Immunity
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The Intracellular Sensor NOD2 Induces MicroRNA-29 Expression in Human Dendritic Cells to Limit IL-23 Release

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http://dx.doi.org/10.1016/j.immuni.2013.08.035

SUMMARY

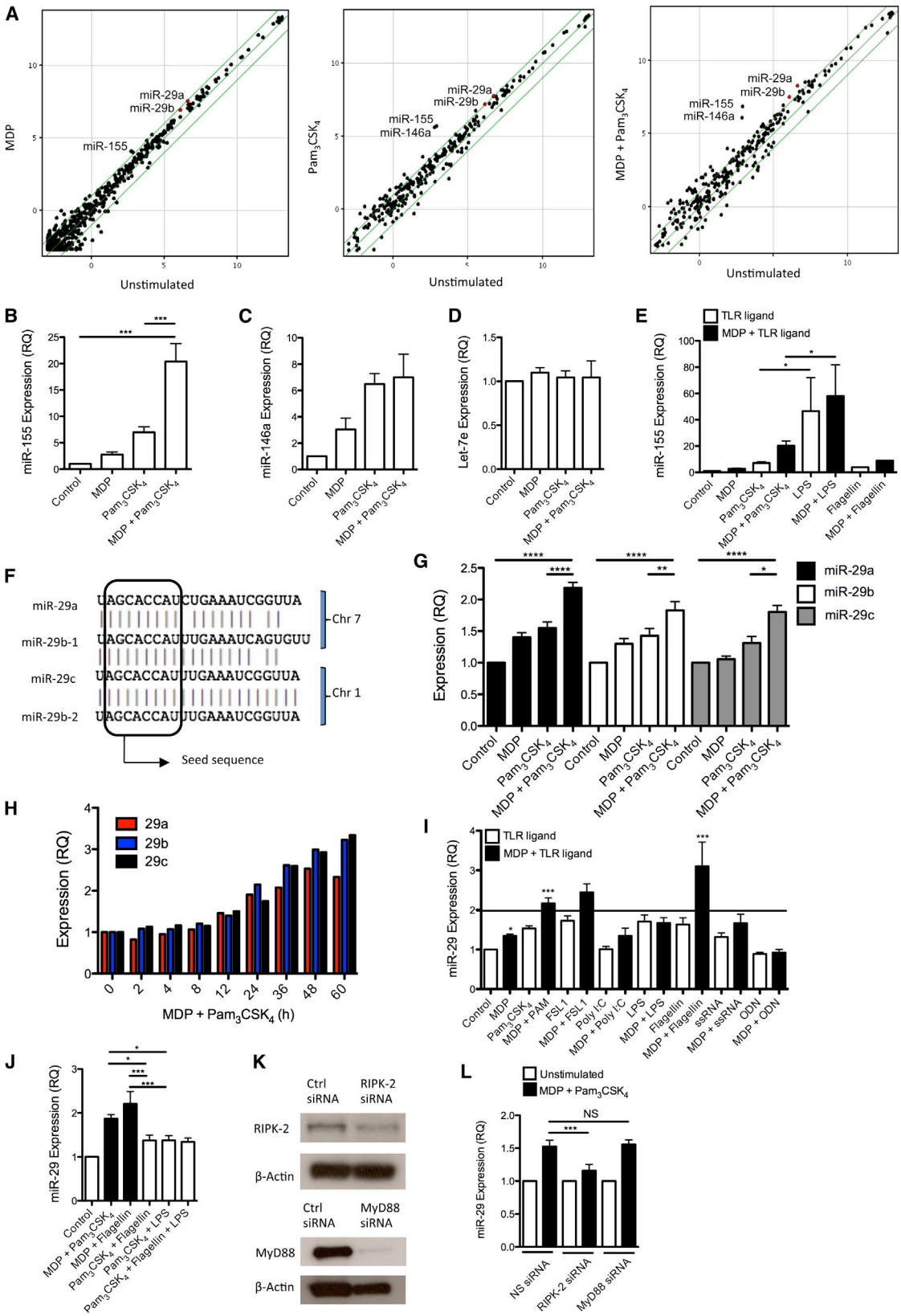
NOD2 is an intracellular sensor that contributes to immune defense and inflammation. Here we investigated whether NOD2 mediates its effects through control of microRNAs (miRNAs). miR-29 expression was upregulated in human dendritic cells (DCs) in response to NOD2 signals, and miR-29 regulated the expression of multiple immune mediators. In particular, miR-29 downregulated interleukin-23 (IL-23) by targeting IL-12p40 directly and IL-23p19 indirectly, likely via reduction of ATF2. DSS-induced colitis was worse in miR-29-deficient mice and was associated with elevated IL-23 and T helper 17 signature cytokines in the intestinal mucosa. Crohn's disease (CD) patient DCs expressing NOD2 polymorphisms failed to induce miR-29 upon pattern recognition receptor stimulation and showed enhanced release of IL-12p40 on exposure to adherent invasive *E. coli*. Therefore, we suggest that loss of miR-29-mediated immunoregulation in CD DCs might contribute to elevated IL-23 in this disease.

