

Abstract

Imaging flow cytometry is an emerging imaging technology that combines features of both conventional flow cytometry and fluorescence microscopy allowing quantification of the imaging parameters. The analysis of protein posttranslational modifications by glycosylation using imaging flow cytometry constitutes an important bioimaging tool in the glycobiology field. This technique allows quantification of the glycan fluorescence intensity, co-localization with proteins, and evaluation of the membrane/cytoplasmic expression. In this chapter we provide the guidelines to analyze glycan expression, particularly the β1,6 GlcNAc branched N-glycans, on the membrane of intestinal T cells from inflammatory bowel disease patients.

Key words *N*-Glycosylation, T-Cell Receptor, T-cells, Ulcerative colitis, Imaging flow cytometry

1 Introduction

Glycosylation is an important protein posttranslational modification being fundamental for the regulation of proteins biological functions both in homeostasis and in pathological conditions. The development of novel accurate assays for analysis of this key biological process is of utmost importance in the glycobiology research field. Flow cytometry is a widely used technique that allows measuring fluorescence intensity in many events, but that lacks the possibility of analyzing imaging parameters. On the other hand, fluorescence microscopy is not ideal to analyze and quantify parameters from a large number of cells. The invention of the ImageStream system, which combines features of fluorescence microscopy and flow cytometry, overcomes these limitations opening new opportunities to explore a range of applications with utility in the glycoscience research field. This technique presents an extraordinary throughput, thus allowing fast acquisition of imaging data [1]. Furthermore the IDEAS data analysis software has the capacity to

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calculate several quantitative features that allow the graphical identification of populations based in several variables (fluorescence intensity, cell size, shape, texture, co-localization of multiple probes, among others) [1]. Therefore, imaging flow cytometry technique becomes a remarkable tool for studies related with cellular morphology, internalization, cell signaling, co-localization, and trafficking (see specific applications in: https://www.amnis.com/applications.html). Additionally, we can even create/define masks to easily identify our targets, for instance, to identify co-localization of probes at the cell membrane.

Taking advantages of the combined parameters of imaging flow cytometry, we have analyzed the levels of membrane TCR branched N-glycans comparing different ulcerative colitis (UC) patients and controls. Furthermore, we provide here an example for analysis of co-localization between a lectin-recognizing glycan and a protein receptor (TCR). Using imaging flow cytometry we evaluated the co-localization of the L-PHA lectin (*Phaseolus Vulgaris Leucoagglutinin* lectin that specifically recognizes the β1,6 GlcNAc branched N-glycan structures) with surface TCR α/β on purified intestinal lamina propria T lymphocytes. Interestingly, this bioimaging technique has validated results obtained from the canonical molecular techniques used to assess proteins glycosylation (such as Western blot and immunoprecipitation approaches) [2]. In this chapter we provide a comprehensive approach on how to use imaging flow cytometry in glycoscience, particularly in studying *N*-glycosylation on the membrane of T cells.

2 Materials

2.1 Reagents for Cell Staining

1. Phosphate buffered saline (PBS) 1×.
2. FACS Buffer: PBS 1× with 0.1 % sodium azide and BSA 2 %.
3. Antibodies: anti-TCR α/β mAb (clone BW242/412, mouse IgG2b) conjugated with R-phycoerythrin (PE) (Miltenyi-Biotech); Fluorescein *Phaseolus vulgaris* Leucoagglutinin (L-PHA/FITC) (Vector Laboratories); mouse IgG2b anti-human conjugated with PE (Immunotools); Streptavidin-conjugated fluorescein isothiocyanate (FITC) (Caltag Laboratories).

2.2 Equipment

Imaging flow cytometer equipped with one laser and one camera (ImageStream^X, from Amnis, now part of Millipore). This equipment can have more lasers and cameras for more complex analysis.

3 Methods

This Imaging flow cytometry protocol involves three main parts: (1) Staining of cells with the probes of interest; (2) acquisition of data on an imaging flow cytometer; (3) data analysis and statistics.

3.1 Cell Staining

Cells can be stained with a protocol typically used for traditional flow cytometry, but a careful titration of the labeling agents (dyes or antibodies) is even more crucial. As with any imaging experiment, one should aim for good signal-to-noise ratio while avoiding saturation. This is particularly important when dealing with more than one fluorophore excited by the same laser: the settings that can be changed during acquisition (laser power and selected filters) will affect visualization of all stainings, and thus it is crucial to tune labeling for optimal imaging. The following protocol is described for already optimized dilutions of probes (*see* **Note 1**).

