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"Identification of adherent-invasive *Escherichia coli* (AIEC) virulence determinants leading to activation of pathogenic Th17 cells in Crohn's disease."

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1. RIASSUNTO

La malattia di Crohn (CD) è una malattia infiammatoria cronica intestinale caratterizzata da frequenti recidive a causa di una continua e inappropriata attivazione del sistema immunitario gastrointestinale in individui geneticamente predisposti. Ad oggi, è comunemente accettata l'ipotesi che il CD si sviluppa a causa dell'iperattivazione delle cellule T intestinali in risposta ad un'alterata composizione del microbiota.

In particolare, la patogenesi di questa malattia è stata correlata ad un significativo aumento di linfociti Th17 secernenti IFN-γ, e ad un concomitante arricchimento del patotipo *E. coli* aderente-invasivo (AIEC) nella mucosa dei pazienti Crohn. Inoltre, in un recente lavoro non ancora pubblicato, abbiamo descritto un nuovo sottotipo di Th17 definiti patogenici (pTh17) attivati selettivamente da AIEC. Tuttavia, è ignoto il meccanismo molecolare con cui AIEC induce il differenziamento e attivazione delle cellule pTh17.

Per questo motivo, una libreria di 10,000 mutanti nel ceppo AIEC-LF82 è stata generata e testata per trovare mutanti con ridotta capacità di indurre la secrezione da parte delle cellule dendritiche umane (DC), delle citochine polarizzanti IL-23/IL-1β, coinvolte nel differenziamento delle cellule pTh17. I nostri dati mostrano che AIEC-LF82 è in grado di persistere e replicare all'interno delle DC derivate da paziente CD, inducendo un'eccessiva produzione di IL-23, ma non IL-1β, rispetto alle DC isolate da donatore sano (HD). Tra i 10,000 mutanti testati, 22 hanno significativamente ridotto la secrezione di IL-23 da parte delle DC derivate da HD, di cui 13 hanno mantenuto la loro capacità di indurre una minor secrezione di IL-23 anche da parte delle DC derivate da CD, confermando la predisposizione genetica dei pazienti CD nell'iperattivazione della risposta infiammatoria. Inoltre, questi 22 mutanti hanno mostrato diversa abilità di persistere all'interno delle DC e di indurre la secrezione di IL-1β, dimostrando che diversi fattori di AIEC sono coinvolti nella sua sopravvivenza intracellulare e nella stimolazione della secrezione di IL-23 e IL-1β. Inoltre, i nostri dati hanno dimostrato che la riduzione dei livelli di IL-23 non è sufficiente per prevenire l'attivazione dei linfociti pTh17, infatti solo 6 mutanti sui 13 selezionati hanno mostrato una ridotta capacità di attivare questo subset patogenico di cellule T. Infine, il sequenziamento dei mutanti di LF82 ha permesso di individuare i fattori di virulenza di AIEC che sono direttamente coinvolti nell'attivazione dell'asse IL-23/Th17 che possono essere utilizzati come bersaglio per lo sviluppo di nuove terapie per prevenire selettivamente l'attivazione dei linfociti T patogenici.

2. ABSTRACT

Crohn's disease (CD) is a chronic inflammatory bowel disease characterized by a relapsing-remitting clinical behaviour resulting from inappropriate and persistent activation of the gut mucosal immune system in genetically predisposed individuals. So far, the accepted dogma is that CD arises from the overly activation of tissue-resident T-cells in response to a dysbiotic composition of the gut microbiota. In particular, the pathogenesis of CD has been linked to a significant enrichment of pathogenic IFNγ-producing Th17 cells with a concomitant selective and significant enrichment of adherent-invasive *E. coli* (AIEC) pathotype. Notably, in a recent yet unpublished work, we described a new subset of pathogenic Th17 cells (pTh17), selectively enriched in the gut of CD patients that are specifically activated by AIEC strain. However, the molecular mechanism by which AIEC induces pTh17 trans-differentiation and activation is still totally unknown. To this aim, a library of 10,000 AIEC-LF82 mutants was generated and screened in order to identify AIEC-virulence determinants specifically involved in pTh17 generation, looking for mutants with an impaired ability to trigger the secretion of the polarizing cytokines IL-23/IL-1β, by human dendritic cells (DCs), linked to pTh17 differentiation.

Our data demonstrated that AIEC-LF82 is able to persist and replicate within CD-derived DCs, promoting a significantly higher release of IL-23, but not IL-1β, compared to DCs isolated from healthy donors (HD). Among 10,000 mutants tested, 22 strains significantly reduced IL-23 secretion in HD-derived DCs, while only 13 mutants out of these 22 strains maintained their ability to reduce IL-23 secretion also in CD-derived DCs, thus confirming the genetic diversity and predisposition of CD-derived immune cells to a higher inflammatory response. Moreover, these 22 mutants differently persisted within DCs and displayed very different levels of IL-1β secretion, thus indicating that distinct AIEC-antigens are involved in promoting its intracellular survival and in triggering IL-23/IL-1β secretion. Interestingly, only 6 mutants out of 13 with a compromised capacity to stimulate IL-23 secretion in CD-derived DCs significantly reduce the generation of pTh17 cells, demonstrating that a strong decrease in IL-23 secretion is not enough to totally prevent the differentiation of pTh17 cells. Finally, sequencing of LF82-mutants showed that only a restricted number of AIEC-pathways are involved in the activation of IL-23/pTh17 axis. The identification of these AIEC-determinants, directly linked to IL-23 hypersecretion and to the activation of pTh17 could pave the way for the development of new and more efficient therapeutic strategies able to prevent the activation of pathogenic T cells leaving protective ones unaffected in CD.

3. INTRODUCTION

3.1. Crohn's Disease: Pathophysiology and Epidemiology

Crohn's disease (CD) is a chronic immune-mediated disorder of the gastrointestinal tract with multifactorial aetiology (Torres et al., 2017). CD, together with Ulcerative Colitis (UC), is one of the main forms of Inflammatory Bowel Disease (IBD), but while the former can affect all the segments of the gastrointestinal tract, in particular the terminal ileum and colon, the latter starts in the rectum and extends towards colon. In contrast to UC, that is often restricted to mucosal layer, CD is characterized by transmural inflammation resulting in fistula, fibrosis and stenosis within 10 years of diagnosis (Hibi & Ogata, 2006; Kobayashi et al., 2020).

The prevalence of CD is higher in the industrialized world, in particular in Western Europe and North America and in Ashenaku Jewish, its incidence ranges from 3 to 20 cases per 100,000 person-years (Feuerstein et al., 2017). CD condition typically manifests around age 30 and has two peaks, the first occurring between ages 20 and 30 and the second occurring around age 50 and there is no association with gender (Torres et al., 2017).

The pathophysiology of CD is under control of several contributing factors, including genetic susceptibility (Van Limbergen et al., 2013), environmental factors (Ng et al., 2013), and gut dysbiosis (Ahmed et al, 2016) that lead to an abnormal immune response and compromised epithelial barrier function (Wang et al., 2016).

3.2. Genetic risk factors

Our knowledge of the genetic causes of IBD has significantly improved during the last few decades. This is a result of advancements in genetic testing and DNA sequencing technology, which have made it possible to conduct numerous genome-wide association studies (GWAS) and identify novel single nucleotide polymorphisms (SNPs) (McGovern et al., 2015).

In 2001 was identified the first susceptibility gene for CD, the Nucleotide-binding oligomerization domain containing 2 (NOD2) (Hugot et al., 2001).

NOD2, also known kas CARD15, is an intracellular pattern recognition receptor (PRR) member of the Nod-like receptor (NLR) family that recognizes the muramyl dipeptide (MDP), a component of the peptidoglycan cell wall of bacteria. In the intestine, NOD2 is highly expressed in intestinal epithelial cells and in myeloid cells, such as macrophages and dendritic cells, but expression is low in T cells (Al Nabhani et al., 2017). Short-chain fatty acids (such as butyrate), hormonal vitamin D, bacterial component (such as LPS) and pro-inflammatory cytokines (such as TNF-α) stimulate the expression of NOD2 (Sidiq et al., 2016).

Three main variants or polymorphisms in NOD2 gene, located within or close to the LRR domain that recognize MDP, were highly associated with susceptibility to CD (Sidiq et al., 2016). NOD2 mutations induce increased susceptibility to colonization by pathogenic bacteria, indeed patients with mutant variants of NOD2 have altered microbiota in the ileum (Lala et al., 2003). The balance between tolerogenic and pro-inflammatory response is important to maintain homeostasis in the gut, in which the presence of the microbiota induces a physiological inflammation. NOD2 mutations in CD patients lead to an altered immune response to the microbiota, hampering the integrity of intestinal epithelium (Jiang et al., 2013). Moreover, it has been demonstrated that the activation of autophagy is related to the bacterial detection by NOD proteins. NOD2 activates autophagy by the interaction with ATG16L1 (autophagy-related 16-like 1), another gene in which were found several polymorphisms associated with CD. It has been shown that the ATG16L1 deficiency abolishes the ability of cells to form autophagosomes, which leads to the disruption of antigen uptake and an insufficient enteric bacterial clearance along with a hyper-inflammatory state of increased secretion of IL-1β and IL-6 (Conway et al., 2013). The most common variants associated to ATG16L1 is the TA300A, which has to have a crucial role in the response to bacteria and the consequent impaired innate immune response and leads to the generation of antigen-specific $CD4^+$ T-cell responses (Cooney et al., 2010). The importance of autophagy in CD development is also supported by the presence of other SNPs associated with other genes involved in autophagy, such as IRGM (immunity-related GTPase family M), in fact changes in its expression levels modulate cellular autophagy of internalized bacteria (McCarroll et al., 2008).

In addition to these frequent mutations found in genes involved in the innate immunity, there are several polymorphisms in genes involved in the adaptive immunity. Indeed, several SNPs in IL-23R gene were studied, in particular the variant rs1004819 was found associated with CD susceptibility (Borecki et al., 2019) and the variant rs11209026 was associated with a greater number of surgeries (Hoffmann et al., 2021). The IL23R risk locus is strictly correlated to T-helper 17 (Th17) signaling, indeed the IL23R gene encodes the receptor complex (IL23R) for the pro-inflammatory cytokine IL-23, formed of p19 and p40 subunits. The interaction between IL23 and IL23R induces the activation of Janus Kinase 2 (JAK2), with the subsequent phosphorylation of signal transducer and activator of transcription 3 (STAT3) and STAT4, that promotes the transcription of other pro-inflammatory cytokines (Pastor-Fernàndez et al., 2020). This increased transcription of pro-inflammatory cytokines results in the differentiation of $CD4^+$ T cells into pro-inflammatory Th17 cells, which are essential for antimicrobial defences (Atarashi et al., 2010). The identification of SNPs also in CCR6 and STAT3 genes, point out the importance of IL-23/Th17 axis in CD pathogenesis (Waterman et al., 2011).

However, the genetic component of illness heritability only accounts for 13.1% of the total (Uniken Venema et al., 2017), underlining the importance of the other contributing factors for the development of CD.

3.3. Alteration of Gut Microbiota

The Human Microbiome Project (HMP) in 2012 has opened to scientific community new knowledge about the disease and new target to fight them. To maintain a healthy condition, the mutual symbiotic relationship between the gut microbiome and the human host is fundamental, indeed the host provides a nutrient-rich habitat and residency for the microbiota, while the gut microbiota supports the host through various physiological functions (Flint et al., 2012). Any alteration of these equilibrium condition, caused by changes in gut microbiota structural composition is defined as “dysbiosis” and is associated with a variety of gut disorders, including CD (Figure 1) (Buttò et al., 2016).

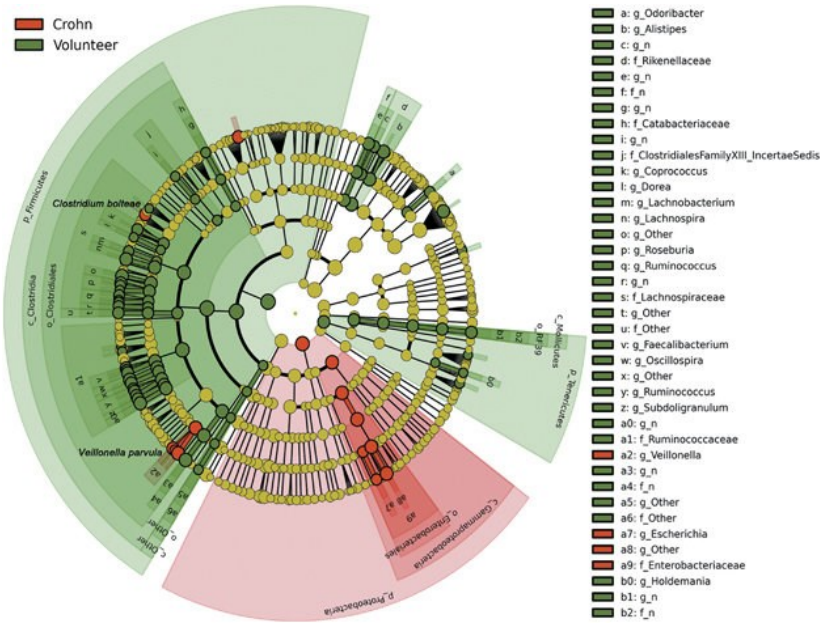


Figure 1: Gut bacterial taxa diversity between CD and control groups. Cladogram representing the microbial composition of CD patients and controls comparing the sequencing data of 16S rDNA. Red and green indicate respectively the taxa enriched in CD or control group. Each circle's diameter reflects the relative abundance of the corresponding taxon (Perez-Brocal et al., 2013).

The genetic background of the host, together with nutrition and stress, has a significant impact on the microbiota's composition (Sekirov et al., 2010). The microbiota composition of CD and UC patients are often studied together, finding that the dysbiosis pattern more closely connected with IBD patients is an increase of pathogenic bacteria, in particular species belonging to Enterobacteriaceae, and a decrease in commensal bacteria diversity, principally Firmicutes and Bacteroides (Li et al., 2014). However, looking into the differences among microbiota composition

of CD and UC, it has been observed that a gain of more pathogenic ones to the detriment of beneficial microorganisms is more associated with CD (Figure 2) (Pascal et al., 2017).

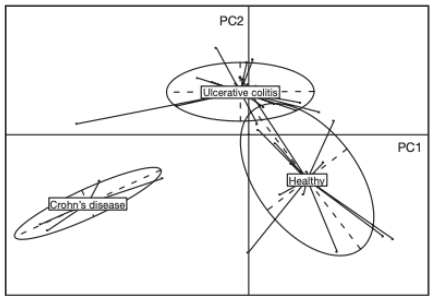


Figure 2: Comparison of bacterial species abundance between healthy individuals, CD and UC patients.
The figure represents the analysis of the principal bacterial components of fecal sample of healthy individuals, CD and UC patients. A clear separation between CD and UC- and healthy-clusters is observed, more significant between CD and UC, compared to UC and healthy individuals (Qin et al., 2010).

Indeed, dysbiosis in CD patients is characterized by decrease of commensal bacteria involved in butyrate and other SCFA production, in particular several studies report the lower relative abundance of *Faecalibacterium prausnitzii* belonging to the Clostridium cluster IV (Khan et al., 2012), while it is present in UC patients, making it a potential marker to discriminate patients with UC from patients with CD (Figure 3) (Pascal et al., 2017).

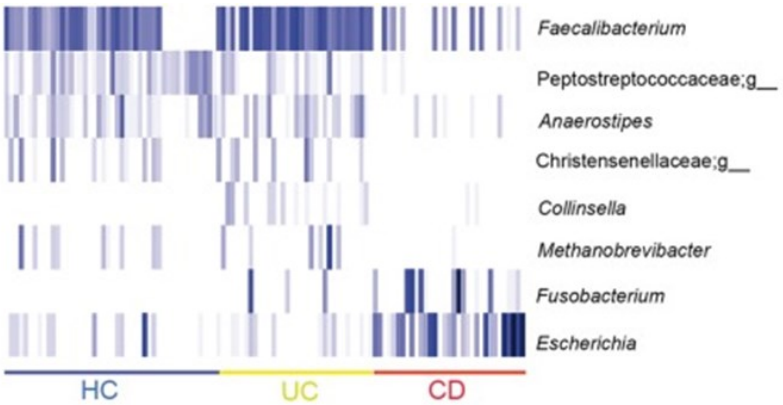


Figure 3: Gut bacterial genera diversity between Healthy Control (HC), Ulcerative Colitis (UC) and Crohn's Disease (CD) groups in faecal samples. Each bar represents the presence of each microbial group for each subject. The gradient of colours for the bars corresponds to white=absent, clear blue=low abundance and dark=high abundance (Pascal et al., 2017).

Potential pathogenic microorganisms, termed pathobionts, increased in CD belong to the class of Actinobacteria and Gammaproteobacteria (Caparròs et al., 2021), with a specific enrichment of *Enterobacteriaceae*, in particular a mucosa-associated adherent-invasive *Escherichia coli* (AIEC) has been identified associated to CD (Bumagart et al., 2007; Darfeuille-Michaud et al., 2004). The higher rates of pathobionts in the gut of CD patients, lead to a persistent and continuous activation of the immune response, contributing to the chronic inflammation typical of CD condition. Indeed, reducing exposure to gut pathogenic bacteria, manipulating microbiota composition, has been

shown to widely alleviate inflammation (Rashed et al., 2022). Therefore, fighting chronic intestinal inflammation targeting exclusively pathobiont enriched in CD patients, represents an efficient therapy strategy.

3.4. Adherent-invasive Escherichia coli (AIEC)

AIEC strains are classified as pathobionts because, as a result of the adaptive evolution of their genome in a particular and receptive host, they promote inflammatory disorders (Nash et al., 2010; Miquel et al., 2010). However, it has not been identified any virulence factor of AIEC which are usually present in pathogenic species (Darfeuille et al., 1998) and, furthermore, genetic determinants that distinguish commensals from AIEC have remained unknown. An *E. coli* strain is defined AIEC when, tested on *in vitro* assays, it displays ability to adhere and invade intestinal epithelial cells (IEC) (Bodeau et al., 1999), as well as, to survive and replicate within macrophages without inducing host cell death (Glasser et al., 2001). AIEC is often linked with the ileum rather than the colon and compared to healthy controls, in which AIEC prevalence ranges from 0% to 19%, CD patients have a mucosal prevalence of AIEC bacteria that ranges from 21% to 62% (Darfeuille et al., 2004; Lòpez-Siles et al., 2022).

In CD patients, the protective mucus layer that covers the intestinal epithelium is more penetrable by bacteria, due to specific receptor that are abnormally expressed in the epithelium. AIEC strains secrete a protease, vat-AIEC, which reveals a mucinolytic activity that contributes to the increasing spread of bacteria through the mucus layer and adhesion to IEC (Dharmani et al., 2009; Gibold et al., 2016). AIEC shows a hypermotile phenotype, due to an insertion sequence upstream *flhDC*, the master flagellar regulator (Elhenawy et al., 2019), that together with the principal component of flagellum, encoded by *fliC* gene, provide AIEC the ability to adhere and invade IEC (Barnich et al., 2003). The role of flagella in driving adhesion to IEC is not exclusive, but it is linked also to their ability to induce pro-inflammatory response, in fact flagellin is recognized and triggers the activation of toll-like receptor 5 (TLR5) and of the cytoplasmatic Nod-like receptor family member NLRC4. It has been observed that the AIEC infection leads to an upregulation of these receptors, provoking an increased secretion of pro-inflammatory cytokines, as IL-8, IL-6 and IL-1β secretion (Subramanian et al., 2008; Carvalho et al., 2008). On the contrary, a recent study demonstrates that AIEC is able to regulate flagellum production, in order to avoid the hyperactivation of the immune response, promoting its persistence in the gut (Sevrin et al., 2022).

Among the different strategies of AIEC bacteria to interact with IEC, the main one is mediated by FimH that interacts with the Carcinoembryonic antigen-related cell adhesion molecule 6

(CEACAM6) expressed on ileal enterocytes (Figure 4) (Barnich et al., 2007). FimH is a two-domain protein at the tip of type I pili that recognizes terminal mannoses on epithelial glycoproteins (Boudeau et al., 2007). Mutations in the *fimH* gene of AIEC have been identified and associated to its higher adhesion ability (Dreux et al., 2013). Notably, CEACAM6 is not expressed by ileal epithelial cells in physiological conditions, while it is overexpressed in the ileal mucosa of CD patients (Barnich et al., 2010), due to AIEC ability to activate the hypoxia-inducible factor- 1 (HIF-1) response, that promotes its own colonization (Mimouna et al., 2011). In addition, FimH was able to bind TLR4, described to be the ligand of lipopolysaccharide (LPS), inducing the secretion of pro-inflammatory cytokines (Mossman K et al., 2008). This pattern recognition receptor (PRR), expressed not only by myeloid and lymphoid cells, but also by IEC, that in healthy condition down-regulate its expression to avoid the exaggerated inflammatory response (Abreu et al., 2001). Moreover, FimH is involved also in the interaction with M-cells through its interaction with glycoprotein 2 (GP2), expressed on the apical membrane of these cells (Hase et al., 2009).

