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CHARACTERIZATION OF MONOGENIC ENTEROPATHIES

Fabienne CHARBIT-HENRION

PhD dissertation in Immunology

Presented and publicly defended on October 14th, 2016

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SUMMARY

Background: Mendelian mutations causing monogenic enteropathies are identified in an increasing number of genes and are responsible for either chronic inflammatory diseases (frequently called VEO-IBD for very early-onset inflammatory bowel diseases) or for congenital diarrheal disorders (CDD). Management of many patients with monogenic enteropathies requires difficult therapeutic decisions and heavy treatments, such as hematopoietic stem cell transplantation for VEO-IBD patients, or total parenteral nutrition and intestinal transplantation for CDD patients. Early molecular diagnosis is crucial to define the most pertinent treatment and increase life expectancy. During my thesis, I introduced in the laboratory big data management tool (e. g. online dedicated database) and applied next-generation sequencing tools (whole exome sequencing (WES) and targeted gene panel sequencing (TGPS)) to a cohort of patients suffering from monogenic enteropathies in order to characterize them phenotypically and genetically.

Methods: My thesis was divided in 4 steps. In Step 1, patients (n=216 in January 2016, n=260 in August 2016) recruited through a French research protocol (Immunobiota, 12 centers) and European network (GENIUS, 33 centers) were phenotypically characterized through an online dedicated database. Following precise phenotyping, molecular diagnoses were obtained by Sanger sequencing of candidate genes suggested by functional tests in Step 2. Step 3 was the adaptation of WES for our cohort of patients (59 patients were sequenced in trio and 11 sequenced by themselves or in duo) and lastly, in Step 4, TGSP was designed and applied to our cohort (173 patients without a molecular diagnosis).

Findings: The cohort gathered 57 patients including 22 with a molecular diagnosis in January 2012, and 216 patients including 70 with a diagnosis in January 2016, corresponding to a global diagnosis rate of 1/3. Approximately 50 new patients are recruited each year, with blood samples taken from each patient, both parents and siblings. During this period, 11 diagnoses were obtained by a phenotype-based approach, with identification of mutations notably in *IL-10R β* (4 patients) and *XIAP* (4 patients). Eleven patients obtained a genetic diagnosis by WES including two siblings with a *MALT1* deficiency responsible for an IPEX-like syndrome. Because of the increasing number of genes involved in monogenic enteropathies, we developed, in collaboration with Genomics, Bioinformatics and Translational Genetics platforms from the Institut IMAGINE, a custom-made TGPS gathering 68 genes responsible for either VEO-IBD or CDD. The sequencing of all negative patients (n=173) on this panel allowed to identify 28 new diagnoses (among which 8 were made in patients included before 2012).

Interpretation: This work lead to the identification of the genetic diagnosis in 1/3 patients. The close investigations of phenotype-genotype correlations highlighted frequent overlaps among monogenic enteropathies. Following completion of this work, we suggest to use TGPS as a first-line genetic test in addition to a precise phenotyping of the patient. Depending on the results, TGPS will either reach an early molecular diagnosis crucial to optimize treatments in a cost-effective manner, or allow to perform further genetic analysis notably by WES.

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ABBREVIATIONS

AIE	Autoimmune enteropathy
AMP	Anti-microbial peptide
ATG16L1	Autophagy related 16-like 1
ATG5	Autophagy related 5
BCL	B-cell lymphomas
BIM	BCL-2-interacting mediator
BM	Bone marrow
CARD11	Caspase recruitment domain-containing protein 1
CBF- β	Core-binding factor subunit beta
CCR4	C-C chemokine receptor type 4
CD	Crohn's disease
CDD	Congenital diarrheal disorders
CGD	Chronic granulomatous diseases
CID	Combined Immunodeficiency
CNS2	Conserved noncoding sequence 2
CTE	Congenital tufting enteropathy
CTLA4	Cytotoxic T lymphocyte antigen-4
CVID	Common variable immunodeficiency
DHR	Dihydrorhodamine
DSS	Dextran sulfate sodium
ED	Epithelial dysplasia
EDA-ID	Anhidrotic ectodermal dysplasia with immunodeficiency
EDA-R	Ectodysplasin receptor
EPCAM	Epithelial cell adhesion molecule
ER	Endoplasmic Reticulum
ESPGHAN	European Society of Pediatric Gastroenterology, Hepatology, And Nutrition
FFAR2	Free fatty acid receptor 2
FOXP3	Forkhead box P3
GALT	Gut-associated lymphoid tissue
GENIUS	GENetically and/or ImmUne mediated enteropathies
GFP	Green fluorescent protein
GGPP	Geranylgeranyl pyrophosphate
GI	Gastro-intestinal
GoF	Gain of function
HIDS	Hyperimmunoglobulinemia D and periodic fever syndrome
HIES	Hyper IgE syndrome
HLA	Human leukocyte antigen
HLH	Hemophagocytic lymphohistiocytosis
HMG-CoA	Hydroxy-méthyl-glutaryl-coenzyme A
HSCT	Hematopoietic stem cell transplantation
IBD	Intestinal bowel disease

IEC	Intestinal epithelial cells
IFN γ	Interferon gamma
IgA	Immunoglobulin A
IL-10	Interleukin 10
IMBT	Immunobiota
iNKT	Invariant natural killer T cells
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IQR	Interquartile range
IRAK-4	IL-1R-associated kinase 4
IRF4	IFN regulatory factor 4
IRGM	GTPase family M
iTreg	Induced Treg
I κ B α	Inhibitor of kappa B, alfa
I κ K	I-kappa B kinase
JAK1	Janus kinase 1
KLRG1	Killer cell lectin-like receptor subfamily G member 1-positive
KO	Knock-out
LAMP2	Lysosomal-associated membrane protein
LC3	Microtubule-associated protein 1 light chain 3
LoF	Loss of function
LRBA	Lipopolysaccharide responsive beige-like anchor protein
LRR	Leucine-rich repeats
LUBAC	Linear ubiquitin chain assembly complex
MAIT	Mucosal-associated invariant T-cells
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAP	Mitogen activated proteins
MCL1	Myeloid leukemia cell differentiation 1
MCP-1	Monocyte chemoattractant protein 1
MDP	Muramyl-dipeptide
meso-DAP	Meso-L-Ala-y-D-Glu, meso-diaminopimelic acid
MHC	Major histocompatibility complex
MIA	Multiple intestinal atresia
miRNA	Micro RNA
MKD	Mevalonate kinase deficiency
MLN	Mesenteric lymph nodes
MUC2	Mucin 2
MVID	Microvillus inclusion disease
MyD88	Myeloid differentiation primary response gene 88
MYO5B	Myosin 5B
NADPH	Nicotinamide adenine dinucleotide phosphate
NFAT	Nuclear factor of activated T-cells
NF- κ B	Nuclear factor-kappa B
NLR	NOD-like receptor
NLRC4	NLR family-Card domain containing 4
NLRP3	NOD-like receptor family, pyrin domain containing 3

NOD2	Nucleotide-binding oligomerization domain 2
NO	Nitric oxide
NPC1	Niemann-Pick disease type C1
OL-EDA-ID	Osteopetrosis and lymphoedema associated to EDA-ID
OR	Odd ratios
OTU	Ovarian tumor domain
PAMP	Pathogen-associated molecular pattern
PAS	Periodic acid Schiff
PI3K	Phosphatidylinositol 3 kinase
PI4KIII α	Phosphatidylinositol 4-kinase III alfa
PID	Primary immunodeficiencies
POLA1	DNA polymerase alfa 1
Poly(IC)	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptors
Rac1	Ras-related C3 botulinum toxin substrate 1
RAG1	Recombination activating gene 1
RhoA	Ras homolog gene family, member A
RIG-1	Retinoid acid-inducible gene-1
RIP1	Receptor-interacting serine/threonine-protein kinase 1
RIPK2	Receptor-interacting serine/threonine-protein kinase 2
ROR γ	RAR-related orphan receptor gamma
ROS	Reactive oxygen species
RUNX1	Runt-related transcription factor 1
SCFA	Short-chain fatty acids
SCID	Severe combined immunodeficiencies
SH3	SRC Homology 3
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosus
SOCS1	Suppressor of cytokine signaling 1
SPF	Specific-pathogen free
SPINT2	Serine Peptidase Inhibitor, Kunitz Type, 2
STAT1	Signal transducer and activator of transcription 1
STING	Stimulator of type 1 IFN gene
STX3	Syntaxin 3
STXBP2	Syntaxin 3 binding protein
TAK1	Transforming growth factor beta-activated kinase 1
TBK1	TANK Binding Kinase 1
TCR	T-cell receptor
TGF- β	Transforming growth factor beta
TGPS	Targeted gene panel sequencing
Th1	T helper type 1
THES	Trichohepatoenteric syndrome
TLR	Toll-like receptor
TNBS	2,4,6-trinitro benzene sulfonic acid
TNF α	Tumor necrosis factor alfa

TRAF2	TNF receptor-associated factor 2
Treg	Regulatory T cells
TTC7A	Tetratricopeptide repeat domain 7A
UC	Ulcerative colitis
UPR	Unfolded protein response
VEO-IBD	Very early onset inflammatory bowel disease
WES	Whole exome sequencing
WGS	Whole genome sequencing
WT	Wild type
XBP1	X-box-binding protein 1
XIAP	X-linked apoptosis inducing protein
XLP-2	X-lymphoproliferative disease
XLPR	X-linked reticulate pigmentary disorder
ZnF	Zinc-finger

INTRODUCTION

PREAMBULE

Intestinal barrier: of mice and men

With a surface of approximately 300-400m², the intestinal mucosa is the largest interface of the human body. It is made of a single layer of epithelial cells supported by a layer of connective tissue called *lamina propria*, and is strongly interconnected with a considerable number of immune cells of hematopoietic origin assembled in the gut associated lymphoid system. Altogether they form a highly regulated barrier able to cope with several challenges. On one hand, the primary function of the intestinal epithelium is the digestion and absorption of nutrients, water and electrolytes. On the other hand, the intestinal barrier must restrict body access to undigested food antigens and to the considerable and complex community of microbes, which settle after birth in the intestinal lumen, where they find the metabolic resources to thrive. Microbial density increases along the gastrointestinal tract and reaches impressive concentrations of 10¹¹ to 10¹² bacteria and archaea per gram of luminal content in the distal gut, which can be seen as the ecological site on Earth the most densely populated by microbes (Cerf-Bensussan and Gaboriau-Routhiau, 2010). As a whole, the intestinal microbiota may contain over 100 fold more genes than the human genome and encode a broad spectrum of enzymatic activities, which can considerably enlarge host metabolic capacities (Gill et al., 2006). Hosts and their microbiota are thus thought to have evolved mutualistic relationships that are largely based on metabolic and energy exchanges. Yet this huge mass of microbes at the intestinal surface must be kept in check. Hosts have evolved multiple mechanisms, which cooperate within a highly dynamic network of interactions implicating both intestinal epithelial cells and immune cells to preserve the symbiotic relationship with the intestinal microbiota, while retaining the ability to recognize and react swiftly against pathogens.

The intricate relationship between hosts and intestinal microbes has been extensively analyzed over the past 20 years in animal models and through physiopathogenic studies of human inflammatory bowel diseases (IBD). It is now admitted that, in polygenic IBD such as Crohn's disease (CD) or ulcerative colitis (UC), inflammation results from abnormal host immune responses to the microbiota. Genome wide association studies have pinpointed over 200 genetic polymorphisms, which may affect the function of host pathways that are important to build and regulate the gut immune barrier (Liu et al., 2015). Yet, altogether these polymorphisms account for less than 14% of the risk to develop IBD, and it is increasingly clear that environmental factors play a central role in jeopardizing host-microbiota interactions (McGovern et al., 2015). Therefore, the exact contribution of genetic variants to inflammation remains difficult to delineate. However, a growing

number of rare Mendelian diseases have and are being identified in children as a cause of severe intestinal inflammation of very early onset (VEO-IBD). Analysis of the expanding number of genes, which can be affected by mutations causing intestinal inflammation thus provides precious information on host pathways indispensable to build and regulate the gut barrier in humans (Uhlir, 2013).

In the INTRODUCTION, I have therefore chosen to illustrate how the different components of the intestinal barrier cooperate to protect the host and to maintain intestinal homeostasis, through the analysis of monogenic enteropathies. This description has been completed, when considered useful, by data concerning selected variants predisposing for human polygenic IBD and by data in mouse models. The first part of this chapter is focused on the role of the epithelium in the gut barrier. The importance of mucus and of two key cellular mechanisms, endoplasmic reticulum stress and autophagy, are discussed. Two monogenic diseases affecting epithelial differentiation and functions, microvillus inclusion disease and congenital tufting enteropathy/epithelial dysplasia, are described. The second part discusses the contribution of innate immune pathways to the gut barrier by highlighting the roles of MyD88, NEMO, NOD2/XIAP and reactive oxygen species production. The third part illustrates how defective regulation of innate immune signaling pathways (overproduction of IL-1 β , STAT3 gain of function, loss of regulation of the NF- κ B pathway in A20 deficiency), lack of regulation by IL-10 or by regulatory T cells, can lead to severe intestinal inflammation. The last part describes syndromes combining defects in epithelial and hematopoietic compartments due to mutations in *TTC7A*, in *TTC37* or *SKIV2L* (tricho-hepato-enteric syndrome) or in *POLA1*. A novel as yet unsuspected role of the nucleic acid homeostasis pathway in intestinal inflammation is highlighted.

I. DEFECTS OF THE EPITHELIAL BARRIER

I.A-The role of epithelium in the intestinal barrier

The intestinal epithelium is made of a single monolayer of epithelial cells (IEC), which arise from stem cells located at the bottom of the crypts. The latter differentiate into several cell types, distributed along crypts and villi in the small intestine and within colonic glands in the large intestine. The majority of IEC are absorptive enterocytes, which predominate in the small intestine where they are essential to the digestion and absorption of nutrients. Goblet cells, specialized in the production of mucins increase in numbers along the gut and are very dense in the colon. Paneth cells, which produce large amounts of antimicrobial proteins (AMPs), are located at the bottom of the crypts of the small intestine, notably in the ileum. Enteroendocrine cells, responsible for the secretion of numerous hormones regulating digestive functions (Peterson and Artis, 2014) and Tuft cells, recently identified as the main source of interleukin 25 (IL-25) in response to parasite infection (Gerbe and Jay, 2016), are interspersed between other cell types. The intestinal epithelium is entirely renewed every 3-5 days and this rapid epithelial cell renewal, one of the highest in the human body, allows rapid restoration of an intact epithelial barrier in case of injury.

The role of the epithelium in maintaining intestinal homeostasis is discussed below through the description of selected models of mice harboring mutations in genes associated with polygenic IBD which impair relationships between hosts and their microbiota and of two monogenic human diseases, microvillus inclusion disease, and congenital tufting enteropathy (also called epithelial dysplasia). The latter are severe epithelial defects that primarily result in loss of intestinal absorption as well as impaired epithelial barrier function.

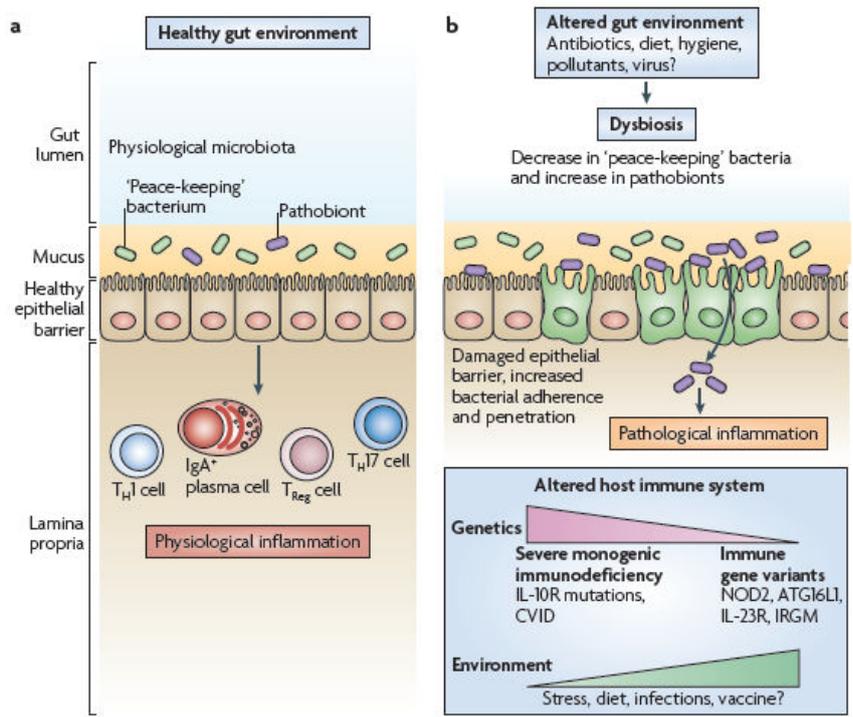


Figure 1, from (Cerf-Bensussan and Gaboriau-Routhiau, 2010)- Schematic representation of host-microbiota interactions in the healthy and inflamed gut*

*All throughout the manuscript, legends of Figures written in italic are copied from the quoted article/review.

To facilitate the discussion of genetically modified mouse models, I have briefly introduced two models of chemically-induced colitis which are widely used to analyze the consequences of gene inactivation in the mouse intestinal barrier.

Dextran sulfate sodium (DSS) colitis

Feeding mice for several days with dextran sulfate sodium (DSS) polymers in the drinking water induces a very reproducible acute colitis. DSS is believed to act via a direct toxic effect on IEC by damaging the mucus layer that is rendered penetrable to bacteria. Owing to the diurnal drinking cycle of mice, the animals can partly recover from the effects of DSS-induced damage during the first days until prolonged bacterial contact with the epithelium causes colitis. It shows the importance of the inner mucus layer in protecting the colon (Johansson and Hansson, 2016). Since DSS induces a severe colitis in mice lacking T and B cells, adaptive immunity is thought to play a minor or no role, and DSS colitis is more particularly useful to study the contribution of innate immune mechanisms to intestinal inflammation (Wirtz and Neurath, 2007).

Colitis induced by 2,4,6-trinitro benzene sulfonic acid (TNBS) or by oxazolone

In these two models, colitis is induced by intrarectal instillation of TNBS (2,4,6-trinitro benzene sulfonic acid) or of oxazolone dissolved in ethanol, after skin pre-sensitization or not. In both models, ethanol is required to break the mucosal barrier, while TNBS or oxazolone induce an adaptive immune response against hapten-modified antigens, either autologous or microbiota derived proteins (Wirtz and Neurath, 2007). The nature of the T cell response is however different, TNBS inducing a T helper type 1 immune response (Th1, dominated by the production of interferon γ (IFN γ) and tumor necrosis factor α (TNF α)) while oxazolone rather induces Th2 cells (producing notably IL-13).

I.A.1. Barrier role of mucus

Mucus is produced by goblet cells that are found along the entire gastrointestinal tract, from mouth to rectum. Goblet cells are however more particularly numerous in the stomach and in the colon. They produce mucins, which are very large and abundantly glycosylated proteins. In addition to their important glycosylation, that enables a “water-holding” effect, mucins can form massive aggregates (McGuckin et al., 2011). Twenty mucin-encoding genes have been reported in humans, the major airway mucins being MUC5A and MUC5B, whereas MUC2 is the main intestinal mucin (Thornton et al., 2008). Two major group of intestinal mucins can be distinguished, transmembrane mucins that form the glycocalyx and gel-forming mucins such as MUC2 that are produced by goblet cells and are released by protease cleavage into the lumen. Work by the group of Johansson and Hansson has shown that the thickness of the mucus layer varied along the digestive tract. In the small intestine, the mucus layer is thin, fluid and even discontinuous, likely to facilitate nutrient absorption. In contrast, in the colon, where the density of bacteria increases considerably, mucus is organized into two layers, one central fluid layer, in which bacteria can thrive and forage for mucin-derived sugars, and one external very thick layer almost impermeable to bacteria at steady state (Johansson and Hansson, 2016).

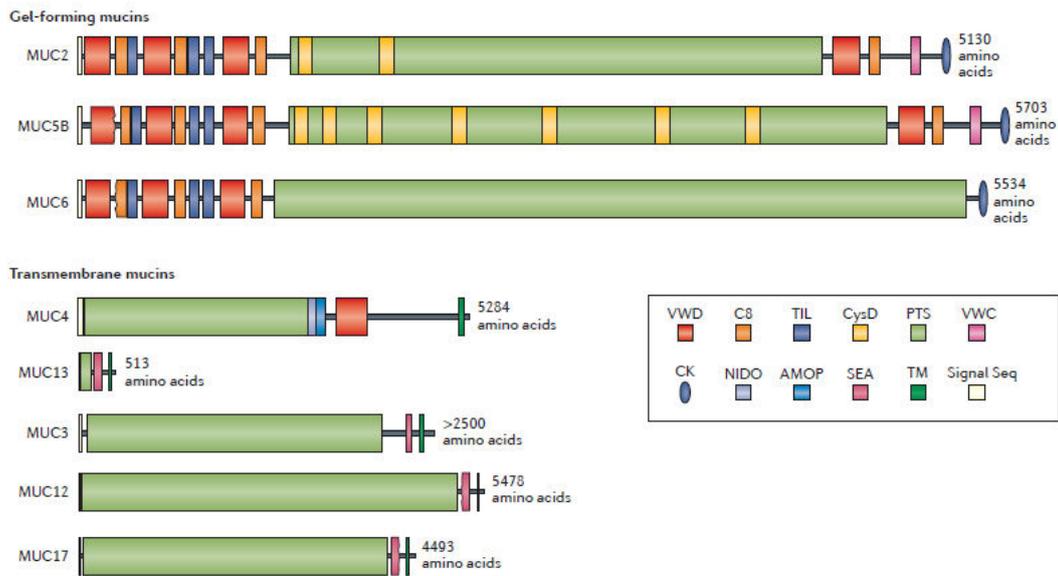


Figure 2, from (Johansson and Hansson, 2016): The domain structures of gel-forming and transmembrane intestinal mucins expressed in the small intestine. The proline, threonine and serine (PTS) domains become heavily O-glycosylated to form the mucin domains. The non-PTS parts of the gel-forming mucins are rich in cysteine amino acids and form compact structures. AMOP; adhesion-associated domain; C8, conserved 8 cysteines domain; CK, cysteine knot domain; NIDO, nidogen domain; Signal Seq, signal sequence domain; SEA, sea urchin sperm protein, enterokinase, and agrin domain; TIL, trypsin inhibitor-like cysteine rich domain; TM, transmembrane domain; VWC, Von Willebrand factor type C domain; VWD, Von Willebrand factor type D domain.

The key role of mucins in the maintenance of gut homeostasis was demonstrated in 2006 with the first description of *Muc2*^{-/-} mice. These mice developed spontaneous colitis at 5 weeks of age which aggravated with time. Histological analysis showed mucosal thickening, increased proliferation, and superficial erosions. Strikingly, analysis with microbial probes revealed many bacteria adhering to the intestinal mucosa, demonstrating the key role of the mucus in allowing bacterial segregation from the intestinal surface. *Muc2*^{-/-} mice were more sensitive to DSS colitis than heterozygous *Muc2*^{+/-} or wild type (WT) mice, with observation of many crypt abscesses instead of mucosal ulcerations. Notably, even though heterozygous *Muc2*^{+/-} mice were less sensitive to DSS than *Muc2*^{-/-} mice, they were also more sensitive to DSS than WT mice, pointing out a dose-effect (Van der Sluis et al., 2006).

Mucus deficiency in human intestinal diseases?

Colonic expression of MUC2 is markedly diminished in UC patients (Van Klinken et al., 1999), along with the loss of glandular cells observed during chronic inflammation. As in mice, the loss of MUC2 is associated with enhanced bacterial adhesion to the colonic mucosa, thereby resulting in excessive microbial signaling to epithelial cells and increased risk of microbial translocation across

epithelium. The loss of MUC2 thus participates to a vicious circle, which maintains colonic inflammation. However, there has never been yet any example in humans of monogenic enteropathy due to mutations in mucins genes. This may be explained by some functional redundancy between mucins in humans. Thus, mucin genes contain long regions of repeated sequences that are rich in GC nucleotides and share important homology (Thornton et al., 2008). Of note, any whole exome sequencing (WES) reveals at least 5-10 rare variants in mucin genes. Ascribing a causative effect to one of these variants is extremely difficult since nobody carries the “normal” sequence of all genes. Yet, as discussed below, other genetic factors can impair mucus production in humans, and may thereby predispose to intestinal inflammation.

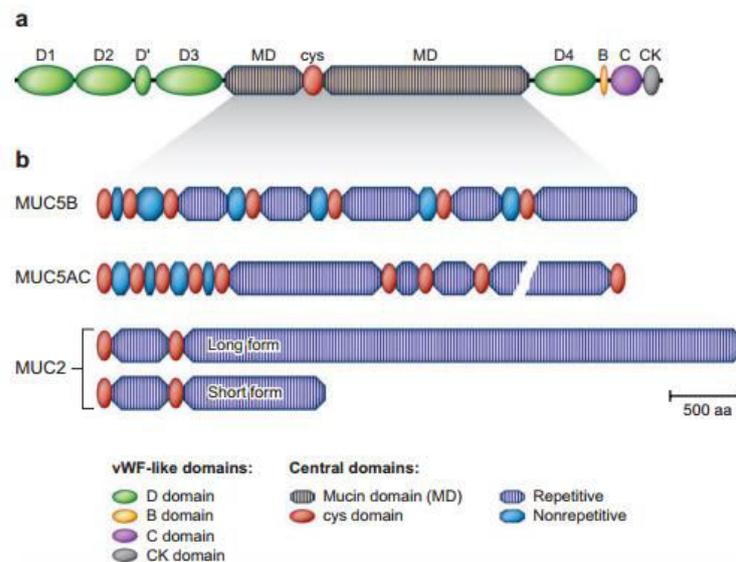


Figure 3, from (Thornton et al., 2008) – Repetitive domains in mucins

I.A.2- Role of endoplasmic reticulum stress in epithelial barrier function

The endoplasmic reticulum (ER) is the cellular organelle in which proteins are synthesized, modified (for instance by glycosylation), and folded (notably by establishing disulfide bonds). Accumulation of improperly folded proteins causes ER stress that triggers the unfolded protein response (UPR). This response aims at restoring protein-folding homeostasis by three main mechanisms: i) transient reduction in protein translation; ii) increase in folding capacity; iii) initiation of apoptosis when ER stress cannot be resolved (Janssens et al., 2014).

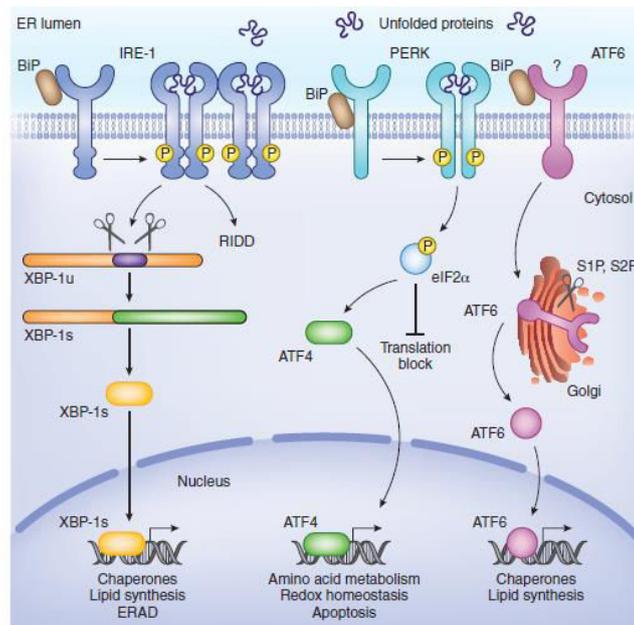


Figure 4, from (Janssens et al., 2014) – The three UPR pathways in humans

One of the three UPR pathways activates the splicing of XBP1 (X-box-binding protein 1), a transcription factor that induces expression of chaperone molecules required for correct protein folding. All cells that produce large amounts of proteins such as goblet cells, Paneth cells and plasma cells are therefore prone to ER stress. Kaser and colleagues engineered mice deleted of *Xbp1* specifically in IEC. These mice exhibited ER stress and spontaneous enteritis with complete loss of Paneth cells and reduction of goblet cells, associated with a considerable increase in activation of the transcription factor (nuclear factor-kappa B) NF- κ B. When challenged by DSS, *Xbp1*^{-/-} mice displayed more severe colitis with increased areas of mucosal erosions, edema, and cellular infiltration along with increased crypt loss compared to WT littermates, whereas heterozygous mice displayed an intermediate phenotype (Kaser et al., 2008). Interestingly, overexpression of HLA-B27, a protein prone to misfolding in humanized rats (human leukocyte antigen B27, expressed with human β 2-microglobulin in transgenic rats) leads to spontaneous colitis and arthritis, thereby mimicking inflammatory disorders observed in a subset of HLA-B27 expressing individuals (Milia et al., 2009). Currently, no human monogenic disease has been described in genes related to ER stress or implicated in UPR. However, *XBP1* variants predisposing to both CD and UC have been found, suggesting that unresolved ER stress might predispose to intestinal inflammation in humans (Adolph et al., 2012).

I.A.3- ATG16L1 and the role of autophagy in epithelial cells

Autophagy is one of the most evolutionary conserved cellular processes triggered by fasting. It is a complex process involving multiple proteins which activates the formation of vesicles containing cytoplasm and cytoplasmic organelles, and their fusion with lysosomes where these components can be degraded. Through this “self-digestion”, cells can salvage nutrients and maintain vital cellular functions during fasting. Cells can eliminate damaged organelles, misfolded proteins, but also invading microorganisms in a process using similar cellular mechanisms called xenophagy. Numerous diseases have been linked to autophagy defects, including neurodegenerative, liver, cardiac, tumoral and dysimmune diseases (Levine and Kroemer, 2008).

Following the identification of a non-synonymous coding variant of *ATG16L1* (Thr300Ala) predisposing to adult IBD, Cadwell and colleagues were the first to generate mouse models to study the role of Atg16L1 but also of Atg5, which form a complex involved in the formation of autophagosomal vesicles. Since complete inactivation of either gene was lethal, they engineered mice with hypomorphic defective protein expression of Atg16L1 or Atg5 restricted deletion to IEC. They observed that Atg16L1- and Atg5-deficient Paneth cells displayed marked abnormalities in the granule exocytosis pathway, attested by disordered, diminished or diffuse lysozyme staining. Their examination of biopsies from CD patients homozygous for the *ATG16L1* risk allele showed similar abnormalities in Paneth cell granules (Cadwell et al., 2008).

Two years later, Cadwell and colleagues used the same *Atg16L1* hypomorphic mice to suggest that intestinal inflammation happened as the consequence of a “multi-hit” process. Unexpectedly they observed that *Atg16L1* hypomorphic mice, rederived in specific-pathogen free (SPF) animal facility, were indistinguishable from WT littermates and failed to display aberrant packaging of the granule protein lysozyme in Paneth cells. In this SPF setting, they demonstrated that Paneth cell abnormalities were triggered by persistent norovirus infection. Triggering was strain specific as non-persistent strains did not induce Paneth cells abnormalities. Persistent-strain infected mice had also to be challenged by DSS to aggravate inflammation. Conversely treatment with either TNF α or IFN γ blocking antibodies, or broad spectrum antibiotics, drastically reduced inflammatory lesions. Overall, the authors suggested that IBD occur along a “multi-hit” model involving genetic predisposition and several environmental triggers which cooperate to ultimately induce inflammation mediated by Th1 cytokines (Cadwell et al., 2010).

The role of the *ATG16L1* (Thr300Ala) was further investigated by the group of R. Xavier, who developed knock-in mice homozygous for this variant. These authors showed that the variant promoted cleavage by caspase 3 and 7 and thereby resulted in lesser amount of functional ATG16L1 protein. They observed that mice raised in SPF conditions displayed not only Paneth cells abnormalities comparable to those described by Cadwell et al, but also enlarged goblet cells

reminiscent of those described in mice with epithelial inactivation of ATG5. In this model in which the variant was expressed in all cells, the authors also observed defective bacterial clearance and increased IL-1 β secretion by macrophages infected with *Salmonella typhimurium*. Yet curiously, they did not report any evidence of intestinal inflammation (Lassen et al., 2014). The exact role of ATG16L1 deficiency in intestinal inflammation remains however incompletely understood and is likely to involve other cells than IEC. Cadwell and coworkers have recently shown that mice with hypomorphic ATG16L1 displayed an intestinal hyperimmune signature notably characterized by enhanced transcription of type 1 IFN genes in response to infection by *Citrobacter rodentium*. Unexpectedly, this conferred protection against this enteropathogen. Protection was abolished by eliminating macrophages (Marchiando et al., 2013). Along the same line, Mazmanian and coworkers have recently shown that ATG16L1^{-/-} macrophages produced more inflammatory cytokines upon activation by bacterial products and were less efficient in triggering IL-10 production by regulatory T cells than WT cells. This effect was ascribed to a role of ATG16L1 in the non-canonical autophagy pathway and was comparable to the one observed in macrophages lacking nucleotide-binding oligomerization domain 2 (NOD2). This intracellular sensor of bacteria-derived muramyl-dipeptide (MDP), which was the first genetic predisposing factor implicated in human IBD (see below) was shown to physically recruit ATG16L1 (Chu et al., 2016). Altogether these data indicate that ATG16L1 dysfunction may predispose to intestinal inflammation via multiple mechanisms involving epithelial cells but also immune cells of hematopoietic origin. Recent work in humans further indicated that ATG16L1 is involved in the negative control of the mitochondrial antiviral signaling pathway and that production of type 1 IFN was abnormally increased in colon cancer cells harboring the *ATG16L1* (Thr300Ala) mutation, an effect correlated with increased survival to colon cancer (Grimm et al., 2016). It is not excluded that this mechanism may conversely predispose to protracted intestinal inflammation.

Autophagy and human intestinal diseases?

In humans, susceptibility to CD conferred by the risk allele *ATG16L1* (Thr300Ala), is relatively weak with an odd ratio of 1.38 (McGovern et al., 2015). In addition, the variant is found in up to 50% in European-derived populations. Yet, it is interesting to stress that a common exonic (c.313C>T) variant in a second gene involved in autophagy, Immunity-related GTPase family M (IRGM) was also shown to predispose to CD. The exonic variant is thought to jeopardize the regulation of IRGM transcription by microRNA196 (miRNA196) up-regulated in CD tissues. Recent work indicated that IRGM is a partner of both NOD2 and ATG16L1 and plays a central role in the activation of the core autophagy machinery (Chauhan et al., 2015). Presently, no human monogenic disease has however been ascribed to an autophagy defect.

I.A.4- Monogenic enteropathies with predominant epithelial defect

Congenital diarrheal disorders (CDD) are a vast group of monogenic enteropathies that usually start within the first days or weeks of life, or even *in utero*. They affect primarily or exclusively IEC functions. They are characterized by an intractable diarrhea which may be isolated or part of a syndrome. This severe diarrhea leads rapidly to life-threatening dehydration and serum electrolyte imbalance. CDD are often associated with intestinal failure requiring long-term parenteral nutrition or even bowel transplantation. Their phenotype and genotype have been extensively characterized (Canani et al., 2015). Chronic diarrhea is ascribed to either secretory or osmotic mechanism. Secretory diarrhea is caused by an excessive electrolytes and water flux from the intestinal mucosa towards the luminal content. Microvillus inclusion disease (MVID) or congenital tufting enteropathy/epithelial dysplasia (ED) are typical secretory diarrheas. Osmotic diarrhea is due to the presence of undigested and/or non-absorbed nutrients, which attract water flux. Osmotic diarrhea may be improved by dietary changes, notably by excluding nutrients that cannot be digested. CDD can be divided into three subsets: defects in enterocyte structure, defects in digestion/absorption, or defects in enteroendocrine cells differentiation. I will only describe CDD due to defects in enterocyte structure, which highlight the barrier role of the epithelial monolayer.

I.A.4.1- Microvillus inclusion disease (MVID)

MVID is an autosomal recessive monogenic enteropathy characterized by a profuse, watery intractable diarrhea (up to 150ml/kg/day water loss at birth) that can even start *in utero* with the formation of hydramnios. Mutations were first described in *MYO5B*, a member of the myosin family (Muller et al., 2008; van der Velde et al., 2013). The defect in enterocyte structure in MVID is the loss of the apical brush border associated with intracellular microvillus inclusions (Canani et al., 2015). MVID diagnosis is made by examination of small bowel biopsies that show accumulation of periodic acid Schiff (PAS) reactivity (thick and blur PAS staining at the apical side), and/or abnormal expression of the brush border enzyme CD10 in the cytoplasm of enterocytes. Visualization of microvillus inclusions can be further demonstrated by electron microscopy. Even though *MYO5B* is ubiquitously expressed, extra intestinal symptoms are rare, perhaps due to partial redundancy of *MYO5A* and *MYO5C* in other organs (van der Velde et al., 2013). Nevertheless severe cholestasis can be observed in some patients, due to abnormal polarization of hepatocytes (Girard et al., 2014).

WES of MVID patients with normal MYO5B sequence and milder phenotype led to identify mutations in Syntaxin-3 (STX3) (Wiegerinck et al., 2014), an apical receptor that regulates protein trafficking and vesicle fusions in IEC. In addition, a mild MVID phenotype has been reported in patients with familial hemophagocytic lymphohistiocytosis (HLH) type 5 due to mutations in STX3 protein binding 2 (STXBP2, also known as MUNC18-2) (Stepensky et al., 2013). Accumulation of PAS-positive granules and thicker CD10 staining at the apical side of enterocytes as well as abnormal kidney epithelial cells were observed, accounting for the severe watery diarrhea and nephropathy described in these patients. Intestinal and kidney defects persisted after correction of the lymphocyte defect by hematopoietic stem cell transplantation (HSCT) indicating that loss of function of STXBP2 is responsible for abnormal cellular trafficking not only in cytotoxic T cells but also in polarized epithelia.

Mouse models of MVID

Several mouse models recapitulate intestinal epithelial lesions in MIVD. In 2007 Sato and colleagues generated *Rab8A*^{-/-} mice, which displayed diarrhea and progressive wasting after 2.5 weeks. They died at 5 weeks, likely from intestinal failure as their small intestines were swollen and contained undigested milk. As Rab8 was thought to regulate basolateral transport in polarized kidney epithelial cells, the authors examined basolateral and apical markers in IEC. Unexpectedly, basolateral markers (like Low-Density Lipoprotein receptor and Na⁺, K⁺ATPase) showed proper localization whereas apical markers (dipeptidyl peptidase IV, alkaline phosphatase, sucrase-isomaltase, and oligopeptide transporter 1) were markedly decreased in the apical membrane and accumulated intracellularly. Kidney epithelial cells and hepatocytes were normal. Mice exclusively lacking *Rab8A* in IEC displayed the same phenotype. Examination of IEC from conditional *Rab8A*^{-/-} mice with mosaic expression of Rab8 found large sub-apical vacuoles identical to those in fully deficient *Rab8A*^{-/-} mice in Rab8-negative but not in Rab8-positive IEC. Electron microscopy examination of IEC from *Rab8A*^{-/-} mice showed marked shortening of microvilli at three weeks, enlarged organelles with electron-dense materials strongly positive for the apical marker dipeptidyl peptidase IV but also for the endosome/lysosome marker LAMP2 (lysosomal-associated membrane protein) while Rab8 did not colocalize with this marker in WT IEC. The authors concluded that Rab8 deficiency prevented normal polarization and resulted in mislocalization of brush border peptidases and transporters to lysosomes, where they were degraded. Digestion and absorption were massively impaired, leading to rapid malnutrition and death. Because of the similarities between phenotypes in *Rab8A*^{-/-} mice in MVID patients, the authors sequenced *RAB8* but failed to identify any mutation in 3 MVID patients (Sato et al., 2007).

In 2012, specific inactivation of *Cdc42* in IEC led to the delayed development of MVID in 10% of the mutant mice, which died at 6 months of age with an average body weight reduction of 60-70%, compared to WT littermates. Apart from large intracellular vacuolar structures, epithelial cells displayed additional features of MVID, notably cell division defects, reduced capacity for clonal expansion and differentiation into Paneth cells, and increased apoptosis in stem cells. It was shown that *Cdc42* deficiency impaired Rab8 activation and its association with multiple effectors. The authors suggested that defects of the stem cell niche might cause MVID (Sakamori et al., 2012).

In 2014, mice with selective intestinal deletion of *Rab11a* were engineered, as ubiquitous *Rab11a* deletion was lethal during embryogenesis. *Rab11a*^{-/-} mice started to die one week after birth and displayed histological features of MVID with cytoplasmic accumulation of apical shorter microvilli and microvillus inclusion bodies. In addition, Rab8a was mislocalized. As Rab11a was also mislocalized in *Rab8a*^{-/-} mice and in one *MYO5B*-mutated patient, the authors pointed out functional relationships between Rab11a, Rab8a and myosin Vb *in vivo* (Sobajima et al., 2014).

No patients with mutations in *CDC42* or *RAB11A* have been reported yet.

The first intestine-specific *Myo5b*-deficient (*Myo5b*^{fl/fl}; *Vil-CreERT2*) mouse model was only generated very recently. Mice developed severe diarrhea within 4 days after tamoxifen induction. They recapitulated histological hallmarks of *MYO5B* deficient patients with subapical accumulation of PAS and alkaline phosphatase staining, almost complete absence of apical microvilli, appearance of microvillus inclusions, and enlarged intercellular spaces by electron microscopy (Schneeberger et al., 2015). Using a tamoxifen inducible model, Weis and colleagues further showed that *Myo5B* loss at 8 weeks of age reduced duodenal brush border enzymes but induced much less prominent microvillus inclusions than at 2 weeks of age, suggesting that *Myo5b* deficiency may differ with age (Weis et al., 2016).

Altogether, human MVID and related mouse models have allowed unravelling the complementary roles of *Myo5B*, Rab8 and Rab11 in the establishment of polarization in IEC, which is essential for their proper absorptive function.

I.A.4.2- Epithelial dysplasia (ED)

Epithelial dysplasia (ED), also known as congenital tufting enteropathy (CTE), is another CDD due to a structural defect of epithelium. As MVID, ED patients display severe, watery, intractable

diarrhea since birth with severe failure to thrive. ED can be isolated or part of a syndrome with superficial punctate keratitis and choanal atresia.

ED was first ascribed to recessive autosomal *EPCAM* mutations in 2008 in patients with chronic diarrhea only. EpCAM (for epithelial cell adhesion molecule) is a transmembrane protein, located at IEC basolateral membrane, which regulates cellular adhesion and proliferation. Examination of small bowel biopsies shows villus atrophy and crypt hyperplasia. Observation of epithelial tufts made of round-shape IEC in teardrop-like formation is considered to be pathognomonic (Sivagnanam et al., 2008). Additional features include increased expression of desmoglein, and increased length and number of desmosomes. As ED patients get older, they can display *lamina propria* T-cell infiltration, even sometimes in the absence of visible tufts (Goulet et al., 2007). Mouse models provide some clues to why EpCAM deficiency might mimic dysimmune enteropathy (see below).

The c.498insC *EpCAM* mutation, which abolishes protein expression, results in the most severe phenotype. This mutation is shared by families originating from Kuwait and Qatar. Delimitation of the minimal common haplotype suggests a founder effect 5,000-6,000 years ago (Salomon et al., 2011). Patients with expression of a truncated protein display a milder phenotype, which permits weaning from parenteral nutrition in late childhood (Salomon et al., 2011; Sivagnanam et al., 2008; Sivagnanam et al., 2010). The only extra-intestinal symptom reported is polyarthritis in a few patients.

Mutations in a second gene called Serine Peptidase Inhibitor, Kunitz Type, 2 (*SPINT2*) have been described as a cause of ED. Such mutations were initially described in patients with syndromic congenital sodium diarrhea, e. g. associated with anal or choanal atresia, hypertelorism and corneal erosions. Mutations in *SPINT2*, and notably the common c.488A>G mutation, were subsequently described in patients displaying the same symptoms but also epithelial tufts reminiscent of those observed in patients with *EPCAM* mutations (Canani et al., 2015; Heinz-Erian et al., 2009).

Mouse models of ED

Analysis of mice harboring hypomorphic *Spint2* mutations indicated that SPINT2, a transmembrane serine protein inhibitor also called hepatocyte growth factor activator inhibitor type 2, is involved in organogenesis and in epithelial homeostasis by regulating multiple cellular processes, including bioavailability of growth factors, ion fluxes and paracellular permeability (Szabo et al., 2009).

In 2009, Nagao et al reported that *EpCAM*^{-/-} mice died *in utero* by E12.5 with growth retardation, delayed development, and prominent placental abnormalities (Nagao et al., 2009). In 2012, two

other groups engineered *EpCAM*^{-/-} mice, which were alive at birth. Mice generated by Guerra and colleagues however failed to thrive, and died soon after birth because of hemorrhagic diarrhea. Histological features of ED were found, including intestinal tufts, villous atrophy and colon crypt hyperplasia, whereas other organs were normal. The authors reported loss of membrane localization and increased intracellular accumulation of E-cadherin and β -catenin (Guerra et al., 2012). E-cadherin is the main intestinal cadherin. It is localized along the basolateral surfaces and apical junctions of IEC and is essential to intercellular adhesion between epithelial cells and epithelial integrity. Of note, mice expressing a dominant negative N-cadherin mutant that prevents surface localization of endogenous E-cadherin, displayed severe inflammation with polymorphous infiltration of *lamina propria* (Hermiston and Gordon, 1995), reminiscent of the inflammatory features described in some ED patients.

EpCAM^{-/-} mice generated by Lei et al also died shortly after birth. These authors described reduced expression of tight junction proteins Claudins 2, 3, 7, and 15 and loss of claudin-7. Tight junctions in intestinal epithelium were morphologically abnormal with a disrupted network. Sulfo-NHS-biotin injected into the intestinal lumen of E18.5 *EpCAM*^{-/-} mice abnormally diffused at the lateral membrane, a result ascribed by the authors to down-regulation of claudins. Claudins being responsible for paracellular permeability, the authors further showed that Na⁺-selective paracellular permeability was reduced while Cl⁻-selective permeability remained normal, reminiscent of the findings in the *Claudin-15* mutant mouse (Lei et al., 2012). Lastly, in 2014, Sivagangam's laboratory and colleagues used Cre-LoxP recombination to engineer mice lacking exon 4 of *EpCAM*. *EpCAM*^{-/-} mice died shortly after birth with growth retardation and histological features of ED including epithelial tufts, enterocyte crowding, altered desmosomes, and intercellular gaps. EpCAM and Claudin-7 expression were low and the two proteins failed to colocalize, a result confirmed in ED patients. Permeability and intestinal cell migration were increased. The authors suggested a pathogenic scheme in which reduced expression of EpCAM and claudin-7 resulted in the development of histological abnormalities including tufting, intercellular gaps, increased desmosomes, and villous atrophy, which secondary lead to increased proliferation and migration of IEC, as well as to loss of barrier function. The exact role of claudin 7 remains however unclear and the defect in EPCAM may impair desmosomes and intercellular adhesion independently of claudin 7 (Mueller et al., 2014).

I.B- Defective innate immunity at the intestinal barrier

I.B.1- Defects in pattern recognition receptors (PRR)

Pattern recognition receptors (PRR) are innate receptors that recognize pathogen-associated molecular patterns (PAMPs) of microorganisms. They include notably Toll-like receptors (TLR), NOD-like receptors (NLRs), and RNA helicases like RIG-1 (retinoid acid-inducible gene-1). Many mouse models of PRR defects have been engineered. Unexpectedly, individual PRR defects did not lead to profound immunodeficiency nor to spontaneous intestinal inflammation. Similarly, monogenic diseases due to mutations in genes coding for PRR display mild or absent phenotypes. These observations point out the important redundancy, which exists between PRR to prevent infections (Alain Fischer and Antonio Rausell, *submitted*) and control intestinal homeostasis. Therefore only three molecules will be discussed in this chapter. The first one is MyD88 (for myeloid differentiation primary response gene 88), a scaffold protein bridging most TLR with intracellular signaling. The second one is NEMO/I κ B γ indispensable to switch on the NF- κ B pathway downstream most PRR. The last molecule is NOD2, as rare variants in this PRR represent the main genetic risk factor for CD in Caucasians, and as it is an important partner of X-linked apoptosis inducing protein (XIAP) in which loss of functions mutations are one monogenic cause of severe colitis in humans (see below).

I.B.1.1- MyD88

MyD88 is a key downstream adaptor coupling IL-1 and IL-18 receptors and all TLR except TLR3 to NF- κ B and mitogen-associated protein (MAP) kinase signaling pathways.

In 2006, Rakoff-Nahum and colleagues engineered *Il-10^{-/-}/MyD88^{-/-}* mice. They showed that *Il-10^{-/-}/MyD88^{-/-}* mice were completely free of all signs of intestinal disease throughout more than 1.5 years, contrary to *Il-10^{-/-}* mice that developed enterocolitis, mild to moderate weight loss, and increased mortality. These results led the authors to suggest that chronic colitis in *Il-10^{-/-}* mice is related to the loss of regulation of immune responses triggered by recognition of commensals by TLR via the MyD88-dependent signaling pathway (Jobin, 2010; Rakoff-Nahoum et al., 2006).

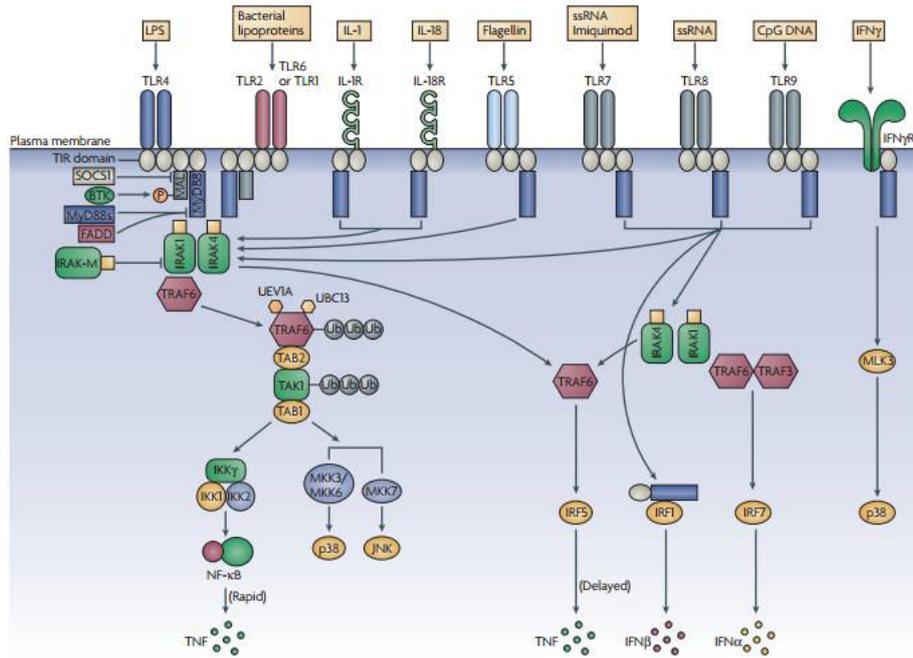


Figure 5, from (O'Neill and Bowie, 2007) – MyD88 at the crossroads of TLR pathways

In 2010, Asquith et al further studied MyD88 contribution to intestinal barrier homeostasis. Following *H. hepaticus* infection, intestinal and systemic inflammation was dramatically reduced in *Rag*^{-/-}/*MyD88*^{-/-} mice compared to *Rag*^{-/-} mice (lacking recombinant activating gene, thus with complete absence of T and B cells). The luminal burden was reduced in the prior mouse model. Then, they generated bone marrow (BM) chimeras from irradiated *Rag*^{-/-} mice that received bone marrow from either *Rag*^{-/-} or *Rag*^{-/-}/*MyD88*^{-/-} mice and infected them with *H. hepaticus*. They found a complete absence of intestinal and systemic inflammation in mice reconstituted with MyD88-deficient BM, demonstrating that hematopoietic, and not epithelial cells, were responsible for transmitting the MyD88-dependent inflammatory response following bacterial infection (Asquith et al., 2010). In keeping with Rakoff-Nahum et al findings, WT mice infected with *H. hepaticus* developed severe typhlocolitis after treatment with anti-IL-10R blocking antibody whereas *MyD88*^{-/-} mice did not.

Even under strict SPF conditions, *Rag*^{-/-}/*MyD88*^{-/-} mice died progressively (30% survival at day 25 after weaning) without signs of wasting or dysimmunity, whereas *Rag*^{-/-}/*MyD88*^{+/-} mice had 100% survival. In chimeric mice with MyD88-deficiency in the hematopoietic system only, survival was normalized, highlighting an important and distinctive role of MyD88 in IEC homeostasis. *Rag*^{-/-} reconstituted with *MyD88*^{-/-}-BM maintained normal anti-microbial peptides (AMP) expression after *H. hepaticus* infection, demonstrating that epithelial MyD88 signaling efficiently drives AMP expression in the colon (Asquith et al., 2010). Taken together, these data indicate that MyD88

activates a cytoprotective signaling pathway in IEC, but triggers inflammatory responses in response to *H. hepaticus* infection in innate immune cells of hematopoietic origin (Jobin, 2010).