INTRODUCTION

NOD2 is a cytosolic pattern recognition receptor (PRR) that controls immunity against intracellular bacteria and inflammatory responses. NOD2 recognizes muramyl dipeptide (MDP), an integral component of bacterial cell walls, and is expressed in monocyte lineage cells, intestinal epithelial cells, and Paneth cells. Three polymorphisms in this gene are present in 40% of Western Crohn's disease (CD) patients (Cuthbert et al., 2002), causing amino-acid substitutions Arg702Trp and Gly908Arg and the frameshift FS1007insC, all found within a leucine-rich repeat region that is responsible for MDP recognition (Inohara et al., 2003).

The molecular mechanism by which NOD2 functions is not completely defined; in particular, the mechanism by which it signals in dendritic cells (DCs). Like other PRRs, it can induce NF-κB activation (Ogura et al., 2001), but in comparison with PRRs, such as the Toll-like receptors (TLRs), this effect is rather weak (Uehara et al., 2005). Large-scale gene-expression studies have shown that NOD2 can synergize with other PRRs in differential gene regulation and that this synergy is lost in cells expressing Crohn's variant NOD2 (Uehara et al., 2005; van Heel et al., 2005; Yang et al., 2001). NOD2 plays a key role in amplifying release of certain proinflammatory cytokines in this context, particularly interleukin-1β (IL-1β), IL-6, and IL-23, from DCs and macrophages (van Beelen et al., 2007; Kobayashi et al., 2005). IL-6 and IL-23 are required for induction of T helper 17 (Th17) $CD4^+$ T cells, a response important for antimicrobial immunity at mucosal surfaces and a hallmark of the inflammatory response in Crohn's. The significance of the IL-23 and Th17 cell pathway for Crohn's pathogenesis is highlighted by genetic studies, with polymorphisms in *IL23R*, *IL12B* (encoding IL-12p40), *STAT3*, *JAK2*, and *TYK2* all contributing to disease predisposition (Franke et al., 2010). A key role for IL-23 in intestinal inflammation has been demonstrated in both innate and T cell-dependent experimental models of colitis (Yen et al., 2006; Uhlig et al., 2006). IL-23R signaling in T cells leads to enhanced Th17 accumulation, reduced differentiation of $FoxP3^+$ T cells, and reduced T cell IL-10 production (Ahern et al., 2010). In innate colitis, IL-23 directs expression of IL-17 and induction of pathology via innate lymphoid cells (ILCs) (Buonocore et al., 2010). IL-23 is increased in mucosa of IBD patients (Liu et al., 2011) and increased ILCs are present (Geremia et al., 2011), emphasizing the importance of this axis in controlling inflammation in colitis.

IL-23 is produced by dendritic cells and macrophages and its release is mediated by NOD2 in combination with TLRs (Lyakh et al., 2008). It is important that PRR signaling pathways inducing effector cytokines such as IL-23 are tightly regulated so that homeostasis can be restored at the termination of an immune



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response. One way in which that might be achieved is via microRNAs (miRNAs), key regulators of gene expression whose main function is to repress target messenger RNA (mRNA) levels in mammalian cells. Studies of miRNA expression induced through TLR stimulation have demonstrated that they function as negative regulators of innate immune responses by targeting key signaling proteins and cytokines (Ceppi et al., 2009; Taganov et al., 2006; Bazzoni et al., 2009; Sheedy et al., 2010; Tili et al., 2007). We hypothesized that NOD2 might modulate miRNA expression to downregulate inflammatory responses induced after receptor stimulation and that NOD2 variants associated with Crohn's might be deficient in this process, leading to inadequate arrest of antimicrobial effectors at the end of an immune response.