Each experiment must include different samples: the staining of interest, a negative control (isotype control if using antibodies) and single stained cells for compensation, when analyzing more than one color.

1. After isolating lamina propria T lymphocytes (LPLs), following an adapted protocol [3], wash cells with RPMI by centrifuging at 800 × *g*, 5 min, 4 °C.
2. Remove supernatant.
3. Resuspend pellet with 1 ml FACS buffer and keep on ice.
4. Divide resuspended pellet in 4 Eppendorf tubes (*see* **Note 2**) for: (*a*) negative control, (*b*) double staining (anti-TCR and L-PHA), (*c*) single staining TCR, and (*d*) single staining L-PHA, for compensation.
5. Centrifuge at 300 × *g*, 10 min, 4 °C.

Blocking

6. Discard supernatant.
7. Resuspend samples with 500 μl of FACS buffer.
8. Incubate for 30 min, on ice.
9. Centrifuge at 300 × *g*, for 10 min, 4 °C.
10. Discard supernatant.

Staining

11. Resuspend samples in 100 μl FACS buffer.
12. Add 10 μl PE-conjugated anti-TCR antibody to tubes *b* and *c*, or the equivalent amount of isotype control to the negative control sample (*a*).
13. Incubate for 30 min, on ice and in the dark.

14. Wash with 500 μl FACS buffer by centrifuging at 300×*g*, 10 min, 4 °C.
15. Repeat washing step (*see* **Note 3**).
16. Prepare the lectin 4 μg/ml: 1 μl L-PHA-FITC in 500 μl FACS buffer.
17. Incubate samples *b* and *d* with 50 μl of prepared lectin and control *a* with 50 μl of FACS buffer.
18. Incubate on ice for 30 min, in the dark.
19. Prepare a streptavidin-FITC 1 μg/ml solution (from stock 1000 μg/ml): 1 μl Streptavidin-FITC in 1000 μl FACS buffer.
20. Incubate samples *a*, *b*, and *d* with 50 μl of streptavidin solution.
21. Incubate on ice for 30 min, in the dark.
22. Wash with 500 μl FACS solution by centrifuging at 300×*g*, 10 min, 4 °C.
23. Repeat washing step.

Fixation (after staining, see **Note 4***).*

24. After the previous step, discard supernatant.
25. Fix cells with 100 μl formaldehyde 4 %, 20 min, room temperature, in the dark.
26. Centrifuge at 300×*g*, 10 min, 4 °C.
27. Wash with 500 μl PBS 1×.
28. Centrifuge at 300×*g*, 10 min, 4 °C.
29. Repeat the washing step.
30. Discard supernatant.
31. Resuspend in 60 μl of PBS 1× (*see* **Note 5**) and maintain at 4 °C, in the dark until acquisition in the ImageStream cytometer (*see* **Note 6**).

3.2 Acquisition of Data by Imaging Flow Cytometry

Images of isolated cells can be acquired on a 6-Channel ImageStream^X imaging flow cytometer equipped with one laser (Amnis, EMD Millipore), using the INSPIRE software.

1. Initialization of equipment.
   Initialize the ImageStream by following your facility's rules: typically, you need to ensure all containers are filled with appropriate solutions (beads, sterilizer—you can use 10 % bleach, cleanser, debubbler—you can use 70 % isopropanol, rinse—ultrapure water, sheath—filtered PBS 1×, also add some bleach to the waste container) and you may need or not to calibrate.

Preparation of samples immediately before acquisition:

2. To filter samples, start by adding a 2 μl drop of PBS 1× to the inside edge of a 1.5 ml tube.
3. Place a 70 μm cell strainer on top of the tube.
4. While exerting some pressure, pipette the cell suspension on the cell strainer, aiming for the place where you added the PBS 1× drop (*see* **Note 7**).

3.3 Sample Acquisition

1. Start by acquiring the sample with all stainings, to allow you to adjust the equipment settings.
2. Create a classifier based on the bright-field image, so that only events with an area above a certain size will be included in the analysis. In this case, we choose an area higher than 20, measured in the bright-field image.
3. Adjust the laser power and choose the best filters for your image. These settings should be defined in such a way that images are not saturated (*see* **Note 8**).
4. Once all settings are defined, you can start acquiring your data. Ensure that you set up the number of events to acquire to at least 10,000 events.
5. Analyze firstly your stained samples and negative controls, and then proceed for the single stained controls for compensation.
6. Before acquiring samples for compensation enable the option "Comp settings," and change the classifier for the channel you will be visualizing (as you will not be able to see any bright-field image).
7. After finishing acquisition, proceed with sterilization and cleaning protocols for your equipment.

