The Relationship Between miR-29, NOD2 and Crohn's Disease

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Abstract Crohn's disease (CD) is a chronic inflammatory bowel disease with a complex aetiology that includes genetic susceptibility and the gastrointestinal microbiome and results in an aberrant Th17 inflammatory response. NOD2 is an intracellular sensor that responds to bacterial cell wall peptidoglycan and contributes to immune defense. Polymorphisms in the NOD2 gene predispose to Crohn's disease, with the largest effect of any of the known genetic risk factors. We have found that wild-type NOD2 controls the expression of miR-29 in human dendritic cells (DCs). miR-29 regulates the expression of a number of immune mediators including the IL-23 cytokine subunits IL-12p40 and IL-23p19. CD patient DCs expressing NOD2 polymorphisms fail to induce miR-29 and show enhanced IL-12p40 release on exposure to adherent invasive *E. coli*. Moreover in a murine model deficient in miR-29, a more severe Th17-driven colitis is established after DSS administration. Therefore, we suggest that the loss of miR-29-mediated immunoregulation in CD-variant NOD2 DCs contributes to elevated IL-23 and aberrant Th17 response in this disease.

Abbreviations

AIEC	Adherent-invasive *Escherichia coli*
APC	Antigen presenting cell
ATG16L1	Autophagy related 16-like 1
CARD15	Caspase recruitment domain family member 15
CD	Crohn's disease
Chr	Chromosome
CLR	C-type lectin receptors

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DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DSS	Dextran sodium sulphate
F. prau	*Faecalibacterium prausnitzii*
Foxp3	Forkhead box P3
GI	Gastrointestinal
GWAS	Genome-wide association study
HD	Human defensin
IBD	Inflammatory bowel disease
IFN	Interferon
IL	Interleukin
IL-12B	Interleukin 12B/IL-12p40
IL-23R	Interleukin-23 receptor
IRGM	Immunity-related GTPase family, M
JAK2	Janus kinase 2
KO	Knock-out
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MDDC	Monocyte-derived dendritic cell
MDP	Muramyl dipeptide
miR	microRNA
MyD88	Myeloid differentiation primary response gene 88
NFκB	Nuclear factor kappa B
NK	Natural killer
NLR	NOD-like receptor
NOD2	Nucleotide-binding oligomerisation domain containing 2
Pam_3CSK_4	Synthetic triacylated lipoprotein—TLR1/2 ligand
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PRR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
RIPK-2	Receptor-interacting protein kinase 2
RORγt	RAR-related orphan receptor gamma
STAT3	Signal transducer and activator of transcription 3
T-bet	T-box transcription factor
Th1/17	T helper 1/17
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Wnt	Wingless
WT	Wild-type

1 Introduction

Crohn's disease (CD) is a form of chronic inflammatory bowel disease (IBD). It typically presents in young people (peak onset between years 10 and 20), with the highest incidence in the western world with a rising incidence as nations move towards a western lifestyle and environment [1]. CD usually manifests clinically with a combination of abdominal pain, weight loss, and diarrhoea. The clinical course is variable, from a fairly mild self-limiting illness through to severe disease resulting in multiple surgeries, short gut syndrome and severe malabsorption. Although there have been substantial improvements in medical therapy, there exists a significant therapeutic gap as evidenced by the modest sustained remission rates with anti-TNF therapy [2] (currently the most potent available therapy), plus the requirement for at least one surgery in the majority of patients (up to 80 % in ileal disease [3, 4]). The transmural granulomatous inflammation typical of Crohn's can involve any part of the gastrointestinal (GI) tract from mouth to anus, but usually affects the terminal ileum, colon and/or perianal regions.

The aetiology of Crohn's disease is complex and multifactorial, with the best evidence suggesting that it involves a combination of genetic susceptibility, a disordered GI tract microflora (dysbiosis), and possibly a third as yet poorly characterised environmental factor, or factors.