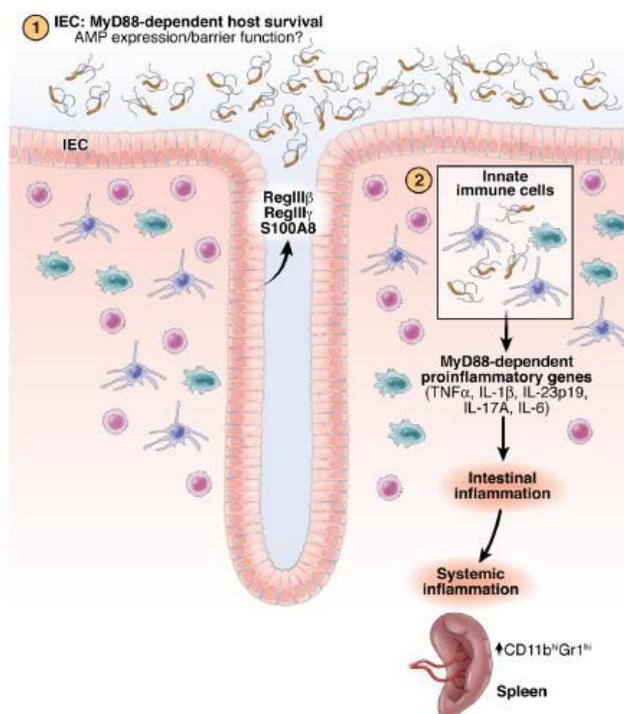


Figure 6, from (Jobin, 2010)- Dual function of MyD88 signaling in the intestine

Autosomal recessive mutations in *MyD88* were first described in 2008. Heterozygous carriers were free of symptoms. As discussed above, loss-of function *MyD88* mutations had relatively mild consequences with only narrow susceptibility to some pyogenic bacterial infections (*Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* responsible for invasive meningitis, sepsis, arthritis, osteomyelitis, and abscesses) while resistance to common fungi, parasites, viruses was normal. Of note, deficiency in IRAK-4 (IL-1R-associated kinase 4), situated immediately downstream MyD88 in the signaling pathway, see figure 5) phenocopied *MyD88* deficiency. In keeping with a preponderant role of innate immunity in early childhood, *MyD88* and *IRAK-4* deficient patients displayed their first invasive bacterial infection before the age of two (90% cases). In contrast, susceptibility to invasive bacterial infections disappeared after 10 years of age, probably thanks to the development of adaptive immune responses. Noninvasive infections mostly of the skin and upper respiratory tract were however observed in adult patients, suggesting that the protective role of MyD88 described in murine IEC may also exist in human epithelial cells (Picard et al., 2011).

Recently, analysis of transcriptome from *MyD88* mutated patients revealed drastically reduced responses to TLR2 agonists (90% less than those of healthy subjects). TLR2 being known to be

crucial for the recognition of Gram-positive bacteria (*S. pneumoniae* and *S. aureus*), this result explains at least partially the narrow range of infections displayed by *MyD88*-mutated patients (Alsina et al., 2014).

I.B.1.2- NEMO/I κ K γ

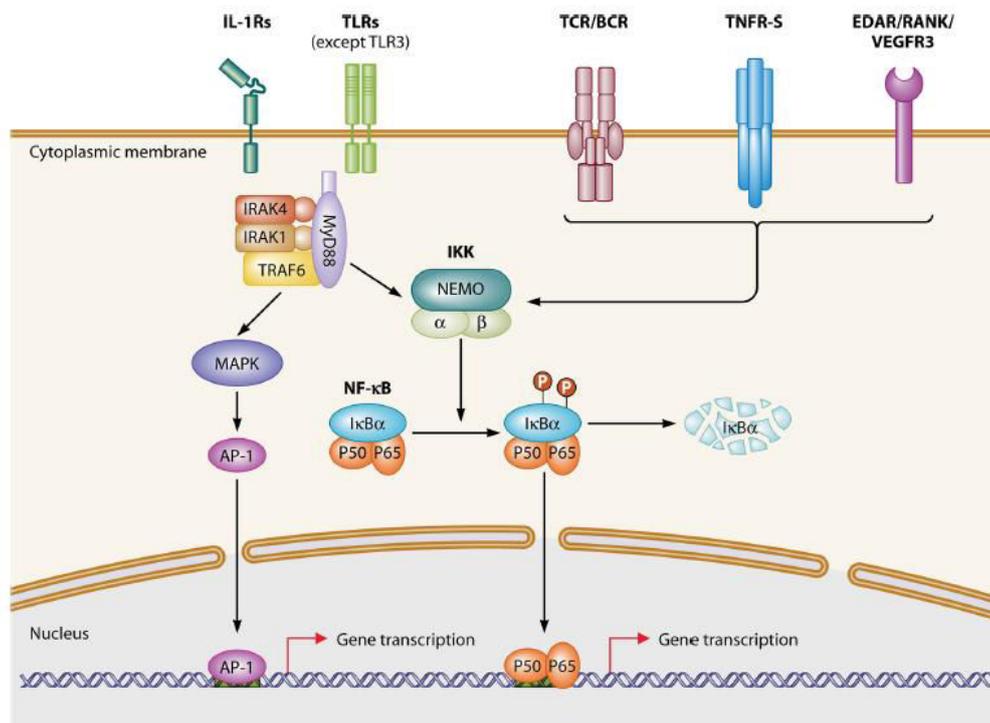


Figure 7, from (Picard et al., 2011)- NEMO signaling

Gender-conditioned phenotypes in human NEMO deficiency

The I κ B kinase enzyme complex (I κ K) is the core element of the NF- κ B pathway. Specifically, I κ K phosphorylates the inhibitor of kappa B α (I κ B α) which, at steady state, is bound to NF- κ B transcription factors and masks their nuclear localization signals, keeping them sequestered in an inactive state in the cytoplasm. Phosphorylation results in the dissociation of I κ B α from NF- κ B, which can then migrate into the nucleus and activate transcription of multiple genes, participating in numerous cell processes and notably in innate and adaptive T cell responses.

Mutations in *IKBKG* gene, which encodes for NEMO/I κ K γ subunit were first described in 2000 in familial incontinentia pigmenti. This monogenic skin disease is an X-linked dominant disorder prenatally lethal in males. Affected females displayed highly variable abnormalities of skin, hair, nails, teeth, eyes and central nervous system. NF- κ B activation was defective in skin fibroblasts from affected fetuses. Affected females survived because of X-chromosome dizygoty and

negative selection of cells carrying the mutant X-chromosome. The most common mutation reported in this article was a large deletion of *NEMO* gene encompassing exons 4 to 10, which resulted from recombination between two repeated regions of strong homology located in intron 3 and the 3' part of exon 10 respectively (Smahi et al., 2000).

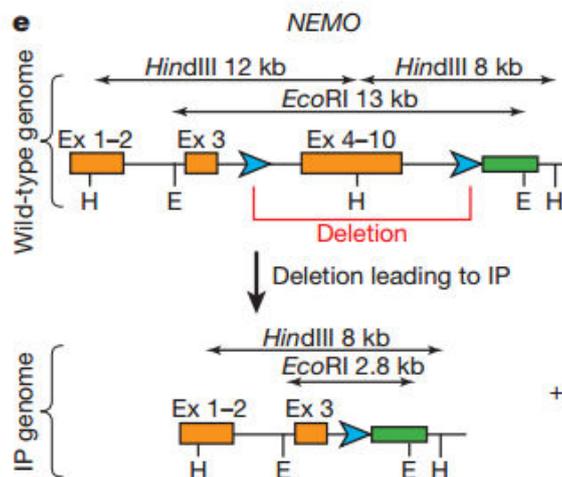


Figure 8, from (Smahi et al., 2000) – Recombination event leading to *NEMO* partial deletion

In 2001, Döffinger and colleagues linked *NEMO* mutations to another syndrome called X-linked recessive anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) that arises in males. They reported hypomorphic mutations in the gene *IKBK*G in 12 males with EDA-ID and in two patients with osteopetrosis and lymphoedema associated to EDA-ID (OL-EDA-ID). As germline loss-of-function mutations in *IKBK*G are lethal in male fetuses, the authors showed that EDA-ID and OL-EDA-ID mutations impaired but did not fully abolish NF- κ B signaling. Notably, they linked patients' abnormal immune features to impaired cell responses to lipopolysaccharide (LPS), IL-1 β , IL-18, TNF α and CD40L. Therefore, loss of regulation of NF- κ B signaling is responsible for combined developmental and immunological defects (Doffinger et al., 2001).

Incontinentia pigmenti and EDA-ID form a syndromic continuum as attested by some case reports. The first one described a female patient with incontinentia pigmenti who suffered from immunodeficiency because of a persistent expression of the mutated X-chromosome found in peripheral blood cells until the age of 3.5 years, after which the X-chromosome inactivation pattern was completely skewed (Martinez-Pomar et al., 2005). The second one described an insertion responsible for a premature stop codon (at position 49). Surprisingly, the affected boy displayed only a severe immunodeficiency. Puel and colleagues showed that reinitiation of translation happened from a methionine located immediately downstream the stop codon, resulting in a truncated *NEMO* protein that was sufficient for normal fetal development of epithelia but insufficient for normal immune function (Puel et al., 2006).

Since 2000-2001, more than 100 male patients with EDA-ID have been reported. EDA-ID male patients represent 90% of NEMO-deficient patients. EDA syndrome comprises sparse hair, abnormal teeth (conical teeth, tooth agenesis), and hypohidrosis, and results from defective signaling downstream the ectodysplasin receptor (EDA-R). Immunodeficiency in NEMO mutated patients most frequently includes impaired antibody response resulting in broad susceptibility to infections, in particular invasive pyogenic bacteria (in 90% of NEMO patients) and atypical mycobacteria (in 40%). The most common infections are sepsis, deep tissue abscesses, and recurrent pneumonia with bronchiectasis, gut infections, and meningitis. Defective antibody production is reminiscent of that observed in patients with mutations in *CARD11* (Caspase recruitment domain-containing protein 11) and *MALTI* (Mucosa-associated lymphoid tissue lymphoma translocation protein 1), two scaffold proteins of the signalosome complex, which connects the B cell receptor to NF- κ B (Turvey et al., 2014), pointing out the non-redundant role of NF- κ B in B cell activation.

Almost all patients display chronic diarrhea, but intestinal inflammation and epithelium damage were not well characterized in most reports. However, follow-up of the patients with severe phenotype who underwent HSCT point out the role of NEMO in epithelial cells. Five of them survived and presented engraftment and correction of their immunodeficiency, but the preexisting colitis was not cured. When colonic biopsies were performed before and after transplantation, the authors reported no improvement in intestinal inflammation, even following adequate engraftment (Fish et al., 2009; Pai et al., 2008). Thus, HSCT can correct the immune defect, but not epithelial cell defects, which may sustain persisting intestinal inflammation (Picard et al., 2011). Mouse models have helped to gain further insight into pathophysiology of NEMO-mediated intestinal inflammation and the respective role of NEMO in epithelial and hematopoietic cells.

NEMO-deficient mouse models recapitulate human phenotypes and help unravel intestinal inflammation mechanisms.

The first *NEMO*^{-/-} mice were generated in 2000, parallel to the first descriptions of human monogenic diseases associated with NEMO deficiency. *NEMO*^{-/-} mice generated by Rudolph and colleagues died prenatally (E12.5-E13.0) from severe liver damage due to apoptosis. NF- κ B DNA-binding activity in response to TNF α , IL-1, LPS, and Poly(IC), and I κ B α degradation, were undetectable in murine embryonic *NEMO*^{-/-} fibroblasts (Rudolph et al., 2000). Male *NEMO*^{-/-} embryos obtained by a second team died similarly around E12-E13 with massive hepatocytes apoptosis, while female heterozygous *NEMO*^{+/-} mice developed a massive skin defect visible soon after birth (3-4 days), with areas of absent pigmentation, massive granulocyte infiltration, hyperproliferation, and increased apoptosis of keratinocytes. The extent to which the skin was

affected varied between different mice, probably due to random pattern of X inactivation. Out of 22 animals, 19 died between 6 to 10 days after birth. The three surviving mice gradually recovered with progressive disappearance of scaling skin areas, presumably through clearing of NEMO-deficient keratinocytes. Taken together, the mouse model recapitulated the human phenotype called *incontinentia pigmenti* (see above) (Schmidt-Supprian et al., 2000).

In 2007, Nenci and colleagues generated mice with specific ablation of NEMO in IEC. Mice developed spontaneous pancolitis starting at 1-2 weeks of age. Inflammatory infiltrate evolved with age, starting by an infiltrate predominantly made of innate cells (dendritic cells and granulocytes) at 2 weeks-old, followed by abundant lymphoid follicles with massive dendritic cells, granulocytes and CD4⁺ T cells infiltrate at 12 and 36 weeks. Histological examination of colonic samples revealed apoptotic lesions and extensive epithelial destruction suggesting a local disruption of the barrier integrity. Moreover, the authors showed the presence of bacteria within *lamina propria*, in close proximity to areas displaying defects in epithelial integrity likely promoting bacterial translocation. Analysis of colon samples from newborn, 1-, 2- and 3-weeks-old mice showed that disruption of epithelial integrity, bacterial translocation into the mucosa and subsequent recruitment of neutrophils was first observed in 1-week-old mice, with progressive development of the disease over time. Of note, small bowel samples from 6-weeks old mice were normal. Analysis of AMP secretion showed that cryptdin expression was only mildly reduced in 2-week-old mice and unaffected at 6 weeks of age, while β -defensin-3, the expression of which is induced by bacteria in mouse colonic epithelial cells, was significantly downregulated at each time point compared with littermate controls. To investigate NEMO role in interactions with microbiota, the authors crossed the *NEMO*^{IEC-/-} mice with mice lacking MyD88, an adaptor molecule required for signaling by most members of the IL-1R/TLR family (see above). Macroscopic and immunohistological analysis of colons from double knock-out (KO) mice aged between 6 to 20 weeks revealed no sign of colitis, demonstrating that TLR-mediated bacterial recognition (and/or IL-1 signaling pathway) has a critical role in inducing intestinal inflammation in *NEMO*^{IEC-/-} mice. Similarly, double KO mice generated by crossing *NEMO*^{IEC-/-} mice with *TNF receptor-1*^{-/-} mice did not show any macroscopic or histological signs of colitis (mice between 6–10 weeks of age), demonstrating that TNF signaling was crucial for disease induction (Nenci et al., 2007).

I.B.1.3- NOD2

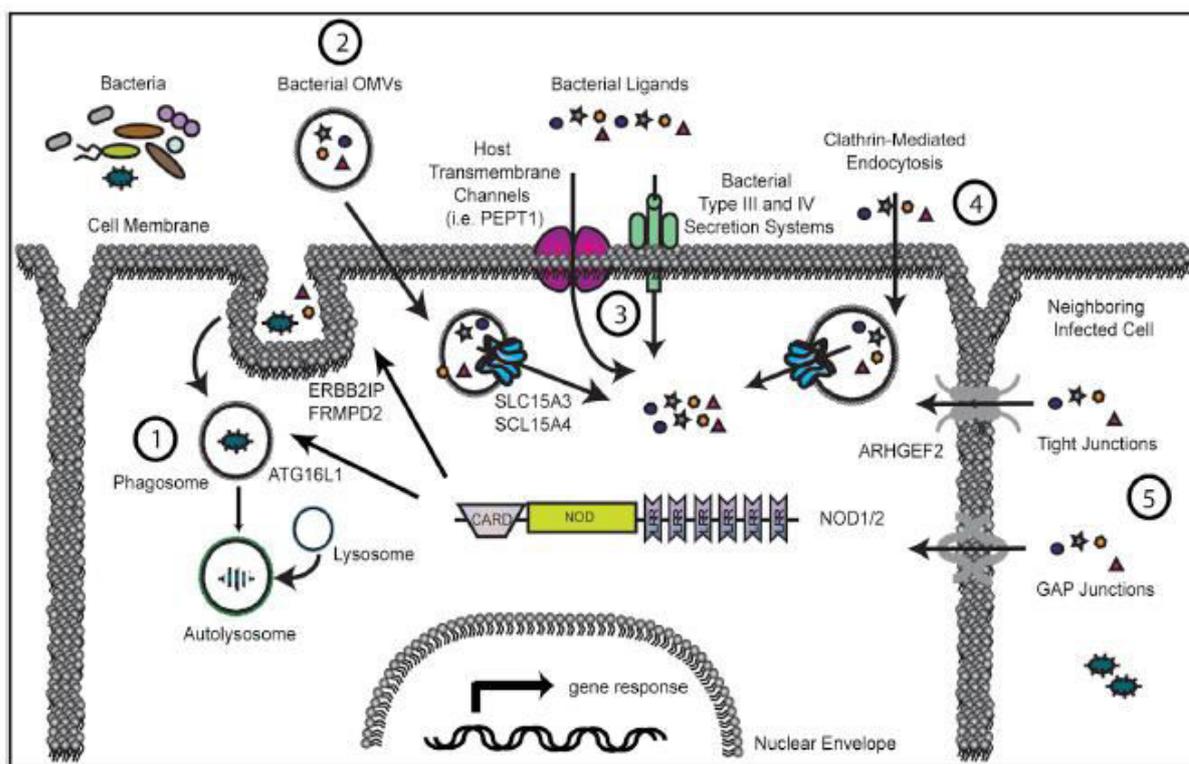


Figure 9, from (Caruso et al., 2014)- Potential Mechanisms for Bacterial Recognition by NOD1 and NOD2

NOD2 encodes an intracellular PRR. This multidomain scaffolding protein is made of two CARD, a nucleotide-binding oligomerization domain (NOD), and multiple leucine-rich repeats (LRRs) that can bind MDP. This small molecule results from the degradation of peptidoglycans, key components of the bacterial wall of both Gram positive and Gram negative bacteria. Of note, *NOD2* shares strong homology with a second intracellular PRR called *NOD1*, which binds Meso-L-Ala-y-D-Glu, meso-diaminopimelic acid (meso-DAP), a distinct fragment of peptidoglycan only found in Gram negative bacteria. While *NOD1* is ubiquitous, *NOD2* is expressed in hematopoietic cells and in some IEC, mainly Paneth cells and stem cells. When MDP is delivered into their cytosol, binding to *NOD2* triggers self-oligomerization, recruitment of receptor-interacting serine/threonine-protein kinase 2 (RIPK2), and activation of the serine/threonine kinase TAK1 (for transforming growth factor β -activated kinase 1). TAK1, in turn, activates NF- κ B and MAPK pathways. A similar cascade is activated upon binding of meso-DAP to *NOD1*. A great number of studies has documented the role of *NOD2* and of *NOD1* in pathogen recognition and immunity (Caruso et al., 2014). Yet, as discussed above, due to the redundancy between PPR, *in vivo* consequences of *NOD1* or *NOD2* deficiency on pathogen clearance remain mild. Following demonstration in 1996 of the first locus of susceptibility to CD on chromosome 16, three low

frequency coding variants (R702W, G908R and L1007fs) were identified in the LRR coding region of *NOD2* gene as well as more minor variants located in the NOD domain. At least one of these variants is present in 30–40% of CD patients compared with 6–7% in European controls. The three main variants have individual odd ratios (ORs) of 2–4 but ORs reach 20–40 in individuals carrying homozygous or compound variants (Ek et al., 2014). Yet the majority of individuals carrying these variants do not develop disease and *Nod2*^{-/-} mice or knock-in mice homozygous for the CD-associated L1007fs *NOD2* variant do not develop spontaneous intestinal inflammation.

While most CD-associated variants were shown to impair NOD2 activation by MDP, the mechanisms by which these variants predispose to intestinal inflammation remain unclear. Several non-exclusive mechanisms have been suggested : i) Impaired bacterial recognition and clearance by macrophages and polynuclear cells, promoting aberrant proinflammatory activation of other pathways, ii) Impaired production of α -defensins by Paneth cells, weakening the epithelial barrier and fostering excessive activation of intestinal immune cells of hematopoietic origin. But observations in *Nod2*^{-/-} mice have however provided contradictory results, iii) Impairment of a putative negative regulatory function of NOD2 on TLR-mediated Th1 cell response in intestine. Yet macrophages from *Nod2*^{-/-} mice do not seem to produce more IL-12 in response to bacterial stimulation, pleading against this hypothesis. iv) Impaired recruitment and activation by NOD2 of ATG16L1. As discussed above, this protein, with a key role in the formation of the auto-phagosome, is also linked to CD. NOD2 was shown to recruit ATG16L1 at bacterial entry site and to promote its activation. Accordingly, CD-associated variants are defective in ATG16L1 recruitment and exhibit impaired bacteria-induced autophagy. As discussed above impaired activation of ATG16L1 may also impair defensin production by Paneth cells. As shown recently, it can also increase the proinflammatory functions of dendritic cells in response to NOD2 activation by bacteria-derived outer membrane vesicles and reduce their ability to induce IL-10-producing T cells (Chu et al., 2016). Altogether, these data allow to perceive how NOD2 variants may impair host-microbiota interactions. Interestingly, if mutations in *NOD2* have not been involved in monogenic cases of IBD, such cases have been ascribed to loss of function mutations in the partner protein XIAP.

I.B.2- XIAP

XIAP encodes for the X-linked member of the IAP family, which comprises evolutionary conserved proteins with anti-apoptotic function. Besides a role in apoptosis, XIAP is an E3 ligase that is recruited upon NOD1 and NOD2 activation by their respective ligands and can polyubiquitinates RIPK2 involved in NOD2 and NOD1 signaling (see above). Loss of XIAP interactions with NOD1

and NOD2 is thought to play a preponderant role in colitis development in a subset of XIAP-deficient patients.

The double-sided phenotype of XIAP deficiency in human disease

Since 2006, over a hundred patients with XIAP deficiency have been reported (Aguilar and Latour, 2015). XIAP defect was first incriminated as the cause of HLH, severe EBV infections and lymphomas and referred to as XLP-2 (X-lymphoproliferative disease) due to similarities with XLP-1 related to *SH2D1A* mutations. XIAP mutations are also a known cause of monogenic chronic colitis. XLP-1 and 2 deficiencies differ in two main aspects: there is no lymphoma in XLP-2-deficient patients, and chronic colitis is extremely rare in XLP-1 deficiency. Altogether, HLH and splenomegaly occur in half of XIAP deficient patients, whereas 25-30% have a severe and refractory CD-like that can be the first and only symptom. The intestinal phenotype is very close to typical CD with possible involvement of the entire digestive tract and, sometimes, epithelioid granuloma in biopsies. In a large IBD cohort, *XIAP* mutations were found in 4% of patients with CD of pediatric-onset (before 16 years old, (Zeissig et al., 2015)). Early onset is however not a reliable criterion to pinpoint XIAP-related colitis as age at onset spreads out from the first months of life to 41 years (Aguilar et al., 2014).

Whereas HLH and severe EBV infections seem related to excessive apoptosis and deficiency in iNKT (invariant natural killer T cells) and MAIT cells (Mucosal-associated invariant T-cells), XIAP-related colitis was rather ascribed to impaired signaling downstream NOD2 and NOD1. Thus, monocytes from XIAP deficient patients failed to produce IL-8 upon MDP or meso-DAP stimulation (Aguilar and Latour, 2015). The key role of monocytes (and thus probably of NOD2) is supported by results in female-carriers with CD-like inflammation in whom monocytes preferentially expressed the *XIAP*-mutated allele (Aguilar et al., 2014; Dziadzio et al., 2015). Moreover, the importance of the XIAP pathway in the hematopoietic compartment is emphasized by the fact that HSCT can cure XIAP-related colitis (Worthey et al., 2011).

***Xiap*^{-/-} mice do not recapitulate the human phenotype**

Mice deficient in XIAP through homologous gene targeting were generated in 2001. They were viable and did not display any obvious phenotype. Even though XIAP was first reported to inhibit apoptosis *in vitro* through binding with active caspases 3, 7 and 9, the authors were unable to detect any defects in induction of caspase-dependent or independent apoptosis in cells from the gene-targeted mice, but they observed increased protein levels of c-IAP1 and c-IAP2, suggestive of a compensatory mechanism (Harlin et al., 2001).

Apart from its anti-apoptotic function, XIAP is involved in the NF- κ B signaling pathway downstream NOD2 stimulation. Upon MDP binding, NOD2 oligomerizes and assembles a multiprotein signaling complex that includes RIPK2, cIAP1, cIAP2, and TRAF2 (TNF receptor-associated factor 2). This induces conjugation of K63-linked ubiquitin chains on RIPK2 previously ascribed to cIAP1/2. Damgaard and colleagues showed that XIAP could be copurified with NOD2, and that it functions as an ubiquitin ligase in NOD2 signaling. XIAP ubiquitin ligase function is provided by its C-terminal RING domain as attested by loss of NF- κ B activation upon MDP-stimulation in *in vitro* models with XIAP modified protein that either lacks the RING domain (XIAP ^{Δ RING}) or carries a missense mutation impairing the RING domain activity (XIAP^{F495A}). Furthermore, the authors showed that ubiquitination of RIPK2 is mediated by XIAP. Degradation of cIAP1/2 upon cells incubation with a chemical compound called LBW-242 led to decreased ubiquitination of RIPK1 but not of RIPK2, whereas ubiquitination of the latter was lost if XIAP was deleted, with or without cIAP1/2. At last, they demonstrated that all three subunits of the linear ubiquitin chain assembly complex (LUBAC), made of HOIP, HOIL-1 and SHARPIN, were recruited to the NOD2 signaling complex upon ubiquitination of RIPK2 by the RING domain of XIAP, and were necessary for NF- κ B activation (Damgaard et al., 2013).

Recently, Schwerd et al described patients suffering from Niemann-Pick disease type C1 (NPC1), a metabolic disorder with impaired lysosomal lipid storage leading to defective autophagy. Some NPC1 patients presented an early-onset colitis with epithelioid granuloma. The authors showed that NPC1 patients with colitis had an impaired bacterial handling similar to patients' with *NOD2* CD susceptibility variants or to *XIAP* mutations. Bacterial clearance was rescued in NPC1 patients' macrophages *in vitro* after pharmacological activation of autophagy. But, contrary to *NOD2* or *XIAP* patients, cytokines production upon NOD2 stimulation by MDP was intact. Thus, antibacterial autophagy induction and pro-inflammatory cytokines production upon MDP stimulation are two independent signaling pathways downstream NOD2 (Schwerd et al., 2016).

Taken together, two important lessons are provided through the study of XIAP deficiency in humans as in mice: IBD-like inflammation ascribed to severe monogenic disorder may have low penetrance rate of 20-25%; and mouse models may not recapitulate the human phenotype. The incomplete penetrance of XIAP deficiency in humans suggests that environmental factors or unknown modifier genes may participate in XIAP-related intestinal inflammation.

I.B.3- Defects in production of Reactive Oxygen Species

Production of reactive oxygen species (ROS) is often viewed as a dangerous counterpart of aerobic metabolism that can lead to inflammation and severe tissue damage. Yet, analysis of patients with enzymatic defects affecting ROS production in phagocytes and epithelial cells highlights their key and non-redundant contribution to host anti-microbial defense and to intestinal homeostasis.

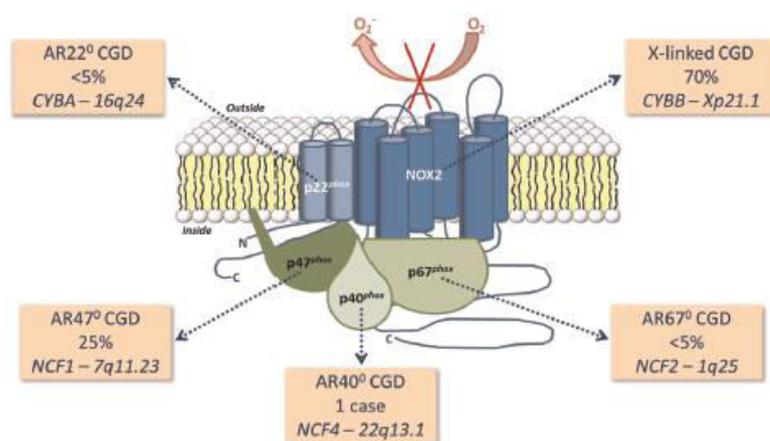


Figure 10, from (O'Neill et al., 2015)- NADPH oxidase complex in phagocytic cells

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox/Duox) are the main enzymes responsible for the production of ROS: superoxide (O_2^-) or hydrogen peroxide (H_2O_2). In humans, this family comprises seven members (Nox 1-5; Duox1/2) which form multi-proteins complexes with small GTPases (Rac 1/2).

In phagocytic cells, the enzymatic core of the transmembrane NADPH complex is made of the heavy chain NOX2, (alias gp91^{phox}) encoded by the X-linked gene *CYBB*, and the light chain p22^{phox} (encoded by the *CYBA* gene). Its activation by NCF1 (alias p47^{phox}) catalyzes the transfer of a single electron from NADPH to oxygen, generating superoxide into the extracellular or intraphagosomal space. NCF1, along with NCF2 and NCF4 (also known as p67^{phox} and p40^{phox}) are cytosolic regulating components of the NOX2 complex along with RAC1 and RAC2 GTPases. The release of ROS from phagocytes is known as the respiratory/oxidative burst which is essential to their bacterial killing ability. The other Nox/Duox members (NOX1; 3-5, Duox1/2) are almost exclusively expressed in non-phagocytic cells (see Table 1) (Jackson et al., 1995; O'Neill et al., 2015; Sareila et al., 2015).

Gene name	Protein name	Other name	Expression
PHAGOCYtic CELLS			
<i>CYBB</i>	NOX2	gp91 ^{phox}	Phagocytes, umbilical vein endothelial cells, coronary microvascular endothelial cells, cardiomyocytes, central nervous system, endothelium, fibroblasts, skeletal muscle, hepatocytes and hematopoietic stem cells
<i>CYBA</i>	CYBA	p22 ^{phox}	Lymphoid tissues, coronary arteries
<i>NCF1</i>	NCF1	p47 ^{phox}	Neutrophils
<i>NCF2</i>	NCF2	p67 ^{phox}	Kidney, neutrophils
<i>NCF4</i>	NCF4	p40 ^{phox}	Mononuclear cells
<i>RAC1</i>	RAC1		Neutrophils
<i>RAC2</i>	RAC2		Hematopoietic cells
NON-PHAGOCYtic CELLS			
<i>NOX1</i>	NOX1	p65 ^{MOX}	Colon and vascular smooth muscle, prostate, uterus, neurons, astrocytes and microglia
<i>NOX3</i>	NOX3	MOX-2	Inner ear. Lower levels liver, lung, spleen
<i>NOX4</i>	NOX4		Kidney, liver, ovary, eyes, smooth muscle cells, fibroblasts, keratinocytes, osteoclasts, endothelial cells, neurons
<i>NOX5</i>	NOX5		Spleen, testis, mammary glands and cerebrum, prostate, lymphatic tissue, endothelial cells
<i>DUOX1</i>	DUOX1		Thyroid, cerebellum and lungs, ileum, colon, Respiratory tract epithelium
<i>DUOX2</i>	DUOX2		Stomach, duodenum, ileum, jejunum, Colon, pancreatic islets, Tracheal and bronchial epithelium, Thyroid and prostate
<i>DUOXA1</i> <i>DUOXA2</i>	DUOXA1 DUOXA2		Activators of DUOX1/2, expressed in same cell types
<i>NOXO1</i>	NOXO1	Nox organizer 1, p41NOX	Testis, small and large intestines, liver, kidney and pancreas
<i>NOXA1</i>	NOXA1	p67phox-like factor	Widely expressed. Detected in pancreas, liver, kidney, spleen, prostate, small intestine and colon

Table 1. Gene names, protein names and sites of expression of Nox/Duox proteins family.

Double burden of immunodeficiency and autoimmunity in CGD patients

CGD is a group of primary immunodeficiencies (PID) caused by mutations affecting one of the genes of the NADPH oxidase complex. This rare monogenic disorder (prevalence of 1/200 000 to 1/250 000) is inherited as either an autosomal recessive (*CYBA*, *NCF1*, *NCF2*, *NCF4*) or an X-linked trait (*CYBB* gene, renamed Nox2, responsible for 70% of CGD cases) (O'Neill review 2015). Recessive *NCF1* mutations are mainly due to recombination between *NCF1/p47-phox* gene and its two highly homologous pseudogenes favored by a two-nucleotide deletion (c.75_76GT) in exon 2 (Roesler et al., 2000). Patients commonly display recurrent and life-threatening bacterial and fungal infections in early childhood that are ascribed to deficient generation of ROS by phagocytes. Susceptibility to infections can be cured by HSCT (Cole et al., 2013). In addition, approximately 30% patients display a CD-like phenotype. Marciano et al reported a higher frequency of CD-like

phenotype in CGD patients with *CYBB* mutations (89%). The median age of onset of gastrointestinal symptoms (GI) was 5 years, spreading out from 1 to 30 years (Marciano et al., 2004). Bearing in mind the close similarity of GI symptoms in adult CD and CGD patients, Jaggi et al undertook the screening of 120 CD patients (including 4 with a history of abdominal or liver abscesses) with a dihydrorhodamine (DHR) test (Jaggi et al., 2012). Surprisingly, none of the patients had CGD. Nevertheless, performing DHR test is recommended in case of VEO-IBD (Uhlig et al., 2014).

Importance of NADPH oxidase in the epithelial compartment has been recently demonstrated in VEO-IBD patients, with the discovery of mutations in *NOX1* or *DUOX2*. Patients with either *NOX1* or *DUOX2* mutations had ROS production levels divided by 2 to 4, demonstrating that both enzymes are not redundant or only partially as deficiency in one of them is sufficient to trigger severe phenotype (Hayes et al., 2015). These rare patients prove how essential the production of ROS by IEC is for the maintenance of gut mucosal homeostasis, extending to humans finding in drosophila, in which loss of Duox function in gut epithelial cells led to increase susceptibility to ingested microbes and mortality (Ha et al., 2005).

What is the pathogenesis of CD-like inflammation in CGD patients? First, defective ROS production impairs bacterial killing as in autophagy defects. Accumulation of partially degraded bacteria in phagocytes could lead to granuloma formation and hyperinflammation due to persistent cell activation. In parallel, ROS can induce apoptosis of inflammatory cells that limits inflammation (O'Neill et al., 2015). Recent results obtained by de Luca and colleagues also suggest defective autophagy and increased IL-1 β production in CGD patients. They notably showed that macrophages from CGD mice (deficient for p47^{phox}/Nox2 or p40^{phox}/NCF4) and blood monocytes from CGD patients displayed minimal recruitment of microtubule-associated protein 1 light chain 3 (LC3) to phagosomes. This was associated with overproduction of IL-1 β . Injection of Anakinra, an IL-1 β receptor antagonist, protected CGD mice from TNBS induced colitis and from invasive aspergillus infections, and simultaneously increased autophagy. As well, *in vitro* treatment of monocytes from CGD patients with Anakinra doubled LC3 recruitment (23% to 51%). Based on these results, two CGD patients with severe colitis were treated with Anakinra and showed rapid and sustained improvement (de Luca et al., 2014).

Apart from severe infections, CGD patients are prone to develop a wide range of autoimmune and dysimmune symptoms, such as autoimmune thrombocytopenia, idiopathic thrombocytopenic purpura, rheumatoid arthritis, immunoglobulin A (IgA) nephropathy, sarcoidosis, or systemic lupus erythematosus (SLE). Furthermore, discoid lupus or lupic skin affections are commonly reported in female-carriers of X-linked CGD (De Ravin et al., 2008). Could hyperinflammation related to impaired bacterial killing be responsible for these autoimmune features? It rather seems to involve

another mechanism as suggested by Kelkka and colleagues. They observed, in CGD patients as well as in *Ncf1^{m/lj}* mice, a prominent type 1 IFN response signature that was accompanied by elevated autoantibody levels (three out of six patients displayed SLE-associated anti-double-stranded DNA, anti-Th/To ribonucleoprotein, and/or vasculitis associated anti-bactericidal permeability increasing protein antibodies). Furthermore, they identified significant deposits of IgG and complement factor C3 within kidney glomeruli of naïve *Ncf1^{m/lj}* mice, aged between 8 and 16 weeks and backcrossed on a Balb/c genetic background, which is known to be more susceptible to induced model of lupus. Lastly, the authors raised the question of the possible participation of bacterial or viral infections (as type 1 IFN are key actors in antiviral immunity) in the induction of autoimmunity. Of note, all WT and six out of eight *Ncf1^{m/lj}* mice bred in a conventional animal facility were positive for anti-murine norovirus antibodies. Expression analysis of spleen samples from mutated mice bred in a germ-free facility recapitulated the type 1 IFN signature. Thus, upregulation of type 1 IFN was not dependent on microbial antigens but likely of endogenous origin (Kelkka et al., 2014). More recently, the same team reported a higher susceptibility to collagen induced arthritis of germ-free *Ncf1^{m/lj}* mice as compared to WT. Germ-free or pathogen-free mutated mice displayed similar arthritis incidence and severity, and disease course was not altered by antibiotics treatment (Wing et al., 2015).

Mouse models of CGD

Mice with defective NADPH oxidase family in phagocytes

The two first CGD mouse models were created in 1995 by disrupting *p47^{phox}* (*Ncf1*) and *Cybb* genes expression. Male *Cybb^{-/-}* mice recapitulated the human phenotype with loss of ROS production by neutrophils and increased susceptibility to severe infections with *S. aureus* and *Aspergillus fumigatus* (Pollock et al., 1995). As well, *Ncf1^{-/-}* mice exhibited decreased ROS production by leukocytes, leading to ineffective bacterial killing. They died of severe infections (50% dead mice at week 14) and granulomatous lesions were noted (Jackson et al., 1995). *Cyba^{-/-}* (*p22^{phox}*) mice also lacked NADPH oxidase function. Surprisingly, these mice also suffered from balance disorder caused by aberrant development of gravity-sensing organs (Nakano et al., 2008).

Interestingly *Ncf1^{-/-}* mice displayed auto-immune and dysimmune features reminiscent of those reported in CGD patients, with increased sensibility to induced collagen-arthritis. Mice were however protected from experimental autoimmune encephalomyelitis, an induced model of multiple sclerosis (Jackson et al., 1995; van der Veen et al., 2000).

Hulqvist and colleagues studied mice with *Ncf1* loss-of-function due to a specific splice mutation previously described in C57BL/6J mice. The mutation, a single nucleotide change

(A>C) located at position -2 before exon 8, led to decreased expression of a truncated protein lacking eight amino acids in the second SRC Homology 3 (SH3) domain required for interactions with Ncf2. Loss of NADPH oxidase function was attested by the absence of ROS production by neutrophils. These mice (called *Ncf1^{m1J}*) were backcrossed with B10.Q mice, widely used in experimental models for rheumatoid arthritis and multiple sclerosis. *Ncf1^{m1J}* mice showed increased severity in collagen-induced arthritis with enhanced IgG and delayed-type hypersensitivity responses against type II collagen. Moreover, female *Ncf1^{m1J}* developed spontaneous severe arthritis during the post-partum period, similarly as in humans, with antibodies against type II collagen that exhibited the same fine specificity as in collagen-induced arthritis. Surprisingly, compared to *Ncf1^{-/-}* mice, *Ncf1^{m1J}* displayed an increased susceptibility to experimental autoimmune encephalomyelitis (Hultqvist et al., 2004).

Sareila and colleagues tried to reconcile controversial results in *Ncf1^{-/-}* mice and *Ncf1^{m1J}* by reproducing collagen induced arthritis and psoriatic arthritis in mice on a pure B10.Q background. As expected, induction of ROS during both diseases was less in *Ncf1* deficient mice than in WT littermates, and increased signal transducer and activator of transcription 1 (STAT1) expression was taken as evidence of an IFN signature. Unexpectedly, female *Ncf1^{-/-}* mice were protected from collagen-induced arthritis whereas *Ncf1^{m1J}* females developed severe disease. This difference was blunted by ovariectomization of *Ncf1^{-/-}* female. These findings confirmed that, even on a similar genetic background, littermates with a targeted gene KO or with a naturally occurring loss-of-function mutation can display different features. How to explain these discrepancies? *Ncf1^{-/-}* mice were engineered by targeted gene disruption through the replacement of exons 1-2 with a cassette in a reverse orientation. Seven transcripts of *Ncf1* have been reported, of which three are protein coding. All protein-coding variants, as well as a transcript considered to be subjected to non-sense mediated decay, include exons 1 and 2 that are intentionally deleted in the targeted KO model. Another transcript containing exon 2 should not be generated either. The authors suggested that one of the two remaining transcripts could possibly exist in the KO mice even though they are not believed to be expressed as protein. In *Ncf1^{m1J}* mice, three alternatively spliced *Ncf1* mRNA exist but none of them can be translated into a functional protein. Whether a distinct *Ncf1* mRNA repertoire in KO and in *Ncf1^{m1J}* may explain differences in outcomes remains to be demonstrated (Sareila et al., 2015).

Mice with defective NADPH oxidase family in non-phagocytic cells

Deficient mouse models of most proteins of the Nox/Duox family NADPH oxidases do not exhibit obvious phenotype apart from *Nox3* and *Duox2* deficiencies. However, some information can be gathered.

Most of the components of the NADPH oxidases family have a restricted expression pattern. Nox3 is expressed in the inner ear only and is required for the proper function of the vestibular system. *Nox3*^{-/-} mice suffered from severe balance and spatial orientation defects.

Nox4 was first identified in kidney epithelial cells, but its expression pattern includes endothelial cells, vascular smooth muscle, fibroblasts, cardiomyocytes, skeletal muscle, osteoclasts, adipocytes, neurons, and microglia. Nonetheless, *Nox4*^{-/-} mice did not show any gross phenotype, either spontaneous or in chronic kidney diseases models including diabetes-induced kidney injury, unilateral ureter ligation, and 5/6 nephrectomy (Sirokmany et al., 2016).

Duox1 and Duox2 (dual oxidase 1 and 2) were first cloned from thyroid gland. They must interact with activator proteins DuoxA1 and DuoxA2 for proper function. Autosomal recessive mutations in *DUOX2* have been described since 2002 in congenital hypothyroidism, either permanent or transient, demonstrating its key role in thyroid hormone biosynthesis (Hayes et al., 2015). Mice with a naturally-occurring missense *Duox2* mutation, or with simultaneous KO of *DuoxA1* and *DuoxA2*, developed congenital hypothyroidism similar to the human phenotype (Sirokmany et al., 2016).

Apart from its thyroid function, DUOX2 has another fundamental role as it is, with NOX1, one of the two main sources of ROS along the gastrointestinal tract. NOX1 is essentially expressed in the colon, caecum, and ileum, whereas DUOX2 can be found in all segments of the gut. NOX1 and DUOX2 are the catalytic subunits of multimeric, membrane-bound enzymes that generate ROS, similarly as the NOX2 complex in phagocytic cells (Hayes et al., 2015). Of note, the presence of two different NOX/DUOX enzymes in colon epithelial cells highlights the importance of regulated ROS production in the colon (Sirokmany et al., 2016). *Nox1* deficient mice did not display any obvious phenotype, but pathology examination of their colon revealed massive conversion of IEC into goblet cells at the cost of absorptive colonocytes. This difference was due to a Nox1-dependent concerted repression of phosphatidylinositol 3 kinase (PI3K)/AKT/Wnt/Beta-catenin and Notch1 signaling pathways (Coant et al., 2010). ROS production by Nox1 oxidase could also contribute to colonic epithelial repair, as impaired mucosal healing was reported in an IEC-specific *Nox1*^{-/-} model (Leoni et al., 2013).

Taken together, and apart from some organ specific isoforms like NOX3, human and mice data strongly suggest that ROS functions are extremely diverse and independent from each other, from essential anti-microbial properties to immune regulatory function against the development of autoimmunity.

I.C- Defects in the effector functions of the adaptive immune system

Immunodeficiencies that severely impair T cell effector functions and/or the production of antibodies such as Severe Combined Immunodeficiencies (SCID) are revealed by severe opportunistic infections by microbes crossing body surfaces, and notably intestinal mucosa, attesting the importance of the adaptive immune system to build an efficient gut barrier. Moreover, SCID are almost invariably accompanied by chronic diarrhea. Nonetheless, digestive symptoms are never, or very rarely, the main symptom in SCID patients, clinical presentation being dominated by life-threatening infections. Therefore, SCID have appeared out of scope of the present Introduction. Immunodeficiencies affecting immunoregulation of the adaptive immune system, in which digestive symptoms are very frequently on the front line, are discussed in the following chapter.

II. DEFECTS IN REGULATION OF THE INTESTINAL BARRIER

As illustrated above, immune responses resulting in “physiological inflammation” are essential to sustain the gut barrier and to restrict body access to intraluminal microbes and antigens. The expanding number of monogenic disorders impairing regulation of innate and/or adaptive immune responses that result in severe intestinal inflammation illustrates the multiplicity of the mechanisms created by hosts to finely adjust gut immune responses and avoid immune runaway. Regulation depends on both intrinsic check-points within signaling cascades and extrinsic mechanisms acting on innate and/or adaptive immune cells.

II.A- Defects in intrinsic regulation of signaling pathways

II.A.1- Hyperinflammation due to over production of IL-1 β

Auto-inflammatory monogenic diseases are characterized by overproduction of IL-1 family cytokines (IL-1 β and IL-18) and of inflammatory markers (C reactive protein, serum amyloid A). They are responsible for recurrent episodes of fever and for a spectrum of inflammatory symptoms. Colitis with CD-like features are observed in two of them, Mevalonate kinase deficiency (MKD) and gain of function mutation in NLR family-Card domain containing 4 (NLRC4), both briefly discussed below.

MKD is due to loss-of-function mutations in the mevalonate kinase gene (*MVK*) that encodes for a protein involved in the metabolism of cholesterol. *MVK* is the second enzyme in the synthesis pathway common to cholesterol and non-sterol isoprenoids and is located directly downstream of hydroxy-méthyl-glutaryl-coenzyme A (HMG-CoA) reductase. Non-sterol isoprenoid end products are involved in the prenylation of proteins. Prenylation (also known as isoprenylation or lipidation) is the addition of hydrophobic molecules, such as non-sterol isoprenoid (farnesyl pyrophosphate or geranylgeranyl pyrophosphate (GGPP)) to a protein, thus affecting its activity or cellular location (Mulders-Manders and Simon, 2015).

MKD encompasses two overlapping syndromes: i) hyperimmunoglobulinemia D and periodic fever syndrome (HIDS), characterized mainly by adenopathy, hepatosplenomegaly, abdominal pain, skin rash, and arthralgia, ii) mevalonate aciduria, a more severe presentation with psychomotor retardation, cerebellar ataxia, and facial dysmorphism, which leads to early death. Severity seems to depend on the amount of residual enzymatic activity (Mulders-Manders and Simon, 2015). MKD is one of the first inflammasome-related disorders linked to VEO-IBD. In a French survey of 50 patients, 69% displayed chronic diarrhea, including 40% for whom severe diarrhea was present at disease onset. Interestingly, the most common mutation p.Val377Ile was found in asymptomatic patients as well as in patients with severe phenotype (Bader-Meunier et al., 2011).

How can *MVK* deficiency be linked to overproduction of IL-1 β ? A role for defective protein prenylation was suggested since deficiency in GGPP leads to IL-1 β overproduction. IL-1 β overproduction was notably ascribed to defective prenylation and activation of RhoA (for Ras homolog gene family, member A), resulting in increased activity of Rac1 (for Ras-related C3 botulinum toxin substrate 1) and Akt/protein kinase B. Inactivation of RhoA was able to induce IL-1 β mRNA transcription independently of caspase-1 activity (Mulders-Manders and Simon, 2015).

In 2014, two teams described simultaneously patients with heterozygous gain-of-function mutation in *NLRC4*, an intracellular NLR whose stimulation recruits and activates caspase-1 within the inflammasome (Canna et al., 2014; Romberg et al., 2014). Physiological secretion of IL-1 β and IL-18 requires two signals: the first one is obtained upon stimulation of transmembrane innate receptors like TLR and induces the expression of pro-cytokines; stimulation of NLR is the second signal and induces procaspase-1 autoproteolysis and cleavage of pro-IL-1 β and -IL-18 into active cytokines. Patients described by Romberg et al displayed periodic fever, neonatal-onset enterocolitis and increased macrophage cell death. On the contrary, patients described by Canna et al suffered from early-onset recurrent fever and macrophage activation syndrome. Phenotype expression of *NLRC4* mutation is therefore variable with an incomplete penetrance. Of note, enterocolitis resolved around 1 year of age. Romberg et al suggested that intestinal inflammation may be induced by a “constant signal 1” delivered during microbiota colonization. Specific treatment of Canna’s patient with IL-1 receptor antagonist had positive results allowing steroids weaning. The authors suggested that IL-18 may be another therapeutic target as its concentration remained high even despite treatment with IL-1 receptor antagonist (Canna et al., 2014; Romberg et al., 2014).

II.A.2- Intestinal inflammation induced by STAT3 gain of function mutations

Following cytokine receptor activation (either with pro or anti-inflammatory cytokines like IL-6 or IL-10 respectively), JAK1 (Janus kinase 1) phosphorylates STAT3 which dimerizes itself and translocates to the nucleus to induce the transcription of either pro or anti-inflammatory pathways. Three subgroups of STAT3 mutations have been described: constitutive LoF leading to immunodeficiency with Job's syndrome/Hyper IgE syndrome (HIES) (Minegishi et al., 2007); somatic GoF leading to lymphoproliferation and neoplasia (Koskela et al., 2012); and constitutive GoF mutations, resulting in autoimmune disorders, combining frequently auto-immune enteropathy (AIE), endocrinopathy and cytopenia (Flanagan et al., 2014; Haapaniemi et al., 2015; Milner et al., 2015). The vast majority of *STAT3* GoF mutations were *de novo*. Their first identification originated from the exploration of a cohort of patients suffering from either polyautoimmunity and/or early-onset type 1 diabetes (Flanagan et al., 2014). But, as for patients with LRBA or CTLA4 deficiencies (see below), age of onset for *STAT3* GoF mutations is wider than suggested by this first report and spreads over the first two decades. Approximately 70% patients suffer from AIE and 25% from hypogammaglobulinemia. As for the autoimmune symptoms, they are highly variable even within multiplex families with individual members displaying variably diabetes, thyroiditis, vitiligo, arthritis, sclerodermia, auto-immune hepatitis, and auto-immune pneumopathy. Moreover, *STAT3* GoF mutations seem to have incomplete penetrance. Compared to *STAT3* somatic GoF described in lymphoma, T effectors cells in constitutive GoF did not display spontaneous STAT3-dependant signal but rather an excessive response upon stimulation. No lymphoma has been reported in patients with constitutive *STAT3* GoF mutations yet, but their onset might be feared as patients get older (Flanagan et al., 2014; Haapaniemi et al., 2015; Milner et al., 2015). Of note, recent work in the laboratory has identified somatic GOF *STAT3* mutations in a subset of IL-15 dependent innate T like lymphomas developing in patients with celiac disease, highlighting how STAT3 GoF might foster lymphocyte activation and expansion in the cytokine-rich intestinal environment (Ettersperger et al, *in press*).

Available mouse models do not recapitulate observations in humans. Mouse models with constitutive GoF mutation of *Stat3* have not been described yet. Mouse models with cell-restricted *Stat3* LoF mutations have been reported, as *Stat3*^{-/-} mice died during embryogenesis. Unexpectedly as compared to the human phenotype, mice with specific disruption of *Stat3* in macrophages and neutrophils developed a spontaneous colitis with age. At 20 weeks, colon samples displayed depletion of goblet cells, marked polymorphous inflammatory infiltrate of the *lamina propria*, frequent crypt abscesses, few mucosal ulcerations and no granuloma. Interestingly, when these mice

were crossed with *Tlr4*^{-/-} or *MyD88*^{-/-} mice, they developed a milder colitis. It was therefore suggested that the colitis was dependent on the loss of regulatory effect of IL-10 that counterbalances the continuous stimulation of immune cells by food antigens and microbiota (Wirtz and Neurath, 2007).

II.A.3- Inflammation due to loss of NF-κB regulation in *A20* deficiency

A20, coded by *TNFAIP3*, is an inducible and broadly expressed cytoplasmic zinc finger protein that inhibits NF-κB activity and TNF-mediated programmed cell death. Many *A20* polymorphisms have been linked with dysimmune multifactorial human diseases, and notably with CD (see figure 11) before the recent demonstration that *A20* haploinsufficiency is a monogenic cause of severe inflammation in humans. Since *A20*-deficient mice have been investigated before the human monogenic disease and display prominent intestinal lesions not yet well described in humans, they will be discussed first.