3.4 Data Analysis and Statistics

Analysis was performed with IDEAS 5.0 (Amnis, EMD Millipore) as follows (Fig. 1):

1. A compensation matrix can be created by choosing "create a new matrix" under "Compensation," and following the wizard. Select the files corresponding to single stained controls. At the end, verify whether images are properly compensated by using the "preview images" tool in the "create compensation matrix" dialog box.
2. For analysis, several wizards are available. Here, we advise starting with the co-localization wizard. Briefly, this wizard guides the user through the most common steps: uploading of raw data files (.rif) and compensation matrix to create compensated data files (.cif) and data analysis files (.daf); gating focused

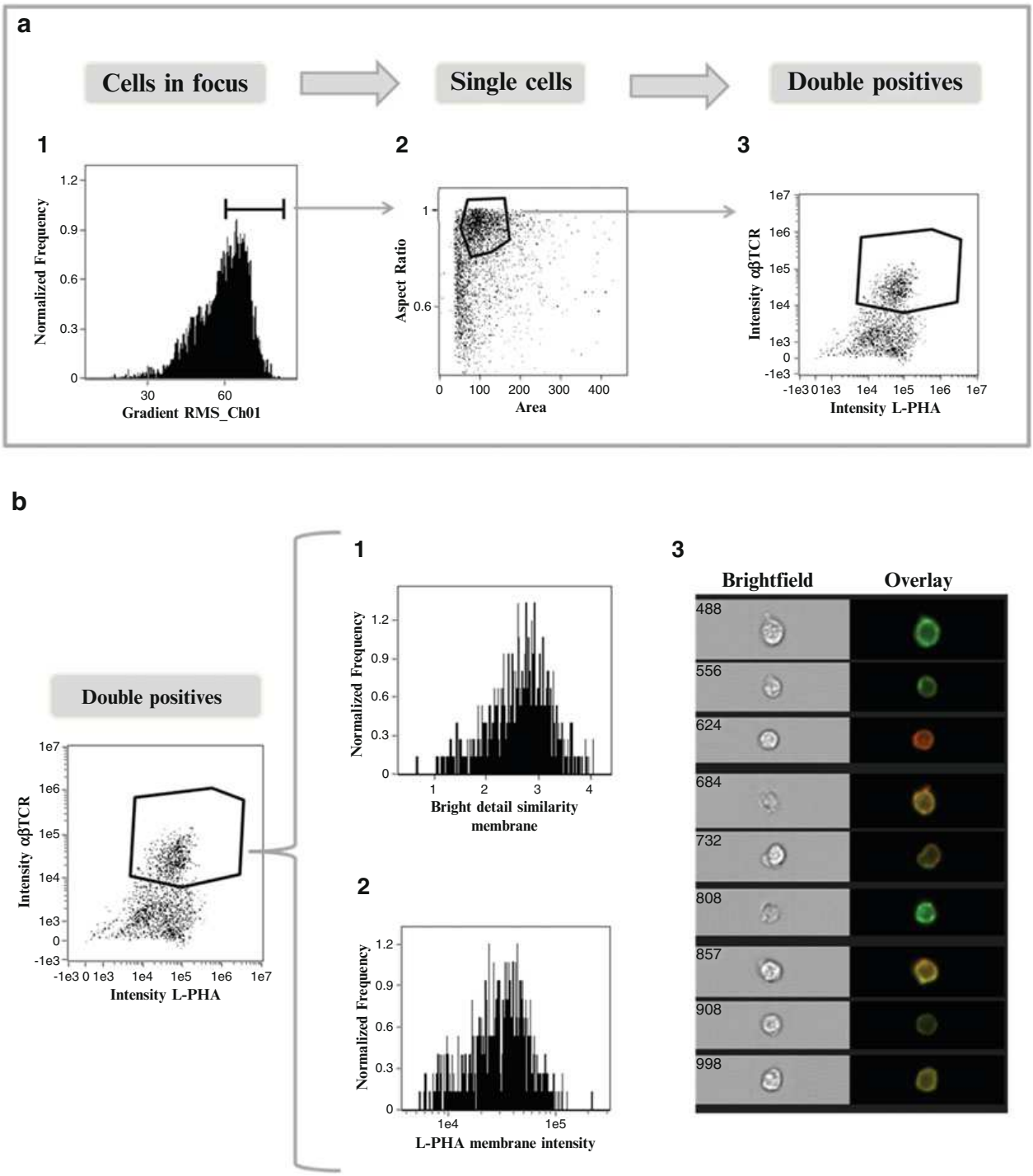

Fig. 1 Schematic representation of the analysis using an example of data acquired after staining for L-PHA and TCR. (**a**) Sequence of analysis steps. (1) A histogram for gradient RMS in bright-field images (Channel 01) can be used to gate for cells in focus. (2) A scatter plot of aspect ratio/area in Channel 1 (bright-field) is used to gate for single cells, within focused events. (3) The intensity of α/β TCR and L-PHA staining is used to gate on double positive cells within focused single cells. (**b**) Graphic representation of parameters analyzed from the double positive cells selected. (1) The level of co-localization at the membrane was quantified as the bright detail similarity. (2) Histogram represents the intensity of L-PHA staining on the membrane of α/β TCR^+ $L\text{-}PHA^+$ cells. (3) Panel showing examples of single cell bright-field (Ch01) and merged (TCR in *red* and L-PHA in *green*) images, showing co-localization between TCR α/β and L-PHA

events; gating single cells (*see* **Note 9**); gating in populations of interest (in this case, TCR-FITC+ cells); calculation of co-localization, or the Bright Detail Similarity between the two probes (L-PHA and anti-TCR) (*see* **Note 10**).

3. Besides calculating co-localization, one can proceed to other analysis in the same file. In this case, the intensity of L-PHA staining specifically located at the membrane of TCR α/β positive events can also be determined. For that, it is necessary to create a new mask by going to Analysis and then Masks (*see* **Note 11**). Here, a new mask to depict the cell membrane was created. This mask uses the functions dilate and erode ("dilate and not erode") and can be based in the bright-field or in a fluorescence image of the membrane.
4. Then create a new feature by going to Features under the Analysis menu. Here, we were interested in evaluating the intensity of L-PHA staining specifically in the cell membrane. A histogram plot for this new feature can then be generated.
5. Finally, create a statistics report to include all relevant information: percentage of TCR+ cells, mean bright detail similarity, mean fluorescence intensity of L-PHA at the membrane, etc..