There is good observational evidence for a genetic component to disease susceptibility, from the documentation of familial clustering by Burrell Crohn himself [5] to the monozygotic twin disease concordance rate of nearly 50 % [6] and the high percentage of patients with a positive family history [7]. Over the past 15 years genetic studies have identified an increasing number of polymorphisms associated with CD, and currently there are 163 loci associated with IBD [8]. The modern era of IBD genetics was initiated by the discovery of mutations in the caspase recruitment domain family member containing 15 (CARD15), or nucleotide-binding oligomerisation domain containing 2 (NOD2), gene in the IBD1 locus on chromosome 16 [9].

CARD15/NOD2 (henceforth NOD2) encodes a cytosolic innate immune receptor and is a member of the NOD-like receptor (NLR) family. NOD2 is triggered by bacterial cell wall muramyl dipeptide (MDP) [10, 11], which is expressed by both gram +ve and gram −ve organisms. This aligns with another central tenet of CD susceptibility—that of the influence of the GI tract bacteria or microbiome. The GI tract is home to a staggering quantity of bacteria—at their most dense the bacteria number 10^{12}/ml. Coupled with this is the startling fact that these bacteria are only physically separated from GI tissue residence/invasion by a single layer of epithelial cells and a covering of mucus (varying in depth from approximately 200 to 700 μm). In many respects, it is interesting that we do not develop overt inflammation of the GI tract more frequently.

Nevertheless, we are dependent on the development and the presence of a normal gut flora for a healthy gut epithelium, for digestion and for the development and function of the immune system [12]. Defining what constitutes a 'normal' gut

flora is still a work in progress and depends in part on what is sampled (faeces or mucosa-associated bacteria) [13] and on other factors such as ones underlying genotype [14], diet [15], age [16] and any recent antibiotic use. The colonisation of the GI tract begins at birth (with commensals from the mother's vagina or skin, depending on delivery via birth canal or C-section respectively), and then slowly develops over 2–3 years into that consistent with an adult faecal signature. Some elements of the commensal bacterial flora, such as *Faecalibacterium prausnitzii* [17, 18] (or *F. prau*) in humans, or certain Clostridial species in mice [19], appear to have crucial anti-inflammatory properties. Conversely, the presence of 'pathobionts' such as adherent-invasive *Escherichia coli* (AIEC) [20–22] correlates with an increased risk of ileal CD.

There is observational and experimental evidence that the presence of the microbiota is necessary in driving, if not definitively initiating, GI inflammation. In humans, antibiotics can ameliorate CD [23], faecal stream diversion is an effective therapy for Crohn's [24, 25], and lamina propria T cells from CD patients are reactive to gut flora [26]. Murine models of colitis are for the most part dependent on the presence of the microbial flora to develop colitis (an exception being the anti-CD40 model). Moreover, under certain conditions, it is possible to transfer a colitogenic flora from one mouse model to another [27].

Patients with Crohn's disease have an altered microbiome or 'dysbiosis', and disease severity corresponds with the degree of difference from healthy controls [28]. The main difference in CD appears to be that the microbial community is more unstable over time and less diverse, for example, with the loss of Bacteroidetes and certain Firmicutes, and an increase in Proteobacteria [29]. It remains unclear how much of this dysbiosis is a primary aetiological event, and how much is secondary to an already established inflammation.

The nature of the aberrant inflammatory response in Crohn's has been demonstrated genetically, in murine models and in human studies. The significance of IL-23 and the Th17 pathway is highlighted by polymorphisms in *IL23R*, *IL12B* (encoding for IL-12p40), *STAT3*, *JAK2* and *TYK2* [30]. Both innate and T cell-dependent models of murine colitis demonstrate a key role for IL-23. In innate colitis, IL-23 directs the expression of IL-17 from innate lymphoid cells [31]. In T cells, IL-23R signalling leads to enhanced Th17 polarisation and reduced $FoxP3^+$ T cell differentiation and IL-10 expression [32]. Finally, in human studies, IL-23 expression is increased in the mucosa of IBD patients [33].