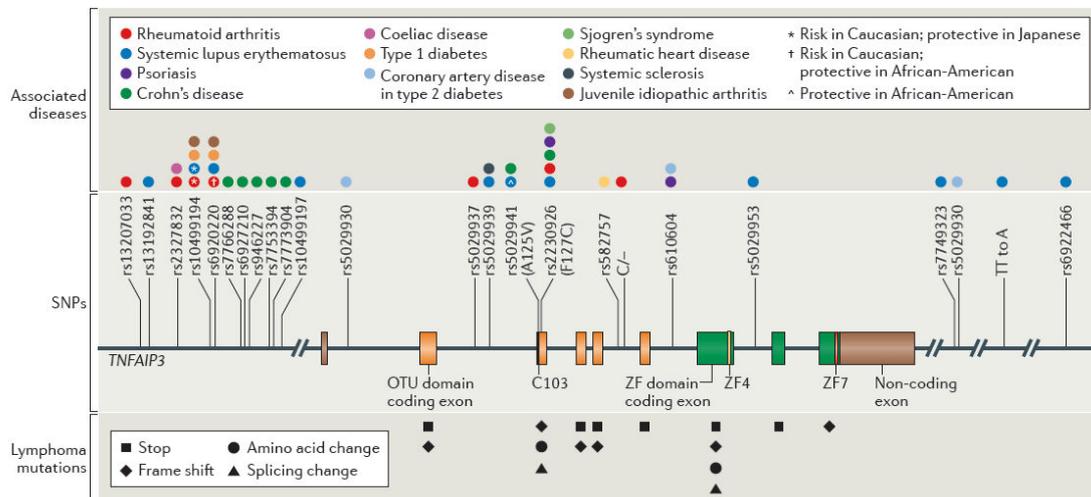


Figure 11, from (Ma and Malynn, 2012)– Polymorphisms in *A20* associated with human diseases

Histological examination of 3-6-weeks-old *A20*^{-/-} mice revealed severe inflammation and tissue damage in multiple organs, including liver, kidney, intestines, joints, and bone marrow. *A20*^{-/-} mice developed cachexia and premature death. They also succumbed to sublethal doses of LPS and TNF. Double mutant *A20*^{-/-} x *Rag1*^{-/-} mice developed granulocytic infiltration, cachexia, and premature death with similar frequency and severity as *A20*^{-/-} littermates, indicating that lymphocytes are not required for *A20*-inflammation. Skin sections revealed thickened epidermal and dermal layers without inflammation, pointing out a role of *A20* in regulating skin differentiation. This finding is

in keeping with the role of NF- κ B in skin differentiation as stressed in patients with NEMO syndrome (see above). The authors showed that absence of A20 resulted in prolonged NF- κ B responses to TNF due to insufficient synthesis of I κ B α mRNA and protein. They concluded that A20, which mRNA expression dramatically and rapidly increased upon TNF stimulation in all tissues, is essential for limiting inflammatory responses and terminate TNF-induced NF- κ B responses *in vivo* (Lee et al., 2000).

Table 1 | Mouse phenotypes resulting from cell type-specific ablation of A20 expression and the related human diseases

Cell type	Genetic modification	Mouse phenotype	Related human disease	Refs
B cells	Cre-mediated deletion of <i>Tnfaip3</i> in cells that express CD19	Germinal centre and plasma cell dysplasia; production of autoantibodies; renal immunoglobulin deposition; B cell resistance to FAS-mediated cell death	Systemic lupus erythematosus	18,19,60
DCs	Cre-mediated deletion of <i>Tnfaip3</i> in cells that express CD11c	DC activation; expansion and activation of T cell and myeloid cell populations; colitis; spondyloarthritis	Inflammatory bowel disease	43
DCs	Cre-mediated deletion of <i>Tnfaip3</i> in cells that express CD11c	DC activation; expansion of T cell and plasma cell populations; increased uptake of apoptotic cells by DCs; autoantibody production; nephritis	Systemic lupus erythematosus	62
Macrophages and granulocytes	Cre-mediated deletion of <i>Tnfaip3</i> in cells that express lysozyme M	Increased IL-6 production; production of collagen-specific autoantibodies; protection against influenza A virus infection	Rheumatoid arthritis	67,68
Intestinal epithelial cells	Cre-mediated deletion of <i>Tnfaip3</i> in cells that express villin	Hypersensitivity to experimental colitis	Inflammatory bowel disease	69
Intestinal epithelial cells	Villin-driven expression of a <i>Tnfaip3</i> transgene	Protection against DSS-induced colitis	Inflammatory bowel disease	72
Keratinocytes	Cre-mediated deletion of <i>Tnfaip3</i> in cells that express keratin 14	Epidermal hyperproliferation; hair and skin defects; sebaceous gland hyperplasia	?	73

DC, dendritic cell; DSS, dextran sulphate sodium; IL-6, interleukin-6.

Figure 12, from (Ma and Malynn, 2012)- Cell-specific models of A20 deficiency

Since the first description of *A20*^{-/-} mice, many cell-type specific models have been engineered (see figure 12). Model of specific inactivation in IEC, responsible for intestinal inflammation, is discussed below.

Specific A20 ablation in IEC (in *Tnfaip3*^{fllox/fllox} villin-Cre mice) leads to an increased susceptibility to DSS-induced colitis and TNF-induced inflammation. Susceptibility to DSS colitis was rescued by TNF receptor 1 deficiency, suggesting that A20 may protect IEC from TNF-induced apoptosis during acute damage. The effect of A20 loss in IEC was most dramatic during intestinal recovery immediately after the removal of DSS treatment, suggesting a role for A20 in tissue repair (Ma and Malynn, 2012). Moreover, overexpression of A20 in IEC seems to be beneficial for mucosal intestinal homeostasis, both in *in vitro* model and in transgenic mice. Thus, shortly after LPS challenge, IEC become tolerant to restimulation with LPS, but not with IL-17 or UV for instance. The state of tolerance, which lasts approximately 24h, coincides with LPS-induced expression of A20. In 2009, Wang and colleagues showed *in vitro* in murine IEC that small interfering RNA (siRNA) silencing of A20 prevents tolerance, whereas overexpression expression of A20 blocks responses to LPS, but not to IL-17 or UV. They found that A20 levels in small intestine epithelial cells are low at birth or following gut decontamination with antibiotics, but high under conditions

of bacterial colonization. Furthermore, A20 is prominently localized to the luminal interface of villus enterocytes in adult rodents. The authors suggested that A20 could play a role in intestinal epithelium tolerance of to TLR ligands and bacteria (Wang et al., 2009).

In 2011, Kolodziej and colleagues engineered transgenic mice (called villin-TNFAIP3 mice) that constitutively overexpressed a *Tnfaip3* transgene in IEC, to assess the direct role of A20 expression. They compared them to full *Tnfaip3*^{-/-} mice. Forty-five minutes after LPS injection into the peritoneal cavity, *Tnfaip3*^{-/-} mice had greater intestinal permeability compared to WT littermates, while villin-TNFAIP3 transgenic mice were protected from increase in permeability. This protection was notably due to prevention of occludin loss from the apical surface after LPS administration. Indeed, upon LPS treatment, WT mice displayed marked reduction of occluding compared to villin-TNFAIP3 mice. Moreover, the authors showed in cultured human IEC cell line (HCT116) that TNFAIP3 deubiquitinated polyubiquitinated occludin and regulated tight junction dynamics through both TNF-induced and myosin light chain kinase-regulated signals. Thus, they suggested a role of A20 in promoting intestinal epithelial barrier integrity through tight junction proteins regulation (Kolodziej et al., 2011).

Recently, it was shown that A20 plays also a role in the regulation of NLR-pyrin-domain containing 3 (NLRP3) inflammasome, independently of its role in NF-κB regulation. Vande Walle and colleagues engineered myeloid-cell-specific deletion of A20 by crossing *A20*^{fllox/fllox} mice into lysozyme M (LysM)-Cre-recombinase-expressing mice. Mice developed a spontaneous erosive polyarthritis that resembles human rheumatoid arthritis. The authors showed that inflammation was dependent on Nlrp3 inflammasome and IL-1 receptor signaling. In response to soluble and crystalline Nlrp3 stimuli, macrophages lacking A20 displayed increases in basal and LPS-induced expression of Nlrp3 and proIL-1β, in Nlrp3 inflammasome-mediated caspase-1 activation, in pyroptosis, and in IL-1β secretion. In contrast, Nlr4 inflammasome activation was similar in mutated and WT mice. To confirm that A20 regulated NLRP3, *Nlrp3*^{-/-} mice were crossed into *A20*^{myel-KO} mice, leading to a marked reduction of IL-1β secretion in *A20*^{myel-KO} *Nlrp3*^{-/-} compared to *A20*^{fllox/fllox} mice. *A20*^{myel-KO} *Nlrp3*^{-/-} and double-KO mice with caspase-1 deletion (*A20*^{myel-KO} *Casp1*^{-/-}) were significantly protected from rheumatoid-arthritis-associated inflammation and cartilage destruction compared to *A20*^{myel-KO} mice. Thus, the authors suggested that A20 is a novel negative regulator of Nlrp3 inflammasome activation (Vande Walle et al., 2014).

Haploinsufficiency of A20 is a monogenic cause of severe inflammation in humans

Recently, Zhou et al described six patients with A20 monogenic disease due to *TNFAIP3* heterozygous mutations, resulting in haploinsufficiency. Patients displayed early-onset systemic inflammation, arthralgia and/or arthritis, oral and genital ulcers reminiscent of Behçet's disease,

and ocular inflammation. One patient exhibited severe early-onset autoimmunity with lupus-like phenotype and central nervous system vasculitis (Zhou et al., 2016). Patients' phenotype is reminiscent to the one observed in mice, either complete KO that displayed persistent NF- κ B activation, spontaneous multiorgan inflammation and early lethality or heterozygous KO that developed autoantibodies with aging (Ma and Malynn, 2012). A20 protein consists of an N-terminal ovarian tumor (OTU) domain followed by seven zinc-finger (ZnF) domains (see figure 13). All six mutations were predicted to impact deubiquitinase function. Five mutations mapped to the OTU domain, which mediates the deubiquitinase activity, resulting in truncated proteins of similar length. The sixth one mapped to the ZnF4 domain, which recognizes Lys63-linked ubiquitin chains and is essential for A20 ubiquitin ligase activity.

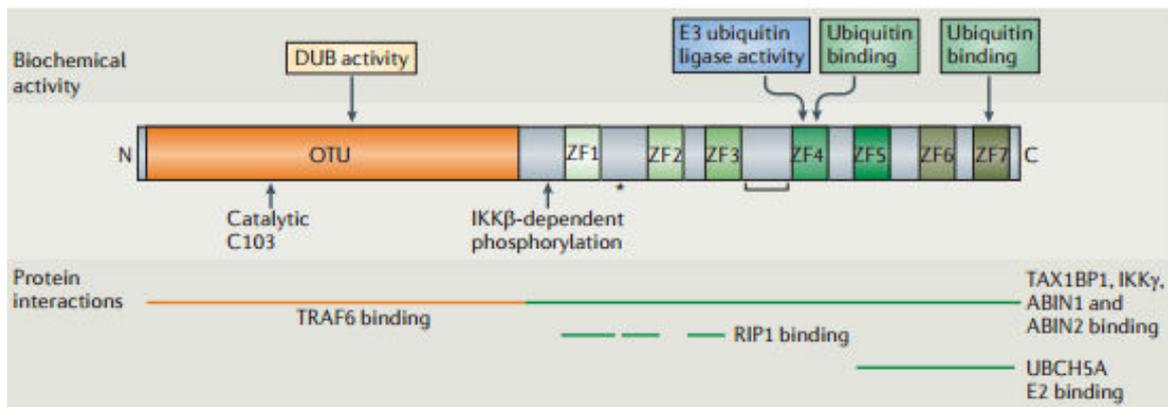


Figure 13, from (Ma and Malynn, 2012)– A20 functional domains

In vitro, protein expression of WT A20 was reduced in patients' mononuclear cells and fibroblasts, whereas mutant proteins were not detectable. By luciferase assay, overexpression of mutated proteins failed to suppress TNF-induced NF- κ B activity. The authors demonstrated excessive activation of NF- κ B upon TNF-stimulation, as attested by increased I κ B α degradation, p65 nuclear translocation, and proinflammatory cytokines expression. A20 inhibits NF- κ B pathway through deubiquitination of specific proteins. As expected, the authors showed defective removal of Lys63-linked ubiquitin from TRAF6 (TNF receptor-associated factor 6), NEMO and RIP1 (Receptor-interacting serine/threonine-protein kinase 1) after stimulation with TNF. At last, they confirmed recent mice studies implicating A20 role in regulation of NLRP3. Indeed, patients exhibited constitutive activation of NLRP3 inflammasome with consecutive increase in secretion of active IL-1 β and IL-18. These results justified the test in one patient of an IL-1 β receptor antagonist, which led to positive results. Of note, *NLRP3* mutations are a cause of monogenic auto-inflammatory disease without intestinal inflammation (Zhou et al., 2016).

II.B- Defects in extrinsic immunoregulatory mechanisms

II.B.1- Defects in IL-10 signaling pathway

Mouse models

IL-10 is a key immunoregulatory cytokine able to inhibit pro-inflammatory responses of immune cells and thus limit tissue damage. Its unique role in gut homeostasis started to be deciphered with the description of the first KO mouse model in 1993 (Kuhn et al., 1993). *Il-10*^{-/-} mice raised under conventional conditions developed after weaning (4-8 weeks) a spontaneous lethal enterocolitis with weight loss and anemia. In this model, the entire gastro-intestinal tract was affected, mostly its upper part, with a massive lymphoplasmocytic infiltrate and some multinucleated giant cells (also called granuloma). LPS-stimulated spleen cells from *Il-10*^{-/-} mice produced more inflammatory cytokines, such as IL-6 and TNF α , than WT animals, reflecting the loss of inhibitory effect of IL-10. Excessive activation of Th1 cells in *Il-10*^{-/-} mice was demonstrated by the efficacy of anti-IFN γ antibody in preventing disease in young mice (Berg et al., 1996), or by the efficacy of monoclonal antibodies against IL-12 or TNF α after bacterial infection (Kullberg et al., 1998). Genetic background was shown to impact the severity of the disease, with BALB/c strain being the most affected as compared to C57BL/6J strain (Berg et al., 1996). Surprisingly, in *Il-10*^{-/-} mice raised in SPF condition, a milder phenotype was reported with colonic lesions only, the other aspects of the phenotype (weight loss, anemia, histological findings) remaining identical to those bred under conventional conditions (Berg et al., 1996; Kuhn et al., 1993). Moreover, germ-free *Il-10*^{-/-} mice did not develop colitis and were undistinguishable from germ-free WT mice (Sellon et al., 1998).

This last observation raised the question of the influence of the microbiota on the development of intestinal pathology in *Il-10*^{-/-} mice. Analysis of *Il-10*^{-/-} mice colonized by *Helicobacter hepaticus* (HH) led to controversial results. Kullberg et al reported that HH triggered colitis in SPF *Il-10*^{-/-} mice and that colitis could be prevented with neutralization of IFN γ and IL-12 (Kullberg et al., 1998). Unexpectedly, these results were not reproduced by Dieleman et al in 2000, who could not trigger enterocolitis in *Il-10*^{-/-} mice with infection by HH (oral gavage and enema) (Dieleman et al., 2000). Of note mice in the first but not in the second study originated from Taconic farms. Furthermore, in the first study, HH infection (intraperitoneally or intragastric) was done in conventional animal facility that may have been contaminated by other interfering pathogens. The triggering role of disease was then tested in *Il-10*^{-/-} mice mono-associated with a spectrum of bacteria. *Pseudomonas fluorescens*, *Candida albicans*, or *Lactobacillus lactis* failed to induce colitis whereas *Enterococcus faecalis*, *E. coli*, or *Bifidobacterium animalis* were sufficient to induce colonic inflammation (Shouval et al., 2014). But, these microbial species did not induce

inflammation of stomach and small bowel which was observed in the first study of *Il-10*^{-/-} mice (Rennick and Fort, 2000). Recently, HH ability to induce colitis in *Il-10*^{-/-} mice was once again investigated by Yang et al (Yang et al., 2013). They noted that mice bred in Hanover facility were resistant to HH infection as compared to mice bred in MIT animal facility, and were able to link this observation to profound differences in their microbiota composition. Notably, analyses of MIT's mice microbiota displayed presence of a pathobiont called *Bilophila wadsworthia*. Interestingly, expansion of *B. wadsworthia*, a sulfite-reducing enteric bacteria, can be stimulated by high fat diet (Devkota et al., 2012), demonstrating how dysbiosis induced by Western-type diet may foster intestinal inflammation in predisposed *Il-10*^{-/-} mice.

Which cell types are responsible for the development of enterocolitis in *Il-10*^{-/-} mice? Th1 CD4⁺ T cells were first implicated. In the T-cell transfer mouse model, colitis did not occur in mice treated systemically with recombinant IL-10 (Powrie et al., 1994). Transfer of lamina propria CD4⁺ but not of CD8⁺ from *Il-10*^{-/-} mice in *Rag2*^{-/-} recipients induced colitis (Davidson et al., 1996). In 2008, Rubstov et al ascribed colitis development to defective IL-10 production by regulatory T-cells (Treg). Using mice with restricted deletion of IL-10 in Tregs, they observed that mice did not develop auto-immunity, a result in keeping with the phenotype of *Il-10*^{-/-} mice, but they developed chronic inflammation in colon and lungs. It suggests that IL-10 is particularly needed at body interfaces to adjust microbiota-induced inflammatory responses (Rubtsov et al., 2008). Finally, Hoshi et al demonstrated in 2011 that, in *Il-10*^{-/-} mice, bacterial sensing by gut macrophages through MyD88 triggered colitis whereas bacterial sensing by epithelial cells did not (Hoshi et al., 2012).

In 1997, Kotenko et al identified the CRFB4 chain as being the accessory chain within the IL-10 receptor complex and thus renamed it IL-10R β chain (Kotenko et al., 1997). One year later, in 1998, Spencer et al described the first *Il-10r β* ^{-/-} mice. Only 60% of these mice developed spontaneous colitis and splenomegaly around 12 weeks of age, if bred in conventional facilities. But, contrary to *Il-10*^{-/-} mice, they displayed neither stomach or small bowel lesions, nor severe anemia. These mice were engineered on a mixed 129Sv/Ev x C57Bl/6 genetic background. Even though IL-10R β is widely expressed and is necessary not only for the IL-10 but also for the IL-22, IL-26, and IFN- λ receptors, its deletion in mice lead to an inflammation restricted to the gut (Spencer et al., 1998). *Il-10r α* ^{-/-} mice generated by Pils et al, on a C57BL/6 background and in a SPF facility, did not develop spontaneous colitis (alike *Il-10*^{-/-} mice generated on the same strain by the authors). These mice were more sensitive to DSS colitis than WT-mice but less than *Il-10*^{-/-} mice. This observation raises the question of an additional effect of IL-22, IL-26 and/or IFN- λ deficiency in the *Il-10r β* ^{-/-} mouse model (Pils et al., 2010).

Recently, the question of which cell type is the major target of IL-10 has been addressed. Li et al engineered mice (C57BL/6 strain) with a selective deletion of *Il-10ra* in macrophages (*Il-10ra^{Mdel}*). These mice were more susceptible to DSS-colitis and recapitulated the phenotype reported in *Il-10^{-/-}* and *Il-10r^{-/-}* mice. In this specific mouse model, TNF α production was increased and its neutralization by monoclonal antibody slightly improved colitis-scores similarly to *Il-10ra^{Mdel}* and WT mice. Compared to controls, *lamina propria* macrophages from *Il-10ra^{Mdel}* produced greater amounts of nitric oxide (NO) and ROS. Following this observation, *Il-10ra^{Mdel}* treated with NOS inhibitor (aminoguanidine hydrochloride) or with ROS scavenger (N-acetyl-L-cysteine) were significantly improved, whereas these treatments did not have a significant impact in WT-mice. Thus, inhibition by IL-10 of NO and ROS production by macrophages is key to contain excessive inflammation after lesions of the intestinal epithelial barrier (Li et al., 2014).

IL-10 signaling deficiency is the archetype of monogenic colitis in humans

Defective IL-10 pathway is the archetype of VEO-IBD with predominant colitis. The first patients with IL-10 receptor (IL-10R) or IL-10 deficiency were described by Glocker et al in 2009 and 2010 respectively (Glocker et al., 2010; Glocker et al., 2009). So far, 61 patients have been reported, among whom only five have an IL-10 deficiency, and 34 and 21 have IL-10R α or IL-10R β deficiency respectively. In one patient, IL-10R deficiency is indicated but not the affect subunit (see Table 6 in SUPPLEMENTARY DATA). Mutations were homozygous or compound heterozygous in 43 and 17 patients respectively. Six patients carried large deletions or duplications (deletion of *IL-10R β* exon 2 in P5 in (Pigneur et al., 2013); deletion of *IL-10R α* exons 1 to 3 in P5 and heterozygous deletion of *IL-10R α* exons 2 to 4 in P6 in (Engelhardt et al., 2013); deletion of exon 3 only (P1 and P2) or associated with duplication of exon 6 in *IL-10R β* (P3, see related article below in the RESULTS section)). When tested, protein expression is always absent, except for one patient with a compound heterozygote mutation in *IL-10R α* (p.T84I; p.R101W, (Mao et al., 2012)) and one patient with a compound heterozygous mutation in *IL-10R β* (deletion of exon 3 and duplication of exon 6, P3 see related article below in the RESULTS section). These two patients had a defective IL-10 pathway.

Disease onset has been reported from the first days of life to 6 years, with a median age of onset of 1.5 months (interquartile range= 0.5-4 months). Contrary to intractable diarrhea, there is a few weeks/months delay in IL-10 and IL-10R deficiency before disease onset. Bearing in mind that germ-free *Il-10^{-/-}* mice do not develop colitis, it suggests that microbial colonization is needed to induce inflammatory responses that will not be kept in check due to defective IL-10 pathway.

Clinical phenotype is very homogeneous between patients with IL-10, IL-10R α and IL-10R β deficiency and penetrance is complete. All patients display bloody chronic diarrhea and perianal

lesions (fissure, fistula and abscess) except P3 reported by Oh SH et al (Oh et al., 2016) who is homozygous for a synonymous mutation that leads to skipping of exon 4 of *IL-10R α* . Endoscopy investigations objectify severe colitis with deep ulcerations and skip lesions that often evolve into diffuse pancolitis. Pathology findings include polymorphic infiltrates, crypt abscesses, and epithelioid granulomas. Involvement of the upper part of the intestinal tract is rare, with only seven patients reported with gastritis and nine with ileitis. Folliculitis is the most common extra-digestive symptom, reported in 21 patients (*IL-10R α* = 10; *IL-10R β* = 11). Its comparable representation in patients deficient for either the α or the β chain suggests a mechanism independent of IL-22. Other dysimmune symptoms include arthritis (n=7), hear loss (n=4), auto-immune hepatitis (n=1), Kawasaki syndrome (n=1), osteoporosis (n=1), bilateral hydronephrosis (n=1) emphysema (n=1), growth hormone deficiency (n=1) and food allergy to cow's milk protein (n=1). It is important to note that, except one patient with an auto-immune hepatitis, auto-immunity symptoms and auto-antibodies were absent in patients with an IL-10 defective pathway, in contrast to *Il-10^{-/-}* mice in which colon-reactive antibodies were reported (Davidson et al., 1996).

Repeated and severe bacterial or viral infections are reported in one third of the patients (n=22). Due to the heavy immunosuppression required to control intestinal inflammation, it is difficult to define whether IL-10 defect itself can lead to an immunodeficiency.

The last hallmark of IL-10R defect is the occurrence of EBV-negative B-cell lymphoma with no difference between deficiencies in α or β chain. The latter B-cell lymphomas (BCL) are monoclonal and highly proliferative, with monomorphic large B cells. Their onset was reported in 10% (see Table 6) to 36% (Neven et al., 2013) of the patients. Neven et al showed that BCL displayed constitutive activation of NF- κ B and defective local T-cell immune response, as reflected by the lack of infiltrative granzyme B⁺ T cells. The authors remarked that such lymphomas were never described in *Il-10^{-/-}* mice. Interestingly, BCL can occur in patients with *STAT3* (the transcription factor downstream IL-10R) LoF mutations although less frequently, suggesting that IL-10 and its signaling pathway participates in B cell homeostasis in humans.

Validation of mutations in IL10R can be easily made by demonstrating either lack of IL-10 mediated *STAT3* phosphorylation and/or loss of IL-10 inhibitory effect on pro-inflammatory cytokines production by mononuclear cells upon stimulation by LPS (Begue et al., 2011).

IL-10 and IL-10R deficient patients are always refractory to immunosuppressive treatments. Due to life-threatening colitis, some patients underwent a total colectomy, mostly before identification of their molecular diagnosis. But importantly, IL-10R deficient patients can be cure by HSCT (Neven et al., 2013; Pigneur et al., 2013; Shouval et al., 2014). Even though IL-10 R is widely expressed, the correction of the defect in the hematopoietic compartment is sufficient, a result in keeping with mice studies demonstrating that intestinal macrophages are the key target of IL-10 (Li

et al., 2014). Interestingly, HSCT has the same favorable outcome in patients deficient for α or β chain of the IL-10R, even though the IL-10R β chain is the accessory chain for the receptors of IL-22, IL-26 and IFN- λ . Thus, contribution of defects in IL-22, IL-26 and IFN- λ signaling to the human phenotype seems limited, perhaps due to redundancy in these cytokines functions in humans, or due to compensatory mechanisms.

II.B.2- Defects in generation and/or functions of regulatory T-cells

II.B.2.1- Lessons from FOXP3 deficiency in mice and humans

The hallmark of adaptive immune cells, which appeared with jawed vertebrates, is their ability to generate an immense repertoire of antigen-specific receptors. But the generation of such a diversity of receptors can give rise to pathogenic self-reactive cells as well as lymphocytes directed against food antigens or commensal bacteria. Despite negative selection of most high affinity autoreactive T cells in the thymus, some may escape to the periphery where they need to be neutralized by a specific subset of T cells with regulatory properties called Treg.

The initial evidence of the existence of regulatory T cells of thymic origin expressing CD4 and CD25 was clearly demonstrated by the group of Sakaguchi in 1996. They showed that neonate mice developed severe autoimmunity when thymectomized at day 3 after birth, but not if the thymectomy was performed at day 1 or 7. Thymectomy resulted in absence of circulating “autoimmune preventive” CD25⁺ T cells in periphery leading to the development of autoimmunity by self-reacting T cells. Autoimmunity was rescued by injecting CD25⁺ T cells from adult mice (Asano et al., 1996). In 2001, forkhead box P3 (*Foxp3*), a transcription factor from the forkhead/winged-helix family, was identified as causative of the monogenic defect in scurfy mice, an X-linked mouse mutant characterized by lymphoproliferation, multiorgan infiltration and systemic inflammation in males (Brunkow et al., 2001). At the same time, *FOXP3* mutations were ascribed to human IPEX syndrome (for immune dysregulation, polyendocrinopathy, enteropathy, X-linked), strongly reminiscent of scurfy mice (see below). Lastly, in 2003, Hori and colleagues established that *FOXP3* was the transcription factor of Tregs and was indispensable to their function (Hori et al., 2003). Thus, adoptive transfer of Treg cells in *Foxp3*^{-/-} mice rescued scurfy phenotype. Mice with T cell-specific deletion of *Foxp3* developed an exactly similar disease to mice with germ-line deletion. Mice with specific *Foxp3* deletion in cell types other than T-cells, such as thymic epithelial cells, dendritic cells, or macrophages, did not develop any dysimmune symptoms. Tregs were

shown to be crucial, not only in neonates, but throughout the life span of normal mice, as chronic deletion of Tregs (in a *Foxp3^{DTR}* model induced by diphtheria toxin) in adult mice led to fatal lympho- and myeloproliferative syndrome (Josefowicz et al., 2012a). The contribution of FOXP3 transcriptional program in Tregs was studied notably with *Foxp3^{GFPKO}* mice, in which green fluorescent protein (GFP) followed by a stop codon was inserted in the FoxP3 gene downstream elements regulating FoxP3 expression but before exons 2 and 3. This model allowed the dissociation between Foxp3-dependent signals, and signals preceding and promoting Foxp3 expression. GFP⁺ T cells from *Foxp3^{GFPKO}* mice exhibited some *Foxp3⁺* Treg cells characteristics, such as high level of CD25 and CTLA4 (for Cytotoxic T lymphocyte antigen-4), absence of proliferation after T-cell receptor (TCR) engagement, or inability to produce IL-2. But, *Foxp3^{GFPKO}* Treg cells were completely devoid of suppressor activity, were able to produce IL-4 and IL-17, and could proliferate in response to limited TCR/CD28 activation (Gavin et al., 2007).

How are Tregs generated?

Thymic Tregs (also called natural or conventional by some authors) were demonstrated to be positively selected in the thymic medulla following high affinity interactions with self-peptide-MHC complexes (major histocompatibility complex) presented by thymic stromal cells, and through accessory and survival signals (notably CD28 stimulation and IL-2 signaling). This model was further refined by the work of two teams in 2008. They showed that following high-affinity TCR engagement, Treg precursors upregulated CD25 (α chain for the IL-2 receptor) to allow activation of IL-2 signaling pathway, leading to STAT5 upregulation and FOXP3 induction through binding to conserved noncoding sequence 2 (CNS2) (Burchill et al., 2008; Lio and Hsieh, 2008). Central role of STAT5 in FOXP3 induction was supported by multiple evidence. First, specific deletion of *Stat5* in double-positive thymocytes resulted in decreased number of *Foxp3⁺* thymocytes. Secondly, expression of constitutively active STAT5 resulted in increased Tregs number and rescued Tregs number in absence of IL-2 or of second signal (*CD28^{-/-}* mice).

The IL-2/STAT5 axis was further shown to inhibit Th17 differentiation. Indeed, CD4⁺ T cells, either from *Il2^{-/-}* mice or from WT mice previously treated with antibody blockade of IL-2, cultured with transforming growth factor β (TGF- β) and IL-6, were more prone to differentiate into IL-17 producing cells compared to WT or untreated cells. Similarly, proportion of Th17 cells was significantly increased in CD4⁺ T cells from *Stat5^{-/-}* mice as compared to WT (Laurence et al., 2007). Lastly, in a mouse model with inducible Foxp3 deletion, Wang and colleagues established that Tregs might help to establish efficient Th17 response after gut infection most likely by consuming IL-2 (Wang et al., 2014). Thus, IL-2 has multiple roles in Treg homeostasis: STAT5-mediated transcription of FOXP3, promotion of cell survival, and opposing differentiation of CD4⁺

T cells into Th17 cells (Josefowicz et al., 2012a). Thymic Tregs were suggested to represent 70–90% of mouse Tregs in blood and peripheral lymphoid organs, using markers such as Helios (also known as IKZF2) and neuropilin 1 preferentially expressed on thymic Tregs (Liston and Gray, 2014).

Peripheral or induced Tregs (iTregs) can be differentiated from naïve CD4⁺ T cells in response to non-self antigens, and in conditions of suboptimal costimulation with high concentrations of TGF- β . These conditions are met within mucosal tissues. Notably, gut-associated lymphoid tissue (GALT) and Peyer's patches are sites favoring iTregs generation specific of food antigens and commensal microbiota. Induction of Tregs is notably favored upon antigen-presentation by CD103⁺ dendritic cells present in GALT and in gut-draining mesenteric lymph nodes (MLN), which produce TGF- β and retinoic acid. TCR repertoire of iTregs is distinct from that of natural Tregs, and specificity against microbial and food antigens can be demonstrated. Colonic iTregs numbers are diminished in germ-free mice, and can be rescued by colonization with a cocktail of several *Clostridium* species characteristic of commensal microbiota. Contrary to thymic Tregs that require CD28 costimulatory signal for their induction, iTregs need CTLA4 costimulation, as demonstrated by Zheng and colleagues who could not generate iTregs neither from CD4⁺CD25⁻ T cells from *Ctla4*^{-/-} mice stimulated with TGF- β , nor from WT cells pre-treated with anti-CTLA4 blocking antibody (Zheng et al., 2006). Smad3, which is activated in response to TGF- β , forms nuclear complexes with NFAT (for nuclear factor of activated T-cells) that binds to CNS1 in order to induce FOXP3 expression in peripheral iTregs. Accordingly, *Cns1*^{-/-} mice displayed a marked reduction of iTreg population whereas numbers of thymic Treg subset remained comparable to WT mice. Moreover, *Cns1*^{-/-} mice exhibited a Th2 phenotype with allergy and asthma, but did not develop autoimmunity or lymphoproliferation. Of note, colitis was not reported as a prominent symptom either (Josefowicz et al., 2012b). Yet, a role of commensal bacteria in the generation or activation of mouse iTregs has been highlighted by several studies. Short-chain fatty acids (SCFA) produced by microbiota and notably butyrate were shown to induce differentiation of Treg cells *in vitro* and *in vivo*. Moreover *Rag1*^{-/-} mice fed with high fiber diet and thus presumably with high intracolonic concentrations of SCFA, were protected against colitis induced by the transfer of CD4⁺ CD45RB^{high} T cells (Furusawa et al., 2013). Partially overlapping results were reported with propionate (Arpaia et al., 2013). Lastly, two teams reported the induction of iTregs expressing the transcription factor ROR γ (for RAR-related orphan receptor γ) in the colon. Expansion of iTreg/ROR γ subset was enhanced by commensal microbiota species, yet was largely independent of butyrate. It rather involved combined signals from IL-6 and IL-23 via Stat3 and from retinoic acid. Moreover, this derivative of dietary vitamin A was suggested to be able to drive differentiation of ROR γ expressing T cells toward Treg rather than Th17 fate. In the absence of iTreg/ROR γ , Th2-driven responses against helminths were amplified, while Th2-associated pathology was exacerbated. Similarly,

Foxp3-cre.Rorc^{fl/fl} mice, unable to generate the iTreg/ROR γ subset, were more susceptible to TNBS-induced colitis (a Th1/Th17 colitis model). Thus, the microbiota, through induction of iTreg/ROR γ cells, acts as a key factor in balancing immune responses at mucosal surfaces (Ohnmacht et al., 2015; Sefik et al., 2015).

	Thymic Treg	Induced Treg
High-affinity TCR against Ag presentation	Self Ag	Food and commensal microbiota Ag
Costimulatory signal	Thymic epithelial cells	CD103 ⁺ dendritic cells
Cytokinic environment	CD28	CTLA4
FOXP3 transcription factor	IL-2	TGF- β
DNA regulatory sequence	STAT5	Smad3/NFAT
Specific markers	CNS2	CNS1
Protection against	Helios, Neuropilin 1	
	Lymphoproliferation Autoimmunity	Allergy, Asthma, Th2 phenotype

Table 2. Main characteristics of thymic and induced Tregs.

After commitment to Treg lineage, Treg cells must maintain Foxp3 expression to remain functional. Such stability is vital since thymic Tregs bear TCR with high affinity for self-peptide-MHC complexes. They might potentially induce autoimmune lesions if converted into effector T-cells. Induction and maintenance of FOXP3 transcription was shown to be regulated by three CNS elements by Rudensky's team. The authors showed that CNS3 and c-Rel interactions promote the generation of Treg cells in the thymus and in the periphery. CNS1, after activation by TGF- β -NFAT, had a prominent role in iTregs generation in GALT. CNS2 was indispensable to maintenance of Foxp3 expression in the progeny of dividing Tregs through binding to the Runx1-CBF β complex (for Runt-related transcription factor 1/ Core-binding factor subunit β) (Zheng et al., 2010). Formation of CNS2/Runx1-CBF β complexes required demethylation of CpG dinucleotides within the CNS2 locus. Moreover, demethylation of CpG within CNS2 and in the FOXP3 promoter was required for stable FOXP3 expression. In humans, activated effector T cells without any regulatory properties can express low amount of FOXP3. This low expression might be induced by TGF- β . In activated T-cells, CNS2 remained in a methylated and repressed state (Josefowicz et al., 2012a).

Tregs act through several mechanisms involving cell-surface molecules (CTLA4, CD25) or secreted proteins. For instance, CD25, the receptor subunit which confers high avidity to IL-2, is constitutively highly expressed on Tregs and can deprive effector T cells of IL-2, thereby inhibiting their activation and proliferation. Among the secreted proteins (IL-10, IL-35, granzyme B, IL-9,

TGF- β , IL-35), the role of IL-10 produced by Tregs was first assessed. Analysis of mice with specific ablation of *Il-10* in Tregs showed that IL-10 production by Tregs was dispensable to control systemic autoimmunity but was essential to keep immune responses in check at environmental interfaces such as colon and lungs. Similarly, absence of 12p35 or Ebi3, two of the major components of IL-35, impaired Treg that could not control homeostatic proliferation after adoptive transfer into lymphopenic hosts which developed intestinal inflammation. At last, TGF- β produced by Tregs can suppress Th1 inflammation (Josefowicz et al., 2012a).

Treg homeostasis is of extreme importance, as too few Tregs can lead to autoimmunity whereas too many will lead to excessive immune tolerance or anergy (as reviewed in (Liston and Gray, 2014)). This balance is tightly regulated through high rate of proliferation and apoptosis. Half of circulating Tregs undergo division every ten days in both mice and humans, and thus have a higher proliferation rate than conventional T cells in non-inflammatory conditions. This high proliferation rate is counter-balanced by high apoptosis rate, mediated through FOXP3-dependent phosphorylation of the pro-apoptotic protein BIM (BCL-2-interacting mediator). IL-2 can counter BIM pro-apoptotic function by upregulating the pro-survival protein MCL1 (myeloid leukemia cell differentiation 1). IL-2 can also control Treg homeostasis independently of apoptotic proteins, through competition effects. In non-inflammatory condition, Tregs constitutively express CD25, providing a competitive advantage over conventional T cells that express only low-affinity receptor. During infection or inflammation, transient upregulation of CD25 expression by effector T cells allows clonal expansion of activated T cells that consume IL-2, thus leading to temporarily Treg population contraction by depriving them of IL-2 (Liston and Gray, 2014). This mechanism was suggested to explain immune dysregulation induced by septic shock syndrome. Reduced numbers of Tregs which were observed during the pro-inflammatory cytokinetic storm at the acute stage. Conversely, reduced number of circulating effector T cells were observed in surviving patients who display profound immunosuppression, and are at risk of severe infectious complications. Therefore, septic shock syndrome may result from dual Treg homeostasis impairment: a reduction of Treg cells during the acute phase responsible for hyperinflammation, followed by over-filling expansion thus leading to an anergic phase (Venet et al., 2008).

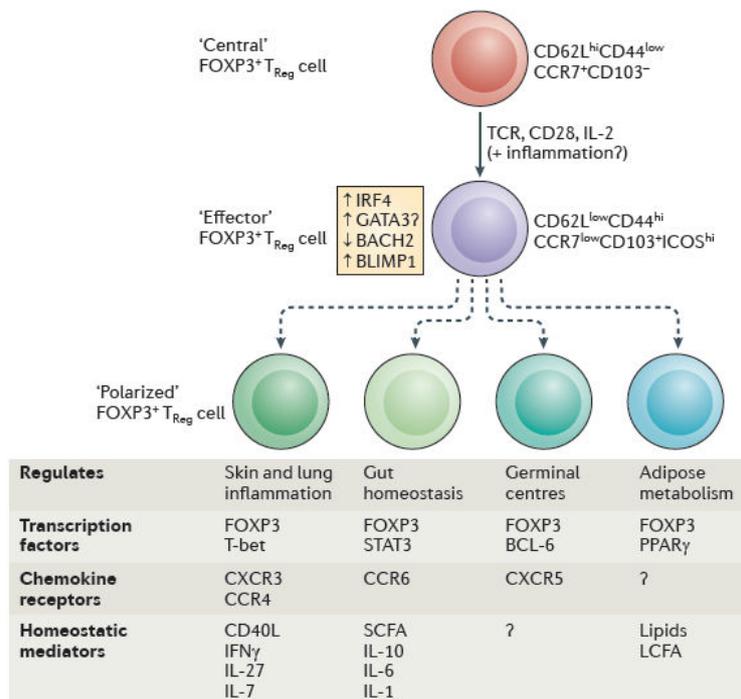


Figure 14, from (Liston and Gray, 2014)– Treg cells activation and differentiation. *BACH2*, *BTB* and *CNC* homologue 2; *BCL-6*, *B* cell lymphoma 6; *BLIMP1*, *B* lymphocyte-induced maturation protein 1; *CCR*, *CC*-chemokine receptor; *CXCR*, *CXC*-chemokine receptor; *GATA3*, *GATA*-binding 3; *ICOS*, *inducible T cell co-stimulator*; *IFN γ* , *interferon- γ* ; *LCFA*, *long-chain fatty acid*; *PPAR γ* , *peroxisome proliferator-activated receptor- γ* ; *SCFA*, *short-chain fatty acid*; *STAT3*, *signal transducer and activator of transcription 3*

Apart from the division into thymic and peripheral Tregs, functional subsets have been individualized recently. Tregs were further divided into “central”, “effector” and “tissue-resident” populations. Central Tregs constitute the majority of Treg cells in the circulation and secondary lymphoid organs. They display circulatory characteristics that are similar to naive conventional CD4⁺ T cells, yet they are not quiescent, with baseline suppressive function and a history of antigen exposure. Most of them express CD62L^{hi}CCR7⁺ allowing their recirculation in lymphoid tissues (Liston and Gray, 2014).

Effector or activated Treg cells are a minor fraction of Treg cells in the circulation and secondary lymphoid organs, and they share phenotypic features with activated conventional T cells. They are CD62L^{low}CCR7^{low}CD44^{hi}CD103⁺KLRG1⁺ (killer cell lectin-like receptor subfamily G member 1-positive). They are thought to have encountered antigens more recently than central Treg cells and show enhanced migration toward non-lymphoid tissues. Shift from central to effector Tregs seems to implicate several transcription factors, notably IFN regulatory factor 4 (IRF4) (Liston and Gray, 2014). Thus, Treg cells from *Irf4*^{-/-} mice failed to downregulate CD62L and to upregulate KLRG1, CTLA4, and CD103 notably, rendering them unable to migrate into non-lymphoid tissues and to suppress systemic Th1 cell responses (Cretney et al., 2011). Setting into motion different

differentiation programs of effector Tregs is important as it induces different migration, homeostasis and functional capacities. For example, Treg cells deficient for CCR4 (C-C chemokine receptor type 4, homing receptor for skin and lungs) could maintain immune homeostasis in secondary lymphoid organs but their inability to traffic to skin and lungs caused severe dermatitis and pneumonitis (Liston and Gray, 2014).

Tissue-resident Treg cells have long-term residence in non-lymphoid tissues, as opposed to short-term migration of effector Tregs through non-lymphoid tissues. One of the well-known polarized tissue-resident subset is the one associated to the gut, with expression of free fatty acid receptor 2 (FFAR2).

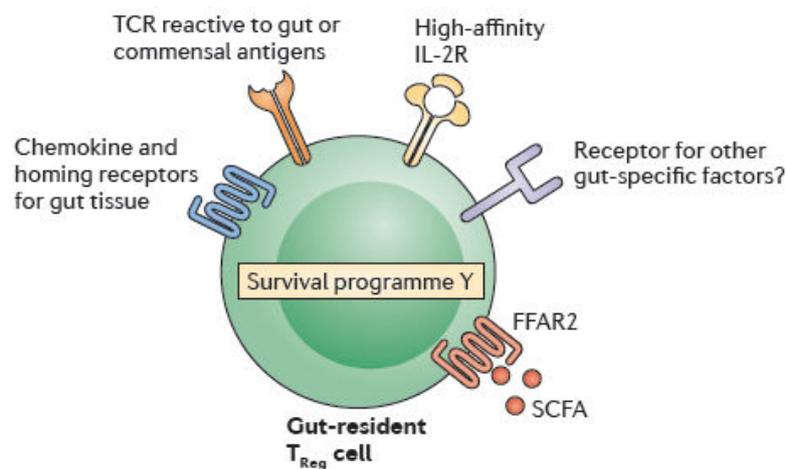


Figure 15, from (Liston and Gray, 2014)– Gut resident Treg cells

Gut-resident Treg cells are the most abundant subset of tissue-resident cells. This subset is essential for the maintenance of intestinal immune homeostasis, particularly in the colon. As discussed above, germ-free mice display a marked reduction in colonic-resident Tregs number that can be rescued by commensal bacterial species. A cocktail of 17 strains of Clostridium bacteria derived from the human microbiota were shown to enhance Tregs abundance upon transplantation in germ-free mice. These strains, which lack prominent toxins and virulence factors, helped expansion and differentiation of Tregs by providing bacterial antigens as well as SCFA in a TGF- β -rich environment (Atarashi et al., 2013). Identification and characterization of gut-resident Tregs reveal how a unique Treg cell differentiation program is established by commensal–host interactions at mucosal surface to maintain immune tolerance (Liston and Gray, 2014).

miRNA might also play a role in function have been shown to impact Tregs in mouse. Ablation of either Dicer or Drosha, two enzymes critical for the generation of mature miRNAs, resulted in fatal autoimmunity indistinguishable from scurfy mice. The suppressor activity of Tregs deficient for Dicer was reduced in non-inflammatory conditions and was completely lost in inflammatory

settings. miRNA were also involved in survival and proliferation of Tregs. miR155 deficiency in Tregs was shown to result in increased suppressor of cytokine signaling 1 (SOCS1) expression and impaired activation of STAT5 in response to limiting amounts of IL-2. miR-146a, another miRNAs prevalently expressed in Tregs, is critical for their suppressor function. miR-146a deficiency in Tregs resulted in fatal IFN γ -dependent immune-mediated lesions in a variety of organs, likely due to augmented expression and activation of Stat1 (Josefowicz et al., 2012a). Currently, no human monogenic disease in Tregs generation and homeostasis has been ascribed to miRNA pathway, but increased utilization of whole genome sequencing might lead to their identification.

***FOXP3*-mutation/IPEX syndrome is the archetype for monogenic autoimmune enteropathy in humans.**

As well as IL-10 pathway defective patients are the archetype of VEO-IBD with predominant colitis, patients with *FOXP3* mutation are the prototype for VEO-IBD with enteropathy and autoimmunity.

The first description of IPEX in humans provided in 1982 by Powel et al who described 17 boys over three generations diversely affected by diarrhea, autoimmune diseases, eczema-like skin lesions and increased sensitivity to infections (Powell et al., 1982). Loss-of-function mutations were subsequently identified in a new member of the forkhead/winged-helix family of transcriptional regulator. The protein first named JM2 (Chatila et al., 2000) was rapidly rebaptized FOXP3 (Bennett et al., 2001; Wildin et al., 2001). Since the molecular characterization of the IPEX syndrome in 2000-2001, more than 100 patients affected by this very rare disease have been described. Their analysis, shown in Table 7 in SUPPLEMENTARY DATA, provides important insight into the role of FOXP3+ Tregs in humans. In the majority of cases symptoms started during the neonatal period (mean onset age: 20 days, range birth-28 months old). Approximately two third of the patients had skin lesions, such as eczema (53%), and more rarely severe exfoliative dermatitis (Wildin et al., 2001), psoriasiform dermatitis or alopecia (Gambineri et al., 2008). Autoimmune disorders were present in approximately 75% of patients, the most common being insulin-dependent diabetes mellitus (57%). Other disorders included cytopenia, notably hemolytic anemia (23%), thyroiditis (19%) and less commonly auto-immune hepatitis (10%) (Gambineri et al., 2008; Moes et al., 2010), nephropathy (18%) (Gambineri et al., 2008; Kobayashi et al., 2001; Moes et al., 2010), lymphadenopathy (Gambineri et al., 2008; Wildin et al., 2001), hepatosplenomegaly (Gambineri et al., 2008), and arthritis (Wildin et al., 2001). Strikingly, one trait common to almost all reported patients was an enteropathy manifested by severe and chronic diarrhea. The enteropathy affecting IPEX patients has often been taken as evidence that Tregs control intestinal responses to the microbiota. Yet, this enteropathy predominated in the small intestine and affected its proximal part;

it was characterized by lesions of total or subtotal villous atrophy and was responsible for malnutrition. In contrast, bloody diarrhea and colitis were rarely reported (15%). Thus Gamberini et al noted colitis in only one out of 14 patients (Gamberini et al., 2008). In a systematic histological study of gastro-intestinal lesions in 12 cases, Patey-Mariaud et al indicated that glandular destruction could affect all parts of the gastrointestinal tract. Yet macroscopic colitis was present only in the three most severely affected patients. These authors qualified gastrointestinal lesions as either graft-versus-host disease-like in 9 patients displaying crypt abscesses or celiac-like in the other patients who presented subtotal villous atrophy without crypt destruction (Patey-Mariaud de Serre et al., 2009). Intraepithelial lymphocyte infiltration, which is a prominent feature of celiac disease, was less pronounced in IPEX, the infiltration predominating in the small intestinal *lamina propria* where it could be associated with eosinophilic infiltrate. Gastritis of variable severity was also reported in one patient (Gamberini et al., 2008; Patey-Mariaud de Serre et al., 2009). Overall, histological description does not plead for microbiota-induced inflammation but is rather consistent with autoimmune and/or food-driven reactions. Pointing to intestinal self-reactivity, the IPEX enteropathy was almost invariably associated with detection of anti-enterocyte antibodies directed against Harmonin, a 75kD antigen expressed in the brush border of the small intestine, in the inner ear sensory hair cells, and in the proximal tubule of the kidney (Lampasona et al., 2013). Antibodies against villin or goblet cells have also been observed. Their role in tissue damage is however uncertain as they can appear after the enteropathy, and epithelial destruction might rather be ascribed to self-reactive T cells. Besides self-reactivity, excessive Th2 responses to food antigens might also contribute to the enteropathy. Thus, 37% of patients displayed hyper-IgE and 20% developed severe allergy, notably food allergies (15%) with IgE antibodies against multiple food allergens. As in patients with an IL-10 defective pathway, approximately one third of reported patients displayed severe and/or recurrent infections. It is however difficult to distinguish possible immunodeficiency from side-effects of immunosuppressive treatments. Currently, HSCT is the only curative treatment. The outcome of the disease is severe and 14% of reported patients died of their disease.

II.B.2.2- IL2RA or CD25 deficiency

IL2RA, also named CD25, encodes the alpha chain of the IL-2 receptor that is necessary for high avidity binding to IL-2. The first human LoF mutation in *IL-2RA* was described in 1997 by Sharfe et al. In keeping with the role of IL-2 in the expansion of both activated T-cells as well as of Tregs, patients combined symptoms observed in T cell deficiencies and IPEX syndrome (Verbsky and Chatila, 2013).

Duodenal histology was also similar with villous atrophy and lymphocytic infiltrate, and serum auto-reactive antibodies (anti-Harmonin, anti-enterocytes, unpublished) were present. Among extra-digestive symptoms, eczema was the most frequent (90%) followed by autoimmune cytopenia and allergies (50%), and diabetes (25%) (Bezrodnik et al., 2014; Caudy et al., 2007; Goudy et al., 2013). Bezrodnik et al reported a female patient with inflammatory lung disease (Bezrodnik et al., 2014). Patients could also display hyper IgE. Roifmann et al showed that autoimmunity could be at least partially ascribed to defective apoptosis of autoreactive clones in the thymus (Roifman, 2000). Later, Caudy et al reported lesser IL-10 production by CD4⁺ T-cells compared to *FOXP3* mutated patients (Caudy et al., 2007). Goudy et al further observed that phosphorylation of STAT5 downstream IL-2 signaling was possible in CD4⁺ T cells from CD25 null patient upon stimulation with high doses of exogenous IL-2 but was always lower than in healthy controls (Goudy et al., 2013).

Yet, IL-2RA deficiency needs to be considered as a severe T cell immunodeficiency as attested by the frequent and severe viral, bacterial or fungal infections (Verbsky and Chatila, 2013). Patients displayed T-cell lymphopenia but normal B-cell phenotype (Roifman, 2000). Lower responses to antigens *in vivo* and *in vitro* contrasted with an increase expression of T-cells activation markers as well as higher cytokines production (IL-6, IL-1 β , IL-12p70, IL-2, IFN γ , IL-4, IL-5, IL-10, IL-17, and TNF α) (Goudy et al., 2013).

Mice defective for IL-2 itself or either α or β chain of its receptor have been extensively studied as models for autoimmunity. Tregs impairment and autoimmunity were comparable to those observed in *Foxp3*^{-/-} mice. Surprisingly, in contrast with humans, they could mount effective immune responses when challenged with infectious agents and superantigens (as reviewed in (Malek, 2008)).

II.B.2.3- LRBA and CTLA4

Common variable immunodeficiency (CVID) was described in 2008 as the most frequent type of primary immunodeficiency, as it may affect up to 1/50,000 inhabitants (Park et al., 2008), with a mean peak of onset around early and mid-adulthood. It is defined by hypogammaglobinemia (low IgG-IgA levels, and low IgM levels in 50% cases), poor antibody response, and higher frequency of infections. Infections have a broad spectrum, and the most common affect the respiratory tract, ear, and sinuses. Autoimmunity is present in 25% of patients and may be the leading symptom. Up to 50% of patients display chronic diarrhea that can be ascribed to *Giardia* enteritis, celiac disease

or IBD phenotype with Crohn-like features (Alangari et al., 2012; Park et al., 2008). It is increasingly clear that the clinical presentation of CVID recovers a large spectrum of distinct molecular defects.

In 2012, Lopez-Herrera and colleagues identified *LRBA* (lipopolysaccharide responsive beige-like anchor protein) as a new gene responsible for CVID and autoimmunity. Since then, multiple reports have contributed to establish the vast heterogeneity of the LRBA phenotype (Alangari et al., 2012; Burns et al., 2012; Charbonnier et al., 2015; Lopez-Herrera et al., 2012) from a mild hypogammaglobinemia to severe IPEX-like presentation. Moreover, age of onset spreads out from early childhood to mid-adulthood. *LRBA* mutations may be difficult to identify by Sanger sequencing since the gene encompasses 58 exons and spans more than 750,000 bp of genomic DNA (Alangari et al., 2012). Most reported patients have no protein expression (Serwas et al., 2015). LRBA deficiency was initially associated to defective B-cell activation and autophagy, and increased susceptibility of B cell to apoptosis (Lopez-Herrera et al., 2012). Charbonnier et al next demonstrated decreased numbers of Tregs, with low expression of FOXP3, CD25, Helios and CTLA4, reduced suppressive function, and increased Tregs apoptosis that could explain at least some of the autoimmune features. *In vitro* induction of peripheral Tregs was however similar to that observed in cells from healthy controls (Charbonnier et al., 2015).