6. An interesting feature of the IDEAS software is the possibility to easily analyze many files with one template. If you have many files to analyze, save the analysis file as a template and perform a batch analysis, by going to Tools, choosing batch data file and defining the input files and template to use.

4 Notes

1. If you do not have any information about using your probe with the available imaging flow cytometer, you should start by titrating it. Perform the protocol described herein, but with different concentrations of probe. The ideal concentration of probe(s) allow you to visualize cells with a good, but not saturated signal (*see* **Note 8**), using the same settings for all probes.
2. Ideally, cells should be counted and divided equally for each condition. But when having a limited number of cells, cell suspension can be divided in the 4 Eppendorfs, as following: 500 μl—double staining TCR-PE/L-PHA; 166 μl—single staining TCR-PE; 166 μl—single staining L-PHA- FITC; 166 μl—negative control (isotype + Streptavidin-FITC).
3. Samples single staining TCR-PE (tube *c*) should be resuspended in 60 μl of PBS 1× and kept on ice until FIXATION.

4. Fixation is a critical step that should be optimized to determine if it is better to fix before or after staining with the specific probes. Furthermore, it must be tested what is the best for fixation (paraformaldehyde, formaldehyde, methanol), since it may influence cells morphology and protein distribution, affecting the quality of images and consequently evaluation of the parameters of interest.
5. Samples are resuspended in 60 μl of PBS 1× because the ImageStream^X will run samples with a minimum of 50 μl. The number of cells per sample advised by the manufacturer is approximately 1×10^6 cells (up to 5×10^6 cells) in a final volume of 50 μl, in a 1.5 ml microcentrifuge tube, but we found that samples with a lower number of cells can still be analyzed, although acquisition will take a long time.
6. Ideally acquisition in the ImageStream cytometer should be performed immediately after staining. As this is not always practical, fixation allows keeping the cells in cold PBS 1× for a couple of days. Imaging should however be performed as soon as possible.
7. Samples with such a small volume are troublesome to filter. Add a 2 μl drop of PBS 1×, that may stay at the edge of the tube or flow down. Either way, it will create a channel for fluid to flow through by capillary action. It is important to avoid having fluid all around the tube as the sample will then not flow through. The first time you do this start by training with water.
8. During acquisition, you can plot in real time the highest intensity in one pixel of your event vs the area of each event on the bottom graphs of the acquisition windows. This allows you to visualize whether your images are saturated.
9. When performing analysis with the IDEAS software an interesting feature is the fact that you can always observe the images to which a dot in a dot plot corresponds. This becomes extremely useful when deciding on where to draw a gate, as it is possible to visualize whether that dot is a cell, cell debris, a doublet, etc.