2 NOD2 and Crohn's Disease

The role of NOD2 within this complex GI environment is an area of active research, and some progress has been made in the 14 years since the gene was identified. NOD2 is constitutively expressed in GI epithelial cells [34], as well as in myelomonocytic cells such as dendritic cells (DCs) [35, 36]. NOD2 has two N-terminal caspase-recruitment domains (CARDs), a central nucleotide-binding

oligomerisation domain (NOD) and a C-terminal leucine-rich repeat (LRR) domain. The polymorphisms that predispose to CD occur in (or adjacent to in the case of R702W) the LRR domain that functions as the MDP ligand-recognition domain. Three single nucleotide polymorphisms (one frame-shift: FS1007insC and two missense: G908R and R702W), account for over 80 % of those identified [37]. Homozygosity, or compound heterozygosity, confer a 17-fold increased risk of developing CD, whilst around 40 % of European CD patients carry at least one mutation [38] versus 14 % of healthy controls. Mutations in NOD2 predispose to terminal ileal CD [38], early-onset disease [37, 39] and possibly a stricturing phenotype [40].

NOD2 activation, on recognition of MDP, is thought to result in oligomerisation [41, 42] and recruitment of an adaptor protein RIPK-2 [43] via a CARD–CARD domain interaction. This leads, via an incompletely understood signalling cascade, to NFκB activation [36] and pro-inflammatory cytokine production. NOD2 is just one of a number of innate immune pattern recognition receptors (PRRs) expressed by antigen-presenting cells such as DCs. Others include the membrane-bound toll-like receptors (TLRs), the C-type lectin receptors (CLRs) and the cytosolic Nod-like receptors (NLRs) of which NOD2 is a member. These PRRs typically respond to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Immune activation by whole microbes is therefore complex, and it is the combination of PRR triggering by a given organism, plus the nature of the cellular microenvironment, that will influence the nature of the subsequent immune response.

Although there is now a large literature on the function of NOD2, the key findings can be summarised reasonably succinctly. Firstly, NOD2 induces autophagy on bacterial recognition in both dendritic [44] and epithelial cells [45], helping to clear the invasive bacteria and (in DCs) facilitating MHC class II antigen presentation. Autophagy is an intrinsic cytosolic process in which the formation of double-membrane vesicles facilitates the degradation of intracellular proteins (macro-autophagy) [46], bacteria (xenophagy) [47] or mitochondria (mitophagy) [48]. The role of autophagy in Crohn's pathogenesis only became apparent when genetic studies revealed polymorphisms in both ATG16L1 and IRGM [30]. The subsequent studies linking NOD2 and xenophagy are important in that they coalesce apparently unrelated CD polymorphisms around the same defective pathway.

Secondly, NOD2 is highly expressed in specialised small intestinal epithelial cells, called Paneth cells [34, 49], which are themselves most numerous in the terminal ileum [50]. Paneth cells are located in the crypts of Lieberkühn, and amongst other functions they secrete anti-microbial peptides, such as defensins. NOD2 polymorphisms are associated with decreased alpha defensin production (HD-5 and HD-6) from Paneth cells [51, 52], raising the question as to whether this is a primary defect in CD pathogenesis. However, there is some continued debate as to whether this apparent reduction owes more to inflammation rather than NOD2 expression [53] or whether the reverse is true [54]. In certain cases, the lack of defensins is due to other pathways altogether, such as defective Wnt signalling [55, 56].

Lastly, as already described, NOD2 is only one of a broad range of innate immune receptors. Large-scale gene-expression studies reveal that NOD2 can synergise with other PRRs, and that this synergy is lost in the presence of CD-variant NOD2 [57–59]. Wild-type NOD2, which in isolation has a relatively weak effect [57], has a key role in amplifying the release of certain pro-inflammatory cytokines, particularly interleukin-1β (IL-1β), IL-6 and IL-23 from DCs and macrophages [60, 61]. It is mooted that this NOD2-driven amplification of TLR responses is in keeping with the cytosolic expression of the NOD2 protein, because MDP stimulation may be indicative of invasive bacterial infection.

