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ABSTRACT

An increase in gut barrier (GB) permeability and abnormal crosstalk between commensal gut microbiota and immune cells underlies the pathogenesis of Type 1 Diabetes (T1D). The gut microbiota plays a pivotal role in triggering this "leaky gut" condition, as suggested by the presence of dysbiosis in T1D patients and at-risk-individuals. Particularly, the presence of LPS-carrying bacteria in children is associated with higher risk to develop T1D but the mechanism through which LPS modulates T1D are unknown. Epithelial Toll-like receptors (TLRs) signaling is important in the cross-talk between the host and the gut microbiota. Under homeostatic conditions, commensal LPS triggers the activation of a tolerogenic TLR4-mediated pathway on intestinal epithelial cells (IEC) through activation of the TRAF3 signaling pathway and promotes Treg cell differentiation thus maintaining immune tolerance and GB integrity; on the other hand, a dysregulated activation of epithelial TLR4 signaling induces the activation of a pro-inflammatory pathway through MYD88-TRAF6 contributing to intestinal inflammation, damage of the GB and breakage of intestinal immune homeostasis.

Here, we tested the hypothesis that loss of GB integrity and intestinal inflammation present in patients and preclinical models of T1D is due to a dysregulated epithelial TLR4 signaling.

Our *in-vivo* and *in-vitro* data show that NOD mice, the spontaneous preclinical model of T1D, carry an inherent overstimulation of the epithelial TLR4-induced TRAF6 signaling pathway under basal homeostatic conditions at the expenses of the tolerogenic TRAF3-mediated signaling pathway. Furthermore, IEC of NOD mice have an impaired response to the commensal LPS *in-vivo* leading to defective FoxP3^{+}Treg cell differentiation, impaired mucus barrier structure and intestinal inflammation compared to non- autoimmune strains of mice (i.e., C57BL/6 mice) as well as diabetes-resistant NOD mice, thus suggesting that the abnormal response to commensal LPS is directly linked to T1D pathogenesis. The abnormal activation of the MYD88-TRAF6 signaling was confirmed in NOD-derived intestinal organoids and is related to altered post-transcriptional regulation mechanisms as demonstrated by decreased expression of miR-146a, a key regulator of the TLR4 signaling, in IEC of both newborn and adult NOD mice compared to the non-autoimmune counterpart. Importantly, our findings in the preclinical model were validated in human T1D patients in which we found an overactivation of the TLR4-induced TRAF6-mediated inflammatory pathway in the small intestinal mucosa.

Here we demonstrate that preclinical models and humans affected by autoimmune T1D have a dysregulated response to commensal LPS that triggers a pro-inflammatory TLR4-signaling pathways possibly leading to damage of GB integrity and reduced intestinal immune tolerance. Further studies are necessary to link the defective response to commensal LPS to activation of islet-reactive T cells and autoimmune pathogenesis of T1D in preclinical models and humans.

TABLE OF CONTENTS

ACRONYMS AND ABBREVIATIONS 4

LIST OF FIGURES AND TABLES 8

INTRODUCTION 9

1. The gut-immune axis 9

1.1. The intestinal immune system *9*

1.1.2 The gut-associated lymphoid tissues (GALT) 9

1.1.3 Lamina propria DC 10

1.1.4 Lamina propria T cells 10

1.2 Microbial recognition by immune cells in the intestinal mucosa *12*

1.2.1 Microbiota recognition by patter-recognition receptors 13

1.2.2. Toll-like receptors 14

1.2.3 The intestinal microbiota drives the functional phenotype of T cells. 16

1.2.4 The induction of immune tolerance during early-life in the gut mucosa 19

1.3 The intestinal epithelium in driving the immune system. *22*

1.3.1 Immune-mechanisms of bacteria separation 22

1.3.2 The cross-talk between the intestinal epithelium and immune cells 24

1.3.3 TLRs signaling within the intestinal epithelium. 27

2. The importance of the gut environment in extra-intestinal autoimmune diseases 29

2.1. Organ-specific autoimmune diseases *29*

2.1.1 The gut microbiota and extra-intestinal autoimmune diseases 29

2.2 The role of the gut in Type 1 Diabetes *31*

2.2.1 Type 1 Diabetes: pathogenesis and risk factors 31

2.2.2 “Leaky gut” in Type 1 Diabetes 33

2.2.3 Gut microbiota and Type 1 Diabetes 35

2.2.4 Microbiota modulate Type 1 Diabetes through Toll-like receptors. 36

2.2.5 The LPS-TLR4 signaling in Type 1 Diabetes 36
AIM OF THE WORK 39
RESULTS 40
The intestinal epithelium of NOD mice is characterized by a defective differentiation of epithelial cells. 40
NOD mice is characterized by a predominant pro-inflammatory epithelial TLRs signaling.. 42
A pro-inflammatory response to nonpathogenic LPS is detected in IE of NOD mice and correlates with defective FoxP3^{+} Treg expansion. 45
NOD mice-derived intestinal organoids present a dysregulated TLR4 signaling *in vitro*. 51
Post-transcriptional regulation on epithelial TLR signaling is compromised in NOD mice. . 56
Pro-inflammatory epithelial TLRs signaling is present in the gut mucosa of T1D patients... 57
DISCUSSION 59
MATERIALS AND METHODS 64
Preclinical studies 64
Mice *64*
Intestinal epithelial cells and mononuclear cells isolation *64*
Ileal murine organoids *65*
Flow cytometry. *65*
In-vivo LPS treatment *66*
ELISA assay *66*
RNA extraction and quantitative real-time RT-PCR *66*
Histology *67*
Diabetes Incidence *67*
Statistical analysis *67*
Clinical studies 67
Study population *67*
Samples collection and processing *68*
RNA extraction and quantitative real-time RT-PCR *68*

Statistical analysis..... *68*

REFERENCES..... 69

ACRONYMS AND ABBREVIATIONS

AMPs: anti-microbial peptides

AP-1: activator protein 1

APC: antigen-presenting cells

APRIL: proliferation-inducing ligand

BAFF: B-cell activating factor

BCL6: B-cell lymphoma 6

CD: celiac disease

CD25: interleukin-2 receptor alpha chain

CD69: cluster of differentiation 69

CSGG: cell surface β- glucan/galactan

DAMPs: damage-associated molecular patterns

DCs: dendritic cells

eGALT: GALT-associated epithelium

FAK: membrane-associated adaptor protein focal adhesion kinase

FMT: fecal microbiota transplantation

FOXP3: forkhead-box-P3

GALT: gut-associated lymphoid tissues

GC: goblet cells

GF: germ-free

GI: gastrointestinal tract

HLA: human leukocyte antigen

IBD: intestinal bowl diseases

iDCs: intestinal DCs

IE: intestinal epithelium

IEC: intestinal epithelial cells

IEL: intra-epithelial lymphocytes

IgAs: immunoglobulins-A

IL-10: interleukin-10

IL-12: interleukin-12

IL-15: interleukin-15

IL-17: interleukin-17

IL-18: interleukin-18

IL-2: interleukin-2

IL-22: interleukin-22

IL-23: interleukin-23

IL-6: interleukin-6

ILC: innate lymphoid cells

ILC1: type 1 innate lymphoid cells

ILC2: type 2 innate lymphoid cells

ILC3: type 3innate lymphoid cells

INF-γ: interferon- γ

iNKT: invariant natural killer T

IRAK: The interleukin-1 receptor associated kinase

IRF3: interferon regulatory factor 3

iTreg: intestinal Treg

LFs: lymphoid follicles

LN: lymph nodes

LP: lamina propria

LPS: lipopolysaccharides

MAIT: mucosal associated invariant T cells

MAMPS: microbe associated-molecular patterns

MHC: major histocompatibility complex

MHC-I: MHC class I

MHC-II: MHC class II

MLCK: myosin light chain kinase

MLN: mesenteric lymph nodes

MS: Multiple Sclerosis

MUC2: mucin-2

MYD88: myeloid differentiation primary response gene 88

NF-κB: nuclear factor κ-light-chain

NOD: Non-obese diabetic

NODs: NOD-like receptors

NOR: diabetes-resistant NOD

NRL9: NOD- LRR- and pyrin domain-containing 6

PD1: programmed cell death protein 1

PDL: PD1-ligand 1

PPs: Peyer's patches

PRRs: patter-recognition receptors

PSA: bacterial polysaccharide

RA: rheumatoid arthritis

REG3γ: C-type lectin regenerating islet-derived protein 3γ

RORγt: RAR-related orphan receptor C

SCFAs: short-chain fatty acids

SFB: segmented filamentous bacteria

SLE: systemic lupus erythematosus

SPF: specific-pathogen-free

T1D: Type 1 Diabetes

TAK1: mitogen-activated protein kinase 7

Tbet: T-box transcription factor TBX21

TBK1: TANK-binding kinase 1

TCR: T cell receptor

Tfh: follicular T helper

TGF-β: transforming growth factor-β

Th: T helper

TIR: Toll/interleukin 1 receptor

TJPs: tight junctions proteins

TLRs: Toll-like receptors

TNFAIP3: TNFα-induced protein 3

TNF-α: tumor necrosis factor alpha

TOLLIP: Toll-interacting protein

Tr1: type 1 regulatory T cells

TRAF3: TNF receptor-associated factor 3

TRAF6: intracellular E3 ubiquitin ligase tumor necrosis factor R-associated factor 6

Treg: regulatory T cells

TRIF: TIR-domain-containing adaptor-inducing interferon-β

TSLP: thymic stromal lymphopoietin

tTreg: thymic Treg

γδ IEL: γδ intra-epithelial lymphocytes

LIST OF FIGURES AND TABLES

Table 1: Clinical data……....57

Figure 1: PRRs-mediated mechanisms of microbial homeostasis in the intestinal mucosa…….14
Figure 2: TLRs pathways…….…….16
Figure 3: Microbiota-induced Th cell subsets in the intestinal mucosa……18
Figure 4: Environmental factors that influence the gut microbiota colonization during infancy…….....21
Figure 5: Antimicrobial peptide production in the intestinal epithelium…….…….24
Figure 6: Cross-talk between IEC and immune cells in homeostasis and inflammation……..26
Figure 7: Microbial activation mechanisms of autoimmunity……..31
Figure 8: Isolation of intestinal epithelial cells……..41
Figure 9: IEC differentiation process pushes toward the absorptive lineage in NOD mice……..42
Figure 10: Pro-inflammatory epithelial TLRs signaling in present in IE of NOD mice…….……43
Figure 11: Epithelial TLRs signaling correlate with disease progression in NOD mice……44
Figure 12: NOD mice present a dysregulated epithelial TLR response to LPS in the ileum……46
Figure 13: NOD mice present a dysregulated epithelial TLR response to LPS in the colon……47
Figure 14: The short-term exposure to LPS induce the local production of inflammatory cytokines in NOD mice…….48
Figure 15: Lack of Treg cell induction in response to low dose LPS stimulation in NOD mice.……..……...49
Figure 16: : The lack of Treg induction correlates with decreased frequency of Treg polarizing DC after shot-term LPS exposure in NOD mice. …….50
Figure 17: Setting mouse-derived intestinal organoids……...…..52
Figure 18: NOD-derived intestinal organoids show a defective maturation of the IE…….…….53
Figure 19: Dysregulated TLR signaling is present in NOD mice-derived organoids……54
Figure 20: LPS induced mucus production in both intestinal organoids lines……..…..55
Figure 21: Dysregulated expression of miR-146a is present in IEC from NOD mice …….56
Figure 22: The gut mucosa of T1D patients is characterized by a pro-inflammatory epithelial TLRs signaling…….….58

INTRODUCTION

1. The gut-immune axis

1.1. The intestinal immune system

The intestinal immune system faces constantly millions of microorganisms and other external antigens at the mucosal level, and constantly needs to distinguish between friends or foes among them. Therefore, it has developed a finely tuned capacity to discriminate commensal microorganisms to pathogens without interfering with the metabolic functions of the microbiota and maintaining a condition called "homeostasis". Besides, the gut microbiota is well known to shape the immune response, from homeostasis to susceptibility to diseases, in the gut but also in extra-intestinal tissues. For these reasons, the intestine is considered as an "immunological organ" and it has elicited increasing interests by immunologists in the past decades, specifically in understanding how it modulates the immune response in health and disease.

1.1.2 The gut-associated lymphoid tissues (GALT)

The intestinal mucosa harbors at least 70% of peripheral lymphocytes and it is considered the largest peripheral lymphoid tissue in the body. It is composed of two functional sides: the gut-associated lymphoid tissues (GALT), that included organized lymphoid structures such as the Peyer's patches (PPs) and numerous lymphoid follicles (LFs) located along the intestinal tract, and the total of innate and adaptive immune cells that lie in the lamina propria (LP) (Ohno H., 2016).

PPs and LFs are organized as small follicles with a consistent number of innate and adaptive immune cells. Dissimilar to systemic lymph nodes, PPs and LFs are not directly linked to the lymphatic system so that antigen sampling occurs directly from the intestinal lumen. Hence, the composition of the GALT-associated epithelium (eGALT) favors the opportunity for bacteria uptake. M cells, for instance, are a subtype of epithelial cells present only in the eGALT and are specialized in the delivery of bacteria to antigen-presenting cells (APCs) such as dendritic cells (DCs) (Ohno H., 2016). The antigens presentation process by M cells is not specific and, due to their low lysosomal enzymatic activity, antigens are not processed but presented intact to APCs. Immunoglobulins A

(IgAs) are produced by secretory B plasma cells located in the germinal centers of PPs and are involved in the clearance of pathogens and toxins in a process called "immune exclusion". Within the germinal center, naïve B cells are in close contact with follicular T lymphocytes, which induce the differentiation of B cells into plasma cells after antigen presentation by DCs (Sage PT et al., 2016).

1.1.3 Lamina propria DC

Intestinal DCs (iDCs) are specialized APC that sample antigens from the luminal contest directly with their dendrites or indirectly through other APC-like cells, such as intestinal epithelial cells (IEC), and engage with T and B cells to induce their differentiation. iDCs migrate from the LP to draining lymph nodes (LN), such as mesenteric LN (MLN), and actively participate in driving the mucosal immune response in either cases of infectious agents or nonpathogen bacteria. Besides, the short lifespan of DCs is a mechanism that intrinsically "limit" the circulation of bacteria into the systemic lymphoid tissues avoiding the activation of adaptive immune cells. This phenomenon is called "immune-ignorance", fundamental for the maintenance of the mutualistic relationship host-microbiota, and takes place mainly into the MLN (Macpherson AJ et al., 2004).

The antigen-presentation by DCs occurs mainly through major histocompatibility complex (MHC) class II (MHC-II) to T cells and through the B-cell activating factor (BAFF) or the proliferation-inducing ligand (APRIL) to B cells. Furthermore, iDCs modulate the differentiation of adaptive immune cells by producing cytokines, for example IL-12 for $CD4^+$ T helper-1 (Th1) cells, retinoic acid (RA) and transforming growth factor-β (TGFβ) for either IgA-producing B cells or intestinal regulatory T cells ($FoxP3^+$ iTreg and IL-10^+ Tr1 cells). Thus, they play a pivotal role for the maintenance of the immune homeostasis at the intestinal level and have the responsibility to recognize pathogens from nonpathogenic bacteria inducing tolerance or inflammatory responses.

1.1.4 Lamina propria T cells

The majority of T cells found in the lamina propria originates from the thymus. Thymic conventional naïve T cells migrate in the intestine where they differentiate into several Th cells subsets in response to various environmental clues. APC and intestinal epithelial cells (IEC) regulate the differentiation of T cells by presenting the antigens captured in

the intestinal lumen. Similarly, some thymic nonconventional naïve T cells differentiate into regulatory T cells (Ma H et al., 2019). iTreg cells differentiated ex novo in the intestine play a crucial role in maintenance of intestinal immune homeostasis. Both tTreg and iTreg express the forkhead-box-P3 (FOXP3)+ (Ito T et al., 2008), however a subtype of regulatory T (Treg) cells that produces IL-10 but does not express FOXP3, defined as type-1 regulatory T cells (Tr1), has been found in the gut, mainly in the small intestine (Maynard CL et al., 2007) and plays a pivotal role in preventing colitis in mouse models (Izcue A et al., 2008). Considering that IL-$10^{-/-}$ mice are high susceptible to colitis (Kühn R et al., 1993) contrarily to $FOXP3^{-/-}$ mice which develop a severe autoimmune condition (Kim JM et al., 2007), it has been assumed that Tr1 IL-10^{+} cells are mostly induced in case of perturbation of intestinal homeostasis and are not constitutively present in the gut mucosa as conventional $FOXP3^{+}$ Treg.

Dietary antigens and commensal bacteria crucially affect the frequency and the function of intestinal iTreg since germ-free (GF) mice or antigen free diet-fed mice have a severe reduced number of intestinal Treg (Kim KS et al., 2016). In this contest, a large body of literature supports the notion that numerous commensal bacterial species induce intestinal $FoxP3^{+}$Treg cell differentiation, such as *Clostridium ramosum* (Atarashi K et al., 2013), *Bacteroides fragilis* (Round JL et al., 2010), *Bacteroides thetaiotaomicron* (Furusawa Y et al., 2014), *Bifidobacterium* and *Lactobacillus* (Dolpady J et al., 2015). Moreover, a randomized clinical trial have identified also *Faecalibacterium prausnitzii* as an intestinal Tr1-inducing bacterial strain (Sokol H et al., 2008).

1.2 Microbial recognition by immune cells in the intestinal mucosa

The human gastrointestinal tract harbors 4×10^{13} microorganisms, including bacteria, fungi, archaea, and virus-like particles, that co-live with the host in a complex symbiotic relationship. The beneficial effects that intestinal microbes provide to the host includes not only enhancing the digestion of food-derived components (such as polysaccharides) but also providing signals for intestinal development, such as epithelial cells maturations and lymphocyte development. Besides, lack of microbiota in gnotobiotic models causes several deficiencies in gastrointestinal function, including reduced epithelial permeability, higher mucus penetrability, reduced epithelial cell proliferation and underdeveloped GALT immune system (Bhattarai Y et al., 2016).

The immune response against intestinal microorganisms depends on the type of bacteria-host interaction. For example, the response against pathogens aims to protect against infectious avoiding in the same time tissue damage; this type of response is associated with the inflammatory "type-1" or "type-3" immunity and with the induction of Th1 or Th17 helper cells. Simultaneously, commensal bacteria are "tolerated" by the immune system in a complex discrimination process that the host develops from the moment of birth and is orchestrated by intestinal Treg cells ($FoxP3^+$ Treg and Tr1 cells). In both cases, the immune response modulates the tissue-resident memory T and B cells, which often develop antigen-specificity towards bacterial antigens (Schären OP et al., 2021). Furthermore, intestinal IgAs have been recently discovered to actively contributing to microbiota tolerance since they promote the delivery of antigens from the intestinal lumen to DC located in GALT, promoting the immune tolerance at the intestinal level (Mantis NJ et al., 2011).

Although most individuals successfully co-lived with their commensal microbiota, the discrimination between harmful or beneficial bacteria is not always perfect as some individuals, for still unknown reasons, develop uncontrolled inflammatory responses against non-pathogenic microbiota. In such cases, the phenotype of memory B and T cells may be mistakenly imprinted causing long-term chronic inflammation linked to autoimmune or immune-mediated diseases (Al Nabhani Z et al., 2019) in the intestine but also at systemic level and in extra-intestinal tissues. For example, the high variability in cancer immunotherapy responses among individuals is thought to be partially attributed

to the gut microbiota composition, and it has been hypothesized that the bacteria-associated immune response may influence tumor surveillance (Lu Y et al., 2022).
Although an increasing number of studies are unravelling the role of gut microbiota in modulating the host's health, the molecular mechanisms by which microbes influence immunity at intestinal and systemic level are still largely unknown.