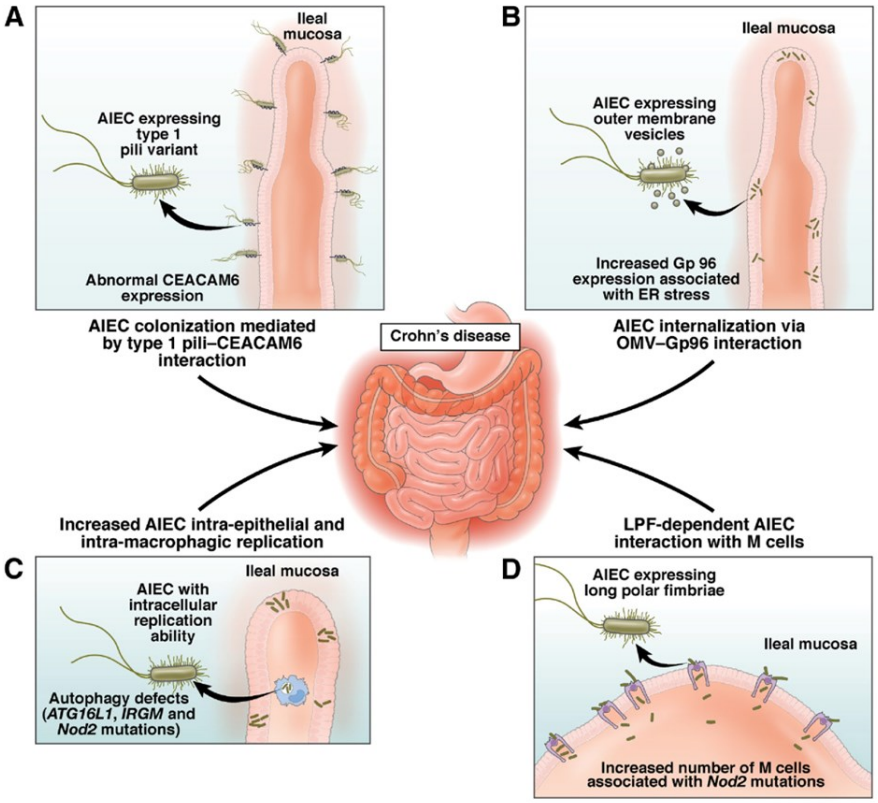


Figure 4: Mechanisms of AIEC virulence linked to the pathophysiology of CD.
A) AIEC colonizes the gastrointestinal tract by the interaction with CEACAM6, overexpressed on the IECs of CD patiets and through type 1 pili variants. B) Gp96, overexpressed on the apical surface of IECs, promotes AIEC invasion interacting with OMV released by AIEC. C) AIEC is able to invade and replicate within host cells with autophagy defects, caused by variants of ATG16L1, IRGM and NOD2 genes. D) In CD patients, AIEC express long polar fimbriae (LPF), that allow the interaction with Peyer's patches and the translocation across membranous/microfold cells (Chassaing & Darfeuille–Michaud, 2011).

In addition to FimH, other factors are involved in the interaction between AIEC and IECs, such as the ChiA that recognizes Chitinase 3-like-1 (CHI3L1), that is described to be upregulated during intestinal inflammation on macrophages and IECs (Mizoguchi E, 2011; Chen et al., 2006; Low 2013).

Moreover, the AIEC ability to form biofilm was associated to its proficiency to adhere and invade IECs, and in particular it was described that σ^E pathway modulates the expression of genes involved in biofilm formation. The involvement of this pathway was described also in other pathogens, like *Salmonella* spp. and is linked to their ability to invade host cells (Chassaing et al., 2013). Recently, it was described in the AIEC strain LF82, that its ability to form biofilm might provide protection to this pathovar against phagolysosomal activity (Prudent et al., 2021).

In addition to the several factors involved in the adhesion to IECs, numerous determinants are implicated in the invasion with these cells. For instance, YfgL lipoprotein encoded by *yfgL* gene has a role in the biogenesis of outer membrane vesicles (OMV) that carry bacterial effectors to the host cells (Rolhion et al., 2005). Once again, genetic susceptibility of CD patients promotes AIEC colonization, indeed OmpA, the major protein on the surface of OMV, binds Gp96, the ER-localized stress response protein, that is overexpressed on the apical surface of IECs of the ileum of CD patients, allowing the AIEC invasion (Figure 4) (Rolhion et al., 2010).

Another gene involved in AIEC invasion within IECs is *ibeA,* that has also a role in the interaction with macrophages, indeed it was described that *ibeA* deletion mutant was unable to invade IECs and survive within macrophages (Cieza et al., 2015).

Other genes that confer to AIEC the ability to survive and replicate within macrophages, without inducing cell death are the oxidoreductase *dsbA* gene and the stress gene *htrA*. It has been described that non-pathogenic *E. coli* strains do not activate the transcription of these genes during phagocytosis, indicating that AIEC bacteria have acquired the ability to regulate the expression of specific genes to survive and replicate within macrophages (Bringer et al., 2005 and 2007). Moreover, AIEC takes advantage of autophagy deficiency of CD patients associated with the polymorphism in NOD2, ATG16L1 and IRGM to persist and replicate within macrophages provoking an increased secretion of pro-inflammatory cytokines (Vazeille et al., 2015) (Figure 4). In particular it has been described, that AIEC persistence within macrophages stimulates TNF-α secretion, which in turn increase the intracellular replication of AIEC, in fact incubation of macrophages with TNF-α antibody impaired intracellular replication (Bringer et al., 2012).

However, while the mechanisms that confer AIEC the ability to survive and persist within macrophages are described, the interplay between AIEC and dendritic cells, a key factor in the activation of the adaptive immune system, is still unknown.

3.5. Innate Immunity in Crohn's disease

The gastrointestinal tract has a luminal surface of 300-400 m^2, representing the largest interface between the host and the environment. Indeed, gastrointestinal mucosa is constantly exposed to food-derived antigens and has to handle pathogenic microorganisms (Haag et al., 2015). The intestinal epithelium with neutrophils, monocytes, macrophages, dendritic cells (DCs) and innate lymphoid cells (ILCs) is the first line of protection against invading bacteria and has the important role to maintain tolerance toward luminal bacterial antigens limiting their exposure to adaptive immune system. In CD pathogenesis, the immune tolerance is compromised due to the altered gut microbiota composition and the genetic predisposition (Geremia et al., 2016).

The mucus layer that covers intestinal epithelium represents the first physical barrier to luminal antigens and it is organized in an internal and an external layer composed by the polymerization of gel-forming mucins, which are secreted by goblet cells. The inner layer is usually sterile, whereas the outer one is permeable and populated by commensal bacteria that find a supportive environment due to the nutrients present in the mucin glycans. MUC2 is one of the major components of the mucus and has an important role in preventing intestinal inflammation, indeed MUC2 knockout mice develop colitis due to the presence of bacteria dealing directly with the intestinal epithelium (Buisine et al., 1999). The second line of defence to bacterial invasion consists of enterocytes and specialized epithelial cells, namely goblet cells and Paneth cells. The integrity of epithelial barrier is preserved by the apical junction complex, composed by tight junctions, desmosomes and adherent junctions. An increased intestinal permeability and defective epithelial barrier was observed in CD patients, although is still unknown whether these variations are the origin or the results of chronic inflammation (Salim et al., 2011).

In addition to the physical barrier, epithelial cells provide protection against bacterial invasion through the secretion of antimicrobial peptides (AMPs), such as α-defensin and β-defensin, the former produced by Paneth cells and the latter produced mainly by epithelial cells. AMPs can be produced constitutively or induced by PRRs expressed on epithelial cells. In CD patients it has been observed a defective production of AMPs, in particular a lower expression of Paneth cell-derived α-defensins and β-defensins HBD2, HBD3 and HBD4 (Wehkamp et al., 2003; Whekamp et al., 2004). Interestingly it has been observed that in CD patients with SNPs in NOD2 the expression of Paneth cell-derived α-defensins is very low (Wehkamp et al., 2004).

The gut epithelium acts as a bridge between the luminal microbiota and immune cells.

The just mentioned PRRs, that recognizes pathogen-associated molecular patterns (PAMPs), are expressed also by macrophages and DCs and the sensing of microbial antigens is fundamental to

maintain intestinal homeostasis. PRRs include the families of TLRs and NLRs, in which were found, as previously described, polymorphisms associated with CD. PRR-induced signaling cascades result in the expression of a range of pro-inflammatory cytokines, which are important not only for the early response to infection, but also for the consequent shaping and activation of the adaptive immune system (Geremia et al., 2014). The signals derived from PRR in antigen presenting cells (APCs) are important for determining whether the antigen presentation leads to activation or cross-tolerance of T cells. Dysregulation of these mechanisms lead to the generation of uncontrolled pro-inflammatory response, that leads to the chronic inflammation typical of CD condition. Indeed, it was described that NOD2 and ATG16L1 variants in CD lead to defective autophagic activity and impaired MHC class II antigen presentation after MDP recognition (Cooney et al., 2010). In particular, it has been described that NOD2 signaling influences CD4 adaptive immune response, and studies have shown that the MDP programs DCs to increase IL-1 and IL-23 secretion, cytokines involved in the differentiation of a pathogenic subsets of Th17 cells (Corridoni et al., 2018). Moreover, other studies demonstrate that NOD2 mutations can lead to the deficiency in TLR2 inhibition with the consequent exaggerated activation of Th1 responses (Joffre et al., 2012). Another NOD2 variant that causes unregulated inflammatory response is the NOD2 3020insC variant, that inhibits the secretion of the anti-inflammatory cytokine IL-10 (Noguchi et al., 2009).

Regarding TLRs, their role in the recognition of commensal microbiota is pivotal for preservation of intestinal epithelial homeostasis and restoration after mucosal damage (Rakoff-Nahoum et al., 2004). Growing evidence underline that also TLRs mediated immune dysfunction are key actors in the pathogenesis of IBD. In particular it was observed that the inhibition of TLR2/TLR6 heterodimer, that recognizes diacylated lipopeptides, induces a reduction of pro-inflammatory cytokines, such as interleukin (IL)-6, IL-23, IL-12, and IL-1β and ameliorate dextran sodium sulfate (DSS)-induced colitis (Shmuel-Galia et al., 2016).

Other studies suggested the important role of TLR1 in providing protection against Gram-negative bacteria and that the absence of this receptor led to chronic immune activation (Kamdar K et al., 2016).

Moreover, polymorphisms were found associated also to TLR4, the first discovered TLR in mammals, that in addition to LPS, as I previously described, binds the type 1 pili variant FimH of AIEC. Although the role of TLR4 in IBD is controversial, indeed some works report that the activation of TLR4 signaling exacerbates DSS-inducing colitis (Lu et al., 2018), while others show the essential role of TLR4 signaling in controlling inflammation in pediatric enterocolitis (Meng et al., 2016).

Even for TLR5, that recognizes bacterial flagellin, it was described a relationship with IBD, indeed TLR5-deficient mice have an altered composition of intestinal microbiota and are susceptible to colitis (Kinnebrew et al., 2012). It has been observed that lamina propria DCs, as the major TLR5 expressing cells in the small intestine, secrete large quantity of IL-23 after bacterial flagellin recognition (Chassaing et al., 2014). Moreover, SNPs in TLR5 was associated with CD in children (Sheridan et al., 2013).

However, it remains unclear whether the dysbiosis is the cause or the consequence of the intestinal inflammation of CD patients, but it is clear that the altered crosstalk between innate immune systems and microbiota, leads to a strong and persistent activation of the adaptive immune system resulting in the chronic inflammation typical of CD.

3.6. Adaptive Immunity in Crohn's disease

Contrary to innate immune system, the response of adaptive immune system is very specific, providing long-term immunity. T cells represent the main actors of adaptive immunity, and, collaborating with each other and with the cells of innate immune system, provide an adequate immune response able to eradicate pathogens. Integrins, such as α4β1, αLβ2, αEβ7 and α4β7, expressed by T cells, allows their migration to the site of inflammation after their activation, that usually occurs in the gut-associated lymphoid tissue (Bertoni et al., 2018).

Clonal proliferation of naïve CD4+ T lymphocytes is influenced by microenvironment and stimulated by the identification of antigen-major histocompatibility complex molecules and specific costimulation, that together with the cytokine milieu drive their differentiation in specific effector cell type, as Th1, Th2 or Th17 (Geremia et al., 2014; Saravia et al., 2019).

Th1, that express the surface marker CXCR3, are specialized in the eradication of intracellular microorganism, they differentiate in the presence of IL-12 signals activating STAT4 that promotes the expression of T-bet, that in turn transcribes *ifng* gene, responsible of the secretion of IFN-γ, that is the signature cytokine of Th1 cells. Moreover T-bet inhibits the expression of GATA-3, the master transcription regulator of Th2 cells. Th2 mediate allergic reactions and counteract parasite infections, they differentiate in the presence of IL-2 and IL-4 and their effector cytokines are IL-4, IL-5 and IL-13 (Gagliani & Huber, 2017).

It is well established the role of adaptive immune system in IBD pathogenesis, and while initial studies suggested a main role for Th1 cells in CD and Th2 cells in UC, the discovery of Th17 cells has revolutionized this Th1/Th2 paradigm, indeed it has been observed that also Th17 cells have a role in the pathogenesis of CD (Heller et al., 2005; Parronchi et al., 1997 Langrish et al., 2005). Th17 cells are mainly present in the gastrointestinal tract in the absence of pathology, their role is to

contribute to the clearance of extracellular bacteria and fungi (Conti et al., 2009). The master transcription factor of Th17 cells is the retinoic acid orphan receptor (ROR)γt that leads to the production of their signature cytokines IL-17A and IL-17B and the expression of the chemokine receptor CCR6 and IL23R. It has been reported that the transcription factor STAT3 regulates the expression of RORγt, crucial for Th17 cells differentiation and development, indeed a decreased RORγt expression leads to enhanced expression of other transcription factors, such as T-bet (Annunziato et al., 2007). Studies on antibiotic-treated or germ-free (GF) mice that the differentiation of Th17 cells is strongly dependent on the gut microbiota (Ivanov et al., 2008; Ivanov et al., 2009).

The role of Th17 cells and related cytokines in IBD pathogenesis is controversial. Indeed, some works report that intestinal Th17 cells provide protection against invading pathogens (Huang et al., 2004), while others show that increased level of IL-17 can drive and aggravate the chronic inflammatory response present in IBD (Korn et al., 2007; Saleh et al., 2019). Moreover, despite the higher levels of IL-17 present in the lamina propria of CD patients, treatment with Secukinumab, anti-IL-17 monoclonal antibody, showed no remission or exacerbation of symptoms (Hueber et al., 2012). A step forward derived from mouse models of autoimmunity, in which was described that Th17 cells are a heterogeneous population, ranging from "classical" Th17 cells, that differentiate in the presence of TGF-β and secrete in addition to the signature cytokine IL-17, the anti-inflammatory cytokine IL-10, to "alternative" Th17 cells, that differentiate in the presence of IL-23 and IL-1β and secrete high levels of IFN-γ and GM-CSF (Ghoreschi et al., 2010) (Figure 5).

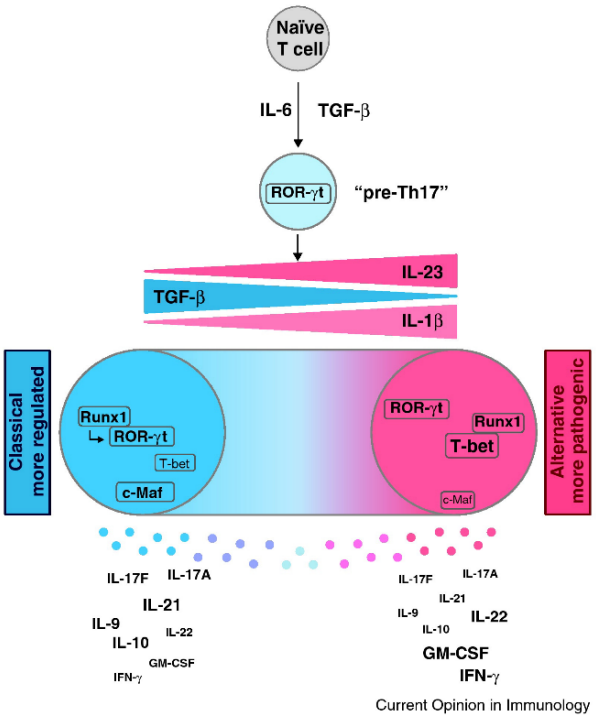


Figure 5: Mouse Th17 cells heterogeneity. Th17 cells comprise a spectrum ranging from the 'classical' Th17 cells, with regulative role, to the 'alternative' Th17 cells, with pathogenic role (Anneli et al., 2011).

Notably, T-cells that co-produce IFN-γ and IL-17 were found also enriched at the active inflammatory mucosal sites in IBD patients, suggesting their pathogenic role in the development of the disease (Globig et al., 2014). It has been proposed that Th17 cells originate from a subset of naïve T cells expressing the lectin receptor CD161 ($CD161^{+}CD4^{+}$ T cell progenitors) (Cosmi et al., 2008). After the initial Th development in the thymus, their functional differentiation takes place when T cells interacts with an activated APC, as DC. Monocytes and DCs that produce high levels of IL-1β, but not IL-12, after stimulation by LPS and peptidoglycan, promote Th17 cells differentiation, contrariwise this process is not promoted by monocytes-derived DCs that secrete IL-12, but not IL-1β after LPS or peptidoglycan stimulation (Galvez 2014; Acosta-Rodriguez et al., 2007). Notably, it has been suggested that Th cells polarization into Th17 cells is promoted within a proinflammatory context in the gut. In particular, while TGF-β and IL-6 are essential for Th17 cells differentiation of naïve T cells, IL-1β and IL-23 induce the development of IFN-γ/IL-17 co-producing T-cells (Wilson et al., 2007; Manel et al., 2008; Bettelli et al., 2006).

3.7. IL-23/Th17 axis in CD

It has become evident the crucial role of IL-23 in the pathogenesis of CD. Indeed, the role of IL-23 is fundamental for the early response to microbe and the crosstalk between innate and adaptive immune system. This cytokine is a member of IL-12 cytokine family and consists of a heterodimer composed by p40 subunit, in common with IL-12, and the p19 subunit. IL12Rβ1 is the

receptor of the shared p40 subunit, while IL23R is the receptor of p19 subunit. The interaction of IL-23 to its receptor leads to tyrosine kinase 2 (tyr2) and Januse kinase 2 (jak2) activation, that leads to the phosphorylation of STAT4 for the p40 subunit and STAT3 for p19 subunit (Parham et al., 2002; Razawy et al., 2018). The activation of STAT3 generates a positive feedback loop that drives expression of genes important for Th17 cells activation and function (Schmitt et al., 2021). Studies have demonstrated the high expression of IL23R in IFNγ/IL-17 co-expressing T cells from CD patients, suggesting again the pivotal role of IL-23/Th17 axis in driving CD pathogenesis (Schmitt et al., 2019). The importance of IL-23/Th17 axis is underlined also by the effect of IL23R polymorphism on Th17 cell function. As previously described several SNPs were found in IL23R gene, however some of them confer protection, while others contribute to the exacerbation of the inflammation. Indeed, it has been observed a reduction in circulating Th17 cells in CD patients carrying the coding variant R381Q, that is a consequence of decreased level of IL-22 and IL-17 after IL-23 stimulation. Moreover, it was observed a reduced IL-23 mediated phosphorylation of STAT3 of T cells from these CD patients in response to an invading pathogen, strong inducer of Th17 cell response (Sarin et al., 2011; Schmitt et al., 2019). On the contrary, the G149R variant of IL-23R gene was associated to enhanced risk for CD, linked to strong activation of IL-23/Th17 axis (Kim et al., 2011).

The unveiled role of IL-23/Th17 axis in the pathogenesis of CD sets the ground for the development of new therapies designed to target immune actors of this pathway.

Also in a work that is currently under revision performed by our research group has emerged the pivotal role of IL-23/Th17 axis in CD pathogenesis (Paroni et al., under revision). In particular in this work, it was discovered a new subset of Th17 cells, which differentiation is mediated by IL-23. This subset, different from conventional Th17 (cTh17 from now on) and also from Th1/17 cells, due to the expression of specific surface markers, is highly enriched in the lamina propria of CD patients, but not in UC and non-IBD control patients, suggesting its pathogenic role in CD. These lymphocytes are defined pathogenic Th17 cells (pTh17 from now on) for their pathogenic phenotype, indeed they co-produce high amount of IFN-γ/IL-17, but not the anti-inflammatory cytokine IL-10, thus mirroring the phenotype of murine alternative Th17 cells (Anneli et al., 2011). Notably, in this work it is shown that pTh17 cells display a strong and specific reactivity against AIEC, that as previously outlined, is an intestinal enterobacteria highly enriched in CD patients. Interestingly, these cells do not show reactivity neither to other intestinal pathogens, such as *Shigella flexneri*, *Salmonella tyhphimurium*, nor to commensal *E. coli* strains, such as *E. coli* Nissle 1917, suggesting that specific AIEC virulence determinants are involved in the activation of pTh17 cells (Paroni et al., under revision). However, the AIEC molecular mechanisms that trigger pTh17

cell activation is still unknow and their identification could pave the way for the development of new therapeutic strategy for fighting the chronic inflammation typical of CD condition.

3.8. Therapeutic strategies for Crohn's disease management

Crohn's disease requires an induction and maintenance program for therapy. The treatment approach is based on disease severity and effectiveness of previous treatment. Corticosteroids, immunosuppressant, as thiopurines and methotrexate and biologics are the most commonly used therapies (Torres et al., 2017).

Corticosteroids are used to treat moderate active disease, however steroid have high dependence potential and show side toxics effects and the lack in the remission maintenance (Yang & Lichtenstein, 2002).

Thiopurines (azathioprine and mercaptopurine) and methotrexate in CD are associated with reduced risk for surgery and are safer and better tolerated than long-term corticosteroid therapy and are effective in treating patients with IBD (Liu et al., 2017). However, an increased risk of malignancies, such as non-melanoma skin cancer and myeloid disorders, is linked with these drugs (Peyrin-Biroulet et al., 2011).

