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1. Proof of concept of microbiome-metabolome analysis and delayed gluten exposure on celiac disease autoimmunity in genetically at-risk infants. Sellitto M., Bai G., Serena G., Fricke WF., Sturgeon C., Gajer P., White JR., Koenig SS., Sakamoto J., Boothe D., Gicquelais R., Kryszak D., Puppa E., Catassi C., Ravel J., Fasano A. PLOSOne 2012; 7(3).

2. Dysbiosis and possible therapeutic approaches: example of celiac disease. G. Serena, A. Fasano. Giorn. Gastr. Epatol. Nutr. Ped 2013; V(5): 34-37.

3. Genetics and the celiac disease: the importance of screening. MM. Leonard, G. Serena, C. Sturgeon, A. Fasano. Expert Review in Gastroenterology and Hepatology 2015; 9(2):209-215.

4. The role of gluten in celiac disease and type 1 diabetes. G. Serena, S. Camhi, C. Sturgeon, S. Yan, A. Fasano. Nutrients 2015; 7(9): 7143-7162.

5. Design of a genomic, environmental, microbial and metabolomic study on celiac disease: an approach to the future of personalized prevention of celiac disease. G. Serena, MM. Leonard, S. Camhi, TB. Huedo-Medina, A. Fasano. Recenti Progr Med 2016; 107: 1-8.

6. Pro-inflammatory Cytokine IFNγ and Microbiome-Derived Metabolites Dictate Epigenetic Switch between FOXP3 Isoforms in Celiac Disease. Gloria Serena, Shu Yan, Stephanie Camhi, Shilpi Patel, Rosiane S. Lima, Anna Sapone, Maureen M. Leonard, Rupa Mukherjee, Barbara J. Nath, Karen M. Lammers, Alessio Fasano. Clinical and Experimental Immunology 2017. 87(3):490-506

First author abstracts:

1. Differences in HLA DQ2/DQ8 haplotype and FOXP3 gene expression between celiac and non-celiac Down syndrome.
   G. Serena, D. Libreri, C. Sturgeon, D. Kryszak, KM. Lammers, A. Fasano
   DDW Annual Meeting, Chicago, IL, May 7-10, 2011.

2. Different expression of FOXP3 gene and HLA DQ2/DQ8 haplotype between celiac and non celiac patients with down syndrome
   G. Serena, D. Libreri, C. Sturgeon, D. Kryszak, KM. Lammers, A. Fasano)
   NASPGHAN Annual Meeting, Orlando, FL October 2011.

3. rs3761547 FOXP3 polymorphism is not related to celiac disease.
   G. Serena, C. Sturgeon, A. Fasano
   Digestive Disease Week Meeting, San Diego, CA May 19th-22nd 2012.

4. Absence of correlation between rs3761547 FOXP3 polymorphism and celiac disease.
   G. Serena, C. Sturgeon, A. Fasano
   NASPGHAN, Salt Lake City, Utah October 2012.

5. Altered expression of FOXP3 Δ2 isoform in active celiac disease
   G. Serena, A. Sapone, KM Lammers, A. Fasano
   MGH Clinical Research day of Boston, MA, October 2013.

6. Celiac disease is associated with increased expression of the alternatively spliced FOXP3 isoform.
   G. Serena, A. Sapone, KM Lammers, A. Fasano
   NASPGHAN meeting in Chicago, IL, October 2013.

7. FOXP3 isoforms expression in gluten related disorders.
   G.Serena. A. Sapone, KM. Lammers, A. Fasano, G. Riegler, L. DeMagistris
   FISMAD congress, Naples, Italy.19-22 March 2014.

8. Expression of the alternatively spliced isoofrm FOXP3 delta2 in celiac disease.
   G.Serena. A. Sapone, KM. Lammers, A. Fasano
   Digestive Disease Week (DDW). Chicago, IL; 3-6 may 2014.

9. Role of microbial derived metabolites and pro-inflammatory cytokine IFNγ in modulating FOXP3 isoforms expression in celiac patients.
   G.Serena , S. Camhi , S. Yan , KM. Lammers , A. Fasano .
   NASPGHAN meeting, Atlanta, GA: 23-26 October 2014.

10. The Microbiome Signature of Autistic Children is Characterized by Decreased Diversity and Dysbiosis
    G. Serena, A. Sapone , MR Iovine, F. Bombace, D. Siniscalco , C. Schiraldi, C. Bravaccio, B. Ma, M. Humphry, J.Ravel, L.de Magistris, A. Fasano
    DDW meeting, Washington DC, May 2015.

Presentations at conferences:

1. "Role of microbial derived metabolites and pro-inflammatory cytokine IFNγ in modulating FOXP3 isoforms expression in celiac patients" at NASPGHAN meeting, Atlanta, GA: 23-26 October 2014.

2. "The microbiome and Celiac disease" at the 16th Annual Postgraduate Nutrition Symposium, Harvard Medical School, Boston, July 2015.

ABSTRACT

Pro-inflammatory Cytokine IFNγ and Microbiome-Derived Metabolites Dictate Epigenetic Switch between FOXP3 Isoforms in Celiac Disease.

Gloria Serena, Doctor of Philosophy, 2017

Thesis advisors: Prof. Alessio Fasano, Massachusetts General Hospital; Dr. Terez Shea-Donohue, Department of Radiation Oncology University of Maryland.

Celiac disease (CD) is an autoimmune enteropathy triggered by gluten and characterized by a strong Th1/Th17 immune response in the small intestine. Treg cells are $CD4^{+}$ $CD25^{++}$ cells that regulate the immune response and maintain immune homeostasis. Given its role in controlling the differentiation and function of Treg cells, FOXP3 has been considered their master transcription factor. Two main isoforms of FOXP3 have been described. Conversely to its counterpart FOXP3 full length (FL), the alternatively spliced isoform FOXP3 Δ2 cannot properly down-regulate Th17 driven immune response. Since the active state of CD has been associated with impairments in Treg cell function, we aimed at determining whether imbalances between the FOXP3 isoforms may be associated with the development of the disease. Intestinal biopsies from patients with active CD showed increased expression of the less functional *FOXP3* Δ2 isoform over FL, while both isoforms were similarly expressed in non-celiac control subjects (HC). Conversely to what we saw in the intestine, peripheral blood mononuclear cells (PBMC) from HC subjects did not show the same balance between the two isoforms. We therefore hypothesized that the intestinal microenvironment may play a role in modulating the alternative splicing of FOXP3 isoforms. The pro-inflammatory

intestinal microenvironment of active patients have been reported to be enriched in butyrate producing bacteria, while high concentrations of lactate have been shown to characterize the preclinical stage of the disease. We show that the combination of IFNγ (the main pro-inflammatory cytokine produced in the intestine of active CD patients) and butyrate triggers the balance between FOXP3 isoforms in HC subjects, while the same does not occur in CD patients. Furthermore we report that lactate significantly increases both isoforms in CD patients when compared to control subjects. Collectively these findings highlight the importance of the ratio between FOXP3 isoforms in CD and, for the first time, mechanistically associate the alternative splicing process with microbial derived metabolites.

Pro-inflammatory Cytokine IFNγ and Microbiome-Derived Metabolites Dictate Epigenetic Switch between FOXP3 Isoforms in Celiac Disease

by
Gloria Serena

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When I initially came to Baltimore for a fellowship, I planned to stay in United States for one year at most. Already after 6 months working in Dr. Fasano's Lab, however, I found myself completely dedicated to my project and I decided to prolong my experience at the University of Maryland by applying to the PhD program in Molecular Medicine. I particularly would like to thank my mentor, Alessio Fasano that allowed me to come here in the United States to pursue my dream of working in research. You have always been supportive of my project advising me when necessary and teaching me how to think independently. None of this would have been possible without your guidance and mentorship. A special thank goes also to Karen. You have been a great help and mentor in the lab. You guided me during my PhD and you have always been a great moral support when the experiments were not working as expected. To all the members of the Fasano's lab, thank you for the suggestions, friendships and numerous coffees that we shared. You are too many to thank one by one, but I will never forget how warmly you accepted me in your lab and how patient you have all been in helping me through these years. Without you my research experience would have been much more boring and less fun. To my committee members, Prof. Jonathan Bromberg, Prof. Amit Golding, Prof. Scott Devine and Prof. Bryan Hurley. Thank you for the constructive comments that you gave me during the several meetings we had. Your expertise and suggestions have been an invaluable part of my scientific growth. A particular thank goes to Prof. Shea-Donohue. You not only have been a very helpful committee member, but you also have supported and helped me during my PhD admission and transfer from Baltimore to

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LIST OF ABBREVIATIONS:

Ag Antigen

AP-1 Activator protein 1

APC Antigen presenting cells

CCL22 CC motif chemokine 22

CCL8 CC motif chemokine 8

CCR10 Chemokine receptor 10

CCR4 Chemokine receptor 4

CCR7 Chemokine receptor 7

CCR8 Chemokine receptor 8

CCR9 Chemokine receptor 9

CD Celiac disease

CDA active celiac disease

CDGF celiac disease in gluten free diet

CNS Non coding sequenses

CREB cAMP response element binding

CTLA4 Cytotoxic T lymphocytes associated protein-4

CXCR3 CXC motif chemokine receptor 3

CXCR4 CXC motif chemokine receptor 4

CXCR6 Chemokine receptor 6

DCs Dendritic cells

EAE Experimental autoimmune encephalomyielitis

FL Full length

FOXP1 Forkhead box protein 1

FOXP2 Forkhead box protein 2

FOXP3 Forkhed box protein 3

FOXP4 Forkhead box protein 4

GI Gastrointestinal tract

GITR Glucorticoid-induced tumor necrosis factor receptor

GPR41 G protein coupled receptor 41

GPR43 G protein coupled receptor 43

GWAS Genome wide association study

HDAC 7 histone deacetylase 7

HDAC 9 histone deacetylase 9

HIF1α hypoxia inducible factor 1 alpha

HLA Human leucocyte antigen

IBD Inflammatory bowel disease

IDO Idoleamine 2,3-dioxygenase

IFNγ Interferon gamma

IL10 Interleukin 10

IL12 Interleukin 12

IL17 Interleukin 17

IL1β Interleukin 1 beta

IL2 Interleukin 2

IL23 Interleukin 23

IL35 Interleukin 35

IL4 Interleukin 4

IL5 Interleukin 5

IL6 Interleukin 6

IL7R Interleukin 7 receptor

ILC innate lymphoid cells

IPEX Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome

IRF4 Interferon regulatory factor 4

LAG3 Lymphocyte activation gene 3

MΦ macrophages

miRNAs MicroRNAs

mTOR mammalian target for rapamycin

NFAT Nuclear factor of activated T cells

NFκB Nuclear factor kappa B

NLRP3 NLR family pyrin domain containing 3

nTregs Natural T regulatory cells

PBMC Peripheral blood mononuclear cells

PSA Polysaccharide A

pTregs Peripheral T regulatory cells

RORαT Retinoic acid-related orphan receptor alpha T

RORγT Retinoic acid-related orphan receptor gamma T

RUNX1 Runt related transcription factor 1

RUNX3 Runt related transcription factor 3

SCFAs Short chain fatty acids

SLE Systemic lupus erythematosus

SMAD3 Smad protein family member 3

SNPs Single nucleotide polymorphisms

STAT3 Signal transducer and activator of transcription 3

STAT5 Signal transducer and activator of transcription 5

T1D Type 1 diabetes

TCR T cells receptor

TGFβ1 tumor growth factor beta-1

TLR1 Toll-like receptor 1

TLR2 Toll-like receptor 2

TLR4 Toll-like receptor 4

TLR5 Toll-like receptor 5

TLR6 Toll-like receptor 6

TLR7 Toll-like receptor 7

TLR8 Toll-like receptor 8

TNFα Tumor necrosis factor alpha

TRAIL-DR5 Tumor-necrosis-factor-related-apoptosis-inducing ligand-death receptor 5

Tregs T regulatory cells

TSDR T specific demethylated regions

Δ2 Delta 2

CHAPTER 1. INTRODUCTION

1.1 Celiac disease

Celiac disease (CD) is an autoimmune enteropathy triggered by gluten in genetically predisposed individuals [1]. The genetic susceptibility for CD has been primarily associated with the HLA (human leucocyte antigen system) DQ2/DQ8. The DQ2 haplotype characterizes the majority of CD patients (95%), while only a minority of them carries the DQ8 (5%) [2]. While the HLA accounts for 40% of the genetic risk for CD, also other genes related to the immune response and to the intestinal permeability regulation have been linked to CD by GWAS (genome wide association studies) [3].

The establishment of gluten as the environmental trigger for CD makes this disease a unique model of autoimmunity. Gluten is a complex molecule present in several grains (wheat, rye and barley) and its link with CD was determined for the first time around 1950 [1, 4]. The two main protein components that characterize gluten, glutenin and gliadin, contain high levels of prolines (20%) and glutamines (40%); this protects them from complete degradation in the GI tract and makes them particularly difficult to digest [5]. Glutenins are monomers that can be subdivided in low and high molecular weights, while gliadins are polymers that are re-divided in α, β, γ and ω gliadins. The role of gluten, and in particular gliadin, as environmental trigger for CD has been demonstrated by several studies *in vitro* where intestinal biopsies and peripheral blood mononuclear cells (PBMC) from CD patients have been shown to express a wide range of pro-inflammatory cytokines upon stimulation with gliadin [6, 7]. The chain of events that lead to the onset of CD after ingestion of gluten is initiated by the physical interaction between partially digested gliadin peptides and the apical side of the small intestine. This

contact triggers the release of chemo-attractant IL8 and pro-apoptotic IL15 from epithelial cells and lamina propria dendritic cells (DCs) [8]. Additionally, the interaction between specific gliadin peptides and the epithelial chemokine receptor CXCR3 (CXC motif chemokine receptor 3) leads to a MyD88 dependent release of zonulin that leads to increased intestinal permeability and antigen trafficking [9]. Upon translocation to the lamina propria, gliadin peptides are pdeamidated by transglutaminase 2 and recognized by different types of antigen presenting cells (APC) [10]. The consequent adaptive Th1/Th17-driven immune response is associated with high production of pro-inflammatory cytokines such as IFNγ (interferon gamma), TNFα (tumor necrosis factor alpha) and IL17 (interleukin 17) that, together, will contribute to a further increase of the intestinal permeability and to the mucosal damage [11] (**Fig. 1**). Presently, many components within the gliadin aminoacidic sequence have been associated with the different steps that characterize the onset of CD. The majority of these epitopes are contained in α- and ω- gliadins and they contribute to the pathogenesis of the disease through their immunomodulatory, gut permeating and cytotoxic properties [12].

The classical presentation of CD is mostly characterized by gastrointestinal (GI) symptoms such as diarrhea, steatorrhea and weight loss. However, particularly in adult patients, other extra-intestinal symptoms including anemia, chronic fatigue and reduced bone density also have been reported [2]. Currently the gold standard treatment for CD is represented by the complete elimination of gluten from the diet. Nevertheless, this therapy seems to fail in a subset of patients (7-30%).

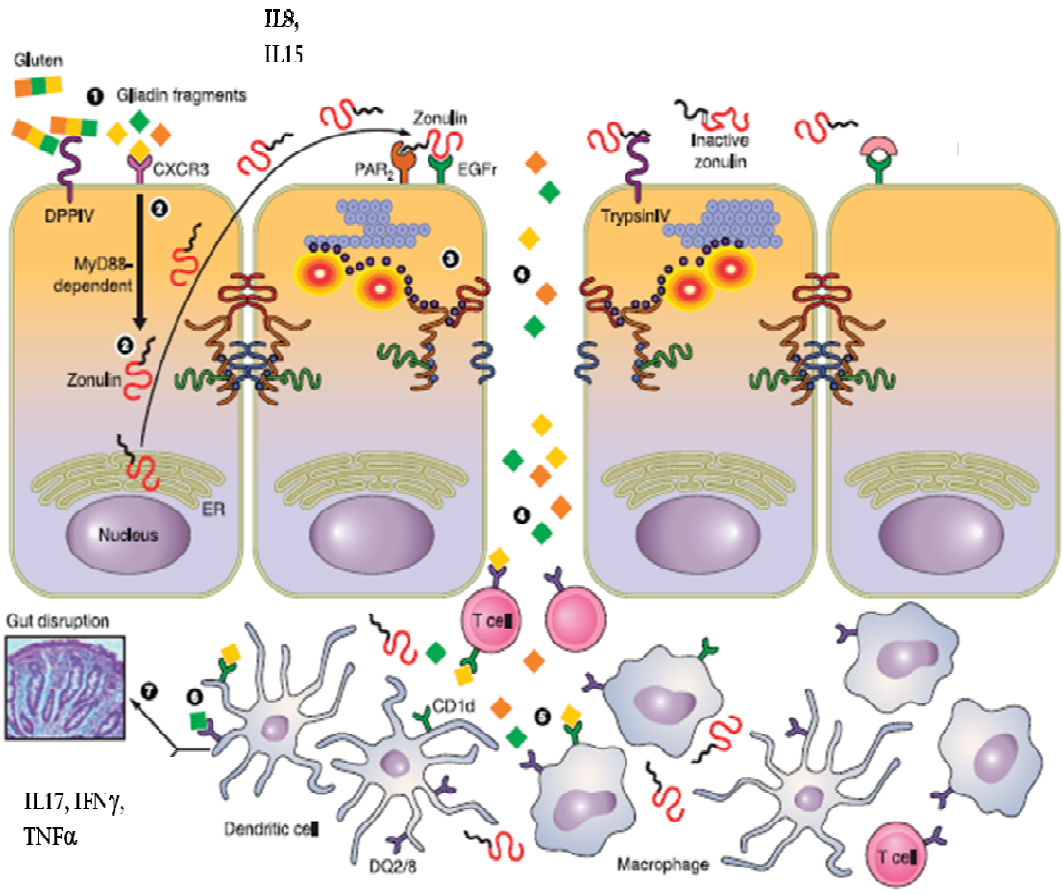


Fig. 1: Mechanisms by which gliadin triggers onset of CD (figure adapted from [10]). Gliadin peptides interact with the apical side of the small intestinal epithelium. This interaction triggers an innate immune response (release of IL8 and IL15) and an increased intestinal permeability (1-3). The translocation of gliadin peptides to the lamina propria activates an adaptive immune response characterized by Th1/Th17 derived pro-inflammatory cytokines that lead to the final mucosal damage (4-7).

The incidence of CD is reported to be approximately 1%, however, recent data show that this prevalence is rapidly increasing worldwide with a particularly high rate of new diagnosis among adult patients [13, 14]. These findings suggest that environmental components, other than the genetic predisposition, contribute to the development of the disease. Although the exact mechanisms that lead to the loss of tolerance to gluten in adulthood have not been elucidated fully, changes in the immune system's regulatory machinery have been suggested to be possible contributors. Paradoxically, CD subjects are characterized by an increased number of Tregs (T regulatory cells) both at peripheral and intestinal level. These regulatory immune cells, however, have been shown to be functionally defective [15-17]. Although it is not yet clear how Tregs in CD fail to properly suppress the immune response, it is now well established that functional, rather than quantitative, defects in the regulatory system, could mediate the final loss of tolerance to gluten. These data also support the hypothesis that additional elements, other than the genetic predisposition, may play a role in the development of CD. Among the suggested environmental factors, the microbiome appears to be one of the most probable candidates.

The intestinal microflora consists of 10^{14} microorganisms that play a fundamental role in the development of intestinal immune homeostasis and physiology [18]. Recently several studies have shown a correlation between the onset of CD and a certain level of GI dysbiosis. Active CD patients present reduced amounts of *Firmicutes* and a higher abundance of *Proteobacteria* and *Bacteroidetes* as compared to healthy subjects [19]. Sanz et al. reported differences in fecal abundance of lactic acid bacteria and *Bifidobacterium* between active CD and controls patients [20], while two other groups

showed that alterations in the microbiome composition were correlated with high production of SCFAs (short chain fatty acids) such as pyruvate and butyrate [21, 22]. The microflora is strictly dependent on the host's diet, therefore it is not surprising that the elimination of gluten has been shown to modulate the microbiome composition of CD patients [23]. Interestingly, however, even after long term treatment, CD patients are reported to maintain a certain level of dysbiosis [19]. The hypothesis that alterations in the microflora may be at least partially causative of CD, and not only a consequence of gluten–induced inflammation, is supported by our proof-of-concept study in which we reported that infants genetically predisposed for CD present a unique microbiota even before the onset of the disease [22]. The microbiome of HLA-DQ2 babies, independently of their diet, was found to be enriched in *Firmicutes* and to be almost totally lacking microbes from the *Bacteroidetes phylum* (**Fig. 2**). Furthermore babies that develop CD or other autoimmune diseases showed an increased production of lactate associated with enrichment in *Lactobacilli spp.* during the preclinical phase of the disease and high levels of butyrate after the onset of the disease (**Fig.3**). These data suggest that, in genetically predisposed subjects, a specific microbiome could trigger the onset of autoimmunity by priming the intestinal microenvironment and that production of different metabolites could contribute to the final pathogenesis of the disease. Due to the unique features of CD, a better understanding of the environmental factors that play a role to its development could give some insights about the mechanisms behind the onset of other autoimmune diseases for which the external triggers are still unknown.

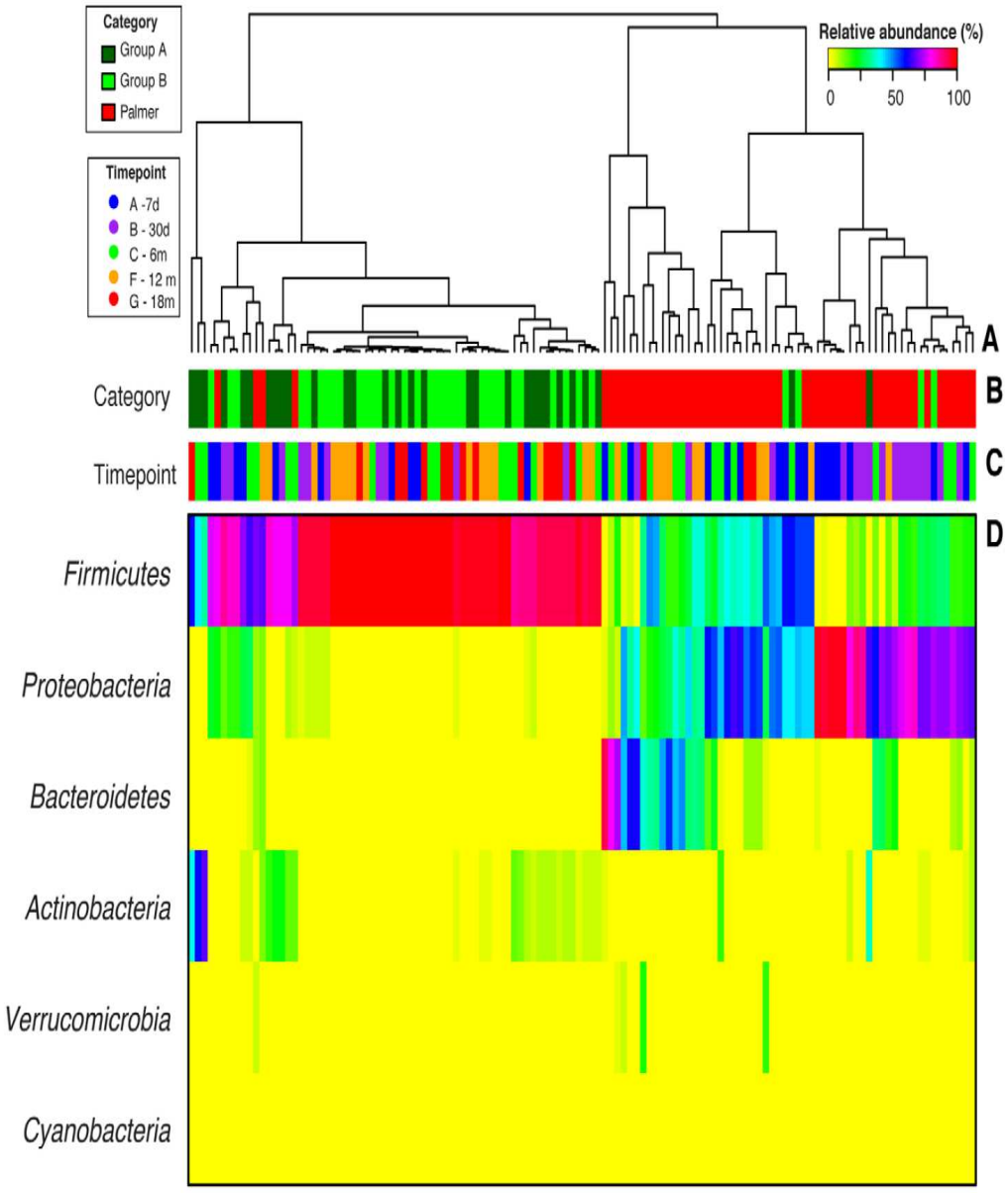


Fig. 2: Microbiome composition in infants genetically predisposed to CD (Figure from [22]). Heat-map representing the bacterial phylum abundance of samples from infants genetically predisposed to CD (HLA-DQ2) that introduced gluten at12 month (Group A) or 6 months of age (group B) and kids from the Palmer study [24].

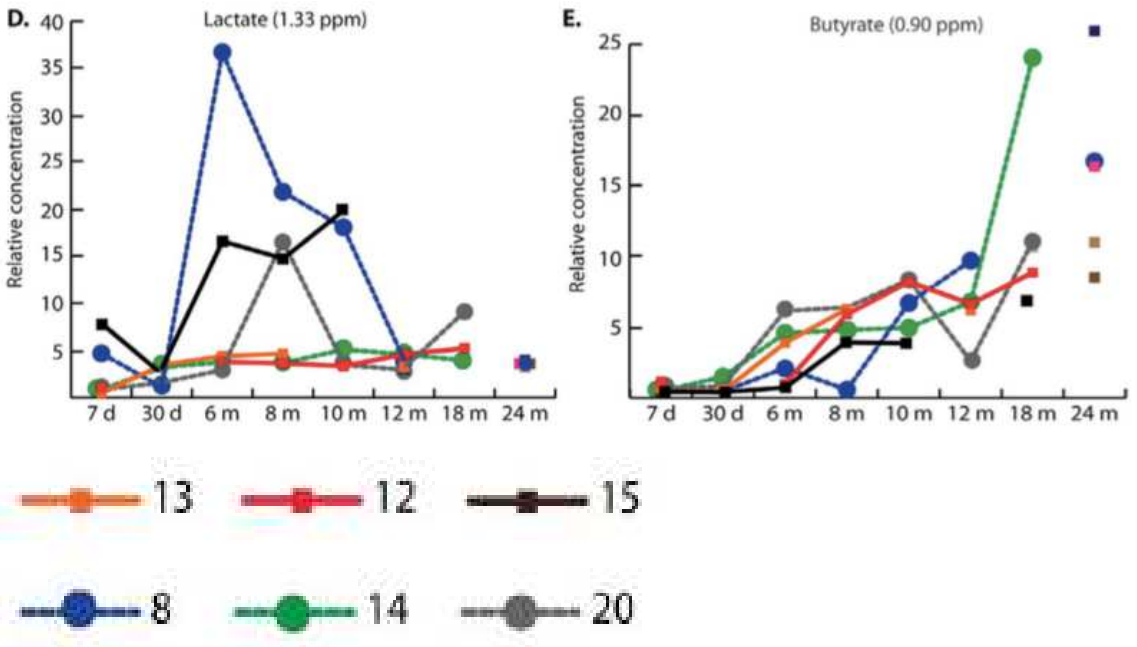


Fig. 3: H-NMR metabolomics analysis (Figure adapted from [22]). Relative concentrations of metabolites lactate and butyrate in infants genetically predisposed to CD (HLA-DQ2) at different time points. Samples 8 and 15 respectively CD and T1D developed after 6 months of age.