1.2.1 Microbiota recognition by patter-recognition receptors

The microbial recognition by immune and non-immune cells is mediated by patter-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NODs), that sense microbe associated-molecular patterns (MAMPS) and accordingly induce transcription of either pro-inflammatory cytokine or type I/type III interferons.
PRRs signaling has been suggested to modulate the microbial colonization since mice deficient for these receptors showed altered microbiota composition, increased mucosal-adhesion bacteria and intestinal inflammation (Wen L et al., 2008; Vijay-Kumar M et al., 2010; Couturier-Maillard A et al., 2013). Besides, the recognition of MAMPS by PRRs results in a finely regulated signaling involving either inflammatory responses against pathogens or tolerogenic responses to non-pathogenic bacteria. Among the beneficial pathways mediated by PRRs, several mechanisms that control the overgrowth of bacteria at the intestinal level exist and are summarized in Figure 1. For example, bacterial flagellin stimulates TLR5 and induces the production of anti-microbial peptides (AMPs) through IL-23/IL-22 axis, mediated by DCs and ILCs. Moreover, peptidoglycan or other microbial metabolites stimulate IEC to produce AMPs through NOD2 and NRL9 receptors respectively. Adaptive immune cells, such as follicular Th cells or IgA-producing B cells, are also regulated by the interaction with specific bacteria, participating to the control on the luminal bacteria composition (Levy M et al., 2017).
Notably, MAMPs from different bacterial species have different immunogenicity on PRRs, thereby stimulating different pathways and altering the balance between tolerogenic and inflammatory signaling. This has been demonstrated, for examples, for the lipopolysaccharides (LPS), whose immunostimulatory activity has been linked to the susceptibility to autoimmune disease such as Type 1 Diabetes in children (Vatanen T et al., 2016), and for the bacterial polysaccharide (PSA), which has a direct impact on the immune system maturation during early life (Mazmanian SK et al., 2005).

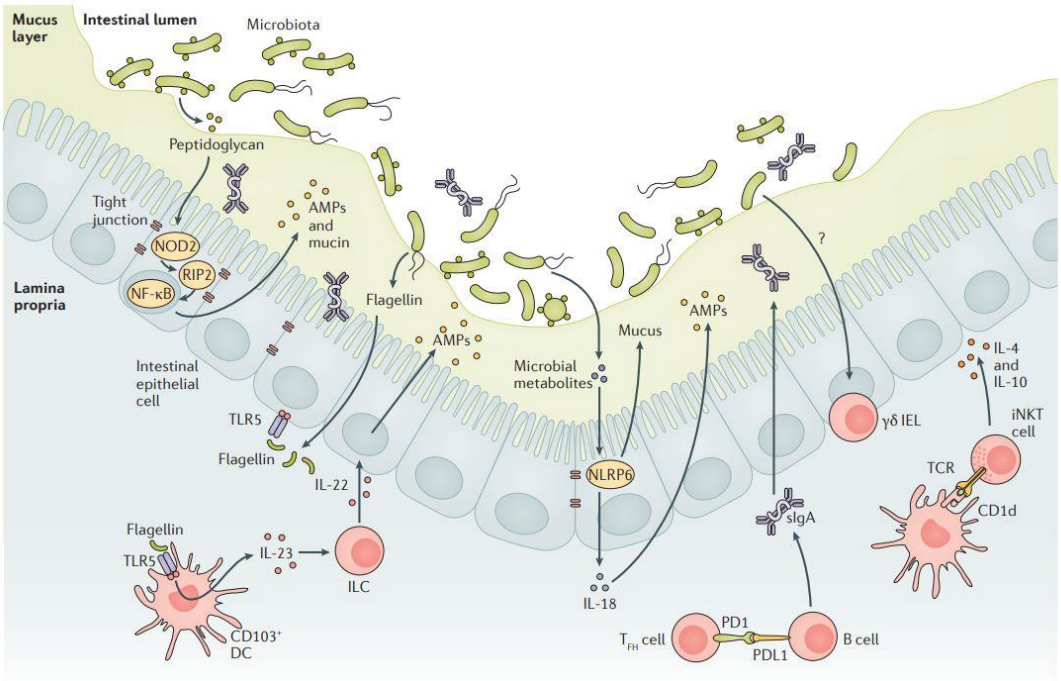


Figure 1: PRRs-mediated mechanisms of microbial homeostasis in the intestinal mucosa.

The nucleotide-binding oligomerization domain-containing protein 2 (NOD2) activation on IEC after interaction with bacterial peptidoglycan induce the production of AMPs via nuclear factor-κB (NF-κB) and kinase receptor-interacting protein 2 (RIP2) signaling. Bacterial flagellin stimulates Toll-like receptor 5 (TLR5) in both dendritic cells (DCs) and IEC to enhance the production of AMPs; specifically, flagellin-stimulated DC produce interleukin-23 (IL-23) which in turn stimulate innate lymphoid cell (ILC) to produce IL-22. The stimulation of the NOD- LRR- and pyrin domain-containing 6 (NLRP6) inflammasome on IEC induce the production of IL-18 and further enhance the AMPs secretion. Additionally, adaptive immune cells participate to control the microbial homeostasis by the secretion of bacteria-specific IgA, mediated by follicular T helper (Tfh) cells and B cells. Likewise, unconventional T cells such as γδ intraepithelial lymphocytes (IELs) and invariant natural killer T (iNKT) cells, which secrete IL-4 and IL-10. Other abbreviations: programmed cell death protein 1 (PD1), PD1 ligand 1 (PDL1), T cell receptor (TCR) (Levy M et al., 2017).

1.2.2. Toll-like receptors

Toll-like receptors (TLRs) are a family of PRRs expressed in both hematopoietic and non-hematopoietic cells, such as innate immune cells, hematopoietic stem cells, epithelial and endothelial cells. They are transmembrane proteins with a ligand-recognition domain, a transmembrane domain, and a Toll/interleukin 1 receptor (TIR) homology domain. TLRs family is divided in 10 receptors in human and 12 in mice, among which some are expressed on the cell surface and others in endosomes. As shown in Figure 2, TLRs

signaling involves the recruitment of several intracellular adaptors that activate transcription factors such as the nuclear factor κ-light-chain enhancers of activated B cells (NF-κB) or the interferon regulatory factor 3 (IRF3) (Aluri J et al., 2021). The myeloid differentiation primary response gene 88 (MYD88) is commonly used by all TLRs, except for TLR3, which utilizes the TIR-domain-containing adaptor-inducing interferon-β (TRIF) adaptor instead. Notably, the TLR4 can recruit both MYD88 and TRIF and the differential triggering generates different responses. The intracellular E3 ubiquitin ligase tumor necrosis factor (TNF) R-associated factor 6 (TRAF6) adaptor, recruited via MYD88, induces the production of pro-inflammatory cytokines through NF-κB, such as IL-1, IL-6, IL-8 and tumor necrosis factor alpha (TNF-α). Contrarily, TRIF activates IRF3 inducing the production of type I interferons (IFNs) via TNF receptor-associated factor 3 (TRAF3). TRAF3 also regulates the Teff/effector function of Treg cell balance and dampens Th effector differentiation. In fact, depletion of TRAF3 impairs $CD4^+$ T cell homeostasis specifically increasing the Th effector cells response (Chang JH et al., 2014; Lalani AI et al., 2015).

In some cases, TRIF also interacts with TRAF6 to activate the NF-κB signaling.

TLRs signaling is pivotal not only in the response against pathogens but also in the maturation of the immune system. For example, TLRs signaling has been shown to regulate the endothelium development during embryogenesis (He Q et al., 2015) and to mediate tolerance against commensal microbes during early-life, when the T cells compartment is highly imprinted by the gut microbiota (Lotz M et al., 2006). In mature immune cells, TLRs is crucial in the initiation of the immune response, specifically against infection. For example, TLRs signaling on APC induce the differentiation of naïve T cells into effector Th cells (Ussher JE et al., 2016). TLRs can also recognize the damage-associated molecular patterns (DAMPs) that is activated by damaged cells during tissue injury, thus regulating the process of tissue repair (Piccinini AM et al., 2010). Furthermore, the TLRs signaling on intestinal epithelial cells (IEC) is crucial in the host's response to luminal bacteria. In this context, dysregulated PRRs signaling is associated with immune defects and susceptibility to immune-mediated diseases. This is particularly true for TLRs, for which several studies in murine models demonstrated their involvement in the pathogenesis of both local inflammatory conditions such IBD (Lu Y et al., 2018) and systemic extra-intestinal immune-mediated diseases such as T1D (Chen

JQ et al., 2016). However, the molecular mechanisms of the complex TLRs signaling in modulating the host's immune response and tissue homeostasis are yet to be determined.

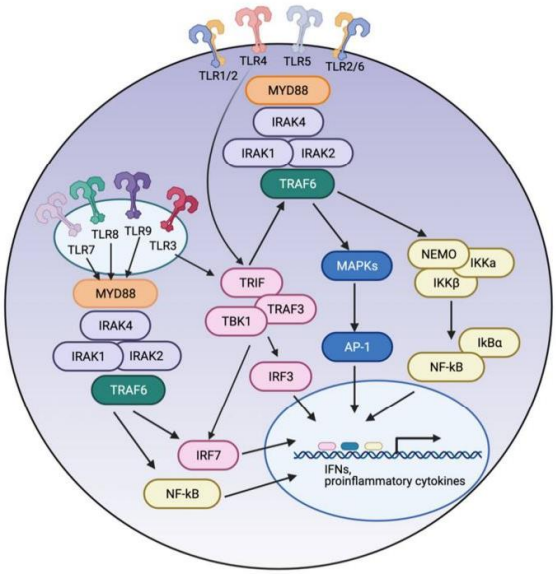


Figure 2: TLRs pathways
TLRs family consist of surface receptors, such as TLR1/2, TRL4, TRL5 and TLR2/6, and endosomal receptors such as TLR7, TLR8, TLR9 and TLR3. MYD88 recruits IRAKs1/2/4 and TRAF6, which activates either MAPKs/AP-1 or NF-κB resulting in the production of proinflammatory cytokines. Conversely, TRIF from either TLR3 or TLR4 recruits TRAF3 and TBK1, which activate either IRF3 or IRF7 to stimulate the production of interferons. Additionally, TRIF can interact with TRAF6 to further stimulate the signaling via MAPKs and NF-κB. (Aluri J et al., 2021)

1.2.3 The intestinal microbiota drives the functional phenotype of T cells.

Studies with GF mice demonstrated that bacterial species modulate the differentiation of intestinal T helper (Th) cells, as shown in Figure 3. This occurs in homeostatic conditions, i.e., in the absence of inflammation, demonstrating that this immune adaptation is instrumental for the cross-talk between the host and the microbiota. Microbial metabolites are capable to impact the functional T cell phenotype. For example, short-chain fatty acids (SCFAs), bile acid metabolites (BAM), PSA and RA (Geuking MB et al., 2020) are Treg inducers while the type of bacterial metabolites that drive effector Th1 and Th17 cell differentiation are less characterized.

Several autoimmune and immune-mediated diseases, in which the pathogenesis is mostly T cell-driven, are often associated with a condition defined "dysbiosis", that refers to

alterations of the microbiota composition and its metabolic profile (Levy M et al., 2017). In this context, IBD represent the best examples of how a dysregulated host-microbiota interactions crucially impact the host's health. IBD is a group of diseases characterized by chronic inflammation of the intestinal mucosa. Although the etiology is complex and still unclear, several lines of evidence show that the microbiota plays a central role in driving the pathogenesis of these diseases (Li Q et al., 2022). For example, it was showed that microbiota-specific memory $CD4^+$ T cells, abundant in healthy individuals, have increased IL-17 production in IBD patients (Hegazy AN et al., 2017).

A paper published in Nature in 2021 elegantly showed that microbiota-specific T cells expand in the thymus during early life after stimulation with microbial antigens carried by intestinal DC (Zegarra-Ruiz DF et al., 2021). Specifically, authors colonized Segmented filamentous bacteria (SFB)-negative specific-pathogen-free (SPF) mice at weaning with SFB and analyzed the T cells population in the thymus, finding an enrichment in SFB-specific $CD4^+$ T cells. These cells were not Treg cells since they did not express neither FOXP3 or the interleukin-2 receptor alpha chain (CD25), but they expressed TCR-β, CD44 and the cluster of differentiation-69 (CD69), showing an antigen-experienced T cell nature (Zegarra-Ruiz DF et al., 2021). These data clearly demonstrate the pivotal role of early-life microbiota in the establishment of a permanent microbiota-specific T cell repertoire. The molecular mechanisms underlying the thymic microbiota-specific T cells expansion are still unclear and it is yet to be determined whether these findings can be translated in humans. Furthermore, although it is widely accepted that microbiota shapes the intestinal Teff/Treg cell balance, the mechanisms underlying the T cell decision to differentiate into a specific functional T cell subset and the type/place of antigenic stimulation for microbiota-specific T cells are yet to be clarified. This mechanism is of high relevance also considering that cross-reactivity between microbial antigens and self-antigens has been demonstrated and poses a potentially high risk to develop autoimmunity in the gut but also in extra-intestinal organs. In fact, studies in murine models showed that specific bacterial antigens mimics self-peptides and are capable to activate self-reactive T lymphocytes specific for self-antigens of pancreatic islets (Tai N et al., 2916;, Sorini C et al., 2019); this mechanism of "molecular mimicry" between gut microbiota and autoimmune T cells potentially underlies the pathogenesis of several extra-intestinal autoimmune diseases (Rojas M et

al., 2018). Therefore, the effect of the microbiota on the intestinal T cells compartment is contest-dependent and it is modulated by a complex interaction between the host's genetic background and the environment, either eliciting beneficial effects or disease pathogenesis.

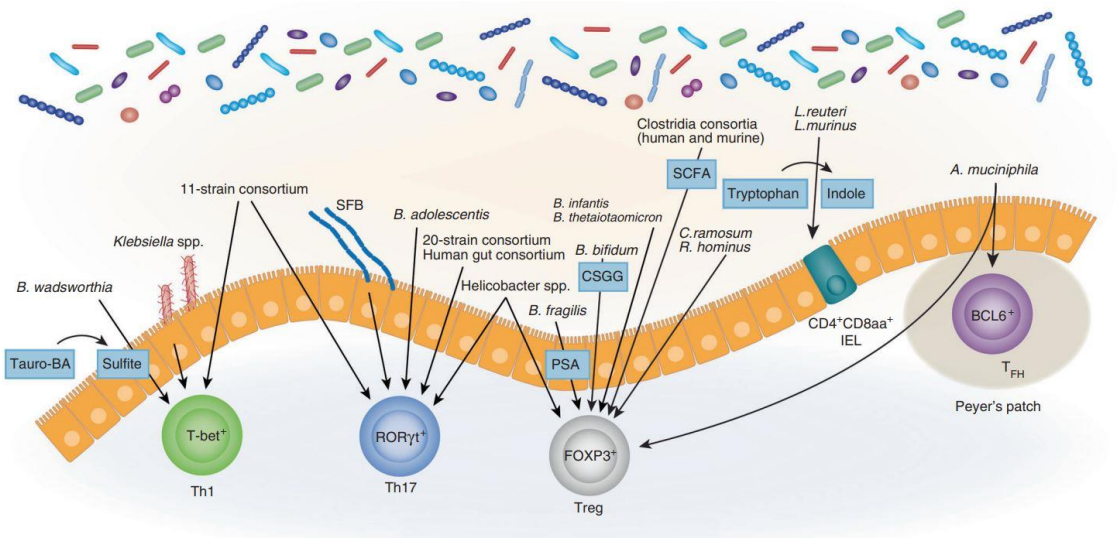


Figure 3: Microbiota-induced Th cell subsets in the intestinal mucosa
Naive CD4$^+$ T cells differentiate into several subsets of T helper (Th) cells after interaction with specific bacteria. For example, Bilophila wadsworthia and its sulfite-derived metabolites such as taurine induce a Tbet$^+$ Th1-oriented differentiation; Klebsiella is also an inductor of Th1 pro-inflammatory response. RORγt$^+$Th17 differentiation is induced by Segmented filamentous bacteria (SFB) and Bifidobacterium adolescentis. Some bacteria, such as Helicobacter, during intestinal inflammation induce a Th17 response while during homeostatic condition are FOXP3$^+$Treg inductor. Bacteroides fragilis and Bifidobacterium bifidum also promote the differentiation of FOXP3$^+$Treg through interaction with polysaccharide A (PSA) and the cell surface β-glucan/galactan (CSGG), respectively. Microbial metabolites such as SCFA, produced by Clostridium strains, Bifidobacterium infantis, Bacteroides Thetaiotaomicron and Roseburia hominis, further influence the Th phenotype inducing FOXP3$^+$Treg differentiation. Lactobacillus reuteri and Lactobacillus murinus metabolize tryptophan to indoles influencing the differentiation of CD4$^+$CD8αα$^+$ IELs. Lastly, Akkermansia muciniphila influence the differentiation of BCL-6+ T follicular helper cells within the PPs and of LP FOXP3$^+$Treg (Geuking MB et al., 2021).

1.2.4 The induction of immune tolerance during early life in the gut mucosa

In human, during the first 3 year of life the microbial composition is highly variable. Early microbiota colonization starts from the moment of birth when neonates are firstly exposed to microbes within the maternal birth canal. The delivery mode influences the early microbiota assembly since vaginally delivered infants show a different microbiota composition of those delivered by cesarian section (Dominguez-Bello MG et al., 2021). Moreover, breast milk contains active antimicrobial factors, such as IgA and lysozyme, conferring additional selection on the microbial colonization, conversely to formula milk, that lacks many of these compounds. Apart from milk-feeding and the birth delivery mode, a neonate is exposed to an additionally large amount of environmental microbes during the first years of life, as summarized in Figure 4, that have a strong impact on the opportunistic colonization of microbiota within the gut (Sprockett D et al., 2018). Hence, the early gut bacterial colonization is crucial for imprinting a stable microbiota in adult life, since the microbiota transmission mothers-to-infants is shown to be more durable than the one from cohabiting individuals, which is temporary (Valles-Colomer M et al., 2023).

During infancy immune cells develop tolerance in response to microbial exposure in a process that involved exclusively the environment and not intrinsic properties of adaptive immune cells. Indeed, infant microbiota colonization plays a pivotal role in educating mucosal immune cells and has the potential to impact the immune system with long-standing consequences, even susceptibility to immune-mediated diseases in adult life, as discussed in the review of T. Gensollen and colleagues (Gensollen T et al., 2016). For example, it was observed that exposure to farm environment during childhood or even pregnancy protects children from developing asthma in adult life; conversely, the use of antibiotics from the first 6 months of life increases the susceptibility to develop allergy disorders (Gensollen T et al., 2016). In both cases the effect was mediated by environmental modulation of the gut microbiota composition. Similarly, in animal models cross-fostering experiments induced a stable shift in microbiota composition in mice (Daft JG et al., 2015) and protected them from developing autoimmune diseases such as T1D (Liang W et al., 2022).

The events regulating gut microbiota composition early in life are fundamental to regulate maturation and self-tolerance, two events that also occur in infancy. In fact, particular

bacterial species have immunomodulatory functions during early colonization, inducing the differentiation of specific T cells subsets such as *Bacteroides fragilis* for Th1 and Th2 (Mazmanian SK et al., 2005) or *SFB* for Th17 (Gaboriau-Routhiau V et al., 2009). Furthermore, B cells function is also dependent on early bacterial colonization since GF mice, although maintain normal number of B lymphocytes, show defective IgA production (Li H et al., 2020). Additionally, innate immune cells such as NK, whose phagocytic activity depends exclusively on TLRs activation, have impaired functionality in GF mice compared to microbiota-colonized mice (Ganal SC et al., 2012), showing that microbiota is fundamental in the development of both innate and adaptive immune system.