We find that NOD2 can regulate miRNA expression in DCs. Of particular interest, NOD2 is required for induction of the miRNA family 29a, 29b, and 29c and induces this family alone or additively with TLR2 or TLR5. By expressing miR-29 mimic and undertaking large-scale gene-expression profiling, we find that miR-29 downregulates IL-12p40/IL-23 and attenuates Th17 $CD4^+$ T cell responses in vitro. We examined Crohn's disease DCs expressing associated NOD2 variants and found they were incapable of inducing miR-29 following NOD2 triggering. This effect was associated with enhanced release of IL-12p40 in response to adherent invasive *E.coli*, bacteria found in increased numbers in the mucosa of CD patients (Martinez-Medina et al., 2009; Darfeuille-Michaud et al., 2004). miR-29a knockout (KO) mice show worsened colitis on DSS challenge, together with raised IL-23 levels and Th17 signature genes in the intestinal mucosa. We have therefore identified expression of the miR-29 family as a new immunoregulatory function of NOD2 in human DCs, and a loss of miR-29 induction in Crohn's DCs might contribute to the abnormal elevation of IL-23 observed in inflamed lesions during this disease.

RESULTS

NOD2 Affects miRNA Expression in DCs and Induces miRNA Family 29a, 29b, and 29c

To explore whether NOD2 triggering by MDP could induce differential miRNA expression in DCs, we stimulated immature monocyte-derived DCs expressing wild-type (WT) NOD2 with MDP and subjected them to miRNA microarray analysis at 24 hr. Only miR-29 was induced upon NOD2 triggering alone (Figure 1A; see Table S1 available online). By comparison, stimulation of TLR2 with Pam_3CSK_4 led to robust differential regulation of miRNAs with strong induction of miR-155 and miR-146, previously described as being induced upon TLR triggering (Taganov et al., 2006; O'Connell et al., 2007) and DC maturation (Lyakh et al., 2008) (Figure 1A; Table S1). NOD2 cross talks with TLR2, and because both these PRRs recognize different components of peptidoglycan, it is likely they would normally be cotriggered upon bacterial recognition. Dual stimulation with MDP and Pam_3CSK_4 led to synergistic differential regulation of a number of miRNAs regulated by TLR2 alone, and in addition greatly increased induction of miR-29 induced by NOD2 (Table S1). Furthermore, we observed differential expression of a number of new miRNAs not observed with single TLR2 or NOD2 activation (Table S1), emphasizing the ability of NOD2 to crosstalk with other PRRs.

To confirm accuracy of the microarray data, we analyzed the miRNAs most strongly induced by NOD2 +TLR2 cotriggering by quantitative PCR (qPCR). NOD2 had a synergistic effect on TLR2 upregulation of miR-155 (Figure 1B), but not miR-146a (for which NOD2 stimulation contributed little to overall induction via TLR2) (Figure 1C) or miR-Let-7e (where little induction occurred across all stimuli used) (Figure 1D). miR-155 is induced by PRR signaling, and the effect of NOD2 on miR-155 induction was examined in comparison with a panel of other PRRs. Activation of TLR4 by lipopolysaccharide (LPS) dwarfed the effect of NOD2 + TLR2 triggering on miR-155 expression, suggesting that NOD2 does not play a major role in miR-155 regulation when DCs encounter microbes (Figure 1E).

miR-29 forms part of a miRNA family expressed from two clusters on chromosomes 1 and 7, and possessing identical seed sequences, therefore targeting the same endogenous mRNAs (Figure 1F). The ability of NOD2 to regulate expression of miR-29 family members after exposure of DCs to MDP, Pam_3CSK_4, or a combination of these two ligands was examined. miR-29a, miR-29b and miR-29c induction after NOD2 + TLR2 stimulation was first detected at 12 hr after stimulation and peaked at around day 3 (Figures 1G and 1H). In contrast to the effect of NOD2 on

Figure 1. NOD2 Regulates miR-29 Family Expression in Human DCs

(A) Representation of differential regulation of miRNA expression observed by miRNA microarray analysis in DCs stimulated for 24 hr with MDP 1 μg/ml (left panel), Pam_3CSK_4 1 μg/ml (middle panel), and MDP + Pam_3CSK_4 combined (right panel). Plots show miRNAs more than 2-fold upregulated by these stimulation conditions, compared with unstimulated DCs.