1.2 T regulatory cells

Tregs were described for the first time 20 years ago. Since then, they have been recognized to be major players in the maintenance of peripheral tolerance and in the prevention of autoimmune diseases [25]. Traditionally, Tregs express high levels of CD25 (alpha subunit of IL2 receptor or IL2Rα) and nuclear expression of transcription factor FOXP3 (Fork-head box protein 3) [26]. Although both molecules have been demonstrated to be necessary for the suppressive function of Tregs, neither of them is exclusive to the Tregs subpopulation in humans. CD25 expression, in fact, is induced in human T effector cells upon activation. Similarly, FOXP3 has been shown to be transiently expressed in non suppressive T cells after TCR (T cell receptor) activation. It is now established that he use of an array of several markers may be useful to phenotipically characterize Tregs. Among the different proposed molecules, CD127 (IL7R interleukin 7 receptor), GITR (glucorticoid-induced tumor necrosis factor receptor) and CTLA-4 (cytotoxic T lymphocytes associated protein-4) are the most common. While the expression of CD127 is down-regulated on Tregs, GITR is highly expressed on resting Tregs and has been reported to be important in promoting their development [27]. CTLA-4 is a negative regulator of T cells activation; it shares 70% homology with CD28 and is involved in Tregs suppressive function by interacting with CD80/CD86 [28, 29]. The co-expression of chemokines receptors CCR4 and CCR8 (chemokine receptor 4 and 8) have been shown to characterize Tregs [30] and studies from Peng et al. and Crellin et al. reported a high expression of TLR8 and TLR5 (toll-like receptors 5 and 8) respectively [31, 32]. Finally, surface expression of TGF-β1 (tumor growth factor beta-1)

has also been reported as characteristic of Tregs and fundamental for their suppressive function [33].

Two main types of Tregs have been described depending on their site of origin. Naturally occurring Tregs (nTregs) develop in the thymus through a high-affinity interaction with self-peptide and MHC class II (major histocompatibility complex class II). They present a wide range of TCRs repertoire specific for self antigens and they play a major role in limiting the onset of autoimmune diseases [34]. In contrast, peripheral Tregs (pTregs) are generated under tolerogenic conditions in the periphery from naïve T cells upon stimulation with foreign antigens. Their TCR repertoire is specific for harmless environmental agents or commensal microorganisms and they play an important role in maintaining tolerance to food and microbiota derived antigens at the mucosal sites. Although definitive markers differentiating nTregs and pTregs have not been identified yet, Baron et al. showed that differences in the methylation status of the non-coding TSDR (Tregs specific demethylated region) located in the promoter of *FOXP3* gene may be useful to distinguish one subpopulation from the other. The authors showed that demethylation in the FOXP3 TSDR was associated only with nTregs, while in induced pTregs, the same region appears fully methylated. These changes in the DNA demethylation status are suggested also to be responsible for the differences in stability of FOXP3 expression between nTregs and pTregs [35].

Recent studies have shown the plasticity between pTregs and pro-inflammatory Th17 cells [36]. Both subsets of T cells play an important role in maintaining the mucosal immune homeostasis and their differentiation is strictly dependent on the surrounding microenvironment. TGFβ is fundamental for the development of both cell types by

inducing the expression of either RORγT or FOXP3. Whether the cells will differentiate towards a pro- or anti-inflammatory subset depends on the presence of other cytokines. IL6 and IL21, for example, are necessary for the differentiation toward a Th17 phenotype. While anti-inflammatory cytokines such as IL10 allow the differentiation toward a Treg population.

Beside cytokines, other molcules such as mTOR and HIF1α (hypoxia inducible factor 1 alpha) have also been shown to be able to modulate the plasticity between Th17 and pTregs. mTor is a protein that positively regulate the differentiation of Th17 cells by inhibiting FOXP3 expression. Similarly, HF1α directly promotes the differentiation of Th17 cells by inducing RORγT expression, while attenuating Treg development and inducing FOXP3 degradation [36]. The anyl hydrocarbon receptor (AHR) is a nuclear receptor that has also been found to play a role in the differentiation between TH17 and pTregs depending on the activating ligand.

It has been shown that Tregs undergo functional characterization by expressing transcriptional factors specific for other T effector cells types and different miRNAs (micro RNAs). Tregs expressing Tbet, CXCR3 and miRNA-146a, for example, accumulate at sites where Th1 mediated inflammation is present [37, 38]. Conversely, Tregs that express IRF4 (interferon regulatory factor 4) and miRNA-182 or STAT3 (signal transducer and activator of transcription 3) are essential for the suppression, respectively, of Th2- and Th17-mediated immune responses [39, 40]. The plasticity of Tregs has been associated also with the expression of different chemokines receptors that allow Tregs to migrate to the “correct tissue”. CD62L and CCR7 (chemokine receptor 7) are linked to migration and retention in lymphoid-tissues [41], Tregs that home to the

skin have been reported to express P- and E- selectin, while CD103 is fundamental for Tregs migration to the gut-associated lymphoid tissue [42, 43]. Finally, CCR9 (chemokine receptor 9), CXCR6 and CXCR4 (CXC motif chemokine receptor 6 and 4) are considered to be important for migration in the small intestine, liver and bone marrow or Payer's Patches [44-47].

All Tregs subpopulations behave similarly and they present a diverse array of suppressive tools (**Table 1**).

MECHANISM OF SUPPRESSION	**MOLECULES INVOLVED**
Release of anti-inflammatory cytokines	IL10, TGFβ, IL35
Induction of apoptosis	TRAIL-DR5, Galectin 1
Cytolysis	Granzyme A, perforin
Metabolic disruption	IL2 consumption through CD25, activation of adenosine receptor 2A through CD39/CD73
Co-inhibitory signals	CTLA4 compete with CD28 for interaction with CD80/CD86
Inhibition of DCs maturation	LAG-3, neuropilin1

Table 1. List of different mechanisms used by Tregs to suppress immune response. Tregs can perform their anti-inflammatory function by suppressing directly T cells mediated immune response (in blue) or indirectly by reducing DCs mediated antigen presentation process (in green).

An important mechanism of suppression used by Tregs is the release of anti-inflammatory cytokines, such as IL10 (interleukin 10) and TGF-β. Although some *in vitro* studies suggest that these cytokines are not essential for Tregs function, their importance for the suppressive activity of Tregs has been demonstrated *in vivo.* Studies using allergy and asthma animal models confirmed that both nTregs and pTregs suppressive functions are, at least in part, dependent on both these cytokines [48-51].

IL35 (interleukin 35) is a newly discovered cytokine that belongs to the IL12 (interleukin 12) heterodimeric cytokines family. It is preferentially expressed by Treg cells and is necessary for their suppressive activity both *in vitro* and *in vivo* [52]. Another mechanism of suppression relevant in Tregs function is cytolysis through release of granzymes. Although these serine proteases are usually found in cytotoxic T lymphocytes and natural killer cells, it has been shown that activated human Tregs can express granzyme A and that their suppressive function can be mediated by granzyme A and perforin through CD18 [53]. Furthermore, Tregs have been shown to induce apoptosis of effector T cells either through TRAIL-DR5 (tumor-necrosis-factor-related-apoptosis-inducing ligand-death receptor 5) or through galectin-1 pathways [54, 55]. Tregs can also display their suppressive activity by mediating metabolic disruption. Their high expression of CD25 allows them to starve effector T cells by consuming local IL2 [51]. Another similar mechanism is represented by the expression of CD39 and CD73. These ectoenzymes suppress effector T cells functions by generating peri-cellular adenosine that activates the adenosine receptor 2A [56, 57]. Finally Tregs can also perform their suppressive activity indirectly by targeting dendritic cells. The direct interaction with DCs has been shown *in vivo* and it involves the co-inhibitory molecule CTLA4. Tregs are able to induce DCs by expressing IDO (idoleamine 2,3-dioxygenase), a strong regulatory molecule that triggers the production of pro-apoptotic metabolites leading to suppression of effector T cells [58]. In addition to CTLA4, LAG3 (lymphocyte activation gene 3) and neuropilin-1 have been proposed to play a role in the Tregs-mediated suppression of DCs by blocking their maturation [51]. Although it is still unclear if Tregs use just a single overriding suppressive mechanism or if they require

multiple processes to maximize their function, it is probable that this choice depends on different factors such as the disease setting, the targeted cell types and the anatomical location [59].

The importance of Tregs in preventing autoimmunity is supported by evidences that show an increased incidence of these diseases later in life and by recent studies that reporting a correlation between age and Tregs prevalence and function [60]. These findings led several groups to examine Tregs frequency in different autoimmune diseases. Studies on systemic lupus erythematosus (SLE) are contradictory with one group showing a decreased frequency and function of Tregs and another showing no significant differences between SLE patients and healthy controls [61, 62]. Patients with rheumatoid arthritis have been reported to have an increased number of circulating Tregs [63], while no differences have been found in patients with multiple sclerosis [64]. Finally, two different groups showed an increased number of Tregs with defective suppressive function respectively in patients with Wegner granulomatosis and autoimmune hepatitis [65]. Abnormal frequencies of Tregs have also been associated with different types of solid tumors such as ovarian, gastric and breast cancer. Several factors may contribute to this increased number of Tregs in cancers. Among them the high expression of CCL22 (CC motif chemokine 22) in tumor cells and macrophages or the hypoxia induced up-regulation of CCL28 (CC motif chemokine 28) have been hypothesized to trigger the recruitment of Tregs through their expression of CCR10 (chemokine receptor 10) and CCR4 chemokine receptors [66]. Given their fundamental role in regulating immune tolerance and tumor growth, Tregs have soon become an interesting target for the

development of new immune therapies against cancers as well as viral infections and autoimmune diseases.

1.3 FOXP3

The role of *FOXP3* gene as an important player in the differentiation and function of Tregs was first discovered in 2001 with studies in the *scurfy* mice [67]. The animals containing *scurfy* mutations developed a multi-organ $CD4^+$ lymphocytes infiltration and an inability to regulate the immune response that caused their death soon after birth. Subsequently, characterization of *FOXP3* as the defective gene in *scurfy* mice led to its association with the genetic defects causing human IPEX (Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) fostering a better understanding of its role in immune regulation [67, 68]. The *FOXP3* gene is located on the short arm of the X chromosome and is composed by 11 exons [69, 70]. Its importance in regulating the immune response has been confirmed by several polymorphisms studies. SNPs (single nucleotide polymorphisms) in the promoter region have been associated with IPEX, type 1 diabetes (T1D) and Graves' disease [67, 71, 72]. Additionally polymorphisms in the intron and exon regions have been correlated to psoriasis and IPEX [73].

The expression of *FOXP3* on Tregs is regulated by different factors. Previously reported positive regulators of *FOXP3* are TGFβ, Foxo proteins, Runt related transcription factor 1/3 (RUNX1/3), retinoic acid, activator protein 1 (AP1) and nuclear factor of activated T cells (NFAT). Cytokines such as interleukin 4 (IL4), IFNγ and interleukin 6 (IL6) have instead are thought to be negative regulators of *FOXP3*. Species

comparison studies revealed the presence of four non coding sequences (CNS) regions. These CNS regions have specific epigenetic features and regulate *FOXP3* gene expression by interacting with other transcription factors (**Fig. 4**).

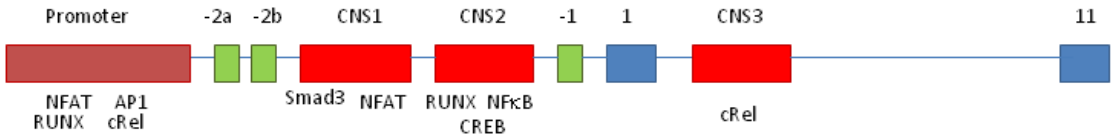


Fig. 4 Conserved non coding sequences (CNS) regions in FOXP3 gene. There are four non coding sequences regions in *FOXP3* gene that includes the promoter and the enhancers CNS1, 2 and 3. These regions contain binding sites for several transcription factors that, together with specific epigenetic features of the regions, contribute to *FOXP3* gene regulation.

In contrast to conventional $CD4^+$ T cells, the *FOXP3* gene in Tregs has a demethylated promoter that can bind to NFAT, AP1, c-Rel, STAT5 (signal transducer and activator of transcription 5) and RUNX1 [35]. These interactions play a key role in the positive regulation and stability of the *FOXP3* gene. The last three CNS regions operate as enhancers and their interaction with other transcription factors is fundamental for *FOXP3* gene expression. CNS1 contains binding sites for NFAT and Smad3 (SMAD family member 3), while CNS2 contains binding sites for NFκB (nuclear factor kappa b), CREB (cAMP response element binding) and RUNX1 [25]. This conserved region is of particular interest because it can be used to distinguish different Tregs subpopulations. Floess et al. in fact, demonstrated that CNS2 is demethylated in thymic derived Tregs, while it is fully methylated in induced Tregs [74]. Finally CNS3 contains c-Rel binding element.

FOXP3 protein is a 431 aa transcription factor that belongs to the forkhead/winged-helix box containing protein P (FOXP) subfamily which includes other three members (FOXP1, FOXP2 and FOXP4). Like the other forkhead subfamily proteins, FOXP3 is characterized by a highly conserved C-terminal forkhead DNA binding domain (exons 9-11), centrally located zinc finger and leucine zipper oligomerization domains (exons 5-7) and finally a N-terminal proline rich domain that enables FOXP3 to interact with other transcriptional repressors or activators (exons 2-4). This last region is unique for FOXP3 and it differs from the glutamine-rich domains that characterize the other FOXP subfamily members [75]. FOXP3 can regulate gene expression by directly binding to the promoter region or by cooperating with other transcription factors (**Fig. 5**). NFAT and RUNX1, for example, are fundamental partners of FOXP3 [76, 77]. NFAT is an important transcriptional regulator involved in T cell activation and homeostasis. In activated T cells, it forms a complex with AP-1 that allows the modulation of gene expression. It has been shown that FOXP3 binds to NFAT through its forkhead domain and that it can compete with AP-1 for this interaction. The cooperation between FOXP3 and NFAT leads to the repression of genes otherwise up-regulated by the complex NFAT-AP1 (for example IL2), but can also lead to up-regulation of other genes highly expressed on Tregs such as CTLA4 and CD25 [76]. FOXP3 interacts with RUNX1 through the loop region between the leucine zipper and forkhead domains. Over-expression of RUNX1 has been associated with an increase of IL2 production in $CD4^+$ T cells while the physical interaction between FOXP3 and RUNX1 leads to the repression of *IL2* gene expression [77]. Other important partners of FOXP3 mediated genes expression are chromatin-remodeling complexes formed by

HDAC7/9 (histone deacetylase 7 and 9) with TIP60 (histone acetyl-transferase) and Eos (a member of the Ikaros family). They have been shown to associate with the repressor N- terminal domain of FOXP3 and to promote its transcriptional function [78]. The proline-rich domain has been shown also to contain the binding site for RORγT and retinoic acid-related orphan receptor (RORαT), two transcription factors critical for Th17 differentiation [79]. This interaction inhibits their ability to activate transcription and, therefore, induces differentiation of $CD4^+$ T cells toward a Tregs rather than a Th17 phenotype.

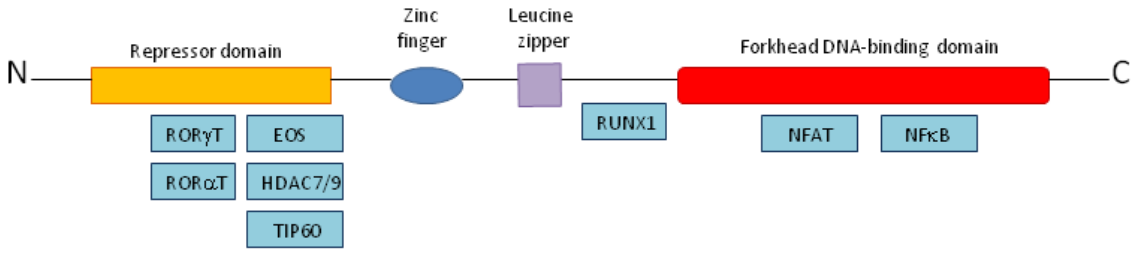


Fig. 5: Structure of FOXP3 domains and its main transcriptional partners. FOXP3 structure is characterized by four functional domains, each of which interacts with different binding partners. The N-terminal repressor domain binds to RORγT and RORαT and to the chromatin remodeling complex formed by HDAC7/9, TIP60 and EOS; RUNX1 binds to a loop region downstream the leucine zipper domain; the forkhead domain binds to NFAT and NFκB.

In contrast to what is described in mice, several FOXP3 isoforms have been found in human Tregs. The two most abundant isoforms are the full length (FL) and the so called "Delta 2" (Δ2) that lacks exon 2. Although both isoforms contribute to Tregs phenotype and confer some suppressive ability to Tregs, they seem to have functional differences

[80]. Exon 2 (amino acids 71-105) is located within the proline rich repressor domain of FOXP3 and contains the binding site for RORγT and RORαT. These two transcription factors positively regulate Th17 differentiation by inducing IL23 receptor. The physical interaction between FOXP3 and RORγ/αT results in the inhibition of ROR-mediated transcriptional activation and, therefore, suppresses the differentiation of $CD4^+$ T naïve cells toward a Th17 phenotype. The lack of exon 2 makes FOXP3 Δ2 unable to interact with the ROR transcription factors and allows the Th17 driven immune response to take place [79, 81, 82]. Furthermore Magg et al. showed that exon 2 contains a nuclear export sequence and that the lack of exon 2 in FOXP3 Δ2 leads this isoform to localize preferentially to the nucleus, compared to its counterpart FL [83]. Alternative splicing is a strictly regulated process and the mechanisms that contribute to the different FOXP3 isoforms are not yet fully elucidated. An Italian group recently showed that glycolysis is important for the generation of human induced Tregs and that the localization of the glycolytic enzyme enolase-1 directly contributes to the induction of FOXP3 FL isoform [84]. Similarly, a group from Sweden showed a correlation between the induction of FOXP3 Δ2Δ7 and the pro-inflammatory cytokine IL1β [85]. FOXP3 Δ2Δ7 is a less common isoform previously described as functionally defective. I*n vitro* experiments in murine cells transduced exclusively with FOXP3 Δ2Δ7 showed that this isoform is unable to confer suppressive capacity to naïve $CD4^+$ T cells and to induce the expression of Tregs markers such as CTLA4 and CD25 [86]. Furthermore, *in vivo* experiments in mice exclusively expressing this spliced isoform exhibited a multi-organ inflammation [87]. This rare isoform has been found to be over-expressed in patients with Sézary Syndrome and in transformed malignant T cells. In addition, FOXP3 Δ2 was found over-

expressed in malignant T cells [88]. The role of FOXP3 Δ2 in autoimmunity is not well defined. Ryder et al. did not find any differences between the two main isoforms expression in patients with arthritis rheumatoid and psoriasis [89], while conflicting data have been reported in inflammatory bowel disease (IBD) patients [85, 90]. A better understanding of the role that this alternative isoform plays within the immune regulation process could give rise to new immune therapies in several domains such as autoimmunity and cancer.

1.4 Alternative splicing: an important regulator of the immune system

Alternative splicing is the molecular process that splices a primary transcript in several ways and generates multiple mature mRNAs [91]. This is a tightly coordinated process that depends on the way the spliceosome binds to the genic splicing sites and on the regulation performed by the heterogeneous nuclear ribonucleoparticle protein. The most common types of splicing are the so called cassette exons in which specific exons are spliced out or included in the final mRNA; the mutually exclusive exons in which only one or the other exon is spliced out; and the alternative 3' or 5' splice event in which one or both ends are altered and the intron retention in which an intron is not excised from the pre-mRNA construct (**Fig. 6**). It is estimated that a wide range of genes (40-60%) are subjected to alternative splicing [92] and many of them are part of the immune system. In particular, alternative splicing has been shown to be important for regulating the response of T cells to antigens, therefore, playing an fundamental role in maintaining T cells homeostasis [93, 94]. CD45 protein exemplifies how alternative splicing can

regulate T cells functions [93]. This molecule is expressed on the surface of T cells as one of three different isoforms (CD45RA, RB and RC) depending on the presence of three variable cassette exons. Naïve T cells express one to two cassette exons (CD45RB, CD45RAB/RBC), while the isoform lacking all three exons (CD45RO) is present on the surface of memory T cells [95]. CTLA4 is another molecule reported to be subjected to alternative splicing (reference).. This co-inhibitory protein is upregulated on activated T cells and contributes to inhibition of cell proliferation and function by binding to CD80/CD86 molecules expressed on APC. A soluble form of CTLA4 protein has been described. It lacks exon 2 that contains the binding sites for CD80/CD86 and, therefore, plays a fundamental role in maintaining immune tolerance and homeostasis [96]. Altered expression of alternatively spliced isoforms has been correlated to several diseases. Increased expression of the soluble form of IL6 receptor, for example, has been linked to an increased severity of asthma, rheumatoid arthritis and systemic sclerosis [97]. Similarly, the expression of the truncated soluble isoform of IL7Rα is significantly higher in subjects with acute lymphoblastic leukemia [98] and spliced isoforms of both IL5 receptor and IL4 have been correlated to asthma [99]. There is a growing interest over the last few decades in the mechanisms involved in the regulation of alternative splicing. Studies in plants have shown that environmental factors can tightly modulate the alternative splicing process and that this regulation depends exclusively on the need of the plants [100]. Furthermore, alternative splicing events in Alzheimer's disease have been related to aging and environmental factors [101]. A better understanding of how external components contribute to epigenetic changes within the cells would promote

identification of additional targets for new therapeutic approaches and may lead to a more personalized medicine.

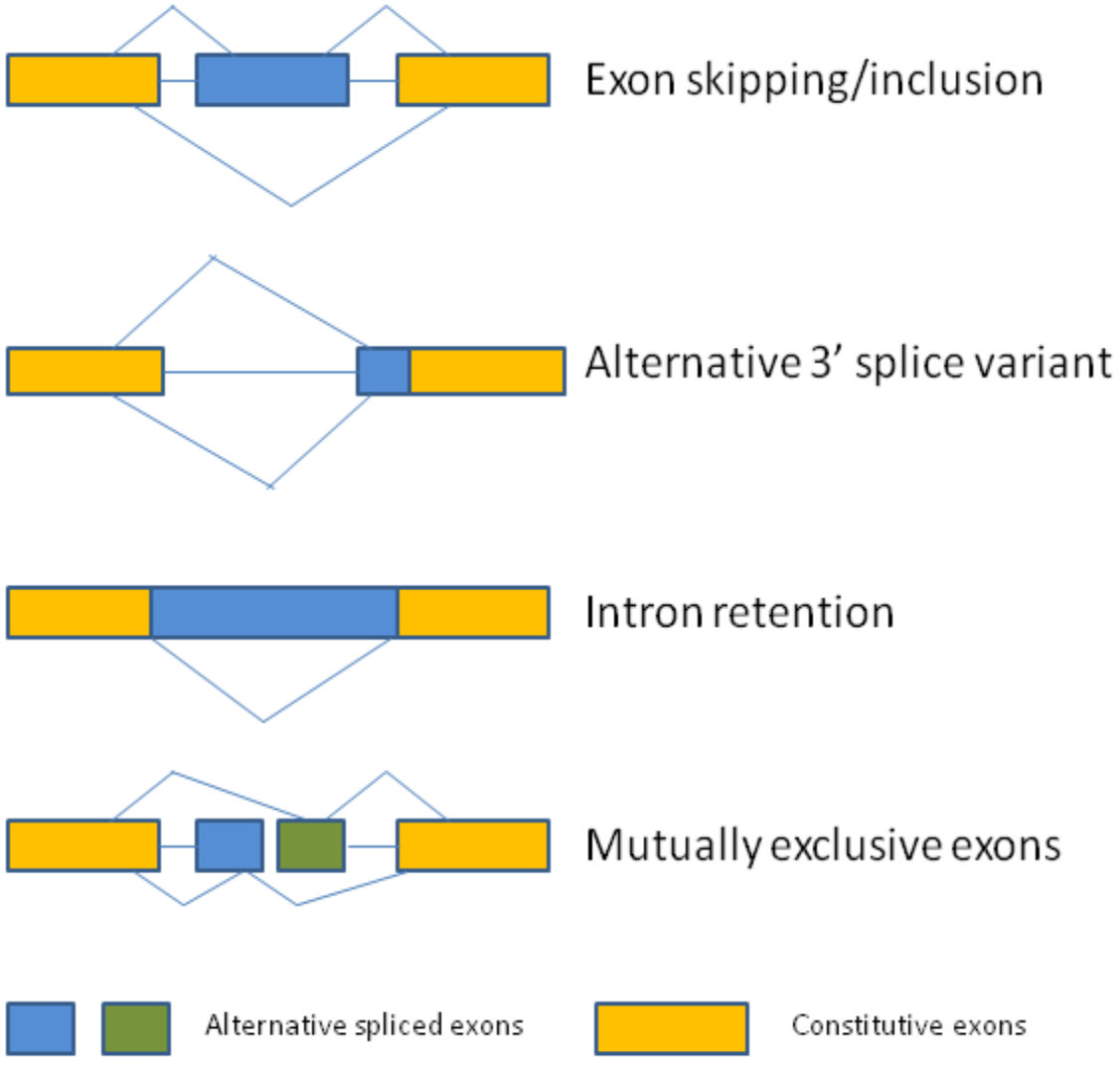


Fig. 6: Schematic representation of alternative splicing. The most common types of alternative splicing consist in the inclusion or skipping of one or more exons variations in the 3' or 5' end, the retention of an intron or the mutual exclusion of one or more exons.