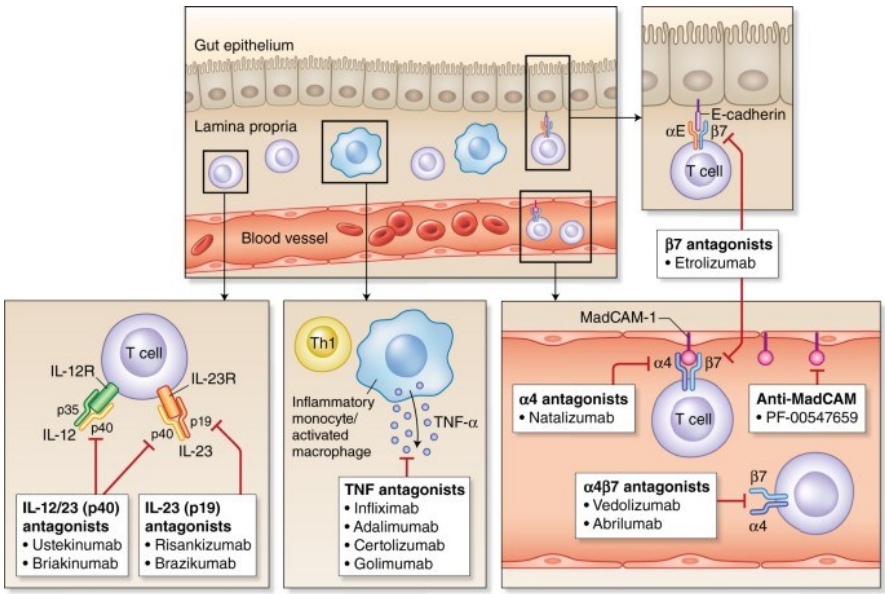


Figure 6: Overview of biological therapies in IBD.
Targets of the current existing biologic agents for IBD treatment (Paramsothy et al., 2018).

In the last 20 years, biologic agents have revolutionized the therapy of IBD, since the monoclonal antibodies have more specific targets compared to corticosteroids and immunosuppressant (Figure 6). The first class of biological agents approved for CD treatment is the anti-TNF-α agents. Four monoclonal antibodies directed to block the cytokine TNF-α are currently approved for IBD treatment, namely Infliximab, Adalimumab, Certolizumab and Golimumab. However, while

Infliximab and Adalimumab are approved for the treatment of both CD and UC, Certolizumab was approved exclusively for CD treatment and Golimumab for UC only (Paramsothy et al., 2018). These anti-TNF-α agents show high efficacy in both gastrointestinal and extra-intestinal manifestation (Fernandes et al., 2015; Vavricka et al., 2014). Even though, IBD patients treated with this class of biologics show enhanced risk of serious infection, but lower than patients treated with steroids. Also, with this treatment was observed an increased risk of malignancy, but still less than treatment with immunosuppressant (Lichtenstein et al., 2012; Nyboe Andersen et al., 2014). However, anti-TNF agents are ineffective in one-third of IBD patients, and relevant number of responders develop resistance during the treatment (Gisbert et al., 2015).

Anti-integrin agents are the second class of biologic approved for IBD therapy, that interfere with leukocyte migration to the site of infection blocking the cell adhesion and migration mediate by integrins. The expression of integrins is upregulated by the presence of pro-inflammatory cytokines, and their blocking, prevent the migration to disease site, including gastrointestinal tract, hampering the pro-inflammatory loop (Ley et al., 2016).

Vedolizumab is a humanized IgG1 monoclonal antibody that block α4β7 that is mainly expressed on cells of gastrointestinal tract, resulting more specific than its precursor Natalizumab that non-specifically targets α4 integrins. Despite Vedolizumab take longer to take effect that anti-TNF-class agents, it has a greater safety profile and responders typically have long-lasting remission (Colombel et al., 2017). A study of 2020 carried out by Coletta, Paroni et al., show a reduction increased level of $CCR6^+$ cells, and thus Th17 and Th1/17 cells, were associated to non-responders, suggesting that these lymphocyte subsets are resistant to this therapy (Coletta et al., 2020).

Given the importance of IL-23/Th17 axis in CD pathogenesis, the effect of several biologics against members of this pathway was evaluated.

Ustekinumab, approved in 2016 for CD treatment, is a monoclonal human IgG antibody that bind the shared p40 subunit among IL-12 and IL-23 cytokines, that as previously described, are involved in the triggering respectively of Th1 and Th17 response (Teng et al., 2015). Ustekinumab shows better efficacy in anti-TNF-α responder patients compared to anti-TNF-α non responder. However, it is now widely accepted that the therapeutic efficacy of this biological drug was linked to its effect on IL-23 blockade (Feagan et al., 2016). Indeed, despite overexpression of IL-12 is observed in CD patients, several studies show the detrimental effects of the genetic excision of IL-12p35 subunit (Paramsothy et al., 2018).

The promising results obtained with ustekinumab, in particular due to the IL-23 inhibition, emphasizes the importance to selectively block activation of Th17 cells, leaving Th1 cells, and their function in microbial defence, unaffected. However, anti-IL17 antibodies, such as secukinumab,

showed no efficacy or even worsen side effects, contributing to exacerbation of the inflammation. This is partially explained by the dual role, protective and pathogenic, of Th17 cells in CD pathogenesis, and increased predisposition to fungal infection due to the absence of IL-17 (Hueber et al., 2012; Paroni et al., under revision).

Beside to the negative effects reported in CD patients treated with Secukinumab, treatment with monoclonal antibody against the IL-23p19 subunit, Risankizumab, has been demonstrated to induce clinical remission in CD patients (Argollo et al., 2019). Risankizumab was recently approved (June 2022) for the treatment of CD, for the successful results obtained in clinical trial about its long-term efficacy and safety. Indeed, it was observed clinical remission and endoscopic response in a high percentage of patients in both the induction and maintenance phase. Risankizumab treatment also achieved the secondary endpoints of clinical response, mucosal healing and corticosteroid-free remission (D'Haens et al., 2022; Choi et al., 2022). Moreover, in our work it has shown that risankizumab treatment resulted in a selective reduction of intestinal pTh17 cells, demonstrating again the link of pTh17 cells with the pathogenesis of CD (Paroni et al., under revision).

Other strategies to counteract IBD inflammation are designed to block JAK proteins. The exaggerated inflammatory condition typical of IBD is characterized by an overproduction of pro-inflammatory cytokines that activate the JAK/STAT phosphorylation pathway. JAK inhibitors act on several inflammatory pathway, however conflicting results was observed in CD and UC patients treated with this class of biologics (Rogler., 2020). Although JAK inhibitors represent an interesting therapeutic strategy to target IL-23 downstream.

3.9. Targeting AIEC for Crohn's disease management

Nowadays it is a brain teaser whether dysbiosis, and in particular AIEC, is the triggering factor of chronic intestinal inflammation or its colonization is the result of genetic susceptibility and pre-existing inflammation. Nonetheless, therapeutic strategies aimed at hampering AIEC colonization could contribute to reduce CD chronic inflammation. Several strategies could be used to manipulate microbiota, such as nutritional interventions, antibiotic therapies, the use of probiotics and the fecal transplantation (Palmela et al., 2017). Currently, the use of traditional antibiotics has been ineffective in CD treatment, due to the lack of specificity against pathogen bacteria. Accordingly, antibiotic treatment contributes to alter microbiota composition (Zimmerman & Curtis., 2019).

On the contrary, probiotics represent a good therapy to prevent the interaction between AIEC and host cells. Indeed, studies on the effect of *Saccharomyces cerevisiae* show its proficiency in hampering AIEC adhesion to IECs due to the interaction between mannose residues of the yeast and type 1 pili of bacteria (Sivignon et al., 2015). Also, members of Lactobacillus genus showed

efficacy to inhibit AIEC survival and growth (Van den Abbeele et al., 2016). On the contrary, we demonstrated that Lactobacillus spp. and Bifidobacterium spp. hamper AIEC virulence and consequently the triggering of IL-23/Th17 axis in UC, but not in CD patients (Leccese et al., 2020).

Another strategy to prevent AIEC colonization of host IECs could be represented by antiadhesive molecules for hampering AIEC colonization of host IECs. For instance, FimH antagonist were designed to saturate the carbohydrate recognition domain (CRD) of FimH, preventing the binding of AIEC to IECs, a fundamental step for AIEC colonization. Low concentrations of these molecules have shown strong antiadhesive potential (Chalopin et al., 2016).

An efficient therapy, that on the contrary to antibiotics, is specific for targeting one bacterial species is the phage therapy. The specific action of bacteriophages limits their side effects on gut microbiota composition. Three virulent AIEC-LF82 bacteriophages were found, and a cocktail composed with these three bacteriophages has shown ability in DSS-induced colitis reduction in mice colonized by LF82 (Galtier et al., 2017).

Recently, the ability of bacteria to transfer antibiotic-resistant genes and pathogenicity islands to other bacteria, was used to remove antibiotic-resistant genes in *Enterocococcus faecalis* (Rodrigues et al., 2019). This result suggests that a conjugative probiotic strain could be used to remove specific AIEC genes. However, has not been identified any genetic determinants that distinguish commensals from AIEC strains and in the light of this, studies aimed to identify AIEC-virulence factors could pave the way for engineering specific conjugative bacteria.

4. AIM OF THE PROJECT

Crohn's disease (CD) is one of the world's most common non-infectious chronic intestinal inflammatory disorder, with a not completely characterized etiology yet. However, the widely accepted dogma is that CD arise from an exaggerated activation of the mucosal immune system in response to an altered composition of the gut microbiota in genetically predisposed subjects. In particular, the pivotal role of IL-23/Th17 axis in CD pathogenesis has recently become evident, shifting the focus of clinical research on the development of new therapeutical strategies aimed to modulate the adaptive immune response. Indeed, several biological therapies were employed to inhibit actors of this inflammatory pathways, like anti-IL17 or antiIL-23 monoclonal antibodies. However, whereas the IL-17 blocking, the signature cytokine of Th17 cells, leads to disease exacerbation in CD patients, the treatment with monoclonal antibody against the cytokine IL-23 has been demonstrated instead to induce clinical remission instead. In this context, we recently demonstrated a strong and selective enrichment of pathogenic intestinal IFNγ-producing Th17 cells (pTh17), that differentiate in the presence of high IL-23 levels from conventional Th17 (cTh17) cells, in the gut of CD patients. Interestingly, intestinal pTh17 cells are strongly and selectively activated by adherent-invasive *E. coli* strain (AIEC), a pathotype strongly enriched in the ileum of CD patients, but not by other commensal *E. coli* strains nor intestinal pathogens. Nevertheless, the role of AIEC in triggering secretion of IL-23, found highly enriched in inflamed gut-tissues of CD patients, through the interplay with dendritic cells (DCs), as well as the molecular mechanism by which AIEC promotes pTh17 generation, are yet completely unknown.

Therefore, the aim of my PhD project was the identification of virulence-determinants by which AIEC is able to trigger the secretion of polarizing cytokines by human DCs, that in turn leads to the differentiation of pTh17 cells and, finally, to find out the AIEC-antigens directly involved in the activation of pTh17 cells. Indeed, in contrast to the well-described molecular mechanisms by which AIEC interacts with human macrophages and intestinal epithelial cells, where specific AIEC virulence-determinants have been described, the interplay between AIEC and DCs as well as the AIEC-determinants that by antigen-presentation lead to pTh17 cells generation, have never been characterized and are the main goals of my PhD project.

5. MATERIALS AND METHODS

5.1. Bacterial Strains and Growth Conditions

The AIEC-LF82 strain used in this project was initially isolated from an ileal biopsy sample of a CD patient (Boundeau et al., 1999). The probiotic strain *E. coli* Nissle 1917 (EcN, Mutaflor; DMS 6601, serotype O6:K5:H1) was used as a non-AIEC control in our study. Bacteria were grown in YESCA medium (10 g/l casamino acids, 1.5 g/l yeast extract) under aerobic conditions at 37°C O/N. For LF82-mutants growth, YESCA medium was supplemented with 50 µg/mL of Kanamycin. Normalization of bacterial concentration (CFU/mL) for infection experiments was performed on O/N cultures. Briefly, O/N bacterial cultures were resuspended to an OD_{600}=1 through spectrophotometer quantification (Genesys 180, UV-Visible Spectrophotometer, Thermo Fisher Scientific, Waltham, Massachusetts, USA), corresponding to $1.47x10^9$ CFU/mL as previously determined, and then diluted to a final concentration depending on the experiments. Bacterial inocula were confirmed by plating serial dilutions onto LB agar or LB agar supplemented with 50 µg/mL of Kanamycin (Kan) for each experiment. Plates were incubated at 37°C O/N.

5.2. Electrocompetent cells

LF82 and TrasforMax EC100D pir-116 (Epicentre Biotechnologies) electrocompetent cells were obtained using the following protocol. Briefly, 1 mL of bacteria O/N culture, was normalized to OD_{600}=1 and inoculated in 100 mL of LB medium at 37°C in shacking condition, until 0.8 OD is reached. Bacterial cells were kept on ice for 30 min and then centrifugated at 5,000 rpm for 10 min at 4°C and washed two times in distilled (dd)H_2O. After centrifugation the pellet was resuspended in 1 mL of glycerol 10%, subdivided in 50 µl/vial (~ $5x10^8$ CFU) and stored at -80°C. An aliquot of competent cells was electroporated with 100pg of plasmid containing an EZ-Tn5 <R6Kγori/KAN-2> Transposon for testing the transformation efficiency that must be > 10^7 CFU/µg of DNA.

5.3. Generation of the library of AIEC-LF82 mutants

The Tn5 transposon mutant library of AIEC-LF82 was generated using EZ-Tn5™ <R6Kγori/KAN-2>Tnp Transposome™ Kit (Epicentre Biotechnologies, Madison, WI, USA) following the manufacturer's protocol. Briefly, LF82 electrocompetent cells were electroporated at 1,500 V with 1µl of the EZ-Tn5 <R6Kγori/KAN-2> Tnp Transposome and recovered for 1h at 37°C in 1 mL of SOC medium (Condalab, Torrejón, Madrid, Spain) in shacking conditions.

Thereafter, 100µl of recovered cells were plated on LB-agar plates supplemented with kanamycin (50µg/mL) and incubated O/N. Selected colonies were picked up from plates and individually inoculated in a 96 well plates containing LB medium supplemented with 22% glycerol and stored at -80°C. In total, through multiple transformation rounds, we generated a library of 10,058 Kanamycin-resistant mutants, covering 2.2 times the whole genome of the AIEC-LF82 strains (4,535 genes) (Rossi, Leccese et al., 2022).

5.4. Rescue Cloning of Transposed Genomic DNA of LF82

Genomic DNA from selected clones were isolated using Quick-DNA bacterial miniprep kit (Zymoresearch, Irivine, California). 1 µg of genomic DNA were digested with NdeI (New England Biolabs -NEB-, Ipswich, Massachusetts, USA) or BglI (NEB) restriction enzyme at 37°C O/N, which recognizes restriction sites outside the transposon, and then digestion reaction was stopped at 65°C for 20 min. After size-checking on 0.8% TAE Agarose gel, digested DNA was ligated using T4 DNA ligase (NEB) at 22°C, and after 24h the reaction was terminated by heating at 65°C for 10 min. TrasforMax EC100D electrocompetent cells were electroporated with 2 µl of the ligation mix at 1500V, immediately resuspended in 1 mL of SOC medium and then transferred into a new sterile tube and incubated at 37°C for 1h in shacking conditions. Transformed bacterial cells were checked by plating on LB agar containing 50 µg/mL of kanamycin. Next, PCR with standard forward and reverse primers provided by EZ-Tn5™ <R6Kγori/KAN-2>Tnp Transposome™ Kit was performed to confirm the transposon presence within the EC100D electrocompetent cells. Finally, DNA from these colonies was isolated using Monarch® Plasmid Miniprep Kit (NEB) and delivered to Eurofins together with the primers included in the Transposome™ Kit for mapping the transposon insertion site.

5.5. Human Peripheral Blood

Following informed agreement, human peripheral blood was collected from Healthy Donors (HD) (n=90) with no history of allergies or immune-mediated diseases, or from patients with active CD (n=54). All CD patients included in this project were not treated with immunosuppressive therapies or antibiotic and were selected according to the disease activity, using Harvey-Bradshow index (HBI) as previously described (Leccese et al., 2020). The study was carried out in compliance with the Declaration of Helsinki protocols and was approved by the local ethical committee (Milano Area B), code 566 2015.

5.6. Generation of Monocyte-Derived Dendritic Cells (MoDC) and Monocyte-Derived Macrophages (MDM)

Human monocyte-derived dendritic cells (MoDC) and monocyte-derived Macrophages (MDM) were obtained as previously described (Leccese et al., 2020). Briefly, heparinized blood samples from HD or CD were used to purify human monocytes by Ficoll density gradient separation and positive selection with $CD14^+$ selection (CD14 Microbeads, Miltenyi Biotec, Bergisch Gladbach; Germany). For generation of moDC, $CD14^+$ cells were resuspended at 10^6 cell/mL in complete RPMI medium (10% heat-inactivated FCS, 1 mmol/L sodium pyruvate, 10 mmol/L non-essential amino acids and 1% penicillin/streptomycin) supplemented with 50 ng/mL of recombinant human granulocyte–monocyte colony stimulating factor (rhGM-CSF, Miltenyi Biotec) and 20 ng/mL of Interleukin-4 (IL-4, Miltenyi Biotec) and seeded into 24-well cell culture plates. Complete culture media supplemented with cytokines were replaced every 3 days. After 7-9 days, MoDC were collected and seeded in RPMI medium without pen/strep into 96-round bottom well plates at the final concentration required for the experiment.

For generation of MDM, $CD14^+$ cells were resuspended at $2x10^5$ cell/mL in complete RPMI medium into flat bottom 96-well culture plates for cell adhesion and supplemented with 50 ng/mL of recombinant human macrophage-colony stimulating factor (rhM-CSF, Miltenyi Biotec). After 2 and 4 days, half of the culture medium were replaced with complete RPMI supplemented with twice M-CSF concentration. On day 7, fully differentiated MDM were washed and maintained in RPMI medium without pen/strep for the subsequent infection experiments.

5.7. Phagocytosis and Intracellular survival assays in MDM and moDC

MDM or moDC were infected with 10^6 CFU/mL of LF82 or EcN (MOI 10:1, 10 bacteria per 1 phagocytic cell). After 1h and 30 min, to allow bacterial phagocytosis, extracellular bacteria were killed by adding 20 μg/mL of gentamycin (Sigma-Aldrich, Darmstadt, Germany). After 1h of gentamycin treatment, infected host cells were washed, and medium was replaced with RPMI supplemented with 2 μg/mL of gentamycin for other 22h. For determining the intracellular bacterial concentration within MDM or MoDCs after 90min (phagocytosis) or after 24h of infection (persistence), MDM or moDC were washed twice with PBS, and then lysed with a solution of Triton X-100 (1% in ddH_20) for 10 minutes. Collected bacterial cells were washed, centrifuged, resuspended in PBS, serial diluted and plated on LB agar plates. CFU were determined after 24h growth at 37°C by viable count. In addition, at 24h post-infection, supernatants of infected moDCs were collected and stored at -20°C for the subsequent detection of cytokines by ELISA assay.

5.8. Infection Assay on intestinal epithelial HT29 cell line (IEC)

HT29 cells were seeded at $8x10^4$ cells per well into 96-well flat bottom plates and grown to confluence (3 days) in complete RPMI medium. Each confluent monolayer was infected with LF82 or LF82 mutants at the final concentration of $1.4x10^7$ CFU/well in RPMI medium without pen/strep. After 3 h of infection, to allow bacterial invasion, IECs were treated with gentamycin 20 µg/mL for 1h, and incubated for additional 3h or 20h in gentamycin 2 µg/mL. At 7h and 24h post-infection IECs supernatants were collected for measuring the amount of CCL20.

5.9. Screening of Tn5 transposon mutant library of AIEC-LF82

LF82 and LF82 mutants were grown in 200µl of YESCA medium or YESCA medium supplemented with 50 µg/mL of kanamycin, respectively, in 96 well plates. After O/N growth, OD600 of LF82 and of each mutant were read in a pleated reader (SAFAS MP96), and the amount of LF82 in order to obtain 106CFU/well (MOI 1:10 for MoDCs experiments) or 1.4x107 CFU/well (MOI 1:17,5 for IECs HT29 experiments) was calculated. Since it was not possible to normalize each of the 10,058 mutants, the infection of host cells with AIEC mutants was performed using the same amount of the parental LF82 strain. As previously described, extracellular bacteria were killed with RPMI supplemented with 20 µg/mL of gentamicin (Sigma-Aldrich) after 90 min for moDCs and after 3h for HT29 cells. At 24h post-infection, cells' supernatants were harvested and stored at -20°C for the subsequent detection of IL-23, IL-1β and IL-12 from moDC and CCL20 from HT29 cells by ELISA assay.

Cytokines concentration (C=OD450) released by moDCs or HT29 in response to each single mutant (M) was first normalized by the corresponding growth rate (G=OD600) of the O/N growth of each mutant strain. The ratio (CGr) of each well was compared with the value of the parental LF82 strain (P) (CGrM/GcrP). Differences in CGr were computed in the form of a log Fold Change (log2(CGrM/CGrP). The top 1% mutants associated with a reduction in log2(CGrM/CGrP) were determined by empirical analysis of the distribution of values.

5.10. Bacterial growth in acid and nutrient-poor medium

LF82, AIEC-mutant strains and EcN were grown O/N in YESCA medium, diluted to OD_{600}=0.02 in Acid and nutrient-poor medium (100 mM bis-Tris, 0.1% Casamino Acids, 0.16% glycerol, and 10 µM $MgCl_2$, and the pH was adjusted to 5.8 with 10M HCl) (Bringer et al., 2007)

and the growth curve, for 16h at 37°C in shacking conditions, was analysed in a microplate reader (SAFAS MP96) (Rossi, Leccese et al., 2022).

5.11. Cell viability assay WST-1

To evaluate MoDCs viability after LF82 or AIEC-LF82 mutants' infection, we used WST-1 kit (Roche) that is based on the cleavage conversion of the faintly red tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) into a dark red formazan dye by metabolically active viable cells.

Briefly, moDCs were infected with LF82 or mutants as previously described and, after 24h of infection, 10 µl of WST-1 was directly added on infected-moDCs for additional 4h at 37°C with 5% CO_2. The formazan dye formed is soluble in aqueous solutions and is directly quantified at 480 nm using the plate reader (SAFAS MP96). Data were analysed using GraphPad (version 9; Software, Inc., La Jolla, CA, USA) and results were expressed as percentage of viable infected moDCs compared to uninfected moDCs taken as 100%.