Even though LRBA is expressed in many cell types, its high expression in immune cells may explain the dominant immune phenotype and the beneficial effect of HSCT (Seidel et al., 2015). Interestingly, new insight into LRBA function may offer novel and less aggressive therapeutic options. Thus, Lo et al recently showed that LRBA deficiency resulted in increased turnover and degradation of CTLA4 in the lysosomal compartment of both Treg and T effector cells. Supporting a key role of LRBA via modulation of CTLA4 expression, they observed that LRBA deficient patients were markedly improved by Abatacept, a CTLA4 agonist. Moreover, *in vitro* data demonstrated significant upregulation of CTLA4 upon lysosome inhibition by chloroquine (Lo et al., 2015), a 50 year-old drug largely used in the past against malaria and which can also improve some patients affected by SLE (Lee et al., 2011).

Ctla4 germ-line deletion was first described in mice in 1995. *Ctla4*^{-/-} mice displayed fatal proliferation of T cell blasts that infiltrated all organs (spleen, lymph nodes, liver, heart, lung, pancreas), a phenotype reminiscent of the human monogenic disease identified 20 years later (Waterhouse et al., 1995). Selective deletion of *Ctla4* in Tregs impaired *in vivo* and *in vitro* suppressive function of Tregs and also resulted in spontaneous development of systemic lymphoproliferation, fatal T cell-mediated autoimmune disease, and hyperproduction of IgE in mice

(Wing et al., 2008). Of note, heterozygous deletion of *Ctla4* did not induce any specific phenotype in mice.

CTLA4 haploinsufficiency was almost simultaneously reported in humans by two independent studies. Clinical presentation was heterogeneous with features of CVID, autoimmunity, dysregulation of Tregs, progressive loss of B cells associated with an increase in autoreactive B cells and infiltration by immune cells of non-lymphoid organs. It is important to note that Tregs numbers were normal, but their functions (suppression of T effector proliferation, CTLA4 binding, CD80 endocytosis) were impaired. As in *LRBA* mutated patients, age of onset was variable from infancy to 40 years. CTLA4 haploinsufficiency has also an incomplete penetrance, as reported by Schubert et al who identified 8 unaffected adults. These healthy carriers did not display any symptom but their Tregs showed reduced expression of CTLA4 as in symptomatic patients (Kuehn et al., 2014; Schubert et al., 2014). In keeping with the fact that CTLA4 expression is only reduced, symptomatic patients could be improved by CTLA4 agonist (Lo et al., 2015).

Identification of *LRBA* or *CTLA4* deficiencies in a subset of CVID patients illustrate how molecular dissection of complex clinical syndromes can help to design rationale based tailor-made treatments.

III. OVERLAPPING SYNDROMES: LESSONS FROM MUTATIONS IN *TTC7A* AND TRICHO-HEPATO- ENTERIC SYNDROME

III.A- *TTC7A*

TTC7A (tetratricopeptide repeat domain 7A) was incriminated first in patients suffering from multiple severe atresia (MIA) and combined immunodeficiency (CID) (Bigorgne et al., 2014; Chen et al., 2013; Samuels et al., 2013). Of note, Samuels et al noticed that only some of their MIA patients displayed immunodeficiency.

Patients with MIA-CID linked to *TTC7A* mutations displayed profound lymphopenia (T, B, NK cells), low IgG, IgA, IgM serum concentrations, increased IgE level, and hypoplastic thymus. Pathology examination pointed out severe epithelial cell abnormalities in thymus and along the gastrointestinal tract. In intestine, epithelium was pseudostratified with apoptotic lesions of the glands, and with villous atrophy in the small bowel. *Lamina propria* was infiltrated by immune cells, mainly macrophages and eosinophils. These abnormalities were observed even shortly after birth. Analysis of intestine-derived organoid cultures by Bigorgne et al demonstrated inversion in cell polarity that could be reversed after inhibition of Rho kinase. Accordingly, apical expression of villin and alkaline phosphatase in intestinal biopsies were extremely low. These findings shed some light in the previously unknown function of *TTC7A*, which appears to regulate Rho kinase activity in order to maintain apical polarity, growth and differentiation of intestinal and thymic epithelial cells (Bigorgne et al., 2014).

Shortly after the implication of *TTC7A* in MIA-CID, two teams ascribed *TTC7A* deficiency to severe VEO-IBD. Avitzur et al identified five patients: one girl with fatal enterocolitis and immunodeficiency that started immediately after birth but without intestinal atresia, two siblings with diarrhea and VEO-IBD without atresia nor immunodeficiency, as well as two siblings with MIA-CID. These observations highlights phenotypic heterogeneity in *TTC7A* deficiency. VEO-IBD, a slightly milder phenotype as compared to MIA-CID, was ascribed to hypomorphic mutations with partial protein expression. The authors showed that *TTC7A* interacts with phosphatidylinositol 4-kinase III α (PI4KIII α) which is highly expressed at the cell surface of IEC and immune cells. Loss of *TTC7A* resulted in mislocalization of PI4KIII α and reduced amount of

phosphatidylinositol-4-phosphate (Avitzur et al., 2014). Interestingly, induced KO of *Pi4kca* (analog of PI4KIII α) in mice led to fatal intestinal failure within 8-10 days with degeneration and necrosis of IEC from stomach to colon, a finding reminiscent of the histological features in *TTC7A*-mutated patients (Vaillancourt et al., 2012).

Then, Lemoine et al reported patients with equivalent VEO-IBD phenotype, except for the addition of alopecia, onychopathy, and autoimmunity (autoimmune hepatitis, hemolytic anemia, psoriasis, type 1 diabetes, and thyroiditis) (Lemoine et al., 2014). Remarkably, *Ttc7^{-/-}* in mice (also known as flaky skin model) results in severe psoriasis-like dermatitis with anemia, hyper IgE and lupus-like anti-dsDNA auto-antibodies (Helms et al., 2005).

Some patients underwent HSCT. Chen and Samuels patients experienced severe recurrence of MIA post transplantation (Chen et al., 2013; Lemoine et al., 2014; Samuels et al., 2013). As well, severe VEO-IBD in Lemoine's F5 patient was not resolved by HSCT despite full donor chimerism and the absence of skin or liver graft-versus-host manifestations. Recently, post-HSCT outcome of four additional patients was reported. All survived with a correction of their immunodeficiency but not of their intestinal disease (Kammermeier et al., 2016b). As demonstrated by these studies, *TTC7A* deficiency leads to a severe disease in both hematological and epithelial compartments. Therefore, even though the immunodeficiency could be at the forefront, HSCT should not be recommended in these patients, or with extreme caution.

III.B- THES and *POLA1*: implications of RNA/DNA metabolism

Syndromic diarrhea/Trichohepatoenteric syndrome (THES) is an autosomal-recessive monogenic enteropathy with a wide range of symptoms. Most emblematic symptoms include early and intractable diarrhea, hair abnormalities, and immunodeficiency. Its incidence is estimated at 1 in 400,000 –500,000 live births (Hartley et al., 2010).

THES phenotype is heterogeneous between patients, and can also evolve with age. Patients were often born with marked growth retardation (80% patients), and developed intractable, watery diarrhea within the first weeks or months of life. Of note, diarrhea does not start immediately at birth contrary to MVID or CTE. Patients required long-term parenteral nutrition but some could be weaned, as evidenced by Fabre et al who observed that 10% patients could be weaned at 5 years and 40% at 10 years in their French survey. Hepatic lesions involving hepatomegaly, fibrosis, siderosis, or even cirrhosis, were found in half of the patients. It was difficult to know whether they were related to parenteral nutrition or an intrinsic symptom of the disease (Fabre et al., 2014).

Histological features of intestinal biopsies were completely aspecific and did not correlate with diarrhea severity. Normal mucosa, mild villus atrophy or subtotal villus atrophy is found in 25%, 50% and 25% of patients respectively. Hartley et al reported reduced expression or mislocalization of apical ion transporters. Moreover, VEO-IBD phenotype with predominant colitis was reported in some patients. Thus, Busoni and colleagues recently described six patients with THES and VEO-IBD. Every patients had pancolitis, associated with ileitis (n=1), panenteritis (n=2), or perianal disease (n=2). They poorly responded to IBD therapeutics, including mesalazine, steroids, immunomodulators, and biological therapy (Busoni et al., 2016; Fabre et al., 2014; Hartley et al., 2010).

Hair abnormalities are the second hallmark of THES. Patients displayed woolly, coarse, sparse and easily removable hair, with *trichorrhexis nodosa* (71%). Facial dysmorphia included hypertelorism, broad flat nasal bridge, and prominent forehead. Skin abnormalities (50%) consisted of xerosis, 'café-au-lait' spots and angioma. Approximately 60% patients presented a mild retardation, with normal brain magnetic resonance imaging when performed (Fabre et al., 2014; Hartley et al., 2010).

The third hallmark of THES is immunodeficiency. Similar to intractable diarrhea and hair abnormalities, all patients displayed immunological defects but their severity was variable. The most frequent immunological trait was Ig deficiency. In the French survey, 9 patients required Ig supplementation, and only 4 could be weaned between 4 to 17 years. Consequently, patients displayed frequent severe infections, sometimes fatal, and poor response to vaccinations. Remarkably, hemophagocytic syndrome was reported in 60% patients of the French cohort. Some patients also exhibited platelets abnormalities without hemorrhagic symptom (Fabre et al., 2014; Hartley et al., 2010).

Other symptoms were less frequent: cardiac malformations (tetralogy of Fallot, ventricular and atrial septal defects), inguinal and umbilical hernia, delayed puberty, tooth abnormalities (narrow pointed teeth and dysplasia), congenital glaucoma, canalicular stenosis, hypothyroidism, chronic regenerative hemolysis without anemia, inadequate response to insulin with hyperinsulinism, or even delirious episodes (Fabre et al., 2014; Hartley et al., 2010).

Molecular basis of THES is known since 2010 and 2012 with the implication of *TTC37* and *SKIV2L* respectively (Fabre et al., 2011; Hartley et al., 2010).

TTC37 (tetratricopeptide repeat domain 37, also known as thespin) is abundantly expressed in vascular tissues, lymph node, pituitary, lung, and intestine, but absent in liver (Fabre et al., 2011). *TTC37* is the human ortholog of *Ski3p* in yeast that has been involved in RNA decay. Destruction of RNA is performed either by the exonuclease XRN1 for RNA in the 5'-to-3' direction or by the exosome multiprotein complex for RNA in the 3'-to-5' direction. Ski proteins are involved in the exosome-mediated mRNA decay. In six patients with THES and wild type sequence of *TTC37*,

Fabre and colleagues sequenced another co-factor of the Ski-complex called *SKIV2L*, and were able to identify recessive mutations in all of them. Interestingly, patients with *TTC37* or *SKIV2L* mutations share a similar phenotype (Fabre et al., 2012).

The essential role of *TTC37* or *SKIV2L* for the maintenance of RNA homeostasis could explain the abundance of symptoms in THES. Moreover, RNA decay is equally essential to anti-viral immunity. Anti-viral immunity, triggered by recognition of viral nucleic acids by innate immune sensors, leads to activation of type 1 IFN pathway as well as RNA decay. But, like any immune response, it has to be kept in check to avoid inappropriate activation by endogenous nucleic acids. Mutations in genes involved in nucleic acids sensing and metabolism can lead to immunodeficiency (*TRIF*, *STAT2*, *TLR3*, *UNC-93B* for instance) as well as autoinflammatory syndromes due to spontaneous hyperactivation of the type 1 IFN pathway (Starokadomskyy et al., 2016). The archetype of this recently identified group of dysimmune disorders (often called “interferonopathies”) is the Aicardi-Goutières syndrome (AGS), a rare genetic disorder that affects the brain, skin and other organs. Approximately half of AGS patients carry mutations in genes encoding one of the three subunits of the RNase H2 complex (Crow and Manel, 2015). Moreover, some severe familial cases of systemic inflammatory and autoimmune conditions including lupus have been ascribed to dominant gain-of-function mutation in *TMEM173*, which encodes stimulator of type 1 IFN gene (STING), a key signaling molecule in cytosolic DNA-sensing pathways (Jeremiah et al., 2014). Recently, Eckard et al investigated *SKIV2L* function in mice and in humans. They found that *SKIV2L* limited activation of RNA innate sensors. Loss of *SKIV2L* led to excessive production of type 1 IFN after UPR stress *in vitro*. Likewise, patients with *SKIV2L* deficiency exhibited a type-1 IFN signature (upregulation of type-1 IFN related genes). However such signature was not observed in *TTC37* deficient patients. These results raised several questions. First, do *TTC37* and *SKIV2L* contribute to the RNA decay in a similar fashion? Second, was activation of the type-1 IFN pathway relevant in the THES pathogenesis? Lastly, as high IFN signature has been linked to autoimmunity in humans, why *SKIV2L* mutated patients did not exhibit autoimmunity? The authors suggested that, as *SKIV2L* deficient lymphocytes exhibited lower survival rate after stimulation, some lymphocyte dysfunction could prevent autoimmunity development (Eckard et al., 2014).

Last year, another monogenic disease has been linked to nucleic acid metabolism pathway. The authors showed that mutations in *POLA1* gene was the cause of X-linked reticulate pigmentary disorder (XLPDR), a primary immunodeficiency with recurrent infections and sterile inflammation in various organs. *POLA1* encodes for the catalytic subunit of DNA polymerase α which participates to the Pol α -primase complex. This complex synthesizes RNA:DNA primers, which are essential

for DNA replication and cell survival. XLPDR starts within the first few months of life with development of recurrent pneumonias, bronchiectasis, chronic diarrhea, and failure to thrive. This immune deficiency is associated with a broad range of symptoms, including diffuse skin hyperpigmentation with distinctive reticulate pattern evident by early childhood, hypohidrosis, corneal inflammation and scarring, enterocolitis that resembles IBD, and recurrent urethral strictures. Skin lesions and hypohidrosis are reminiscent of EDA-ID caused by NEMO mutations. By performing whole genome sequencing in four unrelated probands, the authors identified a shared A-to-G variant in an intron of *POLA1* located in the XLPDR-linkage region. Furthermore, DNA samples from other unrelated probands harbored the same A>G variant. The authors showed that the mutation led to a decrease in *POLA1* mRNA and protein expression. This finding was established in fibroblasts and lymphoblastoid cell lines from patients, although these cell lines did not display cell replication abnormalities. The authors also showed that the A>G intronic mutation created a new splicing site that resulted in a novel transcript containing a novel exon (called 13a by the authors, cf figure). Since low concentration of normal protein was detectable, the authors concluded to hypomorphic *POLA1* mutation.

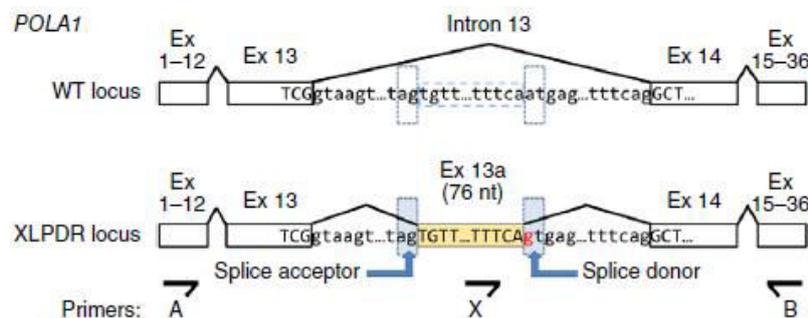


Figure 16, from (Starokadomskyy et al., 2016)- Intron 13 and flanking exons (Ex) of wild-type *POLA1* (top) and XLPDR *POLA1* (bottom), showing the mutated residue (red), the location of the aberrantly spliced exon (yellow; exon 13a) and primers (A, X, B) used for RT-PCR

Patients exhibited recurrent infections of lungs and respiratory tract with pyogenic bacteria as well as atypical mycobacteria, but no other type of invasive infections. Immunological work-up did not reveal gross abnormalities, apart from suppressed production of IL-17A, perhaps explaining their susceptibility to pyogenic infections, and reduced production of IFN- γ , likely promoting mycobacterial infections.

By comparing transcriptomic profiles, the authors could demonstrate strong upregulation of IFN pathway-related genes in XLPDR patients. They showed that, even in baseline conditions, *POLA1* deficient patients' fibroblasts, or HeLa cells depleted in *POLA1* by siRNA, displayed robust phosphorylation of TBK1 (for TANK Binding Kinase 1), and increased phosphorylation of p65

ascribed to increased phosphorylation and activation of both I κ K1 and I κ K2. Blockade of TBK1 with a specific kinase inhibitor was sufficient to prevent the IFN signature. As expected, patients exhibited a strong IFN transcriptional signature in whole blood. The authors ascribed autoinflammatory manifestations affecting gut, cornea, and urinary tract to chronic production of type 1 IFN. In XLPDR fibroblasts, POLA1 deficiency led to decreased level of cytosolic RNA:DNA hybrids. Introduction of RNA:DNA purified from cytosol into cells silenced for *POLA1* was able to normalize IRF target genes expression. Taken together, the authors concluded that reduced expression of POLA1 led to constitutive activation of IRF- and NF- κ B-dependent genes, which resulted in a strong type-1 IFN response (Starokadomskyy et al., 2016).

Even though molecular bases and pathogenesis of THES or *POLA1* deficiency are only partially unraveled, they point out possible contribution to intestinal epithelium homeostasis of pathways rarely implicated such as the one controlling nucleic acids metabolism. Furthermore, *POLA1* mutation discovery highlights whole genome sequencing utility to elucidate the genetic basis of rare diseases, since this mutation had previously escaped detection because of its intronic position.

IN CONCLUSION:

The first part of the manuscript describes key pathways involved in human intestinal homeostasis through the prism of human intestinal diseases, and more particularly of monogenic diseases with known molecular bases. In addition, selected mouse models and experimental studies have been discussed when useful to gain mechanical insight. Although not exhaustive, this description highlights how mutual interactions between the intestinal epithelium and its associated immune system participate in building and maintaining a healthy intestinal barrier. Multiple molecules and pathways are engaged into a dynamic and finely-tuned dialogue. This dialogue is essential to allow hosts to cope with the billions of microbes present in the intestinal lumen, while simultaneously remaining tolerant to the broad spectrum of dietary and self-antigens present locally. As discussed, interactions with the microbiota drive and maintain, all along life, a low-level of physiological inflammation, which is indispensable to build an efficient gut barrier able to segregate symbiotic microbes and pathogens into the intestinal lumen. Conversely, multiple immunoregulatory mechanisms cooperate to keep inflammation in check and prevent onset of tissue damage. Even if the system is built for resilience, inadvertent changes in host environment and/or genetics can tip the scales toward pathogenic inflammation. In the second part of the manuscript, I will describe my efforts to further dissect key pathways involve in human intestinal homeostasis, through the building of a cohort of patients with putative Mendelian intestinal disease and their investigation by functional and genetic studies.

RESULTS

The main objective of my thesis was to characterize phenotypically and genetically an important cohort of patients suffering from severe and early-onset intestinal disease putatively due to Mendelian disorders. Therefore, I invested myself in the recruitment of patients through the French research protocol called IMMUNOBIOTA and through the European GENIUS group from ESPGHAN (European Society of Pediatric Gastroenterology, Hepatology, And Nutrition). As these patients are extremely rare, I designed a dedicated database in collaboration with Nicolas Garcelon, bioinformatics engineer specialized in database and datamining, to optimize the patients' phenotypic characterization. The characterization of the current cohort is described in the first RESULTS section.

At the beginning of my PhD training, some inflammatory monogenic enteropathies were already well-known, mainly IL-10 signaling pathway defects, IPEX syndromes due to *FOXP3* or *IL2RA* mutations, and XIAP deficiency. Thus, patients in the cohort were functionally screened for these three etiologies, which led me to help identify the molecular defects in some patients as well as described two novel mutations in the *IL-10R β* gene (see related article in the second RESULTS section). Patients whose functional tests were negative were then selected based on phenotypic description to be further investigated with whole exome sequencing (WES). This next-generation sequencing technique was started to be used when I began my Master internship in January 2012. Meanwhile, owing to the impressive development of WES in many teams, new genes and pathways were identified in monogenic enteropathies throughout my PhD on a regularly basis. Thus, realization of WES on trio (patient and both parents) from 2014 allowed us to identify novel mutations in genes unknown when WES was launched, but unfortunately already published at the time of their identification: *TTC7A* (1 patient), *MALT1* (2 siblings, see related article in the third RESULTS section), *STAT3* gain of function (2 families, see related poster in the SUPPLEMENTARY DATA section). Given the multiplicity of genes described in monogenic enteropathies as well as their phenotypical overlaps, it was decided that I would set up a custom-made targeted gene panel sequencing (TGPS) in order to better select WES candidate patients by facilitating molecular diagnosis. Our own TGPS was thus designed in collaboration with Sylvain Hanein from the Translational Genetic platform. Its first version encompassed 68 genes in which mutations had been described to be involved in monogenic enteropathies, either with chronic intestinal inflammation or epithelial defects. From July 2015 to April 2016, the DNA of every patients in our cohort was sequenced by TGPS, allowing identification of molecular diagnoses in 28 patients in whom phenotypic characterization, functional tests and often WES had failed to do so (see related article in the last RESULTS section).

I. PHENOTYPIC CHARACTERIZATION OF PATIENTS WITHIN THE COHORT

The main goal of my thesis being the characterization of patients suffering from orphan disease, I invested myself in their recruitment as well as in building an efficient datamining tool to optimize their characterization.

At the beginning of my Master internship (January 2012), the laboratory cohort had gathered 57 patients included since 2009. Today, at the end of my thesis, the cohort gathers approximately 260 patients with 50 new patients included per year (see figure 17).

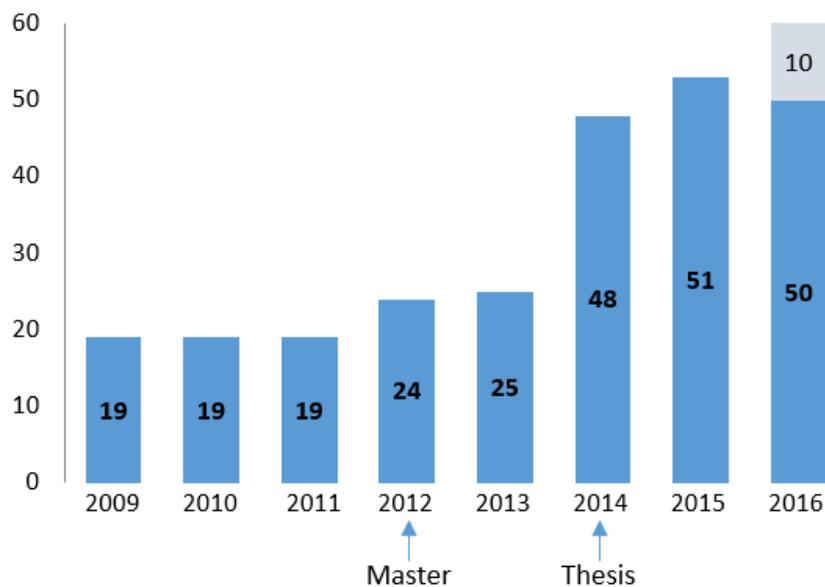


Figure 17. Patient recruitment. Fifty patients were included at the end of August 2016. Based on our experience from the previous years, we may expect to include approximately ten new patients until the end of 2016 (these predicted inclusions are in light blue).

Patients were either included through the French research protocol called IMMUNOBIOTA (initiated in March 2014 with the support of the Advanced ERC grant IMMUNOBIOTA obtained by N. Cerf-Bensussan) or through the European GENIUS group (for GENetically and/or ImmUne mediated enteropathieS) from ESPGHAN (European Society of Pediatric Gastroenterology, Hepatology, And Nutrition) initiated in 2009 by F. Ruemmele. IMMUNOBIOTA is a multicentric

protocol which gathers currently 12 centers (the opening of a thirteen center in an adult hospital is presently being discussed) allowing to study patients for ten years with a five years inclusion period.

To optimize the phenotypic description of patients, I designed a dedicated database in collaboration with Nicolas Garcelon, bioinformatics engineer specialized in database and datamining. The IMMUNOBiota protocol allowed using this secured database since its opening in March 2014. The IMMUNOBiota database thus contains a number sufficient of patients' medical reports to allow extraction of significant information. Since the GENIUS database only obtained French regulatory and ethical approvals in December 2015, it does not contain enough data even if presently directly accessible online (www.database.genius-group.org) or via the GENIUS group website (www.genius-group.org). However, we infer that patients' description from the GENIUS database will not differ much from the IMMUNOBiota one. Nonetheless, the main characteristics of the entire cohort, are described in the article on Targeted Gene panel sequencing (TGPS) (see RESULTS section 4, in Material and Methods subsection).

I.A- Description of the IMMUNOBiota Cohort

The clinical form that I have designed to collect medical data comprises approximately 150 items. Herein only the main characteristics of the IMMUNOBiota patients are summarized. From April 2014 to August 2016, 274 subjects were included, including 84 patients and 190 healthy relatives (parents and siblings). Among patients, 45% were girls. Among the 76 included families, 12 had parents who shared some consanguinity. The median age of disease onset was 1 year, with an interquartile range (IQR) of 0.25-4 years. All patients displayed gastrointestinal symptoms (bloody or watery chronic diarrhea, abdominal pain, failure to thrive) and many exhibited a wide range of extra-intestinal symptoms, among which: allergy (against food allergens: 5 patients, respiratory allergens: 4, and skin allergy: 4), autoimmunity (diabetes mellitus: 1; arthritis: 2; autoimmune hemolytic anemia: 3; autoimmune thrombopenia: 4; uveitis: 1), skin diseases (eczema: 14; folliculitis: 3; psoriasis: 4), and neurological developmental delay (6 patients). Endoscopies ascertained inflammatory lesions in stomach, duodenum, ileum and colon in 46, 52, 50, and 56 patients respectively. Eleven patients displayed perianal involvement. The following histological features were observed at least once in patients: granuloma (38% of patients), villus atrophy (53%), inflammatory infiltrate (granulocytes 57%, eosinophils 56%, and monocytes 54%), ulcerations (54%), and crypt abscesses (54%). Dosages of serum Ig showed low concentrations of IgG, IgA, and IgM in 10, 7, and 6 patients respectively. Two patients displayed global hypogammaglobinemia. Twelve patients had high serum IgE. Patients displayed a wide range of

autoantibodies (see Table 3). Regarding the treatments, enteral nutrition was required at least once in 30 patients, and parenteral nutrition in 28. Medical drugs are summarized in Table 4. Of note, 14 patients were described as steroid-dependent, and anti-TNF α biotherapies were considered to give only partial or no effect in 12 patients out of 30 receiving Infliximab (7 with partial effect), and in 8 patients out of 15 receiving Adalimumab (5 with partial effect). Surgery was performed on ileum, colon or perianal region in 7, 4, and 5 patients respectively.

Auto-Antibodies	Nb of patients
ANCA	46
ASCA	42
ANA	26
Anti Harmonin (ex- AIE 75kd)	26
Anti-Enterocytes	18
Anti-DNA	14
Anti-LKM1	14
Anti-Mitochondria	11
ASMA	10
Anti-TG	10
Anti-Islets of Langerhans	8
Anti-Colonocytes	6
Anti-TPO	5

Table 3. Summary of main auto-antibodies found in IMMUNOBIOTA patients. Nb: Number; ANCA: anti-Neutrophil Cytoplasmic Antibody; ASCA: anti-*Saccharomyces Cerevisiae* Antibody; ANA: anti-Nuclear Antibody; LKM1: liver kidney microsomal type 1; ASMA: anti-Smooth Muscle Antibody; TG: Thyroglobulin; TPO: Thyroperoxidase

Anti-Inflammatory		Nb of patients
	Mesalamine	20
	Salazopyridine	2
Immunosuppressors		
	Steroids	39
	Thiopurin	37
	Methotrexate	8
	Tacrolimus	8
	Sirolimus	3
Biotherapy		
	Infliximab	30
	Adalimumab	15
	Vedolizumab	4
	Ustekinumab	3

Table 4. Summary of main medical treatments used in IMMUNOBIOTA patients. Nb: number

I.B- Molecular characterization of the cohort

When I started in January 2012, 22 patients had a molecular diagnosis, which had been obtained by Sanger sequencing following functional tests selected on the basis of patients' phenotype. At the end my thesis, through functional tests, WES and/or TGPS, 70 patients (among them 11 are included in the IMMUNOBIOTA study) have now obtained a molecular diagnosis (see Table 5).

Diagnosis made...	Before 2012	Following functional tests only	Through WES	Through TGPS
FOXP3	11	2	(1)	4
IL2RA	2		(1)	
IL-10Rα and β	6	5	(1)	
XIAP	3	4	(1)	3
ICOS			1	
LRBA			2	3
TTC7A (2)	1 (2')		1	1
MALT1 (2)			2	
STAT3 GoF (2)			4	
MYO5B			1 (3)	1 (3')
TTC37 (4)				1
SKIV2L (4)				3
EPCAM				1
SI				1
NEUROG3				1
NCF1				2
NLRC4				1
STAT1				1
CTLA4				2

Table 5. Molecular diagnoses of the patients (until January 2016). WES: whole exome sequencing; TGPS: targeted gene panel sequencing; (1): patients sequenced by WES were functionally screened before sequencing; (2): these genes were not described in monogenic enteropathies when the WES was launched; (2'): molecular diagnosis obtained by Centre d'Etudes des Déficits Immunitaires, Hôpital Necker-Enfants Malades; (3): MVID possibility had been discarded after normal electron microscopy; (3'): PAS had not been tested due to clear inflammatory lesions on the biopsies; (4): patients displayed the typical phenotype, TGPS was used to fasten molecular diagnosis.

But, at the time of redaction of the manuscript, we are waiting for the results of the TGPS launched at the beginning of August 2016 in 50 patients included since January 2016. Graphs in Figure 18 indicate molecular diagnoses in the cohort recruited until January 2016 (i. e. all patients who were screened by TGPS or who had obtained a molecular diagnosis before TGPS).

When TGPS does not lead to identification of molecular diagnosis, selected patients are further investigated by WES performed at least in trio. WES analyses allowed us to select one candidate

gene likely disease causing in four distinct families. The selected mutations are currently under functional validation (see CONCLUSION and PERSPECTIVES section).

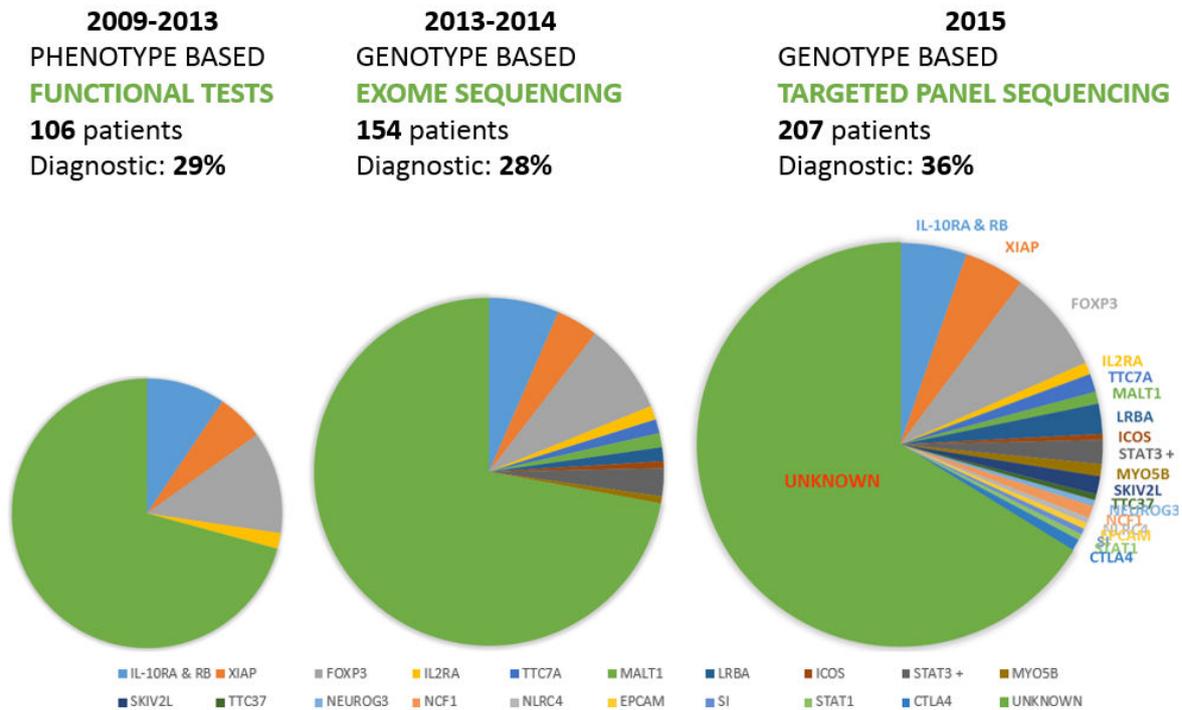


Figure 18. Graphs showing molecular diagnoses obtained in the patients recruited until January 2016.

II. FIRST IDENTIFICATION OF A MUTATION WITH A FOUNDER EFFECT IN *INTERLEUKIN- 10 RECEPTOR 2 GENE*

MANUSCRIPT IN PREPARATION

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The authors declare no conflict of interest.

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AUTHORS CONTRIBUTION

FCH, FR and NCB designed the study; BN, IL, JAD AND NG were in charge of the patients and acquired clinical data; FCH, BB, AS, FRL, MCS, CP, SP AND ZL performed experiments; FCH, and NCB wrote the manuscript that was reviewed by all authors.

FOR MORE INFORMATION

<http://www.institutimagine.org/en/research/23-research-labs/119-laboratory-of-intestinal-immunity.html>

www.genius-group.org

ABSTRACT: 248 words

Objective: Herein, we aimed at describing two novel mutations of the *IL10R2* gene in three unrelated patients of Portuguese origin who displayed very early onset colitis.

Methods: Response to interleukin 10 (IL-10) was determined in peripheral blood mononuclear cells (PBMC) stimulated by lipopolysaccharide by measuring inhibition of interleukin 8 secretion by ELISA. Sequences of genomic and complementary DNA of *IL-10R1* (encoding IL-10R α chain) and *IL-10R2* (encoding IL-10R β chain) and microsatellite analysis of *IL-10R2* gene region were performed by Sanger method. IL-10 receptor expression and STAT3 phosphorylation were studied by flow cytometry in PBMC. Phosphorylation of Tyk2 and JAK1 kinases was analyzed by western blot in Epstein-Barr virus immortalized B cell lines.

Results: All patients showed defective response of PBMC to IL-10. Genomic DNA sequencing revealed a large *IL10R2* deletion spanning exon 3, which was homozygous in patients 1 and 2 but heterozygous in patient 3. Comparable distribution of microsatellites in the *IL-10R2* region confirmed a founder effect. In patient 3, surface expression of IL-10 receptor was normal but there was no phosphorylation of STAT3 and of Tyk2 in response to IL-10. CDNA sequencing revealed *de novo* duplication of exon 6 resulting in frameshift and loss of the intracellular Tyk2-interacting motif encoded by exon 7.

Conclusion: This study provides the first description of an *IL-10R2* mutation with a founder effect and of an *IL10R2* mutation affecting signaling but not expression of IL-10R β . Our results also underscore the importance of functional testing to pinpoint complex genetic mutations of IL-10 receptor.

KEYWORDS

Very-early onset inflammatory bowel disease

Tyk2 kinase

Founder mutation

Intestinal immunity

What is known/ What is new:

What is known?

- Mutations in interleukin 10 receptor (IL-10R) genes are a cause of very early-onset colitis.
- All previously described mutations prevent protein expression, except one mutation in *IL-10R1*.

What is new?

- A large deletion of *IL-10R2* exon 3 is the first described mutation of IL-10 receptor with a founder effect in a specific population.
- Duplication of *IL-10R2* exon 6 is the first described mutation resulting in normal expression but defective signaling of IL-10R β chain with lack of Tyk2 activation.

INTRODUCTION

The key role of Interleukin 10 (IL-10) in intestinal mucosal homeostasis was first demonstrated in mice deficient for IL-10 or its receptor (IL-10R) (1), which developed severe spontaneous enterocolitis. This role has been confirmed in humans with the demonstration that rare cases of severe early onset inflammatory bowel disease (EO-IBD) are due to Mendelian mutations in the genes encoding the two chains of IL-10R or IL-10 itself (2, 3). All affected children display severe colitis and perianal involvement within the first months of life and are resistant to medical treatment, leading in some cases to colectomy. Yet they can be cured by hematopoietic stem cell transplantation (HSCT) (4). Early diagnosis is therefore crucial to define the most pertinent treatment, reduce morbidity and increase life expectancy. One major target of the immunoregulatory effect of IL-10 in intestine are macrophages (5, 6). Therefore, to identify IL-10R deficiency in patients, we have designed a functional test assessing the response of peripheral monocytes to the inhibitory effect of IL-10 (2). This screening method identified three Portuguese patients carrying two new mutations in the β chain of the IL-10R. One mutation consists of a large deletion of exon 3 with a founder effect that abolishes protein expression. The other mutation is a duplication of exon 6 that preserves surface expression of IL-10R β but abolishes the phosphorylation of Tyk2 and thereby the downstream STAT3-dependent signaling cascade.

METHODS

Patients

Medical files were reviewed and informed written consent was obtained for genetic and molecular studies.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll Hypaque (GE Healthcare Velizy-Villacoublay, France, density 1.077 \pm 0.001). PBMC (1 \times 10⁶ cells/mL) were stimulated with 0, 10, or 100 ng/mL lipopolysaccharide (LPS, Sigma, Saint-Quentin Fallavier, France) for 24 hours with increasing concentrations of IL-10 (0-1-10-100 ng/ml) (RD systems, Lille, France) in RPMI 1640 Glutamax supplemented with 1% non-essential amino acids, 1% sodium pyruvate, 1% hepes,

10% inactivated fetal bovine serum, 20ng/mL gentamycine (Invitrogen, Cergy Pontoise, France) and 0.005mM of β mercaptoethanol (Sigma). Supernatants of LPS/IL-10-stimulated PBMC were collected after 24 hours and IL-8 cytokine production was analyzed by enzyme-linked immunosorbent assay (Human CXCL8/IL-8 Duo Set Kit, R&D systems). Epstein-Barr virus (EBV)-cell lines were derived from PBMC cultured in the same culture medium in the presence of EBV, CpG (ODN-2006 type B, CAYLA Invivogen, Toulouse, France), and cyclosporine (Sandimumun, Novartis, Basel, Suisse).

Flow cytometry

To analyze IL-10R β expression, 2.10^5 to 3.10^5 PBMC were stained at 4°C for 30 minutes with PE-labeled anti-IL-10R β antibody, or IgG1 isotype (R&D systems), and CD45-APC, -CD3-PeCy7, -CD19-horizon V450 (BD Bioscience, Rungis, France) and -CD14-FITC (Milteny, Paris) antibodies. To analyze STAT3 phosphorylation 1.10^6 PBMC were stimulated with IL-6 or IL-10 at 25ng/mL (R&D systems) and surface-stained with the same antibody cocktail. After fixation was in fix Buffer I at 37°C for 10 minutes, and permeabilization in perm Buffer III (BD Bioscience) at 4°C for 30 minutes, cells were labeled with anti-phosphorylated STAT3 (pSTAT3) antibody (BD Bioscience). Cells were analyzed on a CANTO II instrument (BD Biosciences) and with FlowJO software (TreeStar Inc, Ashland, Ore).

Genetic analysis

Genomic DNA and RNA were extracted from PBMC using the QIAmp DNA Blood Mini Kit and the RNA Extraction Mini kit respectively according to the manufacturer's protocols (Qiagen, Courtaboeuf, France). cDNA was obtained with QuantiTect Reverse Transcription Kit (QIAGEN). Each exon of *IL-10R1* and *IL-10R2* were amplified from genomic DNA by polymerase chain reaction (PCR) as described (3). *IL-10R2* exon 3 was further amplified from genomic DNA using two primers respectively located in intron 2 and intron 3 (forward: TAAACAGATGTGCCGTCCTC; reverse: TGAGATAAGACTTCACTCTGGTCA). IL-10-R2 cDNA was amplified using two primers located either in exons 2 and 5 (forward: TTCTACAGTGGGAGTCACCT; reverse: AGCTTTGTTCCGATCAG) or in exons 4 and 7 forward: CCCCTGGAATGCAAGTAGA; reverse: ACAAGGGCCAAGACCATCT). PCR products were separated by 1% agarose gel electrophoresis, purified and sequenced by Sanger technique on Genetic Analyzer 3500XL (Applied Biosystems, Foster City, USA).

Microsatellites

The microsatellites profiles enlarging *IL-10R2* were primed by PCR. The following fluorescent primers were used: D21S 262 (Forward: TCTATGAGACAGGGCCAC; Reverse: AAAAAAATATTCCGTGGTTGATTGTTGTT); D21S 1898 (Forward: GCAGGAACAC

TCAGTCTCTTCAG; Reverse: AAAAAAAGCTCCATTAACATTTTAGGCACG); D21S 1254 (Forward: AAATACTGATGATCCTTAATTTTGG; Reverse: AAAAAAAGGTG GCTG AGCGAGAC); D21S 1895 (Forward: AGTCCTACTGATAAACTGTGGGC; Reverse: AAAAAAAGTGTCTCATAAGAACCTACCTGG)

Western blot analyses

EBV-derived cells lines were stimulated with either IL-10 at 25ng/mL, IL-6 at 25ng/mL or IFN α at 100 pM for 15 minutes and protein was extracted for western blot using standard protocols. 40 μ g of lysates were separated by SDS-PAGE gel and transferred to nitrocellulose membranes, followed by incubation with JAK1 antibody (Millipore, ref 06-665), Tyk2 monoclonal antibody T10-2 (Hybridolab, Institut Pasteur), anti-Jak1-phospho-YY1022/23 (Invitrogen), anti-Tyk2-phospho-YY1054/55 (Cell Signaling Technology, Beverly, MA) as described (7).

RESULTS

Patients with defective response to IL-10

Patients 1 and 2 (P1 and P2) were born in Portugal in two distinct consanguineous families while P3 was born in France from unrelated parents of Portuguese origin. P1, P2 and P3 presented diarrhea, rectal bleeding, and perianal lesions since the age of 4 to 9 months. P1 also had severe chronic folliculitis. At diagnosis, endoscopy showed left colonic inflammation (P2) or pancolitis (P1 and P3). P1 was temporarily improved by ileostomy. This clinical phenotype was strongly suggestive of a defect in IL-10 signaling. Supporting this hypothesis, increasing concentrations of exogenous IL-10 failed to inhibit liposaccharide (LPS)-induced IL-8 production by PBMC (Fig 1A).

Identification of two novel *IL-10R2* mutations

Sequencing of *IL-10R2* on genomic DNA revealed a large homozygous deletion of 2980 bp and a 2bp insertion encompassing exon 3 (21: 33275127 to 33278107) resulting in an early-stop codon in exon 4 in all 3 patients (Fig. 1B). The deletion was homozygous in P1 and P2 but heterozygous in

P3. Thus the expected 3.5kb band encompassing exon 3 and the flanking intronic regions were replaced in P1 and P2 by a 0.5 kb band carrying the deletion while P3 displayed the expected 3.5 Kb band as well as the new 0.5 Kb (Figure 1C). The parents of P1 and P2 and only the mother of P3 displayed the two bands, indicating that they were heterozygous carriers of the deletion. P3's father displayed exclusively the 3.5 Kb band (Fig. 1C and data not shown). Microsatellites analyses revealed a 4.7×10^5 bp genomic region from D21S 1898 to D21S 1254 on *IL-10R2* that was common to all individuals carrying delE3, suggesting a founder effect in the Portuguese population.

In P3, sequencing all exons of *IL-10R2* on genomic DNA failed to reveal a second mutation. Moreover flow cytometry revealed normal IL-10R β expression on PBMC. The later result however contrasted with loss of response of monocytes to IL-10 immunosuppression and with complete absence of STAT3 phosphorylation after stimulation by exogenous IL-10 (Fig. 2A) while STAT3 phosphorylation was normal after stimulation by IL-6 (not shown). Therefore *IL-10R2* cDNA was sequenced and this revealed a *de novo* mutation on the paternal allele characterized by duplication of exon 6 (dupE6, Fig. 2B). Duplication induced a frameshift at the end of the first exon 6 which precluded translation of exon 7 and instead resulted in inserting an aberrant 50 amino acid protein sequence (Fig. 2C). As a consequence, the mutated protein should comprise the wild type extracellular and transmembrane domains, followed by an intracellular domain made of the normal first 22 amino acids encoded by exon 6 (instead of 57) fused with a C-terminal aberrant peptide.

Lack of Tyk2 activation in P3

IL-10R is a complex composed of two α chains constitutively associated to Janus kinase 1 (JAK1) kinase and two β chains associated to Tyrosine kinase 2 (Tyk2). Ligand binding to the receptor complex results in the *trans*-phosphorylation of JAK1 and Tyk2, which mediate the recruitment and phosphorylation of STAT3 (8). Western blot analysis of EBV cell lines stimulated by IL-10 showed comparable phosphorylation of JAK1 in P3 and in her parents (Fig. 2D). Phosphorylation of Tyk2 could also be induced in parents' cells by IL-10 or IFN α (another known activator of Tyk2). In contrast, only IFN α but not IL-10 induced Tyk2 phosphorylation in P3 cells (Fig. 2E). Overall these results explain that IL-10 signaling is absent despite normal surface expression of the receptor.

Patients' outcome

All 3 patients have received HSCT. P1 and P2 are free of symptoms after 30 months and 15 months respectively. P3 died recently of severe graft-versus host disease (GVHD) 12 months after HSCT, despite being free of intestinal and perineal symptoms.

DISCUSSION

Herein we describe two novel loss-of function mutations in *IL-10R2* as a cause of early-onset severe colitis. Mutations in IL-10 and in the two chains of its receptor have been reported in approximately 50 patients since 2009 (9-12). In keeping with previous reports, all three patients in our study presented with severe colitis and perianal abscesses and fistula within the first months of life. Unexpectedly, they carried the same large deletion delE3 that prevented any protein expression. Suggesting a founder effect, the 3 patients were born in three unrelated Portuguese families and microsatellites analysis revealed that the deletion was located in a 4.7×10^5 bp genomic region of *IL-10R2* shared by all carriers. This large deletion has never been reported yet, and is, to the best of our knowledge, the first description of an *IL-10R* mutation shared by a particular population. Further studies will be necessary to assess the frequency of this mutation in Portugal. If frequent, it may increase the risk in the Portuguese population of IL-10R β deficiency either due to homozygous delE3 or to the combination of delE3 with a *de novo* *IL-10R2* mutation. In this respect, it is remarkable that P3, who was born in a non-consanguineous family of Portuguese origin and inherited the deletion from her mother, had a *de novo* mutation on the father's allele consisting in a duplication of exon 6 that preserved expression of the transmembrane domain but drastically modified the intracellular domain. Mutations preserving surface expression of IL-10R are uncommon and have been described only for *IL-10R1* in two patients (13, 14). DuplE6 is thus the first example of a mutation preserving IL-10R β cell surface expression but impairing downstream signals. As indicated above, IL-10R β participates in IL-10 signaling via its binding to Tyk2. This tyrosine kinase is also associated with IL-13R α , IL-12R α , gp130 and IFNAR1 (interferon alpha receptor 1). Tyk2 binds to IFNAR1 *via* its FERM (Four-point-one, Ezrin, Radixin, Moesin) homology domain and its Src Homology 2 (SH2)-like domain (15). A recent structural study has revealed the Tyk2-IFNAR1 binding interface and identified the critical residues in both partners (16). Interestingly, the linear IFNAR1 peptide is anchored to Tyk2 *via* residues that are variably conserved in the intracellular domains of the other Tyk2-interacting receptors. In IL-10R β , these amino acids are encoded by exon 7 (Fig. 3A). Consistent with a loss of Tyk2 binding site in the mutated duplE6 that lacks exon 7, IL-10 failed to induce any tyrosine phosphorylation of Tyk2 in EBV cells derived from P3.

Mendelian causes of severe intestinal inflammation are raising growing interest (17) and need to be identified as early as possible to define the best possible therapeutic option. In keeping with previous reports, two patients were successfully cured by HSCT. Digestive symptoms were also cured by HSCT in the third patient but she unfortunately succumbed from GVHD. Of note, exon 3 deletion was difficult to identify by Sanger sequencing on genomic DNA and cDNA sequencing was indispensable to detect the large duplication of exon 6. In contrast, functional analysis of IL-

10 signaling by simple tests such as inhibition of LPS-induced IL-8 production and STAT3 phosphorylation (2) were highly sensitive and specific methods to rapidly pinpoint the molecular defect. These observations underscore the notion that some Mendelian mutations, notably those involving large deletions, can be missed by targeted Sanger sequencing of exons and that functional testing, when simple and robust, remains a method of choice for screening.

In conclusion: a robust and simple functional test enabled to demonstrate loss of response to IL-10 in three patients with very early-onset colitis, leading to the identification of two novel loss-of-function mutations in *IL-10R2* (Fig. 3). One is a large deletion common to three unrelated Portuguese families suggestive of a founder effect. The second is a *de novo* duplication of exon 6, which prevents Tyk2 recruitment by IL-10R β while preserving IL-10R surface expression. Early identification of the molecular defect has been instrumental to indicate HSCT, which is currently the only definitive treatment.

ABBREVIATIONS:

deIE3	Deletion of exon 3
dupIE6	Duplication of exon 6
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
EO-IBD	Early onset inflammatory bowel disease
FERM	Four-point-one, Ezrin, Radixin, Moesin
GVHD	Graft versus host disease
HSCT	Haematopoietic stem cell transplantation
IFN α	Interferon alpha
IFNAR1	Interferon alpha receptor 1
IL-10	Interleukin 10
IL-10R	Interleukin 10 receptor
JAK1	Janus kinase 1
LPS	Lipopolysaccharide
PBMC	Peripheral blood monocyte cells
SH2	Src Homology 2
STAT3	Signal transducer and activator of transcription 3
Tyk2	Tyrosine kinase 2

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FIGURES

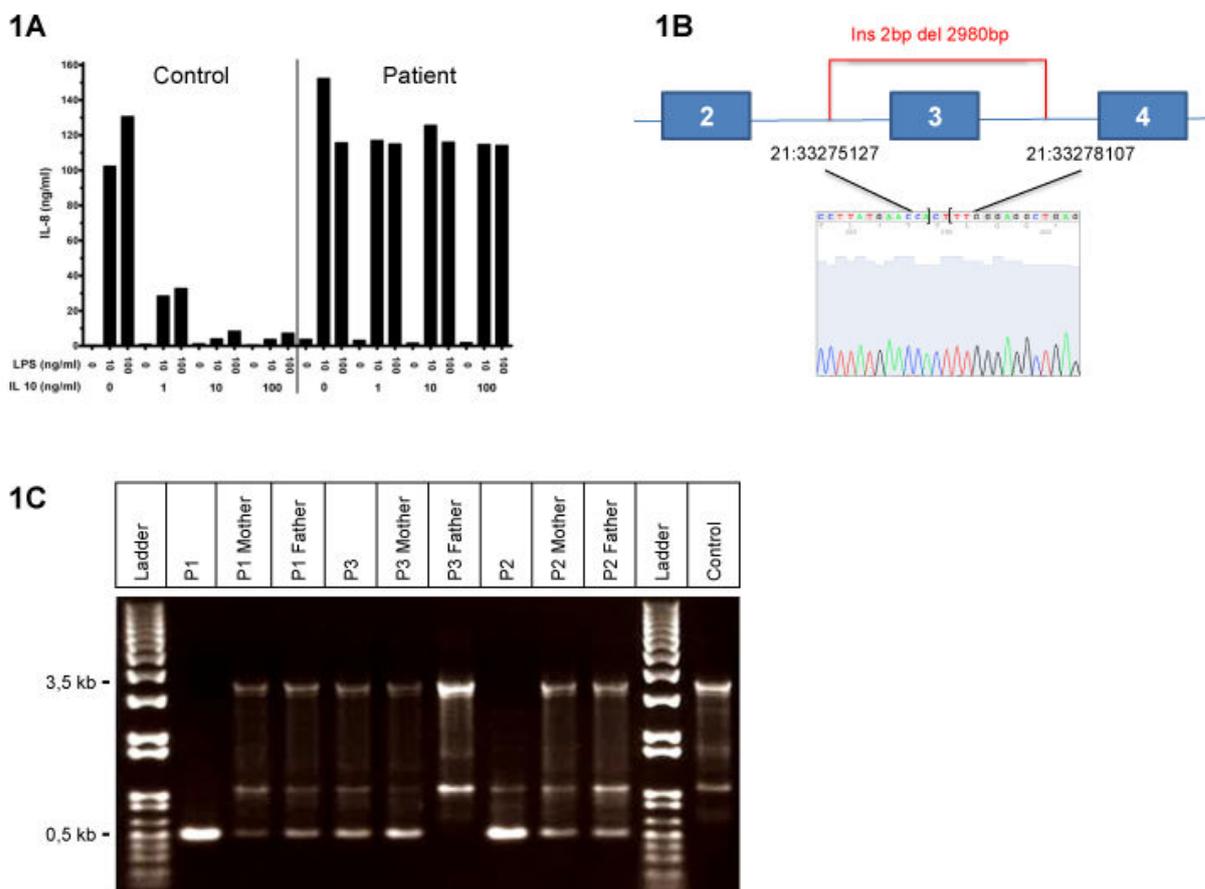


Figure 1: Patients from Portugal share a common deletion of exon 3 in *IL-10R2*

1A: Lack of responsiveness to IL-10 in PBMC stimulated with lipopolysaccharide (LPS) in one patient compared to one control. IL-8 was quantified by ELISA in supernatants after a 24 hour-stimulation at the indicated concentrations of LPS and IL-10. Results were comparable in all three patients. 1B: Scheme showing the exon 3 deletion as defined by Sanger analysis. 1C: Gel analysis of PCR products from genomic DNA with primers flanking the deletion showing the normal 3.5kb band in control, parents and P3 and a short 0.5kb mutated band in patients and parents of P1 and P2.