The exact mechanisms behind the neonatal cross-talk between microbes and the "immature" immune system are still unknown and are the subject of numerous studies in the last decades, also considering the high translational potential of this topic. In this context, non-hematopoietic cells such as intestinal epithelial cells (IEC) have shown a crucial role in orchestrating the complex interaction between microbiota and immune cells during early life. IEC normally interact with both luminal microbes and LP mucosal immune cells thereby expressing PPRs, MHC-II complex and secreting several immune modulators (Peterson LW et al., 2014). The absence of microbiota in GF mice results in impaired IEC functionality such as defective AMPs production (Cash HL et al., 2006) and MHC-II expression (Matsumoto S et al., 1992). Notably, TLRs signaling on IEC have a crucial role in the neonatal host–microbe homeostasis. For instance, during the pre-natal period IEC exhibit a transient status of TLRs activation when exposed to bacterial ligands, regulated by several post-transcriptional mechanisms which trigger hypo-responsiveness to TLR stimuli; the temporary silencing of TLRs signaling facilitates the gut microbial colonization (Lotz M et al., 2006). Since the LPS is a potent activator of several inflammatory genes, it is important to mention a phenomenon called "endotoxin tolerance", described for the first time in the 1946 (Cavaillon JM et al., 2003), that refers to the loss of reactivity against LPS with the aim to prevents tissue damage in response to infections or microbial dysbiosis. In this context, M. Lotz and colleagues showed that it takes place also on IEC, in particular during neonatal period; basically, authors analyzed the LPS-TLR4 signaling in freshly-isolated IEC of fetal, newborn and adult mice and observed that LPS responsiveness is lost in newborn IEC compared to fetal or adult-

derived IEC (Lotz M et al., 2006). Years after, C. Chassin and colleagues demonstrated that this transient negative regulation of the LPS-TLR4 signaling derived from a post-transcriptional mechanism, specifically mediated by microRNA-146a, and it is exclusive to neonatal IEC only. Additionally, authors showed that alterations of this regulation mechanism may result in epithelial damage and disease since oral treatment with anti-miR-146a made IEC susceptible to apoptosis (Chassin C et al., 2010).

These findings show that epithelial tolerance is a further mechanism that occurs during infancy and, together with microbial colonization and immune system development, aim the achievement of a stable and durable intestinal immune homeostasis during life. Alterations in one of those mechanisms during infancy are linked to susceptibility to autoimmune diseases and may represent a therapeutical window for prevention of those diseases.

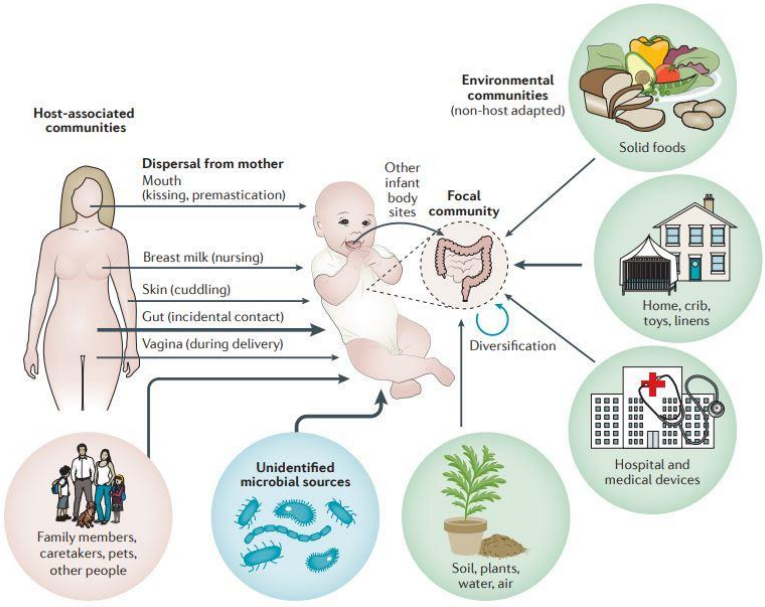


Figure 4: Environmental factors that influence the gut microbiota colonization during infancy.

From the moment of birth, the neonate's gut is exposed to a large among of microbes that came from different sources and have a big impact on neonatal gut bacterial colonization. The maternal microbiota plays a pivotal role on the neonate's microbiota, starting from the passage over the birth canal to the nursing period. Breast-milk further impact the fecal community of the infant, playing important antimicrobial activities. Additionally, the environment where the neonate spends the first years of his life further influence his gut microbial community, including the contact with the microbiota of family member and even pets that co-live in the same house (Sprockett D et al., 2018).

1.3 The intestinal epithelium in driving the immune system.

The homeostatic relationship between the commensal microbes and the host is based on one hand to the controlled sampling of bacterial antigens by the innate immune system and, on the other hand, to the physical separation of luminal microbes from the underling tissue. Therefore, a tiny layer of intestinal epithelial cells (IEC), which forms the intestinal epithelium (IE), physically separates the luminal content with the rest of the intestinal mucosa and orchestrates intestinal homeostasis in a complex network of interaction between the microbiota and immune cells.

IE's structure differs along the GI tract: in the small intestine, where the digestion and absorption of nutrients takes place, finger-like projections called villi protrude to the lumen near invaginations called crypts of Lieberkühn; conversely, in the colon, where the absorption is restricted to water and microbial fermentation products, there are no villi. Mature IEC generate from a cycling population of intestinal stem cells (ISC) located at the base of the crypts, which give rise every 5-8 days to either absorptive or secretory lineages. The secretory lineage includes specifically Goblet cells, enteroendocrine cells, Tuft cells and Paneth cells. All these IEC types collaborate to build a functional IE that regulates the interaction between commensal microorganisms and the host.

1.3.1 Immune-mechanisms of bacteria separation

The interaction between the intestinal epithelium (IE) and luminal bacteria is controlled by several mechanisms. First, a dense layer of mucus covers the epithelial surface and acts as "physical barrier" that is resistant to bacteria penetration. This is particularly evident in the colon, where a double mucus layer restrains luminal bacteria on the outer compartment. Conversely, a single penetrable mucus layer is present in the small intestine to allow the nutrients uptake and absorption. Mucus is mainly composed of mucin glycoproteins produced by specialized IEC called Goblet cells (GC). The lack of the mucin Muc2 in mice causes severe colitis and intestinal inflammation, showing how this mechanism of bacteria-isolation is important in maintaining the symbiotic relationship between the host and the microbiota (Van der Sluis M et al., 2006). Apart from being a physical barrier, numerous studies point out an active role of the mucus in modulating the intestinal homeostasis at different levels. For example, mucus has a large influence on the microbiota composition since creates a microbiological niche where bacteria are selected

to survive. Indeed, some bacterial strain, such as *Bacteroides Thetaiotaomicron*, use mucins as source of energy thereby creating their own habitat within the mucus layer, especially in the colon (Li H et al., 2015). Alterations in the mucus structure, found in inflamed gut, disturb the microbial communities that normally lives within the mucus, inducing the same bacteria to die or to create biofilm within the GI tract. *Akkermansia muciniphila*, for example, expresses several mucin degradation-related genes but lacks mucus binding domains therefore is depleted in individuals with inflamed mucosa such as IBD patients (Png CW et al., 2010). On the other hand, mucus has immunomodulatory functions as the mucin Muc2 interacts with intestinal DCs inducing the proliferation of intestinal Treg cells (Shan M et al., 2013); furthermore, GC act as "sentinel cells" and present antigens directly to LP DCs (McDole JR et al., 2012).

The secretion of antimicrobial peptides is another immune mechanism that controls the bacterial growth within the gastrointestinal tract (GI). AMPs include several peptides such as cathelicidins, C-type lectins and defensins, which can be either constitutively expressed or microbiota-induced, as shown in Figure 5. AMPs are produced by several type of IECs, including GC, enterocytes, and Paneth cells, although Paneth cells produce most of the AMPs. The production of AMPs can be induced by either bacteria through TLRs or cytokines produced by immune cells, such as the IL-23/IL-22 axis (see Paragraph 1.2.1 and Figure 1).

Lastly, bacterial-specific IgAs are secreted by plasma cells within the PPs and contribute to the exclusion of bacteria to penetrate the gut epithelium (Mantis NJ et al., 2011).

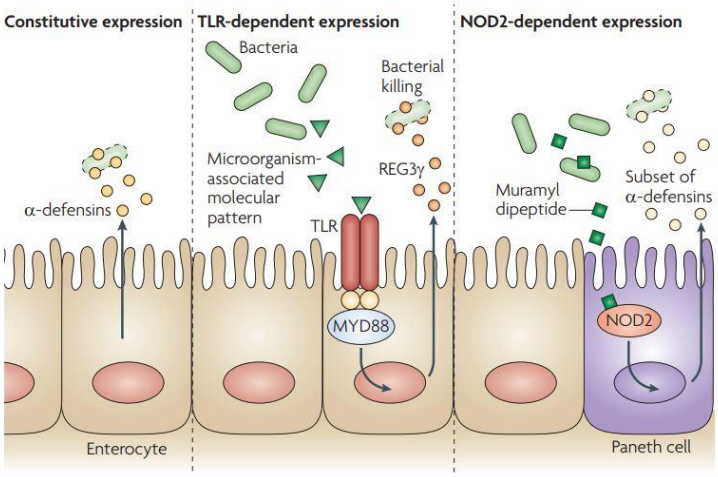


Figure 5: Antimicrobial peptide production in the intestinal epithelium
IEC, mostly enterocytes and Paneth cells, produce several types of antimicrobial peptides (AMPs). For examples, α-defensins are expressed constitutively therefore do not need bacterial signals for their expression. Other type of AMPs, such as C-type lectin regenerating islet-derived protein 3γ (REG3γ) or a particular subset of α-defensins, are released after stimulation of TLR or NOD2 receptors, respectively. Abbreviations: TLR=toll-like receptor; MYD88= myeloid differentiation primary-response protein 88; NOD2= Nucleotide-binding oligomerization domain-containing protein 2 (Hooper LV et al., 2010).

1.3.2 The cross-talk between the intestinal epithelium and immune cells

The ability of IEC to interact with immune cells has recently been discovered and it is believed to play a pivotal role in regulating intestinal homeostasis. Besides, IEC are not only capable of expressing cytokines and chemokines, but they further express the major histocompatibility complexes-class II (MHC-II) molecules thus act as non-conventional APC within the intestinal mucosa. Importantly, this MHC-II expression is mediated by bacteria since IEC from GF mice or from *in vitro* intestinal organoids do not express them (Heuberger C et al., 2021).

Figure 6 summarizes all known mechanisms of IEC-Th cells interaction via MHC-II molecules under both homeostatic and inflammatory conditions. During homeostasis, M. Biton and colleagues observed that IEC interact with Th cells through MHC-II acting as non-conventional APC and, in turn, respond to the cytokines released by Th cells balancing their self-renewal or differentiation activity (Biton M et al., 2018) (Figure 6a). Moreover, it was shown that expansion of the Tregs pool in the intestinal mucosa is independent of DCs activity but is under the control of IEC–Th cell interactions

(Westendorf AM et al., 2009) (Figure 6b). Furthermore, IEC are also capable of releasing MHC II-bearing vesicular structure, like exosomes, which can influence the local adaptive immune phenotype (Karlsson M et al., 2001) (Figure 6c-d).

Interferon-γ (INF-γ) has been identified as a key regulator of MHC-II expression on IEC during inflammation since both human- and mouse-derived in vitro intestinal organoids express MHC-II under INF-γ stimulation (Wosen JE et al., 2019; Farin HF et al., 2014). This means that an increased production of INF-γ by intestinal Th cells boosts the MHC-II expression on IEC, which in turn may increase luminal antigens presentation IEC-to-Th cells (Figure 6e). Multiple studies additionally showed that IEC modulate intestinal immunity through secretion of immuno-modulators. For example, IEC are indirectly capable of inducing IgA-class switching on PPs plasma cells via APRIL secretion through a T cell-independent fashion (He B et al., 2007). The thymic stromal lymphopoietin (TSLP) is also produced by IEC after interaction with bacteria and stimulates DCs to secrete APRIL (He B et al., 2007). Furthermore, TGF-β and RA, key modulators of the tolerogenic $CD103^+$ DC conversion, are also produced by IEC, as shown by *in vitro* experiments with IEC-derived supernatant in which IEC condition DCs to assume a tolerogenic phenotype, releasing IL-10 and IL-6 instead of IL-12 thereby promoting the Th differentiation into Th2 and Treg cells (Iliev ID et al., 2009; Rimoldi M et al., 2005). In addition, IL-25 expression is observed in IEC and controls ILC function in a microbiota-dependent fashion (Sawa S et al., 2011).

In this context, the modulation of gut immunity by IEC occurs also during inflammation by the release of several pro-inflammatory cytokines, playing a crucial role in orchestrate the defense against pathogens (Mahapatro M et al., 2021). For example, parasites and helminths induce the secretion of IL-33 on IEC, which in turn stimulates the ILC population to secrete Th2-polarizing cytokines. Moreover, cytokines from the IL-1 family, such as IL-1β and IL-18, are also released by IEC after activation of the inflammasome and drive the innate immune response after a pathogenic invasion (Mahapatro M et al., 2021).

Overall, although the exact role of IEC in orchestrating the mucosal immune response is still not completely characterized, their physiological role in maintaining the intestinal homeostasis is undoubtedly important, as alterations on IEC activity have been linked to several diseases. This is particularly true in gut-mediated diseases such as IBD and Celiac

disease (CD), in which increased production of inflammatory cytokines in IEC has been detected. For example, IEC isolated from Crohn disease's patients showed dysregulation of TSLP secretion, thus driving DCs to assume a pro-inflammatory phenotype (Rimoldi M et al., 2005). In CD patients, it was shown that IEC firstly produce IL-15 after contact with gluten and thus start the intestinal inflammation impairing the IE barrier structure and the adaptive immune response (Mahapatro M et al., 2021). Notably, impaired IEC function has been detected also in extra-intestinal immune mediates disease; for example, a spontaneous mouse model for autoimmune T1D shows a low-grade intestinal inflammation thereby increased secretion of inflammatory cytokines by IEC (Sorini C et al., 2019).

Collectively, these observations demonstrate that IEC activity shapes the immune responses to luminal flora by interacting with Th cells and together participate to the maintenance of intestinal immune homeostasis. However, more studies are needed to better elucidate the molecular mechanisms of this interaction.

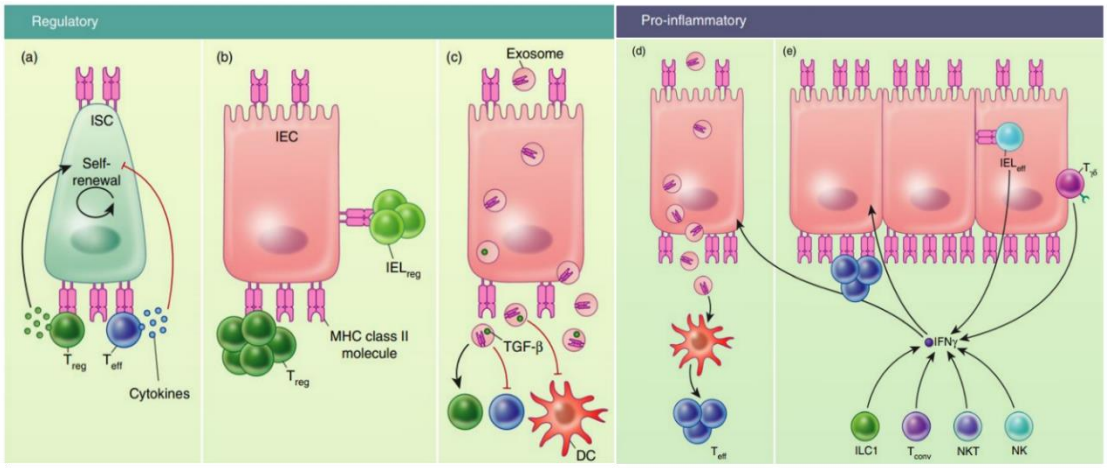


Figure 6: Cross-talk between IEC and immune cells in homeostasis and inflammation *The expression of MHC-II molecules has been described for intestinal epithelial cells (IEC) as an important mechanism of IEC-T helper cells interaction within the intestinal mucosa. (a) Intestinal stem cells (ISC) interact with both T regulatory (Treg) and T effector (Teff) cells through MHC-II and respond to their cytokines inducing self-renewal or differentiation. (b) IEC have been shown to play an important role in maintain the pool of intestinal Treg via MHC-II. (c-d) IEC further interact with immune cells by releasing exosomes; however, whether these exosomes drive either a tolerogenic (c) or inflammatory (d) response is still unknown. (e) Lost of studies showed that INF-γ, released by innate-lymphoid cells (ILC), natural killer (NK), conventional T (T conv) cells or invariant NK (NKT) cells during inflammation, have a key role in upregulating MHC-II expression on IEC (Heuberger C et al., 2021).*

1.3.3 TLRs signaling within the intestinal epithelium.

The functionality of the IE is based on an organized process of differentiation, proliferation and apoptosis of IEC that starts from ISC. These processes are influenced by the microbiota and microbial-derived metabolites. Indeed, IEC express PRRs and thus are capable of interact with MAMPs. The activation of PRR signaling on IEC is fundamental for the intestinal homeostasis and shapes the needs of the intestinal environment in response to several clues. In this context, several studies identified the strong microbiota-dependence on several IE functions. For example, studies with MYD88 or TRIF deficient mice highlighted the role of TLRs in shaping the IE functionality, from AMPs secretion, mucus production, tight junctions' expression to susceptibility to colitis and infectious (Burgueño JF et al., 2020; Stockinger S et al., 2014).

Likewise, TLR signaling on IEC induces the production of cytokines and chemokines modulating the local immune system. For example, it has been demonstrated that epithelial TLRs signaling promotes both the immune cells migration to the lamina propria (Chabot S et al., 2006) and the release of cytokines that modulate DCs phenotype inducing the differentiation of several Th cells (Rimoldi M et al., 2005). Moreover, *SFB* induce IEC to secrete serum amyloid A, which mediates the differentiation of naïve T cells into Th17 cells (Atarashi K et al., 2015). Furthermore, *in vitro* studies shows that IEC secrete TSLP and APRIL after stimulation with LPS and flagellin thus inducing DCs to promote the IgA production on plasma cells (He B et al., 2007).

Overactivation or dysregulation of TLR signaling on IEC has been shown to destroy intestinal homeostasis at different levels, including the IE barrier structure and consequently the local immune responses. Therefore, several mechanisms by which IEC control TLRs activation exist and are crucial to avoid possible overreaction towards the gut microbiota. For example, the epithelial TLR expression is normally very low among the GI tract and is strongly polarized on IEC cells' surface (Price AE et al., 2018). Moreover, several intracellular molecules are expressed by IEC to control the TLR downstream signaling. These intracellular regulators include, for example, the Toll-interacting protein (TOLLIP), which controls the MYD88-dependent NF-kB activation (Capelluto DG et al., 2012) or the TNFα-induced protein 3 (TNFAIP3), which specifically prevents the interaction between TRAF6 and TAK1 (Boone DL et al., 2004).

Collectively, these data show how epithelial TLR signaling is fundamental in shaping both the IE and immune system functionality within the intestinal mucosa. A prolonged dysregulation of epithelial TLR signaling can lead the induction of dysbiosis and to a condition defined “leaky gut”, which has been identified for several immune-mediate diseases, particularly extra-intestinal autoimmune diseases such as Systemic Lupus Erythematosus, Type 1 Diabetes and Multiple Sclerosis (Christovich A et al., 2022).

2. The importance of the gut environment in extra-intestinal autoimmune diseases

2.1. Organ-specific autoimmune diseases

The etiology of organ-specific autoimmune diseases is multifactorial, triggered by a complex interaction between genetic and environmental factors. The breakage of the immune tolerance and activation of autoreactive T cells is the predominant pathogenic mechanism underlying etiopathogenesis of autoimmune diseases (Wahren-Herlenius M et al., 2013). Indeed, genetic risk loci that confers susceptibility to develop autoimmunity are found mainly within the MHC locus. However, epidemiological data clearly indicate that the environment plays a crucial role in promoting the pathogenesis of autoimmune diseases, also considering the dramatic raise in incidence of those diseases in the past decades.

The importance of environmental triggers in autoimmunity emerged in the early 90' from epidemiological studies in transmigratory populations, showing that populations migrating from low incidence to high incidence countries acquire the incidence rate of the host country (Bodansky HJ et al., 1992; Gale CR et al., 1995). To date, one of the explanations for this phenomenon is the so-called "hygiene hypothesis". The hygiene hypothesis originated from the observation that industrialized countries have decreased frequency of infectious diseases associated with a progressive increase in allergic and autoimmune diseases. For instance, the geographical frequency of Type 1 Diabetes (T1D) and Multiple Sclerosis (MS) are the exact opposite of that of infectious diseases such as Tuberculosis and Hepatitis A (Bach JF, 2018). However, the underlying causative mechanisms behind the hygiene hypothesis are still under investigation.