5.12. Phenotypic analysis of AIEC-mutant strains: Biofilm formation, Motility Assay and Adhesion Factors production

Biofilm formation was evaluated using the crystal violet (CV) assay as described previously (Leccese et al., 2020). Briefly, O/N bacterial cultures were diluted to OD_{600}=0.02 in 96-well plates and incubated for 16h at 37°C. Thereafter, plates were washed in ddH_2O and stained with 1% Crystal Violet (CV) solution for 20 min at RT. After 3 washing with ddH_20, adherent bacteria were dissolved in 96% ethanol and quantified at 550 nm in a microplate reader (SAFAS, MP96, Monaco). The adhesion index was calculated as OD550(CV)/OD600 (planktonic culture).

For flagellar motility assay, 3 µl of O/N bacterial culture normalized at OD_{600}=1 was spotted in the middle of a YESCA 0,3% soft-agar plates. The diameter of the area colonized by the bacteria represented the motility and was measured after 8h of growth at 37°C (Migliore et al., 2019).

For adhesion factors detection we evaluated cellulose production with congo red assay. Briefly, 3 µl of O/N bacterial culture normalized at OD_{600}=1 was spotted on YESCA 2% agar medium supplemented with 0.004% Congo red and 0.002% Coomassie blue. Bacteria were grown at 30°C for 24h and after additional 24h at 4°C phenotypes were detected (Rossi et al., 2022).

5.13. LPS extraction, silver staining and stimulation of MoDC

For LPS extraction, $3x10^9$ CFU/mL from LF82, EcN, or each single mutant strains, were collected after overnight growth and centrifugated at 5000 rpm for 10 min at 4°C. Pellets were resuspended in 500 µl of ddH_2O, centrifugated, dissolved in 500 µl of PBS and added an equal volume of phenol (Sigma-Aldrich). Samples were incubated at 65°C for 15 min, shaking every 5 min. After centrifugation at 12,000 rpm for 15 min at 4°C, the aqueous phase was collected and dialysed O/N in PBS using a 6,000-8,000 MWCO cut-off membrane. Samples were freeze-dried (Labconco freeze dryers, Kansas City, MO) and dissolved in ddH_2O to a final concentration of 40 mg/mL.

For LPS structures evaluation, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed using a previously described protocol with some modifications (Tsai and Frash., 1982). Briefly, 18% acrylamide and 0.1% SDS were used to prepare the running gel, while 4.5% acrylamide and 0.1% SDS for stacking gel. Next, 10 µg of each LPS samples and of LPS from *E. coli* O111:B4 (Sigma) as control were loaded on the gel and electrophoresis was performed at 50 V for 2h. Gel was fixed O/N in 25% isopropanol and 7% acetic acid and thereafter oxidised with 0.7% periodic acid in 40% isopropanol for 5 min. Thereafter, gel was washed three times with ddH_2O for 30 min and stained with 20 mM NaOH 0.7% ammonium hydroxide, 0.7% silver nitrate for 10 min. Next, after 4 washings with ddH_2O for 10 min, gel was developed by reduction in 0.02% formaldehyde and 0.26mM citric acid until distinct bands were visible (Sarkar et al., 2014). The reaction was stopped using a solution with 7% acetic acid and, after a further washing in ddH_2O the silver-staining of gel was acquired at Geldoc (Biorad).

To assess the release of pro-inflammatory cytokines by moDCs in response to LPS stimulation, 10^5 moDCs were stimulated with 10 µg/mL LPS isolated from LF82, EcN or LF82 transposon inserted mutants. After 24h of LPS-treatment supernatants were collected for subsequent cytokines detection with ELISA assay.

Finally, in order to evaluate AIEC uptake and persistence in the presence of TLR4 blockade, CD-MoDCs were pre-incubated for 1h with 40µg/mL of anti-hTLR4-IgG1 Neutralizing mAb (Invivogen). Infection with AIEC-LF82 was performed as described in the paragraph 5.7. At 24h supernatant was collected to measure the amount of IL-23 and IL-1β secreted.

5.14. Th17 cell subsets isolation

Human Th17 cell subsets were isolated starting from blood of HD and CD patients. $CD14^-$ cells were stained with a combination of fluorochrome-conjugated monoclonal antibodies (Table 1) and

sorted (FACSAria II, BD Biosciences) according to the expression of following specific surface markers combination: $CD4^+IL\text{-}7R^+CD25^{low}CCR6^+CXCR3^-CCR5^-$ (cTh17 cells) and $CD4^+IL\text{-}7R^+CD25^{low}CCR6^+CXCR3^-CCR5^+$ (pTh17) (Paroni et al., 2017; Paroni et al., under revision).

Antibodies	Clones	Fluorochrome	Companies
Anti-human CD4	(RPA-T4)	BV480	BD
Anti-human CD127 (IL7R)	HIL-7R-M21	BB700	BD
Anti-human CD25	2A3	BB515	BD
Anti-human CCR6	G034E3	APC	Biolegend
Anti-human CXCR3	G025H7	PE	Biolegend
Anti-human CCR5	2D7/CCR5	BV421	BD

Table 1: Monoclonal antibodies used in this study

5.15. Proliferation assay and Antigen-specificity Assay

MoDC from CD patients were seeded into 96-well plates at a final concentration of 10^4cell/well and infected with 10^5CFU/100µl of LF82 or LF82 mutants (MOI 1:10). After 90 min extracellular bacteria were killed by adding 20 µg/mL of gentamycin and, after washing infected-MoDCs were maintained in RPMI supplemented with 2 µg/mL of gentamycin.

For proliferation assay, peripheral cTh17 sorted purified from autologous CD patients and labelled with CFSE 5µM (Biolegend) were co-cultured with infected moDC at 5:1 ratio in RPMI medium without pen/strep supplemented with gentamycin 2 µg/mL and 20U/mL of recombinant human IL-2 (rhIL-2 Biolegend) (Paroni et al., 2017). RPMI medium with gentamycin and IL-2 was replaced every 3 days. After 10 days supernatant was harvested for ELISA assay, while co-cultures if infected-MoDCs-cTh17 were analysed to measure the percentage of proliferating cells using FACSCanto II cytometer (Becton Dickinson, Franklin Lakes, NJ). Results were analysed using FlowJo softwere (BD Biosciences).

For antigen-specificity assay, peripheral cTh17 sorted purified from autologous CD patients were co-cultured with infected moDC at 5:1 ratio in RPMI medium without pen/strep supplemented with gentamycin 2 µg/mL and 20U/mL of recombinant human IL-2 (rhIL-2 Miltenyi Biotec) (Paroni et al., 2017). Complete medium was replaced every 3 days. After 10 days, co-cultures of infected-MoDCs and autologous Th17 cells were analysed for intracellular cytokine detection after a further polyclonal stimulation. Briefly, after 10 days of stimulation with infected-MoDCs or untreated MoDCs, cTh17 cells were stimulated with 0,2µg/ml phorbol 12-myristate 13-acetate (PMA) and

2µg/ml of ionomycin in complete RPMI for 2h, and for further 4h with Brefaldin A at 10µg/ml. Thereafter, cTh17 cells were fixed in PBS 2% Paraformaldehyde (PFA) and permeabilized in PBS 0.5% saponin. For intracellular detection cTh17 were stained with IL-17, IFN-γ and CD40L (Table 2). Analysis was performed with FACSCanto II cytometer (Becton Dickinson, Franklin Lakes, NJ) and analysed using FlowJo software (BD Biosciences).

Antibodies	Clones	Fluorocrome	Companies
Anti-human CD40L	24-31	PE/Cy7	Biolegend
Anti-human IFN-γ	B27	PE	Biolegend
Anti-human IL-17	BL168	APC	Biolegend

Table 2: Monoclonal antibodies used in this study

5.16. Cytokine Analysis

The amount of IL-23 (Thermo Fisher), IL-1β (BioLegend, San Diego, CA, USA) and IL-12 (BioLegend) in supernatants of infected MoDC were analysed by ELISA assay after 24 h.

The amount of TNF-α (Biolegend), IL-6 (Immunotools), IL-10 (Thermo Fisher), IP-10 (R&D Systems, Minneapolis, MN, USA), chemokine (C-C motif) ligand 20 (CCL20/MIP-3α, R&D Systems), IL-23 and IL-1β in supernatants of infected MDM were analysed by ELISA assay after 24 h. CCL20 in the supernatant of IECs HT29 after 7h and 24h of infection was analysed by ELISA assay.

The amount of IFN-γ (R&D Systems) and IL-17 (R&D Systems) in supernatants of infected moDCs co-coltured with cTh17 cells were analysed by ELISA assay after 10 days.

The ELISA plates were read on microplate reader (SAFAS MP96), and data were analysed with Prism software (version 9; GraphPad Software, Inc., La Jolla, CA, USA).

5.17. Statistics

The normality and equality of variances of independent sample groups were evaluated.

Statistical significance of variables of two groups was evaluated using paired or unpaired t-test (Statistical significance was evaluated using paired or unpaired t-test for comparison of two groups with Welch's correction to analyse variables that were not normally distributed) or by one-way ANOVA for comparison of more than two groups (Kruskal–Wallis test to analyze variables that were not normally distributed). All experiments were performed at least 3 times. Significance was defined at p-value < 0.05. Statistics were performed with Prism software (version 9; GraphPad Software).

6. RESULTS AND DISCUSSION

6.1. AIEC survives within human MDM, but does not induce the secretion of polarizing cytokines linked to pTh17 cells differentiation

The interplay between human macrophages and AIEC has been the object of several studies, which report the high proficiency of AIEC to survive and replicate within macrophages, without inducing cell death, leading the secretion of high levels of pro-inflammatory cytokines (Glasser et al., 2021; Bringer et al., 2012; Buisson et al., 2017).

Macrophages are generally the most abundant APCs at sites of inflammation, and act as a bridge between innate and adaptive immunity for their role in the polarization and activation of memory $CD4^+$ T-cells (Arnold et al., 2015). In order to evaluate AIEC ability to induce pTh17 cells activation via macrophages interaction, we tested the interplay between MDM and AIEC, using LF82 as AIEC reference strain, and the commensal probiotic *E. coli* strain Nissle 1917 (EcN) commonly used for IBD treatment as control (Zhao et al., 2022). Our results show that LF82 uptake (2h) and persistence after 24h of infection within MDM is significantly higher compared to EcN; while, in contrast to some literature data (Vazeille et al., 2015), LF82 does not replicate within MDM (Figure 7A). With the aim of investigating the role of AIEC infected macrophages in the polarization of T cell subsets, we analysed the level of proinflammatory cytokines described involved in differentiation processes of memory T cells, released by MDM after 24h of infection. Our results demonstrated that LF82 is not able to induce the secretion of IL-12 or IL-23 by MDM after 24h of infection (Figure 7B), thus strongly indicating that MDM are not involved in activation processes of Th1 or pTh17 cells mediated by these polarizing cytokines. In contrast, we observed high release of IL-6, IL-1β and IL-10 by MDM both in response of LF82 and EcN, thus suggesting that these cytokines are released by MDM not in response to specific AIEC-virulence determinants but most probably in response to some PAMPs. Moreover, we observed higher release of TNF-α by MDM infected with LF82 compared to EcN, in line with the literature (Bringer et al., 2012) and, notably, also significantly higher release of IP-10 (CXCL10) and CCL-20 (MIP-3α) chemokines involved in the recruitment of T cell subsets by binding to CXCR3 (Th1) and CCR6 (Th17), respectively.

Taken together these results indicated that, although the interplay between LF82 and human MDM promotes the secretion of high levels of pro-inflammatory cytokines like TNF-α, one of the main targets of biological treatments currently used in CD therapy (Adegbola et al., 2018), AIEC-infected MDM secrete a low or no amount of IL-1β/IL-23. Therefore, considering the pivotal role especially for IL-23 in driving pTh17 cells trans-differentiation (Paroni et al., under revision;

Schmitt et al., 2021), we did not use MDM for this project, but we focused our attention on dendritic cells to identify AIEC determinants involved in the trans-differentiation of cTh17 into pTh17 cells.

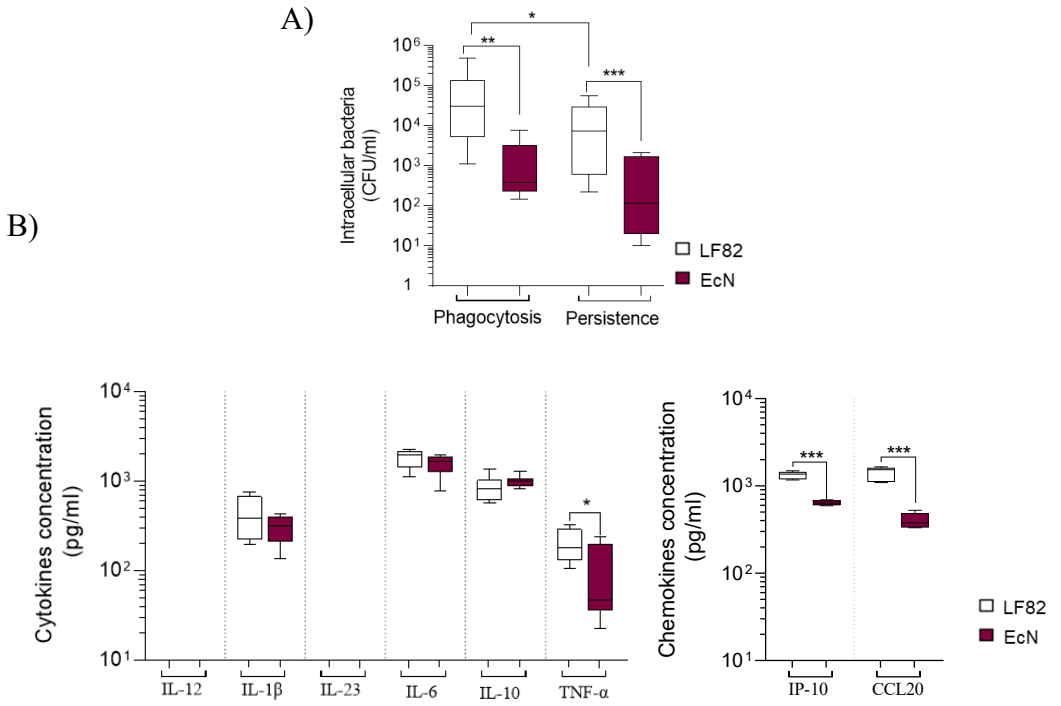


Figure 7: MDM response to AIEC-LF82 infection in comparison to EcN.
*(A) Number of AIEC-LF82 and EcN within MDM derived from HD after 2h (phagocytosis) and 24h (persistence) of infection. (B) Amount of IL-12, IL-1β, IL-23, IL-6, IL-10, TNF-α, IP-10 and CCL20 in the supernatant of infected MDM after 24h of infection. Each experiment was performed in triplicate and data are represented with box-plotes showing the median, range and upper lower quartiles of four independent experiments. Statistical significance was calculated using Welch's test and reported as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

6.2. AIEC persists within moDCs inducing high levels of polarizing cytokines secretion involved in pTh17 cells differentiation

In contrast to the several knowledge about the interplay between AIEC and macrophages, also describing AIEC-virulence factors involved in this interaction (Conte et al., 2014; Bringer et al., 2007; Bringer et al., 2005), little is known about the interaction between AIEC and dendritic cells (Leccese et al., 2020). Therefore, to deeper characterize dendritic cells response to AIEC infection, we infected moDCs isolated from HD and CD patients with LF82 and EcN strains. Our results showed that LF82 is phagocytized by moDCs and persist significantly more than EcN both in HD- and CD-derived moDCs. However, when we compared the number of intracellular bacteria after 2h of infection (phagocytosis) or after 24h of infection (persistence) we observed a significant higher intracellular concentration of both LF82 and EcN in CD-derived moDCs compared to HD-derived moDCs. Indeed, at 2h post-infection the intracellular number of LF82 and EcN were $1.4x10^5$ and $2.8x10^4$ CFU/ml in HD-derived moDCs, and increased to $4.8x10^5$ and $5x10^4$ CFU/ml in CD-derived

moDCs, respectively. These differences were even more pronounced after 24h of infection where the intracellular persistence of LF82 and EcN were $4.4x10^4$ and $4.4x10^3$ CFU/ml in HD-derived moDCs and reached the concentration of $1.4x10^5$ and $1.7x10^4$ CFU/ml in CD-derived moDCs respectively (Figure 8A).

Next, we compared the inflammatory response of DCs isolated from HD and CD patients after 24h of infection with LF82 and EcN. In contrast to what observed in MDM, the higher proficiency of LF82 to persist within moDCs compared to EcN is reflected in its ability to induce the secretion of significant different levels of proinflammatory cytokines. Indeed, we observed significantly higher levels of both IL-23 and IL-1β in the supernatant of LF82- infected moDCs compared to EcN infection, both in HD and CD-derived moDCs (Figure 8B). Especially, it is important to note that compared to HD-MDM, which do not secrete IL-23 and release approximately 450 pg/mL of IL-1β, the amount of these cytokines secreted by HD-moDCs is around 3,800 pg/mL for IL-23 and 3,600 for IL-1β, thus around 3,000 and 10 times more than MDM (Figure 8B).

Conversely to IL-23 and IL-1β, linked to Th17 cells differentiation, that are secreted significantly more in response to LF82 than EcN, the amount of IL-12, linked to Th1 cells differentiation, secreted by moDCs both from HD and CD is significantly higher in response to EcN than LF82, thus indicating the specific role of AIEC in the polarization of Th17 cells.

Moreover, we can observe that while the amount of IL-1β secreted by infected moDCs derived from HD and CD was similar, moDCs from CD secreted a significant higher amount of IL-23 compared to moDCs from HD both in response to LF82 and EcN, reaching a peack of 40,000 pg/mL and 20,000 pg/mL, respectively (Figure 8B).

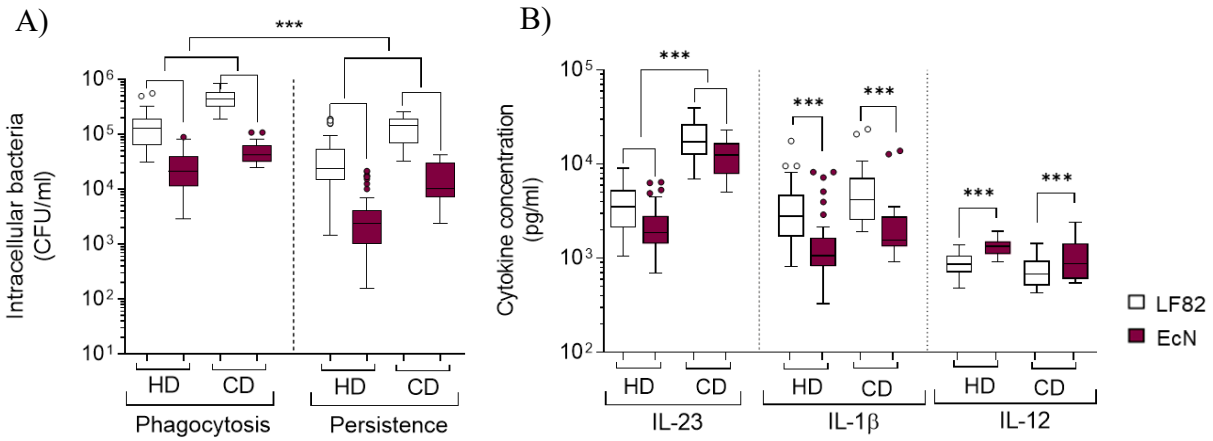


Figure 8: moDCs response to AIEC-LF82 infection in comparison to EcN.
*(A) Number of AIEC-LF82 and EcN within moDCs derived from HD and CD patients after 2h (phagocytosis) and 24h (persistence) of infection. (B) Amount of IL-23, IL-1β and IL-12 in the supernatant of infected moDCs after 24h of infection. Each experiment was performed in triplicate and data are represented with box-plots showing the median, range and upper lower quartiles of at least four independent experiments, outliers are marked as dots. Statistical significance was calculated using Welch's test and reported as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$*

Taken together, our results on the higher LF82 persistence within CD-moDCs and the dramatically higher levels of IL-23 secreted by AIEC-infected DCs, suggest that moDCs from CD patients have a defect in bacterial clearance and have an altered inflammatory response, probably due to the SNPs described in CD patients linked to bacterial response (i.e. NOD2, ATG16L1) (Hoffman et al., 2021; Brain et al., 2013). Nevertheless, the molecular mechanism by which AIEC survives within moDCs inducing high levels of polarizing cytokines secretion, especially of IL-23 which plays a crucial role in CD pathogenesis (Schmitt et al., 2011) and pathogenic Th17 cells generation (Langrish et al., 2005; Paroni, Leccese et al., under revision), is yet completely unknown and must be investigated.

6.3. AIEC persistence within intestinal epithelial cells (IECs) promotes chronic recruitment of Th17 cells

One of the most studied and best characterized virulence mechanisms of AIEC is its ability to adhere and invade IECs (Shawki et al., 2016). Since we previously demonstrated the ability of LF82 to adhere and invade IECs (Migliore et al., 2018), as well as to promote the secretion of high level of chemokine (Leccese et al., 2020), here we compared the virulence mechanism of LF82 with EcN in terms of IEC HT29 cells invasion (4h post-infection), replication (7h and 24h) and inflammatory response. Considering the important role of CCL20 in the recruitment of $CCR6^+$ lymphocytes, like cTh17 and pTh17 cells, as well dendritic cells, within which LF82 is able to persist and promote the release of very high levels of IL-23 (Figure 8A and 8B), thus promoting this circuit of recruitment and activation of pTh17, we focused our attention of this particular chemokine.