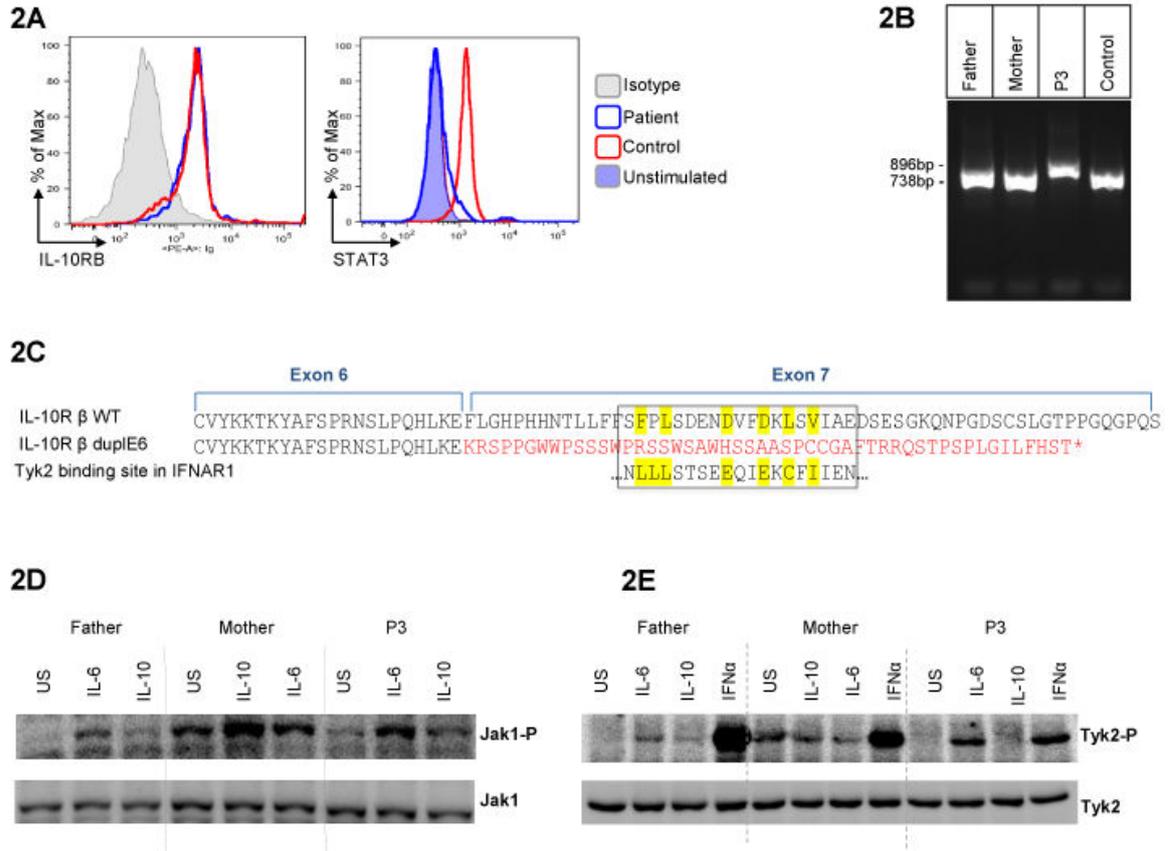


Figure 2: Duplication of *IL-10R2* exon 6 results in loss of exon 7 encoded intracellular domain and prevents Tyk2 activation

2A: Flow cytometry analysis (gated on CD14⁺ cells) showing normal cell surface expression of IL-10R β (left panel) but lack of STAT3 phosphorylation after a stimulation by IL-10 (right panel) in PBMC of P3 compared to one healthy control; 2B: Gel analysis of PCR products from complementary DNA amplified with primers flanking exon 6 showing a 896bp band in P3 instead of the 738bp band observed in the parents and healthy control. 2C: Comparison of protein sequences of normal IL-10R β and of IL-10R β with the frameshift due to the duplication of exon 6. Alignment of the Tyk2 binding site of interferon alpha receptor 1 (IFNAR1) is shown. Amino-acids predicted to be necessary for Tyk2 binding are highlighted in yellow. 2D-E: Western blot analysis of JAK1 phosphorylation after stimulation by IL-6 or IL-10 (2D) and of Tyk2 phosphorylation (2E) after stimulation by IL-6, IL-10 or IFN α in EBV cell lines from P3 compared to her parents. US: unstimulated. These gels are representative of two independent experiments.

3

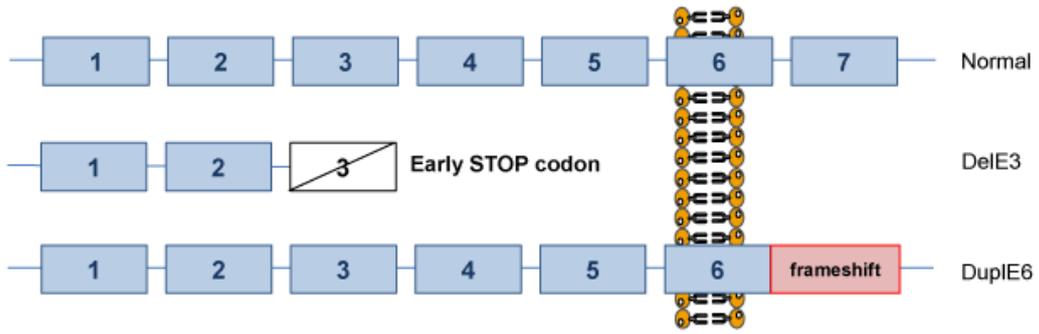


Figure 3: Schematic representation of the two novel mutations described in *IL-10R2*: the deletion of exon 3 (delE3) and the duplication of exon 6 (dupE6)

III. DEFICIENCY IN MUCOSA ASSOCIATED LYMPHOID TISSUE LYMPHOMA TRANSLOCATION 1 (MALT1): A NOVEL CAUSE OF IPEX-LIKE SYNDROME

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ORIGINAL ARTICLE: GASTROENTEROLOGY: INFLAMMATORY BOWEL DISEASE

Deficiency in Mucosa-Associated Lymphoid Tissue Lymphoma Translocation 1: A Novel Cause of IPEX-Like Syndrome

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ABSTRACT

Objective: Early-onset inflammatory bowel diseases can result from a wide spectrum of rare mendelian disorders. Early molecular diagnosis is crucial in defining treatment and in improving life expectancy. Herein we aimed at defining the mechanism of an immunodeficiency-polyendocrinopathy and enteropathy-X-linked (IPEX)-like disease combined with a severe immunodeficiency in 2 siblings born from distantly related parents.

Methods: Whole exome sequencing was performed on blood-extracted genomic DNA from the 2 affected children and their parents on the genomic platform of Institut IMAGINE. Candidate gene mutation was identified using the in-house software PolyWeb and confirmed by Sanger sequencing. Protein expression was determined by western blot. Flow cytometry was used to assess consequences of the mutation on lymphocyte phenotype and nuclear factor-kappa B (NF-κB) activation at diagnosis and after treatment by hematopoietic stem cell transplantation.

Results: We identified a homozygous missense mutation in mucosa-associated lymphoid tissue lymphoma translocation 1 gene (*MALTI*), which precluded protein expression. In keeping with the known function of *MALTI*, NF-κB-dependent lymphocyte activation was severely impaired. Moreover, there was a drastic reduction in Forkhead box P3 (FOXP3) regulatory T cells accounting for the IPEX-like phenotype.

Following identification of the mutation, both children received hematopoietic stem cell transplantation, which permitted full clinical recovery. Immunological workup at 6 and 12 months after transplantation showed normal NF-κB activation and correction of regulatory T cells frequency.

Conclusions: Along with *FOXP3*, *IL2RA*, and *CTLA-4* mutations, *MALTI* deficiency should now be considered as a possible cause of IPEX-like syndrome associated with immunodeficiency that can be cured by hematopoietic stem cell transplantation.

Key Words: auto-immune enteropathy, CBM signalosome, intestinal immunity, extremely early-onset inflammatory bowel disease

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One rare but most severe group of inflammatory bowel diseases concerns extremely young children, for whom the early definition of the most pertinent treatment is crucial to reduce morbidity and increase life expectancy. Early age of onset and disease severity point out to mendelian genetic defects.

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For more information: <http://www.institutimagine.org/en/research/23-research-labs/119-laboratory-of-intestinal-immunity.html>; www.genius-group.org

The authors report no conflicts of interest.

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What Is Known

- Immunodeficiency polyendocrinopathy and enteropathy-X-linked and IPEX-like syndromes can be caused by multiple monogenic defects.
- Mutations in mucosa-associated lymphoid tissue lymphoma translocation 1 gene (*MALT1*) have been described as a cause of severe combined immunodeficiency in 4 patients, 1 of whom improved by hematopoietic stem cell transplantation.

What Is New

- *MALT1* deficiency can lead to an IPEX-like syndrome combining autoimmune enteropathy, dermatitis, hyper immunoglobulin E in addition to a severe immune defect.
- Hematopoietic stem cell transplantation is an effective cure in *MALT1*-deficient patients, correcting both the profound immune defect and the severe autoimmunity.

Accordingly, mutations have been identified in an expanding number of genes (1). Inflammation can predominate in colon as in loss-of-function mutations in the interleukin-10 pathway or in the small intestine as in immunodeficiency polyendocrinopathy and enteropathy-X-linked (IPEX) and IPEX-like syndromes because of mutations in genes indispensable for regulatory T cells differentiation or function (2,3). Herein we used whole exome sequencing (WES) and identified a loss-of-function mutation in mucosa-associated lymphoid tissue lymphoma translocation protein 1 (*MALT1*) as the cause of an IPEX-like syndrome combining autoimmune enteropathy, dermatitis, and hyper immunoglobulin E (IgE) in 2 siblings also displaying severe immunodeficiency.

MALT1 is an intracellular protein constitutively associated with B-cell chronic lymphocytic leukemia/lymphoma 10 (BCL10). *BCL10* and *MALT1* genes were initially identified in recurring translocations that promoted constitutive activation of the canonical nuclear factor-kappa B (NF- κ B) pathway in mucosa-associated B-cell lymphomas (4). It was then shown that BCL10 and *MALT1* can bind 3 members of the caspase recruitment domain (CARD) family of adaptors, CARD9 in myeloid cells, CARD10 in epithelial cells, and CARD11 in lymphocytes and thereby form 3 complexes called CBM signalosomes, which link a large spectrum of membrane receptors to the NF- κ B pathway. The CARD9-containing signalosome is necessary for innate immune responses downstream C-type-lectin receptors and Toll-like-receptors and patients with CARD9 mutations develop severe fungal infections. The CARD11 signalosome is recruited downstream the B- (BCR) and T-cell (TCR) receptors and is indispensable for adaptive immunity. Accordingly, 2 patients with mutations in CARD11 displayed pulmonary infections with *Pneumocystis jirovecii*. Recently, loss of functions mutations in *MALT1* have also been identified in 4 children as the cause of severe combined immunodeficiency (5–7). Herein we report the case of 2 siblings with *MALT1* deficiency and discuss how this immune deficiency can cause IPEX-like syndrome despite profound impairment of lymphocyte activation. In keeping with a recent single report, we confirm that this severe disease can be cured by hematopoietic stem cell transplantation.

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METHODS**Patients**

Two siblings and their parents were studied after informed written consent was obtained for functional and genomic molecular studies. Written consent was obtained for publication of photographs.

Lymphocyte Isolation and Cell Lines

Peripheral blood cells (PBMCs) were isolated on Ficoll HyPaque Plus (GE Healthcare, Velizy-Villacoublay, France). To obtain T-cell lines, PBMC (1×10^6 cells/mL) were stimulated for 3 days with phytohemagglutinin A (PHA) (1 μ g/mL; Sigma, Saint-Quentin Fallavier, France) in RPMI 1640 Glutamax supplemented with 1% non-essential amino acids, 1% sodium pyruvate, 1% HEPES (Invitrogen, Cergy Pontoise, France), and 10% inactivated human serum AB (PAA, Les Mureaux, France). PHA-stimulated PBMCs were next cultured with 50 U/mL recombinant IL2 (R&D Systems, Lille, France) for 2 to 3 weeks. Epstein-Barr virus (EBV) immortalized B cell lines were derived from PBMCs by the Necker Center for biological resources according to a standard procedure.

Phenotyping of Regulatory T Cells and Anti-Enterocyte Antibodies

PBMCs (1×10^6) were surface stained with sCD3-BV510 (OKT3; Sony), CD4-FITC (Leu 3a+3b; BD Biosciences, Rungis, France), CD25-BV650 (MA 251, BD Biosciences), and then intracellularly stained with FoxP3-PE (PCH101; eBioscience) antibody according to manufacturer's instructions. Data were collected with a Fortessa cytometer (BD Biosciences) and analyzed with Flow Jo software (TreeStar, Ashland, OR). Detection of serum antibodies against enterocytes or against the 75 kDa harmonin antigen (8) was performed respectively by immunohistochemistry or radioimmunoassay as described (9).

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Genotypic Analysis

Genomic DNA from peripheral blood cells was isolated using the QIAamp DNA Blood Mini Kit (Qiagen, Courtaboeuf, France) according to manufacturer's instructions. WES was performed on the genomic platform of Institut IMAGINE's. Agilent SureSelect libraries were prepared from 3 μ g of genomic DNA sheared with a Covaris S2 Ultrasonicator. Exon regions were captured using the Agilent Sure Select All Exon V5 (AGILENT, Les Ulis, France) and sequenced using a HiSeq2500 next-generation sequencer (Illumina). Depth of coverage obtained for each sample was around 100 times with >98% of the exome covered at least 15-fold. Paired-end sequences were then mapped on the human genome reference (NCBI build37/hg19 version) using the Burrows-Wheeler Aligner. Downstream processing was carried out with the Genome Analysis Toolkit (GATK), SAMtools, and Picard, following documented best practices (<http://www.broadinstitute.org/gatk/guide/topic?name=best-practices>). Variant calls were made with the GATK Unified Genotyper. All variants were annotated using the in-house software (PolyWeb) developed by Paris Descartes University Bioinformatics platform as described in Fig. 1. All the annotation process was based on the 72 version of ENSEMBL database. Analysis of genome variations was made using PolyWeb. This software allows to filter variants and to eliminate irrelevant and common polymorphisms, to compare exomes of patients and relatives, to detect variations compatible with the different modes of inheritance. Variants were compared

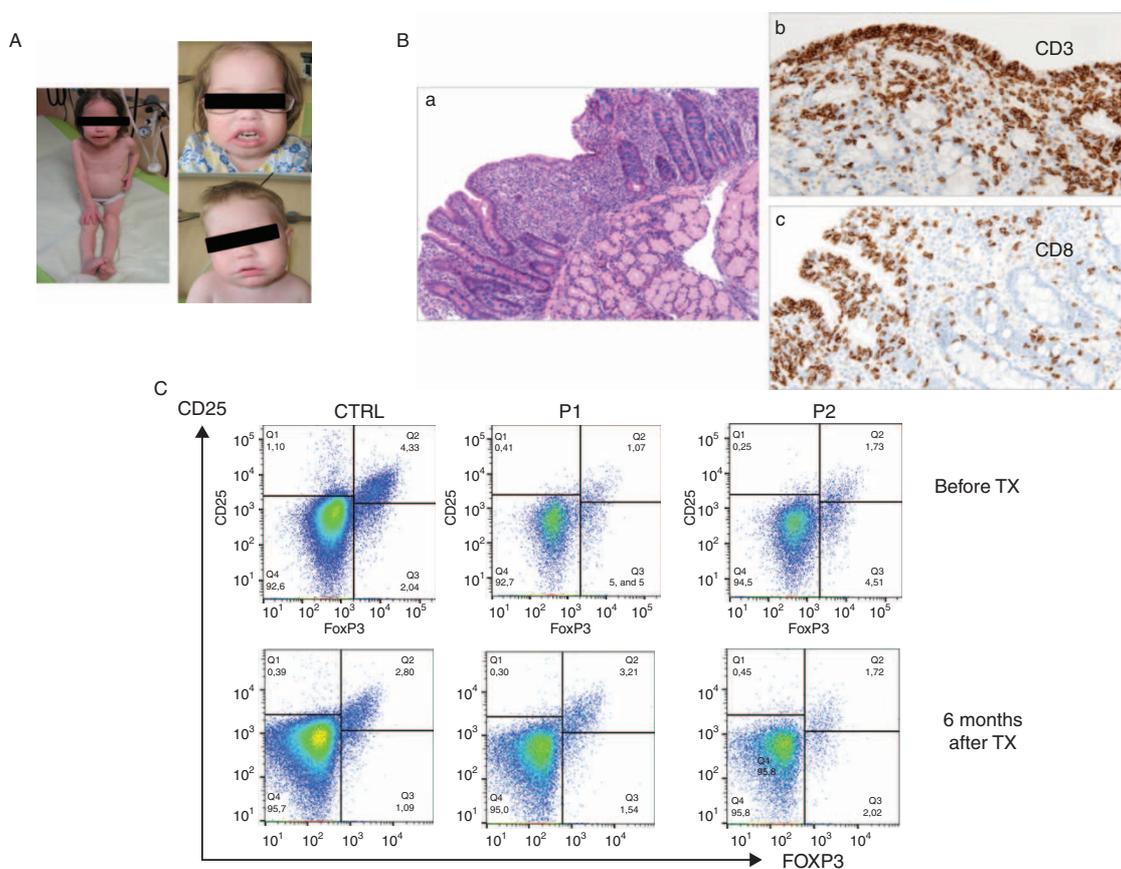


FIGURE 1. Phenotypic characterization of patients with MALT1. A, Photographs depicting failure to thrive in P1 and facial dysmorphism in P1 and P2. B, Duodenal biopsies from P1 at the time of diagnosis: (a) hematoxylin & eosin; (b) anti-CD3 staining; and (c) anti-CD8 staining. C, Multicolor flow cytometry analysis of membrane CD25 and intracellular FOXP3 in blood CD3⁺CD4⁺ T cells in P1, P2, and 1 age-matched control, before and after hematopoietic stem cell transplantation (TX). MALT1 = mucosa-associated lymphoid tissue lymphoma translocation 1 gene.

with the ones already present in US National Center for Biotechnology Information database (10) of SNP, 1000 Genome, and Exome Variant Server databases. The functional consequence of the mutation on the protein function was predicted using 3 algorithms: Polyphen2 (<http://genetics.bwh.harvard.edu/pph2/>), Sift (Sorting Intolerant From Tolerant, J. Craig Venter Institute), and Mutation Taster (www.mutationtaster.org). To confirm the mutation by Sanger sequencing, genomic DNA was amplified by standard techniques using oligonucleotide primers flanking the exon 4 of *MALT1* (forward 5'-TGGGGGAAAAATGTTGCACT-3', lower 5'-CCCCATCCA ACATTCAGCT) using TaqDNA Polymerase (Life Technologies, Saint-Aubin, France). After purification with the QIAquick PCR Purification kit (Qiagen), PCR fragments were sequenced using the same primers by Eurofins on the Genomic Platform of Université Paris Descartes.

PCR and Western Blot

Total RNA (500 ng) extracted from T-cell lines (Rneasy Plus Kit; Qiagen) was retro-transcribed to cDNA with M-MLV retro-transcriptase and a mixture of oligo dT (18) and hexamers (Life

Technologies) and amplified with primers for *MALT1* (forward 5'-CAGTTGCCT AGACCTGGAGC-3' in exon 2, reverse 5'-GCTTCCAACAGCAACACACT-3' in exon 5) and for the house-keeping gene *GAPDH* (forward 5'-GAAGGTGAAGTCCGGA GTC-3' in exon 2, reverse 5'-GAGGGATCTCGCTCC TGGGAAGA-3' in exon 5). To study Malt1 protein expression, 10 × 10⁶ T cells or 4 × 10⁶ EBV-immortalized B cells were lysed in RIPA buffer (Sigma) and 20 μg of proteins were resolved on 4% to 15% SDS-polyacrylamide gels and transferred to PVDF membranes (Biorad, Marnes-la-Coquette, France). Membranes were immunoblotted with a monoclonal antibody against human MALT1 carboxy-terminus (immunogen part Asp701-Thr808, clone MALT1-C, 110 kDa, R&D Systems), and HRP-linked anti-mouse Ig1, or HRP-conjugated GAPDH rabbit antibody (38 kDa, Ozyme, Montigny-le Bretonneux, France) and were revealed using ECL Prime Western Blotting Detection reagent, (GE Healthcare, Velizy-Villacoublay, France) and Molecular Imager Chemidoc (Biorad, Marnes-la-Coquette, France). Bands were analyzed with Image Lab software. To inhibit proteasome activity, EBV-immortalized B cells were incubated in 100 μmol/L of MG132 for 8 hours (Sigma) before western blot analysis.

Intracellular Signaling Assays

PHA-T-cell lines were analyzed after a 4-hour rest without interleukin 2 (IL-2). To study IL-2 induction, T-cell lines were stimulated with 32 nmol/L phorbol-12-myristate-13-acetate (PMA) and 0.5 μ mol/L ionomycin for 18 hours and 10 μ g/mL of Brefeldin A (Sigma) were added for the last 3 hours. Cells (10^6) were then surface labeled with CD3Pe-Cy7 antibody (BD Biosciences), fixed and permeabilized with BDCytofix/Cytoperm Plus Fixation/Permeabilization Solution Kit (BD Biosciences), and intracellularly stained with Alexa Fluor 488 anti-human IL-2 (clone MQ1-17H12; Biolegends, Ozyme). To study I κ B α degradation, T-cell lines were stimulated with 162 nmol/L PMA and (0.5 μ mol/L) Ionomycin for 15 minutes at 37°C, and 10^6 cells were fixed and permeabilized with PhosFlow CytoFix buffer and PermII buffer (BD Biosciences) and then stained with a mixture of anti-I κ B α -PE(clone 25/I κ B α /MAD-3) or isotype control and anti-CD3-BV510 (BD Biosciences). Cells were analyzed on a CANTO II instrument (BD Biosciences) and with Flow Jo software (TreeStar Inc).

RESULTS

Description of Patients

Patient 1 (P1), a 7-year-old girl, and P2, her 4-year-old brother, were born from distantly related parents. Facial dysmorphism was noted from birth (Fig. 1A) and chronic eczema-like dermatitis since the first months of life. After 16 months, P1 experienced multiple severe or even life-threatening bacterial, viral, and fungal infections (Supplementary Table 1, <http://links.lww.com/MPG/A684>). At 2.5 years, she developed severe chronic diarrhea with subtotal villous atrophy, massive duodenal T-cell lymphocyte infiltration (Fig. 1B), and moderate colonic inflammation. Methylprednisolone and tacrolimus induced good clinical response and partial histological recovery, suggesting autoimmune enteropathy despite the absence of harmonin serum autoantibodies that are frequently elevated in AIE, either caused by or not caused by loss-of-function mutations in Forkhead box P3 (FOXP3) (9,11). P2 also presented with chronic dermatitis and repeated infections that were, however, less frequent and less severe than his sister. Because he had no clinical signs of intestinal disease and no anti-harmonin autoantibodies, intestinal biopsy was not performed.

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Both patients displayed low IgM, normal IgG and IgA levels, normal CD20⁺ but reduced CD21 B⁺ cells. Counts of CD4⁺ and CD8⁺ cells, activated (HLA-DR⁺) and memory (CD45RA⁻) T cells were elevated. Proliferative response to PHA was normal, whereas proliferation induced through TCR by CD3/CD28 stimulation was markedly reduced (Supplementary Table 1, <http://links.lww.com/MPG/A684>). Strikingly, both patients showed blood eosinophilia, high IgE, and only 0.7% to 1% of CD4⁺ T cells displayed a CD25⁺FoxP3⁺ phenotype, pointing out to a severe quantitative defect in FoxP3⁺ regulatory T cells (Treg) (Fig. 1C and Supplementary Table 1, <http://links.lww.com/MPG/A684>). Overall these data suggested that the patients had an inborn immunodeficiency combining increased sensitivity to all types of infections and an IPEX-like syndrome.

Identification of a MALT1 Homozygous Mutation

To identify the putative gene defect, WES was performed on genomic DNA from P1, P2, and their parents (Fig. 2A). WES identified 1 single missense c.550G>T variation in exon 4 of the

MALT1 gene, that was homozygous in both affected siblings and heterozygous in their parents (Fig. 2B). *MALT1* is an 824 amino acids intracellular protein, containing an N-terminal death domain, 3 immunoglobulin-like domains and 1 paracaspase domain (Fig. 2C). The missense c.550G>T variation resulted in an aspartic acid to tyrosine substitution at amino-acid position 184 (p.Asp184Tyr) in the first immunoglobulin-like domain of MALT1. Asp184 is highly conserved as the sixth amino acid (EF6) of the E-F helix in proteins of the Immunoglobulin superfamily (12,13) and stabilizes this helix by creating a hydrogen bond with EF3. Its substitution by a tyrosine residue removes the hydrogen bond necessary for proper folding of the immunoglobulin-like domain (Fig. 3A), and should therefore impair protein stability. In keeping with this prediction, the MALT1 D184Y protein was undetectable in PHA-T or EBV cell lines from the patients compared with control and parents, but its expression was restored in the presence of proteasome inhibitor (Fig. 3B and Supplementary Figure 1A and 1B, <http://links.lww.com/MPG/A686>).

As discussed above, MALT1 associates with B-cell chronic lymphocytic leukemia/lymphoma 10 (BCL-10) and, in lymphocytes, with CARD11 to form 1 CBM signalosome that links the TCR and B-cell receptor to the canonical NF- κ B pathway (14). Upon lymphocyte activation, the CBM signalosome stimulates the I κ B kinase complex, leading to phosphorylation and proteosomal degradation of the I κ B α inhibitor and to the release of NF- κ B that can translocate into the nucleus and activate its transcriptional targets. MALT1 has also a paracaspase activity that cleaves negative regulators of NF- κ B and thereby further promotes IL-2 production (15). Confirming the loss-of-function mutation in *MALT1*, degradation of I κ B α and induction of IL-2 in response to PMA and ionomycin were drastically impaired in PHA-T cell lines from both siblings compared with a control cell line and to cell lines from their parents (Fig. 3C and Supplementary Figure 1C, <http://links.lww.com/MPG/A686>).

Treatment of MALT1 Deficiency by Hematopoietic Stem cell Transplantation

Both patients underwent hematopoietic stem cell transplantation (HSCT) with reduced intensity conditioning according to EBMT/ESID guidelines for HSCT for primary immunodeficiencies, consisting of fludarabine (180 mg/m²), busulfan (13–15 mg/kg), and alemtuzumab (0.6 mg/kg). They received peripheral mobilized CD34⁺ cells from two 10/10 HLA-matched unrelated donors. Graft-versus-host disease prophylaxis included cyclosporine and mycophenolate mofetil in both patients. Owing to severe allergic reaction to cyclosporine, graft-versus-host disease prophylaxis was changed to tacrolimus in P2. Post-HSCT cytomegalovirus reactivation was observed in both children and was successfully treated with foscarnet (because of resistance to ganciclovir). Both patients had reactivation despite antiviral prophylaxis that resolved with short-term therapy. P1, who experienced life-threatening infections and severe auto-immune enteropathy, is now free of symptoms and has resumed growth. P2 is also free of symptoms. Chimerism on total blood was 100% in P1 on day 300 and 75% in P2 on day 250 after graft. In both patients, IgM serum levels and T-cell counts were nearly normal, except for a moderate CD4⁺ lymphopenia likely secondary to conditioning by fludarabine (Supplementary Table 1, <http://links.lww.com/MPG/A684>). Frequency of CD25⁺ FoxP3⁺ T cells among peripheral CD4⁺ T cells was comparable with that of control at day 170 and day 400 post-BMT in P1 (Fig. 1C and data not shown). Moreover, I κ B α underwent normal degradation in PHA blasts stimulated by PMA and ionomycin and the later cells produced substantial amounts of IL-2 (Fig. 3C and data not shown).

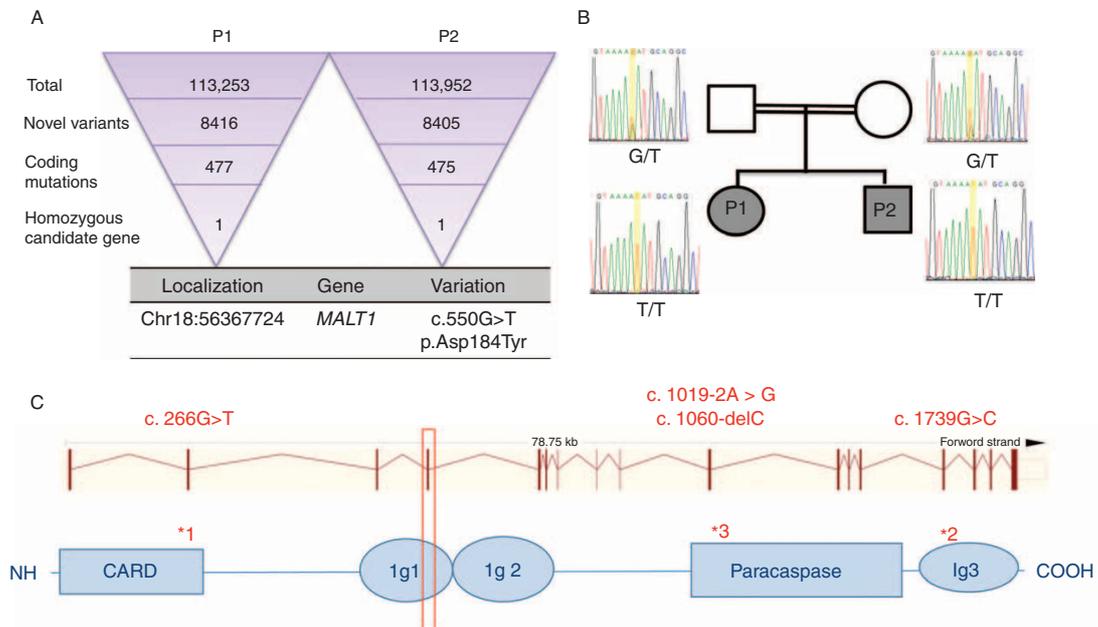


FIGURE 2. Genotypic characterization of patients with *MALT1*. **A**, Whole exome sequencing analysis revealed >110,000 single variant nucleotides (SNVs) per individual. Filtering of SNVs is shown. After removing variations present in dbSNPs, 1000 genomes or exome variant server (EVS) databases and selecting coding variations (exons or essential splicing site), only 1 missense c.550G>T variation in exon 4 of the *MALT1* gene was compatible with recessive inheritance and was present in the 2 siblings. This variation predicted an aspartic acid to tyrosine substitution at amino acid position 184 (p.Asp184Tyr). **B**, Sanger sequencing showing familial transmission of the c.550G>T variation in *MALT1* exon 4. **C**, Localization of the *MALT1* variation c.550G>T (indicated by the red box), compared with the 3 previously reported mutations: *1 = c.266G>T; p.S89I (Jabara et al (5)), *2 = c.1739G>C; p.W580S (McKinnon et al (6)), *3 = c.1019-2A>G, and c.1059C (Punwani et al (7)). CARD = caspase activation and recruitment domain; Ig = immunoglobulin-like domain; *MALT1* = mucosa-associated lymphoid tissue lymphoma translocation 1 gene.

AQ11

Partial recovery of the studied parameters was observed in P2 at day 120 and at day 300 (Figs. 1C and 3C and data not shown).

DISCUSSION

Past studies have demonstrated that IPEX and IPEX-like syndromes can be caused by loss-of-function mutations in *FOXP3*, *IL2RA*, *STAT5b*, and *CTLA-4* genes that are indispensable for Treg generation, homeostasis or function, or from gain-of-function mutations in *STAT3* and *STAT1* that induce T-cell hyperactivation (2,3). In all the latter diseases, effector T cells are not impaired or only moderately impaired in their function. Herein, we highlight how severe defect in Treg in patients with *MALT1* loss-of-function can cause IPEX-like symptoms despite profound impairment of lymphocyte activation.

MALT1 missense mutations have been recently reported in 2 siblings and 2 unrelated children as a cause of severe combined immunodeficiency without lymphopenia (5–7). Mutations were localized in the N-terminal CARD domain of *MALT1*, in its C-terminus or affected splicing, but all abrogated or drastically reduced protein expression (Fig. 2C). Herein, we have identified 2 siblings with a novel missense homozygous mutation that is predicted to prevent correct folding of the first immunoglobulin domain of the protein. We confirmed that, as a consequence, *MALT1* was unstable and rapidly degraded by the proteasome. *MALT1* has a dual function in lymphocytes. As a scaffold protein, it combines with BCL-10 and CARD11 in lymphocytes and forms a

CBM signalosome that is indispensable to activate NF- κ B downstream the T- and B-cell receptors (4). Accordingly, PMA and ionomycin failed to stimulate I κ B α degradation and IL-2 production in T blasts from the 2 patients. In keeping with the profound functional lymphocyte defect and as previously reported in the 4 other cases of *MALT1* deficiency, our 2 patients developed a wide spectrum of severe infections. One striking feature of the disease was, however, an IPEX-like syndrome combining severe eczema-like dermatitis, hyper-IgE and, in 1 sibling, intestinal inflammation with villous atrophy and massive hyperplasia of CD3⁺CD8⁺ intraepithelial lymphocytes. Moreover, both siblings had extremely low counts of FoxP3⁺ Treg. No IPEX syndrome has been formally described in the 4 other reported cases of *MALT1* deficiency, but 2 unrelated children had severe exfoliating dermatitis. Inflammation of the upper digestive tract was noted in the 4 patients and 2 had villous atrophy with increased number of intraepithelial lymphocytes. Increased IgE levels were observed in only 1 case, whereas numbers of FoxP3⁺ Treg were found normal in 1 patient and extremely low in another (Table 1 and Supplementary Table 2, <http://links.lww.com/MPG/A685>) (5–7).

Interestingly, intrathymic but not peripheral generation of natural Treg was found to be profoundly impaired in *Malt1*^{-/-} mice, resulting in drastically reduced numbers of Treg in the peripheral blood of young mice, while this defect was masked by the progressive expansion of peripheral Treg in aged *Malt1*^{-/-} mice (16). Reduced numbers of Tregs were also observed in mice selectively lacking *MALT1* paracaspase activity (*Malt1*^{PM/PM}),

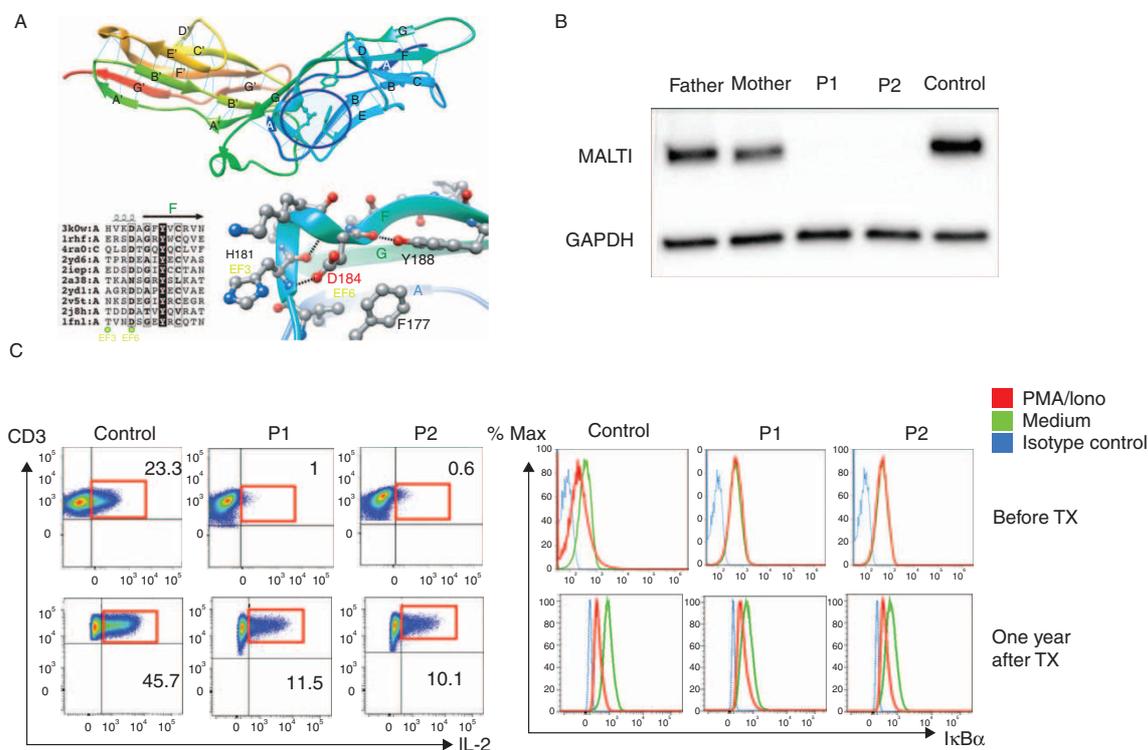


FIGURE 3. Functional consequences of the loss-of-function *MALT1* mutation. A, Model of the E-F helix in the first immunoglobulin-like domain of MALT1 and conservation of acid aspartic at position EF6 in the immunoglobulin superfamily (left panel). B, Analysis of MALT1 protein expression in PHA-T cell lines from P1, P2, their parents, and 1 healthy control. C, Detection of IL-2 (left panel) and IκBα (right panel) by flow cytometry in PHA-T cell lines from P1, P2, and an unrelated control after stimulation with PMA and ionomycin (gated on CD3⁺ T cells) before hematopoietic stem cell transplantation (TX) (upper panel) and after TX (lower panel). IL-2 = interleukin 2; MALT1 = mucosa-associated lymphoid tissue lymphoma translocation 1 gene; PHA-T = phytohemagglutinin T cell; PMA = phorbol-12-myristate-13-acetate.

which is necessary for fine-tuning NF-κB activation (10,17,18). Despite the lack of natural Treg indispensable to protect against autoimmunity, no IPEX-like symptoms were observed in *Malt1*^{-/-} mice but *Malt1*^{PM-/-} mice, which, in contrast to *Malt1*^{-/-} mice, had normal effector T-cell functions, developed hyper-IgE, lymphadenopathy, multiorgan lymphocyte infiltration, and severe autoimmune gastritis alike *Foxp3*^{-/-} mice (19). We suggest that, in MALT1-deficient humans, activation of effector T cells may be less impaired than in *Malt1*^{-/-} mice and therefore be sufficient, if not inhibited by natural Treg, to induce IPEX-like symptoms. Of note

anti-enterocyte (harmonin) autoantibodies, which are present in *FOXP3*^{-/-} patients developing an autoimmune enteropathy, were absent in our *MALT1*^{-/-} patient (8). This negative result is in keeping with the profound B cell defect associated with MALT1 deficiency and confirms that these antibodies are dispensable for intestinal damage.

MALT1 expression is not restricted to the hematopoietic compartment and can form with BCL-10 and CARD10 a signalosome that participates in NF-κB activation downstream G protein-coupled receptors outside the immune system, notably in epithelial

TABLE 1. Comparison of the main phenotypic characteristics of MALT1 deficiency in all reported patients

	Charbit-Henrion et al		Punwani et al (7)	Jabara et al (5)		McKinnon et al (6)	
	P1	P2		P1	P2		
Immunodeficiency	+	+	+	+	+	+	100%
Enteropathy	+	-	+	+	+	+	83%
Low Treg	+	+	+	NA	NA	-	75%
Eczema	+	+	+	-	-	+	67%
High IgE	+	+	-	-	-	+	50%
Dysmorphia	+	+	-	-	-	+	50%

IgE = immunoglobulin E; NA = not available; MALT1 = mucosa-associated lymphoid tissue lymphoma translocation 1 gene; Treg = regulatory T cells.

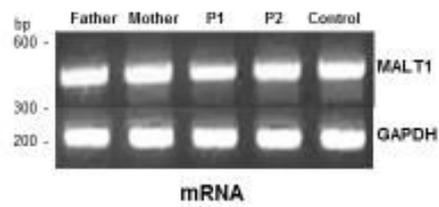
cells (20). The absence of MALT1 in other cell types may thus perhaps account for the dysmorphia observed in the 2 siblings in this study and in 1 previously reported *MALT1*^{-/-} patient (6). Yet MALT1 deficiency seems to affect most dramatically immune cells, highlighting a key nonredundant role of MALT1 in the hematopoietic compartment and pointing out to HSCT as a possible curative treatment. Accordingly, 1 of the 4 reported cases of MALT1 deficiency was successfully treated by HSCT (7). Confirming that HSCT is a pertinent therapeutic option in MALT1 deficiency, HLA-matched HSCT corrected lymphocyte dysfunction and allowed clinical recovery in both siblings in the present study.

In conclusion, our observations confirm and extend previous findings in MALT1 deficient children and highlight the unusual presentation of this extremely rare condition, which combines severe immunodeficiency and IPEX-like syndrome. Analysis of I κ B α degradation after lymphocyte activation provides a simple diagnosis test to orientate genetic testing. Early recognition of this extremely severe disease is indispensable as it can be cured by HSCT.

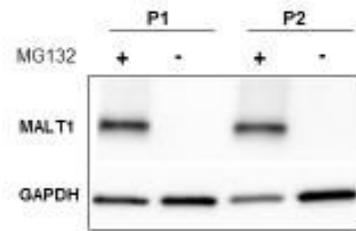
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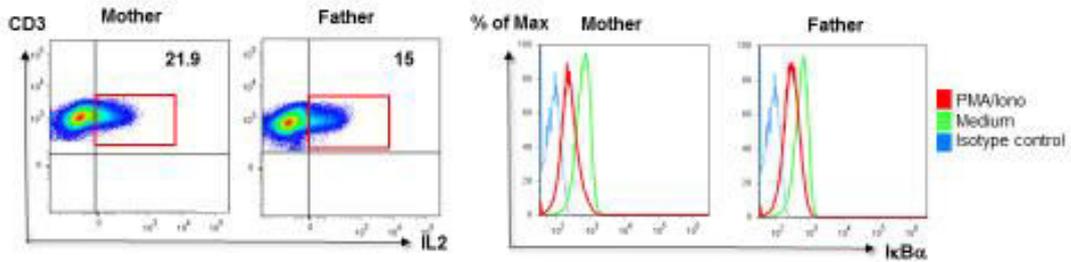
Suppl. 1 A



Suppl. 1 B



Suppl. 1 C



Supplementary Figure 1.

A: Analysis of MALT1 mRNA in PHA-T cell lines from P1, P2, their parents and one healthy control.

B: Analysis of MALT1 protein expression in EBV cell lines from P1, and P2 with or without proteasome inhibitor (MG132).

C: Flow cytometry analysis showing normal IL-2 production (upper panel) and normal IκBα degradation (lower panel) after PMA and ionomycin stimulation in PHA-T cell lines from both heterozygous parents (gated on CD3⁺ T cells).

SUPPLEMENTARY TABLES

	P1		P2		Normal value
	Pre-HSCT	300 days post HSCT	Pre-HSCT	250 days post HSCT	
CD3 +	4218	2410	6498	1830	1100 - 3900
CD4 +	2948	790	4043	600	700 - 2000
CD8 +	1300	1530	2077	1075	500 - 1400
CD19+	512	360	958	959	400 - 1500
CD3+ DR+	19%	46%	9%	35%	3 – 11%
CD16+/CD56+	217	490	657	516	100 - 700
CD4+ CD45RA+	1209	337	2264	180	430 – 1500
	35% CD4+	49% CD4+	59% CD4+	30% CD4+	
CD4+ CD45RA-	1739	351	1779	410	220 – 660
	65% CD4+	51% CD4+	41% CD4+	70% CD4-	
CD4+ RTE	884	282	1819	132	50 - 926
CD4+ FOXP3+	1%	2.4%(D400)	0,7%	1,7% (D330)	3 – 10%
IgG	10,0	5,0	8,8	13,3	5,6 – 14,8 g/l
BrdU⁺ T cells after PHA stimulation	66	52	54	48	29-57%
BrdU⁺ T cells after CD3/CD28 stimulation	42	66	15	24	50-85%

Supplementary Table 1: Phenotypic analysis of lymphocyte subsets in P1 and P2 before and after hematopoietic stem cell transplantation (HSCT). Units: 10⁶ cells/l; RTE: recent thymic emigrants; D330: day 330 post HSCT; PHA: Phytohemagglutinin. T cells proliferation was tested after stimulation by PHA or a mixture of anti-CD3 and anti-CD28 antibodies by flow cytometry using BrdU: 5-bromo-2'-deoxyuridine, an analogue of thymidine incorporated into newly synthesized DNA of replicating cells and anti-BrdU antibody. Incorporation of BrdU was assessed in CD3+ lymphocytes.

Supplementary Table 2: P1's and P2's summaries, compared to previously reported patients, before and after hematopoietic stem cell transplantation (HSCT). HSV-1: Herpes virus type 1; VZV: varicella virus; S. aureus: Staphylococcus aureus; S. pneumoniae: Streptococcus pneumoniae; EBV: Epstein-Barr virus; CMV: cytomegalovirus; RSV: Respiratory syncytial virus; H. influenzae: Haemophilus influenzae; K. pneumonia: Klebsiella pneumonia; CT: computerized tomography; Ig IV: immunoglobulin intravenous; TMP-SMX: Trimethoprim-sulfamethoxazole; GVHD: graft versus host disease; D330: day 330 post HSCT; NA: Not available.

					Punwani D, et al.		Jabara H, et al.	McKinnon M, et al.	
	P1		P2		1 patient		2 siblings	1 patient	
Consanguinity	Yes		Yes		No		Yes	Yes	
	Pre-HSCT	Post-HSCT	Pre-HSCT	Post-HSCT	Pre-HSCT	Post-HSCT	HSCT not done		
Infections									
Skin/mucosa	Candida, HSV-1 Pseudomonas	HSV1	Candida, HSV-1	HSV1	Candida, S.aureus	No		S. aureus HSV-1, VZV	
Pulmonary	S.pneumoniae Pneumocistis jirovecii, EBV, CMV, adenovirus	No	Adenovirus	Adenovirus Clinical bacterial pneumonia	CMV, RSV	No	S.pneumoniae, S.aureus H. influenzae, K. pneumoniae Pseudomonas Candida	S. aureus S.pneumoniae CMV	
Gastrointestinal tract	Salmonella, Campilobacter jejunii rotavirus, adenovirus, EBV, CMV (chronic)	No	Salmonella	No	Clostridium difficile	No	Candida		
Blood	S. aureus, S.pneumoniae	Transient CMV reactivation		Transient CMV reactivation	CMV	No			
Others	HSV-1 keratitis	HSV-1 keratitis		No		No	S.pneumoniae meningitis H.influenzae meningitis CMV (urine)		
Clinical manifestations									
	Poor or delayed growth	Yes	Resolved	No	No	Yes	Resolved	Yes	Yes
	Oral lesions*	Yes	Resolved	Yes	Partly resolved	Yes	Resolved	Yes	Yes
	Eczema	Yes	Resolved	Yes	Partly resolved	Yes	Resolved	Not reported	Yes
	Enteropathy	Severe diarrhea, malabsorption, massive lymphocyte infiltration, subtotal villous atrophy	Resolved	No (no histology)	No	Bloody diarrhea (no histology)	Resolved	Intraepithelial lymphocytosis villous atrophy	Severe intestinal inflammation
	Bronchiectasis	Yes	NA	CT-scan not done	NA	No	No	Yes, respiratory failure	Yes
	Other findings	Dysmorphic facies, severe shortsightedness	NA	Dysmorphic facies Peanut allergy	NA			Mastoiditis	Dysmorphic facies, bone fractures, granulation tissue on vocal cord, larynx, ear canal
Treatments									
	Ig IV	Yes	No	No	No	Yes	Yes	Yes	Yes
	Anti-microbial prophylaxis	TMP-SMX Acyclovir	TMP-SMX Acyclovir	TMP-SMX	TMP-SMX Acyclovir	Yes	No	Yes	Yes
	Immunosuppressive therapy	Methylprednisolone Tacrolimus	No	No	No	Not reported	Not reported	Not reported	Not reported
	Outcome	Alive, well at D400 (8 years)		Alive, well at D330 (5 years)		Alive, well at 7 years		Deceased, respiratory failure, 13 years, 7 years	Alive

ABBREVIATIONS:

AIE	Autoimmune enteropathy
BCL-10	B-cell chronic lymphocytic leukemia/lymphoma 10
BCR	B cell receptor
CARD	Caspase recruitment domain
EBV	Epstein-Barr virus
FOXP3	Forkhead box P3
GVHD	Graft versus host disease
HSCT	Hematopoietic stem cell transplantation
IBD	Inflammatory bowel disease
IL-2	Interleukin 2
IL-10 R	Interleukin-10 receptor
IPEX	Immunodeficiency-polyendocrinopathy and enteropathy-X-linked syndrome
MALT1	Mucosa associated lymphoid tissue lymphoma translocation gene 1
NF- κ B	Nuclear factor-kappa B
PHA	Phytohemagglutinin
PMA	Phorbol-12-myristate-13-acetate
TCR	T cell receptor
Treg	Regulatory T cells
WES	Whole exome sequencing

IV. TARGETED NEXT-GENERATION SEQUENCING PANEL IN MONOGENIC ENTEROPATHIES: AN EFFECTIVE FIRST-LINE GENETIC TEST

MANUSCRIPT SUBMITTED

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TARGETED NEXT-GENERATION SEQUENCING IN MONOGENIC ENTEROPATHIES: AN EFFECTIVE FIRST-LINE GENETIC TEST.

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Research in context

Evidence before this study

If individually, monogenic enteropathies are very rare diseases, they collectively raise difficult diagnosis and therapeutic issues. The number of genes carrying disease-causing mutations has considerably expanded since 2012. Diagnosis is further complicated by the lack of strict phenotype-genotype correlations. Early molecular diagnosis is however crucial in order to reduce mortality and morbidity, notably by defining whether treatment should target the epithelial or the hematopoietic compartment of the intestinal barrier. Next generation sequencing (NGS) techniques provide novel tools to circumvent these difficulties. Whole exome sequencing is valuable to identify novel genetic defects. But, this expensive and time-consuming technique is not tailored for routine diagnosis of known genetic diseases and cannot be applied in single parent households. Targeted NGS (TNGS) is an alternative method designed to simultaneously screen numerous candidate genes in a large number of DNA samples derived from patients sharing overlapping symptoms. Since 2013, several studies have assessed the diagnosis value of TNGS for diagnosing Mendelian diseases, notably of immune or neurological origin. NGS targeting 48 genes, mainly associated with primary immunodeficiencies, was applied in 2014 to a small cohort of 25 patients with very early onset inflammatory bowel diseases (VEO-IBD). Causative mutations were identified in 4 cases without prior molecular diagnosis. In order to investigate the diagnosis value of TNGS in severe enteropathies of putative Mendelian inheritance, we developed, in 2015, a dedicated NGS covering all exons of the 68 genes in which mutations causing monogenic enteropathies had been reported before June 2015 in Pubmed. This tool was applied between August and December 2015 in a large cohort of 185 patients with either VEO-IBD or congenital diarrhoea.

Added value of the study

A first added value of our study is the use of TNGS to screen the majority of genes implicated in monogenic intestinal diseases, including congenital diarrhoeas.

Secondly, and to the best of our knowledge, we have genetically characterised the largest cohort of very early onset intestinal diseases described so far. This large cohort of 216 patients was built through a collaborative network established in 2009 within the GENIUS group (GENetically and/or ImmUne mediated enteropathieS) of ESPGHAN (European Society for Paediatric Gastroenterology, Hepatology And Nutrition) and gathers currently 45 distinct centres, mainly in Europe. This close collaboration was essential to validate molecular diagnoses. We thus feel that this cohort is exemplary in term of European cooperation to improve diagnosis, and care of these severe orphan diseases.

Third, we have compared diagnosis yield by Sanger sequencing of candidate genes and whole exome sequencing to TNGS. In the first 154 patients studied by Sanger sequencing and whole exome sequencing, molecular diagnosis yield was 28%. Using TNGS on patients most of whom were without a diagnosis despite these analyses, we showed that global genetic diagnosis yield of TNGS was 16.2%, raising diagnosis rate in the whole cohort to 33%. In addition, we compared yield of molecular diagnosis depending on clinical presentation and showed that it was much higher in congenital diarrhoea (86%) than in IPEX-like syndromes combining chronic diarrhoea and autoimmunity (30%) or in colites (10%).

Fourth, we describe but also validate novel mutations. Among the thirty-four mutations identified by TNGS, eleven were previously reported as disease causing. But we describe 23 novel mutations and provide functional validation for 18 of them. We thus expect to facilitate molecular diagnosis for future patients.

Finally, we provide the description of patients in whom atypical presentation had prevented phenotype-based diagnosis. Our observations reveal as yet unrecognized phenotypes in some monogenic diseases considered as “archetype”, such as those caused by *FOXP3* and *MYO5B* mutations.