3 NOD2 and miR-29

One of the questions that arise from the cytokine data is why the CD-variant NOD2 should predispose to a pro-inflammatory condition. PRR signalling pathways that induce effector responses, including cytokines, need to be tightly regulated in order to limit bystander damage and terminate the immune response. microRNAs (miRNAs), the main function of which is to target messenger RNA (mRNA), are key regulators of gene expression in mammalian cells. Prior to our investigation, a number of studies of TLR signalling had established a role for miRNAs in the negative regulation of innate immune responses [62–66]. We hypothesised that wild-type NOD2 triggering would contribute to miRNA expression and that the absence of this in CD-variant NOD2 would lead to aberrant cytokine expression and the immunopathology seen in Crohn's.

We used human monocyte-derived dendritic cells (MDDCs) to explore miRNA expression initially after NOD2 and/or TLR2 stimulation [67]. TLR2 responds to Pam_3CSK_4 that, along with MDP, is a component of bacterial cell wall peptidoglycan, meaning that TLR2 is very likely to be co-triggered with NOD2. An miRNA array identified not only the already-described miR-155 and miR-146 as downstream of TLR2 triggering but also miR-29 expression as a novel miRNA in relation to innate immune signalling. Further investigation established that wild-type NOD2 stimulation, and the presence of the adaptor molecule RIPK-2, is critical for the up-regulation of miR-29 and that this is amplified by the co-stimulation of TLR2 or TLR5. Interestingly, this effect is not dependent on the TLR-signalling adaptor molecule MyD88. miR-29 is part of a miRNA family expressed from two clusters on chromosomes 1 and 7 (mir-29a, b and c), and these miRNAs possess identical seed sequences, therefore targeting the same mRNAs. miR-29 expression in MDDCs is detectable from 12 h after NOD2 + TLR2 stimulation and peaks at around day 3. This delayed expression pattern would be in keeping with a role as a negative regulator of immune response, allowing effector mechanisms to work before appropriate termination.

In order to identify potential miR-29 targets in MDDCs, we transfected NOD2- and TLR2-stimulated DCs with miR-29 premiR to artificially increase miR-29 expression. We used large-scale gene-expression microarrays to subsequently

identify a number of differentially regulated genes and went on to validate these by quantitative PCR (qPCR). These genes included a number of pro-inflammatory and immune pathway mediators, and one of the most strongly down-regulated genes identified by this methodology was IL-12p40. IL-12p40 is a predicted target of miR-29 (Targetscan) and is a cytokine subunit of both IL-12 (with IL-12p35) and IL-23 (with IL-23p19). We established that miR-29 directly targets IL-12p40 mRNA via the 3′UTR and indirectly down-regulates IL-23p19, but not IL-12p35.

We went on to demonstrate that NOD2-directed control of miR-29 is relevant at physiological miRNA expression levels. Firstly, we utilised an IL-12p40 3′UTR seed target protector that was designed for miR-29 binding sites. MDDCs transfected with this protector and then stimulated with NOD2 and TLR2 ligands for 24 h express elevated levels of IL-12p40. Secondly, we identified patients with ileal CD and who are homozygous for CD-variant NOD2 polymorphisms. MDDCs from these patients fail to up-regulate miR-29 when NOD2 is triggered and, perhaps more importantly, they express higher levels of IL-12p40 after AIEC infection than their wild-type counterparts. This discrepancy is rescued by the transfection, or replacement, of miR-29 using a premiR.