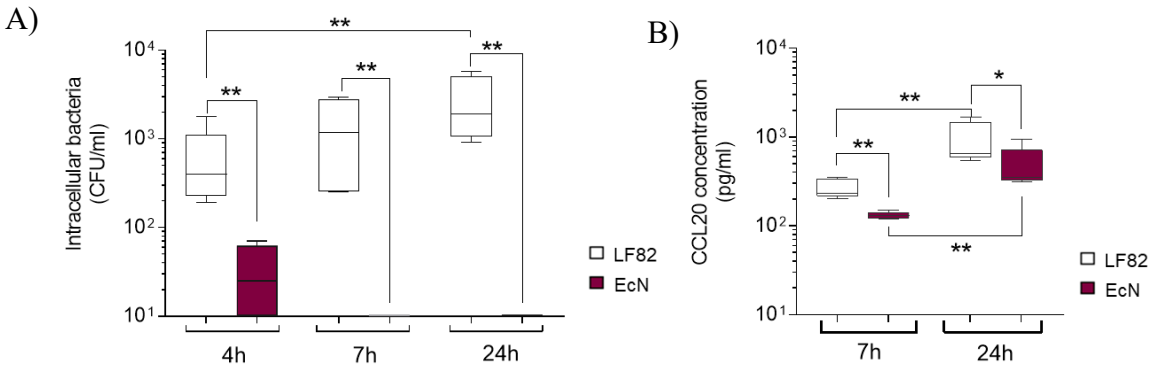


Figure 9: AIEC-LF82 invasion and replication proficiency within intestinal epithelial cells (IECs) and the relative inflammatory response.
*(A) Invasion to (4h) and replication (7h and 24h) within HT29 cell monolayers of AIEC-LF82 and EcN. (B) Amount of CCL20 in the supernatant of infected HT29 cells after 7h and 24h of infection. Each experiment was performed in triplicate and data are represented with box-plots showing the median, range and upper lower quartiles of four independent experiments. Statistical significance was calculated using Welch's test and reported as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$*

Our results confirmed the ability of LF82, but not of the commensal EcN strain to invade and replicate within IECs (Figure 9A). Moreover, we observed a significant higher release of CCL20 by LF82-infected HT29 at 24h post-infection compared to 7h post-infection, consistently with the higher number of intracellular bacteria (Figure 9B).

However, when we compared CCL20 secretion by IECs infected with LF82 and EcN, despite the probiotic strain EcN was not able to invade and persist within IECs, it was however able to trigger the release of CCL20 even if significantly less than LF82. Thus, these data suggest that firstly, the CCL20 release is not dependent by intracellular persistence of bacteria but more likely by the extracellular interaction of IECs with bacterial-determinants and, secondly, indicate that AIEC could modulate the expression of specific and yet unknown genes compared to EcN that during IECs interaction promote the release of very high CCL20 levels.

6.4. First screening and its validation of interaction between DCs and AIEC mutants for the identification of mutants unable to trigger the release of polarizing cytokines

In order to identify the molecular mechanism by which AIEC induces high levels of polarizing cytokines secretion, directly involved in the differentiation and activation of pTh17 cells, we created and screened a library of 10,058 mutants carrying a single mutation compared to the parental strain AIEC-LF82 using EZ-Tn5™ <R6Kγori/KAN-2>Tnp Transposome system. Considering the crucial role of IL-23 in generation of pathogenic Th17 cells described in literature, and the high IL-23 levels secreted by moDCs (Figure 8B) in response to LF82 infection, we performed a first screening on moDCs. In light of the extremely large number of moDCs required for this screening to test all the 10,058 LF82-mutants we firstly used moDCs derived from HD with the purpose to select AIEC-mutants able to block or significantly reduce the secretion of IL-23. In parallel, we also screened all these mutants on HT29 cells detecting the CCL20 level after 24h of infection, in order to assess whether the mutation affecting IL-23 pathway also interferes with CCL20-dependent cell recruitments described above.

Since it was not possible to normalize every 10,058 mutants, the infection for this screening was performed using the CFU/mL correspondence of the parental AIEC-LF82 strain followed by a mathematical normalization as described in the section of materials and methods.

From this first screening, we selected only mutants that induced a significantly lower secretion of IL-23, discarding mutants with a defective growth, compared to the parental LF82 strain. Thus, accordingly to the mathematical model we selected mutants with a value equal to or below the 99th percentile of our library (96 mutants), therefore those mutants under the threshold of -1.26418 $\log_2$

fold change for IL-23 secretion on the basis of the distribution of IL-23 secretion of our library (Figure 10A).

The 96 mutants selected accordingly to the lower IL-23 secretion displayed a completely different pattern of IL-1β or CCL20 secretion: indeed, 5 out 96 mutants with a reduced capacity to trigger IL-23 secretion show also impaired ability to induce IL-1β release (indicated in green in Figure 10B), while other 9 different mutants displayed a defect in the activation of CCL20 pathway (indicated in green in Figure 10C). Therefore, our results indicated that there were no mutants able to impair simultaneously the secretion of IL-23, IL-1β and CCL20, compared to the parental strain LF82, indicating that different virulence determinants are involved in the activation of these three inflammatory pathways.

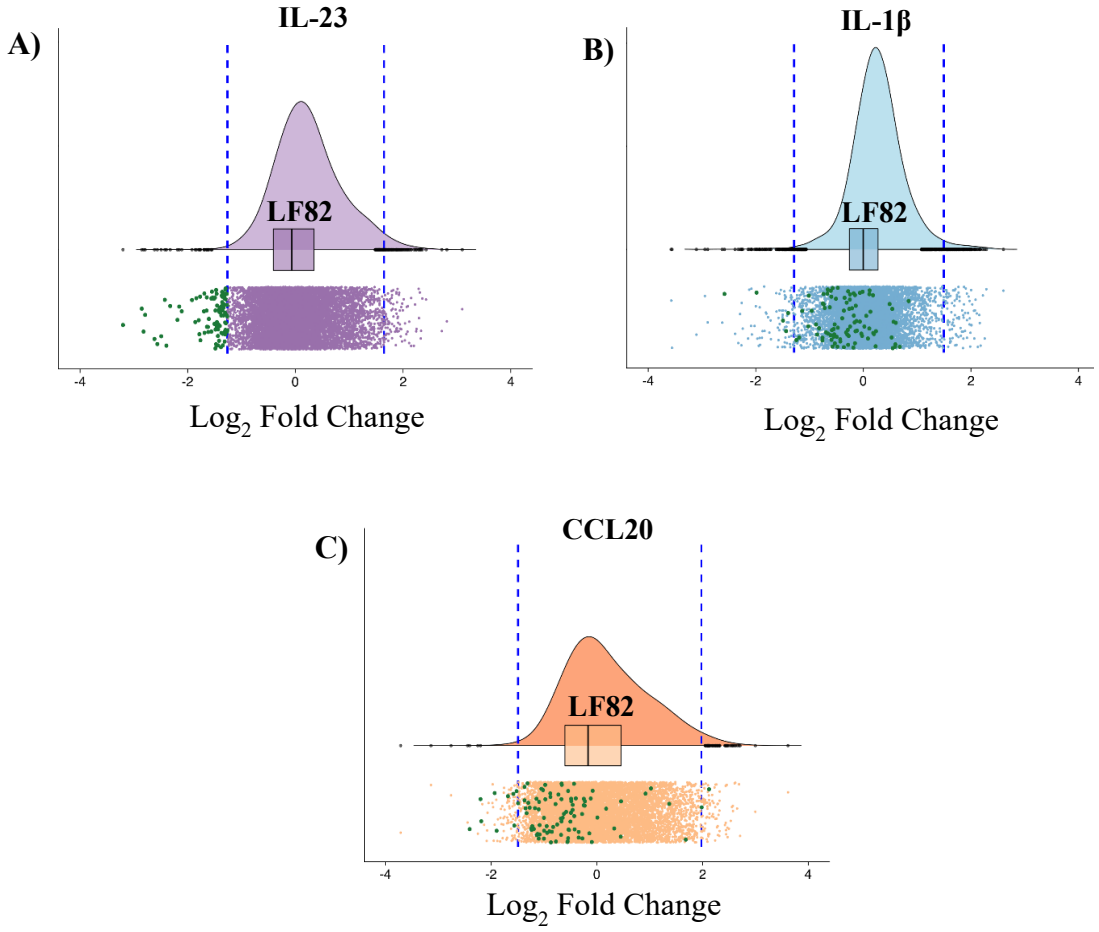


Figure 10: Identification of AIEC-LF82 mutants with impaired ability to trigger immune response.
Distribution of log_2 fold change for differentially cytokine secretion in response to 10,058 mutants of AIEC-LF82. The distribution of log_2 fold change of the ratio of cytokines secretion and O/N growth of IL-23 (A) and IL-1β (B) in the supernatant of infected moDCs and CCL20 (C) in the supernatant of HT29 after 24h of infection with AIEC-LF82 mutants are calculated over the parental strains LF82 taken as zero. Every point represents the log_2fold change value of one of the 10,058 mutants, dotted lines represent respectively -1 and +1 percentile of all the mutants. Green points represent the 96 mutants ≤ -1 percentile of IL-23 secretion. Analysis and graphs were performed using R.

Next, in order to validate the ability of these 96 selected mutants to impair IL-23 secretion compared to the parental LF82 strain, we determined their OD/CFU correspondence and then we repeated the infection of HD-derived moDCs with the effective MOI of 10:1 (10 bacteria for 1 moDC).

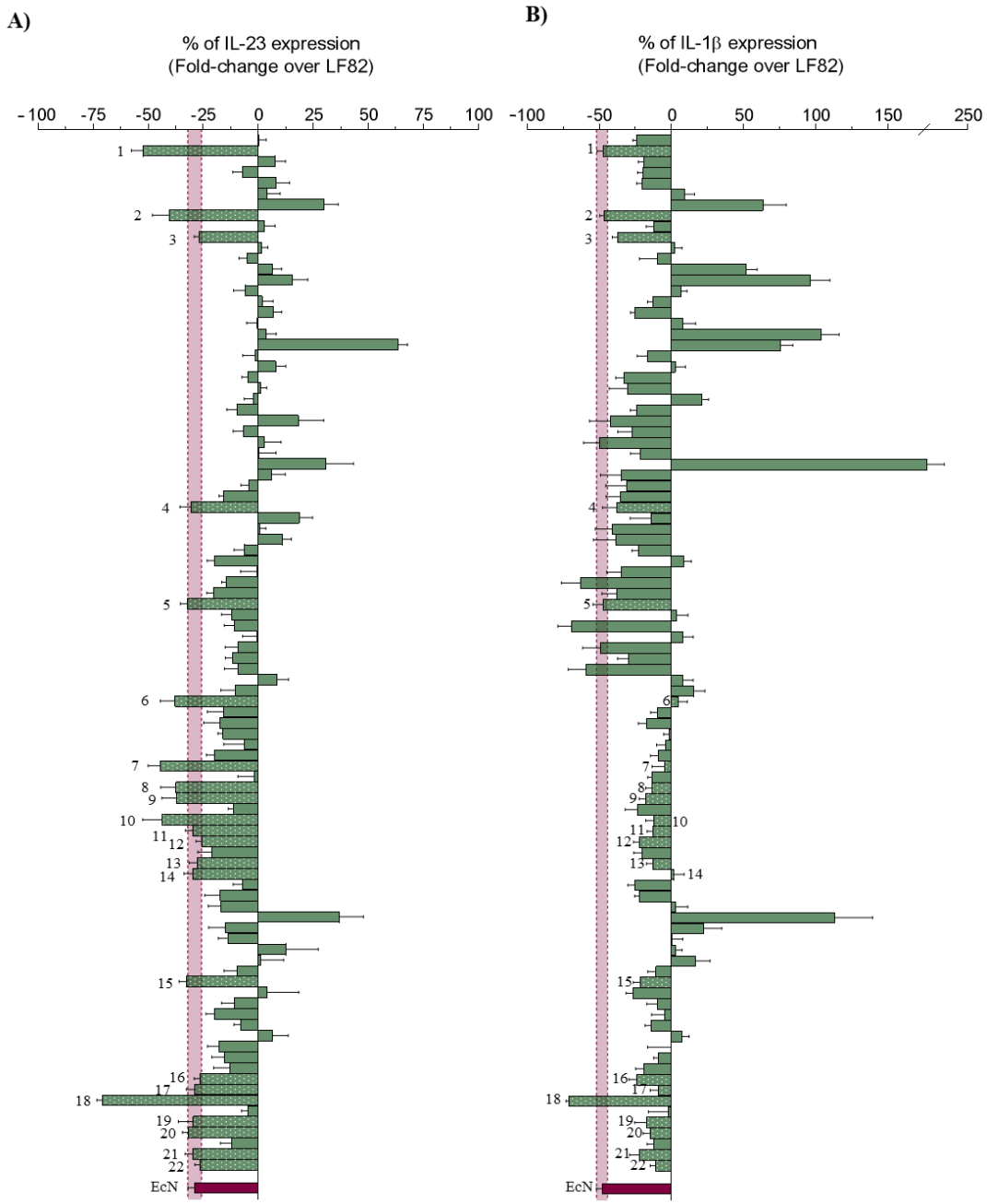


Figure 11: Ability of the 96 AIEC-LF82 mutants from the 1st screening to trigger IL-23 and IL-1β secretion.
Fold-change over LF82 (taken as 0) of the percentage of IL-23 (A) and IL-1β (B) in the supernatant of moDCs derived from HD after 24h of infection with a MOI 10:1 (10 bacteria for 1 moDC) with the 96 mutants identified from the 1st screening. Each experiment was performed in triplicate and data are represented as mean ± SEM of at least three independent experiments. Dot lines represent the mean + SEM and mean –SEM of EcN. The green bars with white points rapresent the 22 mutants with a reduced ability to trigger IL-23 secretion compared to the parental strain LF82, similar to EcN.

Here, we used the probiotic strain EcN for defining the threshold under which the LF82 mutants, even if statistically different from the LF82 parental strain, could be considered defective in the induction of IL-23 secretion. Our results show that 22 mutants out of 96 (Figure 11A, Table 3) confirmed their reduced ability to trigger IL-23 secretion after normalization, inducing a similar or lower level of IL-23 secretion to EcN. In this second set of validation experiments 9 mutants show impaired ability to induce IL-1β secretion like EcN (Figure 11B).

	LF82 mutant		LF82 mutant
1	T6.E4	12	T57.A6
2	T12.G9	13	T59.F7
3	T13.D8	14	T59.F12
4	T31.B12	15	T69.D10
5	T45.G12	16	T74.A5
6	T50.D8	17	T75.E8
7	T52.H10	18	T77.A6
8	T53.A8	19	T82.C9
9	T53.G6	20	T82.D7
10	T56.B7	21	T95.A6
11	T56.E7	22	T96.B8

Table 3: Name of the 22 AIEC-LF82 mutants identified for their reduced ability to trigger IL-23 secretion.

6.5. Correlation between IL-23 secretion and intracellular persistence of AIEC-mutants within HD- and CD-derived moDCs.

To analyse the correlation between IL-23 secretion and the ability of AIEC to persist within phagocytic cells, we tested the ability of the 22 selected mutants to grow in an acid and nutrient poor medium that mimic the phagolysosome vacuole content (Bringer et al., 2015). To this aim LF82, EcN and the 22 AIEC-mutants were grown in acid and nutrient poor medium for 16h at 37°C in shaking condition.

Results obtained with this assay displayed that the growth curves of LF82 and EcN in the acid and nutrient poor medium did not differ, despite their completely different ability to persist within moDCs (Figure 12A), thus indicating that bacterial growth in this acid medium does not correlate with the bacterial proficiency to persist within moDCs. Regarding the growth of AIEC mutants in acid and nutrient poor medium, we detected that 3 mutants (T6.E4, T31.B12, T77.A6) were completely unable to grow, while 5 mutants (T12.G9, T45.G12, T53.A8, T53.G6 and T95.A6) reached the stationary phase even earlier than the parental strain LF82 (Figure 12) thus indicating a selective advantage for these strains in growing in these conditions.

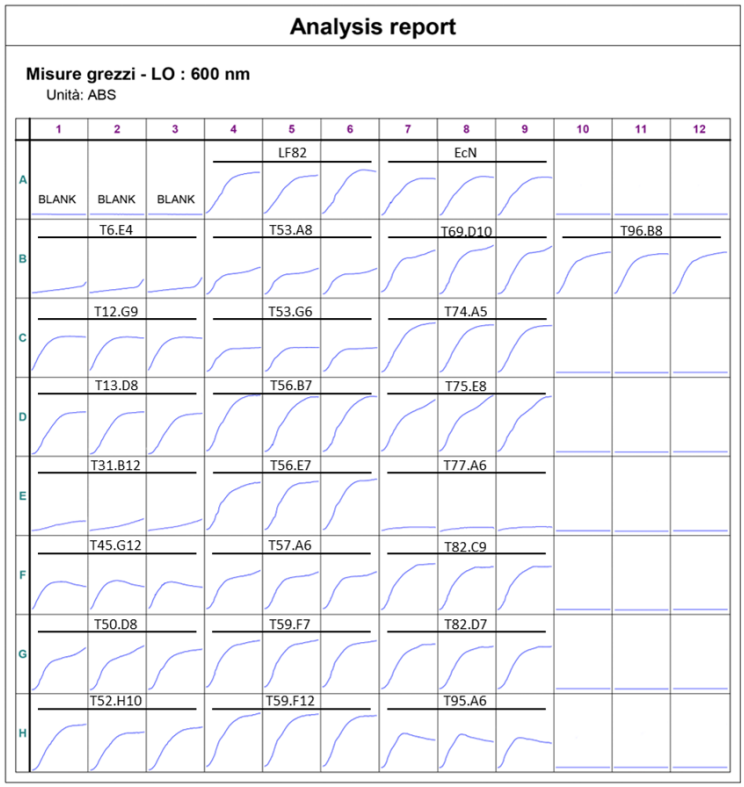


Figure 12: Growth curves for LF82, EcN and LF82 mutants in acid and nutrient poor medium mimicking the phagocytic vacuol content.

Therefore, since our data were in stark contrast to the literature data on the correlation between the AIEC ability to grow in the acid and nutrient poor medium and its persistence within phagocytic cells, we tested the ability of the 22 mutants to persist within moDCs compared to the parental AIEC-LF82 and the probiotic EcN strains. Moreover, as we previously demonstrated that the persistence of LF82 within moDCs derived from CD is significantly higher compared with the persistence within moDCs derived from HD (Figure 8A), we tested the persistence of our selected 22 AIEC- mutants both in HD- and CD-derived moDCs. Our results showed that 9 mutants out of 22 were persisting significantly less within moDCs derived from HD compared to LF82, while in CD-moDCs as many as 12 mutants displayed a defective proficiency to persist within host cells (Figure 13).

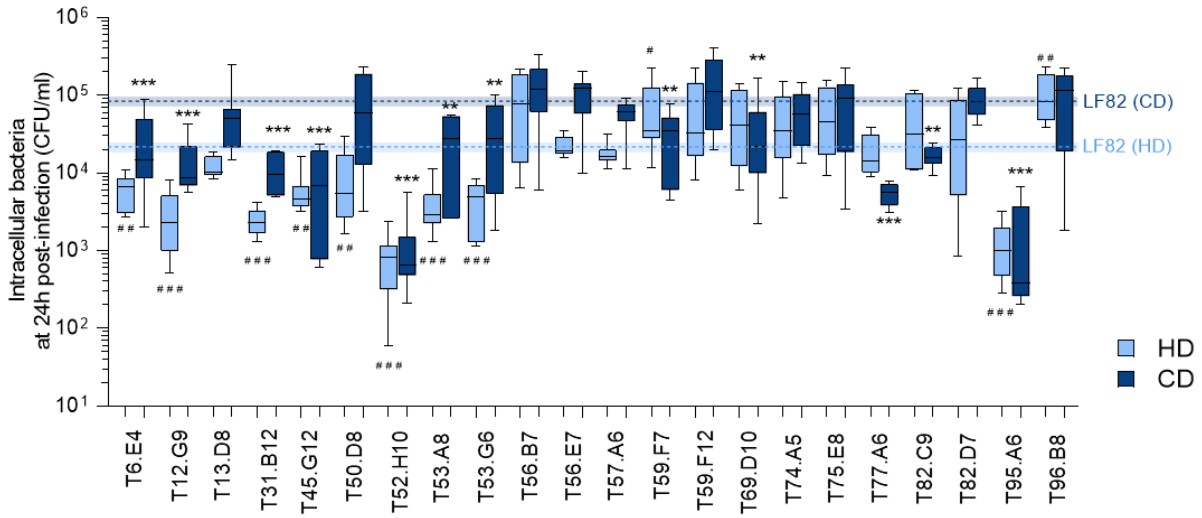


Figure13: Proficiency of AIEC-LF82 mutants to persist within moDCs.
*Number of AIEC-LF82 mutant strains within moDCs derived from HD (light blue) and CD patients (dark blue) after 24h of infection. Each experiment was performed in triplicate and data are represented with box-plotes showing the median, range and upper lower quartiles of at least three independent experiments. Dotted lines represent the mean values ± SEM (halo) of LF82 persistence in HD-moDCs (light blue) and CD-moDCs (dark blue). Statistical significance was calculated using Kruskal-Wallis test and reported as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for moDCs derived from CD patients and as $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ for moDCs derived from HD.*

Moreover, as we previously demonstrated that CD-derived MoDCs secrete significantly more IL-23 compared to HD-derived moDCs (Figure 8B), we assessed whether the 22 selected mutants maintained their defective ability in triggering IL-23 secretion also in CD-moDCs. Our analysis demonstrated that 13 mutants out of 22 maintained their reduced ability to trigger IL-23 secretion compared to the parental LF82 strain also in moDCs from CD patients.

Moreover, our results show that only 5 mutants out of 13 with a defective ability in promoting IL-23 secretion induce also a significant reduction in IL-1β secretion (Figure 14).