2.1.1 The gut microbiota and extra-intestinal autoimmune diseases

The gut microbiota, which is strongly influenced by the environment, play a crucial role in regulating extra-intestinal autoimmune diseases. Metagenomic analysis highlighted a reduction of gut microbial diversity in patients with T1D, MS and systemic lupus erythematosus (SLE) (Bach JF, 2021) and in murine models for MS, T1D and Rheumatoid arthritis (RA) (Berer K et al., 2011; Yoshitomi H et al., 2005; Brugman S et al., 2006). As previously mentioned, the phenotype of intestinal immune cells, such as

$CD4^+$ T helper cells, is shaped by the gut microbiota. For example, SFB are known inducers of Th17 and Th1 cells (Ivanov II et al., 2009), and were found capable to trigger brain autoimmunity in EAE (the preclinical model of MS) (Flannigan KL et al., 2018). However, although the SFB-induced Th17 response promotes autoimmunity in murine models of RA and MS (Wu HJ et al., 2010), SFB colonization prevents T1D in NOD mice (Kriegel MA et al., 2011), suggesting that the influence of microbial species on the progression of autoimmunity is disease-dependent.

Chervonsky AV proposed three possible mechanisms of microbial-induced activation of extra-intestinal autoimmunity (Figure 7): one is the so-called "molecular mimicry" suggesting that microbials antigens "mimic" self-antigens and cross-react with autoreactive T cells triggering their activation; on the contrary, APCs cells can be activated by microbes through TLRs signaling and be induced to present self-antigens expressed at the intestinal level to self-reactive T cells ("bystander activation"); lastly, microbiota activation of APC and ILCs induces cytokines production which in turn promote self-reactive T cells activation ("amplification of autoimmunity by cytokines") (Chervonsky AV, 2013).

To date, whether the disrupt of intestinal microbial community may be the causes or consequences of autoimmune diseases is still under investigation. Fecal microbiota transplantation (FMT) studies have been used to better understand the mechanisms by which microbiota modulate autoimmune diseases. For examples, FMT experiments with microbiota from MS patients accelerated the disease in a transgenic mouse model (Berer K et al., 2017). On the other hand, germ-free NOD mice that received microbiota from T1D-protected mice were in turn protect against the disease (McLeod KH et al., 2018). Thus, some researchers are proposing to use FMT in the treatment of autoimmune diseases, considering that is a therapeutic approach already used for treatment of *Clostridium difficile* infectious (Khoruts A et al., 2014; Allegretti JR et al., 2019). In this context, a preliminary clinical trial of P. de Groot and colleagues has observed that FMT stabilized β-cell function in new-onset T1D subjects (de Groot P et al., 2021). Although this study suggested that the gut microbiota can be therapeutically exploited for treatment of extra-intestinal autoimmune diseases, further studies are necessary to better characterize the beneficial and tolerogenic gut microbiota composition and to dissect the mechanism of microbiota-induced modulation of autoimmune diseases.

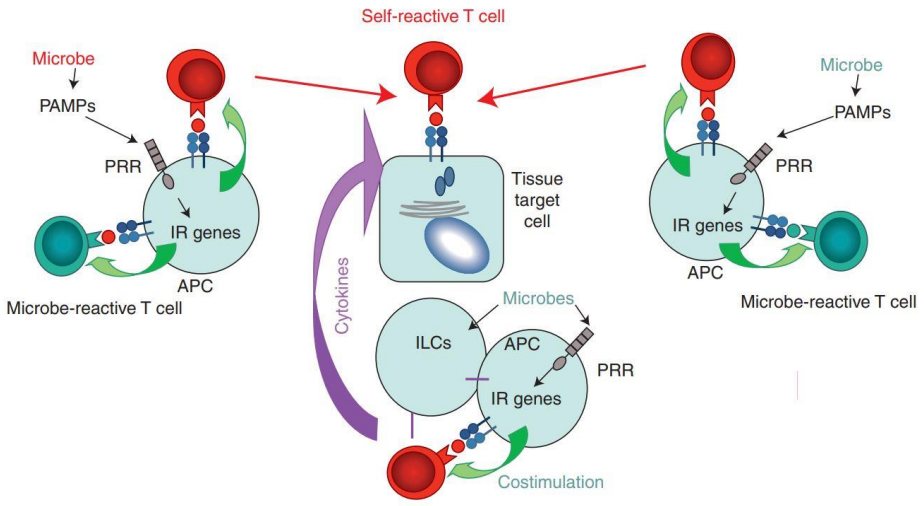


Figure 7: Microbial activation mechanisms of autoimmunity.
The tissue-target cells are destroyed mistakenly by self-reactive T cells. Several mechanisms have been proposed: "Molecular mimicry" (left) is when microbial peptides are similar or identical to self-peptides and activate self-reactive T cells; "bystander activation" mechanism (right) involved the activation of APC by microbial stimulation of TLRs, which in turn activates self-reactive T cells; lastly, the "amplification of autoimmunity by cytokines" mechanism is when APC and ILC, after microbial stimulation, produce cytokines that elicit activation of self-reactive T cells (Chervonsky AV, 2013).

2.2 The role of the gut in Type 1 Diabetes

2.2.1 Type 1 Diabetes: pathogenesis and risk factors

Type 1 diabetes (T1D) is a chronic autoimmune condition which affects mainly children and young individuals. It is characterized by the progressive destruction of the insulin-producing β-cells within pancreatic Langerhans' islets. Since insulin regulates the absorption of glucose into cells, deficit of this hormone leads to damage of body's organs and to many health complications such as cardiovascular diseases, neuropathy, nephropathy, eye disease or lower-limb amputation.

The presence of islet-specific autoreactive $CD4^+$ and $CD8^+$ T cells in the blood of patients confirms the strong autoimmune nature of T1D. Besides, the common explanation for T1D pathogenesis is that β-cells-related autoantigens are mistakenly presented as "non-self" by human leukocyte antigen (HLA) MHC class I and II on APCs to autoreactive

$CD4^+$ T cells, which in turn stimulate B cells to produce high-affinity autoantibodies and diabetogenic $CD8^+$ T cells to acquire cytolytic activity against Langerhans' islets β-cells. The presence of islet autoantibodies in the serum of patients remains the main clinical predictor for T1D diagnosis (Giwa AM et al., 2020). However, the reason why the immune system activates against "self" insulin-producing pancreatic β-cells is unknown. To date, it is widely accepted that the onset of T1D is associated with a complex mosaic of genetic and several environmental factors. Viral and bacterial infections as well as nutritional and ecological factors have been demonstrated to increase the risk of developing T1D, especially in early life. To note, the 10th edition of the IDF Diabetes Atlas estimates 108,3 million of new cases per year in the 0-14 years population, which increase to 149,5 million considering the 0-19 years old population, meaning that the T1D incidence is increasing annually with a 2-5% rate worldwide (Magliano DJ et al., IDF Diabetes Atlas 10th edition).

Genetic plays an important role in T1D etiopathogenesis, making individuals with overexpression of HLA class molecules DR4, DQ8, and DQ2 more susceptible to develop T1D. However, although high-risk haplotypes are present in 90% of children with T1D, only 5% of subject carrying T1D-risk genes develops the disease (Maahs DM et al., 2010). Studies in twins explain how epigenetic and/or environmental factor play a determinant role in triggering T1D. In fact, although monozygotic twins have increased risk to develop islet autoimmunity and T1D compared to dizygotic twins or siblings, the concordance ratio is 50% (Kumar D et al., 1993; Triolo TM et al., 2019), demonstrating that risk genes/alleles are not the only risk factors for T1D occurrence and other epigenetic and environmental factors also play a role. In a review published in Nature in 2021 by Bart O Roep and colleagues, T1D is described as "a disease of the β-cell" speculating that T cells cannot be the only driving-force of the disease. Specifically, authors highlight the role of pancreatic β-cell in dialoguing with the immune system and thus modulating the progression of T1D (Roep BO et al., 2021). This hypothesis is based on several observations that point out the susceptibility of β-cells to metabolic stress under local inflammatory conditions causes for example by virus infectious or intestinal inflammation (Roep BO et al., 2021).

Overall, T1D has a complex etiology since the exposure to several environmental triggers may play a crucial role on its pathogenesis. However, the exact link between

environmental factors and genetic predisposition in the pathogenesis of T1D has not been clarified.

2.2.2 "Leaky gut" in Type 1 Diabetes

Several lines of evidence indicate that the intestinal environment maybe involved in T1D pathogenesis. Clinical observations reveal subclinical enteropathy and increased intestinal barrier permeability not only in the gut mucosa of T1D patients (Secondulfo M et al., 2004; Sapone A et al., 2006) but also in individuals with islet autoantibody-positivity and no hyperglycemia before the clinical onset of the disease (Bosi E et al., 2006), suggesting that alterations of the gut barrier are not diabetes-induced metabolic consequences but may be directly related to its pathogenesis. Based on these observations, Vaarala et colleagues suggested that T1D is the results of a complex interplay involving facets related to the gut, including the intestinal microbiota, the intestinal mucosal barrier, and the intestinal immune system, which interact with each other forming what the authors called "the perfect storm" for the development of the disease (Vaarala O et al., 2008).

Studies in animal models support the hypothesis that alterations in the gut microenvironment influence the outcome of T1D. In this context, the Non-Obese diabetic (NOD) mice, T1D murine model which develops spontaneous autoimmune diabetes (Solomon M et al., 2004), has been largely used for the study of enteropathy and T1D because it shows, as humans, signs of intestinal inflammation and gut barrier damage in the pre-diabetic phase. Specifically, our group and others demonstrated that NOD mice is characterized by increased intestinal permeability and immunological alterations in the gut mucosa several weeks before the clinical onset of the disease (Sorini C et al., 2019; Miranda MCG et al., 2019). Furthermore, the intestinal mucus layer, a key component of the gut barrier, has been documented as altered in T1D (Sorini C et al., 2019; Miranda MCG et al., 2019) and considering its crucial immunomodulatory function in maintaining intestinal homeostasis, its damage may support the proinflammatory environment within the intestinal tract before and during development of T1D.

These data strongly suggest the importance of the intestinal microenvironment in the pathogenesis of T1D, however, how environmental factors and the gut predispose genetically at-risk subjects to develop T1D is still under investigation. Specifically, it is

unknown what cause alterations of the intestinal barrier and whether its damage may be a consequence of an altered microbiota composition or related to inherent genetic/epigenetics defects.

As largely discussed in the previous chapter, the IE is fundamental to regulate the crosstalk between the immune system and the gut microbiota. The alterations of the IE and the presence of a "leaky gut" in patients affected by extra-intestinal autoimmune diseases suggests that this defect could underlying disease pathogenesis. In line with this idea, zonulin, a physiological modulator of intestinal macromolecules trafficking was found altered in the serum of RA and T1D patients (Tajik N et al., 1995; Wood Heickman LK et al., 2020.). Increased intestinal permeability, mostly caused by several factors including diet, microbial dysbiosis and drug, disrupts the gut barrier allowing the translocation of luminal bacteria into the lamina propria thereby causing local inflammation. Microbial compounds, such as LPS, have been also identified as modulator of intestinal permeability. For example, the LPS-TLR4 signaling acts on TJPs opening activating the membrane-associated adaptor protein focal adhesion kinase (FAK) thus increasing intestinal permeability (Guo S et al., 2015). Moreover, *in vitro* experiments with Caco-2 cells identified the myosin light chain kinase (MLCK) as another important mediator of the LPS-induced TJPs opening (Nighot M et al., 2017).

On the other hand, IEC are in closed contact with mucosal immune cells and thus respond to cytokines released in the local environment. Some inflammatory cytokines, such as TNF-α, have been demonstrated to act on TJPs complex by increasing intestinal permeability (Ye D et al., 2011). Therefore, it has been suggested that when local inflammation occurs for example in the intestine due to several factors (i.e. dysbiosis, increased bacterial translocations, uncontrolled inflammatory response), it may trigger the activation of self-reactive lymphocytes, present in genetically-at risk subjects, for either molecular mimicry with translocated bacteria components or bystander activation. In support to this hypothesis, in a transgenic murine model of T1D, the BDC2-5XNOD mice - that do not develop diabetes although >90% of circulating lymphocytes express a transgenic TCR specific for an islet-antigen - islet-reactive lymphocytes can be activated in the gut after induction of colitis and causing T1D (Sorini et al., 2019).

Given the complexity of the gut-immune system interaction, understanding its exact link between intestinal alterations and pathogenesis of extra-intestinal autoimmune diseases

such as T1D remains challenging. Specifically, the involvement of the gut epithelium in extra-intestinal autoimmunity need to be better elucidate.

2.2.3 Gut microbiota and Type 1 Diabetes

So far, a large body of evidence suggests a role of the gut microbiota in predisposing autoimmune diseases including T1D, even if the mechanisms are still under investigation. Apart from studies comparing the microbiota of T1D patients with healthy controls (Vatanen T et al., 2018; Gavin PG et al., 2018), the proof of concept comes from epidemiological data in children at-risk for T1D, which pointed out a direct correlation between gut microbial dysbiosis and the probability to develop the disease. As mentioned, infancy is a critical window of time when the immune system dynamically develops interacting with the new-formed gut microbiota and is also the time when beta cell autoimmunity (i.e., autoantibodies against pancreatic islets) occurs.

The group of M. Knip have studied the role of intestinal microbiota in predisposing T1D in Finland, where the rate of autoimmune diabetes among children has dramatically increased in the past five decades (Siljander H et al., 2019). Specifically, it was observed that some bacteria species, like Bacteroides, produce a type of LPS with immunomodulatory function that during early-life promotes development of T1D in genetically at-risk subjects (Vatanen T et al., 2016). Other studies showed that the *Firmicutes*/*Bacteroidetes* ratio is an early predictive marker of dysbiosis and increased susceptibility to T1D (Giongo A et al., 2011; de Goffau MC et al., 2014; Mejía-León ME et al., 2014).

All these data suggest that acting on microbiota composition during early-life may be a reasonable therapeutic approach to prevent the disease in genetically at-risk individuals. On that purpose, acting on microbiota composition in early life protected NOD mice from development of T1D (Liang W et al., 2022; Dolpady J et al., 2016; Lo Conte et al., 2023). Cross-fostering experiment with diabetes-resistant NOD (NOR) mice restored gut barrier functionality in NOD mice, confirming the importance of microbiota in imprinting the gut homeostasis before weaning (Miranda MCG et al., 2019). However, the different effects that the genetic setup of each individual have on the functionality and composition of gut microbiota must be considered. For examples, the TEDDY study published in 2016 reported that early probiotic supplementation decreased the risk of islet-autoimmunity

only in children with a certain genotype (Uusitalo U et al., 2016), demonstrating the importance of gene-environment interactions in autoimmune settings.

2.2.4 Microbiota modulate Type 1 Diabetes through Toll-like receptors.

Toll-Like Receptors (TLRs) are a family of immune recognition receptors that recognizes bacterial ligands. High expression of TLR2 and TLR4 has been found in monocytes of T1D patients (Devaraj S et al., 2009) suggesting that TLRs signaling plays a crucial role in T1D pathogenesis. Also, the depletion of TLR4 prevents T1D in the streptozotocin-induced diabetic mouse model (Devaraj S et al., 2011), showing that the TLRs-mediated proinflammatory signaling may contribute to T1D pathogenesis. However, administration of the TLR3-ligand Poly I:C protected Bio-breeding diabetes-prone (BBDP) rats from developing T1D in a dose-dependent fashion (Sobel DO et al., 1992), indicating that TLRs signaling may be differentially involved in T1D pathogenesis. Hence, an important open question in T1D research regards the mechanisms through which the TLRs signaling modulates T1D pathogenesis. It has been hypothesized that polymorphisms in TLRs genes may play a role in altering the TLRs signaling thereby causing autoimmune responses. Besides, some studies identified polymorphisms associated with T1D related to TLR4, TLR2 and TLR3 genes (Park Y et al., 2004; Assmann TS et al., 2014). However, other studies showed that there was no correlation between T1D and TLRs polymorphisms because they are restricted only to certain geographical area (Zhang Y et al., 2021). Thus, it was speculated that such T1D-related TLRs alterations may result from interactions with other genes or environmental factors, involving probably complex post-transcriptional regulations. Indeed, epigenetic mechanisms, such as DNA methylation, histone modification or microRNAs expression, regulate TLRs expression without changing the DNA sequence. In this context, miRNA-mediated TLRs regulation has been found in several immune-mediated and autoimmune diseases, including RA and T1D (Banerjee S et al., 2021). However, further studies are needed to better elucidate the TLRs epigenetic-mediated regulation in T1D pathogenesis.

2.2.5 The LPS-TLR4 signaling in Type 1 Diabetes

In the review of W. Bielka and colleagues, LPS is largely discussed as a possible molecular link between gut microbiota, inflammation and T1D. T. Vatanen and

colleagues, by studying the microbiota of children with genetic risk to develop T1D, found that the immunogenicity of different bacterial LPS in earlylife is crucial for the establishment of the endotoxin tolerance and may increase the risk of aberrant immune responses and autoimmunity (Siljander H et al., 2019).

Alterations in the LPS-TLR4 signaling has been documented for T1D. A case-control study revealed higher circulating level of the LPS in the blood of T1D patients compared to non-diabetic individuals (Devaraj S et al., 2009). Moreover, the expression of TLR4 on pancreatic islet β-cells and insulin target tissues such as liver, adipose tissue, and skeletal muscle, supports its relevance in insulin homeostasis and diabetes-associated metabolic disorders (Garay-Malpartida HM et al., 2011).

Studies with knock-out mice demonstrated that the TLR4 signaling has a direct impact on T1D progression. However, studies in NOD mice deficient for the MYD88 protein showed that protection from T1D occurs only when the mice are housed in SPF condition (Wen L et al., Nature 2008), showing that the commensal gut microbiota modulates T1D by stimulating different signals through TLRs.

As mentioned in previous paragraphs, the MYD88 adaptor, common for all the TLRs except for TLR3, induces the production of inflammatory cytokines through the transcription factor NF-kB. On the other hand, the TRIF- signaling, associated with TLR3 and TLR4, apart from the induction of type I interferons, is further involved in T cell homeostasis and specifically Treg cell function (Lalani AI et al., 2015) thus in maintaining immune homeostasis. The balance between MYD88- and TRIF- mediated pathways, which are involved in TRAF6 and TRAF3 signaling respectively, is crucial in avoiding autoimmune reactions and it has been suggested to modulate the outcome of autoimmune diseases, including T1D. To note, TLR4 is the only TLR that can activate both MYD88- and TRIF-dependent pathways, playing a key role in the host's response to microbiota (Zhang Y et al., 2021). Accordingly, MC Simon and colleagues found that T1D development increases after TLR4 deletion in NOD mice, in correlation with higher abundance of *Bacteroidetes*, lower abundance *Firmicutes* and a lower level of circulating SCFAs (Simon MC et al., 2020).

To better clarify the role of TLR4 in the pathogenesis of T1D, M. P. Burrows and colleagues found that the TLR4-downstream adaptor TRIF activate a tolerogenic signaling, protecting NOD mice from T1D development (Burrows MP et al., 2015);

importantly, they suggested that the modulation of T1D by microbes depends on the balance between the mechanisms by the host to control the microbiota and the loss of these controls' mechanisms. De facto, the fine regulation of TLRs signaling is important to maintain mucosal barrier integrity and mucosal immune homeostasis against gut microbiota. In the window of time immediately after birth and before weaning, TLRs regulation is epigenetic-controlled and aims to avoid the TLRs' overactivation to the bacteria that start to colonize the gut tract in that period. In this context, W. Liang and colleagues found an overactivation of TLR4 and TLR2 in the gut on newborn NOD mice, in correlation with gut dysbiosis and increased abundance of the relative TLR4- and TLR2 ligands. The correction of the gut microbiota composition in newborn mice restored gut homeostasis and protected NOD mice from T1D development, showing that alterations in gut microbiota during early life imprints the immune system and may be related to the development of T1D (Liang W et al., 2022).