(B) Quantitative real-time PCR (qPCR) analysis of miR-155 expression in DCs stimulated for 24 hr with MDP, Pam_3CSK_4, or MDP + Pam_3CSK_4 all at 1 μg/ml, compared with unstimulated cells (control) and relative to noncoding small RNA control RNU44. qPCR analysis as for (B) for miR-146a (C) and miR-Let-7e (D).

(E) qPCR analysis of miR-155 expression following stimulation with combinations of PRR ligands alone or in combination as indicated.

(F) Sequence comparison of miR-29a, -29b, and -29c, which form part of two clusters expressed from chromosomes 7 and 1 showing their identical seed sequences.

(G) qPCR analysis of miR-29a, 29b, and 29c in DCs stimulated for 24 hr with MDP and/ or Pam_3CSK_4 (1 μg/ml).

(H) qPCR analysis of miR-29a, 29b, and 29c in MDP + Pam_3CSK_4 1 μg/ml stimulated DCs over time.

(I and J) qPCR of miR-29a expression in DCs stimulated by a panel of PRR ligands alone or in combination as indicated. Ligand concentrations are as follows: MDP, Pam_3CSK_4, LPS, Flagellin, ssRNA, FSL-1 at 1 μg/ml; Poly I:C 10 μg/ml; CpG type A ODN2216 1 μM.

(K) Immunoblot analysis of RIPK-2 and MyD88 expression following transfection of DCs with control non-sense siRNA (NS siRNA) or either RIPK-2 or MyD88 siRNAs.

(L) DCs treated as in (K) were left unstimulated or stimulated for 24 hr with MDP + Pam_3CSK_4 1 μg/ml, and miR-29 expression determined by qPCR analysis. Statistical analysis by one-way ANOVA with Bonferroni post-test, *p = 0.01 to 0.05, **p = 0.001 to 0.01, ***p < 0.001 and ****p < 0.0001. Data are from four biological replicates for (A), and three or more independent experiments for (B)–(L). Error bars show SEM. See also Table S1.