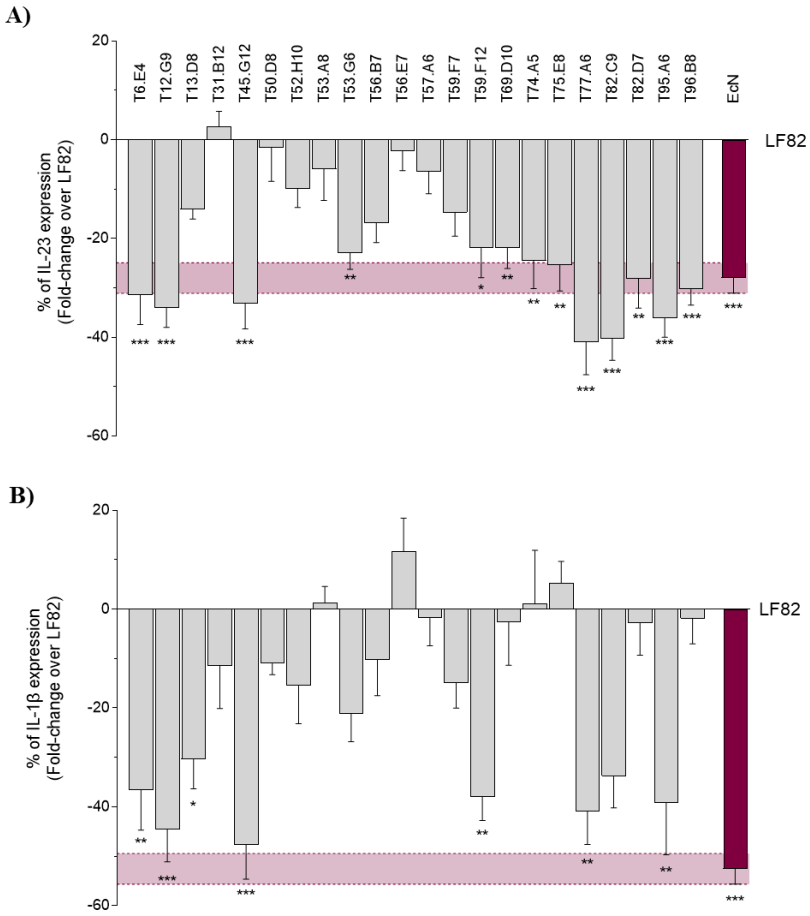


*Figure 14: 13 mutants out of 22 confirmed their reduced ability to trigger IL-23 secretion in moDCs derived from CD. Fold-change over LF82 (taken as 0) of the percentage of IL-23 (A) and IL-1β (B) in the supernatant of infected moDCs from CD with MOI 10:1 (10 bacteria for 1 moDC) after 24h of infection with the 22 mutants identified from the validation of the first screening. Each experiment was performed in triplicate and data are represented as mean ± SEM of at least three independent experiments. Dot lines represent the mean + SEM and mean –SEM of EcN. Statistical significance was calculated using Kruskal-Wallis test and reported as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$*

Next, by analyzing the correlation between the intracellular number of AIEC mutants within DCs and the percentage of IL-23 reduction compared to the parental strain LF82, our data demonstrated that there was no significant correlation between bacteria persistence and IL-23 secretion neither in HD- nor in CD-derived moDCs (Figure 15).

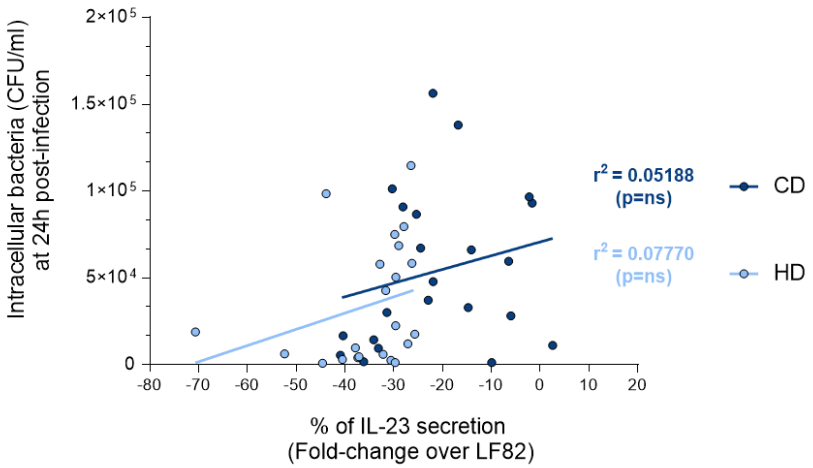


Figure 15: Correlation between AIEC-LF82 mutants persistence within moDC and IL-23 secretion.
Correlation between the persistence of LF82 mutants within moDCs derived from HD (light blue) and CD (dark blue) patients and percentage of IL-23 secreted. Correlation was determined using Sperman's correlation coefficient. No significant correlation between IL-23 secretion and mutants persistence within moDCs neither derived from HD nor derived from CD was observed.

Finally, in order to understand whether the lower persistence of AIEC mutants within DCs, compared to the parental LF82 strain, was due to a higher host-cell mortality we performed a cell-viability assay (WST-1 kit) on CD-derived moDCs after 24h of infection. From this analysis we observed that, neither LF82 nor EcN induced higher mortality of moDCs compared to uninfected moDCs, while only one LF82-mutant, namely the T77.A6, induced a significant reduction of cell viability (Figure 16), thus suggesting that the reduction of IL-23 secretion and the lower number of intracellular bacteria observed for this mutant are most probably linked to a reduced number of viable moDCs than a reduced virulence of this mutant.

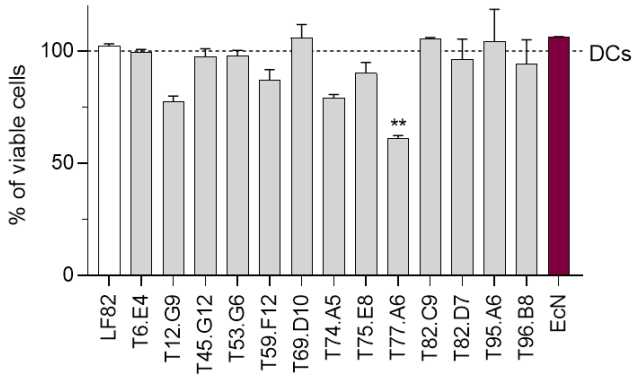


Figure 16: moDCs viability after infection with AIEC-LF82, EcN and AIEC-LF82 mutants.
*Percentage of viable moDCs derived from CD after 24h of infection with LF82, EcN and the 13 LF82 mutants identified from the screening. Each experiment was performed in triplicate and data are represented as mean ± SEM of 3 independent experiments. Dot lines represent the viabilibity of uninfected moDCs taken as 100%. Statistical significance was calculated using one-way ANOVA and reported as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$*

Taken together these results indicated that the release of IL-23 by DCs is independent of the number of intracellular bacteria and that, as demonstrated in human macrophages (Glasser et al., 2001), AIEC survive also within moDCs without inducing host cells death.

Moreover, our results suggest, once again, the dysregulated activation of IL-23/Th17 axis induced by AIEC in the genetic background of CD cells. Indeed, some mutants with reduced ability to trigger IL-23 secretion compared to the parental LF82 in HD-moDCs do not lead to a lower release of IL-23 also in CD-moDCs, indicating that IL-23 is differently triggered in CD derived moDCs by AIEC virulence-factors.

6.6. Identification of AIEC genes and corresponding virulence-determinants involved in the triggering of IL-23 secretion

With the purpose to identify the LF82 mutated genes directly involved in the reduction of IL-23 secretion and consequently in the activation of pTh17 cells, we employed the rescue cloning approach to detect the specific insertion site of the transposon within our selected AIEC-mutants. The sequencing results showed that different pathways are involved in the triggering of IL-23 secretion (Table 4).

# Mutant	Mutated Gene in LF82	Pathway
T6.E4	***aceF*** pyruvate dehydrogenase, E2 subunit	**Metabolic Pathways and Basic cell Processes**
T77.A6	***purA*** adenylosuccinate synthetase	
T82.C9	***rhlB*** ATP-dependent RNA helicase	
T53.G6	***entD*** enterobactin synthetase component D	**Nutritional and/or Environmental stress Response**
T59.F12	***ybaT*** Inner membrane transport protein	
T75.E8	***cpxA*** sensor histidine kinase	
T69.D10 T74.A5	***yhjK*** c-di-GMP phosphodiesterase	**Biofilm Formation**
T82.D7	***visC*** 2-octaprenylphenol 6-hydroxylase	
T96.B8	**kpsS (LF82_p466)** capsular polysaccharide biosynthesis protein	**Capsule synthesis**
T12.G9 T45.G12	***rfaP (waaP)*** lipopolysaccharide core heptose(I) kinase	**LPS structure and composition**
T95.A6	***rfaG (wag)*** lipopolysaccharide glucosyltransferase I	

Table 4: Identification of the EZ-Tn5<R6Kori/KAN-2> transposon insertion site.

Indeed, we found transposon insertion in genes involved in metabolic pathways and basic cell processes (*aceF*, *purA* and *rhlB*), in nutritional and environmental stress response (*entD*, *ybaT* and *cpxA*), in biofilm formation (*yhjK* and *visC*), in capsule synthesis (*kpsS*) as well as in LPS structure and composition, (*rfaG* and *rfaP*). Notably, we found that, among the final 13 selected mutants out of 10,058 tested, in two couple of mutants the transposon insertion resulted in the same gene, namely *rfaP* (T12.G9 and T45.G12 mutants) and *yhjK* (T69.D10 and T74.A5), thus demonstrating the validity of our screening.

In particular, we determined the exact position of the transposon and its orientation on the targeted gene (Figure 17): in almost all AIEC-mutants the transposon was inserted at the beginning or in the middle of the gene, suggesting an induction of a most likely knock-out effect of the gene, except for the mutants T69.D10 and T45.G12, in which the insertion of transposon has fallen at the end of the gene.

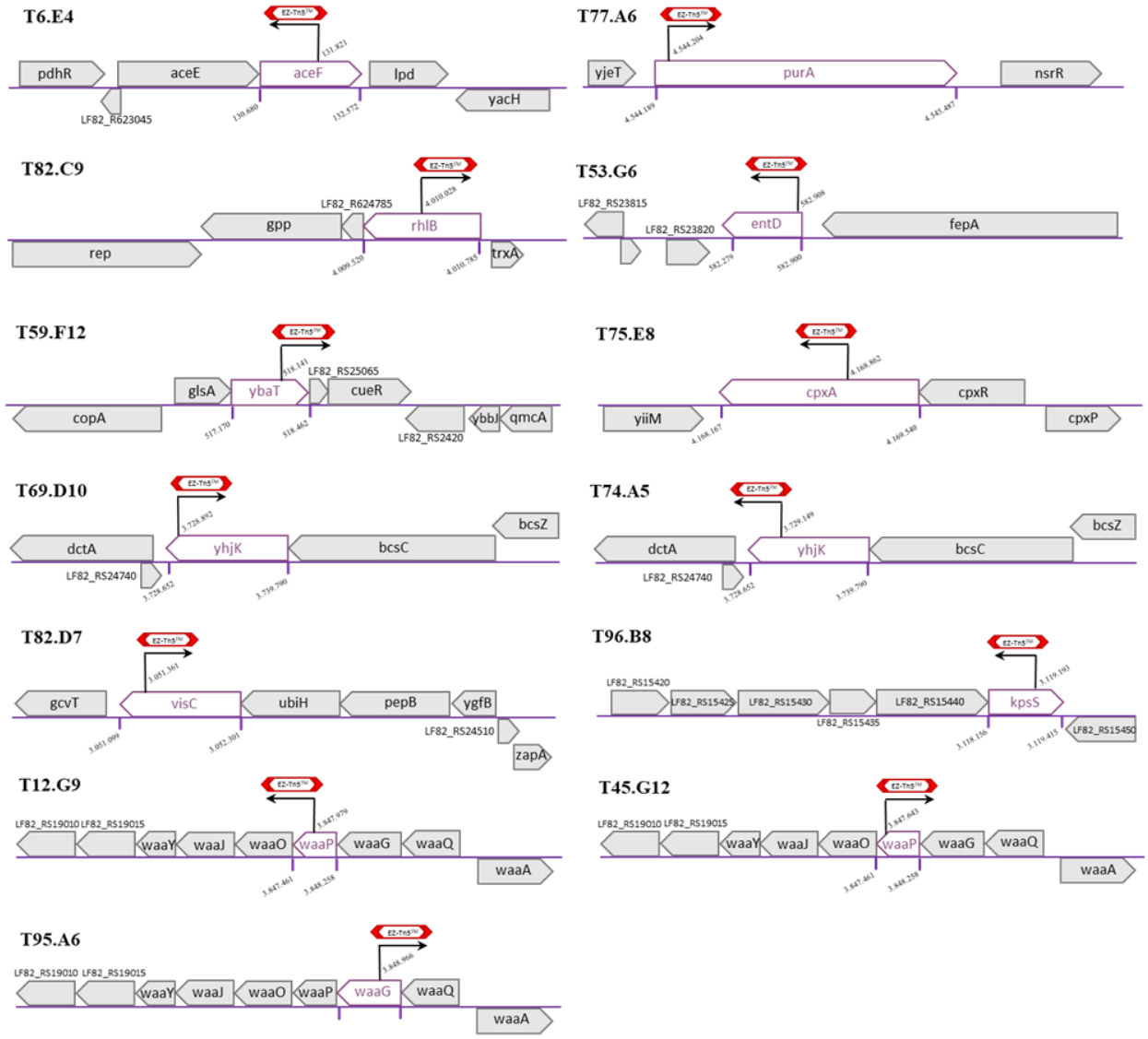


Figure 17: Localization of the EZ-Tn5<R6Kori/KAN-2> transposon insertion in the 13 selected LF82 mutants.

Remarkably, by comparing the correlation between mutation of specific bacterial pathways and the mutants' ability to persist within DCs, we observed that transposon insertions in metabolic pathways (*aceF*, *purA, rhlB*), LPS structure and composition (*rfaP*, *rfaG*) provided a strong inhibition of intracellular persistence in addition to a defective ability to trigger IL-23 secretion. In contrast, mutations in pathways implicated in biofilm formation (*yhjK, visC*), capsule synthesis (*kpsS*), and nutritional/environmental stress responses (*ybaT, cpxA*), with the only exception of the Δ*entD* mutant, did not hamper AIEC intracellular persistence despite a strong IL-23 reduction (Figure 13).

Next, to confirm whether mutations in these pathways have an effect also at the protein levels, we performed in-depth phenotypic analyses focusing on the expression of AIEC-virulence determinants described to be involved in virulence mechanisms. In particular, we tested our mutants for their motility, their ability to form biofilm and to produce curli, three factors commonly associated with AIEC proficiency to adhere to and invade IECs (Migliore et al., 2018; Chervy et al., 2020), in order to investigate their role in the interaction with DCs and the resulting IL-23 secretion.

Regarding biofilm formation, these 13 mutants displayed a very different ability to form biofilm as assessed with the crystal violet assay. Indeed, among strains in which the transposon was inserted in genes involved in biofilm formation (*yhjK* and *visC)* they did not differ from the parental strain LF82 in biofilm formation (Figure 18A), thus indicating that mutations in *yhjK* and visC do not affect the adhesion ability of AIEC. In contrast, we observed that strains affected in LPS structure and composition (*rfaP, rfaG)*, displayed a significantly lower ability to form biofilm similar to the probiotic strain EcN. While, again, among strains with mutations in metabolic pathways or stress responses, we observed both mutants with a significant higher ability to form biofilm (*aceF* and *cpxA),* and one strain (*purA)* with a significant lower ability to form biofilm compared to the parental LF82 strain.

Next, despite several works describe opposite regulation and therefore an inverse correlation between biofilm formation and flagellar motility (Boehm et al., 2010; Guttenplan et al., 2013), our results showed the absence of a strict correlation between these two virulence factors. Indeed, all mutants in LPS biosynthesis pathway (*rfaP* and *rfaG*), resulted both strongly defective in flagellar motility and significantly impaired in biofilm formation, similarly to the probiotic strain EcN (Figure 18A and 18B). On the contrary, several mutants that displayed a reduced motility compared to LF82, like Δ*yhjK*, Δ*rhlB* or Δ*ybaT*, did not differ in terms of biofilm formation in comparison to the parental strain.

Finally, we also screened our mutants for their ability to produce curli, a type of amyloid fiber involved in cell adhesion and invasion (Reichhardt et al., 2015), with the congo red assay (Figure

18C). Our results revealed that both AIEC-LF82 and the probiotic EcN strains, despite their completely different ability to form biofilm or to adhere and invade IECs, displayed a red colony phenotype on congo red agar plates, thus indicating that they were both curli producers. Indeed, in marked contrast with several works that demonstrate a positive correlation between biofilm formation and curli production (Barnhart et al., 2006; Jain et al., 2017), and that also report a selective advantage for curli-producing bacteria in IECs adhesion and invasion (Saldaña et al., 2009), we observed a very intense red phenotype for EcN strain that, instead, was completely unable to adhere or invade IECs (Figure 9A) and produced very low levels of biofilm (Figure 18A and 18C). Moreover, mutants in *purA, rfaG* and *rfaP* genes, that did not produce biofilm, displayed a white-colony phenotype on congo red agar plates, thus indicating that these strains did not produce curli fimbriae.

Interestingly, the role of curli in promoting intestinal inflammation is controversial. Indeed, proteins expressed by pathogenic and commensal bacteria are homologous (Barnhart et al., 2006) and it was described that curli fibers induce an epithelial response through the activation of TLR2/PI3K pathway that lead to the tightening of the barrier, reducing bacterial translocation (Oppong et al., 2013). Conversely, it was shown that curli inhibit the classical complement pathway protecting E. coli against complement-mediated killing (Biesecker et al., 2018). Moreover, it was demonstrated that curli induce the secretion of IL-6 and IL-23 by dendritic cells and macrophages, that induce a Th17 type immune response by the production of IL-17A and IL-22 (Nishimori et al., 2012; Tursi & Tükel, 2018). On the contrary our results showed that there is no correlation between curli production and IL-23 secretion, indeed both curli non producers and producers LF82 mutants, but also EcN, induced a lower production of IL-23.

All together these phenotypic analyses indicate that IL-23 secretion is not strictly correlated with the expression of specific adhesion determinants, in fact although all these 13 AIEC-LF82 mutants have been selected for their reduced ability to trigger IL-23 secretion, they show various and different phenotypes also within the same pathways.

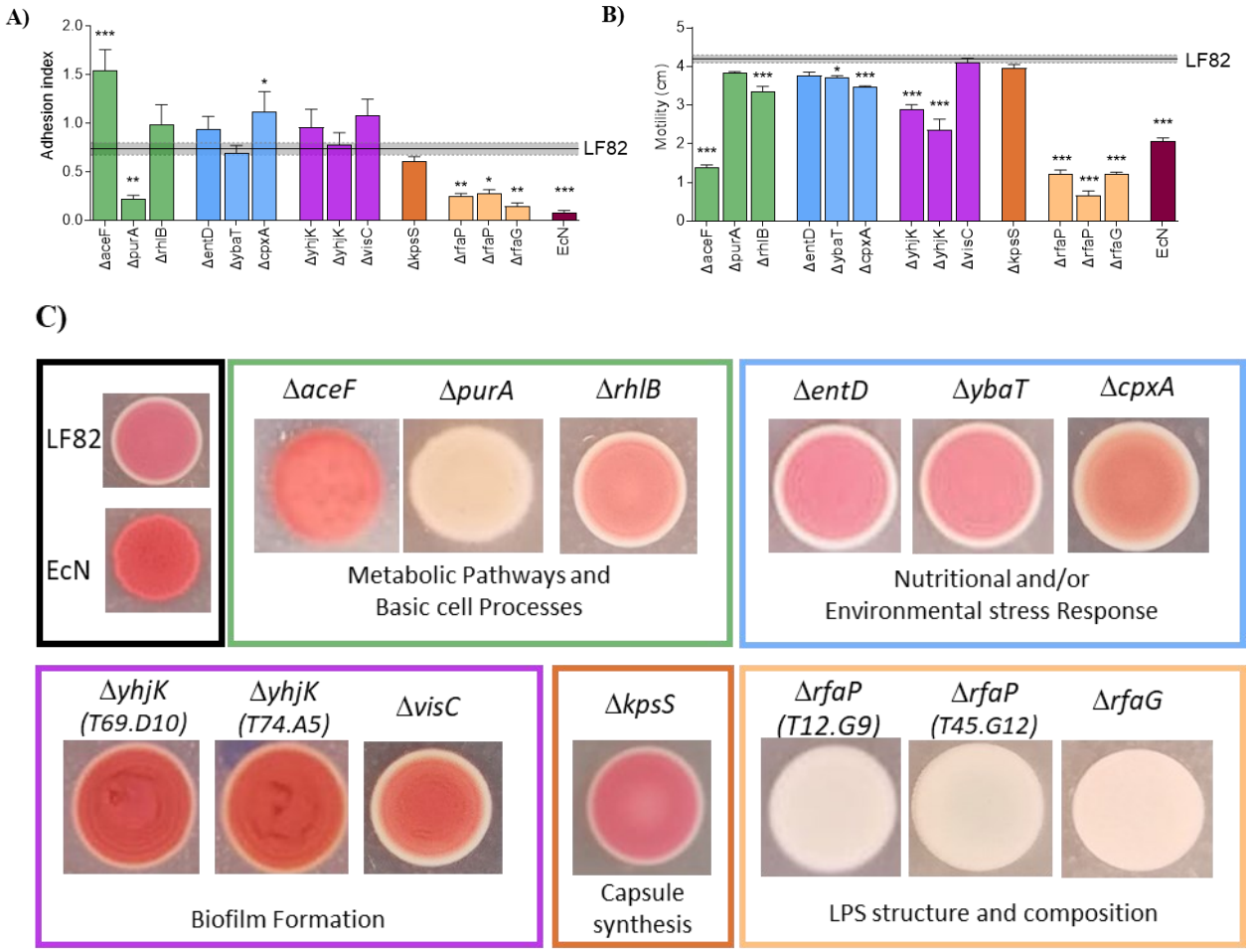


Figure 18: Phenotypic analysis of the 13 selected AIEC-LF82 mutants.
*(A) Quantitative evaluation of biofilm of the 13 selected LF82 mutants in crystal violet (CV) assay and (B) flagellar motility in YESCA/0.3% agar medium. Results are expressed as mean ± SEM of triplicate experiments and dot lines represent the mean of the parental strain LF82. Statistical significance was calculated using one-way ANOVA and reported as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (C) Qualitative detection of curli production on Congo Red agar plates.*

6.7. Correlation between LPS mutation and IL-23/IL-1β secretion

In our functional and phenotypic analysis, among the 13 LF82-mutants with a reduced capacity to trigger IL-23 secretion, the 3 mutants in LPS biosynthesis pathway, namely the lipopolysaccharide glucosyltransferase I (*rfaG*) and the lipopolysaccharide core heptose(I) kinase (*rfaP*), are resulted of particular interest for their concomitant inability to form biofilm, produce curli and persist within CD-derived MoDCs (Figure 13, 18A, 18C).