These data suggest a causal link between alterations in TLRs signaling, modification of the gut microbiota composition and T1D progression, however, the mechanisms underlying TLR4-dependent modulation of T1D pathogenesis are yet to be characterized.

AIM OF THE WORK

Damage of the gut barrier integrity in humans and preclinical models of T1D has been largely documented but it is unclear whether this defect is the cause or the consequence of the disease. The microbiota has been proposed to play a pivotal role in triggering this condition of «leaky gut» since T1D patients and T1D at-risk-individuals have microbial dysbiosis. Besides, alterations of TLRs signaling and their possible involvement in triggering β-cell autoimmunity in T1D have been documented. In this context, several T1D mice deficient for TLRs have been used. However, the limitation of previous studies is that the interaction between the gut microbiota, TLR4 stimulation and the intestinal epithelium (IE), and the influence of the latter on the immune system, has been never considered. Indeed, TLRs are expressed by both immune and non-immune cells, such as intestinal epithelial cells (IEC), that play a crucial role in the crosstalk between the host and the microbiota, starting from the moment of birth to adult life. Thus, we hypothesized that the loss of GB integrity and intestinal inflammation that characterized T1D may be related to a fault tolerance against commensal bacteria and, more specifically, to an altered response of the intestinal epithelial tissue to bacterial components such as the LPS under steady-state conditions. Therefore, the aim of this PhD project was to unravel the role of epithelial TLRs signaling in triggering gut barrier damage and T1D. To address this hypothesis, we conducted experiments in the NOD mice, the spontaneous mouse model of T1D, and validated our results in human ex-vivo intestinal tissue isolated from the small intestine (duodenum) of T1D patients.

RESULTS

The intestinal epithelium of NOD mice is characterized by a defective differentiation of epithelial cells.

Alterations of the gut barrier (GB) have been largely documented in T1D and include aberrant intestinal mucosa morphology together with increased intestinal permeability. These GB alterations precede the clinical outcome of the disease in humans (Bosi E et al., 2006) thus suggesting a causal relationship between enteropathy and T1D pathogenesis. NOD mice, the spontaneous mouse model of T1D, is characterized as humans by GB alterations that correlate with intestinal immune dysregulations and the outcome of the disease (Sorini C et al., 2019). However, the underlying mechanisms of this «leaky gut» condition in triggering T1D are yet to be clarified. Therefore, to investigate what leads to loss of GB integrity in T1D, we firstly performed a basal characterization of IE in the NOD mice. For this purpose, we used a protocol for IEC isolation widely used in literature, based on ethylenediaminetetraacetic acid (EDTA) incubation that allows the IEC recovery from the lamina propria (Gracz AD et al., 2012) (Figure 8A). The purity of the IEC fraction was evaluated by flow cytometry using a monoclonal antibody (mAb) against the epithelial cell adhesion molecule EpCAM and a mAb against the leukocyte common antigen (CD45) for intra-epithelial lymphocytes (IEL) exclusion. We obtained a purified IEC-enriched suspension after two washes (Figure 8B).

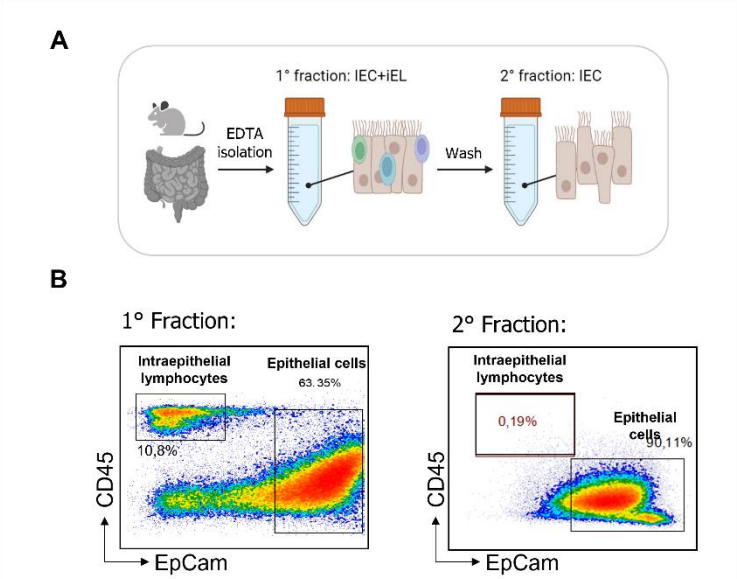


Figure 8: Isolation of intestinal epithelial cells
(A) Protocol for the isolation of intestinal epithelial cells: 1-cm cleaned pieces of murine intestinal tissue were incubated in 30mM EDTA solution at 37°C with shaking for 15 minutes. The first IEC-enriched fraction, containing intra-epithelial lymphocytes (IEL), was centrifugated at 300g to obtain a second pure IEC-enriched fraction. (B) Representative FACS plot analysis of fresh-isolated IEC from the ileum of a mouse. Data were visualized and analyzed with FSCexpress software.

Thus, we extracted RNA from IEC isolated from both NOD female mice and age and sex-matched non-autoimmune control mice (C57BL/6 mice) and analyzed the expression of IEC markers by gene expression analysis through quantitative RT-PCR (qRT-PCR). Firstly, we observed that NOD IE showed a reduced number of ISC markers including OLFM4 and LGR5 (Figure 9A). As already showed by others (Diana J et al., 2013; Sorini C et al., 2019), we also observed a reduced mRNA expression of the Paneth cells marker LYZ1 and of the MUC2 mucin (Figure 9B), suggesting a reduced frequency of secretory IEC in NOD GB. Furthermore, we found a decreased mRNA level of the proliferation marker Ki67 (Figure 9C) that highlights a low proliferation activity in NOD intestinal tissue.

Next, we asked whether the alterations observed in IEC of NOD mice may be related to the differentiation activity of ISC, which is under the control of the WNT and NOTCH pathway (Hageman JH et al., 2020) (Figure 9D). We found an increased activation of the NOTCH pathway at the expense of the WNT pathway in NOD IEC (Figure 9E). These data demonstrate that the IEC differentiation process favors the absorptive lineage rather

than the secretory lineage in NOD mice, that may result in faulty GB and consequently in higher susceptibility to dysbiosis and intestinal inflammation.

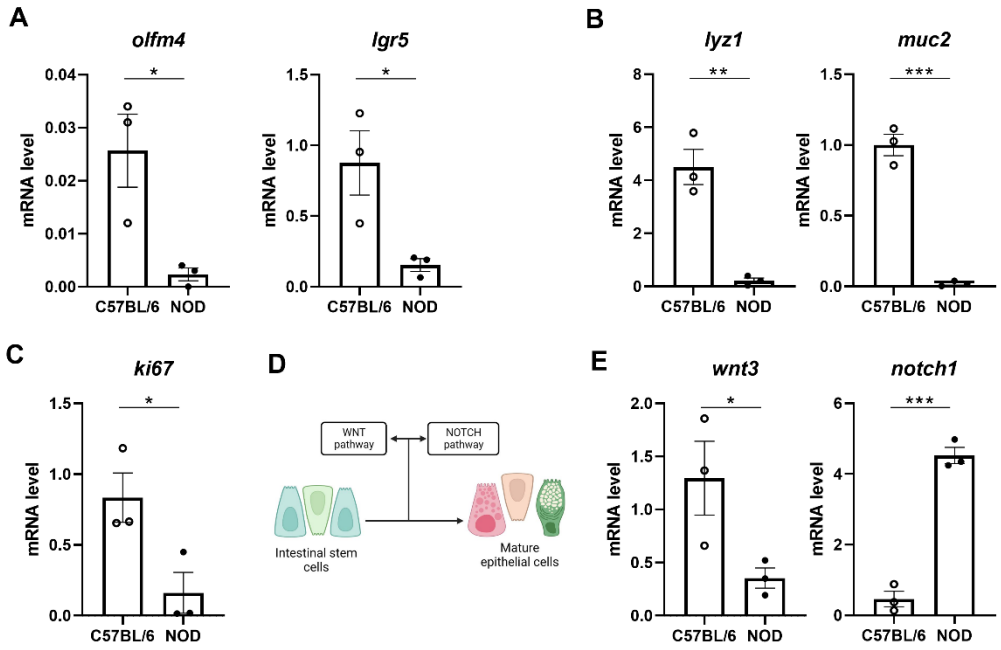


Figure 9: IEC differentiation process pushes toward the absorptive lineage in NOD mice.
*A-C. mRNA level of stem cells markers LGR5 and OLFM4 (A), Paneth cells (LYZ1) and goblet cells (MUC2) markers (B) and proliferation marker Ki67 (C) in fresh-isolated intestinal crypts from 12 weeks-old female C57BL/6 and NOD mice (n=3-4 per group). (D) Schematic representation of the two differentiation pathways of IEC. (E) mRNA level of the differentiation markers WNT3 and NOTCH1, representative of the WNT and NOTCH pathway respectively, in fresh-isolated intestinal crypts from 12 weeks-old female C57BL/6 (B6) and NOD mice (n=3-4 per group). All data are represented as mean ± SD. *p < 0.05, **p<0.01, ***p<0.001.*

NOD mice is characterized by a predominant pro-inflammatory epithelial TLRs signaling.

It has been proposed that microbiota modulates T1D through Toll-likes receptors (TLRs) (Burrows MP et al., 2015) but the modalities are still unknown. Considering that luminal microbes closely interact with IEC, we looked at the epithelial TLR signaling in IEC isolated from a cohort of young NOD female mice and sex- and age-matched non-autoimmune mice (C57BL/6). Specifically, we analyzed the mRNA level of intracellular adaptors MYD88, TRAF6 and TRAF3. MYD88, common to most of TLRs, is associated

with the pro-inflammatory signaling, together with TRAF6; conversely, TRAF3 is associated with the transcription of IRF3, and it has been demonstrated to regulate the function of Treg (Chang JH et al., 2014; Lalani AI et al., 2015).

We found an upregulation of the mRNA level of MYD88 and TRAF6 in our cohort of NOD mice (Figure 10A-B). Contrarily, we did not observe any differences in the mRNA level of TRAF3 (Figure 10C). Additionally, we observed an increased mRNA level of epithelial TLR4 in our cohort of young NOD female mice (Figure 11A). TLR4, which recognized bacterial LPS, it is the only TLR that activates both MYD88-dependent and MYD88-independent pathway (see paragraph 1.2.2) and has been largely studied in T1D (see paragraph 2.2.5). These data suggest that the epithelial TLR4 signaling has a pro-inflammatory tendency in IE of NOD mice.

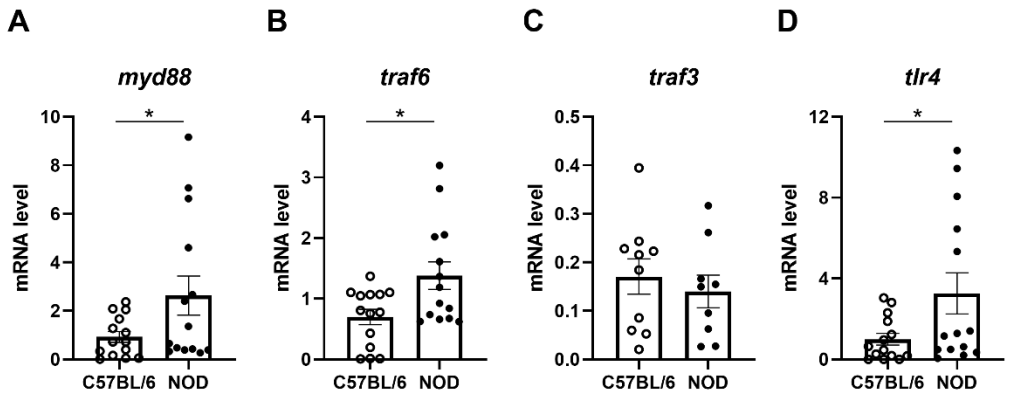


Figure 10: Pro-inflammatory epithelial TLRs signaling in present in IE of NOD mice. *A-C. mRNA level of MYD88 (A) TRAF6 (B), TRAF3(C) and TRL4 (A) in fresh-isolated IEC from 12 weeks-old female C57BL/6 and NOD mice (n=13-14 per group). All data are represented as mean ± SD. *p < 0.05*

Next, to investigate whether the proinflammatory tendency of NOD mice's epithelial TLRs signaling is associate with T1D progression, we measured the mRNA level of MYD88, TRAF6 and TRAF3 in IEC isolated from diabetic NOD mice in comparison with diabetes-resistant NOD mice (10% of female NOD mice do not develop spontaneous diabetes). To this aim, we monitored T1D incidence in a cohort of female NOD mice and separated them into two groups: NOD mice that developed diabetes and NOD mice that, at 30 weeks of age, did not develop the disease thus were considered "diabetes-resistant". We observed that diabetes-resistant NOD mice showed a decreased mRNA expression of

MYD88 and TRAF6 compared to diabetic NOD mice (Figure 11A). Nevertheless, we did not observe any differences in the TLR4 expression between diabetic and diabetes-free adult NOD mice (Figure 11B). The pro-inflammatory epithelial TLRs signaling observed in diabetic NOD mice correlated with an increased ratio of T effector cells (Teff) over Treg (Figure 11C). In addition, our cohort of diabetic NOD mice expressed significantly less MUC2 mucin when compared to diabetes-free adult NOD mice (Figure 11D-E). In line with our findings, previous report showed that resistant Non-Obese diabetic (NOR) mice have a functional GB with proper mucus barrier compared to NOD mice (Miranda MCG et al., 2019). These data suggest that the proinflammatory TLRs signaling may be related to T1D occurrence.

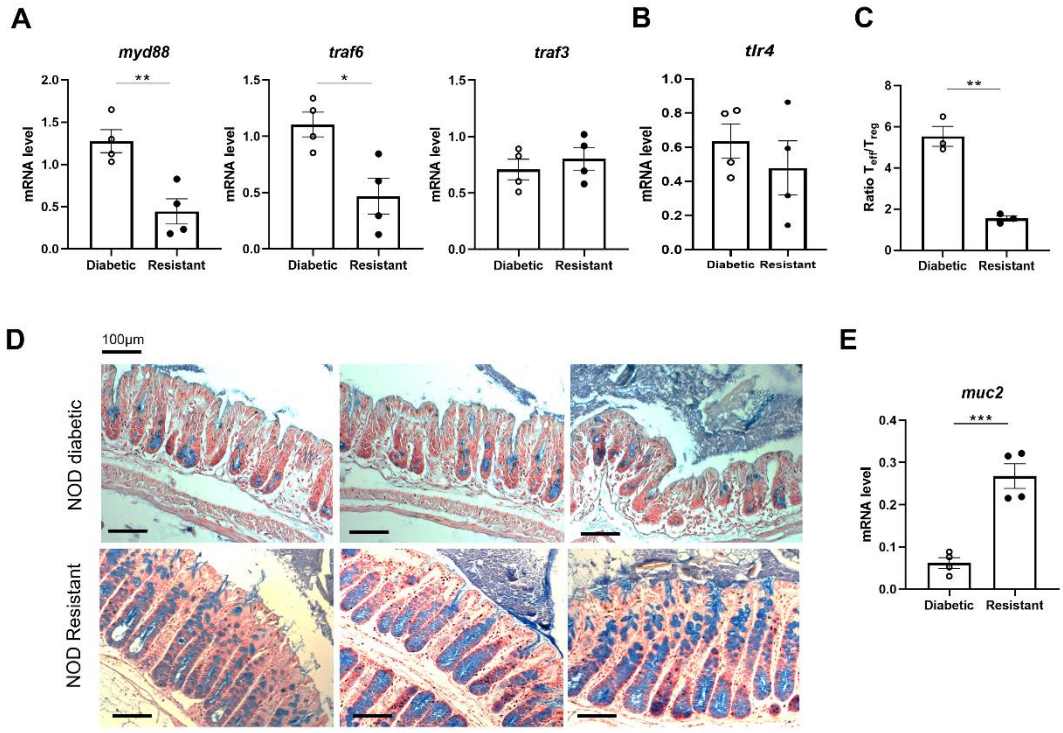


Figure 11. Epithelial TLRs signaling correlate with disease progression in NOD mice. *A-B. mRNA level of MYD88, TRAF6 TRAF3 (A) and TLR4 (B) in fresh-isolated IEC from female NOD mice at different clinical stages: diabetic (18-30weeks old) and resistant (30weeks old); n=4 mice per group. (C) Teff/Treg ratio in the small intestine of diabetic and resistant female NOD mice analyzed by FACS (n=3 mice per group). (D) Alcian-Blue stained colon sections of diabetic (18-30weeks old) and resistant (30weeks old) NOD mice acquired with Zeiss Axio Imager M2m microscope with 10X magnification (100µm). (E) mRNA level of MUC2 mucin in fresh-isolated IEC from female NOD mice at different clinical stages: diabetic (18-30weeks old) and resistant (30weeks old); n=4 mice per group. All data are represented as mean ± SD. *p < 0.05, **p<0.01, ***p<0.001.*

A pro-inflammatory response to nonpathogenic LPS is detected in IE of NOD mice and correlates with defective FoxP3^{+} Treg expansion.

Transcriptional analysis among the gut tract revealed that epithelial MYD88 signaling is only associated with anti-microbial activity against pathogens and that homeostatic host-microbiota interactions are mostly MYD88-independent (Larsson E et al., 2012). In NOD mice, we found that the IE is characterized by a pro-inflammatory MYD88-dependent TLRs signaling in homeostatic basal conditions, i.e., in the absence of pathogens (see previous paragraph). Next, we tested TLR4 response to LPS stimulation. First, we assessed the response of IEC to a short-term low-dose exposure to a nonpathogenic LPS. This *in vivo* treatment has been used by others (Li C et al., 2020) to test the effects of LPS on the intestinal mucus layer. Notably, authors found that low-dose of LPS, that «mimic» a homeostatic condition, increased the thickness of the mucus layer concomitant with the secretion of tolerogenic cytokines such as IL-10. Conversely, a long-term exposure to higher level of LPS, that «mimic» bacterial dysbiosis, leads to loss of GB functions reducing thickness of the mucus layer and leading to secretion of inflammatory cytokines. Moreover, LPS was also identified as an important modulator of the proliferation-to-differentiation balance that occurs within the intestinal crypts and thus is capable to influence the structure of the IE (Naito T et al., 2017).

Therefore, we administered low-dose of a commensal LPS by oral gavage to young NOD female mice and age- and sex-matched C57BL/6 mice for 2 weeks (Figure 12A) to test the "homeostatic" response of IE to LPS. We performed the analysis separating IEC from ileum and colon. The experiment was repeated twice in two different cohort of mice.
We first observed an increased mRNA level of TLR4 and MYD88 in ileum's IEC from both NOD and C57BL/6 mice after LPS exposure (Figure 12B), as expected. Strikingly, we observed that the short exposure to LPS increased the mRNA level of TRAF6 only in NOD mice (Figure 12C). Furthermore, the Muc2 mRNA level was unaffected in NOD mice after LPS exposure, conversely to C57BL/6 mice (Figure 12D). Considering that bacterial LPS is a mucus-inducer in the gut (Smirnova MG et al., 2003) and the process is genetically MYD88-independent (Larsson E et al., 2012), we hypothesized that the TRAF3 adaptor from the MYD88-independent signaling may be involved. Thus, we analyzed the mRNA level of TRAF3 and observed that in C57BL/6 mice TRAF3

increased, concomitant with the increased Muc2 expression, while in the IE of NOD mice this response was completely abolished (Figure 12E).