1.5 Interplay between the immune system and microbiome regulates intestinal homeostasis.

The GI tract represents a permissive site for Tregs to originate and accumulate. While the intestinal pTregs population is shaped by exogenous antigens (Ag) derived from the lumen (for example orally fed Ag) and self-Ag expressed by cells present in the GI tract [102], additional factors contribute to the induction of tolerance in the intestine. TGFβ is highly expressed in the gut and is reported to trigger the expression of FOXP3. Its regulatory function is enhanced by DCs via retinoic acid. T his metabolite of vitamin A has been shown to be fundamental in originating Tregs in the GI tract not only by contributing to FOXP3 expression, but also by inducing the expression of gut-homing receptors on T cells (CCR9 and α4β7) [103]. Tregs present in the GI tract play a major role in regulating the onset oral tolerance and the dualistic relationship between the immune system and the intestinal microflora. The term "oral tolerance" defines the suppression of immune responses to antigens that have been previously administered by the oral route [104]. It consists of two main phases: the first contributes to prevent inflammation and to generate a correct immunological environment while the second establishes an Ag-specific anergy and induction of Tregs.

The importance of communication between immune system and microflora has been well established. The microbiome has been shown to be fundamental for the shaping of the immune system and Tregs contribute to the restraint of normal effector responses to commensal bacteria [105]. The major role that the microbiome plays in regulating Tregs differentiation has been reported by several groups by *in vivo* experiments showing a reduced Tregs population in the intestinal lamina propria of germ-free mice [106, 107]. Furthermore, specific bacteria are associated to Tregs induction

and function. *Clostridium spp*. represents an important component of the gut microbiome. These microorganisms belong to the *Firmicutes phylum* and they are present both in the small intestine and in the colon [108]. *In vivo* experiments in demonstrated that *Clostridium spp*. related to clusters IV and XIVa are significant inducers of colonic Tregs [107]. These bacteria are able to regulate the composition of the immune system by targeting lamina propria DCs. In an inflammatory environment, DCs are conditioned to secrete IL23 and to up-regulate the effector T cells. Conversely, at steady state, *Clostridium* bacteria are able to trigger DCs to produce retinoic acid and, therefore, to induce a regulatory immune response [108]. The role that *Clostridum spp*. play in the intestinal immune-homeostasis has been confirmed also by studies in IBD patients that showed a significant reduction of these bacteria when compared to healthy controls [109]. Round at al. have reported that *Bacteoroides fragilis* is able to induce differentiation of $CD4^+$ naïve T cells into Tregs and to enhance their suppressive function in mice [18]. The regulatory proprieties of this microorganism are related to the production of polysaccharide A (PSA). The recognition of this immunomodulatory molecule by TLR2 expressed on DCs triggers FOXP3 expression and subsequent IL10 release. Treatment with PSA has been shown to prevent and cure experimental colitis in mice and the same regulatory effect has been shown to occur also in human cells [110]. Another example of microorganisms that modulate the immune response is given by *Lactobacilli*. Bacteria belonging to this genus have beneficial effects on the host and are often used as probiotics. The valuable proprieties of *Lactobacilli* depend in part on their ability to produce lactate. *In vitro* studies in monocytes and macrophages have shown that this metabolite reduces TLR4 mediated induction of pro-inflammatory IL1β and

NLPRP3 inflammasome. These results were confirmed subsequently in mice that showed a reduction of organ injury and inflammation [111]. In addition, lactic acid bacterium *Pedicoccus acidilactici* suppress autoimmune encephalitis in EAE mice and to induce IL10 producing regulatory T cells [112]. Furthermore, lactate triggers regulatory phenotype on DCs [113]. Interestingly, the anti-inflammatory properties of this microbial derived metabolite appear to influence only naïve $CD4^{+}CD25^{-}$ cells and not nTregs [114]. Finally, butyrate, propionate and acetate are short chain fatty acids (SCFAs) that are produced from the gut commensal microflora and have been reported to contribute to the modulation of the intestinal immune system. The immune cells express G protein coupled receptors (GPR41 and GPR43) specific for SCFAs, therefore making them particularly susceptible to the modulatory effect of these metabolites [115]. Butyrate in particular has been described as a potent immuno-regulator because of its capability of preventing Ag induced T cells activation and reducing IFNγ production while triggering IL10 release [116]. It has been shown also to modify recruitment of leucocytes to the inflammatory site and to affect *in vitro* neutrophil chemotaxis [117]. Lastly, several groups have reported that SCFAs, and in particular butyrate, are able to induce FOXP3 expression on colonic Tregs by acting as potent deacetylase inhibitor and, therefore, regulating gene expression [118, 119]. Given the importance of the interplay that exists between the regulatory components of the immune system and the intestinal microflora, a better understanding of the mechanisms behind it could lead to possible therapies in order to treat and prevent the onset on several conditions such as allergies, chronic infections and autoimmunity.

1.6 Scope of dissertation

The aim of this dissertation is to determine if alterations in the expression of FOXP3 isoforms may at least partially account for the defects in the regulatory immune system that contribute to the onset and development of CD. In particular my work has focused on measuring the expression of less functional isoform, FOXP3 Δ2, in CD patients. Furthermore, I assessed the interactions between Tregs and microbiome-derived metabolites that have been described previously to characterize CD patients at different stages of the disease [22].

CHAPTER 2. MATERIALS AND METHODS

2.1 Human subjects: Whole blood was obtained by venipuncture from adult patients (between 16 and 65 years old) similarly distributed between both sexes during routine visits to our clinic at Massachusetts General Hospital. They included non-celiac control subjects (HC), celiac patients in remission following gluten free diet (CDGF) with normal duodenal pathology (Marsh 0-II) and active celiac patients (CDA). Small intestinal biopsies were obtained from the second portion of the duodenum according to standard clinical procedure from HC, CDGF and CDA during clinically-indicated upper endoscopic procedures. Patients with CD in the cohort were diagnosed based on pathological evaluation (Marsh III at time of diagnosis) and positive serology results for anti-human tissue transglutaminase IgA antibodies (INOVA Diagnostic) (manufacturer instructions were followed and samples were evaluated positive if values of TtG-IgA $\geq$20 were detected). Patients were considered CDGF if, at the time of specimens' collection, they have been following a gluten free diet for at least 6 months and presented negative serology and normal intestinal mucosa (Marsh 0 to II).

All protocols were approved by the Partners Human Research Committee Institutional Review Board and written informed consent was obtained from all enrolled patients and controls.

2.2 Peripheral blood mononuclear cells (PBMC) isolation*:* PBMC were isolated from whole blood samples by density gradient centrifugation over Ficoll (GE Healthcare). Briefly, whole blood was mixed with the same volume of DPBS 1X (Gibco Life technologies). This mix was then layered on Ficoll (2:1) and centrifuged at room temperature for 30 minutes at 2,100 rpm without brake. The ring containing the PBMC

was collected and washed twice with 10 ml of DPBS 1X at 1,200 rpm for 10 minutes with brake high. Number and viability of cells were determined using Trypan Blue dye. Cells were frozen in 1 ml of complete medium RPMI 1640 Dutch modification (Gibco) supplemented with 1% L-glutamine, 1 % penicillin/streptomycin, 1% sodium pyruvate and 1% non essential amino acid solution (Gibco) with 20% FBS (Sigma-Aldrich) and 10 % DMSO (Sigma) and stored in liquid nitrogen until use.

2.3 Isolation of $CD4^+$ cells from small intestinal biopsies: Four biopsies were collected in ice-cold RPMI 1640 complete medium enriched with 10% FBS. The specimens were then incubated for 20 minutes at room temperature in calcium and magnesium free HBSS 1X (Gibco) and 1mM DTT to eliminate mucus in excess. Biopsies were then incubated twice at 37 °C for 30 minutes under tilting in calcium and magnesium free HBSS 1X and 1mM EDTA to remove the epithelium. Every incubation was followed by a brief wash in in calcium- and magnesium-free HBSS 1X. Finally, the intestinal lamina propria was incubated up for 60 minutes at 37 °C under tilting with RPMI 1640 complete medium enriched with 2% FBS containing 20 µg/ml of DNAse I (Roche) and 1 mg/ml of collagenase (Sigma). After digestion, PBMCs were isolated by Ficoll. Dynabeads $CD4^+$ positive isolation kit was used to isolate $CD4^+$ cells following manufacturer instructions (Invitrogen).

2.4 PBMC stimulation: After isolation, PBMC were cultured ($1*10^6$ cells/ml) for 24 or 48 hours in complete medium RPMI 1640 Dutch modification, supplemented with 10% FBS, 1% L-glutamine, 1 % penicillin/streptomycin, 1% sodium pyruvate and 1% non essential amino acid solution with 1 mg/ml of previously digested gliadin (Sigma), 100

ng/ml of IFNγ (eBioscience), 1 μg/ml lactate (Sigma) or butyrate (Sigma) and combination of IFNγ and butyrate at 37 °C, 5% CO_2.

2.5 Digestion of gliadin: Whole gliadin (Sigma) (100 gr/L) was dissolved in 0.2 M of hydrogen chloride (Sigma) (pH between 1.8 and 2.0) with continuous stirring. Pepsin (1 gr, Sigma) was added and the solution was incubated at 37°C for 4h with continuous stirring (pH adjusted to 7.4). The solution was digested further with the addition of 1gr of trypsin (Sigma) and continuous stir. The solution was then boiled for 30 minutes and lyophilized. Digested gliadin was confirmed negative for endotoxins contamination (Reagent?) and stored at room temperature until use.

2.6 Isolation and stimulation of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells*:* $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were isolated from PBMC with Dynabeads $CD4^+CD25^+$ Treg cells isolation Kit (Invitrogen). $CD4^+CD25^-$ T cells were isolated by negative selection wand stimulated with 1 μg/ml of lactate or with a combination of 1μg/ml of butyrate and 100 ng/ml of IFNγ for 48 hours in complete RPMI 1640 medium enriched with 10% FBS, 50 U/ml of rIL2 (eBioscience), CD3/CD28 dynabeads (Invitrogen). After having negatively selected the $CD4^+CD25^-$ T cells, Treg cells were isolated by positive selection from the remaining cells population, expanded in complete RPMI 1640 medium enriched with 10%FBS, 500U/ml of rIL2, CD3/CD28 dynabeads and cultured in medium or with the different stimuli for 48 hours. Purity of the two T cell populations was higher than 90% and determined by flow cytometry. Briefly, isolated T cells were stained with antibodies PerCP conjugated anti-CD4 (eBioscience) and PE conjugated anti-CD25 (eBioscience). Cells were then permeabilized with FOXP3 Staining Kit (eBioscience) and intracellularly

stained for FOXP3. Detection of $CD4^+CD25^-FOXP3^-$ and $CD4^+CD25^{++}FOXP3^+$ cells was performed with BD Bioscience software.

2.7 Isolation and stimulation of HLA-DR$^-$ cells: HLA-DR$^-$ cells were isolated by negative selection from PBMC of HC and CD subjects with anti-human HLA-DR microbeads (Militeny Biotec) and LS MACS columns (Militeny Biotec) following manufacturer directions. HLA-DR$^-$ cells were then cultured for 48 hours in complete RPMI-140 medium alone or with the addition of 1mg/ml of lactate.

2.8 RNA extraction from biopsies, isolated CD4$^+$ T cells, PBMC and HLA-DR$^-$ cells: Total RNA from PBMC, $CD4^+CD25^-$ T cells, $CD4^+CD25^+$ Treg cells and whole biopsies was extracted with Trizol reagent (Ambion) following the manufacturer's directions. Briefly previously homogenized biopsies and isolated cells were re-suspended in 1 ml of trizol and vortexed for 30 seconds. 100 µl of chloroform (Sigma) was added to the trizol solution and vortexed briefly. After 5 minutes of incubation on ice, the tubes were centrifuged at 4°C for 15 minute at 14,000 rpm and the clear liquid containing the RNA was collected. The same amount of isopropanol (Sigma) was added to the RNA solution and incubated at 4°C for at least 1 hour. The tubes were then centrifuged at 4°C for 20 minutes at 14,000 rpm and the supernatant was discarded. 500 µl of ethanol (Sigma) were used to wash the RNA pellet and the tubes were centrifuged at 4°C for 10 minutes at 10,000 rpm. Finally the supernatants were discarded and the RNA pellet was re-suspended in RNAse/DNase free water (Gibco). Given the small number of cells, RNA from CD4$^+$ T cells isolated from intestinal lamina propria was extracted with RNAse microkit (Qiagen) following manufacturer instructions. Isolated RNA was stored at -80°C until use.

2.9 Reverse transcription and real time RT-PCR: All RNAs were treated with DNAse I to remove traces amounts of DNA following the manufacturer's instructions (Fermentas). Reverse transcription was performed on 1 μg of total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas) and following the manufacturer's instructions. PerfeCta SYBR Green SuperMix (Quanta Bioscences) was used for the Real-Time PCR (in duplicate for each sample) with the following cycling conditions: 10 minutes at 95° C, followed by 40 cycles of 15 seconds at 95° C, 30 seconds at 60° C and 30 seconds at 72° C, finally 1 minute at 95° C and 1 minute at 65° C. Primers sequences used are listed in the **Table 2.**

Total *FOXP3* was detected by primers that amplified a region included between exon 10 and 11 of the gene where no alternative splicing sites have been reported; *FOXP3* FL was recognized by primers that spanned exon 2 and *FOXP3* Δ2 was detected by primers that spanned the junction between exon 1 and 3. The mRNA levels of genes of interest were normalized to the house-keeping gene 18S. Relative expression of the different genes was calculated as $2^{-\Delta\Delta Ct}$ and normalized on house-keeping gene *18S*. To measure the ratio between *FOXP3* isoforms, we determined the relative expression of each isoform and individually calculated their ratio for each sample ($2^{-\Delta\Delta Ct}$ Δ2/$2^{-\Delta\Delta Ct}$ FL or $2^{-\Delta\Delta Ct}$ FL/$2^{-\Delta\Delta Ct}$ Δ2).

GENE	**SEQUENCE**
18s Forward	5'-AACCCGTTGAACCCCATT-3'
18s Reverse	5'-CCATCCAATCGGTAGTAGCG-3'
FOXP3 FL Forward	5'-CCATCCAATCGGTAGTAGCG-3'
FOXP3 FL Reverse	5'-GCCTTGAGGGAGAAGACC-3'
FOXP3 Δ2 Forward	5' CAGCTGCAGCTCTCAACGGTG -3'
FOXP2 Δ2 Reverse	5' –GCCTTGAGGGAGAAGACC-3'
FOXP3 TOT Forward	5'-ATCTACCACTGGTTCACACG-3'
FOXP3 TOT Reverse	5'-CTCCTTTCCTTGATCTTGAG-3'
RORγT Forward	5' –CCTGGGCTCCTGCCTGAC- 3'
RORγT Reverse	5'- TCTCTCTGCCCTCAGCCTTGCC-3'
IL6 Forward	5'-CCAGAGCTGTGCAGATGATGTA-3'
IL6 Reverse	5'- TTGGGTCAGGGGTGGTTATTG-3'
Tbet Forward	5'- GATGCGCCAGGAAGTTTCAT -3'
Tbet Reverse	5'- GCACAATCATCTGGGTCACATT-3'
IL10 Forward	5'-GGGGCTTCCTAACTGCTACA-3'
IL10 Reverse	5'-TCCGAGACACTGGAAGGTGA-3'
CTLA4 Forward	5'-TCCTTGATTCTGTGTGGGTTC-3'
CTLA4 Reverse	5'-TTGCAGAAGACAGGGATGAA-3'
IL17A Forward	5'-ACTACAACCGATCCACCTCAC-3'
IL17A Reverse	5'-ACTTTGCCTCCCAGATCACAG-3'

Table 2. Primers sequences of human genes examined by real time RT-PCR.

2.10 Western Blot: Protein was isolated from PBMC before and after stimulation with butyrate and IFNγ by lysing the cells in 300 µl of RIPA buffer (Sigma) and protease inhibitors cocktail (Roche). Cells were agitated for 15 minutes at 4°C and centrifuged at 4,000 rpm for 15 minutes at 4°C. Protein concentrations were measured with DC protein assay (Biorad). Samples were denatured at 95°C for 5 minutes and separated on 4-20% Novex Tris-Glycine gels (Life Technologies) at 100 V for 2 hours. Proteins were then transferred to PVDF membranes (Life Technologies) using X Cell Blot Module (Invitrogen) at 25 V for 2 hours at 4°C. Membranes were blocked with 5% BSA and incubated with isoform non-specific α-FOXP3 (PHC101 eBioscience) at a concentration of 4µg/ml, followed by washing and incubation with secondary HRP-conjugated Antibody (1: 5,000) (GE Healthcare). B-actin protein (α–β actin Thermo-Fisher) was used to normalize the relative protein expression. Protein bands were visualized with Biorad machine by ECL Western Blotting Substrate kit (Abcam). Proteins quantification was performed with the Image Lab software version 5.0 (Biorad).

2.11 Macrophages differentiation and stimulation: $CD14^+$ cells were isolated from PBMC of CD and HC subjects by positive selection using CD14 Microbeads kit and LS MACS columns (Militeny Biotec) and following manufacturer instructions. $CD14^+$ monocytes were then cultured (2 X 10^5 cells/ml) overnight in complete RPMI 1640 medium supplemented with 10% FBS at 37°C, 5% CO_2. After overnight incubation the monocytes were stimulated for 24 hours with 1 µg/ml of lactate or a cocktail of cytokines composed by 20 ng/ml of rIL10, rIL4 (both Biolegend) and TGFβ (eBioscience). Another well without any added cytokines was used as negative control.

2.12 Flow cytometry: FOXP3 protein expression was measured in PBMC before and after stimulation with lactate by flow cytometry. After 48 hours cultured PBMC were washed for 10 minutes at 1,200 rpm with 3 ml of FACS buffer (DPBS 1X + 2mM EDTA + 0.5% FBS). Supernatant was discarded and cells from each well were incubated for 10 minutes at room temperature with 10 µl of FCR blocking (MACS). After that, antibodies PerCP conjugated anti-CD4 and PE conjugated anti-CD25 were used to stain the cells for 15 minutes at room temperature. Anti-IgG1 PercP and anti-IgG2a PE were used as isotype controls. Cells were then permeabilized with FOXP3 Staining Kit and stained with a FITC-conjugated antibody for FOXP3. Anti-IgG1 FITC (eBioscience) was used as isotype control. The number of $CD4^{+}CD25^{++}FOXP3^{+}$cells was determined with BD Bioscience software.

To determine the purity of isolated HLA-DR^{-} cells and CD14+ cells, we blocked the cells with FCR blocking for 10 minutes and stained them respectively with PercCP conjugated anti-human HLA-DR or FITC-conjugated anti-human CD14 antibodies for 15 minutes. IgG1 PerCp and IgG2a FITC were used as isotypes controls. The cells were then processed as previously described and the purity of the cells population was determined by BD Bioscience software (> 90%).

To examine the number of M2 macrophages after culture with different stimuli, we blocked the cells with FCR blocking for 10 minutes and we stained them with FITC conjugated anti-human CD206 antibody for 15 minutes (Biolegend). Anti-IgG1 FITC (eBioscience) was used as isotype control. The number of $CD206^{+}$ cells from each well was calculated with BD Bioscience software out of the gate representing the activated macrophages population based on FSC and SSC values.

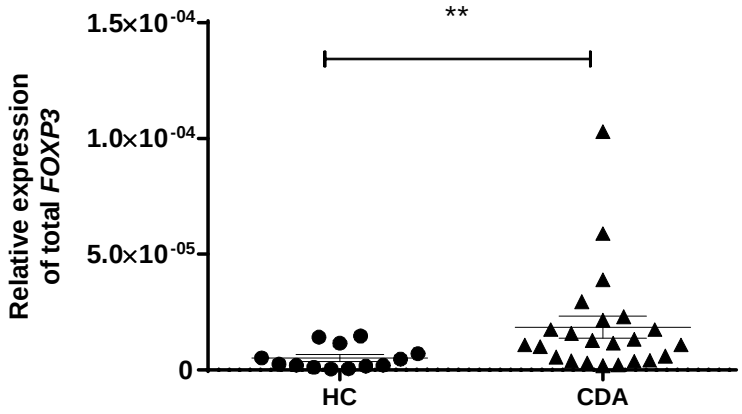
2.13 ELISA: Supernatants from macrophages (MΦ) cultured in medium alone or with lactate or cytokines cocktail were collected and stored at -80 degrees until use. The BD-Opteia IL10 ELISA set (BD Bioscience) was used to determine the concentration of IL10 produced by the cells in different conditions (assay's sensitivity was 7.8 pg/ml). Briefly, the plate was coated overnight with capture IL10 Ab (1:250) at 4°C. The next day the plate was washed three times with wash buffer 1X and incubated for 1h at room temperature with diluents buffer. Following, we incubated the plate with a previously prepared standards and un-diluted samples. The plate was then washed and incubated for 1h at room temperature with detector solution (containing 1:1000 of IL10 detector and 1:250 of SHRPV). Finally the plate was incubated for 30 minutes at room temperature in the dark with previously prepared TBT buffer. After adding stop solution, the absorbance was measured at 450 nm by SpectraMax M5 plate reader.

2.14 Statistics: Depending on the samples distribution and the number of groups to compare, the statistical analysis of the samples was performed by t-test, ANOVA test two-tailed Mann-Whitney test or Krustal Wallis (unpaired samples) and paired t-test, Wilcoxon test or Friedman test (paired samples) as appropriate with the GraphPad software. Standard error of the mean (SEM) was measured to determine the dispersion among data within the same group. In all statistical tests, a *p* value less than 0.05 was considered significant. Correlation between genes' expression was measured by two-tailed Spearman or Pearson correlation analysis depending on the samples distribution.

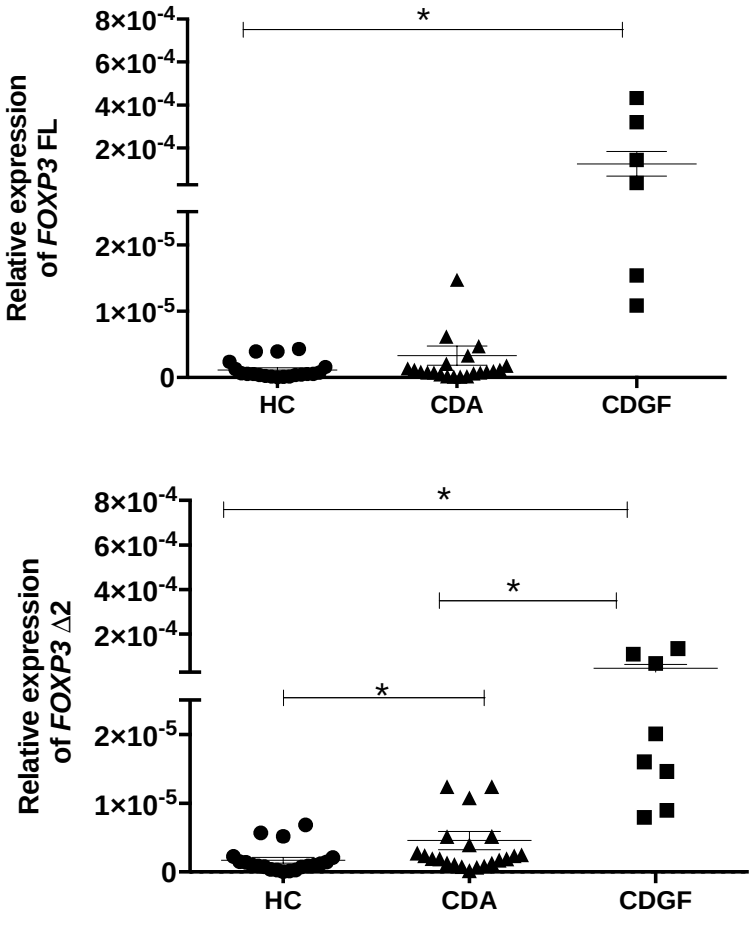
CHAPTER 3: IMBALANCE BETWEEN FOXP3 IFORMS IN ACTIVE CELIAC DISEASE.

3.1 Altered expression of *FOXP3* isoforms, *RORGT* and *IL17A* in the intestine of active celiac patients.

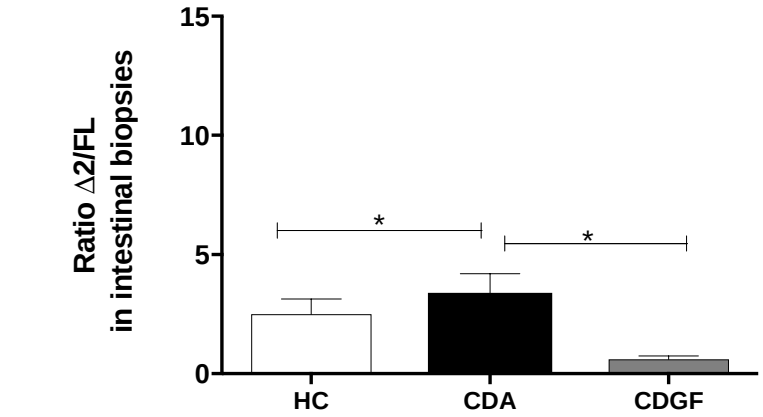
Unlike some autoimmune diseases, CD is characterized by an increased number of Treg cells during the active state of the disease [15]. This suggests that functional rather than quantitative defects of this T cell subset may contribute to the onset and final development of the disease. It has been described that IL15 plays a role in the defective suppressive function of Tregs in celiac patients [8]; however, the exact mechanisms by which this cytokine modulates the immune response are not fully elucidated. Given the inability of FOXP3 Δ2 to down-regulate the differentiation of T cells toward a Th17 phenotype in CD patients, we hypothesized that an over-expression of this less functional FOXP3 isoform is related to the strong Th17 immune response that characterizes the active state of CD. We isolated RNA from whole small intestinal biopsies from HC, CDA and CDGF patients and determined the gene expression of total *FOXP3*, *FOXP3 FL* and *FOXP3 Δ2* by real time RT-PCR. As expected, the expression of total FOXP3 was significantly higher in CDA patients, as compared to HC ($p< 0.005$) (**Fig.7A**). Interestingly, while the expression of *FOXP3 FL* was similar between CDA and HC, the alternatively spliced isoform *Δ2* was significantly higher in CDA patients ($p< 0.05$) (**Fig.7B**). In CDGF patients both isoforms were expressed significantly more than in HC ($p< 0.05$). The analysis between *FOXP3* isoforms within the same groups of patients showed a similar expression between the two isoforms in HC subjects and CDGF patients, while in CDA patients the less functional *FOXP3 Δ2* was significantly more expressed than its counterpart FL ($p< 0.005$) (**Fig.7C**, **D**).