Therefore, in order to analyse whether LPS structure modification in AIEC could have a key role in DCs interaction, promoting its higher phagocytosis and triggering greater amount of IL-23 secretion in comparison to EcN, we performed LPS extraction and analysed its structure through silver staining. Our results showed that the LPS structures of LF82 and EcN strains did not differ (Figure 19B), despite their completely different interaction with moDCs both in terms of phagocytosis,

persistence and inflammatory response (Figure 8A and 8B). Moreover, consistently with mutations in genes of the *rfa* locus, Δ*rfaP* and Δ*rfaG* mutant strains displayed a different LPS structure compared to the parental LF82 strain: indeed LPS profiles of Δ*rfaP* mutants, accordingly to the role of kinases encode by *rfaP* gene responsible for phosphorylation of heptoses (Pagnout et al., 2019) (figure 19A), displayed a lighter inner core structure which is reflected in a shift of the bottom bands at lower molecular weight (Figure 19B).

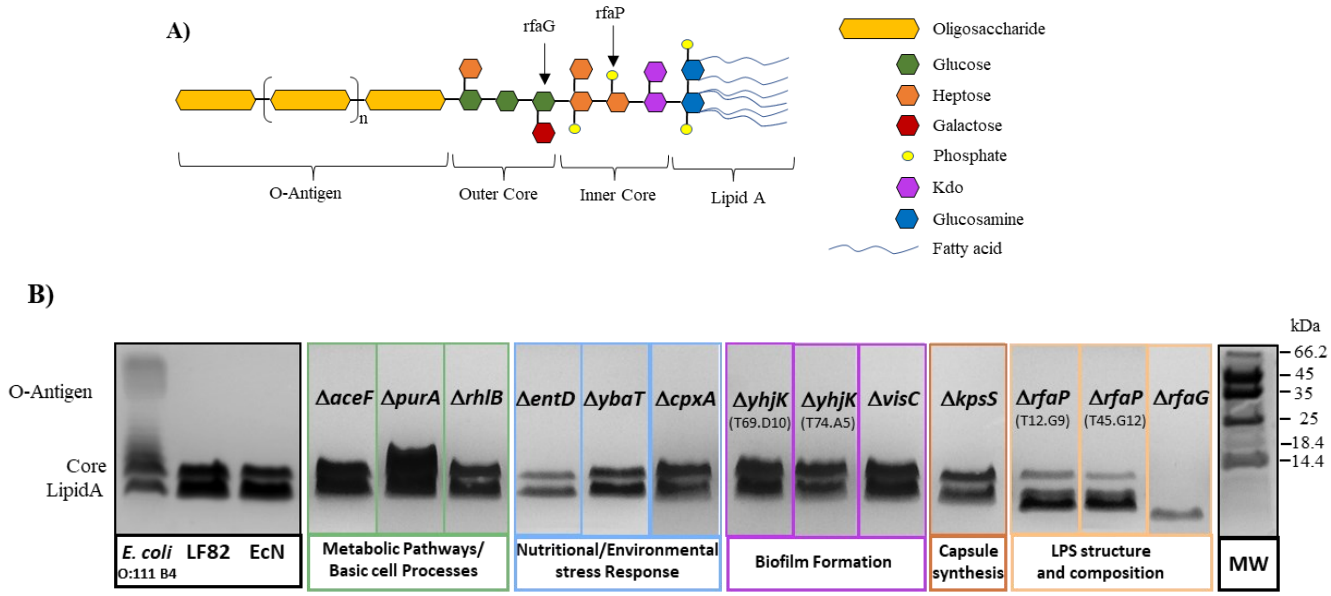


Figure 19: LPS structure and composition. E. coli O111:B4
(A) Schematic representation of LPS structure and composition. (B) Silver stained LPS profiles from strains listed below and above the gels. The bottom bands represent the lipid A and the core, the upper band represent O antigen. Molecular weight ladder is indicated as MW.

Interestingly, our results show that the LPS structures of T12.G9 and T45.G12 strains did not differ, despite they have the transposon inserted in two different positions and in opposite orientation (Figure 17), thus demonstrating the successful knock-out effect of the transposon on *rfaP* gene in both mutants.

Also regarding Δ*rfaG* mutant, consistently with the role of the glucosyltransferases encoded by *rfaG* gene that adds the three glucose residues in the LPS outer core (green hexagons in Figure 19A), and with the position of this gene within the *rfa* operon (Pagnout et al., 2019), we observed a single lower band in the silver staining due to the absence of the outer core structure as well the consequential heptoses phosphorylation carried out by downstream genes in rfa locus.

Notably, also mutant in the central metabolic pathways *purA*, that similarly to Δ*rfaP* and Δ*rfaG* mutants, is a biofilm and curli non-producing strain, and did not persist within CD-derived MoDCs, displayed a different LPS structure compared to the parental LF82 strain.

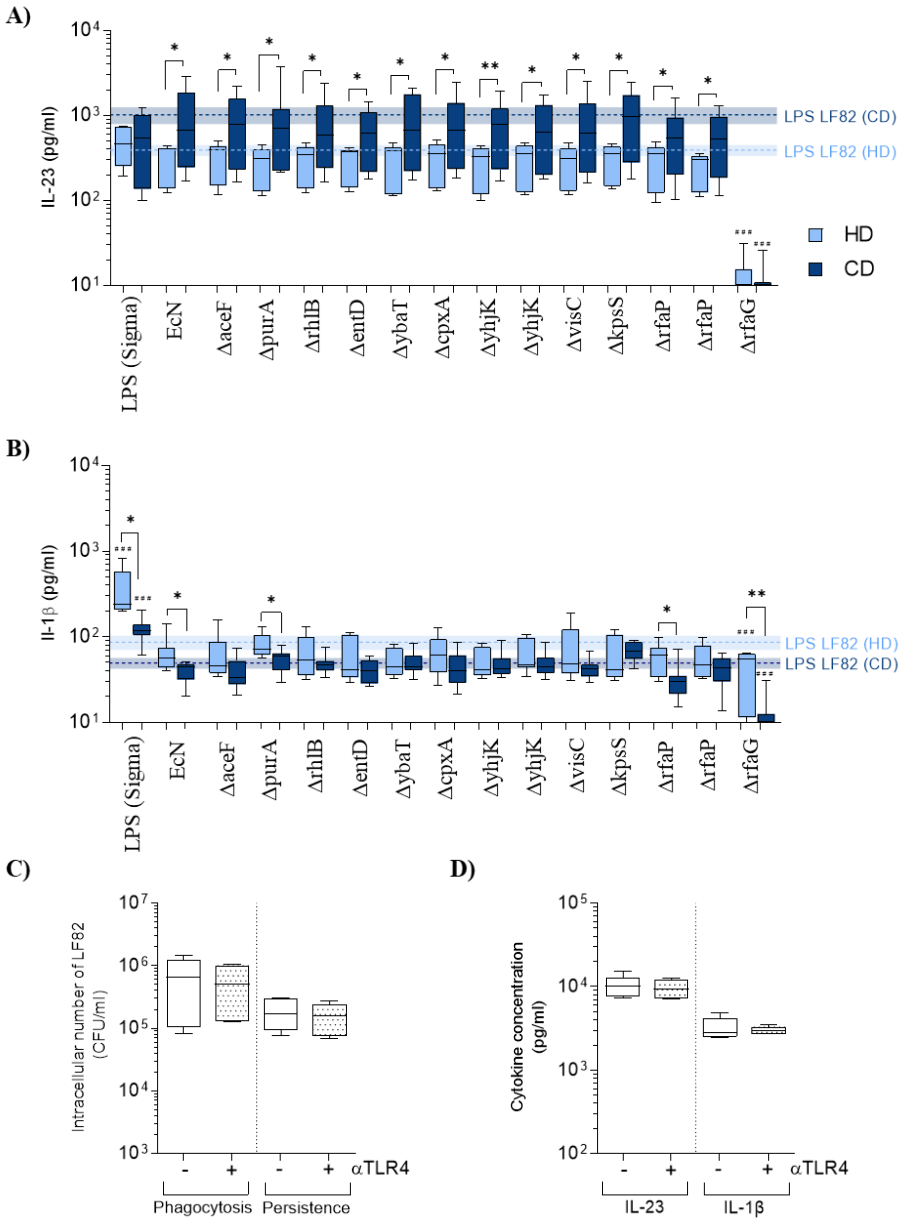


*Figure 20: Immune response to LPS stimulation. Amount of IL-23 (A) and IL-1β (B) in the supernatant of infected moDCs after 24h of stimulation with 10 ug/mL of LPS. Each experiment was performed in triplicate and data are represented with box-plots showing the median, range and upper lower quartiles of at least four independent experiments. Dotted lines represent the mean values ± SEM (halo) of cytokines secreted by HD-moDC (light blue) and CD-moDC (dark blue). Statistical significance was calculated using Welch's t test and reported as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for the comparison between moDCs derived from CD patients and moDCs derived from HD, and as $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ for the comparison between LPS of the mutants and the LPS of LF82. (C) Number of AIEC-LF82 within moDCs derived from CD patients after 2h (phagocytosis) and 24h (persistence) of infection in the presence of absence of anti-TLR4 IgG1 antibody. (D) Amount of IL-23 and IL-1β in the supernatant of moDCs infected with AIEC-LF82 in the presence of absence of anti-TLR4 IgG1 antibody after 24h of infection. Each experiment was performed in triplicate and data are represented with box-plots showing the median, range and upper lower quartiles of three independent experiments. Statistical significance between cells untreated and treated with anti-TLR4 IgG1 was calculated using Welch's test and no significant differences were found.*

Next, in a second series of experiments, in order to definitively evaluate the role of LPS in triggering IL-23 and IL-1β pathways, we stimulated HD- and CD- derived moDCs with LPS extracted from LF82, EcN and from our 13 mutants. Our results demonstrated that CD-derived moDCs secrete higher amount of IL-23 in response to LPS extracted from LF82, EcN or our mutants, but not to the commercial LPS (Sigma) isolated from *E. coli* O111:B4 serotype, compared to HD-derived MoDCs. However, comparing the IL-23 secretion in response to LPS extracted from LF82 or from our mutants, we did not observe any differences neither in HD-derived moDCs nor in MoDCs isolated from CD patients (Figure 20A), in sharp contrast with the results obtained with moDCs in response to live bacteria (Figure 8B). Indeed, the amount of IL-23 secreted in response to LF82 strain alive or to its purified-LPS was around 4,000 and 400 pg/mL, respectively, in HD-derived moDCs; while these values reached a mean of 20,000 and 1,000 pg/ml in CD-derived moDCs (Figure 20A).

Interestingly, among mutants in LPS biosynthesis pathway, only LPS extracted from Δ*rfaG* strain completely inhibited the IL-23 secretion both in CD- and HD-derived moDCs.

Analogously, also the IL-1β levels secreted by moDCs in response to LPS stimulation were extremely lower compared to the levels following the infection with the whole bacterium (Figure 8B). Nevertheless, just like for IL-23, only Δ*rfaG* strain completely hindered the IL-1β secretion both in moDCs derived from HD and CD patients. Moreover, in contrast to IL-23 release, we did not observe significant higher release of IL-1β from moDCs derived from HD compared to moDCs derived from CD, highlighting once again that the signaling pathway of the IL-23 but not of the IL-1β is selectively altered in CD patients (Figure 20B).

Finally, to definitively confirm the subordinate role of LPS as virulence-determinants that promotes higher AIEC phagocytosis and persistence, as well as a greater IL-23 pathway activation, we blocked the interaction between AIEC and the TLR4 in CD-derived moDCs, using a neutralizing antibody.

Our results showed that neither LF82 phagocytosis, nor its persistence within moDCs after 24h of infection, changed in the presence or absence of anti-TLR4 neutralizing antibody. Besides, we did not observe any difference neither in IL-23 nor in IL-1β secretion by CD-derived moDCs in the presence or absence of anti-TLR4 neutralizing antibody (Figure 20C and 20D), thus definitively demonstrating that other pathways of LF82 are activated during its interaction with moDCs promoting its virulence. For instance, as previously mentioned, curli protein represent another PAMP, that is recognized by DCs and macrophages by the TLR2/TLR1/CD14 heterocomplex and that activate NLRP3 inflammasome, inducing a pro-inflammatory response (Rapsinski et al., 2015). In addition to TLR4 and TLR2, also TLR5 play an important role in the interaction between *E. coli*

and phagocytic cells, indeed it has been demonstrated that the flagellin triggers an increased expression of TLR5, in turn increasing the production of IL-1β and IL-6 by phagocytic cells (Carvalho et al., 2008). Moreover, also CEACAM6 could represent another interactor of DCs with AIEC. Indeed, CEACAM family members were found to be expressed not only in epithelial cells, but also in other cell types, as DCs (Glas et al., 2011) and in certain type of tumor it has been described to be overexpressed on DCs (Witzens-Harig et al., 2013). In CD it has been described an increased expression of CEACAM6 (Barnich et al., 2020), that could represent not only a way of entry of AIEC into epithelial cells trough FimH, but also within DCs.

All together these results indicate once again the dysregulated response to bacterial determinants of moDCs derived from CD patients, and that LPS alone is not sufficient to trigger the altered IL-23 secretion observed in CD patients.

6.8. AIEC-LF82 mutants' inability to induce the trans-differentiation and activation of cTh17 in pTh17 cells

In our previous work (Paroni, Leccese, et al., under revision) we demonstrated that peripheral conventional Th17 (cTh17) differentiated into pathogenic Th17 cells (CCR5^{+}Th17 cells, pTh17) when stimulated in vitro with anti-CD3/CD28, that partially mimics stimulation by antigen-presenting cells, and high levels of IL-23. Moreover, we also proved that intestinal pTh17 cells were selectively activated by specific bacterial antigens, like those derived from AIEC-LF82 strain but not by other Enterobacteriaceae like *Shigella* strain. However, the molecular mechanism by which AIEC, but not other *E. coli* strains, is able to selectively activate pTh17 cells has not been characterized yet.

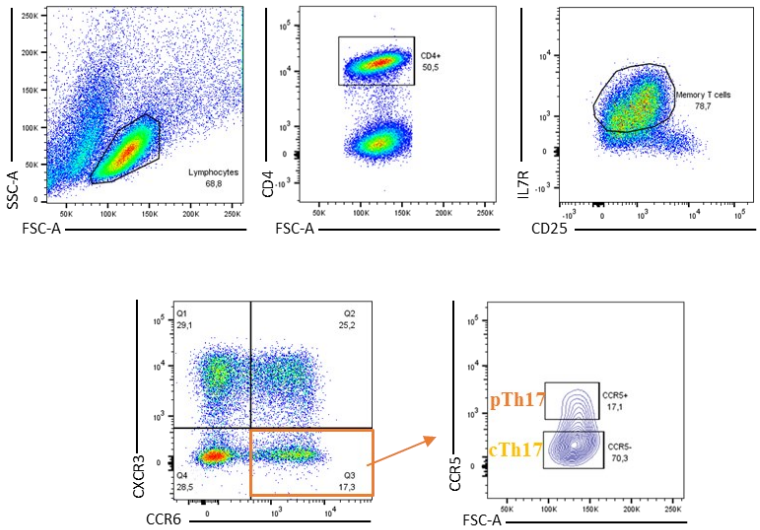


Figure 21: Isolation of cTh17 and pTh17. Gating strategy to identify and isolate CD4+ memory (IL7RHigh CD25low/-) cTh17 (CCR5-) and pTh17 (CCR5+) in the blood of CD patients.

Therefore, starting from the evidence that IL-23 plays a crucial role in the differentiation of pathogenic Th17 cells (Langrish et al., 2015) as well as of our described pTh17 cell subset, and that AIEC is preferentially able to promote very high levels of IL-23 and activate pTh17 cells, here we screened our AIEC-mutants for their ability to hinder the trans-differentiation of cTh17 into pTh17 cells. To this end, in a first series of experiments we analysed our AIEC-mutant strains, selected for their reduced ability to trigger IL-23 secretion, and for their ability to promote activation of IFNγ-producing Th17 cells starting from cTh17 sort-purified from blood of CD patients (Figure 21) through a proliferation assay.

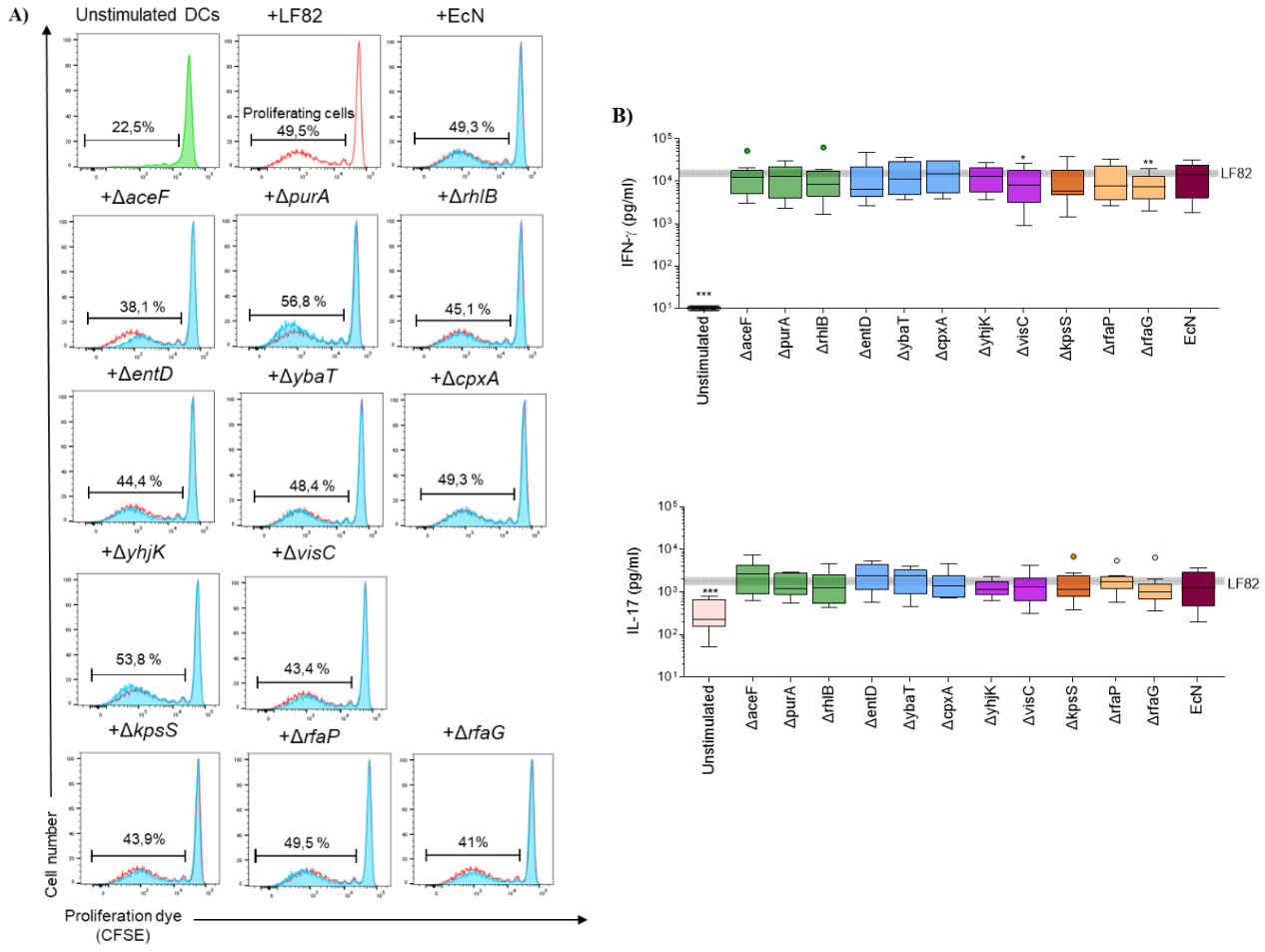


*Figure 22: Trans-differentiation of cTh17 in pTh17 cells. (A) Percentage of proliferating cTh17 cells sorted purified from CD labelled with CFSE co-cultured with autologous moDCs infected with LF82, EcN and LF82 mutants. The level of FITC fluorence in the cell populations evaluated by flow cytometry determines the numbers of generations through which a cell has progressed since the fluorescent label was applied. One representative experiments out of four experiment is shown. (B) Amount (pg/ml) of IFN-γ and IL-17 in the supernatant of Th17 cells co-cultured with autologous moDCs infected with MOI 10:1 (10 bacteria for 1 moDC) after 10 days of infection. Every strain was tested at least four times and data are represented with box-plotes showing the median, range and upper lower quartiles of four independent experiments, outliers are marked as dots. Dotted gray lines represent the mean values ± SEM (halo) of cytokines secreted in response to LF82 infection. Statistical significance was calculated using Welch's test and reported as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$*

Since 2 couples of AIEC mutants resulted mutated in the same gene, and both our phenotypic (biofilm formation, motility, curli production) and functional analysis (persistence within moDCs, IL-23 secretion) did not reveal any difference between the same 2 mutants, we conducted all subsequent functional experiments only with the strains T12.G9 for Δ*rfaP* and T74.A5 for Δ*yhjK*, in which the transposon was inserted in the middle of these genes.