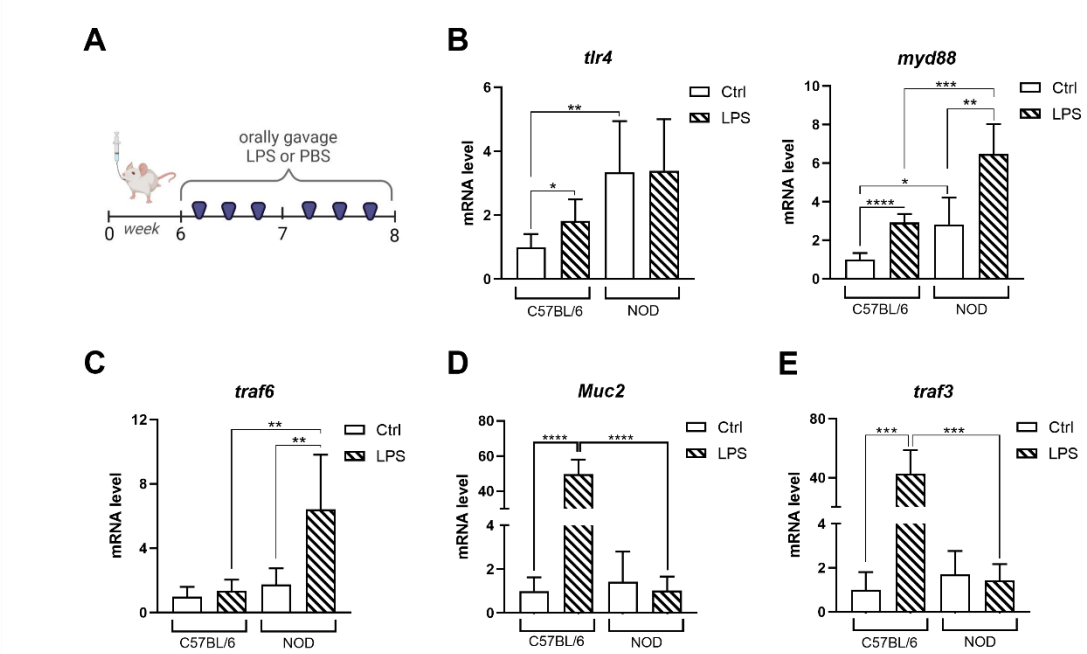


Figure 12: NOD mice present a dysregulated epithelial TLR response to LPS in the ileum.
*(A) In vivo LPS treatment: 6weeks-old NOD and C57BL/6 mice were orally gavage with 200ug/kg of LPS or PBS (n=6 per group) 3 times a week up to 2 weeks; at the end of the treatment mice were sacrificed and IEC from ileum and colon of treated-mice were isolated for gene expression analysis. B-E: mRNA level of TLR4 and MYD88 (B), TRAF6 (C), Muc2 (D) and TRAF3 (E) in ileum's IEC isolated from LPS-treated mice. All data are representative of two independent experiments. Data are represented as mean ± SD. *p < 0.05. , **p<0.01, ***p<0.001, ****p<0.0001.*

We performed all the analysis also in IEC isolated from the large intestine, i.e., the colon of the same cohort of LPS-treated mice. We observed increased mRNA level of TLR4 and MYD88 in both groups after LPS exposure (Figure 13A). However, we also observed that while TRAF6 was unaffected (Figure 13B), mRNA level of TRAF3 dramatically decreased in NOD mice IEC, contrarily to C57BL/6 mice (Figure 13C). In parallel, the mRNA level of Muc2 increased in C57BL/6 mice after LPS exposure, contrary to NOD mice (Figure 13D).

Additionally, we performed a qualitative analysis of the colonic mucus layer that confirmed reduced production of Muc2 in this cohort of NOD mice as previously demonstrated (Sorini et al., 2019) (Figure 13E).

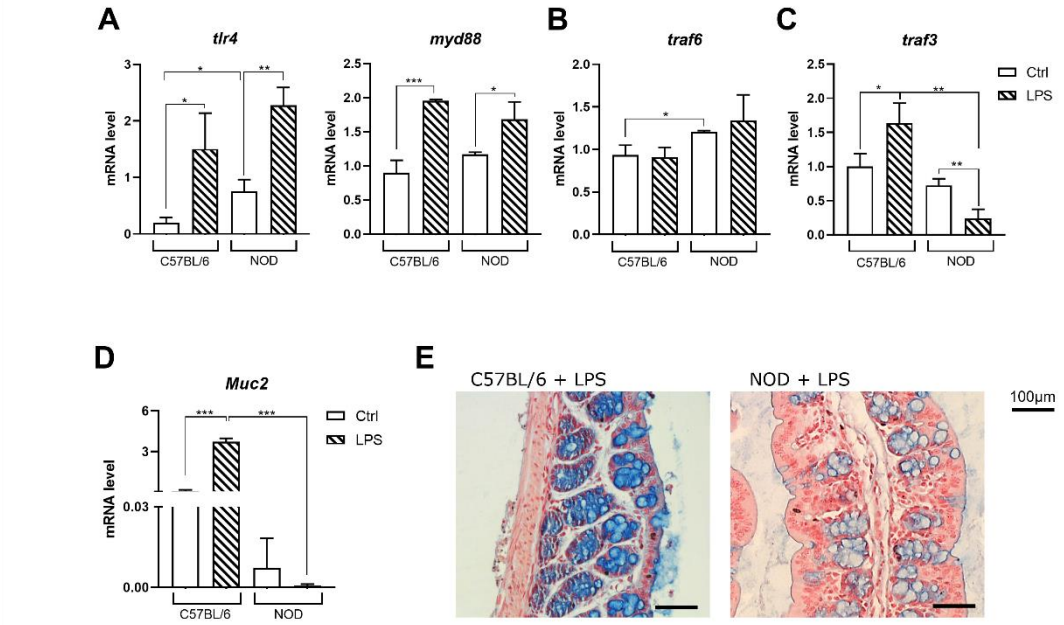


Figure 13: NOD mice present a dysregulated epithelial TLR response to LPS in the colon.
*A-D. mRNA level of TLR4 and MYD88 (A), TRAF6 (B), TRAF3 (C) and Muc2 (D) in in colonic IEC isolated from LPS-treated mice; n=6 mice/group. (E) Alcian-Blue stained colon section of LPS-treated mice acquired with Zeiss Axio Imager M2m microscope with 20X magnification (100µm). All data are representative of two independent experiments. Data are represented as mean ± SD. *p < 0.05., **p<0.01, ***p<0.001, ****p<0.0001.*

Multiple studies showed that IEC modulate intestinal immune cells since they are in close contact with intra-epithelial lymphocytes (iEL). Therefore, we asked whether the epithelial TLR signaling on IEC may have an influence on intestinal immunity. Thus, we cultured IEC and iEL isolated from our cohort of LPS-treated mice and measured the cytokines level in the supernatant after 12h (Figure 14A). We observed an increased level of IL-17a and TNF-α in the supernatant of LPS-treated NOD mice compared to LPS-treated C57BL/6 mice (Figure 14B). High levels of TNFα normally induce apoptosis in IEC and increase intestinal permeability specifically acting on tight junctions (Lee SH,

2015), suggesting that the presence of TNF-α may be the starting point for GB breakage in NOD mice.

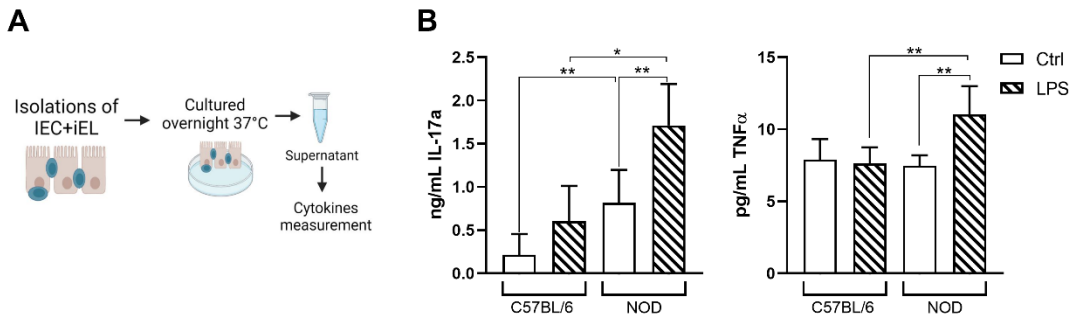


Figure 14: The short-term exposure to LPS induce the local production of inflammatory cytokines in NOD mice.
*(A) LPS-treated mice were sacrificed and intestinal epithelial cells (IEC) with intra-epithelial lymphocytes (iEL) were isolated and cultured at 37°C. Supernatant was collected after 12h for cytokines measurement. (B) IL-17a and TNF-α level measured in IEC supernatant by ELISA assay; n=6 mice/group. Data are represented as mean ± SD. *p < 0.05. , **p<0.01.*

Next, we looked at the phenotype of LP lymphocytes in our cohort of LPS-treated mice. Firstly, we observed that the short-term LPS exposure increased the differentiation of Treg cells in C57BL/6 mice, contrarily to NOD mice in which we did not observe any increased of Treg cells frequency (Figure 15A). In addition, the frequency of Th17 cells increased in NOD mice after LPS treatment (Figure 15B), probably due to the activation of the MYD88-mediated signaling (Figure 15B-C). The decreased Teff/Treg ratio in the intestine of C57BL/6 mice after short-term LPS treatment suggest that this could be a key mechanism to maintain immune tolerance towards gut antigens (i.e., microbial and food antigens) (Figure 15C). On the contrary, the lack of Treg cell induction in response to low dose LPS stimulation in NOD mice indicate a possible defect of intestinal immune tolerance in this strain that favor inflammation and GB damage (Figure 15C). The same finding was observed in the small and large intestine of NOD mice (Figure 15D)

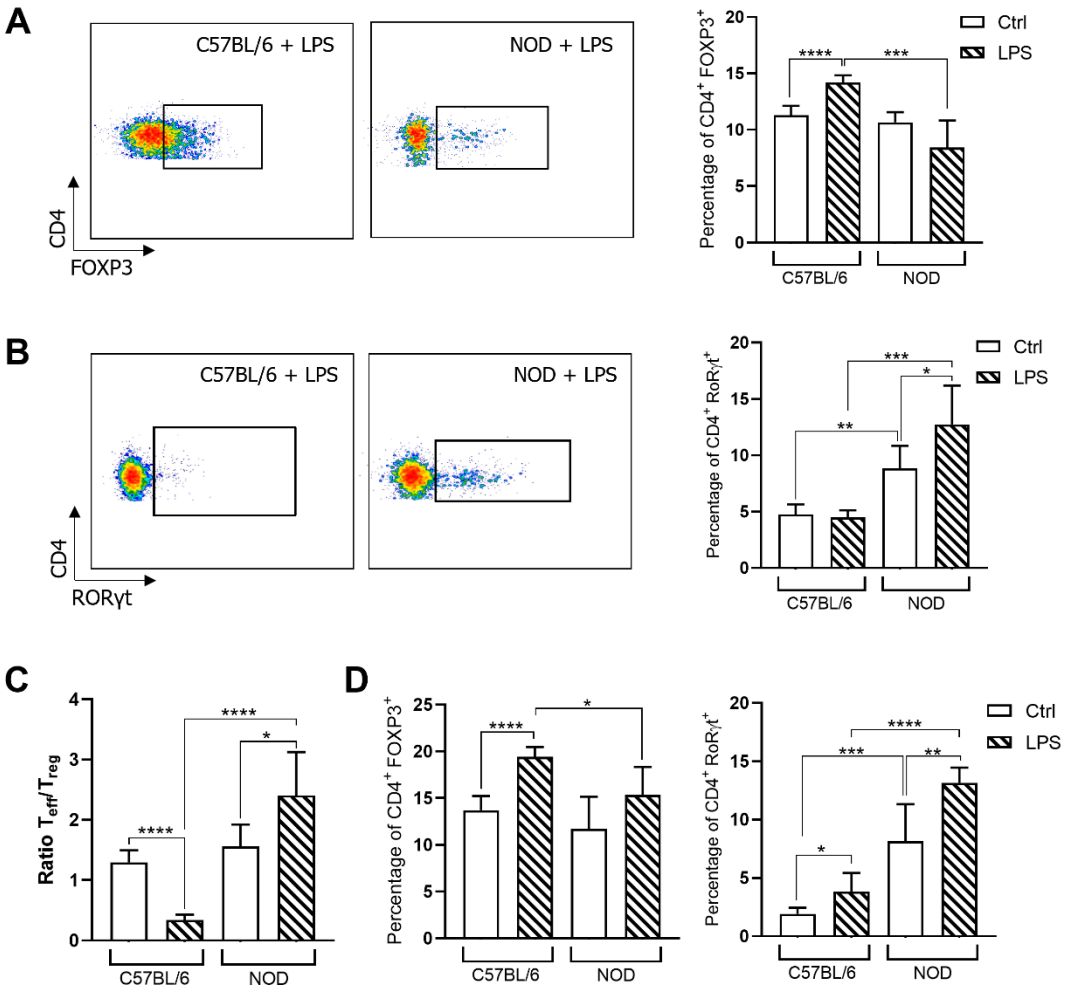


Figure 15: Lack of Treg cell induction in response to low dose LPS stimulation in NOD mice.

*(A) Representative flow cytometry plots (left) and bar graph with mean percentages ± SD (right) of CD4$^+$ FOXP3$^+$ T reg cells in the small intestine LP of LPS-treated NOD and C57BL/6 mice; (B) Representative flow cytometry plots (left) and bar graph with mean percentages ± SD (right) of CD4$^+$ RORγt$^+$ Th17 cells in the small intestine LP of LPS-treated NOD and C57BL/6 mice. (C) Teff over Treg ratio in the small intestine of LPS-treated mice. (D) bar graph with mean percentages of CD4$^+$ FOXP3$^+$ T reg and CD4$^+$ RORγt$^+$ Th17 cells in colonic LP of LPS-treated NOD and C57BL/6 mice. n=6 mice/group. All data are representative of two independent experiments. *p < 0.05., **p<0.01, ***p<0.001, ****p<0.0001.*

We also analyzed the phenotype of Treg- and Th17-polarizing LP DC and observed increased frequency of Treg-polarizing $CD11c^+CD103^+$ DC in C57BL/6 mice in response to LPS stimulation contrarily to NOD mice (Figure 16A). Moreover, we observed increased frequency of Th17-polirizing $CD11b^+CD103^+CCR2^+$ in the LP of NOD mice contrarily to C57BL/6 mice (Figure 16B). The same results were observed in the colon (Figure 16C).

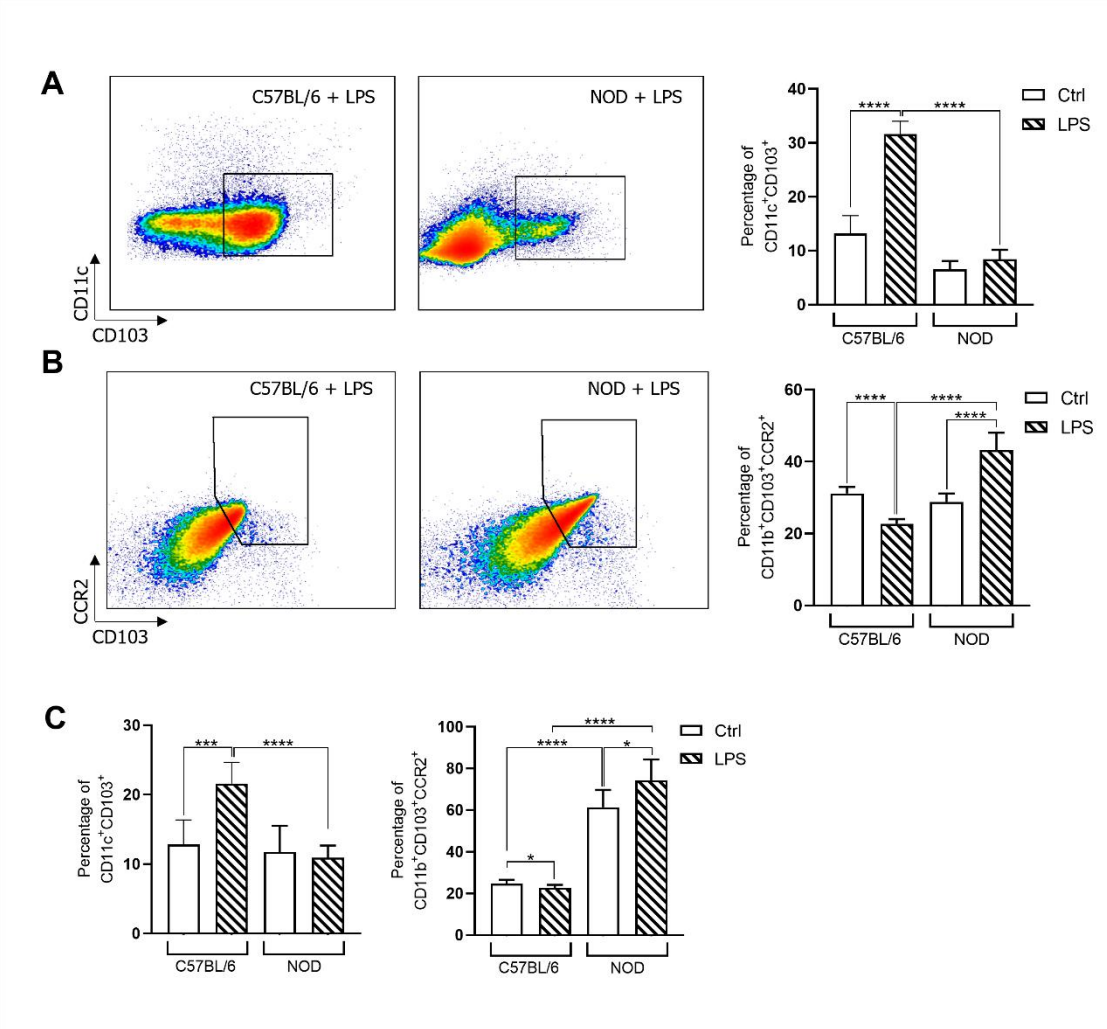


Figure 16: The lack of Treg induction correlates with decreased frequency of Treg polarizing DC after shot-term LPS exposure in NOD mice.

*(A) Representative flow cytometry plots (left) and bar graph with mean percentages ± SD (right) of $CD45^+CD11c^+CD103^+$ Treg- polarizing DC in the small intestine LP of LPS-treated NOD and C57BL/6 mice (B) Representative flow cytometry plots (left) and bar graph with mean percentages ± SD (right) of $CD45^+CD11b^+CD103^+CCR2^+$ Th17-polirizing DC in the small intestine LP of LPS-treated NOD and C57BL/6 mice. (C) Bar graph with mean percentages ± SD of $CD45^+CD11c^+CD103^+$ Treg- polarizing DC and $CD45^+CD11b^+CD103^+CCR2^+$ Th17-polirizing DC in the colonic LP of LPS-treated NOD and C57BL/6 mice. n=6 mice/group. All data are representative of two independent experiments. *p < 0.05., **p<0.01, ***p<0.001, ****p<0.0001.*

NOD mice-derived intestinal organoids present a dysregulated TLR4 signaling *in vitro*.

Our data shows that NOD mice have dysregulations in epithelial TLR4 signaling that correlates with a defective production of intestinal mucus and ineffective differentiation/expansion of intestinal FoxP3$^+$ Treg cells in response to LPS. To better investigate the involvement of the TLR4 signaling in the epithelial differentiation into mucus-production IEC, we developed intestinal organoid cultures by growing and perpetuating stem-cell containing crypts isolated from the intestinal tissue (Figure 17A). Gut organoids cultures derived from studies on ISC made by T. Sato and H. Clevers in 2009 in which they discovered that intestinal Lgr5$^+$ stem cells, under specific condition, can expand *in vitro* differentiating into a single layer three-dimensional structure and containing all the cell types represented in the IE (Sato T et al., 2009; Sato T and Clevers H, 2013). The resulting organoids are characterized by a convoluted morphology with crypt-like projections and villus-like domains as in the real gut (Figure 17B) and, importantly, are genotypically identical to the donor tissue. Following the experimental procedure described in the paper of T. Sato and H. Clevers (Sato T and Clevers H, Methods Mol Biol 2013) with some modifications, we were able to isolate intact and alive ISC-containing crypts (Figure 17C). After the embedding in Matrigel, ISC within the crypts differentiate and proliferate assuming a spheroid-like shape by the 2nd day of culture to a mature convoluted morphology by the 7th day (Figure 17D).