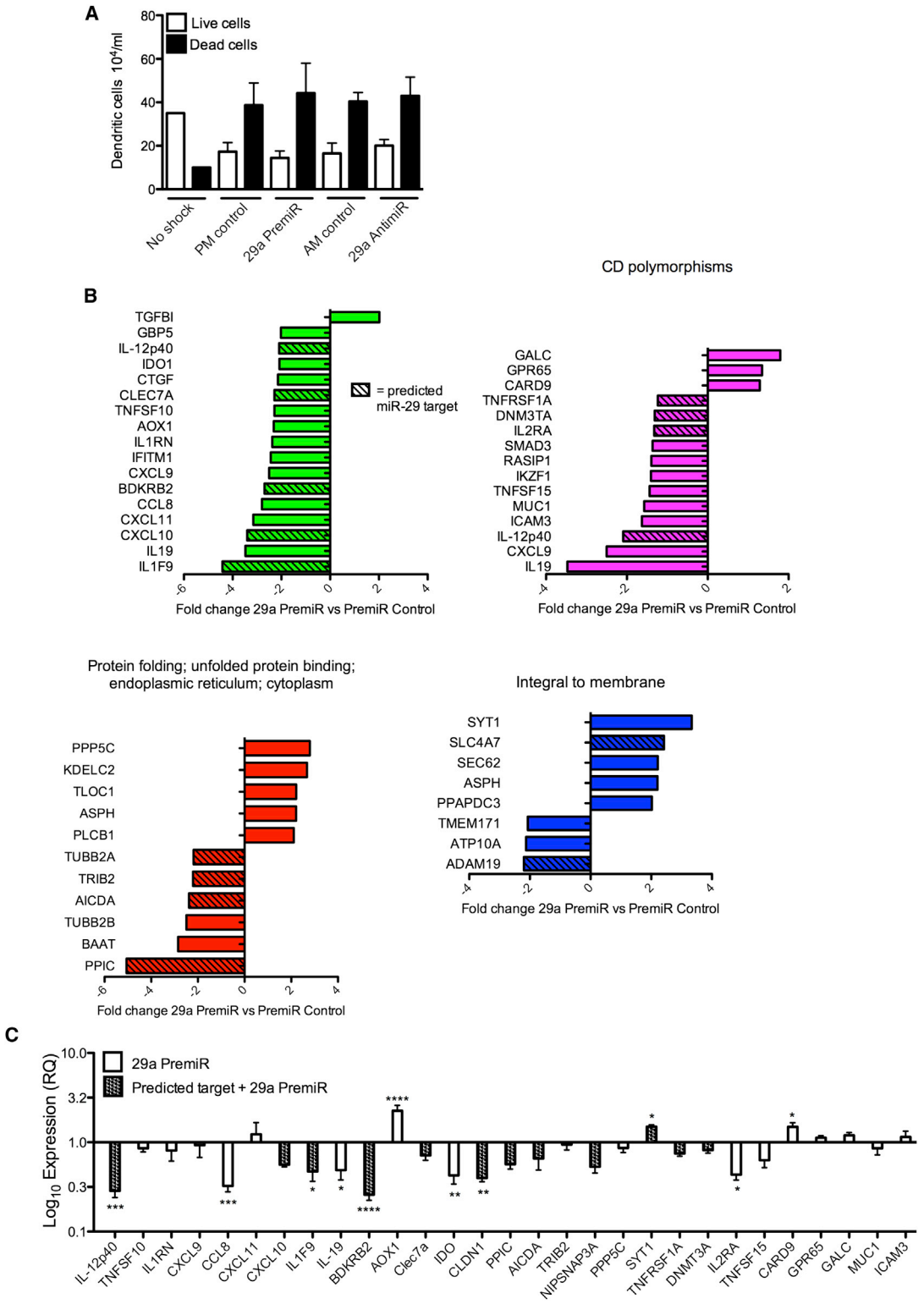

Figure 2. miR-29 Regulates Immune and Inflammatory Mediator Expression in DCs

(A) The ratio of live to dead cells assessed with trypan blue staining, in DCs transfected with miR-29 premiR or antimiR or controls for 24 hr before stimulation with MDP + Pam_3CSK_4 1 μg/ml for 24 hr.

(B) DCs transfected with miR-29 premiR or PM control for 16 hr were then stimulated with MDP + Pam_3CSK_4 1 μg/ml for 8 hr prior to Agilent whole human-gene-expression microarray analysis. Shown are representation of differential gene expression within immune response and inflammatory response genes, Crohn's polymorphisms, protein folding and endoplasmic reticulum (ER) stress response, and membrane proteins.

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miR-155 expression, NOD2 played a dominant role in the induction of miR-29, where greatest upregulation was observed with either NOD2 + TLR2 triggering or NOD2 + TLR5 triggering (Figure 1I). NOD2 was absolutely required for this effect because combined TLR2 + TLR5 triggering did not result in miR-29 upregulation (Figure 1J).

NOD2 signaling requires RIPK-2, but not the TLR adaptor MyD88. To test whether miR-29 expression required either of these signaling mediators for its upregulation upon MDP + Pam_3CSK_4, we knocked down MyD88 or RIPK-2 in DCs by using siRNAs (Figure 1K). We then undertook qPCR analysis of miR-29 expression following MDP + Pam_3CSK_4 exposure and demonstrated a requirement for RIPK-2, but not MyD88, in miR-29 induction (Figure 1L). These data show that NOD2 induces expression of the miR-29 family members 29a, 29b, and 29c over time after MDP treatment of DCs, and requires RIPK-2 to exert this effect.

miR-29 Regulates the Expression of Multiple Inflammatory Genes in DCs Including *IL12B*