Implications of all the available evidence

Given the considerable number of genes in which mutations can result in comparable clinical presentations with very severe outcomes, it is urgent to define rapid, accurate and cost-effective methods for molecular diagnoses of Mendelian diseases. In keeping with several recent studies, we confirm that TNGS is a very efficient method to simultaneously screen numerous candidate genes in a large group of patients and to identify not only single nucleotide mutations but also exonic copy number variations that are difficult to detect by other methods. We demonstrated that our custom-made TNGS provided a sensitive and robust diagnosis method to rapidly identify known monogenic causes of severe chronic enteropathies. Most importantly, we hope that our results will lead to a change in clinical practice. We recommend using TNGS as a first-line genetic test in patients suspected of monogenic enteropathies in order to rapidly reach precise molecular diagnosis, which is crucial to optimize therapy and to preserve life expectancy and quality.

ABSTRACT (299 words)

Background: Individually, monogenic enteropathies are very rare diseases. But, collectively, they raise difficult diagnosis and therapeutic issues, due to the increasing number of genetic defects and the lack of strict phenotype-genotype correlation. As Next-Generation Sequencing (NGS) methods are becoming more affordable, they provide novel genotype-based approaches to establish molecular diagnoses. Herein our goal was to evaluate diagnosis efficacy of a custom-made targeted panel (TNGS) encompassing 68 genes in which mutations are known to cause monogenic intestinal diseases.

Methods: All coding exons of the 68 selected genes were captured by Agilent's SureSelectXT Custom kit, sequenced on a Illumina HiSeq2500 HT system and analysed by dedicated softwares in 185 patients followed up for severe intestinal diseases of putative monogenic inheritance in 45 distinct, mainly European, centres. Diagnosis specificity and sensitivity was assessed in 12 patients with a known molecular diagnosis. 173 patients were analysed to identify mutations in the selected genes.

Findings: All coding exons were properly covered, including *NCF1*, *IKBKKG* and *NEUROG3* known to be difficult to capture. All mutations in the 12 positive controls were identified. A novel molecular diagnosis was established in 28 patients, including three cases with very atypical presentations. In addition, deep coverage (mean: 682 reads/exon) allowed identification in three patients of large deletions and insertions, which were not detected previously by whole exome sequencing, and thus enabled to establish molecular diagnosis.

Interpretation: Custom-made TNGS was an accurate cost- and time-effective tool to screen simultaneously a large number of genes in patients suspected of monogenic enteropathies. Diagnosis yield was 16% in patients without prior molecular diagnosis. Using TNGS as a first-

line genetic test should help to accelerate molecular diagnosis and to optimize therapy indispensable to reduce mortality and morbidity in these very severe diseases.

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INTRODUCTION

Mendelian mutations causing rare but very severe chronic intestinal diseases are identified in an increasing number of genes expressed in hematopoietic immune cells, in epithelial cells or in both. Monogenic enteropathies can be schematically divided into congenital diarrhoeal disorders (CDD), in which mutations severely impair epithelial differentiation or absorptive function, and dysimmune disorders, in which mutations compromise function or regulation of the gut immune barrier. Due to their usual onset within the first months or years of life, dysimmune disorders are often regrouped under the term VEO-IBD for very early-onset inflammatory bowel diseases. Immune disorders can be further divided into IPEX and IPEX-like syndromes (for Immune dysregulation, polyendocrinopathy, enteropathy, X-linked) characterized by prominent autoimmunity, and VEO-colitis as seen in patients harbouring mutations in interleukin (IL-10) pathway or in *XIAP* (X-linked inhibitor of apoptosis protein).^{1,2} Therapeutic management of monogenic intestinal diseases is difficult and often requires heavy treatments, including hematopoietic stem cell transplantation (HSCT) in VEO-IBD, or total parenteral nutrition and intestinal transplantation in CDD.^{2,3} Early molecular diagnosis is crucial to rapidly define the most pertinent treatment and to reduce mortality and morbidity.

Diagnosis of monogenic intestinal diseases is classically based on precise phenotyping of patients followed by selected functional tests and Sanger sequencing of candidate genes.⁴ This strategy however has limitations given increasing evidence of lack of strict phenotype-genotype correlations.⁵ Moreover, the number of genes in which mutations causing severe enteropathies have been identified has considerably expanded, stressing the need to develop novel time- and cost-effective diagnosis approaches.^{2,6} Next-generation DNA sequencing (NGS) methods now provide powerful tools for efficient genotype-based molecular diagnosis. While whole genomes (WGS) and whole exomes (WES) sequencing provide unbiased approaches, they remain relatively expensive and raise difficult challenges for analysis and interpretation. Targeted panel

sequencing, also called TNGS, emerges as a powerful tool for fast and simple screening of genes of interest. Since 2014, TNGS was proven to be valuable to identify primary immunodeficiencies, notably in patients with atypical presentations.^{5, 7, 8} TNGS was also able to identify a few mutations in a small cohort of VEO-IBD patients.⁹ Herein, we have designed a custom-made TNGS enabling to sequence the exons of 68 genes in which mutations causative of monogenic enteropathies have been identified. This tool was used to screen a cohort of 185 VEO-IBD and CDD patients recruited from 45 distinct Paediatric Gastroenterology centres joined in GENIUS group (GENetically and/or ImmUne enteropathies) from ESPGHAN (European Society for Paediatric Gastroenterology, Hepatology And Nutrition). Our goals were to define a diagnosis rate in VEO-IBD and CDD patients using TNGS and to investigate phenotype-genotype correlations in monogenic enteropathies. Our data indicate that TNGS is a cost-effective and robust diagnosis tool which permits to accelerate molecular diagnosis of monogenic enteropathies and choice of rationale-based therapies.

METHODS

Study design and Patients

A working group from ESPGHAN called GENIUS (GENetically and/or ImmUNE mediated enteropathies) was created in 2009 in order to better characterize monogenic enteropathies and to optimize patients' care. GENIUS (www.genius-group.org) gathers Paediatric Gastroenterologists from 45 centres: Czech Republic (n=2 centres), Denmark (n=1), France (n=17), Germany (n=2), Greece (n=1), Hungary (n=2), Italy (n=5), Lithuania (n=1), Netherlands (n=1), Portugal (n=4), Slovenia (n=1), Spain (n=2), Switzerland (n=1), Australia (n=1), Israel (n=1), Tunisia (n=2), Turkey (n=1). Patients suffering from VEO-IBD and CDD were included by each centre after obtaining patients' informed written consent for functional and genetic studies. French patients were included through the Immunobiota research protocol.

Between August 2009 and August 2015, 216 patients were included. In 43 patients, molecular diagnosis was established by Sanger sequencing of candidate genes selected on the basis of clinical phenotyping and functional testing, or by WES performed on trios (patient and both parents). Genomic DNA samples from 12 of the latter patients and from the remaining 173 patients were analysed by the custom-made TNGS described below. One hundred out of 173 children were boys (58%). Seventeen children were born from consanguineous parents. All cases were single cases except patients 10 and 11 and patients 25 and 26, who were siblings. Age at onset ranged between birth and 16 years, with a median age of 12 months. More than 75% of the patients were 3 years old or younger at the beginning of their disease (interquartile range 4-36 months). Clinical diagnoses before TNGS were CDD in 7 (4%) and VEO-IBD in 166 patients, among which 27 displayed IPEX or IPEX-like syndrome (16%) and 139 had colitis (80%).

DNA preparation

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMC) isolated by Ficoll HyPaque Plus (GE Healthcare, Velizy-Villacoublay, France), from umbilical cord tissue (patient 25) or from whole blood lysed by BD lysis buffer (BD Biosciences, Le Pont de Claix, France) using the QIAamp[®] DNA Blood Mini Kit (Qiagen, Courtaboeuf, France).

Targeted Next Generation Sequencing

Genomic DNA libraries were generated from 1 µg DNA sheared with a Covaris S2 Ultrasonicator using SureSelect^{XT} Library PrepKit (Agilent, Garches, France) on the Genomic Platform of Institut IMAGINE. 1140 regions of interest (ROI) encompassing all exons of the 68 selected genes were captured by hybridation with biotinylated complementary 120-pb RNA baits designed with SureSelect SureDesign software (H. sapiens, hg19, GRCh37, February 2009). Targeted ROI were pulled out with magnetic streptavidin beads, PCR-amplified using indexing primers and sequenced on an Illumina HiSeq2500 HT system. Data analysis was performed with Paris Descartes University / Institut IMAGINE's Bioinformatics core facilities. Paired-end sequences were mapped on the human genome reference (NCBI build37/hg19 version) using the Burrows-Wheeler Aligner. Downstream processing was carried out with the Genome Analysis Toolkit (GATK), SAMtools, and Picard, according to documented best practices (<http://www.broadinstitute.org/gatk/guide/topic?name=best-practices>). Variant calls were made with the GATK Unified Genotyper based on the 72 version of ENSEMBL database. Genome variations were defined using the in-house software PolyDiag, which eliminates irrelevant and common polymorphisms based on frequencies extracted from public databases: US National Center for Biotechnology Information database of SNP (dbSNP), 1000 Genome, Exome Variant Server (EVS), and Exome Aggregation Consortium (ExAC, <http://exac.broadinstitute.org>). Consequences of mutations on protein function were predicted

using 3 algorithms: Polyphen2 (<http://genetics.bwh.harvard.edu/pph2/>), Sift (Sorting Intolerant From Tolerant, J. Craig Venter Institute) and Mutation Taster (www.mutationtaster.org).

Gene validation

Histology (HE, PAS staining) and immunochemistry (EpCAM staining) was performed on formalin-fixed intestinal biopsies. EPCAM was revealed with clone 323/A3 (Acris Antibodies GmbH, Montluçon, France) as described.¹⁰ **Phenotypic and functional characterisation of FOXP3 T cells** were performed as described.^{11, 12} **XIAP intracellular staining** was performed on 2×10^6 PBMC fixed-permeabilized using the Intraprep Permeabilization Reagent kit (Beckman Coulter, Villepinte, France) and incubated with anti-XIAP antibody (clone 28, BD) or isotype control antibody (mouse IgG1, Sony Biotechnologies, Weybridge, UK) and with secondary PE-goat anti-mouse IgG1 antibody. For extracellular staining, cells were incubated with anti-CD3-Bv510, anti-CD14-Pe/Cy7, anti-CD11c-APC, anti-CD86-Bv421 (Sony), and anti-CD19-Bv711 (BD). **Analysis of Interleukin 8 (IL-8) secretion** by PBMC stimulated with N-acetyl-muramyl-M-alanyl-D-isoglutamine hydrate (MDP, Sigma, St. Quentin Fallavier, France) or lipopolysaccharide (LPS, Sigma) was performed as described.¹³ For **Western blot analyses**, cell lysates and immunoblots were performed according to standard protocols. Proteins were detected with monoclonal antibodies against human NCF1 (Santa Cruz, sc-14015 phox-p47, Dallas USA), LRBA (clone HPA023597, Sigma) or XIAP (clone 28, BD), HRP-linked secondary antibodies or HRP-conjugated GAPDH rabbit antibody (Ozyme, Montigny-le Bretonneux, France) and ECL Prime Western Blotting Detection reagent (GE Healthcare).

RESULTS

Quality control of targeted next generation sequencing

The custom-made TNGS covered 1140 ROI encompassing all coding exons of 68 genes in which mutations causative of VEO-IBD or CDD have been reported (see full list in Appendix A). All ROI were covered, including *NCF1*, *IKBK*G and *NEUROG3*, known to be difficult to capture. DNA samples from all patients were sequenced between August 2015 and April 2016 in three consecutive runs. Run 1 included DNA samples from 12 patients with a known molecular diagnosis and from 30 patients without diagnosis. Runs 2 and 3 included 52 and 91 samples respectively, all from patients without diagnosis. Mean coverage of the panel was 682 reads, and varied depending on the number of samples sequenced by run with medians of 826, 629 and 593 reads in runs 1, 2 and 3 respectively. ROI with the smallest coverage were the first exons of *DGATI* (50 to 200 reads) and *MALTI* (10 to 100 reads), likely due to their high content in GC nucleotides which impairs capture; as well as exon 7 of *ITCH* (20 to 200 reads) and exon 1 of *APOB* (20 to 160 reads) due to the presence of repeated regions within their sequence.

As shown in Table 1, all mutations previously detected by Sanger sequencing in the 12 patients used as validation samples were identified by TNGS, including a complex compound heterozygous defect in *IL10R2* (VS12). This complex genetic event consisted in large deletion of exon 3 on the maternal allele and duplication of exon 6 on the paternal allele, which had been detected by sequencing of complementary DNA only. Altogether, TNGS efficiently detected, not only point mutations, but also exonic copy number variants.

Novel diagnoses of Mendelian enteropathies by targeted gene panel sequencing

TNGS of genomic DNA was performed in 173 patients without a prior molecular diagnosis. As shown in Table 2, TNGS identified 21 missense mutations, 3 small insertions/deletions

resulting in frameshift, 8 nonsense mutation, and 5 large deletions (P6, P10, P11, P25, P26). Mutations were either homozygous (n=8), compound heterozygous (n=8), X-linked (n=8), or dominant heterozygous (n=4) in 4 patients with *NLRC4*, *STAT1* or *CTLA4* mutations. Mutations were identified in 28 patients in *CTLA4* (n=2), *EPCAM* (n=1), *FOXP3* (n=4), *IL10R1* (n=1), *LRBA* (n=3), *MYO5B* (n=2), *NCF1* (n=2), *NEUROG3* (n=1), *NLRC4* (n=1), *SI* (n=1), *SKIV2L* (n=3), *TTC37* (n=1), *TTC7A* (n=1), and *XIAP* (n=4).

Among the 34 mutations identified by TNGS, 11 were previously reported as disease causing. Validation was performed to confirm mutations that have not been described yet. *SKIV2L* (P3, P9, P24) and *TTC37* defects (P18) were confirmed by microscopical demonstration of *trichorrhexis nodosa* in hair samples (not shown), *MYO5B* deficiency by periodic acid-Schiff (PAS) staining of duodenal biopsies (P17, P21), and of *EPCAM* by immunochemistry on intestinal biopsies (P15). *FOXP3* mutations were validated by demonstrating reduced frequency of CD4⁺ CD25⁺ CD127⁺ FOXP3⁺ regulatory T cells (Treg) (P2 and P8) or abnormal Treg suppressive function (P1). *LRBA* deficiency was confirmed in P26 by loss of protein expression. This diagnosis was considered as highly probable in P19 despite normal *LRBA* protein expression as all algorithms predicted the damaging effect of the two mutations on protein function. No change in surface expression of *CTLA-4*, previously described in regulatory and activated T cells from *LRBA* deficient patients was observed in either P26 or P19.¹⁴ *XIAP* mutations were authenticated by showing impaired IL-8 production upon MDP stimulation, and either total lack of protein in two boys with hemizygous mutations (P6, P12) or reduced protein expression in the monocytes of the girl with a heterozygous mutation (P7). Functional validation was not performed in P14 and in P16. P14 displayed a heterozygous mutation in *NLRC4* predicting a truncated protein lacking 274 out of the 368 amino acids composing the leucin-rich regulatory domain, a result consistent with dominant gain-of-function effect. P16 displayed compound mutations in the gene encoding sucrose-isomaltase:

one was a known disease-causing point mutation and the second predicted a short truncated protein of 124 instead of 1827 amino-acids. Overall, TNGS identified a molecular diagnosis in 28 patients, yielding a 16.2% diagnosis rate. Diagnosis rate was much higher in patients with CDD (86%) than in patients with IPEX-like syndromes (30%) and colitis (10%). Median disease duration until TNGS was 7.5 years (range 1-18 years) highlighting severe delay before diagnosis in many patients.

Added value of TNGS for diagnosis of atypical presentations

Description of the 28 cases and of the observed mutations is given in Table 2. Importantly, in three patients (P1, P21, P7), atypical presentation had prevented phenotype-based diagnosis. The first atypical patient (P1, Table 2) displayed pancolitis since 10 months of life (Figure 1A). Hyper IgE and serum anti-harmonin antibodies were suggestive of IPEX syndrome, but the lack of severe duodenal lesions and the absence of auto-immune symptoms apart from mild eczema were unusual. Moreover, frequency of peripheral Treg was normal and their expression of FOXP3 comparable to that of controls (Figure 1B). TNGS identified a novel hemizygous *FOXP3* mutation, (c.152G>A; p.Arg51Gln), which was predicted to prevent N-terminal cleavage of FOXP3 into the 41 KD peptide detected in the chromatin fraction of murine activated Treg. In mice, processing of FOXP3 into a peptide lacking both C and N-termini was shown to be necessary for optimal suppressive activity but the impact of N-terminus cleavage alone remained unclear.¹⁵ Sustaining the hypothesis that N-terminus cleavage is important for the function of FOXP3 in human Treg and confirming diagnosis of IPEX syndrome, T cell proliferation was less efficiently suppressed by Treg from P1 than from controls (Figure 1C). The second atypical patient (P21) was a boy who had intractable diarrhoea since one month of age, and was dependent on total parenteral nutrition. Extensive investigations, including endoscopy and histology, were considered as negative. At 3 years, he developed Crohn-like

inflammatory disease, which was initially characterized by ulcerations in sigmoid and subsequently progressed into pancolitis and terminal ileitis. TNGS identified two novel variants, V1 c.4853-1G>C and V2 c.5313+3A>G, both affecting essential splicing sites in the *MYO5B* gene. Biopsies were re-examined with PAS staining, which confirmed the diagnosis of microvillus atrophy (Figure 1D). The third atypical patient (P7), now 17 year-old, had developed severe pancolitis at 6 months. Her 7 years older sister and her deceased maternal uncle had been diagnosed with severe Crohn's disease. Her disease was also very severe and had relapsed despite multiple treatments, including azathioprine, methotrexate and anti-TNF α monoclonal antibodies. TNGS revealed a heterozygous *XIAP* mutation (c.758C>G; p.Ser253*) which introduced an early-stop codon at the end of the second exon. This mutation was previously described as causative of colitis in a male patient.¹⁶ Since *XIAP* is carried by the X chromosome, most affected individuals are male. IBD has however been described in two heterozygous female carriers of *XIAP* mutations although presentation was less severe than in P7. Disease was ascribed to the predominant expression of the *XIAP*-mutated allele in monocytes. In keeping with the latter observations, *XIAP* expression was selectively decreased in the monocytes of P7 (Figure 1E).¹⁷

Added value of TNGS for detection of exonic copy number variants

The effectiveness of TNGS in detecting exonic copy number variants (ECNV) was first demonstrated in validation sample VS12, in which TNGS readily detected a large deletion of *IL10R2* removing exon 3 and a duplication of exon 6 (see above). Moreover, in two sets of siblings, TNGS identified mutations in *NCF1* and *LRBA* which had not been identified by WES performed prior to TNGS. In the first family, P10, a 17 year-old girl, and her brother P11, aged 20, displayed Crohn-like disease affecting all segments of the digestive tract since the age of

10 and 3 years respectively. Granuloma was however only observed once and only in one biopsy from P10. Both patients also suffered from chronic severe eczema. In contrast, they had never experienced any severe bacterial infection apart from liver abscess in P10 while under anti-TNF α therapy. Due to disease severity, both patients had undergone ileostomy. WES was performed in both siblings and their parents in 2013, with inconclusive results. TNGS revealed a homozygous extended deletion of the *NCF1* gene, starting from exon 2, which was ascribed by Sanger sequencing to the c.75_76GT deletion in exon 2 commonly described in patients with autosomal recessive chronic granulomatous disease.¹⁸ Lack of NCF1 protein was confirmed by Western Blot in PBMC-derived macrophages (not shown). Loss of NADPH activity was demonstrated by dihydrorhodamine test (not shown). In the second non-consanguineous family, P25 and P26 presented with severe IPEX-like syndrome since the very first months of life. P25 died before 9 months. His sister, P26, now 7 year-old, was treated by sirolimus and azathioprine with good efficacy. An older brother was free of symptom. WES, performed in P26 and her parents in 2014, had identified two variants in *LRBA*, both inherited from the mother. One variant (c.2835_2838delAGAA) led to a frameshift from the amino-acid number 945 while the second variant (c.1401G>A; p.Met467Ile) was predicted to be tolerated by all algorithms. Since no variant was identified in the father, *LRBA* was not further considered. TNGS was performed on DNA extracted from PBMC of P26 or from umbilical cord of P25 respectively. TNGS confirmed the two maternal variants V1 and V2 and revealed an extended deletion from exon 3 to exon 48 carried by the paternal allele. Lack of LRBA protein was confirmed by Western Blot (not shown).

DISCUSSION

Monogenic enteropathies are individually very rare diseases, which collectively raise difficult diagnosis and therapeutic issues due to their considerable diversity and severity. Herein, we show in a large cohort of 185 patients from 45 Paediatric centres participating in ESPGHAN that TNGS is a sensitive, accurate, and time-effective technique to obtain molecular diagnosis. We propose that TNGS should become one first-line diagnosis test to investigate putative cases of monogenic enteropathies.

Since monogenic enteropathies are rare diseases, Paediatric Gastroenterologists from ESPGHAN created the GENIUS group in 2009 with the goals of implementing clinical knowledge and of fostering development of therapeutic strategies based on precise molecular diagnosis. In the first 106 patients recruited until end of 2012, Sanger sequencing of candidate genes, selected on the basis of clinical presentation and functional testing, yielded diagnosis in 29% cases, mainly affected by mutations in *IL10RI/2*, *XIAP* and *FOXP3*. In 70 patients without diagnosis, WES identified 12 additional patients with causative mutations in genes previously implicated in monogenic enteropathies. Overall, rate of molecular diagnosis was 28% in 154 patients. Yet, WES is time- and cost- consuming. We reasoned that custom-made TNGS should allow us to simultaneously screen numerous candidate genes in a large number of patients, and thereby to accelerate diagnosis in a cost-effective manner. 173 patients without molecular diagnosis and 12 positive controls with known mutations were tested on a targeted panel of 68 genes in which mutations can cause CDD or VEO-IBD. Our TNGS covered 100% of all ROI, including ROI of genes difficult to sequence by NGS (*IKBKG*, *NCF1*) or by Sanger method (*NEUROG3*), and permitted to confirm all known molecular defects, both single nucleotide and exonic copy number variants. Diagnosis yield was 16.2%, raising diagnosis rate in the whole cohort of 216 patients to 33% (Figure 2A). This rate is comparable to that recently reported by

Kammemeier et. al. in 68 patients with VEO-IBD.¹⁹ Importantly, among the 173 patients sequenced by TNGS, 111 did not have any molecular diagnosis despite prior Sanger sequencing of candidate genes or WES. TNGS allowed to reach genetic diagnosis in 17 of them (15%), stressing the efficiency of this technique. Thus, TNGS allowed molecular diagnosis in several patients with atypical presentation in whom phenotype-based approach had failed. Moreover, TNGS revealed one large deletion in *NCF1* not detectable by the commercialized capture kit (Agilent V5) used for WES and one large deletion of *LRBA* difficult to detect by WES. Strikingly, diagnosis rate was much higher in CDD (86%) than in IPEX-like syndromes (30%) and in colites (10%). Discrepancy cannot be ascribed to heterogeneous coverage of targeted genes, or to insufficient depth of coverage. Likely, molecular dissection of CDD is currently more advanced than that of immune-mediated disorders.³ We therefore expect that an updated version of the panel, designed to include untranslated regions (UTR), enhancer regions and novel genes in which enteropathy-causing mutations have been recently identified, will enable to increase diagnosis rate of monogenic diseases by TNGS. It is however possible that colites may not all be monogenic diseases, notably in patients without familial history and in the eldest children included in the cohort. Yet, given increasing evidence of *de novo* mutations, of incomplete penetrance, and/or of very delayed disease onset in patients with loss-of function mutations in *XIAP*, *CTLA4*, *LRBA*, or gain-of-function mutations in *STAT3* or *NLRC4* (Figure 2B),^{13, 14, 20-22} we suggest that access to TNGS screening should not be too stringent and should be considered in patients with severe intestinal diseases refractory to all types of treatments, regardless of familial history or of age of onset.

While efficient to pinpoint molecular defects in genes of interest, TNGS requires rigorous analysis. In our study, elimination of meaningless variants was considerably helped by an in-house software, which predicts consequences on protein structure or function and compares frequency of observed variants with those present in databases, notably ExAC, which provide

sequence of 60 000 exomes from individuals of different ethnic origins and Institut Imagine database.²³ The latter in-house base covers information from over 8000 exomes from precisely phenotyped patients with genetic diseases and their families. Interpretation of novel rare variants remains however challenging when they do not impact protein expression. Consequently, and unless the variant has already been reported as disease-causing, functional validation is essential to prove causality. Herein, novel mutations were validated by testing expression or protein function (Table 2). Thus, performing selected assays to validate variants detected by TNGS was more time and cost-effective than performing broad functional work-up to orientate Sanger sequencing of candidate genes. Moreover, mutations identified by TNGS could be easily confirmed by Sanger method with primers targeting the zone of interest. By circumventing the need of full sequencing of several candidate genes, and notably very large genes such as *LRBA*, which encompasses 58 exons, TNGS can thus save considerable amounts of time and money. We therefore suggest that TNGS is currently the best first-line test to rapidly screen patients for the extensive list of known monogenic intestinal diseases. If negative, patients can next be explored by unbiased but more complex and expensive sequencing methods such as WES and WGS to identify putative novel genetic defects. Early and precise molecular diagnosis is crucial to choose rapidly the most pertinent therapy and to preserve life expectancy and quality. Unfortunately, in our cohort, diagnosis was too late to propound HSCT before colectomy in a boy carrying a *XIAP* mutation.²⁴ In contrast, 10 patients with mutations in *FOXP3*, *XIAP* or *NCF1* are now candidates for HSCT. Moreover, patients with *CTLA4* or *LRBA* mutations are now considered for treatment by CTLA-4 agonist.¹⁴

In conclusion, TNGS is an accurate and time-effective diagnosis tool that can be used as a first-line genetic test to screen putative monogenic enteropathies and to accelerate molecular diagnosis indispensable to optimize patients care. Implementing the list of genes to be studied

upon discovery of novel enteropathy-causing genes is expected to progressively increase sensitivity and diagnostic yield.

AUTHOR CONTRIBUTIONS

FCH, FR and NCB designed the study; FCH and SH were in charge of designing the TNGS; OA and CF performed TNGS; FCH, MP, RDL, JN analysed TNGS data; BB and SR performed experiments; JB performed histology experiments; FRL and EL did the regulatory T cells and LRBA analyses; SL performed XIAP experiments; FR, AF, CP, FRL, SL reviewed data; FCH, and NCB wrote the manuscript that was reviewed by all authors. Members of GENIUS group (marked with an *) were in charge of the patients and acquired clinical data.

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CONFLICT OF INTEREST:

The authors do not declare any conflict of interest.

Tables

Table 1. Validation of the custom-made targeted next generation sequencing.

VS	Sex	Gene	Inheritance	Reads (reference/mutated)	Mutation
VS1	M	<i>ICOS</i>	Homozygous	(3/804)	c.90delG
VS2	M	<i>MYO5B</i>	Homozygous	(4/960)	c.3488T>G
VS3	F	<i>MALT1</i>	Homozygous	(2/894)	c.550G>T
VS4	M	<i>XIAP</i>	X-linked	(38/576)	c.219G>A
VS5	M		X-linked	(1/377)	c.968G>A
VS6	M	<i>FOXP3</i>	X-linked	(1/362)	c.1170T>A
VS7	F	<i>TTC7A</i>	Compound heterozygous	(399/372)	c.1467-2A>G
				(375/394)	c.1469C>T
VS8	F	<i>LRBA</i>	Homozygous	(1/574)	c.6862delT
VS9	F	<i>STAT3</i>	Heterozygous	(386/432)	c.1201A>G
VS10	M	<i>IL10R1</i>	Homozygous	(0/790)	c.1495C>T
VS11	M	<i>IL10R2</i>	Homozygous	(1/987)	c.421G>T
VS12	F		Compound heterozygous	(509/943) 54%	delE3
		(1400/888) 158%		dupLE6	

VS: validation sample; (3/804): 3 reads with the reference nucleotide and 804 with the mutation; delE3: deletion of exon 3; dupLE6: duplication of exon 6; M: male; F: female; for the *IL10R2* genetic events: 509 and 1400 reads in the patient as compared to the mean depth of coverage of 943 and 888 respectively.

Table 2. Novel molecular diagnoses obtained by targeted next generation sequencing.

ID	Sex	Onset (months)	Phenotype	Gene	Inheritance	Mutation	Protein	Reported in	Validated mutation
1	M	10	Colitis	FOXP3	X-linked	c.152G>A	p.Arg51Gln	-	Yes
2	M	4	IPEX-like	FOXP3	X-linked	c.816+5G>C	5_ex8	-	Yes
3	M	0	CDD	SKIV2L	Homozygous	c.1396T>G	p.Trp466Gly	-	Yes
4	M	36	Colitis	TTC7A	Compound heterozygous	V1 c.1001+2T>G V2 c.1433T>C	V1 2_ex7 V2 p. Leu478Pro	25	Yes
5	M	1	CDD	NEUROG3	Homozygous	c.319C>A	p. Arg107Ser	26	-
6	M	0	Colitis	XIAP	X-linked	Deletion of exons 2 and 3		-	Yes
7	F	6	Colitis	XIAP	Heterozygous	c.758C>G	p.Ser253*	16	Yes
8	M	9	IPEX-like	FOXP3	X-linked	c.1190G>A	p.Arg397Gln	27	-
9	F	0	CDD	SKIV2L	Homozygous	c.3187C>T	p.Arg1063*	-	Yes
10	F	120	Colitis	NCF1	Homozygous	Deletion from exon 2		-	Yes
11	M	36	Colitis	NCF1	Homozygous	Deletion from exon 2		-	Yes
12	M	1	Colitis	XIAP	X-linked	c.993_997delAGAAC	fs, stop exon 4	-	Yes
13	M	0	IPEX-like	FOXP3	X-linked	c.816+7G>C	7_ex8	28	-
14	M	2	Colitis	NLRC4	Heterozygous	c.2145T>A	p.Tyr715*	-	No
15	M	6	Colitis	EPCAM	Compound heterozygous	V1 c.412C>T V2 c.425+5G>T	V1 p.Arg138* V2 5_ex3	29	Yes
16	M	7	Colitis	SI	Compound heterozygous	V1 c.3370C>T V2 c.5234T>G	V1 p.Arg124* V2 p.Phe1745Cys	30	No
17	F	0	CDD	MYO5B	Compound heterozygous	V1 c.1117G>T V2 c.2110_2111insT	V1 p.Glu373* V2 fs from Phe 704	-	Yes
18	M	0	CDD	TTC37	Homozygous	c.4572G>A	p.Trp1524*	-	Yes
19	M	4	IPEX-like	LRBA	Compound heterozygous	V1 c.4591T>G V2 c.5893C>G	V1 p.Phe1531Val V2 p.His1965Asp	-	No
20	M	NA	Colitis	XIAP	X-linked	c.295G>T	p.Glu99*	31	-
21	M	1	Colitis	MYO5B	Compound heterozygous	V1 c.4853-1G>C V2 c.5313+3A>G	V1 -1_ex37 V2 3_ex38	-	Yes
22	F	25	Colitis	STAT1	Heterozygous	He c.629G>A	p.Arg210Lys	32	-
23	M	NA	Colitis	IL10RA	Homozygous	c.170A>G	p.Tyr57Cys	33	-
24	M	0	CDD	SKIV2L	Homozygous	c.1312G>A	p.Glu438Lys	-	Yes
25	M	1	IPEX-like	LRBA	Compound heterozygous	V1 c.2835_2838delAGAA V2 c.1401G>A V3 deletion exons 3-48	V1 fs from Glu 945 V2 p.Met467Ile V3 deletion exons 3-48	-	Yes
26	F	1	IPEX-like	LRBA	Compound heterozygous	V1 c.2835_2838delAGAA V2 c.1401G>A V3 deletion exons 3-48	V1 fs from Glu 945 V2 p.Met467Ile V3 deletion exons 3-48	-	Yes
27	F	192	IPEX-like	CTLA4	Heterozygous	c.567+1G>T	1_ex3	-	Yes
28	F	156	IPEX-like	CTLA4	Heterozygous	c.208C>T	p.Arg70Trp	22	-

ID: patient identification number; M: male; F: female; V1: variation 1; fs: frameshift; NA: not available

Figures Legends

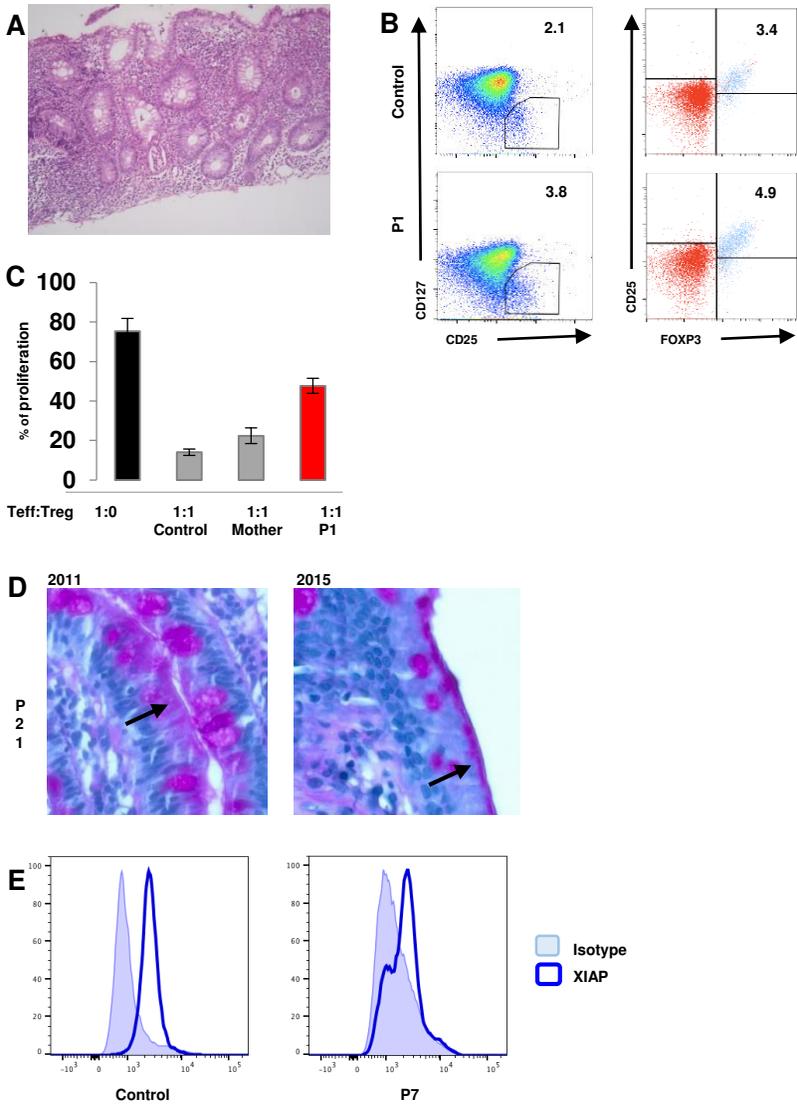
Figure 1. Functional validation of molecular defects identified by TNGS. A: HE staining of colon biopsies from P1 (optical zoom x100). B: Flow cytometry analysis of regulatory T cells (Treg) in P1 and one healthy control. C: Analysis of the suppressor activity of CD4⁺CD25⁺ T cells (Treg) from P1, his mother and one healthy control on the proliferation of control CD4⁺CD25⁻ T cells (Teff). D: PAS staining of duodenal biopsies (2011 and 2015) from P21 (x400). Arrows point out the thick abnormal PAS staining of the brush border, typical of microvillus atrophy. E: XIAP intracellular staining by flow cytometry, gated on CD11c⁺CD14⁺ cells, on P7 and one healthy control.

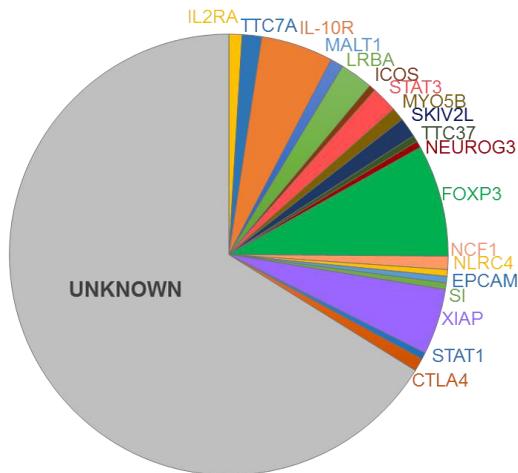
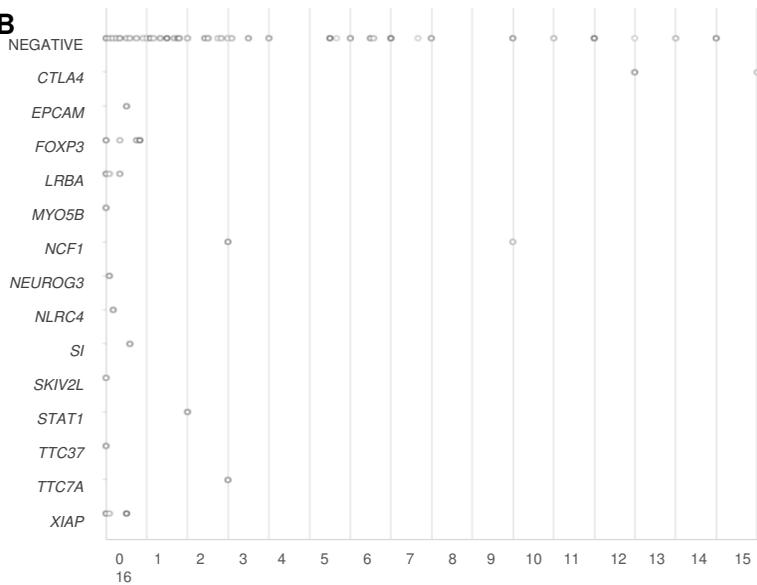
Figure 2. Genetic characterization of the GENIUS cohort. A: Schematic representation of molecular diagnoses obtained in the GENIUS cohort of 216 patients. B: Schematic representation of age at onset of the 185 patients analysed by TNGS, depending on their genotype.

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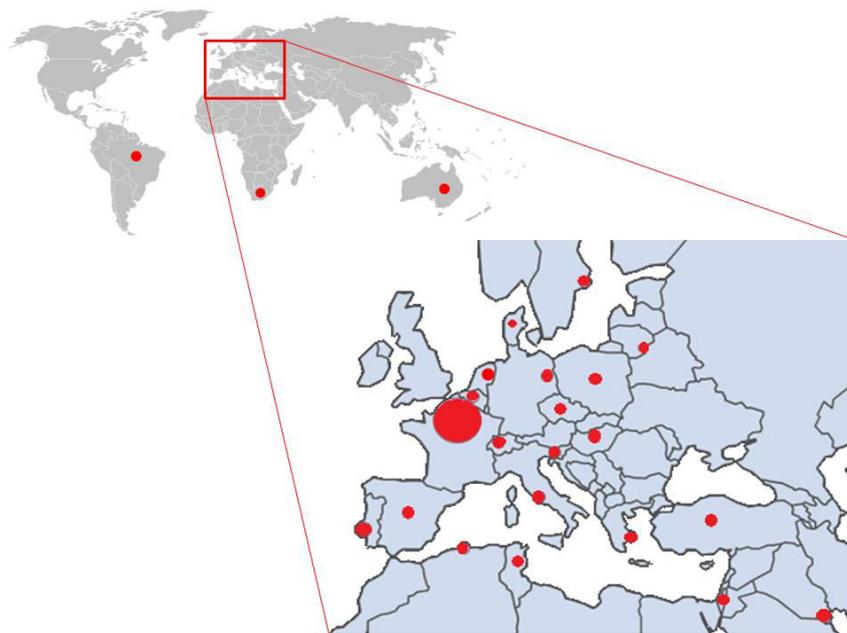


A**B**

DISCUSSION

The main objective of my thesis was to participate in the molecular dissection of key pathways in the gut barrier through phenotypic and genetic characterization of patients suffering from severe and early-onset intestinal disease putatively due to Mendelian disorders. This work was initiated during my Master internship in January 2012. It was part of a novel research axis for the laboratory at a time when the team was offered to join Institut Imagine, a novel institute dedicated to the study of rare monogenic diseases.

Since this study was based on the analysis of patients with very rare diseases, I invested much effort in organizing their recruitment. Since 2014, I am the study coordinator of the IMMUNOBIOTA research protocol. When this protocol was initiated in April 2014, it was planned to include a total of 360 subjects during the five-year inclusion period, including 120 patients (among whom approximately 50% were already included in the prior laboratory cohort) and at least the two parents, and, if possible and pertinent, siblings or any other relatives (healthy or affected). Currently, in August 2016, after 26 months of inclusions, IMMUNOBIOTA gathers 274 subjects including 84 patients in 76 families. Therefore, inclusion rate is beyond expectation. Outside France, patients have been/are recruited through the GENIUS working group of ESPGHAN, initiated by Frank Ruemmele in 2011. All in all, as mentioned in the TGPS article, we collaborate with 45 different centers mostly in European countries (see figure 19), which has allowed a steady rate of inclusions of approximately 50 new patients per year for the past three years.



Figures 19. Participating countries in our cohort.

My second major effort has been to obtain precise phenotypic characterization of included patients. To do so, and to optimize datamining in this cohort of rare patients, I had the chance to collaborate with Nicolas Garcelon, Imagine bioinformatics engineer specialized in database and datamining. Together, we built a dedicated database which contains over 150 fields which can next be used to perform statistical analyses, to extract medical reports, or even to build a timeline for each patient summarizing disease progression (see Figure 20). The database is accessible online, directly (www.database.genius-group.org) or via the GENIUS group website (www.genius-group.org), with personal login and password. Each participating center has access to its own patients' data and all database tools, but cannot access other centers data. The home page of the database offers pooled statistical results from all centers.

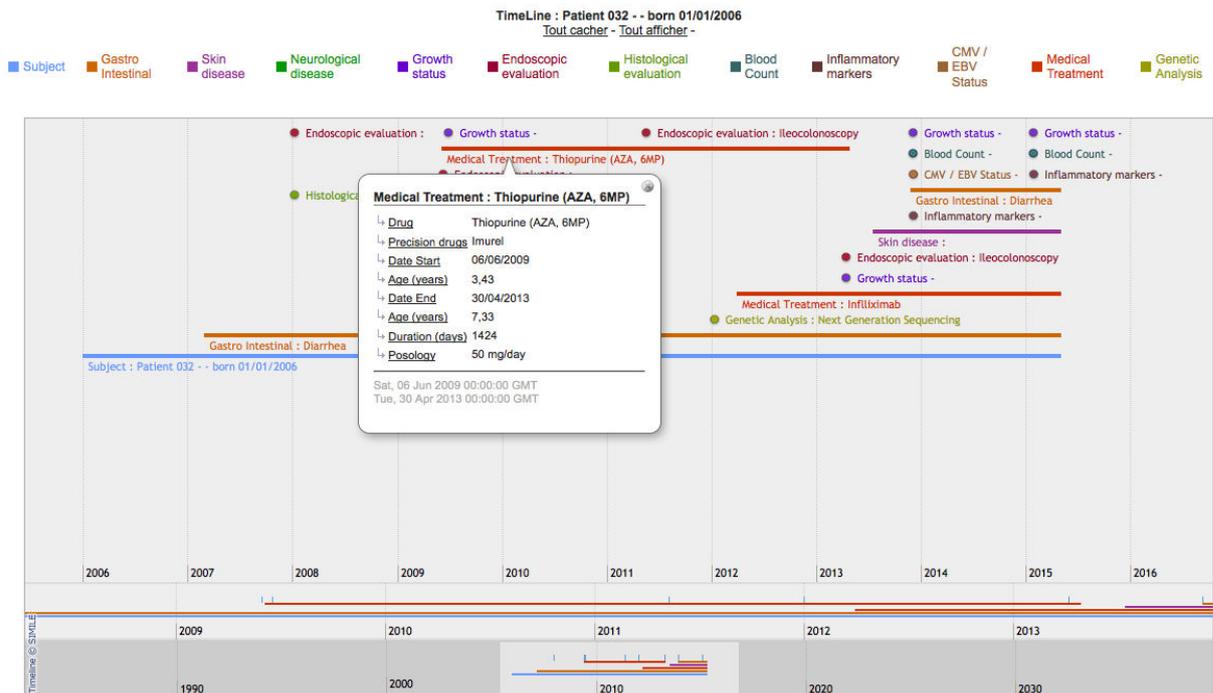


Figure 20. Screen shot of a patient's timeline generated by the database.

Our cohort is comparable to VEO-IBD cohorts described in the literature. In the IMMUNOBIOTA (IMBT) study, as well as in the complete cohort, median age at disease onset is one year. 75% of patients were 3 year-old or younger in the complete cohort versus 4 year-old or younger in the IMBT cohort. VEO-IBD was defined as disease onset before the age of six years (Muise et al.,

2012), in accordance with published cohorts (Kammermeier et al., 2014; Kammermeier et al., 2016a; Kotlarz et al., 2012; Ruemmele et al., 2006). Comparison with a recently described VEO-IBD cohort of 62 patients (Kammermeier et al., 2016a) reveals many similarities, except age at disease onset. Indeed our age criterion for inclusion is not as stringent as in Kammermeier's cohort in which patients were included only if disease started before two. We have chosen to include patients in IMBT if disease starts before the age of six or later in case of familial history suggestive of Mendelian inheritance. We observed the same sex ratio (male 55%), but had two to three times less patients issued from consanguineous parents (Kammermeier et al: 29%; IMBT: 15%; global cohort: 10%), or from multiplex families (Kammermeier et al: 15% versus IMBT: 10% of patients with at least one sibling affected with IBD). Gastrointestinal characteristics bear some similarities: perianal disease was present in 15% of patients in Kammermeier et al versus 13% in IMBT; extensive disease (defined by involvement of both upper gastrointestinal tract and colon) was present in 55-60% of patients in both cohorts. Yet, the two cohorts differ by the frequency of small intestinal histological lesions, villus atrophy being observed in 53% of cases in the IMMUNOBIOOTA cohort versus 30% of cases in the Kammermeier's cohort and the frequency of granulomas which were detected in 24% patients in the Kammermeier's cohort versus 38% in IMMUNOBIOOTA. No granuloma was observed in any of the patients with known "monogenic" diseases identified in the Kammermeier's cohort. Regarding treatments, both cohorts are comparable in terms of need for parenteral nutrition (40% in Kammermeier et al versus 33% in IMBT) or for biotherapy (39% patients in Kammermeier et al required infliximab and/or adalimumab, while 35% patients received infliximab and 18% adalimumab in the IMBT study). At last, molecular diagnosis rate is similar (31% in Kammermeier versus 33% in our global cohort).

Taken together, the cohort of patients built in the laboratory since 2009 is equivalent to other VEO-IBD cohorts. For the past three years, the cohort has a strong and steady rate of inclusion achievable through tight collaborations with many centers in France and in Europe.

At the beginning of my PhD training, some molecular causes of inflammatory monogenic enteropathies had already been identified, mainly IL-10 signaling pathway defects, IPEX syndromes due to *FOXP3* or *IL2RA* mutations, and XIAP deficiency. Therefore, every new patient in the cohort was functionally screened for these three etiologies depending on clinical phenotype (colitis versus autoimmune enteropathy), in collaboration with two teams of Institut Imagine:

Frédéric Rieux-Laucat's lab for Tregs phenotype or suppressive function assay, and Sylvain Latour's lab for XIAP deficiency (protein expression, apoptosis assay and NKT phenotyping is performed by Sylvain Latour's team, whereas functional assay with MDP stimulation is done by our team in patients from both teams). Functional tests led me to help identifying mutations in *FOXP3*, *IL-10R α* , *IL-10R β* , and *XIAP* in 2, 1, 3 and 3 patients respectively. Patients in whom functional tests were negative were then selected for further analysis by WES, with a priority given to familial and/or most severe cases.

For WES, DNA sequencing was/is performed on the Imagine genomic platform. After quality control and initial sequence alignment by Paris-Descartes/Imagine bioinformatics, precise interrogation of the sequences is performed using a dedicated software designed by this platform. Since my M2 internship and all along the thesis, I have invested considerable effort in learning and refining our use of this interface to interpret WES data in the context of a cohort with almost no consanguineous or multiplex families, and with very heterogeneous phenotypes. No gene could be identified in the first 10 WES performed in 2012-2013, likely due to insufficient gene capture and weak depth of coverage as well as to the fact that only index cases were sequenced, precluding the elimination of the multiple intrafamilial non-pathogenic variants.

Starting from 2014, all WES were performed at least on trios (patient and both parents) and sometimes also on other family members (for example, WES was performed on two affected children, two healthy siblings and both parents in a consanguineous family). Yet, parallel WES studies were performed by many research teams studying primary immunodeficiencies as well as VEO-IBD and monogenic enteropathies, leading to identification of novel genes and pathways involved in monogenic enteropathies throughout my PhD. Thus, when we first identified disease-causing mutations by WES in 2014, comparable findings had just been published (which were not described when WES was launched). In total, WES allowed the identification of 11 genetic defects in 59 patients analyzed in trios or more (18.6% diagnosis rate). This work led to report the case of two siblings affected by a novel *MALTI* mutation as their phenotype (IPEX-like syndrome, abnormal Tregs numbers, and favorable outcome of HSCT) completed the description of previously reported patients.

Yet, obtaining a molecular diagnosis by WES on a gene already implicated in monogenic enteropathy is expensive and time-consuming. Furthermore, considering that overlapping phenotypes were, and are still, more and more frequently reported, and that the list of genes involved in monogenic enteropathies is constantly increasing, we came to the conclusion that another

genomic diagnosis tool was needed to screen patients before performing WES. We also reasoned that genetic approach offered some practical advantages compared to functional testing, as DNA handling and sequencing is less challenging than the processing of peripheral blood monocytes or biopsies (Uhlir et al., 2014).

Therefore, in collaboration with Sylvain Hanein from the Translational Genetic platform, I set up a first version of a custom-made TGPS, which encompassed 68 genes involved in monogenic enteropathies, either inflammatory or related to epithelial defects. 185 patients were sequenced with this version, as well as 50 patients from Frederic Rieux-Laucat's cohort of patients with autoimmunity. A second version now encompasses 100 genes including recently described enteropathy-causing genes (*A20*, *DUOX2*, *NOX1*...), genes involved in monogenic pancreatic diseases, in chronic intestinal pseudo-occlusion syndrome, and in CVID, as well as genes known to carry the heaviest risk factors for adult polygenic IBD (*NOD2*, *ATG16L1*, *IRGM*, *IL23R*, *FUT2*). Fifty new patients, recruited since January 2016, are currently sequenced on the TGPS version 2. We have not received their data yet.

As discussed in the TGPS article, this technique is more effective than WES in term of diagnosis. Notably, TGPS is cost and time-effective and permits better capture and depth of coverage thus allowing detection of some mutations (notably deletions and insertions) missed by WES. Yet of course, it cannot pinpoint variant in new candidate genes. Based on our experience, we suggest that TGPS must be used as a first-line genetic tool, and then followed by validation of identified mutations or WES if negative. In Necker-Enfants Malades hospital, we have successfully initiated a multidisciplinary meeting after each TGPS run (three for TGPS version 1). During these meetings, which gathered pediatric and adults gastroenterologists, researchers from the lab, pathologists as well as experts in immunodeficiencies, each candidate mutation pinpointed by TGPS was discussed in the context of the medical file in order to decide collegially its pertinence and how to perform functional validation (if the mutation was not already described as disease-causing in the literature). Indeed, implication of novel variants not previously reported as disease-causing requires rigorous interpretation and functional validation since computational mutation prediction can be misleading. For example, most protein prediction algorithms are based on interspecies conservation and sometimes on biochemical properties but they do not integrate 3D structure putative changes and do not give indication on whether the mutated amino-acid belongs to a functional domain or not. Thus, two disease causing mutations in *IL10RA* were considered as "tolerated" by SIFT algorithm while PolyPhen classified them as "damaging" (Kotlarz et al., 2012).

Among the patients in whom a molecular diagnosis was identified by TGPS, half of them suffered from a disease that evolved since 7.5 years and up to 18 years. Such a long delay in diagnosis can be highly detrimental for patients with monogenic enteropathies that are often severe and refractory diseases requiring aggressive therapies. Along this line, one patient with *XIAP* mutation was diagnosed too late to benefit from HSCT and avoid colectomy (see TGPS article). Moreover, emergence of tailor-made therapies able to specifically target the molecular defect is a compelling reason to reach molecular diagnosis as early as possible. For instance, VEO-IBD patients due to auto-inflammatory disorders like *MVK*, *NLRC4* or *A20* mutations could benefit from IL-1 β receptor antagonist whereas IPEX-like patients with *CTLA4* or *LRBA* mutations could be highly improved by CTLA4 agonists. Another argument to establish an early genetic diagnosis is the possibility to anticipate some complications or extra-intestinal manifestations. For example, *XIAP* mutated patients are particularly prone to HLH, IL-10R deficient patients can develop B-cell lymphoma, and autoimmunity is prone to arise in patients carrying mutations in genes involved in IPEX-like syndromes (Uhlig et al., 2014). At last, obtaining a molecular diagnosis is essential to any genetic counselling that could be sought by some families.

When suspect monogenic enteropathy?

As written above, VEO-IBD and CDD raise difficult diagnosis and therapeutic issues due to their considerable diversity and severity. Establishing an early molecular diagnosis is crucial to optimize patient's care.