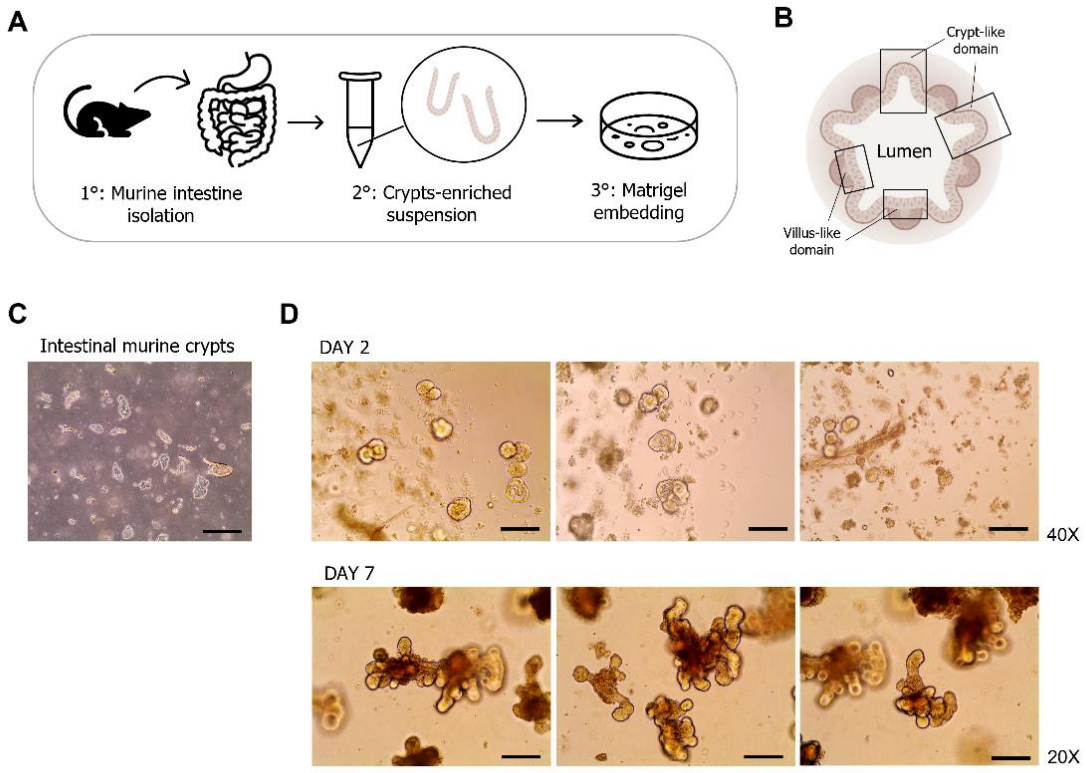


Figure 17: Setting mouse-derived intestinal organoids.
(A) Protocol for growing ileal-intestinal organoids: cleaned segments of ileum are processed to obtain crypts-enriched suspension with alive ISC. Several fractions of crypts are embedded in Matrigel and let them grow. (B) Schematic representation of the structure of an intestinal organoid, characterized by a convoluted morphology with crypt-like projections and villus-like domains; IEC have a polarized orientation, with the apical side to the lumen-like area and the basal side to the Matrigel. (C) Representative light microscope image of fresh-isolated intestinal crypts (40X magnification). (D) Representative light microscope images of intestinal organoids during the growth stages: at day 2, they have high proliferating activity and show a spheroid-like shape (40X magnification); at day 7, they reached a differentiated convoluted morphology with clear crypt-like projections (20X magnification).

We next isolated intestinal crypts from adult NOD female mice and sex- and age-matched C57BL/6 mice and established several NOD-derived and C57BL/6-derived intestinal organoid lines. Our first observation was that NOD-derived organoids grew differently from their counterparts derived from non-autoimmune C57BL/6 mice, assuming a spheroid-like shape instead of the usual convoluted morphology with crypt-like projections (Figure 18A). Accordingly, we detected a decreased mRNA level of the

proliferation marker Ki67 in NOD-derived organoids compared to C57BL/6-derived intestinal organoids lines (Figure 18B) as an indication of less proliferation capacity. In addition, we observed a decreased expression of the stem cell marker LGR5 in NOD-derived organoids (Figure 18C). These preliminary findings suggest that an inherent defect in the maturation of the IE is present in NOD mice and may be responsible for the loss of gut barrier integrity (Sorini et al., 2019).

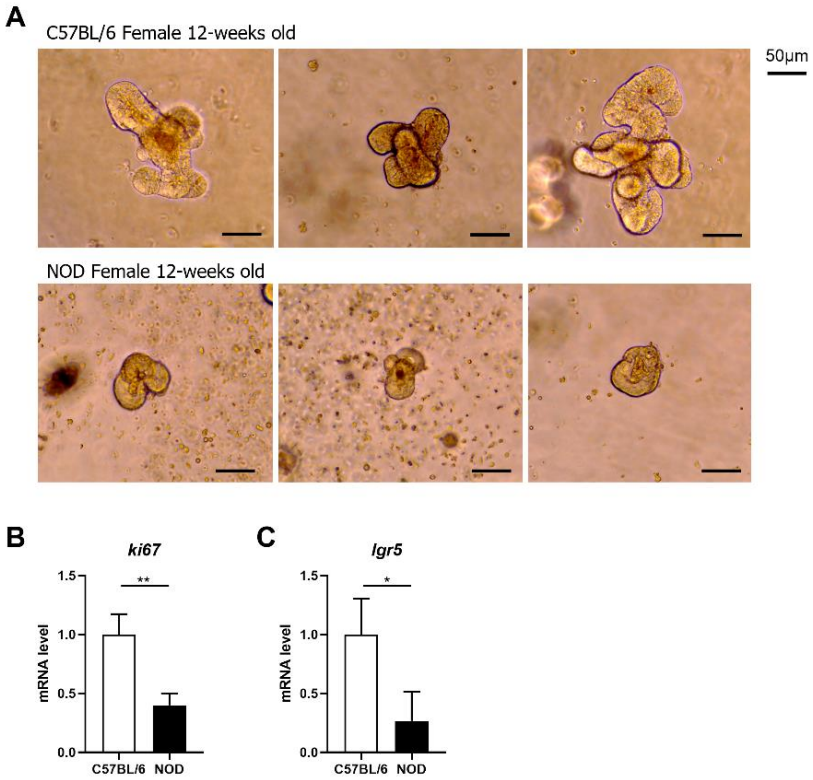


Figure 18: NOD-derived intestinal organoids show a defective maturation of the IE. *(A) Representative light microscope images of ileal-intestinal organoids (40X magnification) derived from 12weeks-old female C57BL/6 and NOD mice (n=3 organoids lines per group). C-D: mRNA level of proliferation marker KI67 (B) and the stem cells marker LGR5 (C) on C57BL/6 and NOD mice-derived ileal organoids. Data are represented as mean ± SD. *p < 0.05., **p<0.01.*

To better characterize the epithelial TLR response in NOD mice, we challenged our lines of intestinal organoids with LPS *in vitro* (Figure 19A). Surprisingly, we observed that our NOD-derived organoids lines showed an upregulation of TLR4 in absence of any stimulation, contrarily to C67BL/6-derived counterparts that increased the epithelial TLR4 expression only after LPS stimulation (Figure 19B). Moreover, LPS induced a the predominant up-regulation of MYD88 and TRAF6 in our NOD-derived organoids lines (Figure 19C) contrary to organoids from non-autoimmune mice, confirming the dysregulating TLR signaling in NOD mice that previously observed ex-vivo in freshly isolated intestinal tissues.

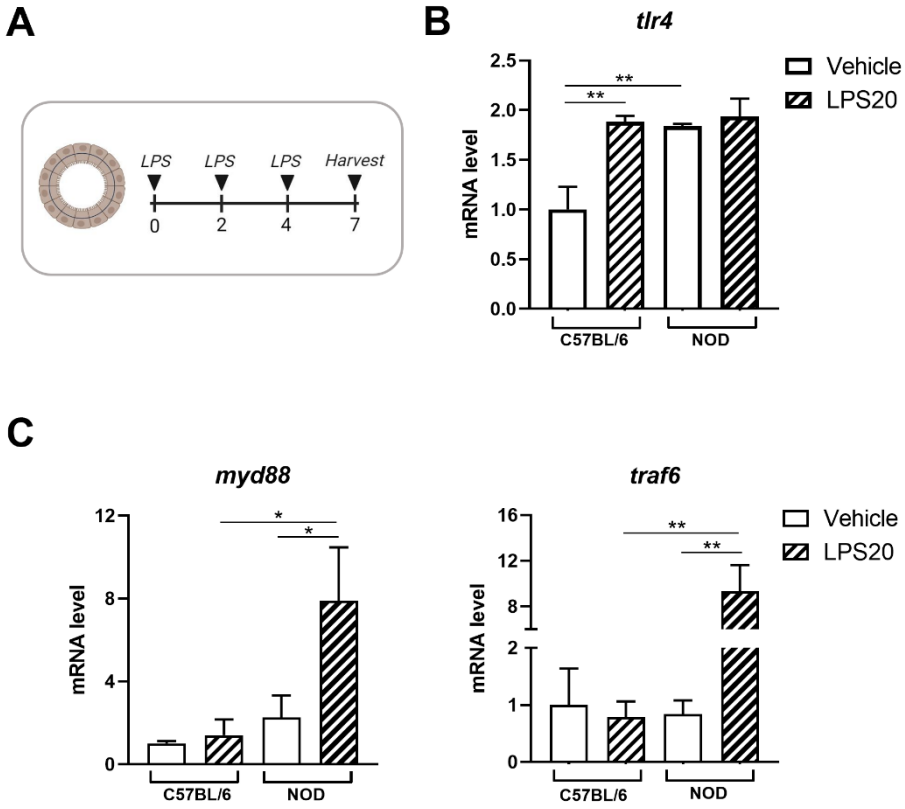


Figure 19: ***Dysregulated TLR signaling is present in NOD mice-derived organoids***
*(A) Ileal-intestinal organoids derived from 12weeks-old female C57BL/6 (WT) and NOD mice (n=3 organoids lines/group) were treated with 20ng/mL of LPS or Vehicle (medium) for 7 days prior to gene expression analysis. B-C: mRNA level of TLR4 (B), MYD88 and TRAF6(C) on C57BL/6- and NOD mice-derived ileal organoids treated with LPS or Vehicle for 7 days. All mRNA levels were calculated using ΔΔCT method and express as fold change over C57BL/6 mice. Data are representative of two independent experiments. Data are represented as mean ± SD. *p < 0.05., **p<0.01.*

Next, we assessed the mucus production after LPS stimulation. LPS induced mucus production in our intestinal organoids lines, as shown by the light microscope images in figure 20A. *In vitro* stimulation with LPS re-established a correct mucus-producing capacity in NOD-derived organoids (Figure 20B), contrarily to *in vivo* data (Figure 12D-13D). In addition, our NOD-derived organoids further re-established the expression of TRAF3, as C57BL/6-derived organoids (Figure 20C). Our finding suggest that although a predominant MYD88/TRAF6 response in NOD mice is genetically related, the expression of TRAF3 and Muc2 production are regulated at epigenetic level.

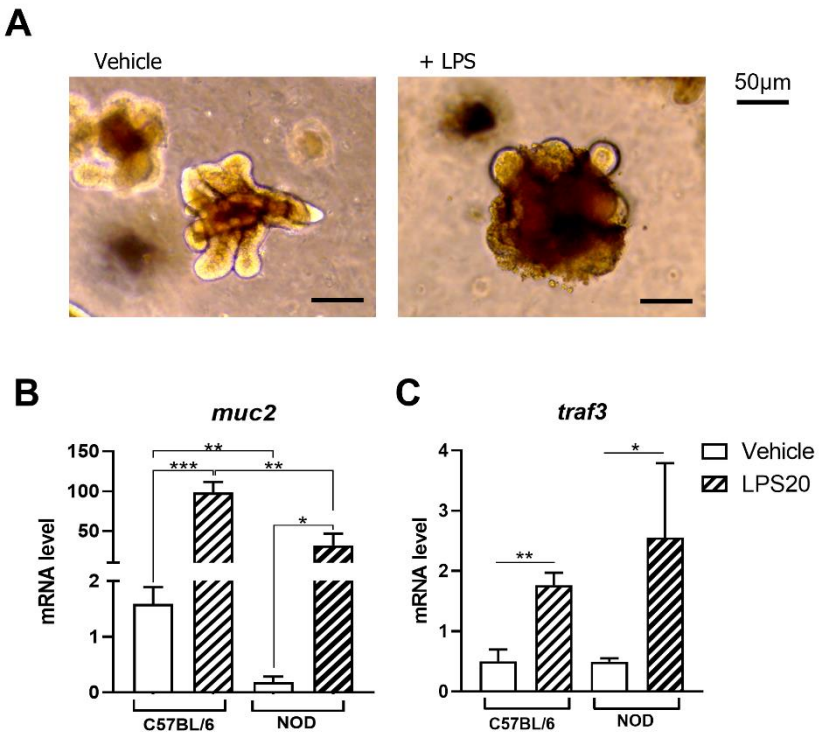


Figure 20: LPS induced mucus production in both intestinal organoids lines.
*(A) Representative light microscope images of ileal-intestinal organoids (40X magnification) treated with vehicle or LPS after 7 days. B-C: mRNA level of MUC2 (B) and TRAF3 (C) on C57BL/6- and NOD mice-derived ileal organoids treated with LPS or Vehicle for 7 days. All mRNA levels were calculated using ΔΔCT method and express as fold change over C57BL/6 mice. Data are representative of two independent experiments. Data are represented as mean ± SD. *p < 0.05., **p<0.01.*

Post-transcriptional regulation on epithelial TLR signaling is compromised in NOD mice.

The upregulation of epithelial TLR4 that occurs in NOD mice from our *in vivo* and *in vitro* experiments leads to the hypothesis that the molecular mechanism that control the TLRs signaling are defective. Indeed, epithelial TLRs signaling is under the control of several post-transcriptional mechanisms that, for the TLR4 signaling, are called «LPS tolerance» (Nahid MA et al., 2011). Among these mechanisms, one mediated by miRNAs occurs specifically during early life and imprints the epithelial TLRs response to bacteria. Specifically, miR146a has been identified as a key regulator of the «LPS tolerance» during the neonatal period since alterations in this process cause epithelial TLR4 signaling dysregulations during adult life (Chassin C et al., 2010). Moreover, miR146a expression has been detected also in adult IEC and generally controls inflammatory responses (Garo LP et al., 2021). Thus, to investigate whether the dysregulated TLR4 signaling that we found in adult NOD mice is related to alterations in the post-transcriptional mechanisms of the «LPS tolerance» process, we measured the miRNA level of specifically miR-146a in IEC isolated from newborn 1-weeks-old female NOD- and C57BL/6- pups. We found that IEC from female newborn NOD mice have a decreased expression of miR-146a compared to sex and age-matched newborn C57BL/6 mice (Figure 21A). Moreover, we found that IEC from adult female NOD mice did not express miR-146a, contrarily to C57BL/6 mice (Figure 21B)

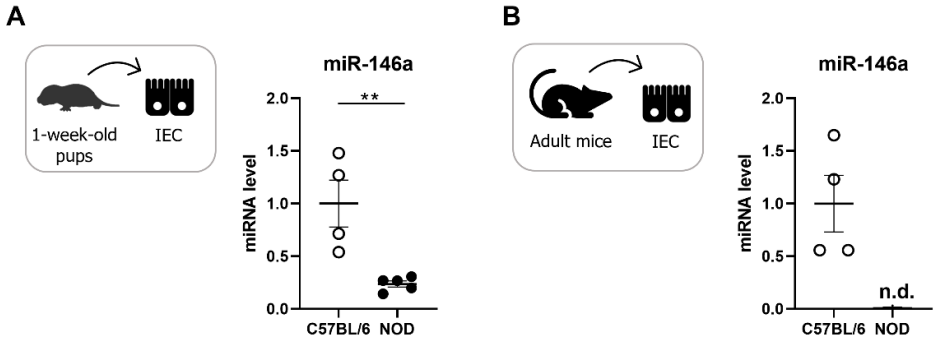


Figure 21: Dysregulated expression of miR-146a is present in IEC from NOD mice
(A) miRN-146a level measured in IEC isolated from newborn 1-weeks old female NOD and C57BL/6 mice (n= 4-5 per group). (B) miRN-146a level measured in IEC isolated from 5-weeks old female NOD and C57BL/6 mice (n= 4 per group). miRN-146a relative abundance was calculated against the snor202 expression. Data are represented as mean ± SD.

Pro-inflammatory epithelial TLRs signaling is present in the gut mucosa of T1D patients.

Epithelial TLRs signaling is crucial in maintaining tolerance to luminal bacteria and thus in avoiding the overactivation of the local immune system. Particularly, our data show that the preclinical model of T1D, the NOD mice, have defective tolerance to nonpathogenic LPS resulting in upregulation of the MYD88 proinflammatory signaling. An inflamed gut associates with dysregulations in TLRs signaling, as specifically observed in IBD and other intestinal inflammatory conditions (Szebeni B et al., 2008). Altered TLRs signaling has been found in PBMC from T1D patients (Meyers AJ et al., 2010) but it has never been studied in human intestinal tissue. Thus, we collected duodenal biopsies from a cohort of T1D patients and healthy individuals that underwent on EGDs procedure (see clinical data in Table1) and extracted RNA for gene expression analysis (Figure 22A).

Cohort	*n*	Age (years)	Age (mean ± SD)	Sex (women/men)	Other autoimmune disease
Healthy	16	25-67	43,3 ± 13,5	10/6	NA
T1D	19	18-57	32,73 ± 12,5	13/6	Celiac disease (*n*=5)

Table 1: Clinical data
All individuals recruited in the study underwent EGD for diagnostic purposes (e.g., clinical suspect of IBD not confirmed by histological examination of intestinal tissue)..

Firstly, we found increased mRNA level of TRAF6 in the gut mucosa of T1D compared to healthy controls (Figure 22B); as NOD mice, no differences in TRAF3 expression was found (Figure 22C). In addition, we detected high mRNA level of IL-1β cytokine in gut mucosa of T1D patients (Figure 22D), that is a sign of tissue inflammation. Indeed, high level of IL-1β acts directly on tight junctions affecting intestinal permeability (Al-Sadi R et al., 2010) and correlate with intestinal inflammation (Rawat M et al., 2020). Lastly, we analyzed the expression of TLR4 and found an increased mRNA level in T1D patients (Figure 22E).

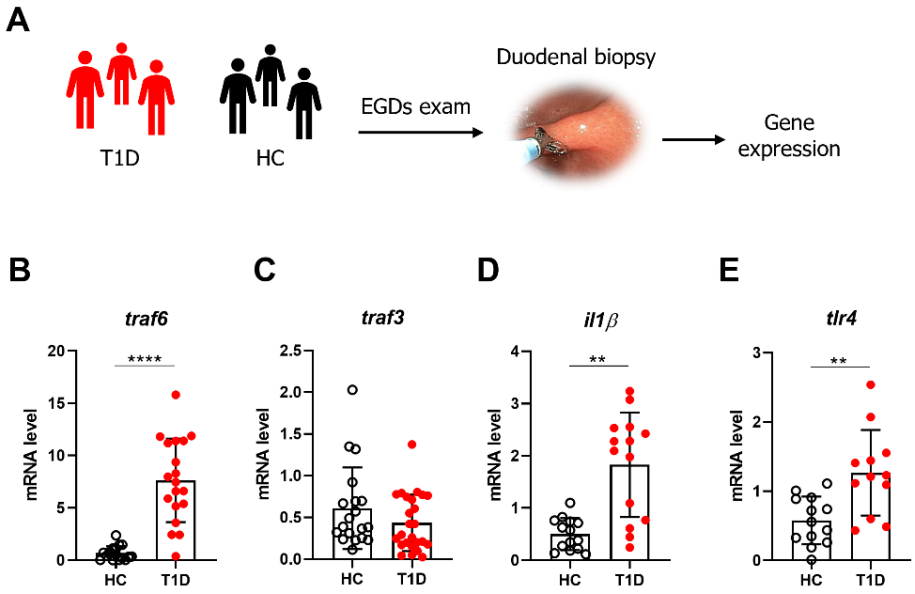


Figure 22: The gut mucosa of T1D patients is characterized by a pro-inflammatory epithelial TLRs signaling.