miRNAs act by translational inhibition, followed by deadenylation and decay of their target mRNA (Djuranovic et al., 2012; Bazzini et al., 2012). NOD2 + TLR2-stimulated DCs, transfected with miR-29 premiR to artificially increase miR-29 expression, were examined by gene-expression microarray analysis to identify potential target genes among mRNAs that were differentially regulated. miR-29 affects expression of genes controlling cell death, such as MCL-1 (Mott et al., 2007). However, the miR-29 premiR, or an antimiR (antagomir) blocking miR-29 activity, did not significantly alter rate of cell death, as identified by Trypan blue staining (Figure 2A). Large-scale gene-expression profiling was undertaken of NOD2 + TLR2-stimulated DCs transfected with either miR-29 premiR or control. We determined differential gene regulation as significant if there were a 2-fold change in expression in biological replicates in comparison with controls. The differentially regulated mRNAs included clusters of genes previously described as functioning in immune or inflammatory pathways, protein folding and unfolded protein response, membrane proteins, and as IBD susceptibility genes (Figure 2B). We confirmed a number of these putative targets as being differentially regulated by miR-29 by qPCR (Figure 2C). These include CCL8, Bradykinin receptor 2 (BDKR2), Claudin-1 (CLDN-1), Indoleamine 2,3-dioxygenase (IDO), and IL-2 receptor alpha chain (IL-2RA). miR-29 upregulates Aldehyde oxidase 1 (AOX1) and CARD9. One of the most strongly downregulated genes identified by this methodology was IL-12p40. Thus miR-29 exerts broad control over a number of inflammatory and immune pathway genes, including the known IBD susceptibility gene, IL-12p40.

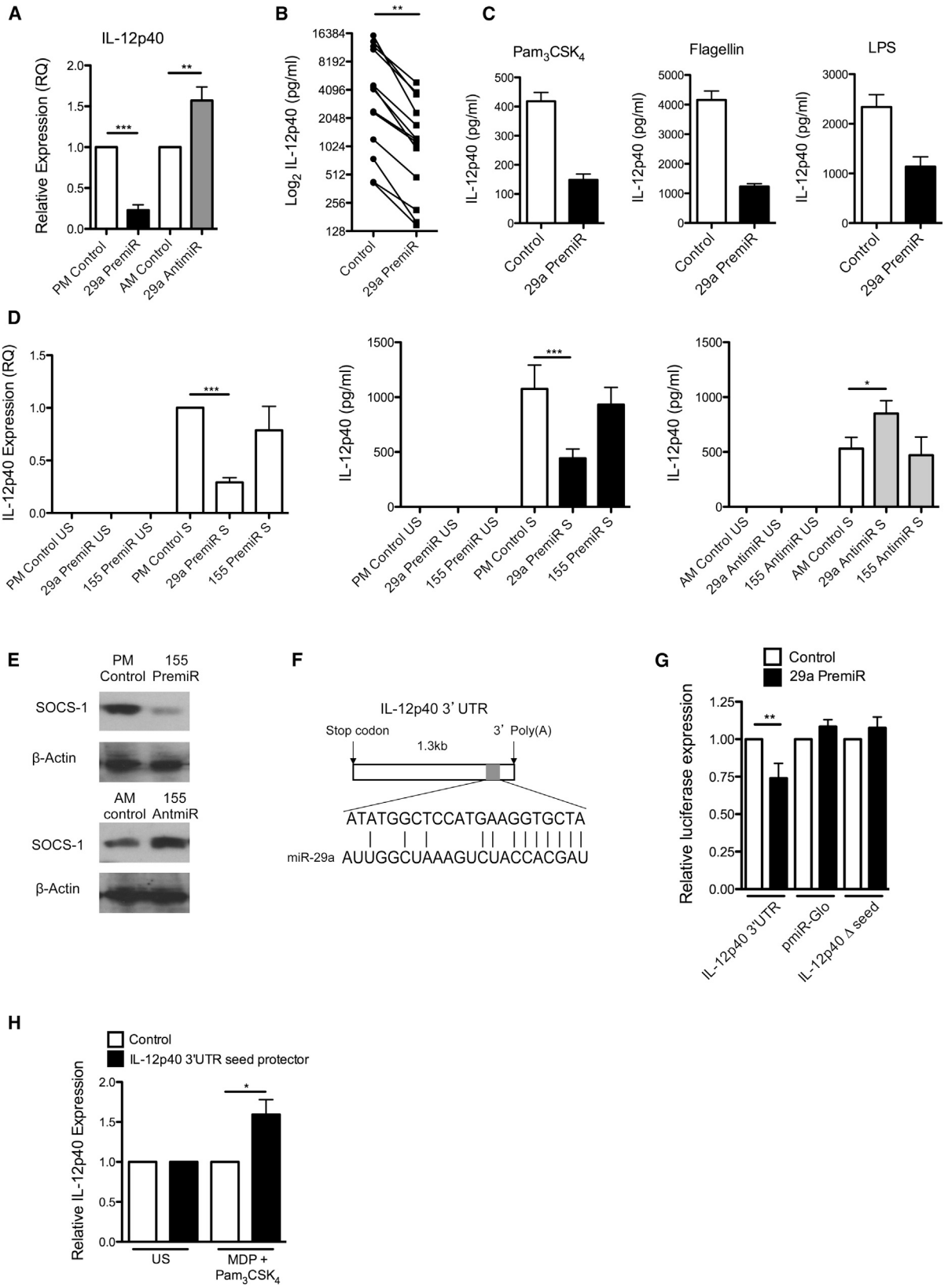
miR-29 Targets and Downregulates IL-12p40