Therefore, what are the key criteria that can be inferred from descriptions of both known monogenic enteropathies as well as of our cohort of patients and that should lead to suspect a monogenic disorder?

One feature, most frequently advertised is the early age at onset. Most CDD start at birth, sometimes in the following months. The majority of VEO-IBD start before two years. IBD starting before the age of six is more likely due to monogenic defects than cases of later onset and genetic investigation needs to be considered (Muisse et al., 2012). Yet delayed onset after six years does not allow to eliminate a causative Mendelian defect. Thus several monogenic disorders, especially those recently described as monoallelic diseases (such as *NLRC4*, *CTLA4*, or *STAT3* GoF) can appear until the age of 40 (see figure 21 from (Uhlig et al., 2014) as well as figure 5 in TGPS article).

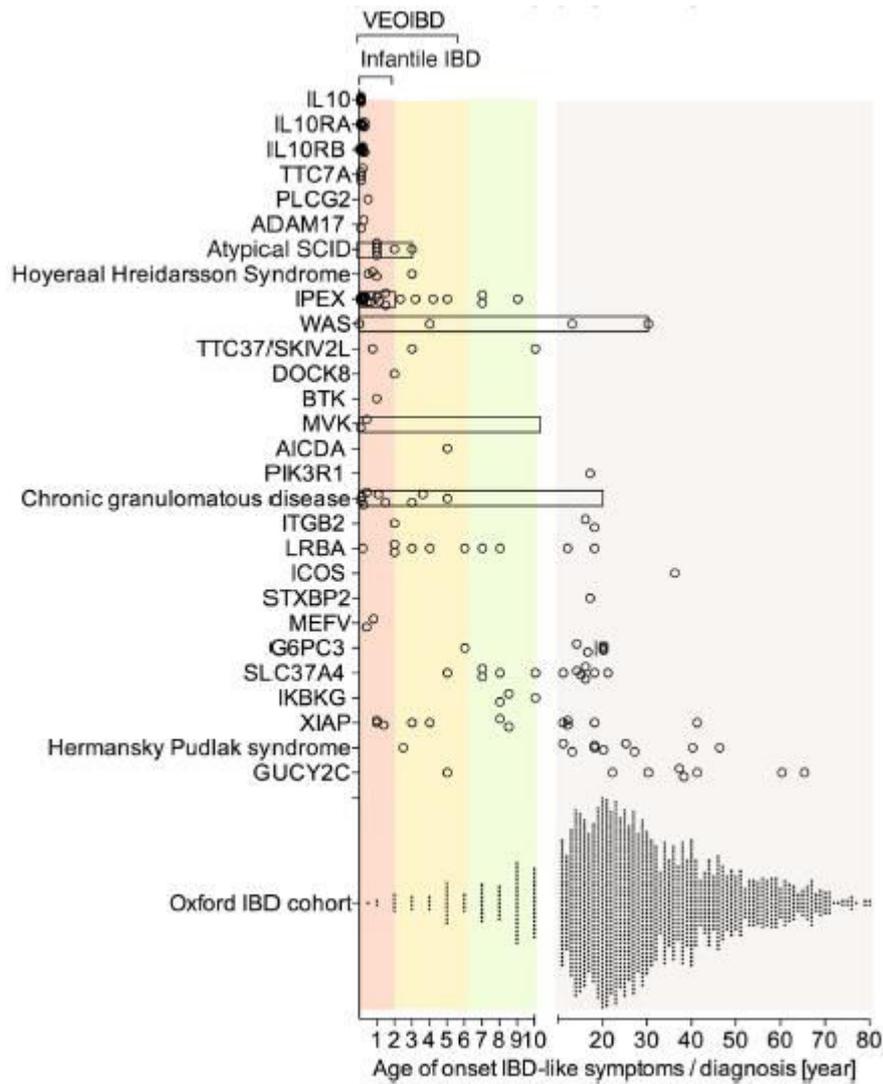


Figure 21, from (Uhlir et al., 2014)- Age of onset of IBD-like symptoms in patients with monogenic diseases. Multiple genetic defects are summarized in the group of atypical SCID, Hoyeraal–Hreidarsson syndrome, CGD, and Hermansky–Pudlak syndrome. By comparison, an unselected IBD population is presented (Oxford IBD cohort study; pediatric and adult referral-based IBD cohort, $n = 1605$ patients comprising CD, UC, and IBD unclassified [IBDU]). Symbols represent individual patients. Bars represent the age range of case series if individual data were not available.

In cohorts of patients with a disease onset predominantly before six years of age, the current diagnosis rate is around 33%. Of course, the later the disease starts, the less likely it is to be a monogenic defect. As screening tools like TGPS are quite cost and time-effective, would it be manageable to use it in large cohorts, as pediatric IBD or even adult IBD? In this case, the workload and amount of money necessary would be important, and the diagnosis rate would drop tremendously. But, it may allow the identification of a Mendelian disorder in a very few number of patients who would not have been investigated otherwise, knowing that treatment could be optimized and some complications could be foreseen. All in all, the question is: what is the human and monetary “cost” that we are willing to pay to diagnose one unsuspected patient?

Apart from age at onset, familial history is equally a good clue for the monogenic origin of IBD. Yet, multiplex families are rare, and *de novo* mutations are reported on a regular basis, especially in hemizygous (*FOXP3*, *XIAP*) or heterozygous mutations (*STAT3* and *NLRC4* GoF, *CTLA4* or *A20* haploinsufficiency). But, more importantly, penetrance of many disorders is incomplete and their expression highly variable. For example, only 20-25% of *XIAP* mutated patients display an IBD phenotype. With the massive development of next-generation sequencing in all kind of cohorts, a lower penetrance than previously described is more frequently reported. It even seems possible to be completely resilient to mutations previously thought to be completely penetrant. Indeed, Chen et al identified recently 13 adults carrying mutations for eight severe Mendelian childhood diseases (*CFTR* or *AIRE* mutations for example) by screening 874 genes in 589,306 genomes. This study might raise some critics: almost 500,000 genomes were analyzed by genotyping array and not by whole exome or genome sequencing; individuals were considered as resilient because clinical manifestations of the indicated disease were absent when patients were recruited in one of the 12 compiled genetic studies used but none of them could be recontacted, and none of these studies had collected metadata. Yet, how could we explain their resilience? Through modifier genes? Or “protective gene variants” as suggested by the authors? Identification and extensive analysis of such individuals could lead to exciting new discoveries in terms of treatments (Chen et al., 2016).

Patients suffering from monogenic enteropathies share one common trait regarding their intestinal disease: its severity and resistance to treatments. Kammermeier and colleagues identified three interesting features highly predictive of monogenic etiology: i) extensive disease was reported in 37% “monogenic patients” versus 8% in “non-monogenic patients” (p 0.049); ii) epithelial abnormalities (defined as morphological abnormal IEC or florid apoptosis in IEC) when present was reported in 90% of “monogenic patients” versus 19% in “non-monogenic” ones (p 0.001), and iii) parenteral nutrition requirement was observed in 60% of “monogenic patients” compared to 11% in “non-monogenic” ones (p 0.001) (Kammermeier et al., 2016a). Similarly, resistance to

treatments is often reported (Ruemmele et al., 2006), but it could be overlooked especially in older patients (for example *XIAP* mutated patients or *CGD* patients) who can exhibit a disease course completely similar to severe CD.

Occurrence of atypical symptoms may be of help for recognition of monogenic enteropathies. Macrophage activation syndrome, HLH, intestinal atresia, or hair abnormalities for example are highly suggestive of some genetic defects that will easily trigger specialized investigations. On the other hand, multiple and/or recurrent infections, a clear sign of PID, could be falsely ascribed to immunodeficiency secondary to immunosuppressive treatments, or growth failure could be confounded for malabsorption or systemic chronic inflammation.

All in all, suspicion of monogenic enteropathy should be raised by the association of some characteristics as age at onset, familial history, disease severity and resistance to treatments, as well as occurrence of atypical symptoms.

CONCLUSION and PERSPECTIVES

Molecular diagnosis rate in monogenic enteropathies is globally estimated around 33%, with much higher rates in CDD and IPEX-like syndromes compared to VEO-IBD with predominant colitis. Therefore, even though some of these VEO-IBD patients do not carry monogenic defects, continuing their characterization should lead to new and exciting discoveries, most likely through next generation sequencing techniques. Recently, Zhang, Su and Lenardo estimated that next generation sequencing analysis of 200 trios (child and parents) should generate 200 terabytes of data that would require one to two bioinformatics specialists to extract most relevant candidate variants. Those variants would then necessitate ten to twelve biochemists and immunologists for their validation and investigation (Zhang et al., 2015). During my PhD, I did not get the chance to identify a novel gene in monogenic enteropathy, but not for lack of trying! Mutations selected in four genes were tested extensively but were proved to be false. Learning from our mistakes these past five years, and even if our team is not as big as the “dream team” described above, we have presently selected four candidate genes in unrelated families through WES: gene A is linked to autophagy in a boy with isolated pancolitis, gene B is involved in TGF- β signaling in two siblings from a consanguineous union with several extra-intestinal symptoms, gene C, which encodes an epithelial protein already involved in intestinal inflammation, has been identified in a boy with a severe pancolitis that required a pancolonic resection, and gene D, which belongs to the JAK-STAT pathway, seems to be causative in a girl with severe auto-immune enteropathy. Hopefully, ongoing functional validation will be successful for at least one of them.

Mucosae are privileged interfaces, where host cells are in constant contact with their environment, where a balanced dialogue must be established, where loud and angry speeches must be avoided as well as too soothing ones. Among mucosal tissues, the gut barrier is maybe the most fascinating, when one takes into account the trillions of bacteria and the tons of food antigens that need to be dealt with along an entire life-span. The complexity of interlinked pathways required to maintain a healthy intestinal barrier is humbling. Studying these exciting interactions will keep leading on to important discoveries that will hopefully be translated into new therapeutic options, with the hope of cure for patients suffering from chronic intestinal diseases.

SUPPLEMENTARY DATA

Identification of two novel mutations gain-of-function of STAT3 responsible for severe enteropathies

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INTRODUCTION

Non celiac enteropathies with villous atrophy are rare but severe diseases. They can be divided into two subsets: 1- auto-immune (AI) enteropathy characterized by the presence of anti-enterocyte antibodies; 2- enteropathies without anti-enterocyte antibodies often associated with immunoglobulin (Ig) deficiency and other extra-intestinal manifestations. The pathophysiology of the non-celiac enteropathies and the causative role of predisposing genetic factors remain largely unknown. Whole exome sequencing (WES) is an effective tool to identify single gene mutations responsible for monogenic diseases. Here we report the identification of 2 novel germline mutations of STAT3 responsible for enteropathies without anti-enterocyte antibodies in 3 individuals.

PATIENTS AND METHODS

Two female patients (II.2, V.2) had severe non-celiac enteropathies with villous atrophy. They both had no anti-TG2 antibodies and were negative for the HLA DQ2/DQ8 haplotype. They had no evidence of malignant lymphoproliferation (B and T clonality negative). The search for anti-

enterocyte antibodies by immunohistochemistry and the dosage of anti-harmonin or anti-75kD antibodies were negative. Their clinical characteristics are summarized in Table 1.

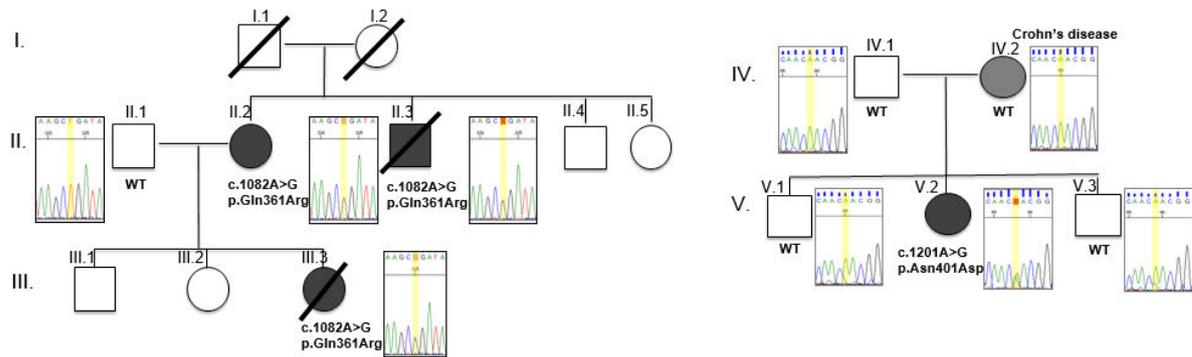
Exome sequencing was performed on DNA obtained from blood mononuclear cells on the platform of the Imagine Institute (Illumina sequencer HiSeq2500). Patient V.2's DNA was sequenced with both parents. Patient II.2's DNA was sequenced with the DNA from her brother, her daughter (both deceased) and from her daughter's father. Data were analyzed with an in-house software developed by the bioinformatic platform of University Paris Descartes. The mutations were confirmed by Sanger sequencing. Suppressor of cytokine signaling 3 (SOCS3) is a major downstream target of STAT3 and a negative regulator of STAT3 signaling. Gain-of-function was assessed by comparing SOCS3 transcription levels in EBV lines from patients, from a healthy control and from a positive control (a patient carrying the gain-of-function mutation p.T716M described in Flanagan et al) with or without stimulation by interleukin 21 (IL-21) at different concentrations (0, 1, 5, 10, 50ng/ml).

	Age of onset	Digestive disease	Auto and dysimmunity
Patient V.2 c.1201A>G p.N401D <i>de novo</i>	5 months	Enteropathy with subtotal villous atrophy	AI thyroiditis, AI pancytopenia, IgG deficiency
Patient II.2 c.1082A>G p.Q361R	25 years	Enteropathy with total villous atrophy, colitis and lymphocytic gastritis	Vitiligo
Brother of patient II.2 c.1082A>G p.Q361R Deceased at 48y	25 years	Enteropathy with total villous atrophy	Polyarthritits, psoriasis, vitiligo, IgG deficiency
Daughter of patient II.2 c.1082A>G p.Q361R Deceased at 11y	3 years	Auto-immune hepatitis	Polyarthritits, scleroderma, heart failure

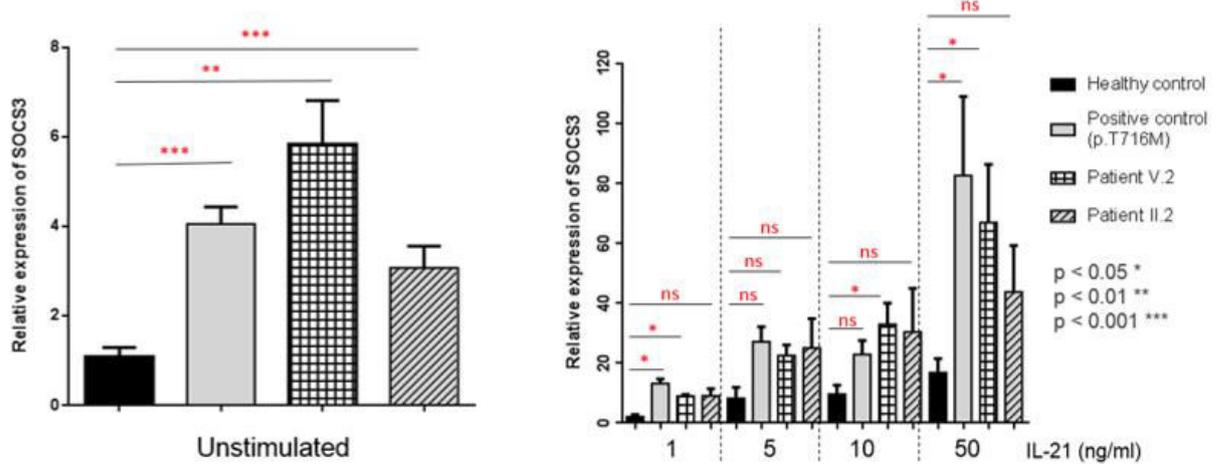
Table 1. Clinical summary

RESULTS

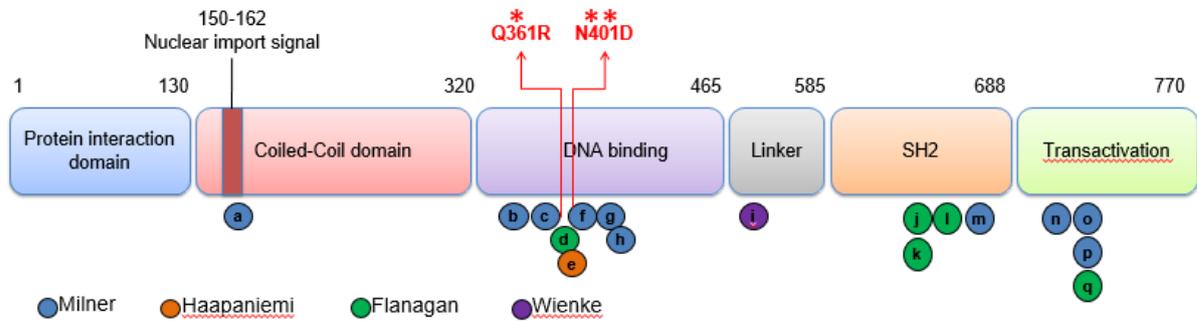
Both mutations are missense heterozygous and have not been described in the literature. The mutation of patient V.2 was not found on her parents strongly suggesting a *de novo* mutation. On the other hand, the mutation of patient II.2 was also found on the WES of her deceased brother and daughter confirming its familial character.



Familial trees of both families with Sanger sequencing results



Augmentation of SOCS3 expression in EBV from both patients



a: R152W; b: Q344H; c:V353F; d: K392R; e: M394T; f: E415K; g: N420K; h: G421R; i:P471R; j-k:N646K; l:K658N; m:T663I; n:A703T; o-p-q: T716M

* II. 2

** V.2

Schematic representation of STAT3 with the two novel mutations and the gain-of-function mutations previously reported

CONCLUSION

Herein are reported two novel gain-of-function mutations of *STAT3* responsible for severe non-celiac enteropathies. This work highlights the broad spectrum of phenotypes associated with this monogenic disease: a wide age of onset (from 5 months to 25 years), a variable severity of the enteropathy, diverse dysimmune and auto-immune symptoms with some variable penetrance. Furthermore it emphasizes the necessity to reach an early molecular diagnosis in severe and/or atypical non-celiac enteropathies which is now easily accessible through next-generation sequencing tools such as targeted gene panel sequencing.

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On the following pages are enclosed three articles written in collaboration during my PhD. In these articles, we did the genetic analysis which led to molecular diagnoses.

- **XIAP article : published**
- **LRBA article: *Epub ahead of print***
- **NEUROG3 article: accepted, *in revision***

Refractory monogenic Crohn's disease due to X-linked inhibitor of apoptosis deficiency

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Dear Editor:

Crohn's disease (CD) is an idiopathic, chronic inflammatory process that can affect any part of the gastrointestinal tract. Susceptibility to disease is influenced by a complex interplay of genetic and environmental factors. Most of the genes thought to be involved in the development of the disease play a role in mucosal immunity, and their products are found on the mucosal barrier epithelium.

X-linked inhibitor of apoptosis (XIAP) deficiency (also known as X-linked lymphoproliferative syndrome type 2, XLP-2) is a rare primary immunodeficiency. Since the disease was clarified as a unique entity in 2006, more than 70 cases have been reported. The main clinical features of XLP-2 are elevated susceptibility to hemophagocytic lymphohistiocytosis, recurrent splenomegaly, and inflammatory bowel disease (IBD) with the characteristics of CD [1].

The first description of severe CD in XIAP-deficiency was published in 2011 [2]. Today, XIAP variants in male patients with pediatric-onset CD represent about 4 % of patients and

are characterized by refractoriness to several treatments [3]. The clinical spectrum of the 27 patients with XIAP deficiency was recently reported [1]. Seven of these patients developed severe IBD resembling CD with clinical findings of granulomatous inflammation, recurrent colonic strictures, severe perianal fistulas, as well as, pancolitis and ulcerations affecting stomach and small bowel [1].

Herein, we describe a patient with XIAP-deficiency with disease onset at 13 years of age with a severe CD-like illness, refractory to several medical and surgical treatments.

A 13-year-old male patient complained of a scrotum abscess without any other symptoms. The past medical history showed a normal length and weight development, infectious mononucleosis, viral meningitis, and Henoch-Schonlein purpura at 3, 6, and 8 years of age, respectively. He had no family history of IBD. A presumptive diagnosis of a CD was made at 13 years of age with Montreal classification A1L2B1p. The patient began therapy with azathioprine (2.0 mg/kg/day). Despite the treatment, 2 years later, he started to complain

Rosa Coelho and Armando Peixoto contributed equally to this work.

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about abdominal pain, bloody diarrhea, and fever. He was admitted at the local hospital and intravenous steroids and antibiotics were started.

At 17 years of age, he was referred to our hospital as he maintained fever and diarrhea with malnutrition despite parenteral feeding for several weeks. At this time, an esophagogastroduodenoscopy showed a normal macroscopic appearance of the stomach and esophagus. The biopsies revealed focal active gastritis with a granuloma. Flexible sigmoidoscopy showed deep ulcerations with friable mucosa up to 30 cm from the anal edge. Perianal examination showed a complex perianal fistulating disease. Broad-spectrum intravenous antibiotics were administered followed by perianal surgery with placement of setons and drains.

One month later, he started infliximab, and as the perineal fistula persisted with drainage after the induction period, a diverting sigmoid colostomy was performed. One month after the abdominal surgery, a colonoscopy through the colostomy showed involvement of the descendent colon with skip lesions characterized by erythema and friability of the mucosa. At this time, the patient was under combo therapy with infliximab and azathioprine. As he maintained active luminal and perianal disease, he started subcutaneous methotrexate 20 mg/week and infliximab was increased up to 10 mg/kg/injection.

A few months after, at the age of 18, he had an anterior uveitis that was solved with topical steroid. After this episode, a scrotum metastatic CD was diagnosed through a cutaneous biopsy from a scrotum lesion and he maintained a destructive perianal disease with a scrotum fistula. After several perianal surgeries, he underwent ileostomy and within 5 months, he was admitted to the intensive care unit with a severe sepsis due to a perianal abscess, leading to the indication of total proctocolectomy which was performed at 19 years. Three months later, he started on weekly adalimumab and 13 months later, a subcutaneous para-umbilical abscess was diagnosed. At this time, the computed tomography-enterography showed a fistulous tract between an ileal loop and the right seminal vesicle. Nowadays, the main symptoms are skin abscesses predominantly localized in the lower limbs, managed with antibiotics and surgical drainage. He maintained a highly destructive perianal disease with some perianal drainage.

At this point, recognizing the severity of CD course, refractory to many medical and surgical treatments, further work-up was conducted to investigate for genetic or acquired immunodeficiency. One of the tests performed was a functional screening assay for XIAP deficiency was performed. In this test, interleukin 8 (IL-8) is measured by ELISA (enzyme-linked immunosorbent assay) after supernatants of lipopolysaccharide (LPS)/muramyl dipeptide (MDP) stimulation. Our patient was unable to produce any IL-8, a result highly suggestive of XIAP deficiency.

The identification of a non-sense mutation p.W323X (c.969G>A) confirmed the XIAP deficiency. This mutation leads to a complete loss of protein expression as shown in a

western blot of T cell blasts lysates. The patient's mother was also tested and found to be heterozygous carrier for the same mutation c969G>A, but she has remained always asymptomatic.

Deficiency of XIAP is a newly recognized disorder with the first case being described in 2006 [2]. Apart from hemophagocytic lymphohistiocytosis syndrome, an IBD-like illness can occur in 26 % of the patients, clinically and histologically indistinguishable from CD [3]. Thus, XIAP-deficiency has been proposed as a Mendelian cause of IBD.

Patients with XIAP-deficiency have a CD-like disorder with a clinical severe course [4].

The methods that allow the diagnosis of XIAP deficiency are as follows: gene sequencing and western blotting of lysates of T cell blasts [3] or peripheral blood mononuclear cells [2]. Flow cytometry can also be used; however, this result is dependent on the protein expression. Actually, gene sequencing is considered the gold standard test to diagnosis a XIAP deficiency, the diagnosis is later confirmed with a protein expression analysis. It is also important to highlight that the functional test allows a prompt screening of the patients before performing gene sequencing.

In our case it was first performed a functional test on the XIAP pathway, and considering the abnormal results, the XIAP gene was sequenced in order to identify the mutation. This diagnosis could be coupled to a test that evaluates the protein expression using western essay or throughout other functional tests (e.g., apoptosis susceptibility).

In our patient, a non-sense mutation was identified by gene sequencing. This mutation was previously described by Zeissig et al. [4]. After that, western blot was used to compare the level of protein expression in T cell blasts from our patient (XIAPW323X) with cells of two healthy donors (control 1 and control 2).

In summary, we wish to highlight the clinical features of XIAP deficiency. This deficiency should be considered in all patients with a severe course of CD and refractoriness to immunomodulators even if the disease apparently starts beyond childhood.

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Atypical Manifestation of LPS-Responsive Beige-Like Anchor Deficiency Syndrome as an Autoimmune Endocrine Disorder without Enteropathy and Immunodeficiency

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Monogenic primary immunodeficiency syndromes can affect one or more endocrine organs by autoimmunity during childhood. Clinical manifestations include type 1 diabetes mellitus, hypothyroidism, adrenal insufficiency, and vitiligo. Lipopolysaccharide (LPS)-responsive beige-like anchor protein (LRBA) deficiency was described in 2012 as a novel primary immunodeficiency, predominantly causing immune dysregulation and early onset enteropathy. We describe the heterogeneous clinical course of LRBA deficiency in two siblings, mimicking an autoimmune polyendocrine disorder in one of them in presence of the same underlying genetic mutation. The third child of consanguineous Egyptian parents (Patient 1) presented at 6 months of age with intractable enteropathy and failure to thrive. Later on, he developed symptoms of adrenal insufficiency, autoimmune hemolytic anemia, thrombocytopenia, and infectious complications due to immunosuppressive treatment. The severe enteropathy was non-responsive to the standard treatment and led to death at the age of 22 years. His younger sister (Patient 2) presented at the age of 12 to the endocrinology department with decompensated hypothyroidism, perioral vitiligo, delayed pubertal development, and growth failure without enteropathy and immunodeficiency. Using whole exome sequencing, we identified a homozygous frameshift mutation (c.6862delT, p.Y2288MfsX29) in the *LRBA* gene in both siblings. To our knowledge, our patient (Patient 2) is the first case of LRBA deficiency described with

Abbreviations: Ab, antibodies; aTPO, antithyroperoxidase; AP, alkaline phosphatase; BEACH, the beige and Chediak-Higashi syndrome-domain; GAD65, glutamat-decarboxylase; IGFBP3, insulin-like growth factor-binding protein 3; IA2, islet autoantibody-2; IAA, insulin autoantibodies; IGE, insulin-like growth factor; LYST, lysosomal trafficking regulator; LPS, lipopolysaccharide; PHA, phytohemagglutinin; PTH, parathormone; SEE, staphylococcal enterotoxin E; TSH, thyroid-stimulating hormone; TG, thyroglobulin.

predominant endocrine phenotype without immunodeficiency and enteropathy. LRBA deficiency should be considered as underlying disease in pediatric patients presenting with autoimmune endocrine symptoms. The same genetic mutation can manifest with a broad phenotypic spectrum without genotype–phenotype correlation. The awareness for disease symptoms among non-immunologists might be a key to early diagnosis. Further functional studies in LRBA deficiency are necessary to provide detailed information on the origin of autoimmunity in order to develop reliable predictive biomarkers for affected patients.

Keywords: autoimmune thyroiditis, lipopolysaccharide responsive beige-like anchor gene, autoimmune enteropathy, stem cell transplantation, genotype–phenotype correlation

INTRODUCTION

A series of monogenic primary immunodeficiency disorders (PIDs) has been described, causing a combination of immunodeficiency and multi-endocrine disorders. A prime example is the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome (APECED) as a monogenic immune disorder caused by mutations in autoimmune regulatory (*AIRE*) gene. Further examples include immunodeficiency, polyendocrinopathy, x-linked (IPEX) syndrome (1), Stat5b-deficiency (2), CD25-deficiency (3), and CTLA-4-haploinsufficiency (4), which all can present with immunodeficiency and endocrine disorders. Mutations in Lipopolysaccharide responsive beige-like anchor (LRBA) gene have been introduced as a PID with a predominant polyautoimmune phenotype (5, 6). Affected individuals typically present with severe enteropathy in early life and may suffer from hypogammaglobulinemia, pulmonary disease, lymphoproliferative disorder (7), and infancy-onset type 1 diabetes mellitus (8). Recently, Alkhairy et al. described autoimmune thyroiditis as a part of the disease symptoms in 3 out of 31 affected patients (9). A predominant endocrine phenotype of LRBA deficiency without symptoms of enteropathy and immunodeficiency has not been described yet. Here, we describe the detailed immunology and endocrine profile of two LRBA-deficient siblings sharing the same genetic mutation with lack of LRBA protein expression. The older sibling (Patient 1) suffered from a combination of complex immune dysregulation caused by LRBA deficiency, whereas the younger sibling (Patient 2) predominantly developed multi-endocrine symptoms with thyroiditis, vitiligo, and growth retardation.

PATIENTS AND METHODS

Patient 1

The third of the four children of consanguineous Egyptian parents presented, at the age of 6 months, with intractable diarrhea resulting in maldigestion and malabsorption. Over the first 4 years of life, the patient required steroid medication. Consecutively, secondary adrenal insufficiency with hypocortisolemia occurred. There was no evidence of underlying growth hormone deficiency or autoimmunity against pituitary gland and testicular tissues. Endocrine features such as growth retardation, delayed puberty, and osteoporosis were related to the severe course of chronic

illness. By the age of 14 years, two episodes of autoimmune hemolytic anemia and one episode of rhabdomyolysis occurred, followed by polyserositis (**Figure 1A**). Initial combinatorial treatment with glucocorticoids and sirolimus resulted in short remission. However, further intensification of the immunosuppressive therapy including administration of azathioprine and tacrolimus as well as splenectomy were necessary to ameliorate autoimmunity. The severe enteropathy continued to be the predominant complaint and remained non-responsive to immunosuppressive treatment (**Table 1**). The functional short bowel syndrome resulted in severe cachexia with a body weight of 33.5 kg at 20 years despite long-term parenteral nutrition. The course of the disease was complicated by secondary end organ damages. The patient suffered from liver disease, esophageal varicose veins, and neuropathy. The option of allogeneic hematopoietic stem cell transplantation (alloHSCT) combined with short bowel and liver transplantation was considered at that stage. Intensified immunosuppression led to severe recurrent infections refractory to

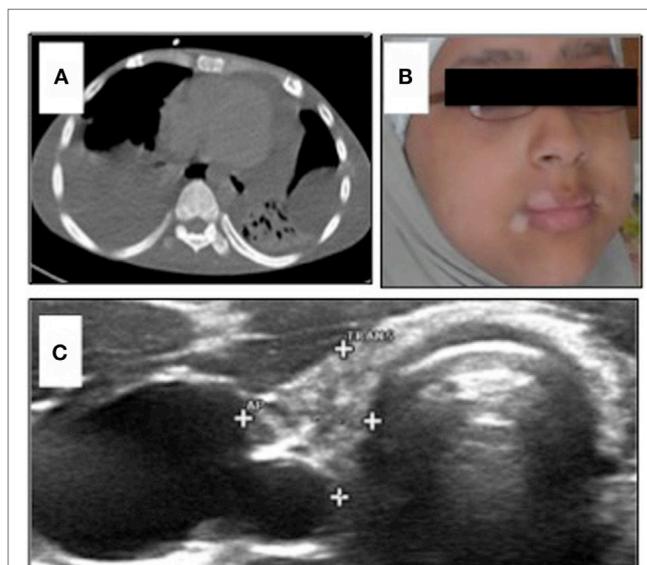


FIGURE 1 | (A) Chest CT-scan of Patient 1 showing severe polyserositis with pleural and pericardial effusions; **(B)** patient 2 with progressive perioral vitiligo without other abnormalities in her face; and **(C)** ultrasound of the thyroid gland in Patient 2 showing a hypotrophic thyroid gland with 2.5 ml volume and several nodules <5 mm.

TABLE 1 | The course of the disease and clinical complications.

	Immunosuppression
Patient 1	
6 months: severe pan-colitis, failure to thrive	Steroids
2 years old: chronic diarrhea, TPN-dependent	
5 years old: hearing loss	
10 years old: subclavian vein thrombosis	
14 years old: AI-hemolytic anemia, rhabdomyolysis	Splenectomy
15 years old: severe cachexia, liver failure	Ciclosporine
17 years old: recurrent GI-bleeding (ICU)	Tacrolimus
20 years old: neuropathy, seizure	Sirolimus
21 years old: 2x AI-hemolytic anemia, AI-thrombocytopenia, and polyserositis	Azathioprine
21 years old: chronic osteomyelitis (complete destruction of the joint)	
22 years old: respiratory failure, death	
Patient 2	
12 years old: AI-thyroiditis, starting on L-thyroxine	None
14 years old: vitiligo	None
15 years old: 2x decompensated hypothyroidism	None

antibiotic treatment and surgical intervention. The patient lost his lower leg due to chronic osteomyelitis at the age of 21 years prior to scheduled from a HLA-identical family donor. The patient died due to recurrent septic complications and respiratory failure after lower leg amputation prior to the transplantation.

His initial laboratory results showed mild CD4⁺-lymphopenia [CD4 505/ μ l (NR 700–1300)] with normal distribution of CD8⁺ T-cells, NK-cells (CD3⁺CD56⁺), and B-cells (CD19⁺). IgA deficiency was observed with normal IgG, slightly reduced IgM, low tetanus vaccine antibodies, and normal hepatitis B vaccine antibodies. Thyroid hormones, growth factors, and PTH were within the normal range with low levels of vitamin D [<5 ng/ml (NR 20–30)] (Table 2). Sanger sequencing showed no *FoxP3* mutation indicative of IPEX syndrome, and the presentation was evaluated as IPEX-like.

Patient 2

The younger sister of Patient 1 was admitted to the endocrinology department at the age of 12 years with decompensated hypothyroidism [TSH > 200 mU/l (NR 0.5–3.6)]; fT4 0.1 ng/dl (NR 0.9–1.6), related growth retardation ($<P3$), pubertal arrest, and progressive perioral vitiligo (Figure 1B). Her past medical history was unremarkable with regard to relevant infections and gastrointestinal symptoms. We observed high thyroid antibody titers [aTPO-ab > 2818 IU/ml (NR < 35); TG-Ab > 3000 IU/ml (NR < 40)]. Low IGF-1 [170 ng/ml (NR 190–805)] and IGFBP3 [2.5 μ g/ml (NR 2.2–4.6 μ g/ml)] were detected with normal level of cortisol in plasma. Serum-calcium and phosphate were at the lowest normal range. We observed low vitamin D [<4 ng/ml (NR 20–45)], elevated AP [173 U/l (NR 47–119)], and elevated PTH [764 pg/ml (NR 15–65)]. These parameters normalized after oral calcium and vitamin D supplementation without evidence of malabsorption. Bone density – measured by peripheral quantitative CT-scan – was within the lower normal range. Ultrasound evaluation revealed a small thyroid gland [2.5 ml (NR 5.7–13.3)]

TABLE 2 | Immunology and endocrinology work-up in two siblings with LRBA deficiency syndrome.

Lab results	Normal range	Patient 1	Patient 2
WBC	4–11/nl	9.0	6.5
Hb	11–15 g/l	11.2	10.7
Thrombocytes	200–400/nl	289	185
Lymphocytes (CD3 ⁺)	2–4.8/nl	3.7	2.3
Granulocytes	1.8–7.1/nl	4.7	3.9
CD4 ⁺ T-cells	700–1400/ μ l	505 ^a	594
CD8 ⁺ T-cells	200–900/ μ l	314 ^a	585
Naive CD4 ⁺ (CD4 ⁺ CD45RA ⁺ CD62L ⁺)	220–873/ μ l	n.a.	185
Naive CD8 ⁺ (CD8 ⁺ CD45RA ⁺ CD62L ⁺)	100–470/ μ l	n.a.	368
DNT (CD3 ⁺ TCRab ⁺ CD4 ⁻ CD8 ⁻)	0–5%	n.a.	5%
Regulatory T-cells (CD4 ⁺ CD25 ⁺ CD127 ^{low})	4–12%	n.a.	7.1%
Effector memory T4 (CD4 ⁺ CD45RO ⁺ CD62L ⁻)	4–20%	n.a.	9%
Central memory T4 (CD4 ⁺ CD45RO ⁺ CD62L ⁺)	8–50%	n.a.	48%
B-cells (CD19 ⁺)	100–500/ μ l	104 ^a	164
Naive B-cells (CD19 ⁺ CD27 ⁻ IgD ⁺)	150–515/ μ l	n.a.	220
Switched memory B (CD19 ⁺ CD27 ⁺ IgD ⁻)	5–77/ μ l	n.a.	20
Non-switched memory B (CD19 ⁺ CD27 ⁺ IgD ⁺)	2–77	n.a.	36
NK-cells (CD3 ⁺ CD56 ⁺)	90–600/ μ l	95	90
IgG	590–1400 mg/dl	1159	1220
IgM	50–317 mg/dl	45	44
IgA	70–250 mg/dl	<7	17
IgE	<100 U/ml	1.2	1
Coombs test	negative	+++ ^a	–
Anti-thrombocyte-ab	negative	–	+
Anti-granulocyte-ab	negative	++ ^a	+
TSH	0.5–3.6 mU/l	1.7	200
fT4	0.9–1.6 ng/dl	1.4	0.1
PTH	15–65 pg/ml	24 ^a	>700
25-OH-vitD	20–30 ng/ml	<5^a	<4
Calcium	2.1–2.55 mmol/l	2.1	2.44
IgfBP3	2.2–4.6 μ g/ml	2.1 ^a	2.5
HGH	0.14–14 ng/ml	n.a.	1
IGF-1	190–805 ng/ml	466 ^a	170
TG-ab	<40 IU/ml	n.a.	>3000
aTPO-ab	<35 IU/ml	76^a	2818
Adrenal-ab	negative	–	–
TRAK	<1 U/l	0.03 ^a	0.04
HbA1C	4.8–5.9% Hb	4.9	5.1
IFT ANA	$<1:10$	1:160^a	1:320
GAD-ab	<50 mGAD/ml	n.a.	<50
Anti-IA2-Ab	<8 U/ml	$<8a$	<8
Insulin-ab	<0.4 IU/ml	$<0.4a$	<0.4
Anti-gliadin-ab	<15 U/ml	12 ^a	35
Anti-transglutaminase-ab	<12 U/ml	10 ^a	1

Bold, abnormal values; n.a., not available.

^aValues were obtained at 14 years of age.

with multinodular (<5 mm) texture, transformed in accordance to ongoing thyroid autoimmunity (Figure 1C). Since there was no biochemical evidence of underlying growth hormone deficiency, the progressive growth retardation in this patient was related to long-term preexisting hypothyroidism. Following initiation of medical substitution, fT4 and TSH normalized subsequently.

Echocardiography revealed a marked pericardial effusion resolving after thyroxin substitution. On further follow-up, bone age remained significantly retarded. Furthermore, slightly elevated anti-gliadin-IgG [35 U/ml (NR < 12)] was measured in presence of normal anti-transglutaminase antibodies. WBC showed mild thrombocytopenia with detectable anti-thrombocyte antibodies [185/nl (NR 200–400)] and slightly reduced hemoglobin [Hb 10.1 g/dl (NR 11–15.5)].

The lymphocyte subset analysis showed mild CD4⁻ lymphocytopenia [594/μl (NR 700–1400)] with normal naive population (CD4⁺CD45RA⁺CD62L⁺) and normal distribution of effector memory (CD4⁺CD45RO⁺CD62L⁻) and central memory (CD4⁺CD45RO⁺CD62L⁺) T-cells, normal B-cells (CD19⁺) with normal naive (CD19⁺CD27⁻IgD⁺), memory (CD19⁺CD27⁺), switched memory (CD19⁺CD27⁺IgD⁻), non-switched memory B-cells (CD19⁺CD27⁺IgD⁺), and normal NK-cells (CD3⁻CD56⁺). Tregs (CD4⁺CD25⁺CD127^{low}) were within the normal range. As in her brother, IgA deficiency was detected [17 mg/dl (NR 70–300)] with normal IgG and slightly reduced IgM levels in plasma (Table 2). After thyroid hormone substitution and vitamin D supplementation, the patient re-started to grow and later experienced delayed menarche at the age of 16 years. The patient is currently 19 years old and on thyroid hormone substitution as well as oral vitamin D and calcium supplements. No immunosuppressive therapy has been initiated yet.

Genetic Analysis Using Whole Exome Sequencing

Genetic analysis was performed after informed consent by the parents. Genomic DNA from peripheral blood cells was isolated using the QIAamp[®] DNA Blood Mini Kit (Qiagen, Courtaboeuf, France) according to manufacturer's instructions. Whole exome sequencing (WES) was performed on the genomic platform of Institut IMAGINE's. Agilent SureSelect libraries were prepared from 3 μg of genomic DNA sheared with a Covaris S2 Ultrasonicator. Exons regions were captured using the Agilent Sure Select All Exon 51Mb V5 (AGILENT, Les Ulis, France) and sequenced using a HiSeq2500 next generation sequencer (Illumina) on the Genomic Platform of Institut IMAGINE, Paris. Depth of coverage obtained for each sample was around 100× with >98% of the exome covered at least 15-fold. Paired-end sequences were then mapped on the human genome reference (NCBI build37/hg19 version) using the Burrows-Wheeler Aligner. Downstream processing was carried out with the genome analysis toolkit (GATK), SAMtools, and Picard, each following documented best practices.¹ Variant calls were made with the GATK Unified Genotyper. All variants were annotated using the in-house software (PolyWeb) developed by Paris Descartes University Bioinformatics platform. All the annotation process was based on the 72 version of ENSEMBL database. Analysis of genome variations was made using the PolyWeb software. Variants were compared to the ones already present in US National Center for Biotechnology Information database (10) of SNP, 1000 Genome, and Exome Variant Server databases.

¹<http://www.broadinstitute.org/gatk/guide/topic?name=best-practices>

The impact on the protein function was predicted using three algorithms: Polyphen 2,² SIFT (Sorting Intolerant From Tolerant, J. Craig Venter Institute), and Mutation Taster.³ To confirm the mutation by Sanger sequencing, genomic DNA was amplified by standard techniques using oligonucleotide primers flanking the exon 46 on the Ensembl transcript ENST00000357115 of *LRBA* (forward 5'-TTTCCCTCCCTATTGGCAGC-3', lower 5'-ACAGCAAGCATCTGAAGGGG-3') using TaqDNA Polymerase (Life Technologies, Saint-Aubin, France). After purification with the QIAquick PCR Purification kit (Qiagen), PCR fragments were sequenced using the same primers by Eurofins on the Genomic Platform of Université Paris Descartes.

Cell Culture and Immunoblotting

PBMC were collected from blood using standard density gradient separation method. Cells were either cultivated right away or frozen and activated upon thawing. T-lymphocytes were activated by staphylococcal enterotoxin E (SEE 0.1 ng/ml) or Phytohemagglutinin (PHA 12.5 μg/ml) and cultured in Panserin, 5% SAB, 1% penicillin/streptomycin, and 1% glutamine medium. At day 3 of culture and further on three times per week, IL-2 (100 ng/ml) was added to maintain cell proliferation. Cell lysates were prepared according to standard methods, separated using 3–8% Tris-acetate gels (Invitrogen), transferred onto PVDF membrane, and immunoblotted with primary antibodies to LRBA (HPA023597, Sigma), Ku70 (MA5-13110, Thermo scientific), and secondary antibodies to rabbit and mouse (Santa Cruz).

RESULTS

Using WES, we identified a total of 14 genes with homozygous variants in both siblings. These homozygous variants included two genes with missense variants predicted to be damaging and one frameshift in the *LRBA* gene. The segregation of the *LRBA* variant was checked and correlated to the disease symptoms, as shown in Figure 2. None of the healthy siblings were homozygous for the *LRBA* variant. Two other homozygous variants were also detected in both affected siblings. The first variant was located in the gene encoding Fibrinogen gamma chain (*FGG* rs775086103; c.620A > G; p.Tyr207Cys). This gene has been reported in context of blood clotting disorders including familial dysfibrinogenemia, hypofibrinogenemia, and thrombophilia. There is no evidence of immunodeficiency in described patients. The second homozygous variant in both siblings was found in the gene encoding for chorionic somatomammotropin hormone 2 (*CSH2* rs549767039; c.-62A > G). This variant was located inside the 5' UTR, which belongs to the non-coding exonic sequence. *CSH2* is expressed mainly in the placenta and utilizes multiple transcription initiation sites. No association is known with immunodeficiency or autoimmunity (Table 3).

Lipopolysaccharide responsive beige-like anchor expression was analyzed by Western blot. Compared to a healthy control, we

²<http://genetics.bwh.harvard.edu/pph2/>

³www.mutationtaster.org

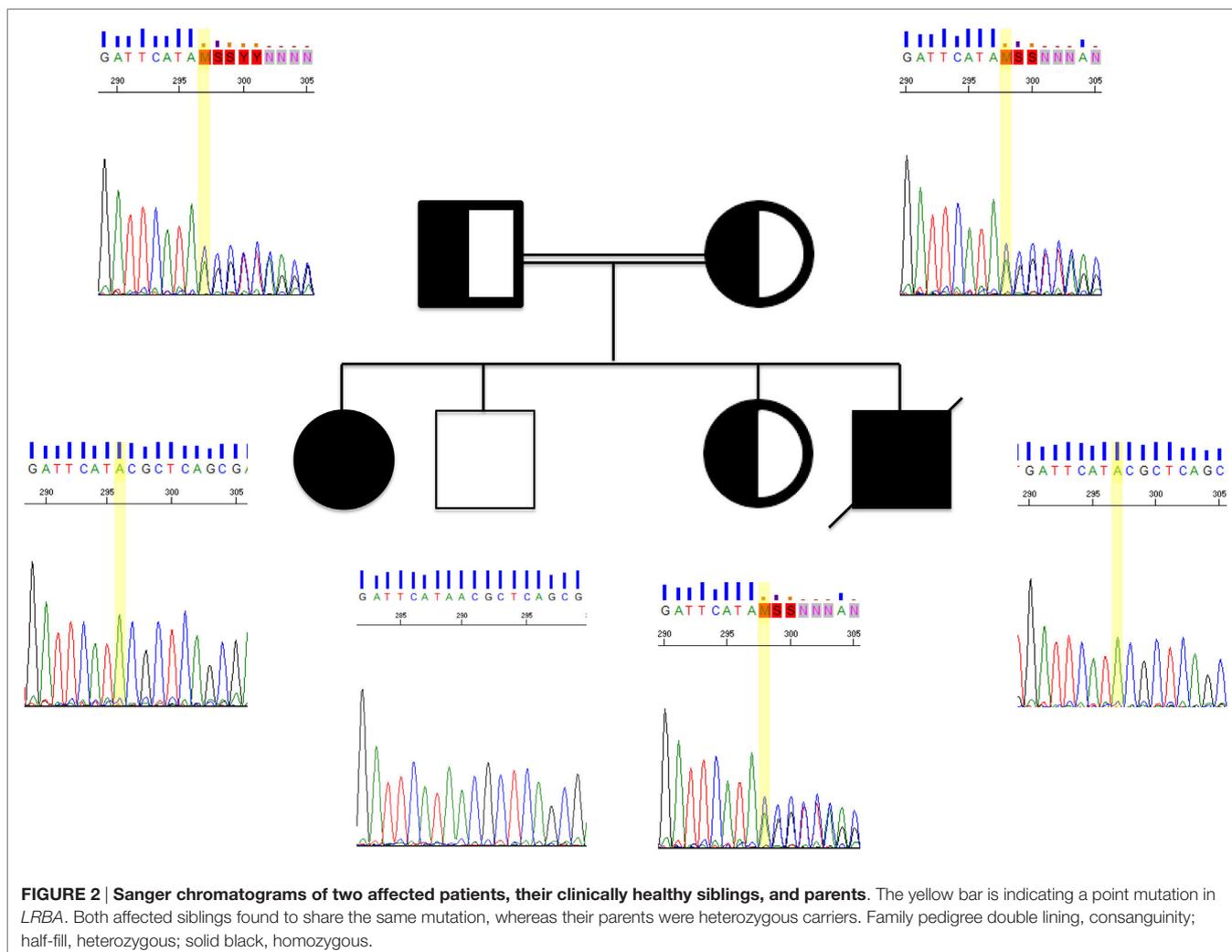


TABLE 3 | List of homozygous variants detected by whole exome sequencing in both affected patients.

Gene	Nucleotide	Amino acid	Chromosome	Gene function
FGG	c.620A > G	p.Tyr207Cys	4q32.1	Fibrinogen gamma chain. Related to familial hypo- and dysfibrinogenemia
CSH2	c.-62A > G	Non-coding exonic	17q23.3	Chorionic somatomammotropin hormone 2, carbohydrate, and protein metabolism during pregnancy
LRBA	c.6862delT	p.Y228MfsX29	4q31.1	B-cell function, lysosomal trafficking, and autophagy

Bold characters highlight the importance of the finding.

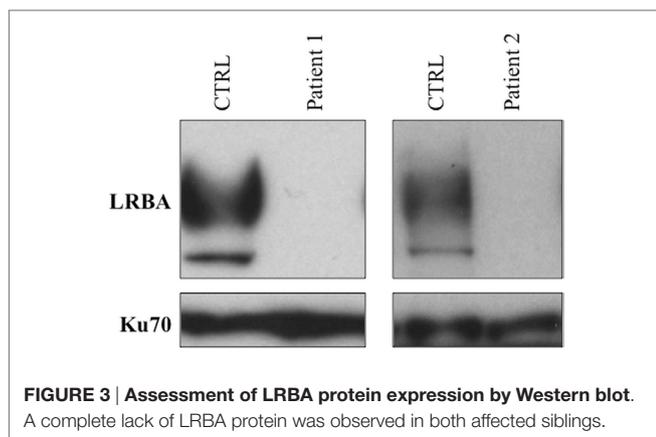
observed a complete absence of LRBA protein in both affected siblings. The pathogenicity of the homozygous variant found by WES was confirmed by the complete lack of the LRBA protein (Figure 3).

Diagnostic work-up of the clinically healthy family members revealed an interindividual variable degree of autoimmune thyroiditis. Both LRBA-heterozygous parents showed low TSH with normal fT3 and slightly increased fT4. Thyroid antibodies (TG-ab and aTPO-ab) were detected in both parents and heterozygous sister in keeping with the diagnosis of a compensated thyroid dysfunction. The autoimmune thyroiditis in heterozygous family members is, in contrast to our Patient 2, associated with an asymptomatic clinical course of thyroid disease. Specific

antibodies against adrenal tissue (21-hydroxylase) as well as antibodies typically associated with type 1 diabetes (GAD65, IA2, IAA) were proven negative in all family members. There was no history of gonadal insufficiency or impaired growth in parents or siblings. The immunology parameters were within the normal range for all of them.

DISCUSSION

We observed a heterogeneous clinical manifestation of LRBA deficiency in two siblings of a consanguineous family with a frameshift mutation, resulting in truncation of the LRBA protein. Our patient (Patient 2) presented with predominant endocrine



symptoms of hypothyroidism, growth retardation, delayed menarche, vitiligo, and rickets but without type 1 diabetes mellitus and enteropathy or immunodeficiency.

Pediatric patients with primary immunodeficiencies may present with variable and combined multi-organ symptoms in early childhood. Heritable monogenic PID with a predominant autoimmune phenotype has overlapping symptoms with the clinical spectrum of endocrine disorders (10). Whereas some of the patients suffer from a classic combination of symptoms, others might initially and exclusively present with endocrine symptoms causing a delay in diagnosis and treatment. Early diagnosis of the underlying disease is important in order to provide optimal treatment but may be a challenge in such patients. Within the spectrum of autoimmune endocrine diseases, LRBA deficiency has to be considered in addition to IPEX, CTLA-4-haploinsufficiency, CD25⁻, and Stat5b-deficiency.

Lipopolysaccharide responsive beige-like anchor is a member of the beige and Chediak–Higashi syndrome (BEACH)-domain containing protein family. It has a significant homology with LYST, another member of BEACH family (11). LRBA is involved in critical cellular interactions such as apoptosis and autophagy and plays a fundamental role in the regulation of immune system. However, the detailed pathomechanism underlying the variable symptom complex in LRBA deficiency is not completely understood yet.

Maintaining the balance between immunity and autoimmunity requires a complex interaction of activating and inhibiting factors. Firstly described in 1987, the glycoprotein CTLA-4 (12), mainly localized in intracellular vesicles of Tregs, is a key regulator molecule in this cascade (13). By acting as an early checkpoint, CTLA-4 has a major influence on maintaining self-tolerance (14, 15). There is evidence that LRBA controls CTLA-4 expression and thereby supports its transendocytotic function by preventing its degradation in recycling lysosomes (16). In support, Tregs of LRBA-deficient patients expressed lower intracellular and membrane levels of CTLA-4 without an alteration of the CTLA-4 mRNA. Two LRBA-deficient patients with detectable moderate residual LRBA protein were reported to have higher CTLA-4 levels suggesting a quantitative correlation between CTLA-4 deficiency and residual LRBA protein in LRBA-deficient patients (16).