**
1.5×10-04
1.0×10-04
5.0×10-05
0
Relative expression of total FOXP3
HC
CDA

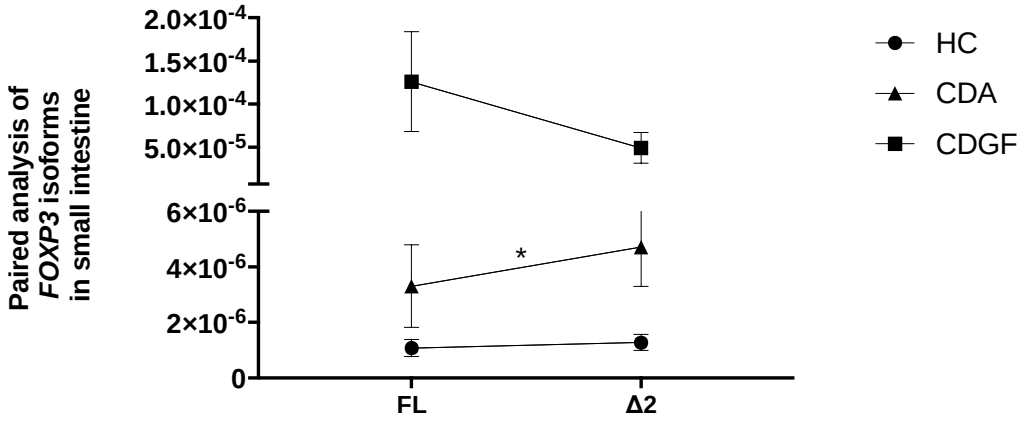
(A)


*
8×10-4
6×10-4
4×10-4
2×10-4
2×10-5
1×10-5
0
Relative expression of FOXP3 FL
HC
CDA
CDGF
*
*
*
8×10-4
6×10-4
4×10-4
2×10-4
2×10-5
1×10-5
0
Relative expression of FOXP3 Δ2
HC
CDA
CDGF

(B)



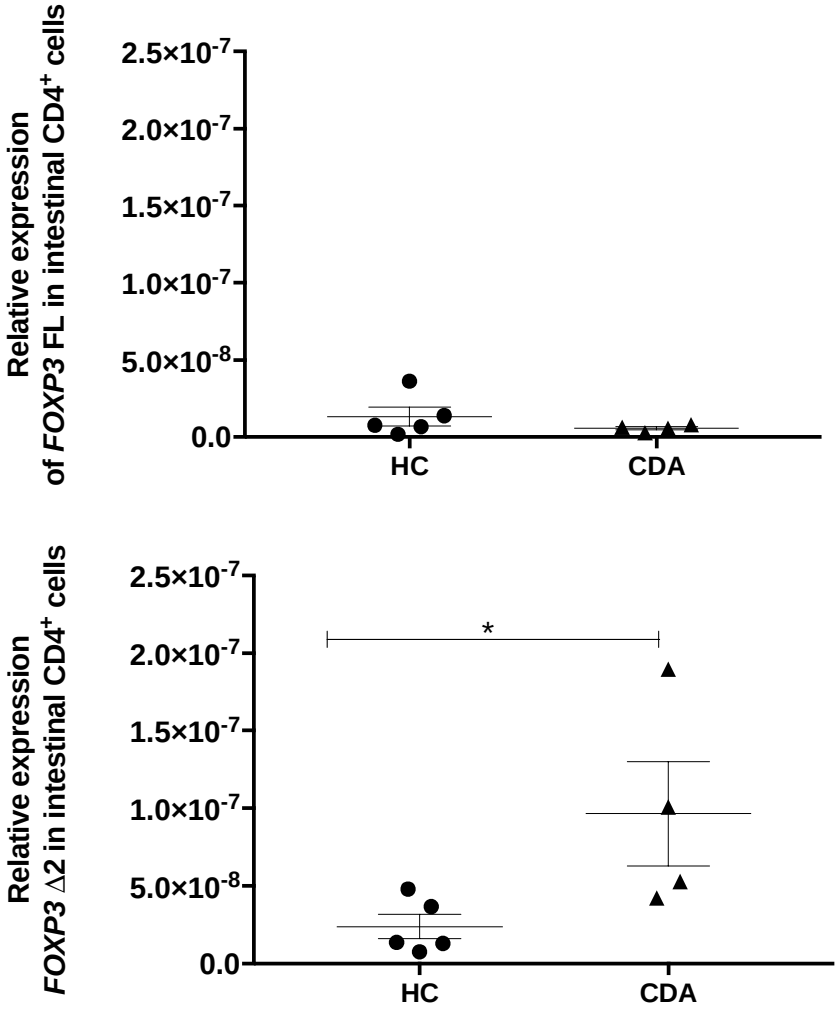
(C)



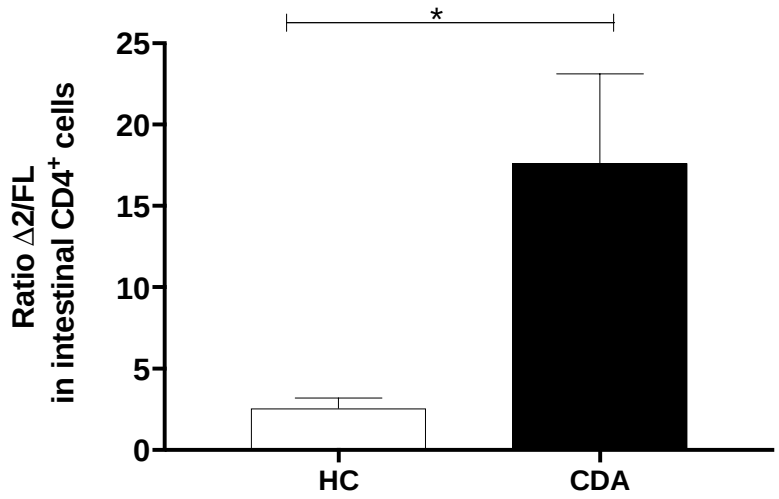
(D)

Fig. 7 Increased expression of *FOXP3* Δ2 in whole small intestinal biopsies of CDA patients. Relative expression of total *FOXP3* and each *FOXP3* isoform was measured in HC (*n*=20), CDA (*n*=22) and CDGF (*n*=8) subjects by real time RT-PCR (A,B). The ratio between the two isoforms (Δ2/FL) was calculated individually for each subject and compared among the three groups of patients. The bars show the mean among ratios ± SEM in HC (white), CDA (black) and CDGF (gray) subjects (C). Paired analysis was used to determine the differences between the two isoforms within the same group of patients (D). Each dot represents the mean among the mRNA expression values ± SEM. Statistical comparisons were made using Kruskall -Wallis test (A,B,C) and Friedman test (D). * $p< 0.05$, ** $p< 0.005$.

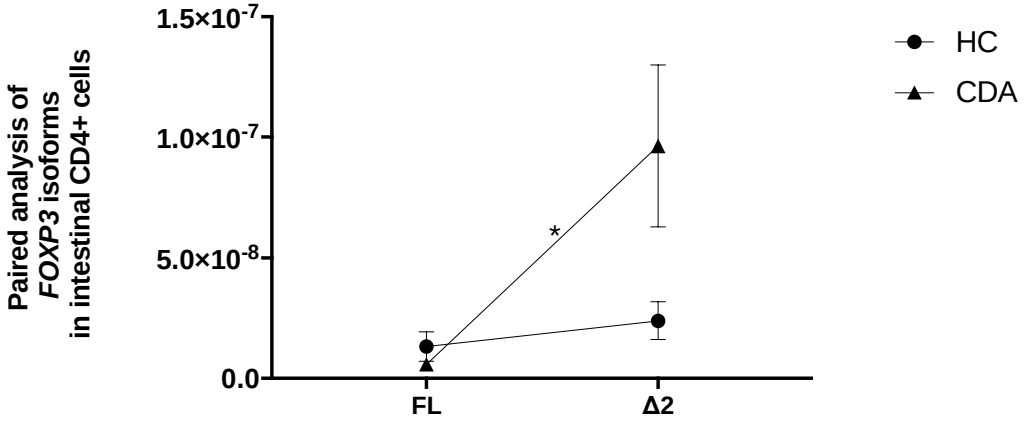
Experiments on $CD4^+$ cells isolated from the lamina propria of duodenal biopsies confirmed that the differences that observed between *FOXP3* isoforms ratio was due to this specific population and not to other FOXP3 expressing cells (**Fig. 8**).



(A)



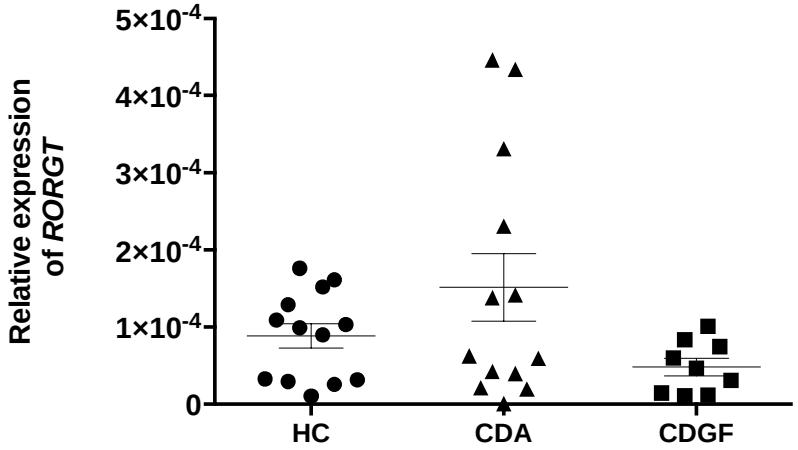
(B)



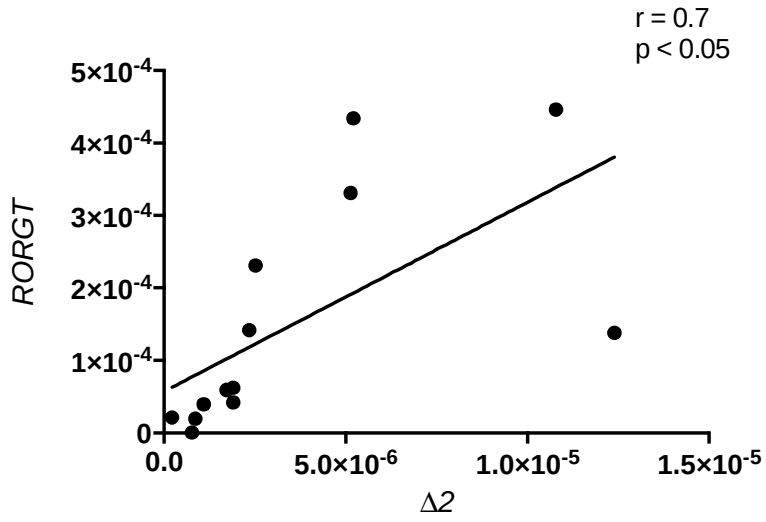
(C)

Fig. 8: Increased expression of FOXP3 Δ2 in CD4$^+$ cells isolated from small intestinal lamina propria of CDA patients. Real time RT-PCR was used to measure the relative expression of *FOXP3* isoforms in CD4$^+$ cells isolated from small intestinal biopsies in HC (*n*=5) and CDA (*n*=4) patients (A). The ratio between isoforms (Δ2/FL) was calculated individually for each subject and compared between the two groups (B). The bars show the mean among ratios ± SEM in HC (white), CDA (black). Paired analysis was used to further determine the differences between the two isoforms within the same group of patients (C). Each dot represents the mean among the mRNA expression values ± SEM. Real time RT-PCR data were normalized to house-keeping gene *18S*. Statistical comparisons were made by using Kruskall Wallis test (A,B) and Friedman test (C). * $p<0.05$.

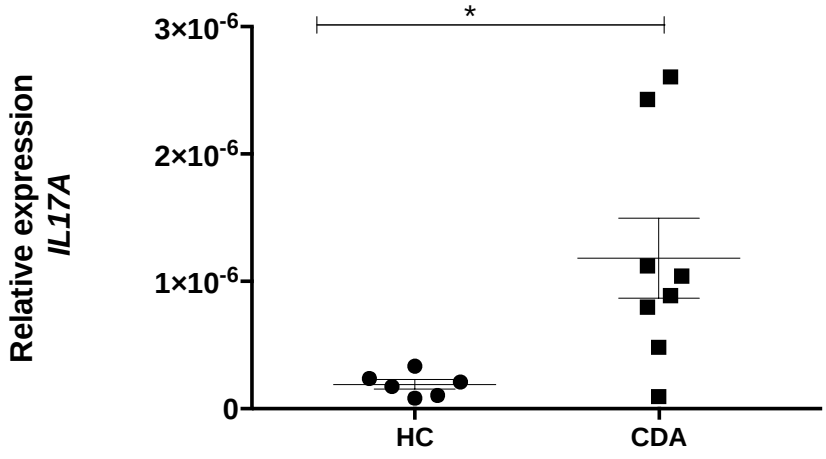
FOXP3 Δ2 has been shown to be defective in down-regulating the expression of RORγT and, therefore, the Th17 driven immune response [79]. We decided to measure the gene expression of *RORGT* in small intestinal biopsies from HC, CDGF and CDA subjects by real time RT-PCR. In CDA patients, *RORGT* expression was higher than in HC and CDGF subjects (**Fig. 9A**) and was positively correlated with *FOXP3 Δ2* expression *($p<0.05$)* (**Fig. 9B**). Measurements of the pro-inflammatory cytokine *IL17A* further confirmed not only the role of Th17 response in CDA patients, but also showed a significant positive correlation between the expression of *IL17A* and the alternatively spliced isoform *FOXP3 Δ2* (**Fig. 9C, D**). Although in a small amount of samples, the same increased expression of *RORGT* in CDA positively correlated with *FOXP3 Δ2* expression was observed also in $CD4^+$ cells isolated from small intestinal lamina propria (**Fig. 10**). Collectively these data highlight the presence of an imbalance between *FOXP3* isoforms in the small intestine of CDA patients. Our findings show that CDA patients are characterized by an increased expression of the less functional FOXP3 isoform that correlates with a higher expression of RORGT and pro-inflammatory cytokine IL17A. Given the defective role of the isoform *FOXP3 Δ2* in down-regulating *RORGT* expression, these findings suggest that the unbalanced ratio between FOXP3 isoforms may contribute to the Th17 induced pathogenesis of CD.


Relative expression of RORGT
5×10-4
4×10-4
3×10-4
2×10-4
1×10-4
0
HC
CDA
CDGF

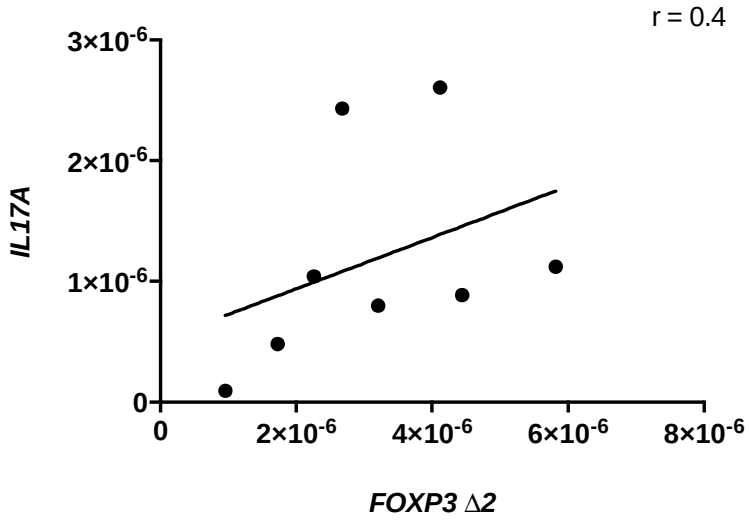
(A)


r = 0.7
p < 0.05
RORGT
5×10-4
4×10-4
3×10-4
2×10-4
1×10-4
0
0.0
5.0×10-6
1.0×10-5
1.5×10-5
Δ2

(B)

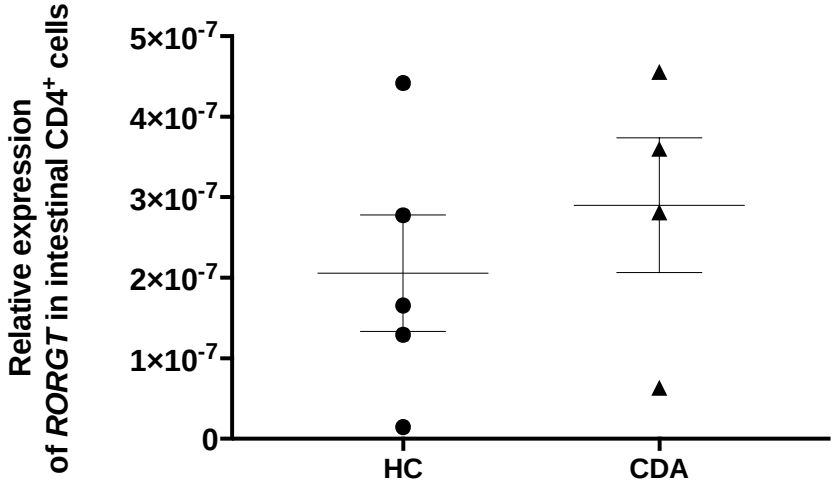


(C)

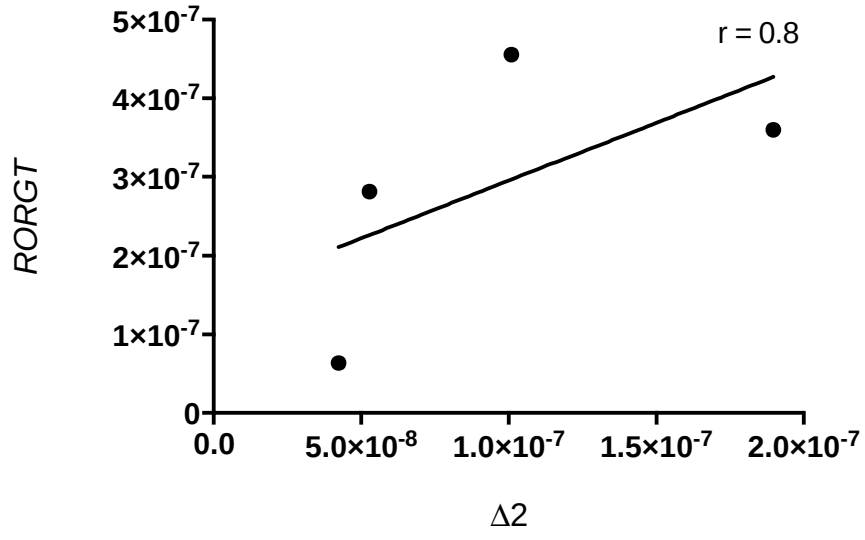


(D)

Fig. 9: Increased expression of *RORGT* and *IL17A* in small intestinal biopsies of CDA patients. Relative expression of *RORGT* was measured in HC (*n*=13), CDA (*n*=13) and CDGF (*n*=9) subjects by real time RT-PCR (A). The positive correlation (r=0.7) between the expression of *FOXP3 Δ2* and *RORGT* was determined in CDA subjects for which we had values for both genes (*n*=13) by two tailed Spearman correlation analysis. Relative expression of *IL17A* was measured in HC (*n*=6) and CDA (*n*=8) subjects by real-time RT-PCR (C). The positive correlation (r=0.4) between *IL17A* and *FOXP3 Δ2* expression was determined in CDA patients (*n*=8) by two tailed Pearson correlation analysis. Real time RT-PCR data were normalized to house-keeping gene *18S*. Statistical comparisons were made using One way ANOVA test (A) and Mann-Whitney test (C).



(A)

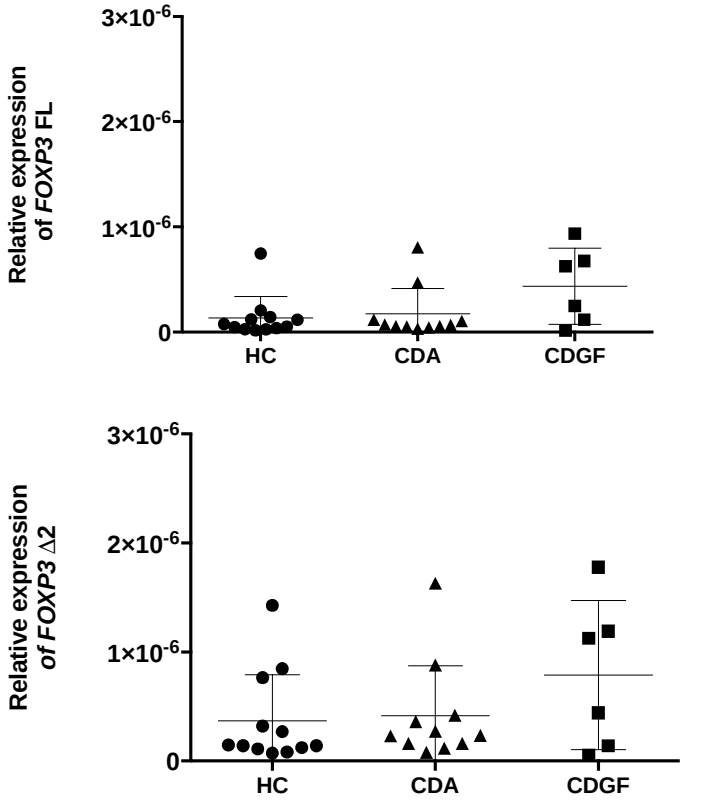


(B)

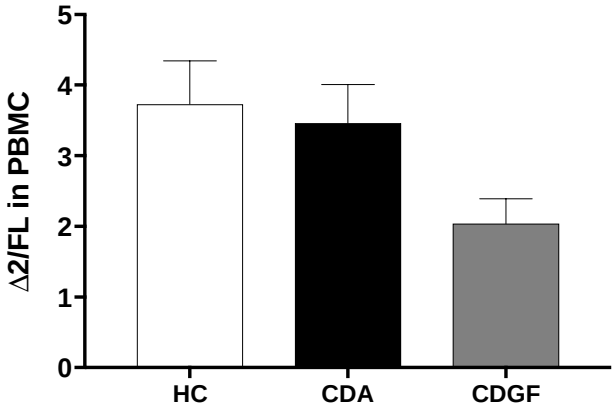
Fig.10: Increased expression of *RORGT* in CD4$^+$ cells isolated from small intestinal lamina propria of CDA patients. Relative expression of *RORGT* was measured in HC (*n*=5) and CDA (*n*=4) patients (A). Correlation between the expression of *RORGT* and *FOXP3 Δ2* was determined on CDA samples by two-tailed Spearman correlation analysis (B). Real time RT-PCR data were normalized to house-keeping gene *18S*.

3.2 The expression of *FOXP3* isoforms on peripheral blood mononuclear cells is similar between celiac patients and control subjects.

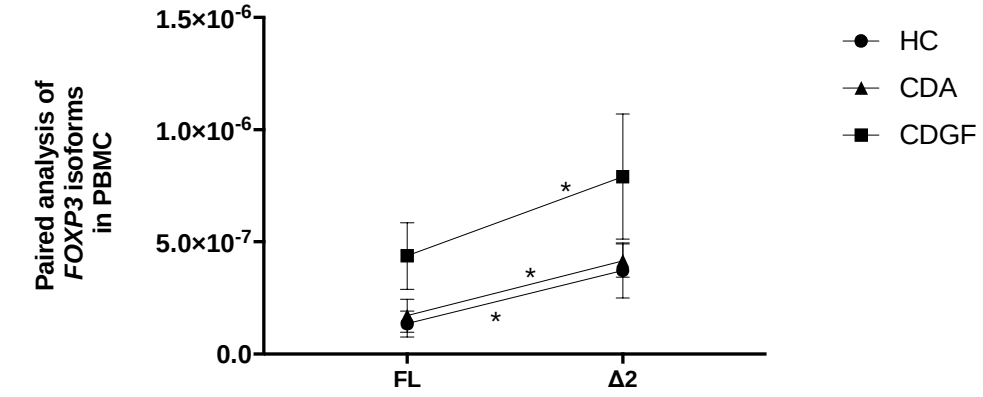
The intestine represents a unique and highly diverse microenvironment and it is not surprising that the immune cells that are located in the intestine do not share the same phenotypes and properties of those circulating in the blood stream. We aimed to test if the differences in *FOXP3 Δ2* expression found in the intestine of CDA patients represented an event restricted exclusively to the intestinal compartment. We measured by real time RT-PCR the gene expression of *FOXP3 FL* and *Δ2* in PBMC isolated from CDA, CDGF and HC patients. From our analyses we did not find any differences among the three groups of patients (**Fig. 11A**). Furthermore, while in the intestine of HC and CDGF the two *FOXP3* isoforms were similarly expressed, the data from PBMC showed that in all groups *FOXP3 Δ2* was significantly more expressed than its counterpart FL (**Fig. 11B, C**). In addition, the expression of *RORGT* was not higher in CDA patients, as compared to HC, emphasizing the importance of the ratio between the two major FOXP3 isoforms in regulating *RORGT* expression (**Fig.11D**). Overall, these findings show that in HC subjects the ratio between FOXP3 isoforms in circulating Tregs is different from the one in pTregs located in the intestine while in CD patients the less functional FOXP3 D2 is more expressed than its counterpart FL in both Tregs populations. We hypothesize that alterations within the gut milieu of CD patients may contribute to the development of the disease by modulating the ratio between FOXP3 isoforms. Given the heterogeneity of the PBMC population, however, more studies focused on the isolated $CD4^+$ peripheral T cells would be useful to confirm these conclusions.