Our results showed that, after 10 days of co-culture with autologous infected-moDCs cTh17 cells significantly proliferated both in response to LF82 but also to EcN compared to non-infected moDCs (Figure 22A). Moreover, when we analysed the secretion of IFN-γ or IL-17 in the supernatants of cTh17 co-cultured with LF82- or EcN- infected DCs, we observed significant higher levels of both IFN-γ and IL-17, compared to unstimulated moDCs, without however any significant difference between EcN and LF82 infection (Figure 22B).

Similarly, also in the presence of moDCs infected with LF82-mutants we observed a strong proliferation of cTh17 without any significant difference compared to the parental LF82 strain, slightly higher in the presence of Δ*yhjK* and Δ*purA strains*, while a slight reduced proliferation was observed only in the presence of Δ*aceF* mutant (Figure 22A). Surprisingly, the analyses of IFN-γ and IL-17 secreted by cTh17 cells stimulated with moDCs infected with AIEC mutants, showed that only Δ*visC* and Δ*rfaG* mutant strains induced a slight but significant reduction of IFN-γ compared to the infection with LF82, suggesting the absence of any correlation with cTh17 proliferation observed in the presence to these mutants.

These results suggested that neither cTh17 proliferation, nor the quantification of IFN-γ and IL-17 in the supernatant of proliferating cTh17 cells were highly suitable for the evaluation of the trans-differentiation of cTh17 into pTh17 cells. Indeed, despite we previously demonstrated that pTh17 cells were exclusively activated by AIEC, with this proliferation assay we observed proliferation of cTh17 and production of high levels of IFN-γ also in the presence of EcN but most of all, we can not distinguish among proliferating cTh17 cells how many of them effectively co-produced IFNγ and IL-17 or maintained the cTh17 phenotype.

For these reasons, in order to quantify the trans-differentiation of cTh17 into pTh17 cells in response to LF82 or AIEC-mutants, we performed an intracellular staining determining the percentage of IFN-γ, IL-17 and IFN-γ/IL-17 co-expression after PMA-IONO stimulation of cTh17 cells co-cultured with infected moDCs. Our results show that, after 10-days of co-culture with unstimulated moDCs, activated $CD40^{+}$cTh17 started to produce IFN-γ and co-produced IFN-γ/17 (Figure 23B) compared to the ex-vivo stimulated cTh17 (Time 0, Figure 23A), confirming also the data obtained with proliferation assay (Figure 22A), but at significantly lower levels in comparison

to pTh17 cells (Time 0, Figure 23A). Notably, cTh17 co-cultured with LF82-infected moDCs co-expressed very high levels of IFN-γ/IL-17, even significantly higher than ex-vivo stimulated pTh17 (Figure 23B), thus definitively demonstrating the AIEC ability to induce the trans-differentiation of cTh17 in pTh17. On the contrary, in response to EcN-infected moDCs we observed that activated $CD40^{+}$cTh17 co-expressed significantly lower levels of IFN-γ/IL-17(Figure 23B), indicating a reduced but not inhibited ability to promote the trans-differentiation of cTh17 into pTh17, probably due to the EcN ability to induce the release of sustained levels of IL-23.

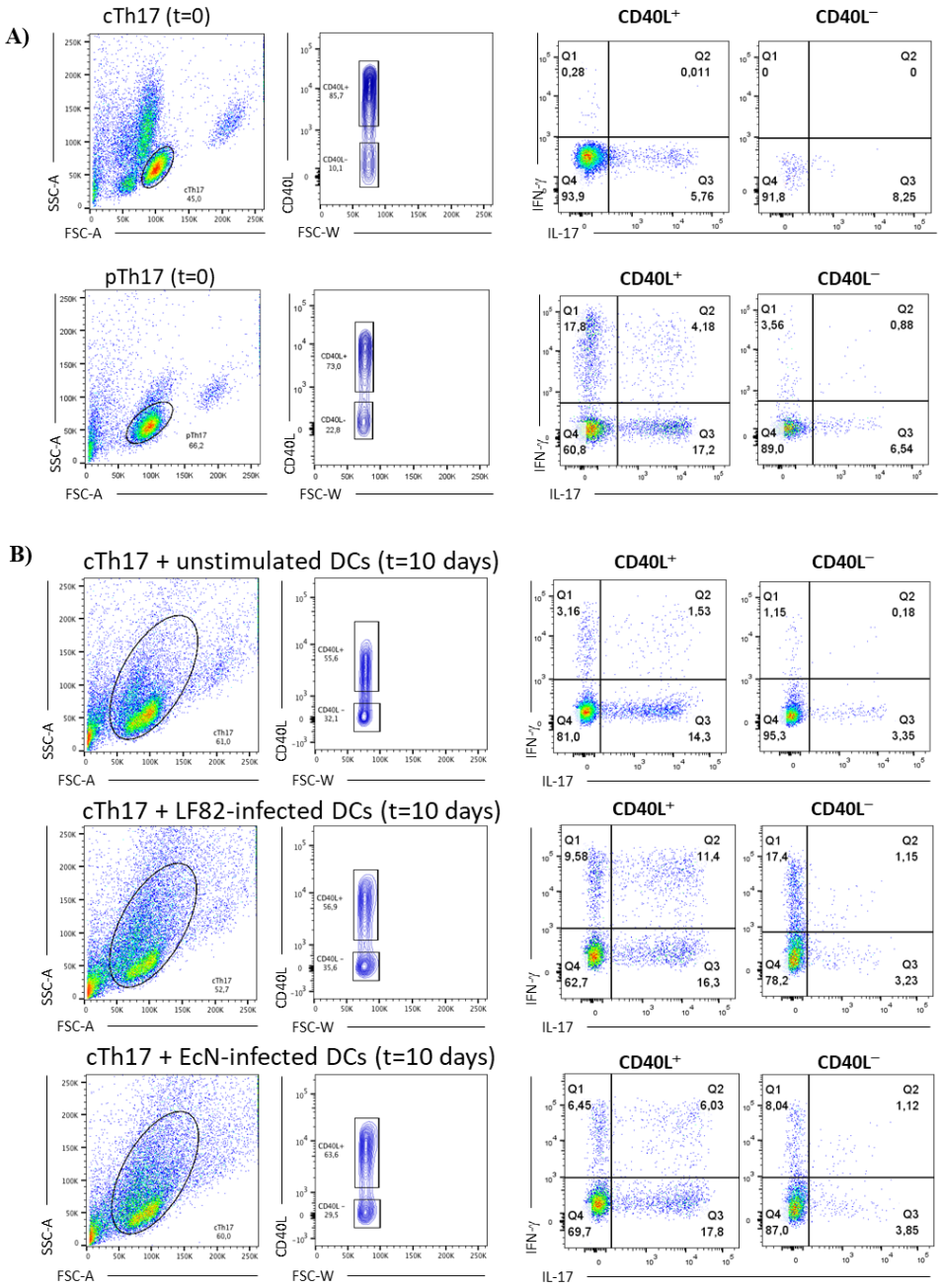
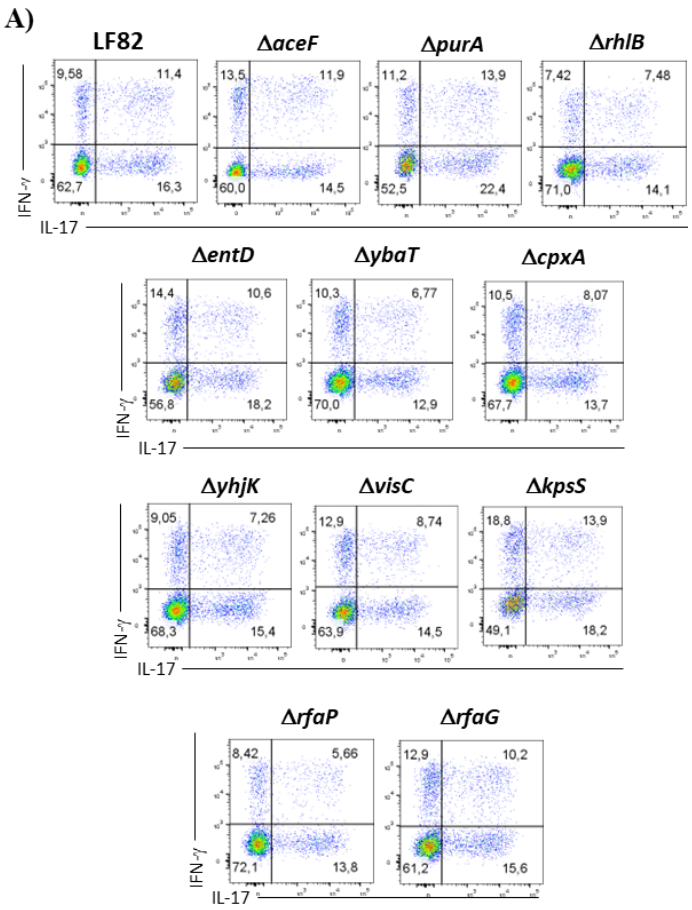


Figure 23: LF82 but not EcN induces the trans-differentiation of cTh17 into pTh17. (A) Frequency of IFN-γ, IL-17 expression and IFN-γ/IL-17 co-expression by CD40L+ and CD40L- ex-vivo stimulated cTh17 and pTh17 cells isolated from blood of CD patients. (B) Frequency of IFN-γ, IL-17 expression and IFN-γ/IL-17 co-expression by CD40L+ and CD40L- ex-vivo stimulated cTh17 co-cultured for 10 days with unstimulated autologous moDCs and LF82 and EcN infected moDCs. One representative experiment out of at least four experiment is shown.

Regarding AIEC-mutants our results showed that, despite all these mutants significantly reduced IL-23 secretion similarly to EcN probiotic strain (Figure 14A), only Δ*rhlB*, Δ*ybaT*, Δ*cpxA*, Δ*yhjK*, and Δ*rfaP* mutant strains were able to induce a significantly lower expression of IFN-γ/IL-17 by activated CD40+cTh17 cells, and therefore diminished the trans-differentiation of cTh17 into pTh17, after 10 days of co-culture (Figure 24A e 24B). Notably, this reduced IFN-γ/IL-17 co-expression was not correlated to a simultaneous significant decrease of IFN-γ- or IL-17- single positive cells, thus indicating that these mutants selectively affect the activation mechanism of pTh17 cells and also explaining to some extent data on total IFN-γ release observed in proliferation assay (Figure 22B).

Surprisingly, some AIEC-mutants like Δ*aceF*, Δ*purA*, Δ*entD*, and Δ*kpsS* strains, despite their lower induction of IL-23 secretion by CD-derived moDCs, resulted unable to significantly reduce IFN-γ/IL-17 co-expression by activated CD40^{+}cTh17 in comparison to the activation with parental LF82 strain (Figure 24A e 24B). In particular, Δ*purA* and Δ*aceF* strains even increased, albeit not significantly, the co-expression of IFN-γ/IL-17 by activated CD40^{+}cTh17, thus suggesting that a strong reduction of IL-23 levels may not be enough to interfere with the trans-differentiation pathway of cTh17 cells into pTh17 cells, but that specific AIEC-antigens play instead a more important role in this process.



B)

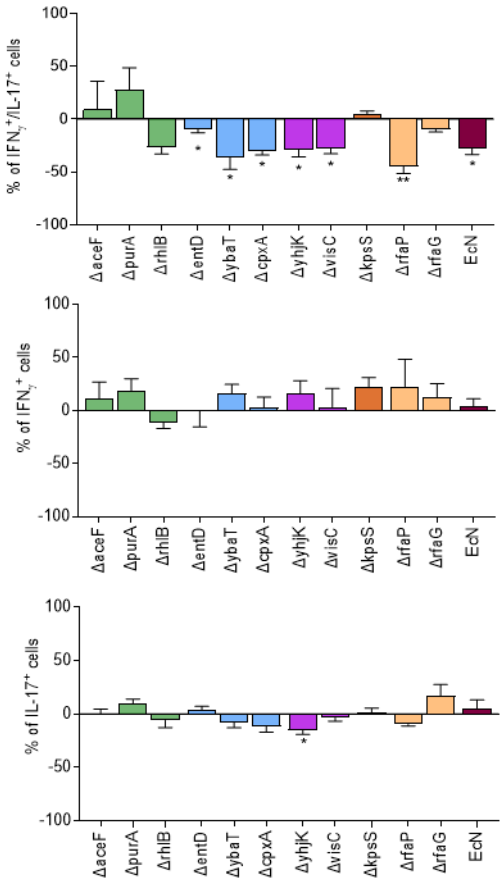


*Figure 24: LF82 mutants role in the trans-differentiation of cTh17 into pTh17. (A) Percentage of IFN-γ, IL-17 expression and IFN-γ/IL-17 co-expression by CD40L$^+$ ex-vivo stimulated cTh17 cells isolated from blood of CD patients and co-coltured for 10 days with infected autologous moDCs. One representative experiment out of at least four experiment is shown. (B) Fold-change over LF82 (taken as 0) of the percentage of IFN-γ and IL-17 in the supernatant of cTh17 co-coltured with infected moDCs from CD with MOI 10:1 (10 bacteria for 1 moDC) after 10 days of infection. Every strain was tested four times and data are represented as mean ± SEM. Statistical significance was calculated using Kruscal-Wallis test and reported as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$*

7. CONCLUSIONS AND FUTURE PERSPECTIVES

The etiopathogenesis of CD is associated with a dysregulated activation of intestinal-resident T-cells against dysbiotic components of gut microbiota. More particularly, besides the strong correlation between higher abundance of AIEC pathovar and mucosal dysbiosis in CD, increasing evidence point to a crucial and pathogenic role for IL-23/Th17-mediated immune response in CD pathogenesis (Schmitt et al., 2021). However, the correlation between AIEC abundance and the dysregulated activation of IL-23/Th17 axis in CD patients has never been fully characterized.

In this work, we identified for the first time different AIEC-virulence determinants directly involved in the hypersecretion of IL-23 by CD-derived DCs, that could be used as novel microbiological targets to prevent the activation of dysregulated inflammatory response in CD patients.

In particular, on the one hand, our results confirm the ability of AIEC strains to survive within macrophages (Glasser et al., 2001) and to also induce a significantly higher release of TNF-α, one of the main targets of current biological therapies in CD patients, compared to EcN probiotic strain. But, on the other hand, our data clearly demonstrated that macrophages do not secrete neither IL-23, nor very high levels of IL-β, required for the differentiation of pathogenic Th17 cells (Langrish et al., 2005; Mailer et al., 2015). Therefore, although the important role of TNF-α in CD chronic inflammation is evident, also supported by some recent works demonstrating that anti-TNF agents hamper AIEC replication within macrophages (Douadi et al., 2022), our data exclude the AIEC ability to promote IL-23-dependent pTh17 cells activation via interaction with macrophages, as also confirmed by recent clinical evidence reporting that IL-23/IL-17 axis is not influenced by TNF blockade (Milanez et al., 2016). For this reason, we focused our attention on DCs demonstrating that AIEC-infected DCs secrete very high levels of IL-23 and IL-1β, in sharp contrast to macrophages, and therefore having a central important role in T cells differentiation and activation. Indeed, our results strongly indicate that the interplay between AIEC and moDCs is a key process for T-cell polarization and that, while AIEC triggers the secretion of high levels of IL-23 and IL-1β, linked to pathogenic Th17 cells differentiation, the probiotic EcN strain is linked instead to Th1 cells polarization through the release of high amount of IL-12. This outcome could also be an additional explanation for the different remission rates and clinical responses reported in CD patients treated with ustekinumab, a monoclonal antibody against the common p40 subunit of IL-23 and IL-12 cytokines, or with monoclonal antibody against anti-IL23p19 like risankizumab (Gecse., 2008; Ma et al., 2019).

In this context, interestingly, our results revealed a specific dysregulated activation of IL-23 pathway in CD patients compared to healthy subjects, displaying a significantly higher amount of IL-23 secretion in response to AIEC selectively in CD-derived DCs, thus pointing to the expression of specific AIEC-virulence determinants exclusively involved in the IL-23/Th17 axis triggering. Hence, with the purpose of unveiling the molecular mechanism by which AIEC selectively induce the dysregulated secretion of IL-23, we generated and screened a library of 10,058 AIEC-mutants both on human DCs and intestinal epithelial cells (IECs). Interestingly, our results demonstrated that AIEC plays a crucial role in the activation of several immunological pathways but with the involvement of different virulence determinants. Indeed, among 10,058 mutants tested, 22 strains significantly reduce IL-23 secretion compared to the AIEC parental strain, 5 out of these 22 also hamper IL-1β release, while different 9 mutants out 22 also inhibit CCL20 secretion, but none of them blocks simultaneously the three pathways thus definitely demonstrating the involvement of different AIEC-determinants in the triggering of IL-1β/IL-23 by DCs and CCL20 secretion by IECs. Therefore, targeting AIEC-virulence factors involved in IL-23 secretion could exclusively prevent the activation of the pathogenic IL-23/Th17 pathway, leaving the CCR6-CCL20 axis unaffected, thus maintaining the homeostatic balance between Th17 and Treg and in turn the mucosal tolerance (Kularni et al., 2017), as well as the chemotactic effect on DCs and macrophages (Kaser et al., 2004).

Furthermore, the AIEC proficiency in triggering hypersecretion of pro-inflammatory cytokines was commonly related to virulence determinants that confer to AIEC strains the ability to invade and persist within host cells. Indeed, several works propose a direct correlation between AIEC virulence, both in term of adhesion and invasion of IECs, as well as AIEC replication within MDM, to its ability to form biofilm (Wine et al., 2010; Prudent et al., 2021). Here, the functional screening of our AIEC-mutant strains demonstrates not only the complete absence of any correlation between IL-23 release and AIEC ability to form biofilm, but also that, in marked contrast to the evidence that biofilm protects AIEC from phagolysosomal attack (Prudent et al., 2021), the absence of any correlation also between biofilm formation and AIEC persistence within DCs. Indeed, we did not observe any significant difference between AIEC mutants with higher (i.e. Δ*aceF*) or lower (i.e. Δ*purA*) capacity to form biofilm compared to the parental AIEC strain, in persisting within moDCs. Moreover, our results also suggest that biofilm formation, in contrast with data reported in literature (Jain et al., 2017), is not strictly associated with curli production and that, neither a different curli production interferes with IL-23 secretion. Indeed, in our hands the probiotic EcN strain produces more curli fimbriae than the AIEC-LF82 strain, as well as the great majority of AIEC mutants with

a reduced ability to trigger IL-23 secretion display a curli-positive phenotype identical to the parental strain. Furthermore, our results indicate also the absence of a strict correlation between biofilm formation and AIEC motility, despite several works describe opposite regulation and therefore an inverse correlation between biofilm formation and flagellar motility (Guttenplan et al., 2013).

In general, from our functional and phenotypic screenings we can assert that there is not any correlation between IL-23 secretion and the expression of main AIEC adhesion determinants, described in literature as the main AIEC virulence factors, such as motility, biofilm formation or curli production. Indeed, AIEC mutants with a defective ability to trigger IL-23 secretion display completely different phenotypes, also indicating that the classical phenotypic characterization is not a valid tool to identify AIEC-virulence determinants involved in the interaction with DCs.

Within the screening of our library, we found that several mutants with a reduced IL-23 secretion possess transposon insertion in LPS biosynthesis pathways and display both a defective ability to persist within DCs and a completely different phenotype compared to parental AIEC-LF82 strain, namely biofilm-negative, non-motile and curli non-producing, caughting our interest to deeper explore the role of LPS in the activation of IL-23/Th17 axis. In particular, since LPS is an essential cell wall component that contributes to the inflammatory cascade by binding the CD14/TLR4/MD2 receptor complex (Mbongue et al., 2022), and that TLR4 blockade has been proposed as an effective therapeutic approach because extensively upregulated in CD (Tam et al., 2021; Kordjazy et al., 2018) , we characterized the inflammatory response of DCs after infection with our IL23-defective AIEC mutants or their LPS. Interestingly, our results clearly show that LPS is not exclusively involved in the triggering of IL-23/IL-1β pathway, since, except for LPS extracted from Δ*rfaG* mutant, there was no difference in the inflammatory response of DC following treatment with LPS extracted from different AIEC mutants.

Moreover, LPS doesn't even have a key role in promoting AIEC phagocytosis and persistence within DCs, given that the TLR4 blocking with neutralizing antibodies completely failed in hampering these processes. Thus, altogether these results indicate that other AIEC-virulence factors are implicated in DCs-interaction and IL-23 triggering, also confirming literature data reporting that LPS-mutants can be associated with changes in other bacterial virulence determinants.

Finally, this work shows for the first time the key pathogenic role of AIEC virulence-determinants in the trans-differentiation of cTh17 into pTh17 cells, demonstrating that specific AIEC-antigens play a more important role compared to IL-23 levels in this process (Langrish et al., 2005). Indeed, despite the AIEC mutants have been selected mainly for their reduced ability to trigger IL-23

secretion in CD-derived DCs, only 6 of them were actually able to induce a significantly lower expression of IFN-γ/IL-17 by activated cTh17 cells, and therefore interfere with the trans-differentiation of cTh17 into pTh17 cells. While, surprisingly, some IL-23-defective AIEC mutants enhance the IFN-γ/IL-17 co-expression, thus indicating that a reduced IL-23 secretion is not enough to abolish the trans-differentiation of cTh17 into pTh17 cells.

In conclusion, we identified 6 mutants with an impaired ability to trigger IL-23 secretion and to promote pTh17 cells trans-differentiation, actually representing novel and unique therapeutic targets that can be used to hamper exclusively the activation of pathogenic Th17 cells in CD patients. Nevertheless, further analyses are required to understand whether, for instance, the deletion of some AIEC genes correlated with an increased pTh17 trans-differentiation can be due to an altered activation or regulation of other connected AIEC virulence determinants during AIEC persistence within DCs, and, finally, to investigate whether a complete inhibition of IL-23, and not only its strong reduction as observed with these AIEC mutants, together with the inactivation of here described AIEC virulence-determinants, could be a better strategy to fully prevent the generation of pTh17 cells.

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