The recent description of the extended phenotype of the disease in 22 genetically confirmed cases of LRBA deficiency showed immune dysregulation (95%), organomegaly (86%), recurrent infections (71%), and hypogammaglobulinemia (57%) as the main clinical complications, whereas 81% of these LRBA-deficient patients had normal T-cell counts, and 73% had reduced Tregs numbers (6). Both patients reported by Schreiner et al. suffered from endocrine symptoms in addition to underlying enteropathy and immunodeficiency (8). Autoimmune thyroiditis was observed by Alkhairy et al. in 3 out of 31 patients with underlying LRBA deficiency syndrome (9). However, none of these patients presented with an autoimmune thyroiditis as the major clinical manifestation. GH deficiency *per se* has not been associated with LRBA deficiency. Indeed, most of the affected patients seem to develop secondary growth failure in addition to their gastrointestinal dysregulation and malabsorption. This is a common feature of a series of other primary immunodeficiencies as well. However, Patient 2 did not suffer from gastrointestinal disease or malabsorption previously. There was no biochemical evidence of underlying growth hormone deficiency. The progressive growth retardation in this patient was related to long-term preexisting hypothyroidism. As observed in this cohort and in additional reports, the clinical course of the disease remains highly variable (6, 9).

The suggested selective regulation of CTLA-4 degradation by LRBA is tempting to compare immune defects of LRBA with CTLA-4-deficient patients. Schubert et al. reported 19 patients with a genetically confirmed CTLA-4-haploinsufficiency (4). Only 12 patients presented with severe clinical manifestations including enteropathy (78%), hypogammaglobulinemia (76%), granuloma (66%), autoimmune thrombocytopenia (35%), and autoimmune hemolytic anemia (28%). Autoimmune thyroiditis was present in two patients as a part of a polyautoimmune disorder. Isolated autoimmune endocrine disorder was not reported in this cohort (4). Recently, Slatter et al. reported the outcome in a group of CTLA-4 deficient patients ($n = 8$) undergoing alloHSCT. Autoimmune endocrine disorders (type 1 diabetes, exocrine pancreas insufficiency and thyroiditis) were reported in two patients as one part of their complex autoimmune disorder. Interestingly, signs of isolated endocrine autoimmunity were observed among family members of these two patients suggesting a minor disease activity in these individuals without being diagnosed as CTLA-4-deficient previously (17).

As mentioned above, the majority of LRBA- and CTLA-4-deficient patients show a relevant Treg dysfunction with immune dysregulation as their main clinical symptom resulting in an IPEX-like disorder. However, a series of proven CTLA-4-deficient individuals are asymptomatic despite disturbed Treg cell suppressive ability (4). As observed in our Patient 2, LRBA deficiency can also present with normal T- and B-cell subpopulations and Treg numbers. Whether there is a correlation between the disease severity and the degree of Treg dysfunction in LRBA-deficient individuals needs to be analyzed in larger cohorts prior to initiation of immunosuppressive treatment. Furthermore, the lysosomal sorting function of LRBA might not be limited to T-lymphocytes and to CTLA-4, opening possibilities to evaluate symptoms of these patients, which are not fully explained by a Treg-restricted dysfunction. To what extent additional

modifiers such as epigenetic or environmental factors might influence the disease outcome in LRBA- and CTLA-4-deficient patients, remains to be analyzed in further studies.

Since first description in 2012, about 50 LRBA-deficient patients have been detected and the number is increasing. Very recently Lévy et al. described two patients suffering from arthritis as their main clinical symptoms (18). We believe that the awareness of the heterogeneity of this disease among non-immunologists might be the key to the early detection and initiation of the treatment of LRBA deficiency. Without appropriate treatment, there is a high mortality in a majority of the affected patients. While some of the patients die early due to enteropathy and complications of the immunosuppressive treatment, others remain stable on systemic immunosuppression with glucocorticoids as single agents or as part of combinatorial therapy (6). Due to the lack of genotype–phenotype correlation in this disease and the clinical heterogeneity, the underlying diagnosis might remain undetected in some patients and lead to delayed treatment. These patients need early multidisciplinary care involving specialists in the fields of endocrinology, gastroenterology, and immunology. Supportive medical therapy of the evolving endocrine insufficiencies is indicated at an early stage with continuous follow-up to detect further organ manifestations. Some patients may remain refractory to standard therapy. Extended immunosuppressive therapy may be necessary for patients with progressive symptoms of the disease and might result in stable disease but also can lead to lethal complications. Recently abatacept, a CTLA-4 fusion protein, was shown to provide improvement in LRBA-deficient patients (16). There is no long-term evaluation of efficacy and safety of the treatment available yet. In patients suffering from threatening autoimmunity, enteropathy and infectious complications of the immunosuppressive treatment early stem cell transplantation could be the only curative treatment option. A few LRBA-deficient patients being treated successfully by stem cell transplantation have been reported (6, 19). Further studies on transplantation in LRBA deficiency are necessary to evaluate the optimal conditioning regimen and transplantation-related problems such as immune reconstitution posttransplantation and the risk of graft versus host disease. Given the severe fatal course of the disease in Patient 1, the establishment of a long-term treatment strategy

including evaluation of a preemptive alloHSCT seems crucial for Patient 2 at this stage.

In light of a quite limited genotype–phenotype correlation, further data on LRBA-deficient patients are necessary to establish predictive prognostic biomarkers. Furthermore, detailed data on LRBA-deficient patients with an endocrine phenotype are necessary to evaluate the long-term risk of endocrine disorder, their outcome, and the optimal early treatment options in these patients.

ETHICS STATEMENT

This study was performed with parental permission and approval by the ethic committee of University Hospital Frankfurt.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and interpretation of data. SB, UP, and PB provided clinical data and wrote the manuscript. EL, FC-H, FR-L, FR, and NC-B provided data on functional testing and genetics.

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PEDIATRICS®

A New Case of Enteric Anendocrinosis: An Extremely Rare Cause of Congenital Malabsorptive Diarrhea and Diabetes Secondary to Mutations in Neurogenin-3

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3 **A New Case of Enteric Anendocrinosis: An Extremely Rare Cause of Congenital**
4 **Malabsorptive Diarrhea and Diabetes Secondary to Mutations in *Neurogenin-3***
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39

40 **Abbreviations:**

41
42 CDD: congenital diarrheal disorders.

43 EA: enteric anendocrinosis.

44 EC: enteroendocrine cells.

45 NEUROG3: neurogenin-3.

46 CD: celiac disease.

47 PN: parenteral nutrition.

48 anti-tTG: anti-transglutaminase.

49 IPEX: immune dysregulation, polyendocrinopathy, enteropathy, X-linked.
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Contributors' Statements:

Marta Germán-Díaz: Dr. Germán-Díaz was involved in the clinical management and follow-up of the patient, wrote and revised the manuscript.

Jaime Cruz-Rojo: Dr. Cruz-Rojo was involved in the clinical management and follow-up of the patient and revised the manuscript.

Yolanda Rodriguez-Gil: Dr. Rodriguez-Gil provided images and descriptions of pathology slides and provided critical revisions for intellectual content.

Fabienne Charbit-Henrion: Dr. Charbit-Henrion performed the genetic testing and revised the manuscript.

Nadine Cerf-Bensussan: Dr. Cerf-Bensussan performed the genetic testing and revised the manuscript.

Javier Manzanares-López Manzanares: Dr. Manzanares-López Manzanares was involved in the clinical management and follow-up of the patient and revised the manuscript.

José Manuel Moreno-Villares: Dr. Moreno-Villares was involved in the clinical management and follow-up of the patient and provided critical revisions for intellectual content.

All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Abstract

Congenital diarrheal disorders (CDD) are a group of rare enteropathies that often present with life-threatening diarrhea in the first weeks of life. Enteric anendocrinosis (EA), characterized by a lack of intestinal enteroendocrine cells (EC) due to recessively inherited mutations in the *Neurogenin-3 (NEUROG3)* gene, has been described as a cause of congenital malabsorptive diarrhea. Diabetes mellitus is also typically associated with *NEUROG3* mutations, be it early onset or a later presentation. Here we report a case of a 16-year-old male patient with severe malabsorptive diarrhea from birth, who was parenteral nutrition (PN) dependent and who developed diabetes mellitus at 11 years old. To the best of our knowledge, only nine cases of recessively inherited *NEUROG3* mutations have been reported in the literature to date. Our patient presents with several remarkable differences compared to previously published cases. This report can contribute by deepening our knowledge on new aspects of such an extremely rare disease.

Review Copy

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3 **A New Case of Enteric Anendocrinosis: An Extremely Rare Cause of Congenital**
4 **Malabsorptive Diarrhea and Diabetes Secondary to Mutations in *Neurogenin-3***
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8 Congenital diarrheal disorders (CDD) are a group of rare enteropathies that often
9 present with life-threatening diarrhea in the first weeks of life¹. The number of well-
10 characterized disorders attributed to CDDs has gradually increased over the past several
11 years, and many new genes have been identified related to CDD²⁻³. One of these new
12 disorders is enteric anendocrinosis (EA), characterized by severe malabsorptive diarrhea
13 and a lack of intestinal enteroendocrine cells (EC) due to recessively inherited mutations
14 in the *Neurogenin-3* (NEUROG-3) gene⁴. Very few cases that mention this disorder
15 have been published to date⁴⁻¹⁰. In this article, we present the tenth case reported, with
16 several remarkable differences compared to previously published cases. This report can
17 contribute by deepening our knowledge on new aspects of such an extremely rare
18 disease.
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33 An 11-day-old male newborn presented with severe dehydration, metabolic acidosis
34 (pH:7.16; bicarbonate: 6.0 mmol/l; base excess: -21) and high-volume watery diarrhea
35 (10 loose stools daily). Prenatal and natal history indicated a healthy pregnancy and
36 delivery, with a birthweight of 3560 g. He had received regular infant formula and
37 breast milk since birth. There was no family history of gastrointestinal disorders or
38 immunodeficiency. Parents were not consanguineous.
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47 Intravenous fluids were started and diarrhea ceased during fasting. Several different
48 types of formula (casein extensive hydrolyzed, galactose and glucose-free formula,
49 amino acid-based formula) were administered in the first months of life but did not lead
50 to resolution of the diarrheal symptoms. He presented a malabsorptive diarrhea with fat
51 malabsorption (fecal fat excretion of 12.3 grams per day) and protein malabsorption
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3 (fecal nitrogen of 2.1 g/d). Comprehensive evaluations for infectious, allergic,
4 immunologic and metabolic causes of diarrhea were all negative. Biopsy of the
5 stomach, esophagus, duodenum and colon revealed normal histologic features. Electron
6 microscopy was also conducted without abnormal findings. The activity of mucosal
7 disaccharidases was within the normal range. At that moment, celiac disease (CD)
8 antibodies were also negative.
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17 He was readmitted on several occasions during the first months of life because of these
18 symptoms. Different treatments including loperamide or pancreatic enzymes were
19 administered without significant improvement. As a result of this diarrhea, he developed
20 severe failure to thrive. Therefore, home parenteral nutrition (PN) was started finally at
21 32 months of life.
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29 Over the following years, he was readmitted on several occasions because of infections
30 related to the central venous catheter and PN was discontinued several times for this
31 reason. Nevertheless, after some months without PN it was resumed due to weight loss
32 and fatigue. He continued to pass 3 to 4 high-volume liquid stools daily.
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39 When our patient was 11 years old, he developed type 1 diabetes with negative
40 pancreatic autoantibodies; therefore insulin treatment was started. At that point, a new
41 evaluation was made. He had 30 g of stools per kilogram of body weight per day with
42 fecal fat excretion of 58.1 g/d and fecal nitrogen of 12.8 g/d. Fecal elastase levels were
43 decreased: 15.1 mg/g (normal, 200-500 mg/g). Both antigliadine and anti-
44 transglutaminase (anti-tTG) antibodies were positive. Antigliadine were 110 U/ml
45 (normal less than 18) and anti-tTG were 150 U/ml (normal less than 10). HLA study
46 was: DR17, DR7/ DQ2, DQ9.
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3 An esophagogastroscopy and a colonoscopy were repeated. Duodenum biopsies
4 revealed moderate to severe villous atrophy. Goblet cells and Paneth cells were present
5 in a normal pattern. Staining of the small intestine with chromogranin A, a
6 neuroendocrine marker, showed presence of EC. With these findings, a diagnosis of CD
7 was made and a gluten-free diet was started. He was also treated with supplemental
8 pancreatic enzymes without improvement. Three years after the starting the diet our
9 patient continued to have a malabsorptive diarrhea. Duodenum biopsies were repeated,
10 revealing mild to moderate villous atrophy (Fig. 1). Immune dysregulation,
11 polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome was suspected, but
12 *FOXP3* mutation analysis was negative. Mutations of *NEUROG3* were investigated and
13 a homozygous mutation (c.319C>A; p.Arg107Ser) was identified. After the
14 identification of the *NEUROG3* mutation, intestinal biopsy samples were reexamined
15 for the presence of EC and compared with control subjects. We found that our patient
16 had a significant depletion of EC. Only 1 cell was identified per 10 crypts in the
17 duodenum and no EC were identified in the colon, while two-three cells were present
18 per crypt in normal mucosa of control patients (Fig. 2,3).
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39 At the time of writing, the patient was 16 years old (weight: 51 kg, -1,7 standard
40 deviation (SD); height: 161.5 cm, -1,4 SD; body mass index 19,55 kg/m²). He eats a
41 regular home diet with no gluten and receives night-time PN 7 times a week. He has
42 good glycemic controls with insulin in the PN.
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48 DISCUSSION

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51 CDD are a group of rare enteropathies related to specific genetic defects, which are
52 generally inherited as autosomal recessive traits. Frequently, infants with these
53 disorders present with chronic diarrhea of sufficient severity to require PN early in life.
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3 In 2010 Berni Canani et al suggested a new classification of CDD according to the type
4 of defect present¹. This classification includes four groups of CDD: defects in digestion,
5 absorption and transport of nutrients and electrolytes; disorders of enterocyte
6 differentiation and polarization; defects of EC differentiation and dysregulation of
7 intestinal immune response¹⁻³. EA, caused by mutations in *NEUROG3*, is included in
8 this classification as a defect of EC differentiation¹⁻³.
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17 *NEUROG3* is a basic helix-loop-helix transcriptional factor that drives endocrine cell
18 fate in both the pancreas and intestine^{11,12}. Studies involving mice have shown that
19 *Neurog3*^{-/-} mice lack endocrine cells in the pancreas and intestine, which is presumably
20 why they die of diabetes during the first few days of life¹³.
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27 The relationship between mutant *NEUROG3* and congenital malabsorptive diarrhea was
28 first described by Wang et al⁴. They reported cases of three children with mutant
29 *NEUROG3* who lacked intestinal EC and have malabsorptive diarrhea. This finding
30 suggested that EC had not only a secretory effect as previously believed, but that they
31 also played a critical role in augmenting nutrient absorption. Nevertheless, the
32 mechanisms were still not completely understood. We indentified the same *NEUROG3*
33 mutation as in one of the Wang et al patients (Arg107Ser). To the best of our
34 knowledge, nine cases of recessively inherited *NEUROG3* mutations have been reported
35 in the literature to date⁴⁻¹⁰. All nine patients had congenital malabsorptive diarrhea and
36 four developed diabetes; two of them had neonatal diabetes and two developed it by the
37 age of 8. Limited clinical information has been reported on two patients. As it can be
38 seen, the number of patients described so far is too small to draw reliable conclusions
39 about the typical clinical picture of this disease. A summary of the clinicopathologic,
40 histologic, immunohistochemical, genetic and prognosis features of the cases reported
41 in the literature is provided in Table 1⁴⁻¹⁰.
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3 There are several differences between our patient and those reported previously. Initial
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5 biopsies taken at the onset of the condition showed normal mucosa both in the small
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7 intestine and colon. However, when the biopsies were repeated years later, the patient
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9 showed moderate to severe villous atrophy. After testing positive for anti-tTG
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11 antibodies and HLA (DQ2), gluten was eliminated from the diet. In spite of these
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13 actions, when new biopsies were performed 3 years later results still showed moderate
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15 atrophy and high levels of antibodies. We verified that the diet was being strictly
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17 followed. Failure to respond to the diet and the clinical data cast doubt on the CD
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19 hypothesis. Specificity of the anti-tTG antibodies is very high, but high levels can be
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21 found in other conditions, such as when there is inflammation at the intestinal mucosa
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23 level or in the presence of other co-existing autoimmune disorders¹⁴. On the other hand,
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25 other causes of villous atrophy such as hypogammaglobulinemia, immunodeficiency,
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27 bacterial overgrowth, and infections were ruled out¹⁵. In the cases described so far only
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29 one presented with villous atrophy at the time of diagnosis⁹. In our case there was no
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31 atrophy at the time of diagnosis either but it could have potentially appeared as the
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33 disease evolved aided by prolonged mucosa damage. It must be kept in mind that our
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35 patient is the oldest reported patient to date, which could help us understand the
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37 behavior of the disease in the long run.
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44 It is important to note that in view of the reported data the degree of EC depletion at the
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46 intestinal level can vary. In our case, a Chromogranin A stain biopsy confirmed the
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48 presence of EC. However, the quantity of these cells was not taken into account, which
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50 resulted in diagnostic delay. Once the genetic diagnosis was obtained the results from
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52 the biopsies were reevaluated, comparing with age matched controls revealing that the
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54 quantity of EC was much lower than normal (1 for every 10 crypts as opposed to 2-3
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56 per crypt as is observed in healthy individuals). Therefore, the presence of this type of
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3 cells in the intestinal mucosa does not rule out the diagnosis, making quantitative
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5 precise counting of the cells necessary, using age matched controls. The quantity of EC
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7 does not seem to be related to the prognosis of the disease. In fact, the
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9 physiopathological mechanism behind the diarrhea remains unclear. Different
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11 hypothesis have been presented (accelerated intestinal transit, exocrine pancreatic
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13 insufficiency, EC involvement in nutrient absorption, etc.). Up to date there are no
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15 effective treatments available for these patients. Among the reported cases only one
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17 partial response to the use of cholestyramine was reported. Our patient had low elastase
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19 levels, prompting the use of a trial treatment with pancreatic enzymes that had no results
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21 and was stopped.
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26 In conclusion, EA should be considered in all patients with unexplained congenital
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28 malabsorptive diarrhea. This diagnosis should be further investigated with
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30 Chromogranin-A staining of intestinal biopsies and a mutation analysis of the
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32 *NEUROG3* gene.
33

34 35 **Acknowledgements:**

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38 We thank Professor Frank Ruemmele, MD, PhD, of Department of Pediatric
39
40 Gastroenterology, Hôpital Necker-Enfants Malades, in Paris; and Fabienne Charbit-
41
42 Henrion, MD, Institut IMAGINE-Inserm UMR 1163, Université Paris Descartes-
43
44 Sorbonne, in Paris; for providing mutation analysis of the *NEUROG3* gene.
45

46 47 **References:**

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Figure 1. After three years of gluten free diet, duodenum biopsies still revealed mild to moderate villous atrophy.

Figure 2. (A) Chromogranin-A staining of normal duodenal mucosa from an age-matched control, showing a normal distribution of endocrine cells, 1-3 enteroendocrine cells per crypt. (B) Chromogranin-A staining of patient with enteroendocrine dysgenesis that shows a significant depletion of enteroendocrine cells, 1 was identified per 10 crypts (original magnification, ×200).

Figure 3. (A) Chromogranin-A staining of normal colonic mucosa from an age-matched control, showing a normal distribution of endocrine cells, 1-3 enteroendocrine cells per crypt. (B) Chromogranin-A staining of patient with enteroendocrine dysgenesis that shows no immunohistochemical reactivity to chromogranin-A (original magnification, ×200).

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Table 1. Clinicopathologic, histologic, immunohistochemical, genetic and prognosis features of the cases reported in the literature to date.

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Table 1. Clinicopathologic, histologic, immunohistochemical, genetic and prognosis features of the cases reported in the literature to date.

Author	Age at diagnosis	Sex	Clinical onset	Diabetes	Histopathologic features	Staining for chromogranin A	Mutation	Clinical course
Wang et al ⁴	35 mo	Male	Malabsorptive diarrhea the first weeks of life	No	Normal intestinal mucosa structure	1 EC in the more than 350 small-bowel crypts examined and none in the colon	Homozygous missense change, p.R107S	He died after a liver-intestine transplant because of sepsis
Wang et al ⁴	8 yr	Male	Malabsorptive diarrhea the first weeks of life	Yes, at 8 yrs	Normal intestinal mucosa structure	1 EC in 100 crypts in the duodenum and 8 in 100 crypts in the jejunum	Homozygous missense change, p.R93L	- PN until 24 months of age - Diarrhea continues - Growth retardation
Wang et al ⁴	9 yr	Male	Malabsorptive diarrhea the first weeks of life	Yes, at 8 yrs	Normal intestinal mucosa structure	1 EC in 100 crypts in the duodenum and 8 in 100 crypts in the jejunum	Homozygous missense change, p.R93L	- PN during 2 months - Diarrhea continues - Growth retardation
Rubio-Cabezas et al ⁵	5 yr	Female	Diabetes and severe malabsorptive diarrhea since birth	Yes, neonatal	Normal intestinal mucosa structure	Complete absence of EC in small bowel and colon	Two heterozygous mutations: p.E28X, p.L135P	- Night-time PN 3 times a week - Diarrhea persists - Normal growth
Pinney et al ⁶	10 mo	Female	Malabsorptive diarrhea since birth	Yes, at 5 ^{1/2} mo	Moderate to severe villous blunting and atrophy	Complete absence of EC in small bowel	Homozygous nonsense mutation, p.E123X	She died at 10 months from chronic liver failure secondary to PN
Oshie et al ⁷	3 mo	Female	Malabsorptive diarrhea since birth	No	NR	Rare EC in small bowel. None per 50 crypts in colon	NR	NR
Sayar et al ⁸	20 mo	Female	Malabsorptive diarrhea started on the 20th day of life	No	Normal intestinal mucosa structure	No EC in the duodenum. 3 EC per 50 crypts in the colon	Homozygous frameshift mutation, p.S171fs	- Home PN 2 days per week - Diarrhea persists - Normal growth
Teetipsatit et al ⁹	10 wk	Female	Intractable watery diarrhea	No	No significant histologic abnormalities of colon. No available small bowel biopsy	Complete absence of EC in colon	NR	NR
Aksu et al ¹⁰	2 yr, 5 mo	Male	Malabsorptive diarrhea since birth	No	Normal intestinal mucosa structure	1 EC per crypt in small bowel	Homozygous missense change, p.R93L	- PN until 18 months of life - Partial response to cholestyramine - Normal growth

Mo: months; Yr: years; Wk: weeks; EC: enteroendocrine cells; NR: not reported; PN: parenteral nutrition.

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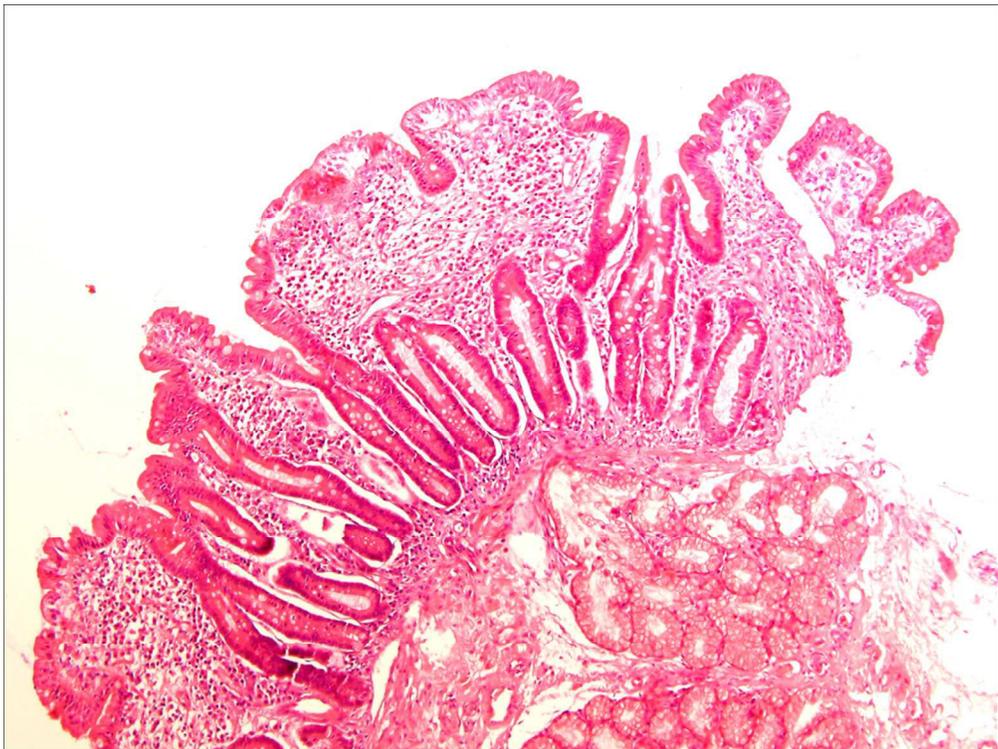


Figure 1. After three years of gluten free diet, duodenum biopsies still revealed mild to moderate villous atrophy.
189x142mm (150 x 150 DPI)

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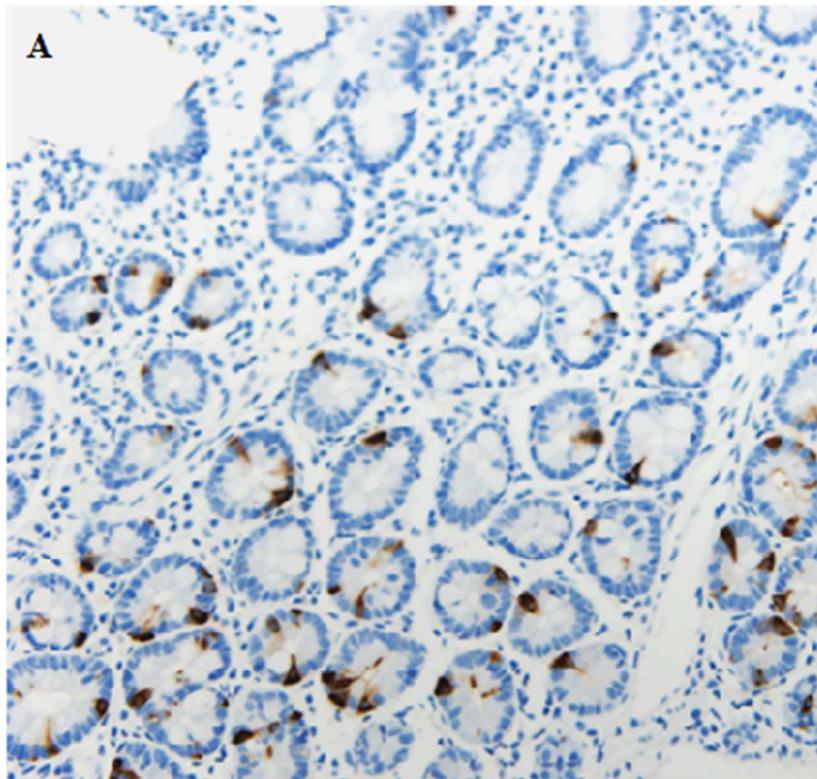


Figure 2. (A) Chromogranin-A staining of normal duodenal mucosa from an age-matched control, showing a normal distribution of endocrine cells, 1-3 enteroendocrine cells per crypt.

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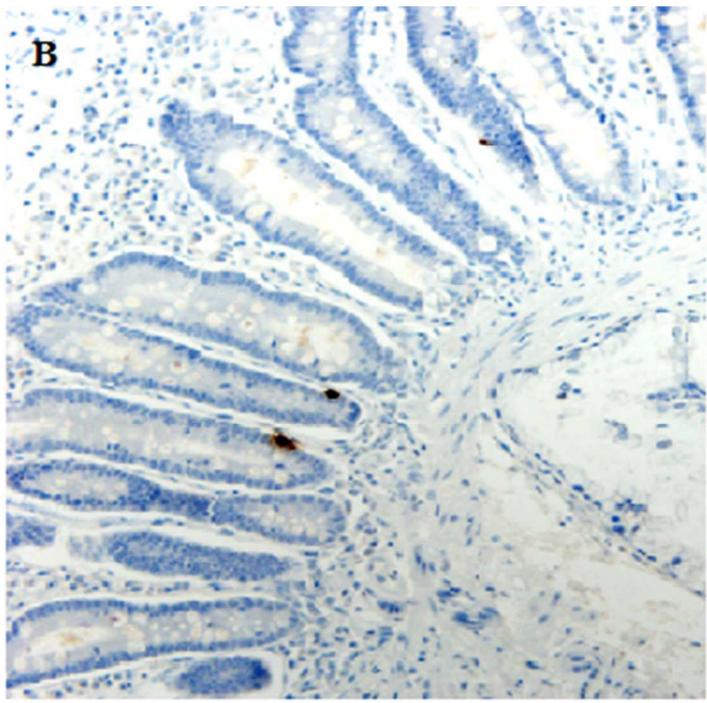


Figure 2. (B) Chromogranin-A staining of patient with enteroendocrine dysgenesis that shows a significant depletion of enteroendocrine cells, 1 was identified per 10 crypts (original magnification, x200).

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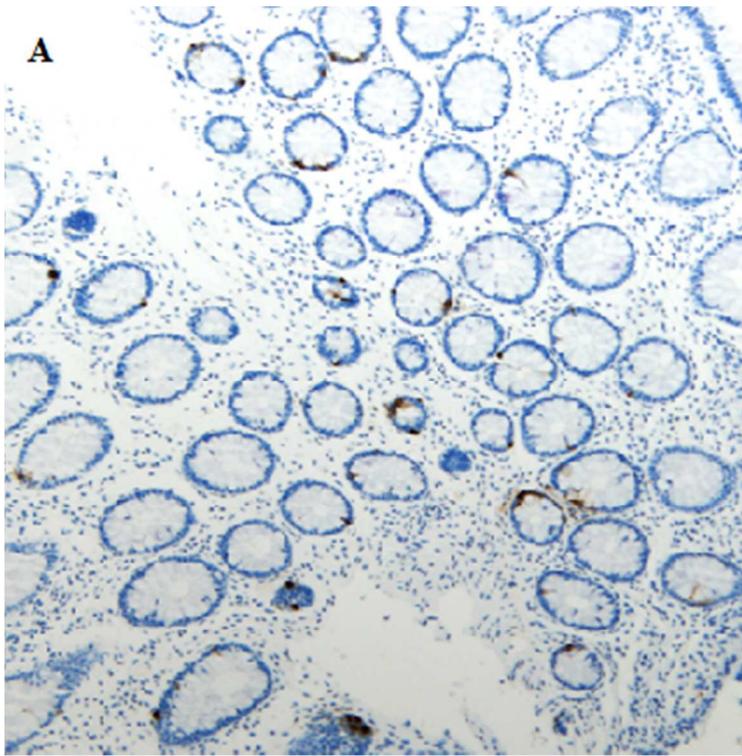


Figure 3. (A) Chromogranin-A staining of normal colonic mucosa from an age-matched control, showing a normal distribution of endocrine cells, 1-3 enteroendocrine cells per crypt.

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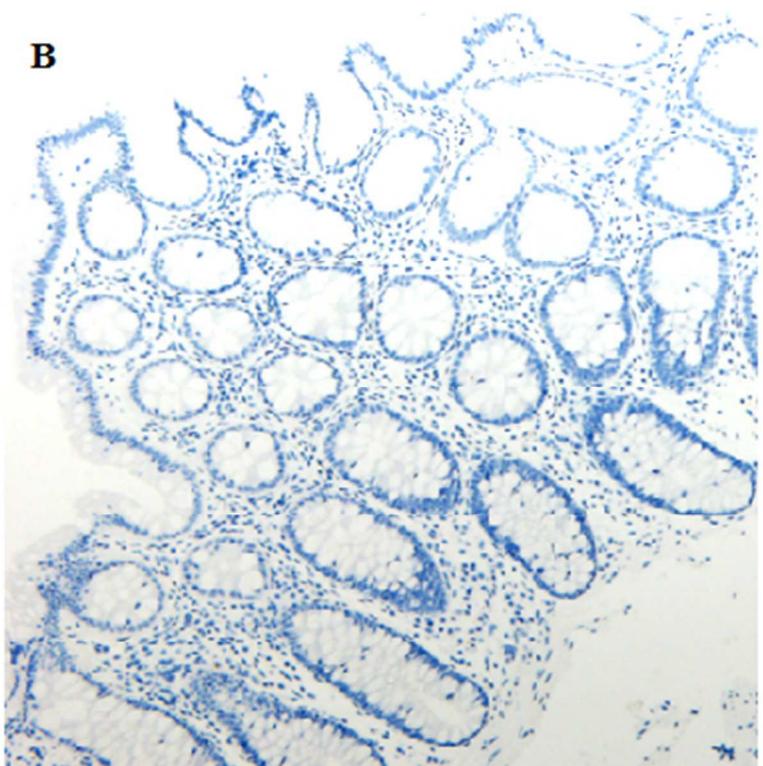


Figure 3. (B) Chromogranin-A staining of patient with enteroendocrine dysgenesis that shows no immunohistochemical reactivity to chromogranin-A (original magnification, ×200).

Table 6. Summary of IL-10 and IL-10R defective patients.

When patients are reported in more than one article, they are reported only once in the chart below. Some full version articles were not available and are thus not included.

M: male; F: female; del: deletion; dupl: duplication; 40d: 40 days; GH: growth hormone; NA: not available

Article	Gene	Mutation	M/F	Disease onset (months)	Esophagus	Stomach	Duodenum	Jejunum	Ileum	Colon	Rectum	Perianal lesions	Folliculitis	Arthritis	Other	Infections	B cell lymphoma
Begue Am J Gastro 2011																	
P3	IL10RB	c.G421T	M	3				0	0	1	1	1					
P11	IL10RA	c.C784T	M	1				0	1	1	1	1					
Personal data																	
F.T.	IL10RA	c.C1615T	M	3								1					
P1	IL10RB	delE3	M	9						1	1	1	1				
P2	IL10RB	delE3; duplE6	F	4						1	1	1					
P3	IL10RB	delE3	M	5						1	1	1					
Glocker NEJM 2009																	
P II-3	IL10RB	c.G477A, p.Trp159X	M	3		0	0	0	0	1	1	1	1	1			
P II-4	IL10RB	c.G477A, p.Trp159X	F	12		0	0	0	0	1	1	1	1	1	Renal abscesses	1	
P II-5	IL10RA	c.G421A, p.Gly141Arg	F	12		0	0	0	1	1	1	1	1		GH deficiency		
???	IL10RA	c.C251T,p.Thr84Ile	M	0,5		0	0	0	0	1	1	1	1				

Article	Gene	Mutation	M/F	Disease onset (months)	Esophagus	Stomach	Duodenum	Jejunum	Ileum	Colon	Rectum	Perianal lesions	Folliculitis	Arthritis	Other	Infections	B cell lymphoma
Pigneur IBD 2013																	
P1	IL10RA	c.585delT, p.S196fsX	NA	4		0	0	0	1	1	1	1				1	
P5	IL10RB	del exon2	NA	0,5		0	0	0	0	1	1	1			2 hear loss	1	
P6	IL10RB	p.Y59C	NA	0,5		0	0	0	0	1	1	1	1			1	1
P7	IL10RB	p.W204C ; p.F269fsX275	NA	0,5		0	0	0	0	1	1	1	1			1	1
P9	IL10RB	g.11930-17413del	NA	0,5		0	0	0	0	1	1	1	1			1	1
P10	IL10RB	c.T229G, p.W100G	NA	4		0	0	0	0	1	1	1	1	1		1	
Glocker 2010 Lancet																	
	IL10	Gly113Arg	F	11					1	1	1	1			moderate hear loss		
	IL10	Gly113Arg	M	3						1	1	NA					
Moran IBD 2012																	
	IL10RA	g.IVS5+2T>C, P206X	F	5					0	1	1	1		1		1	
Mao, Genes and Immunity, 2012																	
	IL10RA	c.251C4T, p.T84I ; c.301C4T, p.R101W	M	0,5						1	1	NA					
Kotlarz Gastro 2012																	
P5	IL10RA	c.C301T, p.Arg101Trp	M	0,25		0	0	0	0	1	1	1	1				

Article	Gene	Mutation	M/F	Disease onset (months)	Esophagus	Stomach	Duodenum	Jejunum	Ileum	Colon	Rectum	Perianal lesions	Folliculitis	Arthritis	Other	Infections	B cell lymphoma
P6	IL10RB	c.G477A, p.Trp159X	M	2		1	1	0	1	1	1	1	1				
P7	IL10RA	c.A170G, p.Tyr57Cys ; c.C349T, p.Arg117Cys	M	1,5		0	0	0	0	1	1	1		1			
P8	IL10RB	c.G197A, p.Cys66Tyr	F	1,5		0	0	0	0	1	1	1	1				
P9	IL10RB	c.C52T (3'UTR)	M	3								NA		1			
P10	IL10RB	c.G611A, p.Trp204X ; c.C689A, p.Ser230X	F	0,6		1	1	0	0	1	1	1	1				
P11	IL10RB	c.G611A, p.Trp204X	F	1		1	1	0	0	1	1	1	1				
P12	IL10RA	c.T506C, p.Ile169Thr	M	0,25		1	1	0	1	1	1	1	1				
P13	IL10	c.G458A, p.Gly153Asp	M	1		0	0	0	0	1	1	1					
P14	IL10	c.G458A, p.Gly153Asp	M	1		0	0	0	0	1	1	1					
P15	IL10	c.G458A, p.Gly153Asp	M	1		0	0	0	0	1	1	1					
P16	IL10RB	c.331+907_574del	M	2		1	1	0	0	1	1	1	1				
Dinwiddie Genomics 2013																	
CMH166	IL10RA	c.C784T, p.Arg262Cys ; c.C349T, p.Arg117Cys	M	6		1	1	0	1	1	1	1			osteoporosis		1

Article	Gene	Mutation	M/F	Disease onset (months)	Esophagus	Stomach	Duodenum	Jejunum	Ileum	Colon	Rectum	Perianal lesions	Folliculitis	Arthritis	Other	Infections	B cell lymphoma
CMH165	IL10RA	c.C784T, p.Arg262Cys ; c.C349T, p.Arg117Cys	M	2	1	1	1	0	0	1	1	1			0	1	
Shim JO, Eur J Gastroenterol Hepatol 2013																	
P1	IL10RA	c.C301T, p.R101W ; c.G350A, p.R117H	F	1					1	1	1	1	1			1	
P2	IL10RA	c.A272G, p.Y91C ; c.C784T, p.R262C	M	1						1	1	1	1				
P3	IL10RA	c.A272G, p.Y91C ; c.C301T, p.R101W	F	0,01						1	1	1	1		pulmonary interstitial emphysema	1	
Engelhardt 2013, JACI																	
P1	IL10RA	c.G350A, p.Arg117His	F	42		0	0	0	0	1	1	0					
P2	IL10RB	IVS3+1G>C	F	1		0	0	0	0	1	1	0			bilateral hydronephrosis		1
P3	IL10RB	c.53detT	F	1		0	0	0	0	1	1	0			hear loss		1
P4	IL10RB	c.G577C, p.Gly193Arg	M	0,3		0	0	0	0	1	1	0			died at 40d	1	
P5	IL10RA	del Exons 1-3	M	2		0	0	0	0	1	1	0				1	
P6	IL10RA	c.A170G, p.Tyr57Cys ; del Exons 2-4	M	3		0	0	0	0	1	1	0					
P7	IL10RA	c.T374G, p.Leu125Arg	F	1		0	0	0	0	1	1	0				1	

Article	Gene	Mutation	M/F	Disease onset (months)	Esophagus	Stomach	Duodenum	Jejunum	Ileum	Colon	Rectum	Perianal lesions	Folliculitis	Arthritis	Other	Infections	B cell lymphoma
Moran IBD 2013																	
P1	IL10RA	g.IVS5+2T>C	F	3					0	1	1	1		1			
Murugan J Clin Immunol, 2014																	
P1	IL10RA	c.368-10C>G	NA	6						1	1	1				1	1
P2	IL10RA	c.368-10C>G	NA	6						1	1	1				1	
P3	IL10RA	c.368-10C>G	NA	6						1	1	1				1	
Shim J Human Genet 2014																	
P1	IL10RA	c.350G4A, p.R117H ; c.272A4G, p.Y91C	M	0,01						1	1	1	3				3
P3	IL10RA	c.272A4G, p.Y91C	F	0,3					1	1	1	1					
P5	IL10RA	c.301C4T, p.R101W ; c.205T4C, p.W69R	F	1						1	1	1					
P6	IL10RA	c.301C4T, p.R101W ; c.784C4T, p.R262C	F	6						1	1	1					
Lee, J Crohns Colitis, 2014																	
P1	IL10RA	c.583T>C; c.1368G>T	NA									NA					
Beser, JPGN 2015																	
P1	IL10RB	c.G477A; p.W159X	NA									NA					

Article	Gene	Mutation	M/F	Disease onset (months)	Esophagus	Stomach	Duodenum	Jejunum	Ileum	Colon	Rectum	Perianal lesions	Folliculitis	Arthritis	Other	Infections	B cell lymphoma
P2	IL10RA	c.T192G; p.Y64	NA									NA					
P3	IL10RA	c.T133G; p.W45Gly	NA									NA					
Gassas, PEDIATR Transplant 2015																	
P1	NA	NA	F	0,3		No				1		1					
Kunkozmas, BMT 2016																	
P1	IL10RA	c.G477A, p.Trp159X	M	2,5						1		1					
Oh SH, JCC, 2016																	
P1	IL10RA	p.R101W; p.T179T	NA							1		1					
P2	IL10RA	p.R101W; p.T179T	NA							1		1					
P3	IL10RA	p.T179T	NA	72						1		NO					

Table 7. Summary of *FOXP3*-mutated patients.

When patients are reported in more than one article, they are reported only once in the chart below. Some full version articles were not available and are thus not included.

AI: autoimmune; AIE: autoimmune enteropathy; GI: gastrointestinal; AI cytopenia: on thrombocytes and/or leukocytes

Article	Mutation	Age at disease onset (days)	Death	AIE	Other GI lesions	Skin lesions	Eczema	Food allergies	Diabetes mellitus	AI cytopenia	AI hemolytic anemia	AI Thyroiditis	AI Hepatitis	Hyper IgE	Infection	Nephropathy	Other
Chatila, JCI 2000																	
5 patients (from 2 families)	P1: IVS9 ; P2-5: c.751_753delGAG, p.delE251			5		5	5	4	5	3				4			
Bennett Immunogenetics 2001																	
	AAUAAA-->AAUGAA																
Bennett Nat Genet 2001																	
	g.G1338A, p.Ala384Thr																
	g.G1338A, p.Ala384Thr																
	g. 1481_1483delCT, p.Z432Thr																
Wildin, Nat Genet 2001																	
	c.C1189T, p.R397W		1	1					1	1		1					
	c.del1290_1309/insTGG		1	1		1	1		1		1				1		1
	c.T1113G, p.F371C			1		1			1		1						

Article	Mutation	Age at disease onset (days)	Death	AIE	Other GI lesions	Skin lesions	Eczema	Food allergies	Diabetes mellitus	AI cytopenia	AI hemolytic anemia	AI Thyroiditis	AI Hepatitis	Hyper IgE	Infection	Nephropathy	Other
	c.G1150A, p.A384T		1	1		1			1	1		1			1		
Levy-Lahad, 2001																	
patient 1		0		1					2	2		1					
patient 2				1													
patient 3				1													
Kobayashi, J Med Genet 2001																	
patient 1	c.del227T, p.fs128X	15		1							1	1					1
patient 2	c.A1087G, p.Ile363Val			1					1			1		1			1
Wildin, J Med Genet 2002																	
patient 1	c.G1040A, p.R347H	90	1	1					1								
patient 2	IVS9 + 459 A>G	42		1		1	1		1	1		1					
patient 3		60		1					1								
Owen 2003																	
II-1		14		1		1	1										
II-2	c.del76T	21		1		1	1						1				
Nieves 2004																	
patient	p.A384T	210		1	colitis	1	1		1								
Tanaka 2004																	
patient	g.T1117G, p.F373V	60		1					1					1			

Article	Mutation	Age at disease onset (days)	Death	AIE	Other GI lesions	Skin lesions	Eczema	Food allergies	Diabetes mellitus	AI cytopenia	AI hemolytic anemia	AI Thyroiditis	AI Hepatitis	Hyper IgE	Infection	Nephropathy	Other
Mazzolari 2005																	
P1	Deletion in promoter region	120		1					1					1	1		
Myers 2006																	
Case 1	c.-7G>T	4	1	1					1						1		
Case 2	p.S390N																
Benedetti 2006																	
Patient 1	c.454+4A>G	18		1		1								1			arthritis
Patient 2	p.T108M	420		1	mild ileitis									1			arthritis
McGuinness 2006																	
Patient	p.A384T	1		1		1	1							1	1		
Bacchetta JCI 2006																	
Patient 1	p.F373A	14		1					1					1			
Patient 2	c.970T>C; p.F324L	120		1		1	1				1			1			
Patient 3	c.3G>A (ATG -> ATA)	20		1					1					1	1		
Gavin 2006																	
IPEX 1	c.210_210 +1delins AC	60		1		1	1		1	1	1			1			
IPEX 2 P1	c.751_753delGAG, p.delE251	180		1		1	1		1		1	1		1			
IPEX 2 P2	c.751_753delGAG, p.delE251	180		1		1	1		1			1		1			

Article	Mutation	Age at disease onset (days)	Death	AIE	Other GI lesions	Skin lesions	Eczema	Food allergies	Diabetes mellitus	AI cytopenia	AI hemolytic anemia	AI Thyroiditis	AI Hepatitis	Hyper IgE	Infection	Nephropathy	Other
Moudgil 2007																	
Patient	c.304_305del	120		1	vomiting	1	1		1		1	1				1	
Fuchizawa 2007																	
P2	p.A384T	60		1		1	1										No treatment apart inhaled corticosteroids at 6 years old
P3	p.A384T	19		1		1	1										No treatment at 15 years old
Rao 2007																	
P1	A>G splice junction mutation in intron 9			1	colitis	1	1	1			1						
P2	c.304_305del			1	colitis	1	1	1			1						
P3	p.C424Y				colitis	1	1	1									1
P4	p.D409G				colitis						1						
Suzuki 2007																	
P1	c.1099T>C, p.F367L	8	1	1		1	1		1	1			1		1	1	
Taddio 2007																	
P1	p.A384T	30		1		1	1		1		1	1					
Burroughs 2007																	
P1	c.1271G>A; p.C424Y	?		1					1								1

Article	Mutation	Age at disease onset (days)	Death	AIE	Other GI lesions	Skin lesions	Eczema	Food allergies	Diabetes mellitus	AI cytopenia	AI hemolytic anemia	AI Thyroiditis	AI Hepatitis	Hyper IgE	Infection	Nephropathy	Other
Lucas 2007																	
P1	?	180		1		1									1		
Torgerson, Gastro 2007																	
patient IV.1	g.4859-6247_del	30	1	1	colitis	1	1	1						1			
patient IV.2	g.4859-6247_del	60				1	1	1						1			
Gambineri JACI 2008																	
patient 1	c.2T>G (ATG -> ACG)	20		1		0			1						1		
patient 2	c.3G>A (ATG -> ATA)	20		1		1	1		1			1		1		adenopathy, splenomegaly	
patient 3	IVS1+2T>G	20		1		1	1		1			1	1		1		
patient 4	g.C543T	20		1		0											
patient 5	g.C543T, p.F324L	20		1		1	1										
patient 6	IVS7+5G>A	20		1		1	1		1								
patient 7	IVS8+4A>G	20		1		1	1		1				1	1			
patient 8	p.P339A	20		1		0			1		1		1			splenomegaly	
patient 9	p.R347H	20		1		1	1	1	1	1			1	1			
patient 10	p.R347H	200			gastritis	1	1		1								
patient 11	p.A384T	20		1		1	1		1		1	1					
patient 12	p.F373A	20		1		1	1		1					1			
patient 13	p.F374C	20			colitis	1	1			1	1			1	1		
patient 14	p.L242P	120		1		1	1							1	1	1	
Costa-Carvalho 2008																	
BBP		1		1		1	1		1			1			1		

Article	Mutation	Age at disease onset (days)	Death	AIE	Other GI lesions	Skin lesions	Eczema	Food allergies	Diabetes mellitus	AI cytopenia	AI hemolytic anemia	AI Thyroiditis	AI Hepatitis	Hyper IgE	Infection	Nephropathy	Other
Yong 2008																	
Patient 1	c.1061del; p.P354Q	840		1		1											
patient 2	p.Q70H	30		1		0				1	1				1		arthritis
Zhan 2008																	
P1	p.T380I	130		1		1		1						1			
Dorsey 2009																	
patient	AATAAA >AATAAG	20		1	colitis	1											
Rubio-Cabezas, 2009																	
patient I	p.V408M	2		0	no diarrhea at 15 years old				1								1
patient IIa	p.V408M	21		1		0			1			1			1		
patient IIb	p.V408M	100		1		0			1			1			1		
patient III	P.R337Q	30	1	1		0			1					1			
patient IV	p.P339A	7	1	1		0			1			1					
patient V	p.L76QfsX53	1	1	1		0			1	1					1		
Hashimura 2009																	
Patient	c.748_750delAAG; p.250Kdel	60		0	no diarrhea	1	1	1	1		1			1	1	1	
d'Hennezel editor NEJM 2009																	
P1	p.A384T		1														
Ohshima, Pediatr Pulmonol 2009																	
P1	c.1150G>A, p.Ala384Thr	30		0	no diarrhea	1	1		0	0	0	0		1	1		

Article	Mutation	Age at disease onset (days)	Death	AIE	Other GI lesions	Skin lesions	Eczema	Food allergies	Diabetes mellitus	AI cytopenia	AI hemolytic anemia	AI Thyroiditis	AI Hepatitis	Hyper IgE	Infection	Nephropathy	Other
elder brother	c.1150G>A, p.Ala384Thr			1		1											
2 maternal cousins	c.1150G>A, p.Ala384Thr					1								2	2		
Moes Gastro 2010																	
patient 1	torgerson 2007	35		1	colitis	1	1	1						1			
patient 2	torgerson 2007	21		1	colitis	1	1	1						1			
patient 3	g.C560T, p.P187L	0		1	colitis	1	1			1		1		1			
patient 4	g.T1121G, p.F374C	0		1	colitis	1	1			1	1			1			
patient 5	c.751_753delGAG, p.delE251	42		1	colitis	1	1				1	1		1		1	
patient 6	c.751_753delGAG, p.delE251	28		1		1	1		1	1	1		1	0			
patient 7	c.C1015G, p.P339A	7		1	colitis	1	1		1		1						
Burroughs JACI 2010																	
P1	c.210 +2del	60		1		1			1		1			1	1		
P2	c.816+7G>C	?		1		1			1			1		1	1	1	growth hormone deficiency
Tourangeau 2011																	
patient	c.748_750delAAG; p.250Kdel	180	1	1		1	1							1	1	1	adenopathy
Bae 2011																	
patient	c.201+1G>A	330		0	no diarrhea				1		1				1	1	

Article	Mutation	Age at disease onset (days)	Death	AIE	Other GI lesions	Skin lesions	Eczema	Food allergies	Diabetes mellitus	AI cytopenia	AI hemolytic anemia	AI Thyroiditis	AI Hepatitis	Hyper IgE	Infection	Nephropathy	Other
Passerini JACI editor 2011																	
P3	IVS1+2T>G	20		1		1	1		1				1		1		
P7	IVS8+4A>G	20		1		1	1		1				1				
P8	p.P339A	20		1		0	0		1		1		1				splenomegaly
P9	p.R347H	20		1		1	1	1	1	1			1				
P11	p.A384T	20		1		1	1		1		1						
P12	p.F373A	20		1		1	1		1								
P14	p.L242P	120		1		1	1								1	1	
P17	p.I346T	20		1		1	0									1	
P18	AATAAA>AATGAA	20		1		1	0										
P20	IVS7+2del	150		1		1	1			1							arthritis
Kobayashi 2011																	
Case 2	c.-23+1G>T			1					1								1
Otsubo 2011																	
patient 5	IVS1+1T>G	180		1	colitis				1								1
An 2011																	
P1	g.13098-13099insA	20	1	1		1	1		1						1	1	
P2	g.13128G>A, p.M370I	14	1	1		1	1		0					1	1	1	
P3	g.11628T>C; p.F324L	26	1	1		0			1						1		adenopathy, splenomegaly
Kasow 2011																	
P1	p.A384T	42		1		1	1				1			1	1		

Article	Mutation	Age at disease onset (days)	Death	AIE	Other GI lesions	Skin lesions	Eczema	Food allergies	Diabetes mellitus	AI cytopenia	AI hemolytic anemia	AI Thyroiditis	AI Hepatitis	Hyper IgE	Infection	Nephropathy	Other
Horino 2014																	
Patient	c.1117T>G	60		1													
Okou D, JPGN May 2014																	
II.1	c.694A>C, p.C232G	infancy		1		1	1	1							1		
II.2	c.694A>C, p.C232G	infancy		1		1	1	1	1						1		arthritis
II.3	c.694A>C, p.C232G	infancy		1	colitis	1	1	1						1	1		
mother	Heterozygous c.694A>C, p.C232G				subtotal colectomy for ulcerative colitis	psoriasis											
mother's brother	Not tested				Crohn's disease	psoriasis											

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