IL-12p40 was the most strongly downregulated gene after expression of miR-29, and it is a predicted to be a direct target of miR-29 by computation algorithms. We therefore further investigated the role of miR-29 in control of IL-12p40. Expression of miR-29 in DCs led to marked downregulation of both IL-12p40 mRNA and cytokine release (Figures 3A and 3B), and this was irrespective of the PRR stimulus used to induce IL-12p40 (Figure 3C). Moreover, blocking the effect of miR-29 with an antimiR in MDP+ Pam_3CSK_4 treated DCs increased IL-12p40 expression (Figure 3A). We transfected varying doses of miR-29 premiR into DCs to ensure the reduction of IL-12p40 observed in the presence of miR-29 occurred with amounts of miR-29 that are physiologically relevant to that induced by NOD2 triggering normally. IL-12p40 was also downregulated after expression of miR-29 premiR at levels of miR-29 similar to that detectable during induction of all three miR-29 family members upon NOD2 and TLR2 stimulation (Figure S1).

miR-155 enhances inflammatory T cell development and Th17-relevant cytokines in mice (O'Connell et al., 2010). We investigated whether miR-155, which is induced strongly on PRR triggering in either murine or human DCs, might affect IL-12p40 expression in human DCs and thus negate the effect of miR-29 on IL-12p40 repression. We observed no effect of miR-155 premiR or antimiR on IL-12p40 mRNA or protein expression in MDP + Pam_3CSK_4-stimulated DCs, indicative of different targeting pathways operative between mice and human cells in this instance (Figure 3D). Neither miR-29 nor miR-155 premiR or antimiR had any effect on IL-12p40 mRNA or protein in unstimulated DCs (Figure 3D). We confirmed that miR-155 premiR and antimiRs were biologically active after DC transfection by examining their effect on a known miR-155 target in human DCs, SOCS-1 (Lu et al., 2011). Figure 3E shows the effect of miR-155 mimic and antimiR on SOCS-1 expression in DCs, as expected from previous reports (Lu et al., 2011).

miR-29 is predicted to directly target the IL-12p40 3′ UTR (Figure 3F). We cloned the 3′ UTR (1.3 kb) of IL-12p40 mRNA into a vector downstream of a reporter gene encoding luciferase. We transfected HEK293 cells with this vector, or control empty vector, along with miR-29 premiR for 48 hr. Reporter gene expression was suppressed by 25% in cells carrying the vector containing the predicted binding site for the miR-29 family (Figure 3G). We confirmed these results by mutating the IL-12p40 3′ UTR seed sequence (Figure S2) and found that suppression of reporter gene expression was reversed in the presence of the mutant 3′ UTR. To compliment this assay, we transfected an IL-12p40 3′UTR seed protector into DCs prior to NOD2 + TLR2 triggering. A miScript Target Protector (QIAGEN) was designed for miR-29 binding sites in IL-12p40 3′ UTR mRNA. Cells were transfected with IL-12p40 miR-29 seed-target protector or negative-control target protector and subsequently stimulated for 24 hr with MDP and Pam_3CSK_4 1 μg/ml. Cells transfected with IL-12p40 seed-target protector induced IL-12p40 expression to a greater extent than cells without the IL-12p40 3′UTR seed-sequence target protector present (Figure 3H) These experiments identify IL-12p40 as a target of miR-29 in human DCs.

(C) qPCR analysis of genes identified as differentially expressed by Agilent microarray analysis versus GAPDH control, calculated by the change in threshold ($\Delta\Delta C_T$) method. DCs were transfected for 24 hr with miR-29 premiR or PM control, prior to MDP + Pam_3CSK_4 1 μg/ml stimulation for 24 hr. Significant results, by one-way ANOVA with Bonferroni post-test (*p = 0.01 to 0.05, **p = 0.001 to 0.01, ***p < 0.001 and ****p < 0.0001). Data are from four independent experiments (A), from three biological replicates for array (B), and four independent experiments for qPCR (C). Error bars show SEM.