(A) Intestinal tissue samples from the duodenal mucosa were collected during EGDs from T1D patients (T1D, n=19) and healthy controls (HC, n=16) and RNA was extracted for gene expression analysis (qRT-PCR). B-E. mRNA level of TRAF6 (B), TRAF3 (C), IL-1β (D) and TLR4(E). The relative mRNA level was calculated using ΔΔCT method and express as fold change over HCs.

DISCUSSION

The GB plays a crucial role in orchestrating the interaction between the gut microbiota and immune cells. Its disruption causes alterations in the communication between the intestinal immune system and commensal microbes and thus in the intestinal homeostasis. Besides, GB alterations are linked to several immune-mediated disorders, including T1D. However, the causative link between GB, microbiota and immune system in the context of T1D have not been fully clarified.

Studies on the T1D mouse model NOD mice showed several signs of enteropathy that occur before the clinical onset of the disease (Sorini C et al., 2019; Miranda MCG et al., 2019) leading to the hypothesis that alterations of the gut microenvironment and, specifically, of GB integrity, may be directly involved in the pathogenesis of the disease. Indeed, autoreactive T lymphocytes can be activated in the gut and cause T1D in a transgenic mouse model after induction of GB damage (Sorini C et al., 2019). The presence of microbial dysbiosis in both T1D mouse models and human (Vatanen T et al., 2018; Gavin PG et al., 2018.) leads to the assumption that microbes play a crucial role in modulating GB structure and intestinal immune system triggering extra-intestinal autoimmunity. Several studies with knock-out mice for TLRs demonstrated that microbes modulate the pathogenesis of T1D through TLRs signaling (Wen L et al., 2008; Burrows MP et al., 2015; Simon MC et al., 2020), but the exact mechanism underlying the modulation of pancreatic autoimmunity by LPS is still unknow. In particular, the abnormal response of the IE to LPS in the context of T1D was never investigated.

Here we showed that alterations of epithelial TLRs signaling are present in preclinical model of T1D and in T1D patients. By isolating IEC from NOD mice, we detected alterations in the IE differentiation process starting from ISC (Figure 9A-9E), also including the pathways that control the differentiation of ISC (Figure 9B). ISC are located in the intestinal crypts and give raise to all the different IEC in response to several stimula that they receive from the external environment to meet homeostatic needs or regenerative demands, including both bacterial and immune stimulation. Importantly, the activation of TLRs on ISC controls crypt dynamics by altering proliferation and apoptosis. Therefore, the molecular signaling that occurs on ISC are fundamental for the generation of a functional GB. In this context, we observed that the IE of NOD mice is characterized by

a basal upregulation of the pro-inflammatory TLRs signaling compared to sex- and age-matched non-autoimmune mice, which include MYD88 and TRAF6 (Figure 10). In addition, our cohort of young prediabetic NOD mice showed also increased epithelial TLR4 expression (Figure 10D). However, looking at the graph plot of figure 10D, the NOD population looks clearly divided into two sub-groups, with 5-6 mice that have increased TLR4 expression compared to C57BL/6 that contribute to the overall statistical significance. This can be due to the young age of these NOD mice: indeed, the T1D incidence rate in NOD mice is 80-90% (Delovitch TL et al., 1997), so when experiments are conducted in young prediabetic NOD mice is impossible to assume which mouse will develop the disease. For this reason, we divided our cohort of NOD mice into diabetic and diabetes-free adult NOD mice, i.e., adult NOD mice that did not develop T1D by 30 weeks of age. By analyzing the epithelial TLR4 expression of diabetic NOD mice vs diabetes-free adult NOD mice, we observed no differences in the mRNA level of TLR4 between the two groups (Figure 11B), contrarily to the intracellular adaptors MYD88 and TRAF6, which significantly increased in diabetic NOD mice (Fig 11A), thus suggesting that unbalanced downstream epithelial TLR4 signaling correlates with the occurrence of T1D, specifically activating the pro-inflammatory signaling rather than the tolerogenic MYD88-independent signaling.

Importantly, TLR4 signaling dysregulations were also found in the gut mucosa of a cohort of T1D patients (Figure 22B-E), specifically including increased level of TLR4, TRAF6 and the inflammatory IEC-related cytokine IL-1β, confirming the involvement of TLR4 signaling in T1D. Besides, alterations in the LPS-TLR4 signaling have been documented for T1D and studies in knock-out mice demonstrated that the TLR4 signaling has a direct impact on disease progression (see paragraph 2.2.5). Specifically, the balance between the two TLR4 related pathway, the MYD88-TRAF6 and the TRIF-TRAF3 signaling, which are involved in the activation of the inflammatory and tolerogenic signaling respectively, plays a pivotal role in modulating T1D (Zhang Y et al., 2021).

Our *in vivo* and *in vitro* experiments with the TLR4-ligand LPS clearly demonstrate the importance of the TLR4-dependent response in the gut epithelium, especially in NOD mice. Specifically, while in C57BL/6 mice a short-term LPS exposure induced the activation of tolerogenic $CD103^+$ DCs (Figure 16A) and the differentiation/expansion of

intestinal FoxP3$^+$ Treg cells (Figure 15A), in NOD mice the LPS-TLR4 signaling induced, contrarily, an inflammatory response, including increased expression of the proinflammatory MYD88-signaling (Figure 12B-C), the production of inflammatory cytokines by IEL (Figure 14B) and increased frequency of Th17 cells (Figure 15B). Commensal LPS specifically regulates the mucus production in the gut by inducing mucin mRNA expression in IEC (Smirnova MG et al., 2003). Likewise, our *in vivo* and *in vitro* data showed that the exposure to low-dose of LPS increased the expression of muc2 mRNA level in C57BL/6 mice (Figure 12B; Figure 20A-B) together with the activation of the TLR4-mediated TRAF3 signaling (Figure 12E; Figure 20C), suggesting a tolerogenic role of the TRAF3-mediated signaling in regulating tissue homeostasis and, specifically, in inducing the mucus production after the stimulation of TLR4 by LPS. IEC from NOD mice, on the contrary, increased the MYD88-TRAF6 signaling after the LPS stimulation *in vivo* at the expense of the TRAF3-mediated signaling (Figure 12), suggesting that alterations in the tolerogenic TLR4-mediated signaling may be linked to T1D pathogenesis. Indeed, while MYD88 knock-out NOD mice are protected from development of T1D (Wen L et al, 2008), NOD mice deficient for both MYD88 and TRIF, which are involved in the TRAF6 and TRAF3 signaling respectively, developed the disease (Burrows MP et al., 2015), suggesting a protective role of the TRAF3-mediated pathway in T1D. However, although several studies with knock-out mice highlighted the crucial role of TLR4 pathway in T1D, further studies are necessary to better clarify how the specific activation of the TLR4-related TRAF3 signaling in IEC is involved in the maintenance of tissue homeostasis and particularly in the pathogenesis of autoimmune diabetes.

TLR4 is an epithelial barrier regulator playing a crucial role in the balance between the inflammatory and the tolerogenic signaling, necessary to maintain a stable gut homeostasis. Besides, studies with transgenic villin-TLR4 mice demonstrated that the over-activation of epithelial TLR4 altered the gut homeostasis by disrupting the IE differentiation and inducing susceptibility to colitis (Dheer R et al., 2016). Nevertheless, TLR4 signaling is fundamental also in the response to epithelial injury and bacterial translocation since TLR4 deficient mice showed severe and acute intestinal inflammation after induction of experimental colitis (Fukata M et al., 2005). In this context, the TLR4-

TRIF signaling is specifically involved in maintaining intestinal epithelial barrier function by promoting AMPs production and mucosal homeostasis (Stockinger S et al., 2014).
For these reasons, TLR4 signaling is finely regulated by a complex network of post-transcriptional mechanisms, some of which are activated by the IEC itself and aim to limit the eventual TLR4 overactivation against the microbiota. These important regulatory mechanisms develop during neonatal period in response to early microbiota colonization, when both immune cells and IEC regulated themselves to establish a stable and durable tolerance against the commensal microbiota. Any disruption of these processes has been linked to the development of immune-mediated disorders during adult life (Uusitalo U et al., 2016). In NOD mice, shaping the microbiota composition during early life protect against T1D (Miranda MCG et al., 2019; Liang W et al., 2022), suggesting that microbiota plays a pivotal role in orchestrating the gut homeostasis before weaning. Notably, our *in vitro* experiments with NOD mice-derived intestinal organoids show defective IEC differentiation and altered TLRs signaling even in absence of any stimula (Figure 20) suggesting that early-life events may have an impact in imprinting the epithelial TLR signaling regulation in adult NOD mice. In this context, others demonstrated that newborn NOD mice are characterized by gut microbial dysbiosis and increased TLR4 and TLR2 expression in the GB before weaning (Liang W et al., 2022), confirming the hypothesis that dysregulation of TLRs signaling may occur during neonatal period in NOD mice.
In this context, the miRN-146a is as a key regulator of the "LPS tolerance" that control the activation of TLR4 in response to luminal LPS in the IE during neonatal period (Chassin C et al., 2010). In addition, miR-146a has been identified as a modulator of the inflammatory responses within the adult gut tract (Garo LP et al., 2021). We found that IEC from adult NOD mice do not express miR-146a contrarily to non-autoimmune control mice (Figure 22B). Moreover, IEC isolated from 1-weeks old newborn female NOD mice have decreased expression of miR-146a compared to sex and age-matched C57BL/6 mice (Figure 22A), showing that dysregulation on the epithelial TLR signaling occurs during the prenatal period and may be the cause of the dysregulated epithelial TLR signaling that we observed in adult NOD mice (Figure 10).
From these results we can speculate that the alterations of the miRNA regulation on TLR4, linked to the neonatal period, may be the causes of the altered GB structure that we observed in adult NOD mice. However, the causative mechanisms behind these

alterations are not clear, specifically whether they can be related to the interaction with a dysbiotic microbiota or other inherent defects. Previous studies demonstrated (Garo LP et al., 2021) that the oral administration of miR-146a reduced tissue inflammation in the murine gut, protecting from tumor-associated inflammation in colorectal cancer. Since adult IEC from NOD mice do not express miR-146a (Figure 22B), this miRNA could be therapeutically exploited to prevent T1D in preclinical models and, if similar down-regulation is found in T1D patients, also in humans as possible therapeutic approach to modulate the TLR4 signaling in the gut, decrease subclinical inflammation and test the beneficial effect on occurrence of beta cell autoimmunity and T1D.

MATERIALS AND METHODS

Preclinical studies

Mice

Female NOD and C57BL/6 were purchased from Charles River Laboratories. All mice were maintained under specific-pathogen–free conditions in the animal facility at San Raffaele Scientific Institute, and all experiments were conducted in accordance with the Institutional Animal Care and Use Committee in accordance with the rules of the Italian Ministry of Health.

Intestinal epithelial cells and mononuclear cells isolation

Intestinal epithelial cells were isolated from the intestine as described previously (Gracz AD et al., 2012) with some modifications. The intestines of mice were isolated, and small intestine and colon separately flushed with PBS and 0,5% Bovine Serum Albumin (BSA, Sigma-Aldrich) to remove feces. Each piece were cut into 1cm sections and incubated with 6mL of Gentle Dissociation Reagent (Stem Cell) at room temperature for 10 minutes with shaking. Epithelial cells were then isolated by vigorously shaking the pieces in DMEM and 0,5% BSA and the supernatant with epithelial cells was collected. After centrifugation of supernatant, the pellet was incubated for 7 minutes at 37°C with TrypLE Express (Gibco) for separations into single-cells suspension. The purity of epithelial cells fraction was checked by FACS. After the recovery of epithelial cells, intestinal mononuclear cells from lamina propria were isolated with Lamina Propria Dissociation kit (Miltenyi Biotec) following manufactory's instructions. The fraction of lymphocytes were purified from mononuclear cells using Percol gradient separation (Sigma-Aldrich). Briefly, cells were resuspended in 5 mL of 40% Percoll (Sigma-Aldrich), and overlaid on 2.5 mL of 80% Percoll in a 15-mL Falcon tube. Percoll gradient separation was performed by centrifugation at 1,000 × g for 20 min at 20 °C. The interface cells were collected and used as intestinal lymphocytes for fluorescence-activated cell sorting (FACS) analysis.

Ileal murine organoids

Ileal murine organoids were generated as described elsewhere (Sato T et al., 2011) with some modifications. Briefly, ileum was isolated and flushed with PBS and 0,5% BSA (Sigma-Aldrich) to remove feces. Each piece were cut into 1cm sections and incubated with 6mL of Gentle Dissociation Reagent (Stem Cell) at room temperature for 10 minutes with shaking. Intestinal crypts were then isolated by vigorously shaking the pieces in DMEM and 0,5% BSA. Supernatant was centrifuge 300*g* 5minutes 4°C to collect crypts containing intestinal stem cells. Collected crypts were counted and almost 900crypts plated in 50uL of Phenol-Red free Matrigel (Corning) per well. Medium were refreshed every 2 days.

Flow cytometry.

Single-cell lymphocytes suspension was resuspended in staining buffer containing PBS, 1% FBS, and 0.09% NaN3, and stained with monoclonal antibodies against surface markers. For intranuclear staining, cells were first stained for surface markers, then fixed and permeabilized using eBioscience FOXP3/Transcription Factor Staining Buffer set (Invitrogen) and finally stained for intranuclear markers. The following antibodies were used: FITC anti-mouse CD4 (RM4-5, Invitrogen), PercP-eFluor710 anti-mouse RoRγt (B2D, Invitrogen), PE-cy7 anti-mouse Tbet (4B10,BioLegend), APC anti-mouse FOXP3 (FJK-16s, Invitrogen), APC-cy7 anti-mouse CD3 (17A2, BioLegend), FITC anti-mouse CCR2 (SA203G11, BioLegend), PE anti-mouse CD103 (2E7, eBioscience), PercP-cy5.5 anti-mouse CD11c (N418, BioLegend), PE-cy7 anti-mouse MHC-II (M5/114.15.2, Invitrogen), APC-cy7 anti-mouse CD45 (30-F11, Invitrogen) and Pacific Blue anti-mouse CD11b (HL3, BD Biosciences).

Single-cell epithelial cells suspension was resuspended in staining buffer containing PBS, 2mM EDTA and 0,5% BSA (Sigma-Aldrich) and stained with antibodies against surface markers. The following antibodies were used: PE anti-mouse CD326 (caa7-9G8, Miltenyi Biotec) and APC-cy7 anti-mouse CD45 (30-F11, Invitrogen). Dead cells were stained with Fixable Viability Dye eFluor 506 (Invitrogen) for exclusion in the analysis. Flow

cytometry was performed using FACSCanto III (BD Biosciences), and data were analyzed with FCS Express V8 software (De Novo Software).

In-vivo LPS treatment

LPS treatment *in vivo* was performed as described elsewhere (Li C et al., 2020): briefly, 6weeks-old NOD, C57BL/6 and BALB/c mice were orally gavage with 200ug/kg of Lipopolysaccharides (LPS) from *Escherichia coli* O55:B5 (Sigma-Aldrich) or PBS (control group) three times a week up to 2 weeks. At the end of the experiment mice were sacrificed and used for downstream analysis.

LPS treatment *in vitro*: ileal intestinal organoids were passaged and after 2 days treated with 20ng/mL of LPS (*Escherichia coli* O55:B5, Sigma-Aldrich) for 7 days; fresh medium (with LPS) was replaced every 2 days. At the end of the experiment, organoids were harvested from the Matrigel and resuspend in lysis buffer for RNA extraction.

ELISA assay

A quantitative measurement of the cytokines TNF-α and IL-17 was performed with commercial kits that used the ELISA method, following manufacturer's instructions. The following commercial kits were used: IL-12 p70 Mouse Uncoated ELISA Kit (Thermofisher) and TNFalfa mouse Uncoated ELISA (Thermofisher).

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from fresh-isolated IEC with NucleoSpin RNA/Protein kit following manufacturer's instructions (Macherey-Nagel). RNA was retrotranscribed with SuperScript III First-Strand Synthesis System following manufacturer's instructions (Life Technologies). Real-time qPCR assay was performed with SYBR Select Master Mix (Life Technologies) using primers purchased from Biorad.

Total miRNA was extracted from IEC of adult and 1-week old mice with the mirVana Kit (Thermofisher) and quantified by NanoDrop spectrophotometer (Thermofisher). miR-146a-specific reverse transcription was performed using TaqMan MicroRNA Reverse Transcription Kit (Thermofisher). qRT-PCR was performed using TaqMan MicroRNA Assay Mix containing PCR primers and TaqMan probes (Thermofisher). Expression values were normalized to snoRNA-202.

Histology

Ileum samples were collected immediately after sacrifice in formalin for fixation. Fixed segments of ileum were embedded in paraffin wax, sectioned at 5 μm, mounted on slides and stained with hematoxylin and eosin (H&E). Images were acquired by Aperio Slide Scanners (Leica Biosystems).

For analysis of the mucus layer structure, segments of the distal colon were fixed in Carnoy's fixative (60% dry methanol, 30% chloroform, and 10% acetic acid), washed in absolute methanol, ethanol, xylene, embedded in paraffin wax and sectioned at 6 μm. The sections were stained with Alcian Blue staining kit, pH 2.5 (Sigma-Aldrich) and images acquired with Axio Imager M2m Light Microscope (ZEISS).

Diabetes Incidence

Diabetes was monitored by weekly measurements of blood glucose levels with a GB35 Ascensia Breeze 2 glucometer (Bayer). The animals were considered diabetic after two consecutive blood glucose measurements ≥250 mg/dL.

Statistical analysis

Statistical significance was calculated using the unpaired two-tailed Student's t test. GraphPad Prism, version 8.0 (GraphPad Software, San Diego, CA), was used for statistical analysis, and values of $p \leq 0.05$ were considered statistically significant.

Clinical studies

Study population

T1D patients and Healthy controls (HC) included in this study were adult male and female individuals who performed esophagogastroduodenoscopy (EGDS) for diagnostic purposes (dyspepsia, gastroesophageal reflux, altered bowel habits, etc.). Exclusion criteria for both T1D and HC were antibiotics and corticosteroids treatments in the three months before EGDS, clinical history of gastroenteritis, gastric ulcer, irritable bowel disease (IBS), inflammatory bowel disease (IBD) and gastric and colorectal cancer (see

Table 1). All individuals signed a written informed consent, complied with the study procedure. The study was approved by the Institutional Review Board of the IRCCS San Raffaele Scientific Institute (Ethical Committee Board), Milan, Italy.

Samples collection and processing

All EGDS were performed at the Gastroenterology Unit of the San Raffaele Hospital (Milan, Italy). Intestinal tissue fragments were collected in RNAlater solution (Invitrogen) and immediately frozen at −80°C until RNA extraction.

RNA extraction and quantitative real-time RT-PCR

RNA was extracted from duodenal biopsies by adding 100μL of chloroform, precipitating the aqueous phase with 300μL of 70% ethanol, and purifying RNA with RNeasy Mini Kit (QIAGEN). RNA was retrotranscribed with SuperScript III First-Strand Synthesis System following manufacturer's instructions (Life Technologies). Real-time qPCR assay was performed with SYBR Select Master Mix (Life Technologies) using primers specific for TLR4, TRAF6, TRAF3 and IL-1β (Bio-Rad) on a ViiA 7 Real-Time PCR System (Life Technologies). The expression of all genes was normalized to the reference gene and the results were calculated using the ΔΔ Ct method.

Statistical analysis

Statistical significance was calculated using the nonparametric Mann-Whitney U test. GraphPad Prism, version 8.0 (GraphPad Software, San Diego, CA), was used for statistical analysis, and values of $p \leq 0.05$ were considered statistically significant.

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