   The bright detail similarity is one of the many built in features that can be used with IDEAS. This index gives a measure of the co-localization of two probes in a defined region. It computes the log transformed Pearson's correlation coefficient of the bright spots with a radius of 3 pixels or less within a masked area in the two input images. More details on how this feature functions can be found in [4].
10. It must be pointed out that this bright detail similarity gives a measure of whether two molecules are within the same area of the cells, but due to resolution limitations, it does not indicate

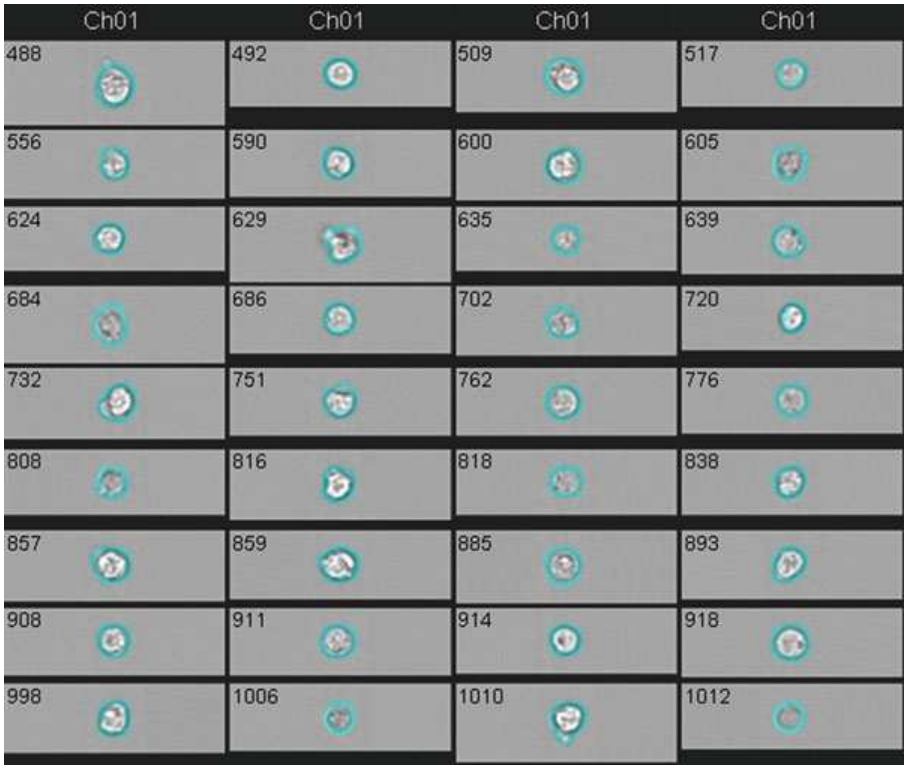

Fig. 2 Panel with examples of bright-field images (channel 1, Ch01) with indication of the membrane mask create (in *light blue*). By creating masks, with IDEAS 5.0 (Amnis, EMD Millipore), we can restrict the analysis to a specific location. The mask membrane created allows quantification of L-PHA staining on the cell membrane of TCR $\alpha/\beta+$ cells from ulcerative colitis patients and controls

whether these molecules interact with each other. For that, other techniques are necessary, such as immunoprecipitation [2] or FRET.

11. If you use a wizard that automatically creates a mask, it is advisable to confirm whether this mask is being correctly applied. For that, go to image properties (icon is the symbol for brightness and contrast manipulation), create a new view that includes your channel of interest with the mask created, go back to the global window and choose that view. Click then on the mask icon and scroll through your cells to see how the mask is being applied (Fig. 2).

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