Relative expression of FOXP3 FL
3×10-6
2×10-6
1×10-6
0
HC
CDA
CDGF
Relative expression of FOXP3 Δ2
3×10-6
2×10-6
1×10-6
0
HC
CDA
CDGF

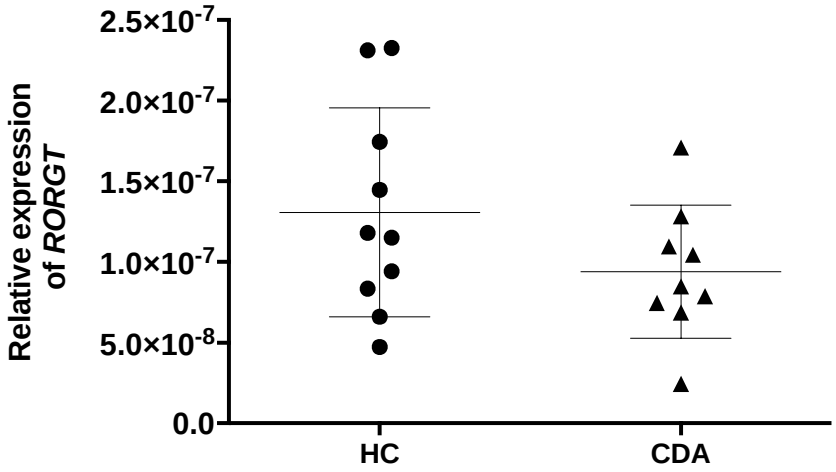
(A)


Δ2/FL in PBMC
5
4
3
2
1
0
HC
CDA
CDGF

(B)



(C)



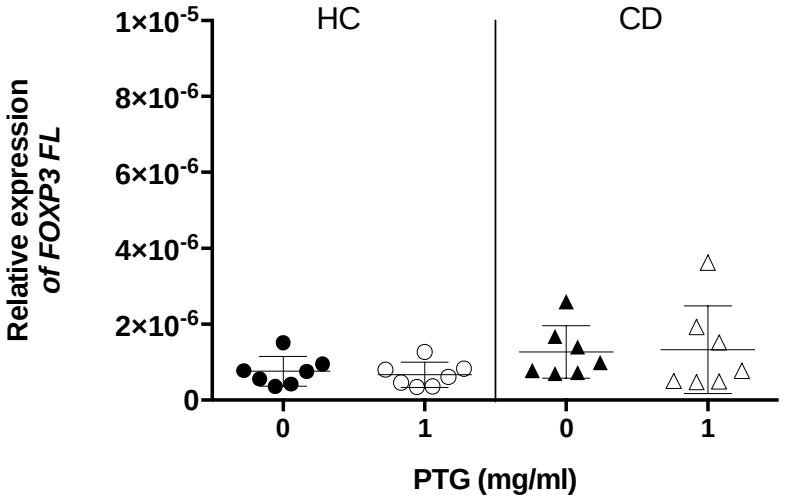
(D)

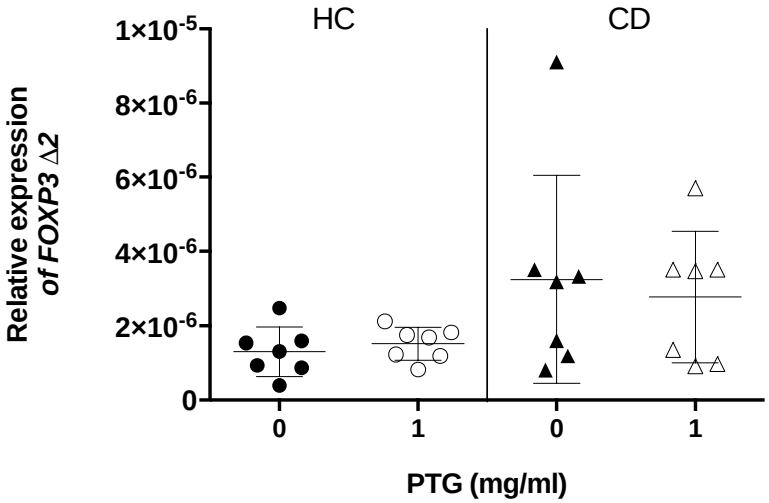
Fig.11: Same expression of *FOXP3* isoforms and *RORGT* between peripheral blood mononuclear cells (PBMC) of CDA and HC subjects. Real time RT-PCR was used to measure the relative expression of both *FOXP3* isoforms separately (A) and *RORGT* (D) HC (*n*=12), CDA *(n*=11) and CDGF (*n*=6). The ratio between the two isoforms (Δ2/FL) was calculated individually for each subject and compared among the three groups of patients. The bars show the mean among ratios ± SEM in HC (white), CDA (black) and CDGF (gray) subjects (B). Paired analysis was used to further determine the differences between the two isoforms within the same group of patients (C). Each dot represents the mean among the mRNA expression values ± SEM. Real time RT-PCR data were normalized to house-keeping gene *18S*. Statistical comparisons were made using Krustal Wallis test (A,B), unpaired t test (D) and Wilcoxon (C). * $p< 0.05$.

3.3 PT-gliadin does not affect the ratio between *FOXP3* isoforms expression.

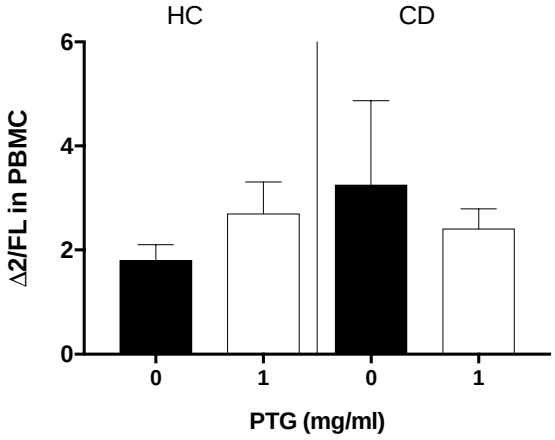
CD represents a unique model of autoimmunity because of the knowledge of its environmental trigger, gliadin [1]. Different gliadin peptides have been shown to have specific roles in triggering the development of CD such as induction of intestinal permeability and cytotoxic or immunomodulatory properties [120].

We hypothesized that gliadin may trigger the intestinal changes in *FOXP3* isoforms that we found between HC and CD patients. We reasoned that this would also explain the absence of *FOXP3* isoforms imbalance that we observed in subjects following a gluten free diet. We stimulated PBMC from HC and CD patients in remission (CD) with gliadin previously digested with pepsin and trypsin (PTG). We then measured the gene expression of the two major *FOXP3* isoforms by real time RT-PCR. There were no differences in *FOXP3* isoform expression upon stimulation with PTG (**Fig. 12A**). Furthermore, both groups of patients showed higher levels of *FOXP3 Δ2* as compared to *FL* (**Fig. 12B, C**). Relative expression of *IL6* was used as positive control to determine the efficacy of the PBMC stimulation with PT-gliadin (**Fig.12D**). Although gliadin is the recognized environmental trigger for CD, our findings suggest that it is not able to modulate the balance between *FOXP3* isoforms within the PBMC population in HC nor CD patients. It is plausible to hypothesize that other components present during the inflammatory process in the intestine of CD subjects may play a role in sustaining the imbalance between FOXP3 isoforms in this group of patients.

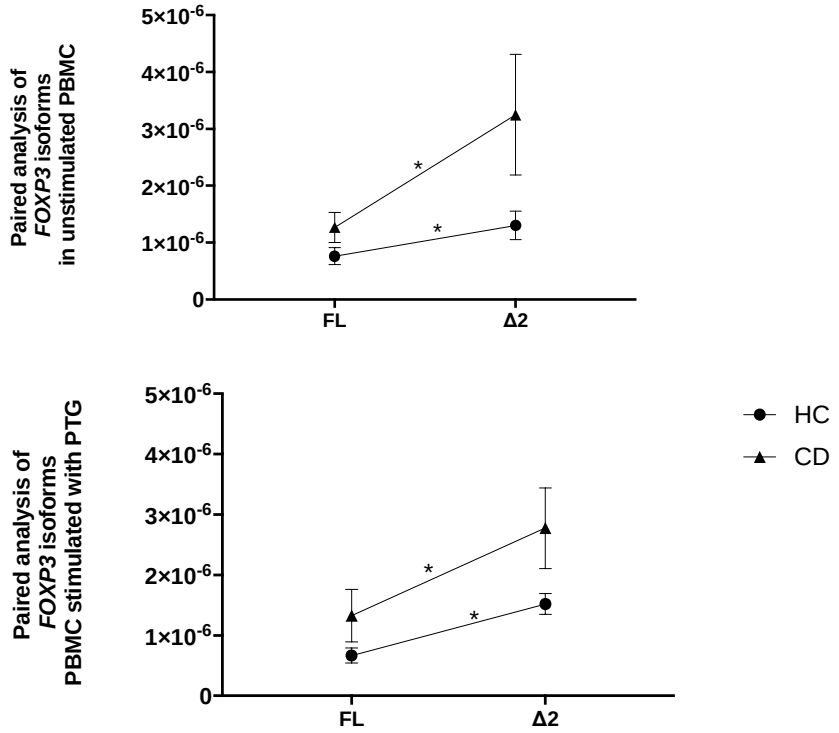

HC
CD
1×10-5
8×10-6
6×10-6
4×10-6
2×10-6
0
Relative expression of FOXP3 FL
0
1
0
1
PTG (mg/ml)


HC
CD
1×10-5
8×10-6
6×10-6
4×10-6
2×10-6
0
Relative expression of FOXP3 Δ2
0
1
0
1
PTG (mg/ml)

(A)


HC
CD
6
4
2
0
Δ2/FL in PBMC
0
1
0
1
PTG (mg/ml)

(B)



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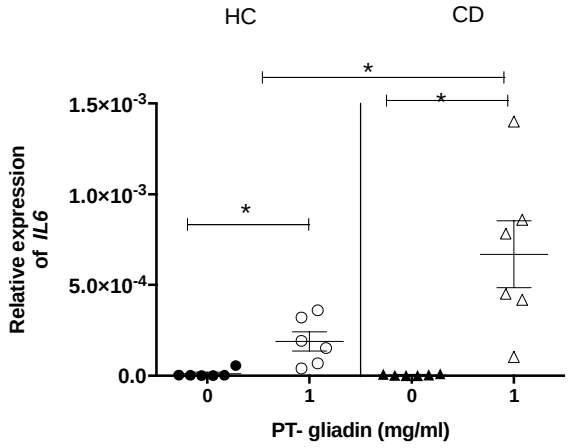


(D)

Fig.12. PT-gliadin does not modulate expression of *FOXP3* isoforms in PBMC. PBMC from HC and CD patients were cultured with medium alone or with PT-gliadin (PTG). Expression of *FOXP3* isoforms was measured before and after stimulation in HC (n=7) and CD (n=7) patients (A). Ratio between FOXP3 isoforms (Δ2/FL) was calculated individually for each subject. (B). Paired analysis was used to determine the differences between the two isoforms within the same group of patients (C). Expression of *IL6* was measured as positive control of PTG-induced stimulation (D). Statistical comparisons were made using Wicoxon test (A,B,D), Mann-Whitey (B,D) and Krustal-Wallis test (C). * p< 0.05.

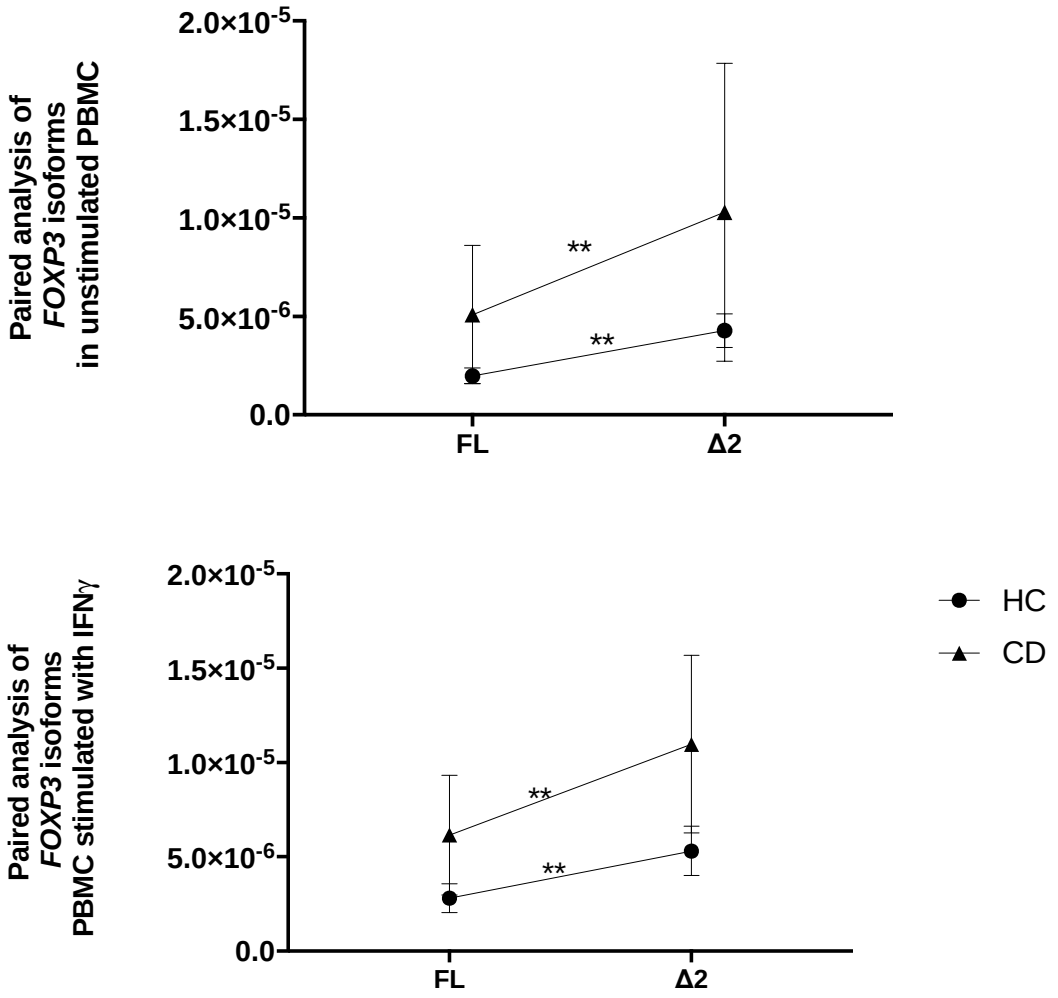
3.4 Summary of chapter 3

We have shown that active CD patients are characterized by an intestinal over-expression of the less functional isoform *FOXP3 Δ2* that positively correlates with a high expression of *RORGT*. Furthermore, our data have also demonstrated that in the intestine of HC subjects the two isoforms are equally expressed while in active CD patients the ratio between *FOXP3 FL* and *Δ2* is skewed toward the latter. These findings suggest that alterations in the ratio between isoforms contribute to the development of CD and, in particular, to the Th17 driven immune response that characterizes active CD patients. Our data on PBMC showed that the altered expression of *FOXP3* isoforms was present exclusively at intestinal level, therefore suggesting a role of the specific gut milieu in modulating the ratio between *FOXP3 FL* and *Δ2*.

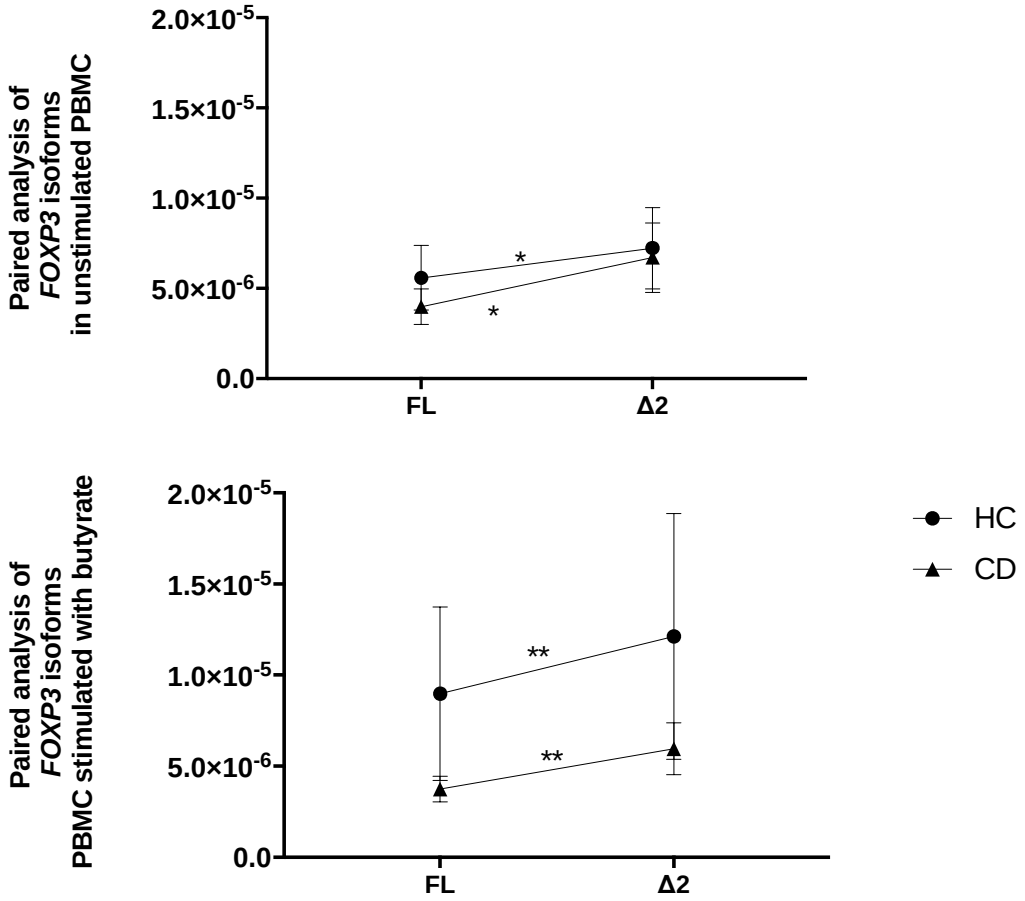
CHAPTER 4. ROLE OF THE MICROBIAL DERIVED SHORT CHAIN FATTY ACID BUTYRATE COMBINED WITH PRO-INFLAMMATORY CYTOKINE IFN-γ IN MODULATING FOXP3 ISOFORMS EXPRESSION DURING THE ACTIVE PHASE OF CELIAC DISEASE.

4.1 Expression of *FOXP3* isoforms is not modulated by stimulation with IFNγ or butyrate.

In CD patients, the digestion of gluten triggers a cascade of events that eventually leads to enteric damage. Thus, the intestinal microenvironment of CDA patients is very different from one found in HC subjects or CD patients in remission. The active state of CD is characterized by a strong Th1 driven pro-inflammatory response that leads to a high production of cytokine IFNγ [6]. In addition, changes in the microbiota composition such as high levels of butyrate and reduced microbiome diversity have also been described in CDA patients. We aimed to determine if this pro-inflammatory cytokine or the microbial derived metabolite alone were able to modulate the expression of FOXP3 isoforms. We isolated PBMC from HC and CD patients in remission and we stimulated them for 48h hours respectively with 1 μg/ml of butyrate or 100 ng/ml of IFNγ □□□□□□□□□□□□□□□□□□□□□□□□ [22, 121]. Our data showed that neither IFNγ nor butyrate alone had any effect in altering the ratio between *FOXP3* isoforms in HC or CD subjects (**Fig. 13**).


Paired analysis of
FOXP3 isoforms
in unstimulated PBMC
2.0×10-5
1.5×10-5
1.0×10-5
5.0×10-6
0.0
**
**
FL
Δ2
Paired analysis of
FOXP3 isoforms
PBMC stimulated with IFNγ
2.0×10-5
1.5×10-5
1.0×10-5
5.0×10-6
0.0
**
**
FL
Δ2
HC
CD

(A)

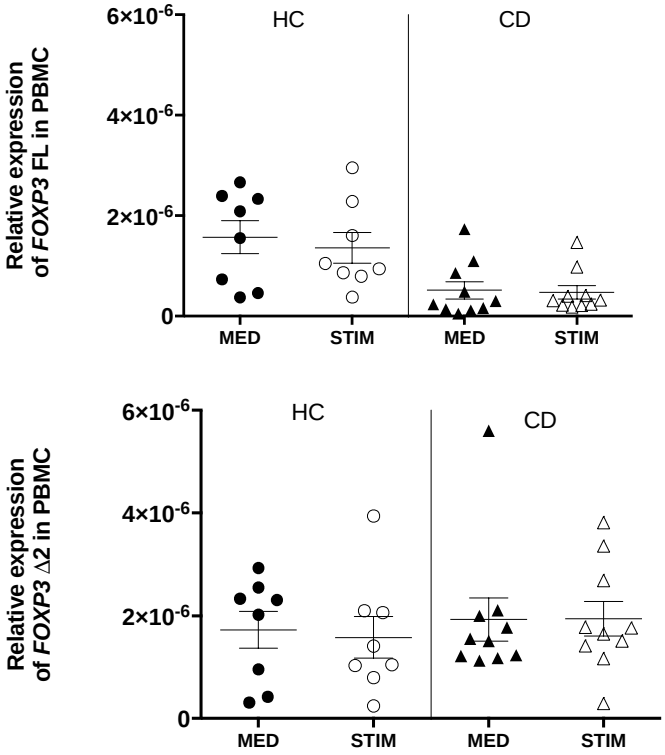


(B)

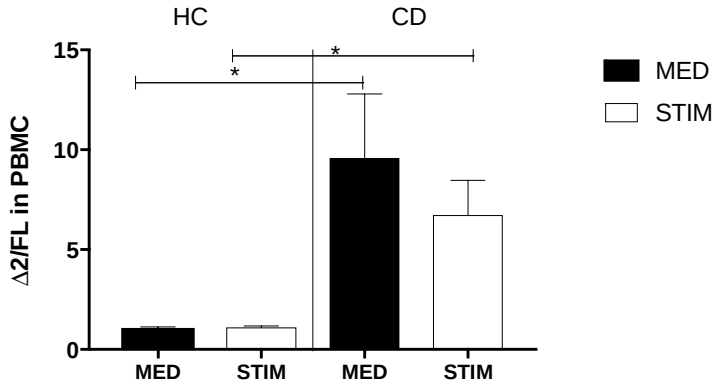
Fig. 13. Stimulation with IFNγ or butyrate alone does not modulate expression of *FOXP3* isoforms in PBMC. Paired analysis was used to determine the differences between the two isoforms' expression within the same group of patients in PBMC from HC (*n*=12 for IFNγ experiment and *n*=19 for butyrate experiment) and CD (*n*=10 for IFNγ experiment and *n*=20 for butyrate experiment) subjects were cultured for 48 hours in medium alone or stimulated with either 100 ng/ml of IFNγ (A) or 1 μg/ml of butyrate (B). Each dot represents the mean among the mRNA expression values ± SEM. Differences between the relative expression of *FOXP3* isoforms within each group was determined in HC and CD in both cultures conditions. Relative real-time RT-PCR data were normalized to house-keeping gene *18S*. Statistical comparisons were made using Wilcoxon test or paired t-Test (A,B). * $p< 0.05$, ** $p< 0.005$.

4.2 Combined effect of pro-inflammatory cytokine IFNγ plus butyrate on modulating the ratio between FOXP3 isoforms expression in PBMC.

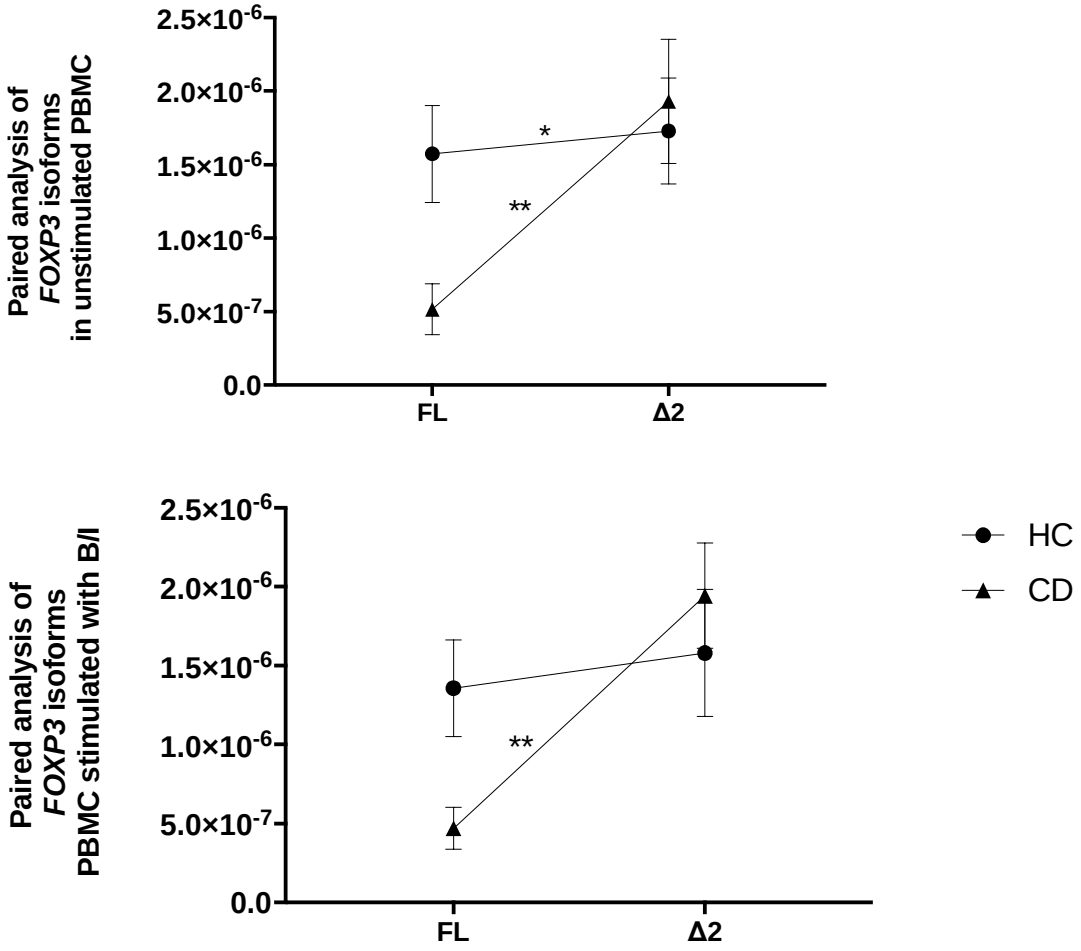
We aimed to see if a combination of the pro-inflammatory cytokine IFNγ and the microbial derived metabolite butyrate is able to trigger changes in FOXP3 isoforms expression. PBMC from HC and CD subjects in remission were stimulated with a combination of 100 ng/ml of IFNγ and 1 mg/ml of butyrate for 48 hours. In both HC and CD PBMC we saw an equal abundance of the two isoforms irrespective of the stimulation with IFNγ and butyrate (**Fig. 14A**). In CD PBMC the ratio between isoforms was higher than HC even without stimulation and the Δ2/FL ratio was not further increased (p<0.05) (**Fig. 14B).** Interestingly the stimulation with IFNγ and butyrate led to a balance between isoforms in HC subjects, while in CD patients Δ2 was maintained more expressed than its counterpart FL after stimulation (p<0.005) (**Fig. 14C**). The balance between *FOXP3* isoforms in HC subjects paralleled with a significant decrease in *RORGT* mRNA expression after stimulation (p<0.05) in these patients but not in CD subjects (**Fig. 14D, E**).