We next explored the role of miR-29 in vivo using mice with a targeted deletion of the *miR-29a/b-1* locus (henceforth mir-29 KO mice). We investigated whether a lack of miR-29 in a murine model alters the development or susceptibility to colitis in a DSS model. miR-29 KO mice show an increased susceptibility to colitis (1.7-fold higher compared to WT littermates) and exhibit more severe pathological scores and weight loss. In keeping with the human in vitro data, we demonstrated a marked Th17 transcriptional signature from inflamed colonic tissue. This included elevated expression of the miR-29-targeted *Il12b* and *Il23a*, as well as the mRNA encoding cytokine IL-17A, and the Th17 subset-determining transcription factor RORγt. This contrasts with the transcription factors GATA-3, T-bet and Foxp3 which are essentially unchanged. Moreover, there is no general change in pro- or anti-inflammatory mediators between wild-type and mir-29 KO mice, with similar colonic expression of *Il1b*, *Tnfa* and *Il6* and *Il10*. In other murine models, miR-29 targets IFN-γ in NK cells, $CD4^+$ and $CD8^+$ T cells [68], and through targeting of T-bet and Eomes in T cells influences Th1 bias [69], but in our model we found no difference in IFN-γ expression or Th1 cell numbers in colonic tissue.

4 Conclusion

In summary, our data suggest that wild-type NOD2 has an important role, via the expression of miR-29, in ensuring that the critical balance of pro- and anti-inflammatory responses is maintained within the GI tract (Fig. 1). There is evidence that IL-23 expression is particularly prominent within the terminal ileum [70], which would align with the known phenotype of NOD2-associated Crohn's. We would propose that the defective expression of miR-29 observed in the presence of

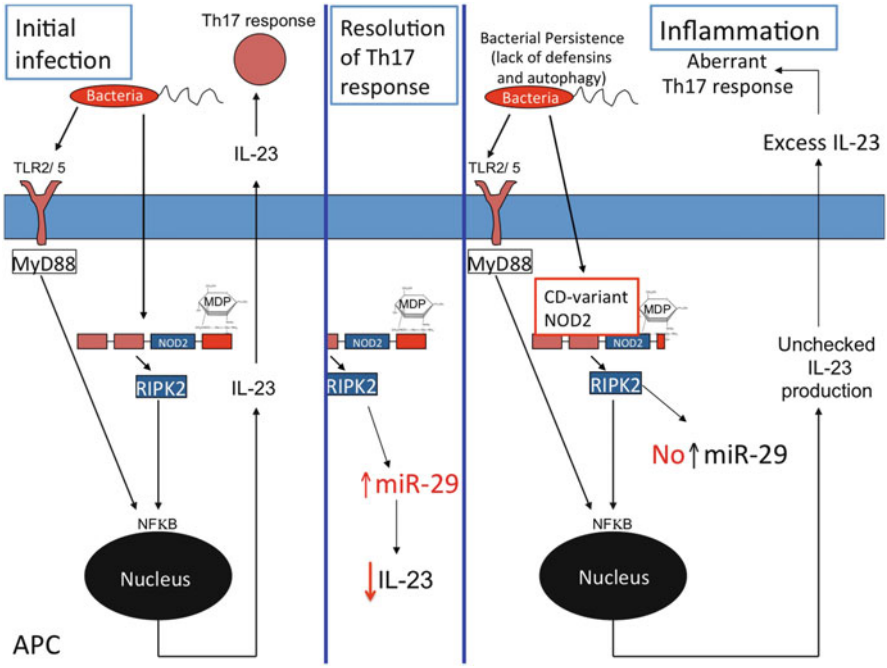

Fig. 1 A representation of the role of NOD2-stimulated miR-29 expression in antigen presenting cells (APCs) such as gastrointestinal DCs. In the *left-hand panel* the cell is challenged by bacterial infection and mounts an appropriate Th17 immune response. The *central panel* details appropriate resolution of this response through delayed miR-29 expression in the presence of wild-type NOD2. The *right-hand panel* depicts the problems that occur in the presence of CD-variant NOD2, with initial bacterial persistence due to defective autophagy, and a subsequently unchecked Th17 response due to lack of miR-29 up-regulation

CD-variant NOD2 should be placed in context of other NOD2 data [71]. In this model excess inflammation in the terminal ileum would result from a dysbiosis (possibly related to defensin deficiency), aberrant bacterial handling and persistence due to defective autophagy and an inability to appropriately arrest the resultant Th17 response due to deficient miR-29 expression.

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