Relative expression of FOXP3 FL in PBMC
6×10-6
4×10-6
2×10-6
0
HC
CD
MED
STIM
MED
STIM
Relative expression of FOXP3 Δ2 in PBMC
6×10-6
4×10-6
2×10-6
0
HC
CD
MED
STIM
MED
STIM

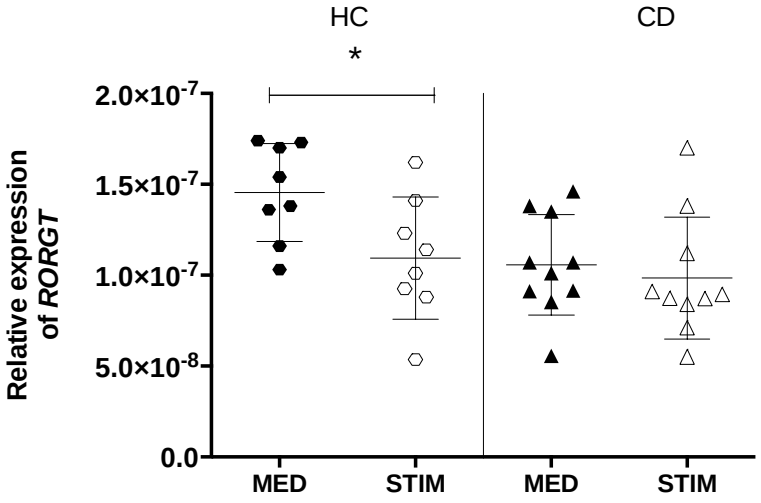
(A)


HC
CD
Δ2/FL in PBMC
15
10
5
0
*
*
MED
STIM
MED
STIM
MED
STIM

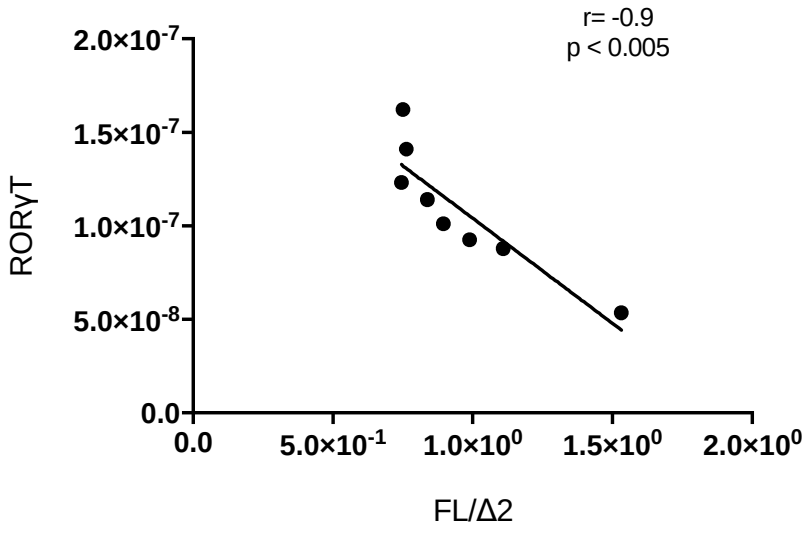
(B)


Paired analysis of
FOXP3 isoforms
in unstimulated PBMC
2.5×10-6
2.0×10-6
1.5×10-6
1.0×10-6
5.0×10-7
0.0
FL
Δ2
*
**
Paired analysis of
FOXP3 isoforms
PBMC stimulated with B/I
2.5×10-6
2.0×10-6
1.5×10-6
1.0×10-6
5.0×10-7
0.0
FL
Δ2
**
HC
CD

(C)



(D)

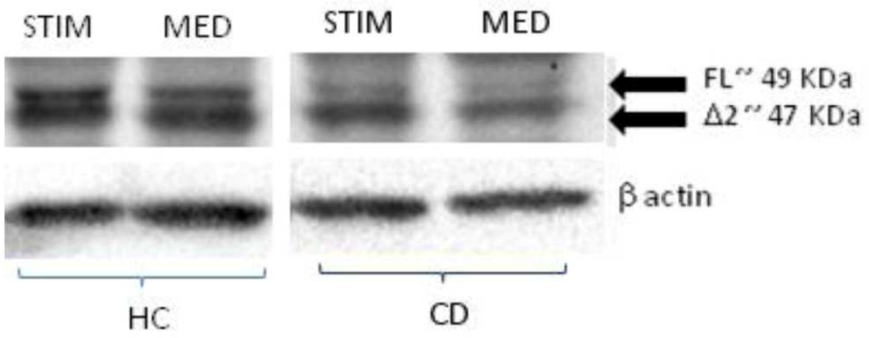


(E)

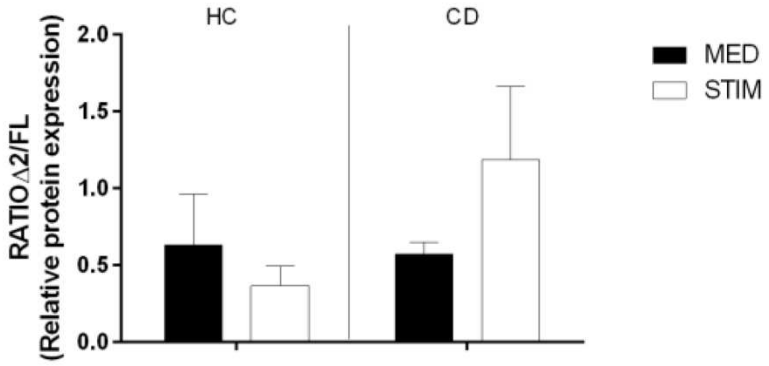
Fig 14. IFNγ and butyrate modulate expression of *FOXP3* isoforms in PBMC of HC subjects. Expression of *FOXP3* and *RORGT* was measured by RT-PCR (A, D). The ratio between the relative expressions of *FOXP3* isoforms (Δ2/FL) was calculated individually for each subject in HC and CD cultured with medium alone (MED) or stimulated (STIM) (B). Paired analysis was used to further determine the differences between the two isoforms' expression within the same group of patients (C). Negative correlation between the expression of *RORGT* and the ratio *FOXP3 FL/Δ2* was confirmed by two-tailed Spearman correlation analysis (E). Statistical comparisons were made using Friedman or ANOVA.* $p< 0.05$, ** $p<0.005$.

We performed a western blot assay to confirm the presence of the two isoforms at protein level in both groups of patients before and after stimulation (**Fig. 15A**). The analysis of FOXP3 proteins showed that stimulation decreased the Δ2/FL ratio in HC, but increased the ratio in CD patients (**Fig. 15B**). In addition, the stimulation of PBMC with the pro-inflammatory cytokine and the microbial derived metabolite triggered a significant reduction in gene expression of anti-inflammatory cytokine *IL10* in both groups of subjects (**Fig.16A**). The same stimuli did not alter the expression of *CTLA4*, another important co-inhibitory molecule characteristic of the Tregs subpopulation (**Fig. 16B**), therefore underlying the influence that the ratio between FOXP3 isoforms exerts on regulating RORγT expression, but not other molecules that characterize Treg cells. Relative expression of *Tbet* was used as positive control to confirm the efficiency of the stimulation (**Fig. 16C**).

Collectively, these data suggest that in HC subjects a pro-inflammatory microenvironment, such as one enriched of IFNγ and butyrate, contributes to establishing the balance between the two major FOXP3 isoforms. Additionally the stimulation of PBMC with IFNγ and butyrate also led to a decreased expression of transcription factor *RORGT*. This response to a "pro-inflammatory like" milieu could represent a compensatory mechanism adopted by PBMC of HC subjects to efficiently respond to inflammation. Our data show that the same stimuli do not trigger a balance between isoforms in cells from CD patients and that in these subjects the defective FOXP3 isoform is sustainably more expressed than FOXP3 FL. This may account for the lack of proper immune response regulation that characterized CD patients and consequently to the development of CD.

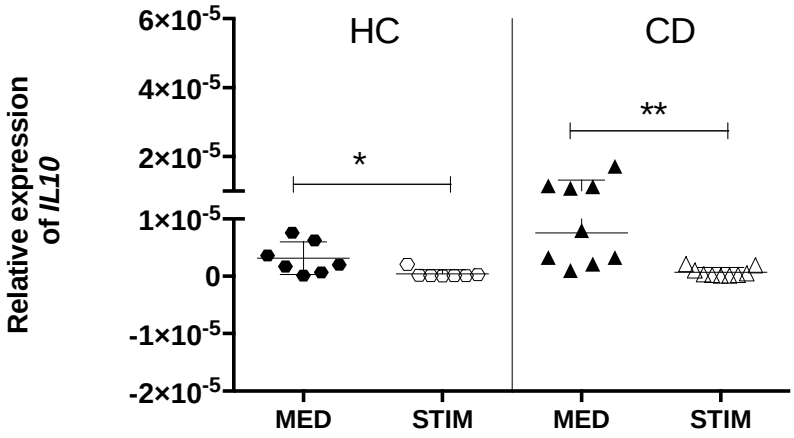


(A)

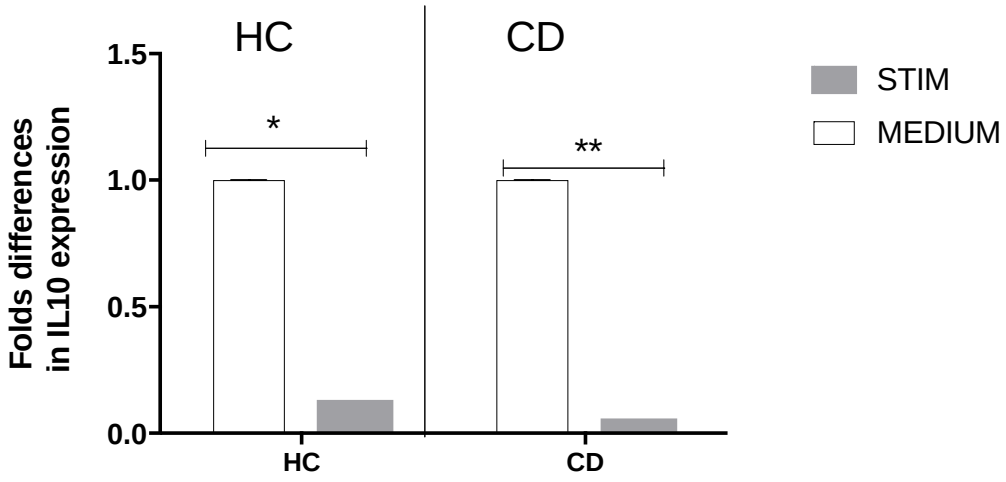


(B)

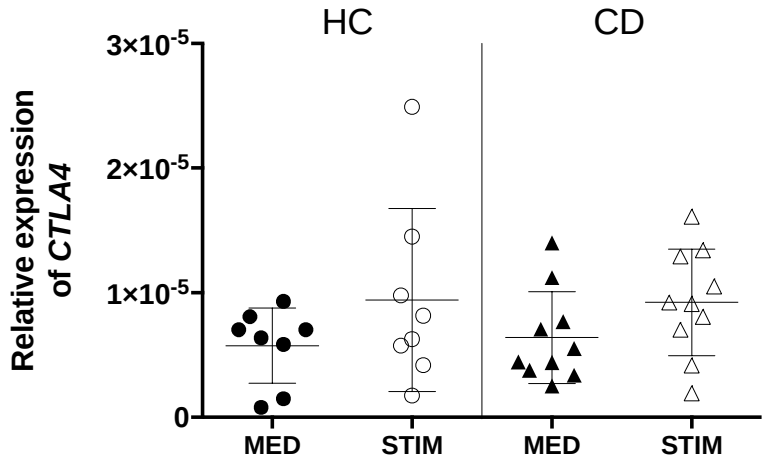
Fig.15. Protein levels of FOXP3 isoforms in PBMC stimulated with butyrate and IFNγ. Protein levels of splice variants were analyzed by western blot using isoform-non specific α-FOXP3 antibody PCH101. The ratio between FOXP3 isoforms (Δ2/FL) was calculated normalizing the relative protein expression of each isoforms to β-actin in PBMC isolated from HC (*n*=3) and CD subjects (*n*=3) before (MED) and after stimulation (STIM) (A). Western blot of FOXP3 protein detection in PBMC cultured with medium alone (MED) or with butyrate and IFNγ (STIM) is representative of three independent experiments on PBMC from HC and CD patients (B).


Relative expression of IL10
6×10-5
4×10-5
2×10-5
1×10-5
0
-1×10-5
-2×10-5
HC
CD
*
**
MED
STIM
MED
STIM

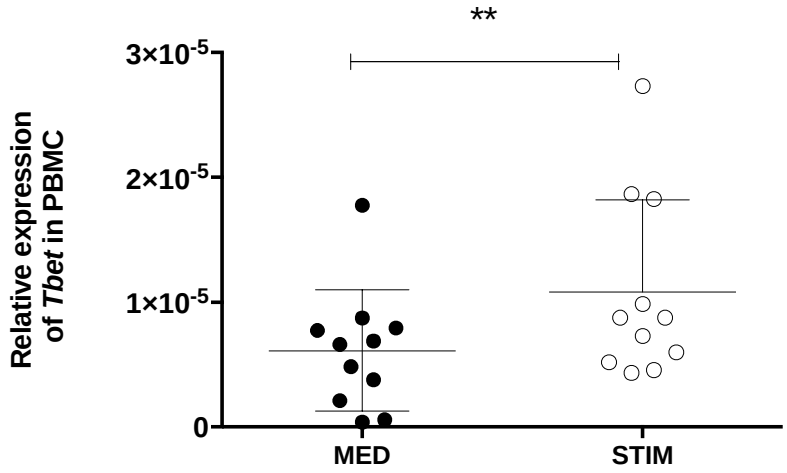
(A)


Folds differences in IL10 expression
1.5
1.0
0.5
0.0
HC
CD
*
**
HC
CD
STIM
MEDIUM

(B)



(C)

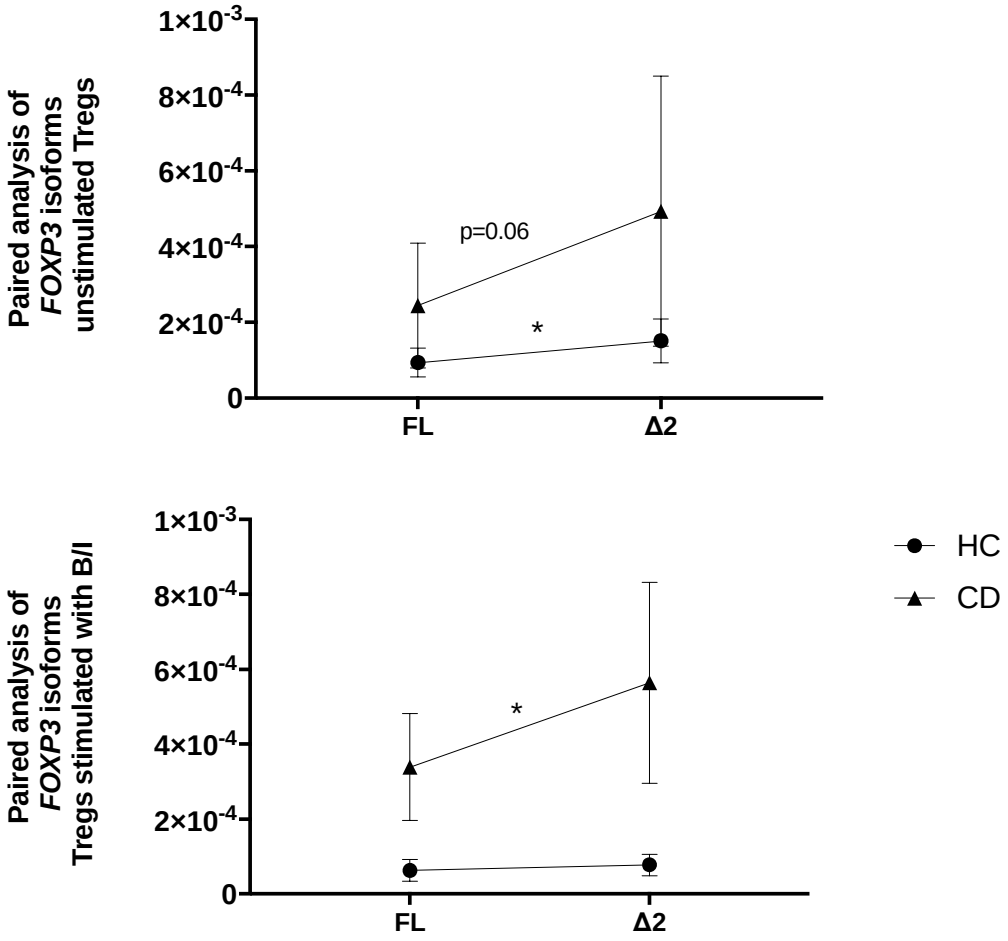


(D)

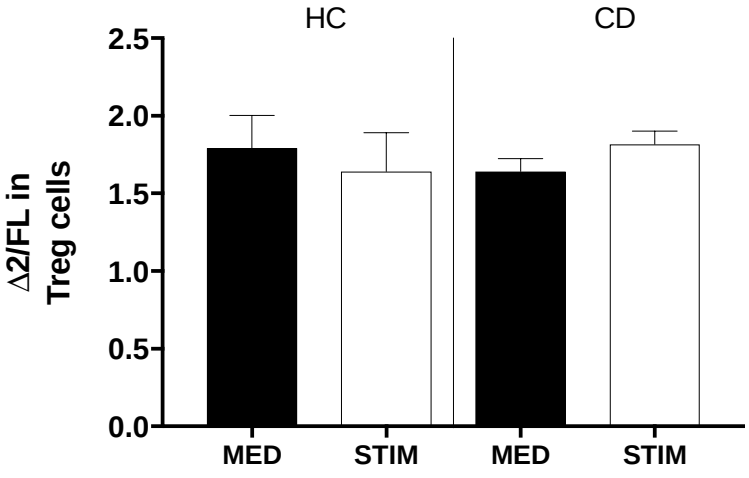
Fig 16. Relative expression of *IL10, CTLA4* and *Tbet* in PBMC stimulated with IFNγ and butyrate. PBMC from HC and CD patients in remission were cultured for 48 hours in medium alone (MED) or stimulated with 100 ng/ml of IFNγ and 1 µg/ml of butyrate (STIM). Relative expression of *IL10* and *CTLA4* was measured by relative real time RT-PCR (A, B) (HC *n*=7 for *IL10* and *n*=8 for *CTLA;* CD *n*=10). Relative expression of *Tbet* was measured in a subgroup of subjects (*n*=11) as positive control to confirm the efficacy of the stimulation (C). Statistical comparisons were made using Wilcoxon, paired t-Test. * $p< 0.05$, ** $p<0.005$.

4.3 Effect of butyrate and IFNγ on the expression of *FOXP3* isoforms in $CD4^+CD25^+$ Treg and $CD4^+CD25^-$ T cells.

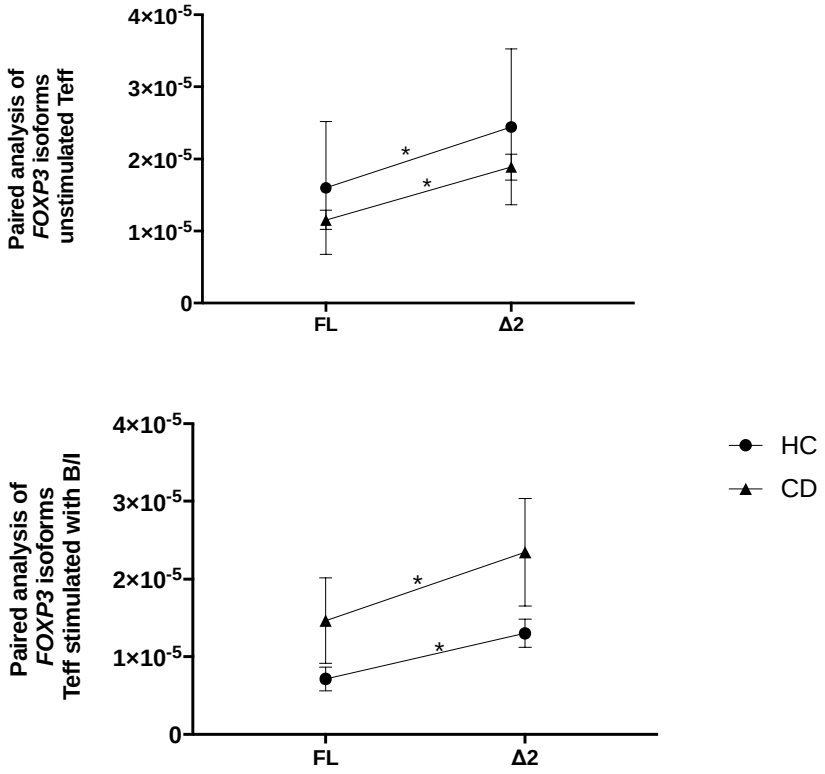
Although for some time FOXP3 has been considered an established master transcription factor for Tregs, its expression can be up-regulated also in $CD4^+CD25^-$ T cells. We aimed to understand the precise cells subset targeted by IFNγ and butyrate. We isolated $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs from HC and CD patients and we stimulated them with a combination of IFNγ plus butyrate. We then analyzed the gene expression of both *FOXP3* isoforms by real-time RT-PCR. Our experiments on Tregs showed that IFNγ and butyrate triggered a balance between *FOXP3* isoforms in HC subjects but not in cells isolated from CD patients, mimicking what we showed previously in PBMC (**Fig. 17A**). We did not see changes in the overall ratio between isoforms in the two groups of patients (**Fig. 17B**). In contrast to what we observed in the Tregs population, our data on $CD4^+CD25^-$ T cells showed that stimulation with IFNγ and butyrate did not trigger a balance between isoforms in HC patients and that expression of *FOXP3* Δ2 remained higher than its counterpart FL in both HC and CD subjects (**Fig. 17C, D**). The purity of different T cells populations was determined by flow-cytometry (**Fig. 18**). Collectively these data confirm our findings on PBMC and show that the pro-inflammatory cytokine, IFNγ and the microbial derived metabolite, butyrate, modulate the expression of *FOXP3* isoforms in HC subjects by directly targeting Tregs. Furthermore our experiments show that the transient expression of *FOXP3* on $CD4^+CD25^-$ T cells does not account for these changes.


Paired analysis of FOXP3 isoforms unstimulated Tregs
1×10-3
8×10-4
6×10-4
4×10-4
2×10-4
0
p=0.06
*
FL
Δ2
Paired analysis of FOXP3 isoforms Tregs stimulated with B/I
1×10-3
8×10-4
6×10-4
4×10-4
2×10-4
0
*
FL
Δ2
HC
CD

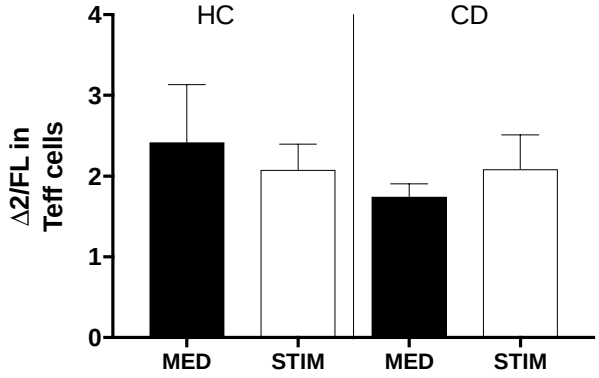
(A)


HC
CD
Δ2/FL in Treg cells
2.5
2.0
1.5
1.0
0.5
0.0
MED
STIM
MED
STIM

(B)



(C)



(D)

Fig. 17: Stimulation with IFNγ and butyrate modulates expression of *FOXP3* isoforms in $CD4^+CD25^+$ Treg and $CD4^+CD25^-$ T cells in CD patients. The effect of butyrate and IFNγ on *FOXP3* isoforms expression was evaluated by RT-PCR on $CD4^+CD25^+$ Treg (HC *n*=6, CD *n*=6) and $CD4^+CD25^-$ Teff cells (HC *n*=8 and and CD *n*=11). Paired analysis was used to determine differences between the two isoforms' expression in both groups of patients respectively in $CD4^+CD25^+$ Treg (A) and $CD4^+CD25^-$ T cells (C). The ratio between *FOXP3* isoforms (Δ2/FL) was calculated individually for each subject in HC and CD (B,D). Statistical comparisons were made using Wilcoxon test and Paired t-test (A,C) and Krustal-Wallis test (B,D). * $p< 0.05$.

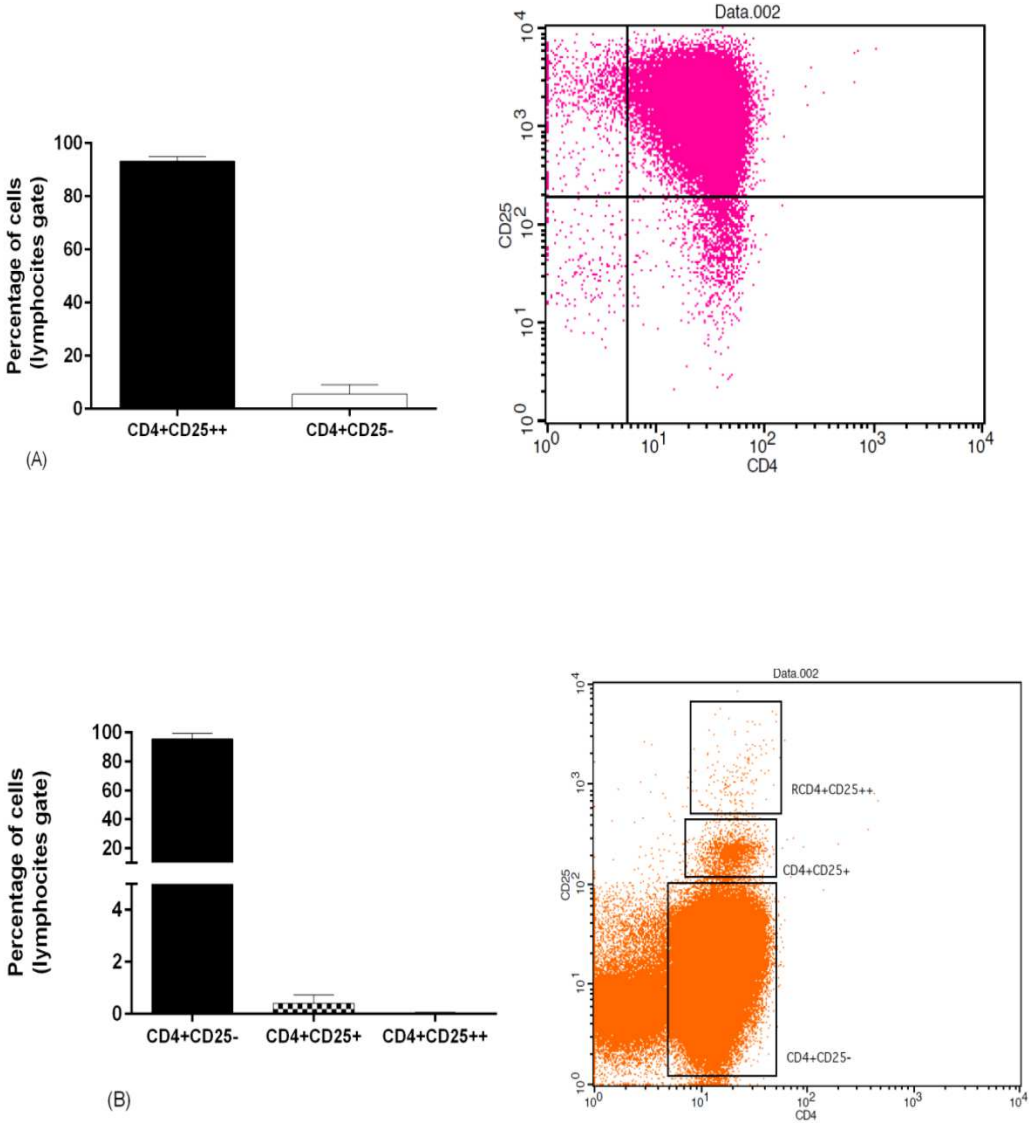


Fig. 18: Flow cytometry analysis of $CD4^+CD25^+$ Treg and $CD4^+CD25^-$ T cells at baseline. The purity of isolated $CD4^+CD25^+$ Treg cells (A) and $CD4^+$CD25- T cells (B) was determined higher than 90% by flow-cytometry. Cells were stained with antibodies PerCP conjugated anti-CD4 and PE conjugated anti-CD25. Detection of $CD4^+CD25^{++}$ and $CD4^+CD25^-$ was performed with BD Bioscience software. Scatter Dot Plot are representative of 2 separate experiments for Treg cells (A) and 3 separate experiments for $CD4^+CD25^-$ T cells (B).

4.4 Summary of chapter 4

Our experiments with IFNγ and butyrate showed for the first time that the process of alternative splicing on Tregs can be modulated by a combination of the pro-inflammatory cytokine and the microbial derived metabolite. In addition, we showed that Tregs from CD patients respond differently than cells from HC subjects to the stimulation. These findings suggest a mechanistic link between immune regulation and the specific gut microenvironment.

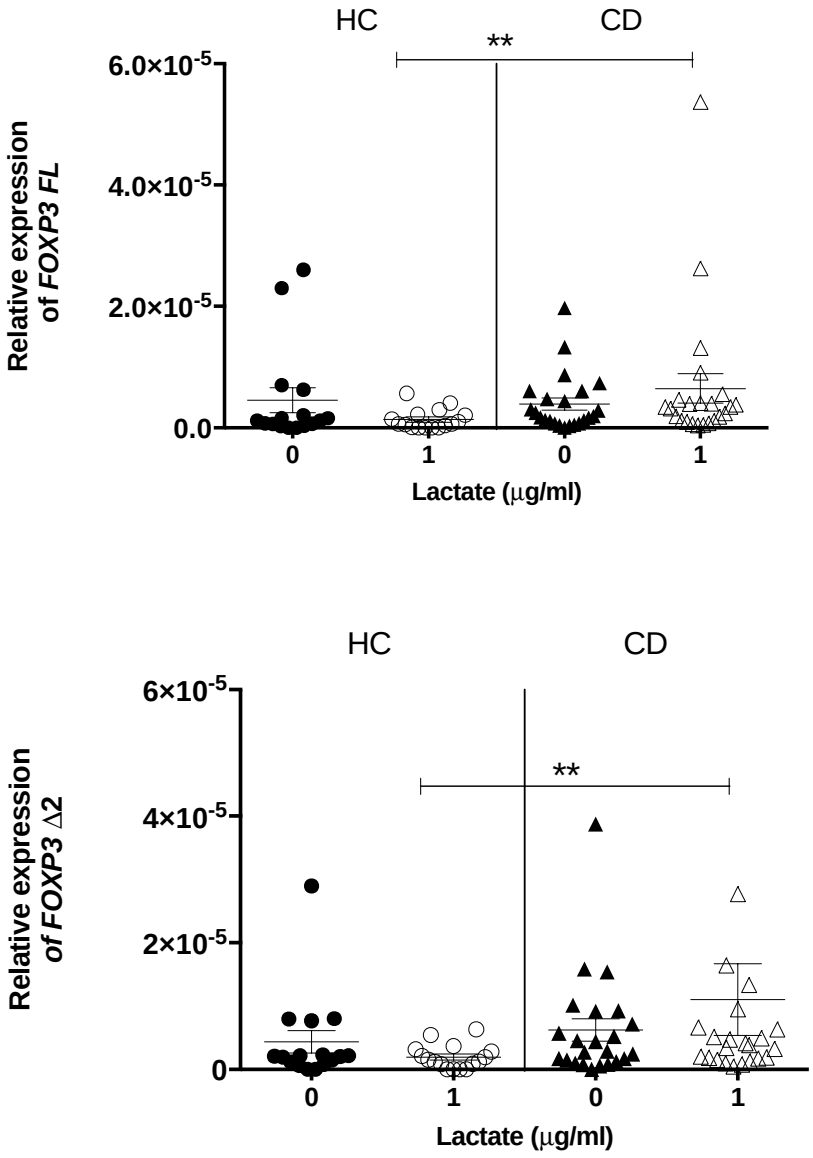
We hypothesize that the balance between FOXP3 isoforms reached in HC subjects may represent a compensatory mechanism that allows Tregs to efficiently regulate the inflammation. Conversely the absence of balance between isoforms that we saw in Tregs from CD patients upon stimulation may account for the up-regulation of Th17 pro-inflammatory immune response that characterize the pathogenesis of CD.

CHAPTER 5. ROLE OF THE MICROBIAL DERIVED METABOLITE LACTATE IN MODULATING FOXP3 ISOFORMS EXPRESSION DURING THE PRECLINICAL PHASE OF CELIAC DISEASE.

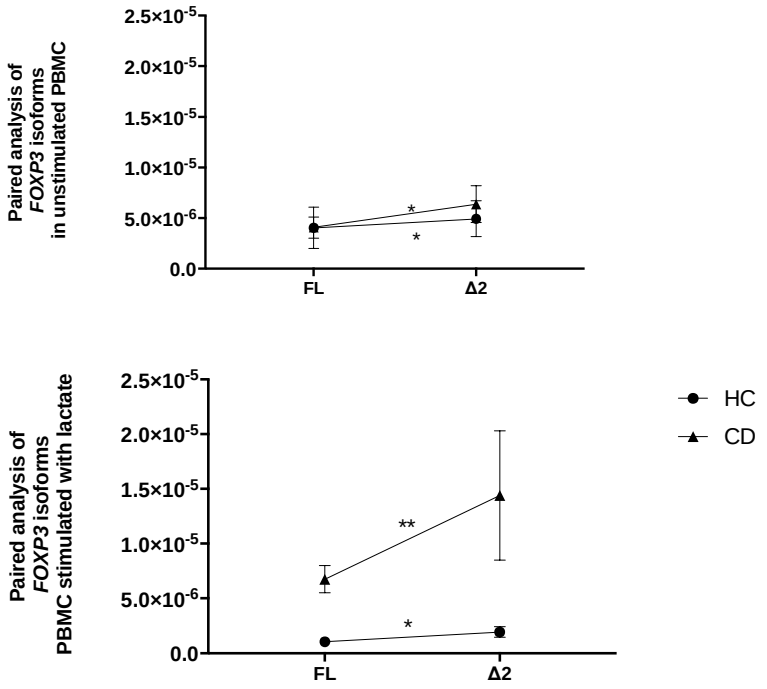
5.1 Microbial derived lactate modulates FOXP3 isoforms expression in PBMC.

We demonstrated previously that prior to the onset of the disease, the microbiome from infants genetically predisposed to CD is characterized by high levels of lactate correlated with a high number of *Lactobacilli spp.* [22]. We aimed to investigate if this microbial derived metabolite may modulate the expression of FOXP3 in CD patients contributing eventually to the onset of the disease. Given the important role of Tregs in regulating the intestinal immune homeostasis from an early age, we hypothesized that this subset of cells may be particularly susceptible to the influence of lactate. We stimulated PBMC from adult HC subjects and CD patients in remission with the microbial derived metabolite and we measured the expression of both *FOXP3* isoforms. Our experiments showed that, upon stimulation with lactate, both *FOXP3* isoforms were significantly increased in CD patients compared to HC subjects ($p< 0.005$) (**Fig. 19A**). In contrast to what we previously found in PBMC stimulated with butyrate and IFNγ, the skewed ratio between the two *FOXP3* isoforms toward *FOXP3* Δ2 was maintained in both groups of subjects ($p< 0.005$) (**Fig. 19B, C**). Flow cytometry analysis confirmed that, after stimulation with lactate, the total expression of FOXP3 increased in CD patients compared to HC subjects ($p< 0.05$) (**Fig. 20**). Surprisingly, the increased expression of FOXP3 in CD patients after stimulation with lactate did not correlate with a higher expression of the anti-inflammatory cytokine IL10 (**Fig.21A**). Furthermore our experiments did not show any differences in the relative expression of transcription factor *RORGT* (**Fig. 21B**). Collectively these data show that PBMC from CD patients respond

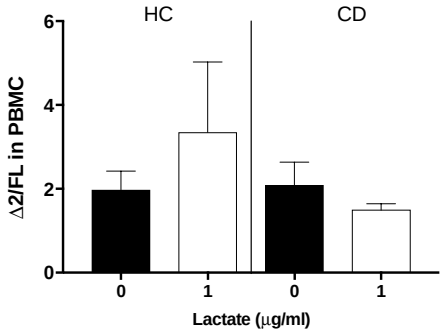
to the stimulation with lactate by increasing FOXP3 expression, while in HC subjects the metabolite does not have the same effect. This suggests that PBMC from the two different groups respond differently to the same microenvironment.



(A)

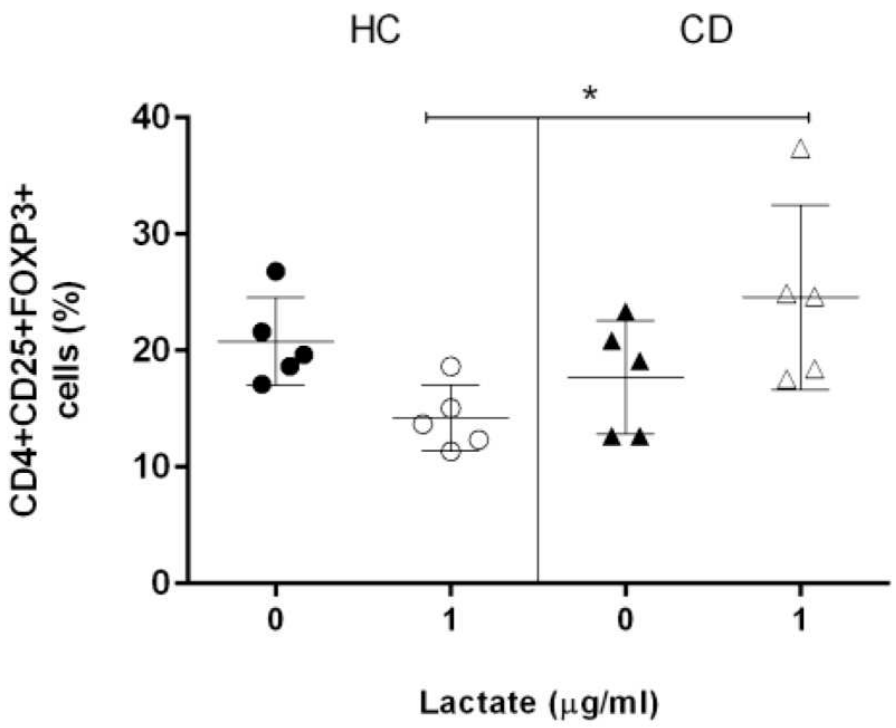


(B)



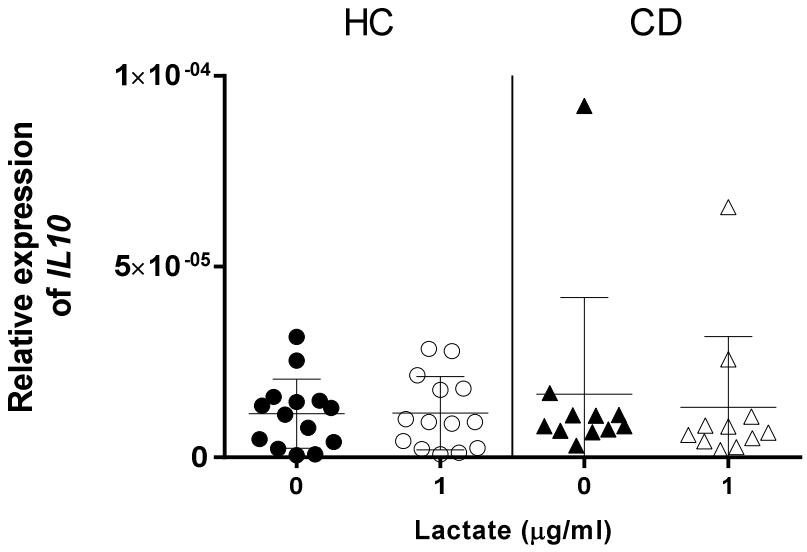
(C)

Fig. 19: Stimulation with lactate modulates expression of *FOXP3* isoforms in PBMC of CD patients. The effect of lactate on *FOXP3* isoforms expression was evaluated by RT-PCR on PBMC from HC subjects (*n*=16) and CD patients (*n*=23) after stimulation (A). Paired analysis was used to determine differences between the two isoforms' expression within each group of patients in both cultures conditions (B). The ratio between the relative expressions of *FOXP3* isoforms (Δ2/FL) was calculated individually for each subject in HC and CD (C). Statistical comparisons were made using Friedman (A, D) and Krustal-Wallis test (B,C). * $p< 0.05$,** $p< 0.005$.

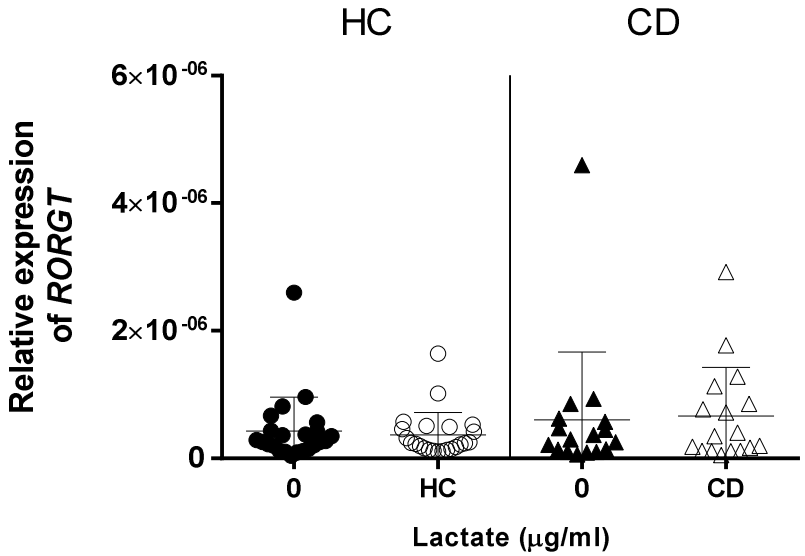


(A)

Fig. 20 Protein expression of FOXP3 in PBMC upon stimulation with lactate. Flow cytometry was used to determine the protein levels of total FOXP3 before and after stimulation in HC (*n*=5) and CD (*n*=5) subjects.



(A)



(B)

Fig. 21: Relative expression of *IL10* and *RORGT* in PBMC upon stimulation with lactate. Real time RT-PCR was used to determine the relative genic expression of *IL10* (A) and *RORGT* (B) before and after stimulation in PBMC from HC (*n*=14 for *IL10* and *n*=23 for *RORGT*) and CD (*n*=11 for *IL10* and *n*=17 for *RORGT*) subjects.

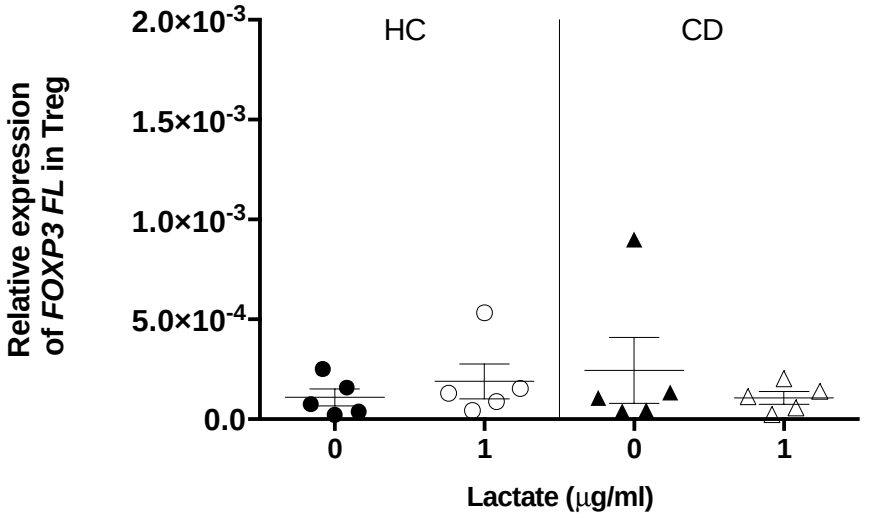
Overall these data suggest that PBMC from CD and HC subjects respond differently to a lactate enriched milieu. Furthermore, they demonstrate that lactate is able to trigger an increase in the total amount of FOXP3 in CD patients, but that this does not necessarily correspond to an increased suppressive function of the cells as demonstrated by the measurements of *IL10* gene expression. Finally, the lack of differences in *RORGT* between the HC and CD subjects, suggests that the ratio between the two FOXP3 isoforms is important in modulating the regulation of this transcription factor rather than the total FOXP3.

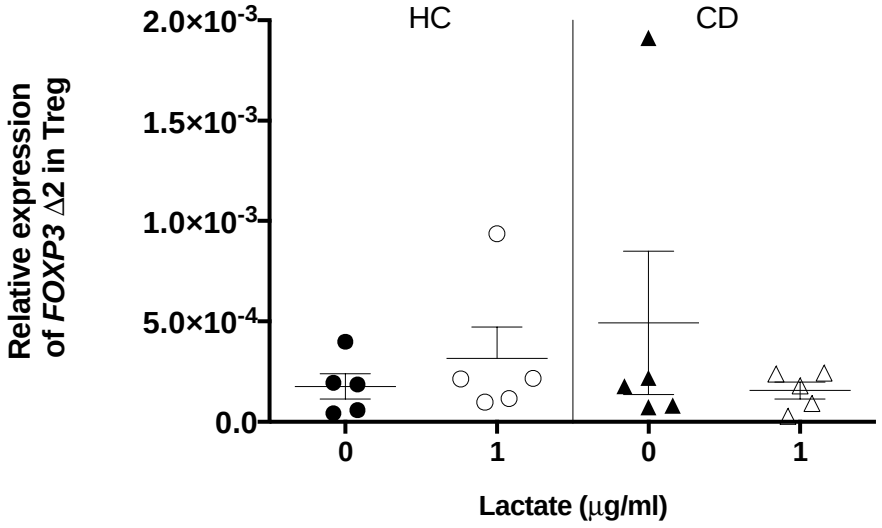
5.2 *FOXP3* isoforms expression in celiac patients on $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells is not modulated by stimulation with lactate.

We sought to better understand how lactate was able to induce the increased expression of both FOXP3 isoforms in CD patients. We isolated $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs from HC and CD patients and stimulated them with the microbial-derived metabolite for 48 hours. From our experiments we found that lactate-stimulated Treg cells from CD patients did not show an increased expression of *FOXP3* isoforms compared to HC subjects (**Fig. 22A**). This, refutes the hypothesis that this microbial-derived metabolite directly targets this subset of cells. Interestingly, lactate stimulation of $CD4^+CD25^-$ T cells led to a higher expression of *FOXP3* FL ($p< 0.05$) in CD patients as compared to HC subjects, but not of *FOXP3* Δ2 (**Fig. 22B**). Taken together these data indicate that lactate does not modulate expression of *FOXP3* isoforms in CD patients by directly targeting Treg cells, suggesting a role of another subset of cells included in the PBMC population as intermediate players between Treg and the metabolite for this modulation.

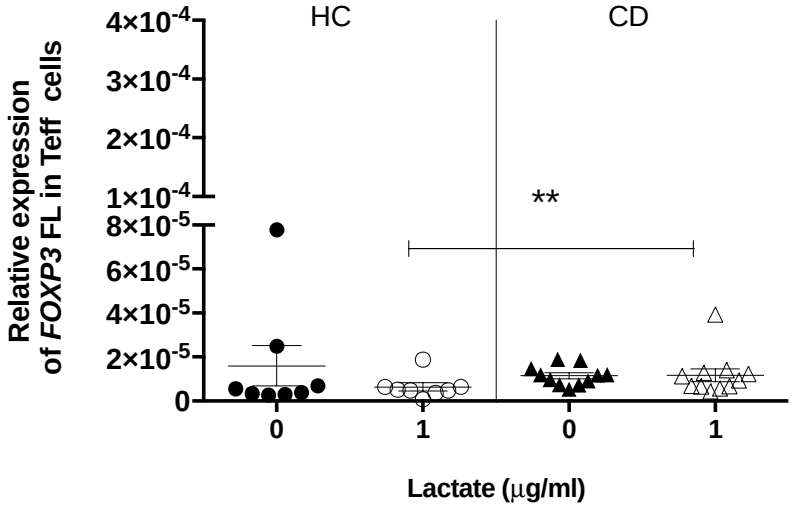
5.3 Modulation of *FOXP3* isoforms expression on Tregs of celiac patients requires HLA-DR^{+} cells.

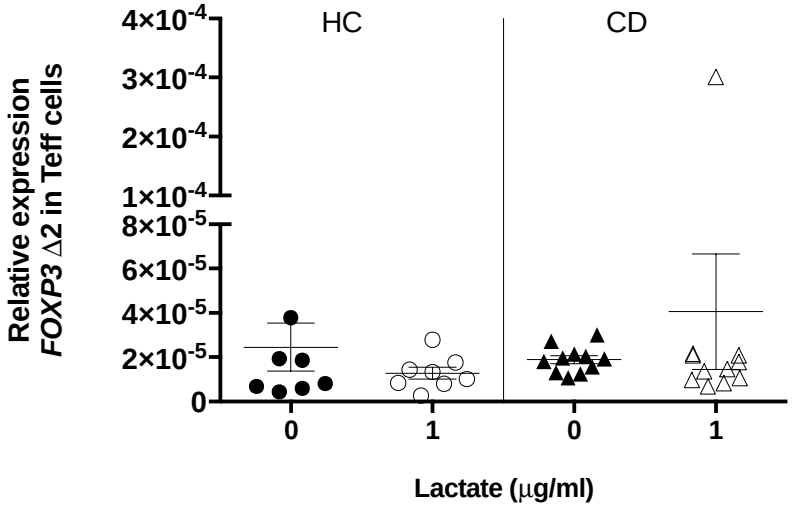
We aimed to investigate which subset of cells is required to modulate the expression FOXP3 isoforms on Tregs following stimulation with lactate. Among the PBMC population, APC cells are particularly susceptible to the surrounding microenvironment and several studies have shown how the microbiome contributes to regulate their function [110, 115]. APC cells are characterized by high expression of the HLA-DR molecule. This allows the cells to trigger an adaptive immune response by recognizing the Ag and presenting it to T cells [122, 123]. We isolated from PBMC of HC and CD patients a subset of HLA-DR^{-} cells that did not include APC, and stimulated them with lactate. We next examined the effect that the metabolite had on these cells by measuring the gene expression of both *FOXP3* isoforms. The purity of HLA-DR^{-} population was determined by flow-cytometry (**Fig.23A**). Our data showed that lactate had no effect of *FOXP3* expression in either group of patients (**Fig. 23B**). These findings suggest that lactate is able to modulate FOX3 expression in CD patients by triggering changes on a subset of HLA-DR+ cells such as APC and that these cells serve as "intermediate" between the metabolite and Treg cells.


Relative expression of *FOXP3 FL* in Treg
2.0×10-3
1.5×10-3
1.0×10-3
5.0×10-4
0.0
HC
CD
0
1
0
1
Lactate (μg/ml)


Relative expression of *FOXP3* Δ2 in Treg
2.0×10-3
1.5×10-3
1.0×10-3
5.0×10-4
0.0
HC
CD
0
1
0
1
Lactate (μg/ml)

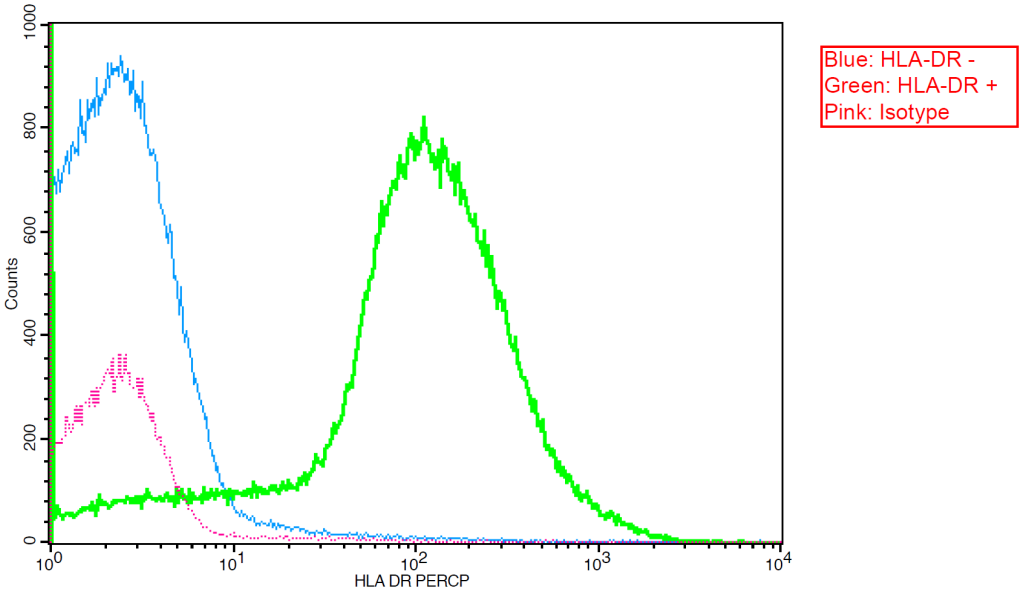
(A)



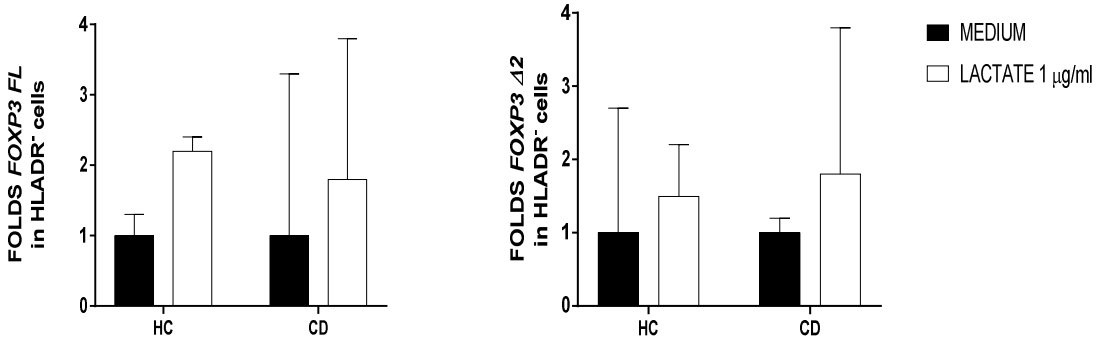


(B)

Fig. 22: Lactate does not modulate expression of *FOXP3* isoforms in $CD4^+CD25^+$ Treg and $CD4^+CD25^-$ T cells upon stimulation with lactate. The effect of lactate on the relative expression of both *FOXP3* isoforms was evaluated by real time RT-PCR in $CD4^+CD25^+$ Treg (HC *n*=6, CD *n*=6) and $CD4^+CD25^-$ T cells (HC *n*=8 and and CD *n*=11). $CD4^+CD25^+$ Treg (A) and $CD4^+CD25^-$ T cells (B) were isolated from PBMC respectively by double positive and negative selection and cultured for 48 hours with medium alone or with 1 µg/ml of lactate. The relative expression of *FOXP3* isoforms was normalized on *18S*. Statistical comparisons were made using Friedman test (A) and Krustal Wallis test (B). * $p< 0.05$.** $p< 0.005$.



(A)



(B)

Fig.23: Lactate does not modulate FOXP3 expression on in HLA-DR$^-$ cells. HLA-DR$^-$ cells were isolated from PBMC of HC (*n*=8) and CD (*n*=6) and stimulated for 48 hours with 1 µg/ml of lactate. Purity of HLA-DR$^-$ subset of cells was higher than 90% by flow-cytometry (A). The relative expression of *FOXP3* isoforms was determined by real-time RT-PCR and represented as FOLDS differences (B). The expression of each isoform in the unstimulated cells (MEDIUM) was considered as 1 fold. The relative expression of *FOXP3* isoforms was normalized on *18S*. Statistical comparisons were made using Friedman test and Krustal Wallis test. * $p< 0.05$.**

5.4 Lactate does not induce alternative activation of macrophages.

MΦ are important APC that represent a bridge between innate and adaptive immune response [124]. Two main types of mature differentiated MΦ have been described: the classically (M1) and the alternatively activated (M2). While M1 contribute to the pro-inflammatory immune response against microorganisms, M2 macrophages display a regulatory function by attenuating the inflammatory response and promoting wound healing [125]. Their maturation is induced by anti-inflammatory cytokines such as IL10 and TGFβ, but also by Th2 cytokines IL13 and IL4 or parasites and fungal cells. M2 macrophages secrete IL10 and can induce functional $CD4^+FOXP3^+$ Tregs via TGFβ [124]. Given the role that lactate was shown previously to play in regulating the immune system [111, 123], we hypothesized that this microbial derived metabolite may represent an additional new M2 inducer, thereby explaining the increased expression of FOXP3 in CD patients. We isolated $CD14^+$ cells from PBMC of HC and CD subjects and we culture them for 24 hours with medium alone, with 1 μg/ml of lactate or with a cocktail of cytokines (IL10, IL4 and TGFβ) as previously described [126]. Flow cytometry was used to determine the purity of the $CD14^+$ cells (**Fig. 24A**). We determined the percentage of M2 as $CD206^+$ cells by flow cytometry. Our analysis did not show any differences in the percentage of M2 $CD206^+$ between MΦ cultured in medium alone or stimulated with lactate in either CD or HC subjects (**Fig.24B**). The lack of differences in regulatory MΦ population was also confirmed by measuring IL10 production (**Fig. 24C**). Collectively our findings show that M2 induction cannot account for the increased expression of FOXP3 in PBMC of CD patients stimulated with lactate

and suggest that this microbial-derived metabolite triggers cells population other than MΦ.

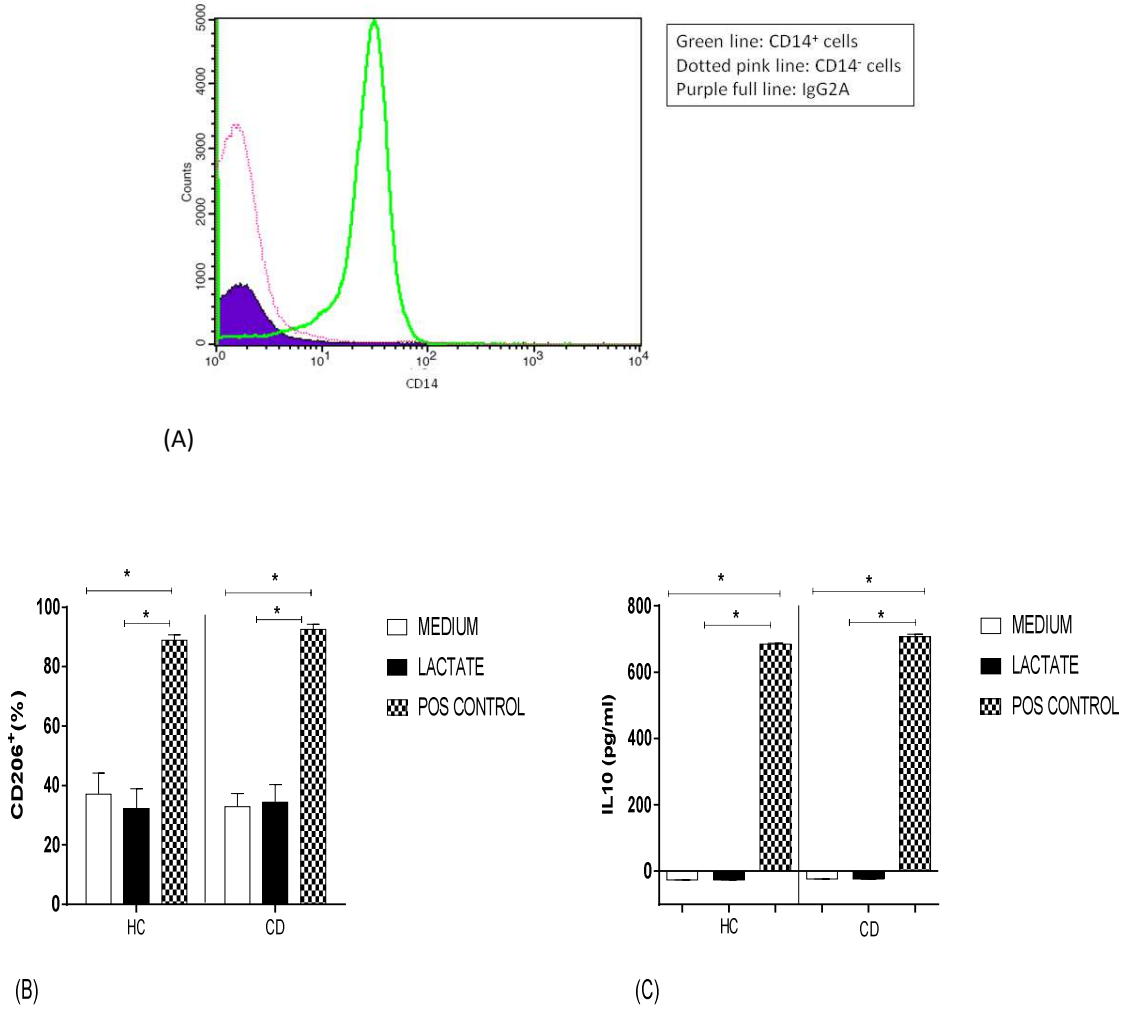


Fig.24: Detection of CD206$^+$ M2 macrophages and production of IL10. Flow cytometry was used to determine the percentage of CD206$^+$ M2 macrophages. Purity of CD14$^+$ isolate cells for subsequent macrophages differentiation was determined by flow cytometry (A). Mature macrophages (MM) sub-population was gated depending on SSC and FSC parameters. Percentage of CD206$^+$ cells was measured within the MM gate in cells from HC (*n*=7) and CD (*n*=7) patients that were cultured in medium alone, stimulated with 1 µg/ml of lactate or with positive control (cocktail of IL10, IL4 and TGFβ) (B). Differences in IL10 production were measured by ELISA in the three different culture conditions (C).

5.5 Summary of chapter 5.

We have shown that lactate triggers an increased expression of both FOXP3 isoforms in CD patients compared to HC subjects. Our data on different T cells subsets, however, suggest that this microbial-derived metabolite does not directly modulate Treg cells, but probably triggers a subpopulation of APCs. The experiments on macrophages show that lactate does not induce M2 marker expression on these cells suggesting the involvement of other APCs subpopulations. We also have also showed that lactate did not lead to an increase in anti-inflammatory *IL10* or *RORGT* expression. Taken together, these data suggest that innate alterations in the immune cells of CD patients contribute to the differences in how they respond to their surroundings.

CHAPTER 6. Discussion.

Celiac disease represents a unique model of autoimmune enteropathy because the external trigger, gliadin, as well as the genetic predisposition, HLA DQ2/DQ8, are well known [1]. The rapid worldwide increase in the incidence of CD patients that has characterized the last decades [127, 128], however, suggests that additional environmental factors may contribute to the onset of the disease.

For many years, CD was considered to be exclusively a pediatric condition, but it is now well established that this autoimmune disease can develop in patients at any age. The mechanisms that trigger the loss of tolerance to gluten later in life are still not fully understood. Tregs have been shown to play an important role in regulating immune homeostasis and in controlling chronic immune responses in the GI tract [26]. Given the increased number of Tregs that characterizes CD [17], it is plausible to hypothesize that qualitative, rather than quantitative, defects in this T cells subpopulation, contribute to the final development of the disease.

FOXP3 is a transcription factor necessary for the suppressive function and differentiation of Treg cells and, in humans, it can exist as full-length isoform (FL) or as the alternatively spliced isoform FOXP3 Δ2 [80]. This latter isoform lacks exon 2 and has been shown to be defective in inhibiting the master regulator for Th17 cells, transcription factor RORγT [79]. We reported that in the intestine of CDA patients the less functional isoform *FOXP3* Δ2, but not its counterpart FL, is expressed at significantly higher levels than in HC. Interestingly, we also found that the relative amount of *FOXP3* Δ2 in the gut mucosa of CDA patients was higher than the FL, while

the two isoforms were similarly expressed in HC subjects and CDGF patients. These findings suggest that the imbalance between isoforms, rather than the total amount of FOXP3, contributes to the defective function of Tregs in CDA patients. Our experiments confirmed that the over-expression of *FOXP3* Δ2 versus FL in CDA patients was positively correlated with higher mRNA levels of *RORGT* and the pro-inflammatory cytokine *IL17A*. Given the role that the Th17 pro-inflammatory immune response plays in the pathogenesis of CD [129, 130], it is plausible to hypothesize that the imbalance between FOXP3 isoforms plays a major role in the development of active CD by failing in down-regulating Th17 driven immune response.

Although FOXP3 Δ2 has been shown to be defective in inhibiting Th17 pro-inflammatory immune response, its exact function is still matter of debate [79, 80]. For instance it is yet unknown if the two isoforms are expressed contemporarily on the same cells or if each one of them characterizes a different subset of Tregs. Both isoforms are expressed at high levels on Treg cells and the exact function of the less functional FOXP3 Δ2 is still unknown. A better understanding on how these isoforms are expressed on Tregs would provide new insights on their role in regulating the immune system. While we have reported that both isoforms are equally expressed in the intestinal samples of HC and CDGF patients, our data from PBMC did not show the same balance and we did not find differences in *RORGT* expression among the three groups of patients (HC, CDGF and CDA). Collectively these data suggest that the unique features of the intestinal microenvironment contribute to modulate the expression of FOXP3 isoforms in pTregs. More experiments on Tregs isolated from the intestine lamina propria are necessary to better understand and investigate the mechanisms that regulate the alternative splicing

process in the intestine. Given the plasticity that characterizes the Th17 and pTregs, this would allow to take in consideration new specifically targeted therapeutic approaches for the diseases for which Th17 driven immune response plays a major role. The deficiency of FOXP3 Δ2 in down-regulating RORγT appears to be an intrinsic characteristic of this isoform. A better understanding on how the plasticity between Treg and Th17 plays a role in the pathogenesis of CD may help to clarify how the over-expression of FOXP3 Δ2 ultimately contributes to the loss of tolerance to gluten in predisposed subjets.

T cells have been shown to be particularly susceptible to surrounding signals [131], therefore, it is not surprising that a cell population may have different phenotypes depending on their microenvironment. The intestinal milieu is populated with commensal bacteria that play a fundamental role in shaping the immune system by interacting directly with immune cells to either promote or down-regulate inflammatory processes [132]. Several studies have reported that intestinal dysbiosis is associated with CD and that this may contribute to the development of the disease [133-135]. Our findings demonstrated the combination of the pro-inflammatory cytokine, IFNγ, plus microbial-derived butyrate (both present in the intestine of CDA patients) created a gut micro-milieu that established a balance between the two FOXP3 isoforms in HC subjects. The same balance between isoforms was not present in PBMC from CD patients after stimulation. Additionally we have shown that the same effect was not seen when the PBMC were stimulated with only one of the two molecules. All together these findings suggest for the first time a synergistic action between IFNγ and the butyrate and underscore the strong connection between the immune system and the microflora. The role of butyrate in regulating the immune system has been previously described. This

short chain fatty acid has been shown to induce FOXP3 expression and Treg cell differentiation in the colonic lamina propria and to promote *FOXP3* promoter acetylation [115, 118, 136]. Although the production of SCFAs such as butyrate mainly characterizes the colon, their presence has been reported also in the cecum and the small intestine [137]. Goverse et al. have shown that this specific metabolite is able to induce tolerogenic DCs activation via HDAC inhibition in the small intestine epithelial cells [138]. *In vivo* experiments in mice have shown that this SCFA triggers Tregs induction and ameliorates the development of colitis by enhancing histone H3 acetylation in the promoter and non-coding sequences regions of *foxp3* locus [118]. Furthermore Zhang et al. have reported the role of butyrate in regulating the Treg/Th17 balance therefore preventing IBD development in mice [139]. Similar butyrate mediated anti-Th17 effects have been also found in human cells. Similarly to butyrate, also IFNγ has been reported to contribute to be able to induce of Tregs differentiation [138].

Our experiments on different subsets of T cells (namely $CD4^{+}CD25^{-}$ T effector cells and $CD4^{+}CD25^{++}$ Treg cells) suggest that the butyrate and the pro-inflammatory cytokine target directly Treg cells.

The hypothesis that a specific microenvironment during gut homing may influence the alternative splicing machinery has been already suggested by Mailer et al. In their study, the authors showed that patients affected by Crohn's disease display an increased expression of the less common FOXP3 isoform lacking exon 7 and that pro-inflammatory cytokine IL1β was inducing the alternative splicing [85].

Collectively our experiments show how cells from CD patients respond differently than cells from CD subjects to the stimulation with IFNγ and butyrate. Based on our findings,

it is tempting to hypothesize that in HC subjects the stimulation with IFNγ and butyrate may mimic a pro-inflammatory environment that triggers the balance between *FOXP*3 isoforms therefore representing a compensatory mechanism used by Treg cells to become more efficient at responding to mucosal inflammation. Tregs cells from CD patients respond differently to the two stimuli and we do not see the same balance between isoforms. This may account for the lack of proper anti-inflammatory response and the subsequent chronic mucosal inflammatory process that characterizes the active state of the disease despite the high number of Treg cells.

Although our experiments suggest that Treg cells are directed affected by the combination of IFNγ and butyrate, they do not address the pathways by which the modulation of FOXP3 isoforms occurs. Several G protein coupled receptors have been shown to specifically recognize butyrate [140, 141], given their epithelial expression however, their activation would not account for the direct effect of the SCFA on Tregs. Microbial components and metabolites have been reported to trigger several TLRs on either T conventional cells and Tregs. TLR2, TLR1, TLR6 are similarly expressed on both Tregs and T effector conventional cells. In addition, Tregs express significantly more TLR4, TLR5, TLR7 and TLR8 compared to T effector cells [142]. While the effect of TLR4 activation on Tregs is still under debate, it has been shown that TLR5 binding to Tregs is able to induce FOXP3 expression and their suppressive function [32]. Similarly, also TLR2 has been demonstrated to play fundamental role in regulating Tregs expansion and function [143]. Given the crucial role that TLRs play in the regulation of T cell differentiation and the link existing between microbiome and immune system, it is plausible that butyrate may act as a TLR ligand. Therefore it will be interesting to

examine the effect that this microbial derived metabolite has on the TLR expression and how this effect may influence the balance between FOXP3 isoforms. The hypothesis that the changes on FOXP3 isoform expression observed after stimulation with butyrate and IFNγ may be due to variations in TLRs activation is supported by reports showing that IFNγ can positively or negatively influence TLR signaling [144]. This pro-inflammatory cytokine has been reported to prime macrophage responses to TLRs agonists. Furthermore, it was shown to promote TLR signaling capabilities by increasing ligand-binding properties, enhancing signaling capacity and promoting TLR activation.

In a proof of concept study we have previously shown that infants genetically predisposed to CD present an increased production of lactate associated with enrichment in *Lactobacilli spp*. in the preclinical phase, followed by a sharp drop of both just before the onset of the disease. The same metabolomic profile was not detected in kids that did not develop CD [22]. The human GI microbiota has many functions such as limiting the growth of potential pathogenic microorganisms, regulating GI development and contributing to the breakdown of dietary products [105, 145]. The intestinal immune system, in turn, plays an important role in establishing the tolerogenic environment to commensal bacteria [146] and the microbiome itself is fundamental in shaping the immune system [147, 148].

We hypothesized that lactate may trigger changes in FOXP3 expression in CD patients. Our experiments show that, upon stimulation with lactate, PBMC from CD patients in remission expressed higher level of both *FOXP3* isoforms compared to non celiac HC subjects. These data confirm our previous findings and underscore how CD patients and HC subjects respond differently to the same microenvironment. Since

Lactobacilli spp. are known to enhance the integrity of the intestinal barrier and to protectively shape the immune system [149, 150], it is tempting to speculate that the altered *FOXP3* expression in response to lactate in CD patients may represent a preclinical compensatory mechanism in CD at-risk patients to down-regulate the inflammation. This theory is supported by the evidence that longitudinal studies of infants at-risk for CD have shown that, in the preclinical phase, the onset of the autoimmune process, evidenced by the appearance of anti-tissue transglutaminase auto-antibodies, is dynamic and reversible. Indeed, 50-80% of these individuals show normalization of the auto-antibody titers without any dietary intervention [151, 152]. Interestingly, the increased expression of FOXP3 did not correlate with higher levels of *IL10.* Although Tregs can effectively suppress the immune response through mechanisms other than IL10, the lack of increased expression of the anti-inflammatory cytokine suggest that these Tregs are not fully functional.

Our data on T effector and Treg cells suggest that lactate does not directly modulate *FOXP3* expression, but it probably triggers other cells such as APC. This hypothesis is supported by several studies showing the effect of Lactobacilli or their metabolite on APC. Goodman et al. reported that the probiotic family of *Lactobacilli* contains suppressive motifs that interact with APC signals [132]. Additionally, *Lactobacillus crispatus* has been reported to to induce human DCs proliferation thereby conferring an anti-inflammatory phenotype [113].

Our experiments with lactate showed that this microbial-derived metabolite does not influence FOXP3 expression through an action on MΦ. It is plausible to hypothesize that DCs, another important subset of APC, may be involved in the mediation process

between intestinal microbiota and immune system. Several studies have already shown that lactate producing bacteria are able to modulate DCs function. *Bifidobacterium Infantis* has been reported to be able to trigger DCs in inducing anti-inflammatory $FOXP3^+$ and IL10 secreting Tregs [153]. Furthermore, several lactic acid producing bacteria have the capability of regulating DCs activation and to subsequently polarize T cells activity toward Th1/Th2/Treg response *[154]* Eslami et al. have recently found that *Lactobacillus Crispatus* confers anti-inflammatory properties to DCs, leading to induction of $CD4^+CD25^+FOXP3^+$ Tregs and IL10 production [113]. An interesting lactate receptor candidate would be GPR81. This G - protein coupled receptor has been previously reported to be specific for lactate and expressed in other subsets of APC such as MΦ [155]. It will be of great interest to determine if lactate triggers changes in other APC such as the DCs population that are able to modulate FOXP3 isoforms expression. This not only would allow us to better understand the interplay between immune cells and microbial derived metabolites, but will also give important insights on how a high production of lactate by the increased number of *Lactobaccilli spp* reported in the preclinical phase of CD may contribute to the onset of the disease.

From our data on intestinal Treg cells and the findings on how metabolites are able to modulate the expression of FOXP3 isoforms on peripheral Treg cells we hypothesize that in CD patients the following sequence of events may occur (**Fig. 25**): Treg cells in the intestinal compartment, are influenced by a specific gut microenvironment. Cells from HC or CD subjects are differentially modulated by the stimuli that characterize the intestinal microenvironment and this leads to a different expression of the two isoforms between the two groups of subjects. The preclinical phase of CD is characterized by a

high production of lactate that triggers an increased expression of FOXP3 in CD patients compared to HC. This microbial-derived metabolite, however, is not able to induce increased expression of anti-inflammatory cytokine IL10. Furthermore it does not directly target Treg cells, but probably triggers changes on a subset of APC. The increased intestinal permeability and the consequent pro-inflammatory microenvironment related to microbiota dysbiosis (such as excessive butyrate production) characterize the active state of CD [113]. This unique microenvironment triggers an efficient anti-inflammatory immune response in HC subjects by directly modulating the balance between FOXP3 isoforms, while intrinsic defects on Treg cells do not allow this balance to occur in CD patients. The sustained over-expression of FOXP3 Δ2 in CD patients impedes the efficient suppression of Th17 mediated immune response to occur and, therefore, partially contributes to the final outcome of chronic inflammation that characterizes them.

Although intriguing, our model still needs more experiments to be confirmed. pTregs located in the intestine differ from nTregs that mostly populate the peripheral blood. Therefore it would be of great interest to isolate pTregs from the lamina propria of the intestine and stimulate them with the different metabolites and cytokines to confirm that specifically enriched milieu contribute to the modulation of FOXP3 isoforms in intestinal Tregs. Furthermore it is necessary to take in consideration how other immune cells that are located in the intestine such as neutrophils and recently ILCs (innate lymphoid cells) [156] may contribute to modulate the alternative splicing process. Finally given the important role that the pTregs have on regulating oral tolerance and the effect that dietary

Ag have been shown to have on Tregs differentiation [137], it would be of great interest to further investigate if diet can influence the expression of FOXP3 isoforms.

In conclusion, our study demonstrates for the first time the existence of a direct and mechanistic link between microbiota-derived metabolites, mucosal inflammatory processes, and changes regulating *FOXP3* isoform expression. Furthermore it also underscores the multi-factorial nature of CD and provides a set of new possible targets for the prevention of CD in genetically-susceptible individuals.

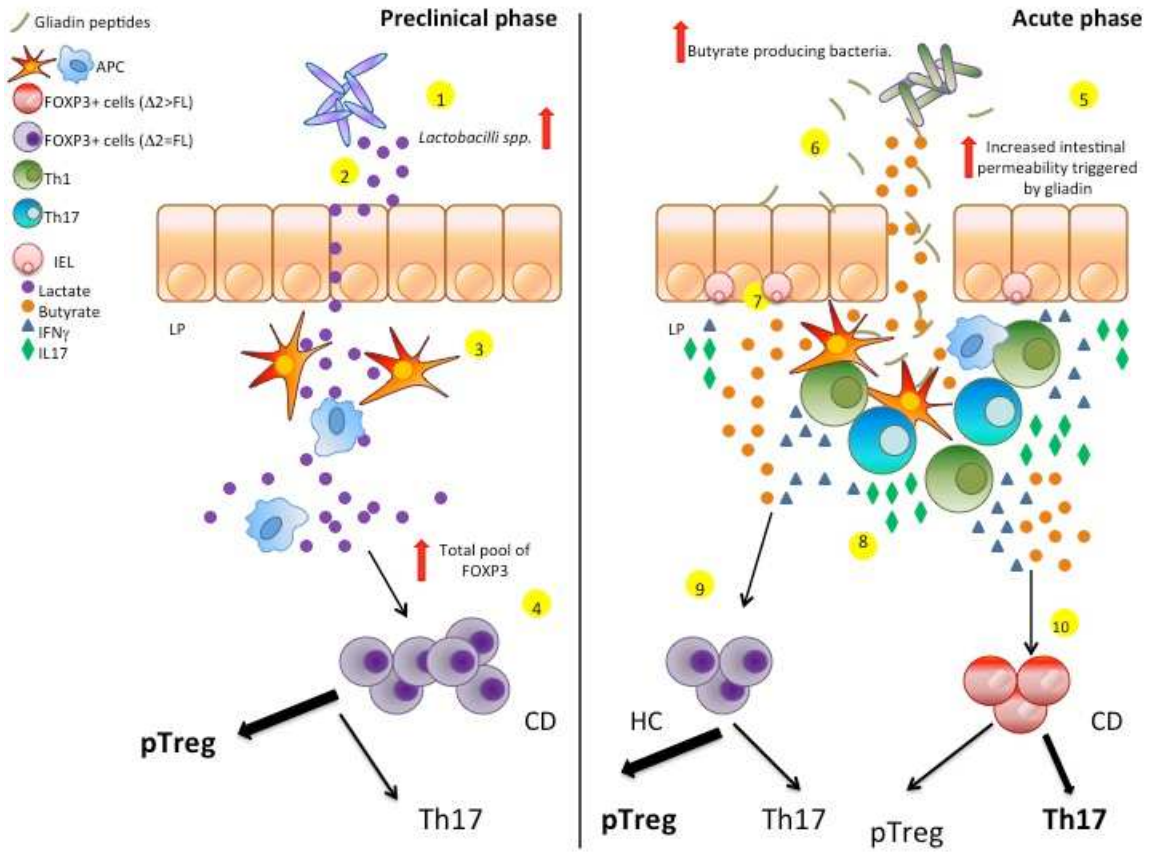


Fig. 25: Proposed sequence of events contributing to the development of CD. Increased production and enrichment in *Lactobacilli spp.* has been shown to characterize the preclinical phase of individuals genetically predisposed to CD (1, 2). The interaction between this microbial derived metabolite and APC triggers a regulatory phenotype on the cells (3) that, in CD patients, will lead to an increase in the total pool of $FOXP3^+$ cells compared to HC as a possible compensatory mechanism to maintain high level of functional $FOXP3^+$ FL isoform (4). During the active state of the disease the small intestine of CD patients is characterized by a drop in lactate levels, an increased intestinal permeability due to the interaction between gliadin and epithelial cells (5), and high production of butyrate from the microbial flora (6). The increased intestinal permeability allows gliadin peptides to translocate to the lamina propria of the small intestine where they are recognized by APC (7). This triggers an adaptive immune response Th1/Th17 driven characterized by high production of pro-inflammatory IL17 and IFNγ. In HC subjects the local micro-milieu characterized by this pro-inflammatory environment and microbiome-derived excess butyrate causes FOXP3 a equilibrium between the two FOXP3 isoforms (9). Conversely in CD patients , conversion of naïve CD4 T cells to pTregs is reduced due to persistent over-expression of FOXP3 Δ2 (less functional) over FL, leading to chronic inflammation and, eventually, autoimmune enteropathy (10).

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