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miR-29 Downregulates IL-23

IL-12p40 is a subunit of both IL-12 and IL-23, the other subunits being IL-12p35 and IL-23p19, respectively. We investigated whether miR-29 controlled expression of either of these other two subunits that act in concert with IL-12p40. miR-29 premiR downregulated expression of IL-12p40 and IL-23p19, but not IL-12p35 (Figure 4A). miR-29 also downregulated IL-23p19 protein released from DCs upon NOD2 + TLR2 triggering (Figure 4B). We tested the specificity of our results by analyzing whether miR-29 overexpression affected the expression of other cytokines involved in Th17 cell or immunoregulatory responses. miR-29 did not affect expression of IL-6, TGF-β, or IL-10 mRNA (Figure 4C) or of IL-10 and IL-6 secretion (Figures 4D and 4E). In contrast to observations in murine DCs (O'Connell et al., 2010), we observed no effect of miR-155 premiR or antimiR on IL-23p19 expression (Figure 4F). As for IL-12p40, miR-29 only affected expression of IL-23p19 mRNA and protein after DCs had been stimulated to induce IL-23 by PRR ligands (Figure 4F).

We then investigated the mechanism by which miR-29 downregulates IL-23p19 expression in DCs. miR-29 is predicted to directly target the IL-23p19 3′ UTR (MicroCosm), although only via a weak seed-sequence pairing (Figure 4G). We used an IL-23p19 3′ UTR luciferase reporter assay to assess the effect of miR-29 on IL-23p19 3′UTR. However, there was no demonstrable effect on luciferase expression (Figure 4H) suggesting that miR-29 targets IL-23p19 indirectly. We next examined the microarray data obtained when miR-29 was expressed in DCs to find potential mechanisms for indirect targeting of IL-23p19 by miR-29. ATF2 and SMAD3 mRNAs were both downregulated after overexpression of miR-29 premiR in DCs, and both act as transcriptional activators of IL-23p19 in macrophages (Al-Salleeh and Petro, 2008). We therefore determined whether downregulation of ATF2 or SMAD3 affected expression of IL-23p19 mRNA in NOD2 + TLR2 stimulated DCs. We used siRNAs to reduce expression of ATF2 (Figure 4I) or SMAD3. ATF2 knockdown reduced IL-23p19 expression in stimulated DCs (Figure 4J), whereas SMAD3 knockdown had no effect (data not shown). We then transfected DCs with miR-29 premiR or control and found that miR-29 expression resulted in effective downregulation of ATF2 protein expression (Figure 4K). miR-29 expression in HEK293 cells did not target ATF2 in a 3′UTR luciferase assay, suggesting the effect of miR-29 on ATF2 is indirect (Figure S3). These results indicate that miR-29 downregulates IL-23p19 indirectly, at least in part via downregulation of its transcriptional activator ATF2.

Repression of IL-23 by miR-29 Decreases IL-17 Production in DC and T Cell Cocultures

The functional relevance of miR-29 repression of IL-12p40 and IL-23p19 on the magnitude of Th17 responses was then explored. DCs were transfected with miR-29 premiR or control before DC coculture with $CD4^+$ T cells. IL-17 production by T cells was then assessed at 72 hr. This revealed significant reduction in IL-17 production from cocultures where DCs expressed miR-29 (Figure 5A). The effect on IL-17 production mediated by miR-29 was comparable to that observed with anti-IL-23 blocking antibodies (Figure 5B) and could be reversed by addition of recombinant IL-23 to DC T cell cocultures (Figure 5C). In contrast to its ability to downregulate IL-17 release from DC T cell cocultures, DC-expressed miR-29 had no effect on interferon-γ (IFN-γ) expression (Figure 5D).

Exacerbated, Th17-Associated Intestinal Inflammation in miR-29-Deficient Mice

Because IL-23 drives colitis in animal models (Yen et al., 2006; Uhlig et al., 2006), we investigated whether lack of miR-29 lowers the threshold for development of intestinal inflammation in vivo. Unlike in human cells, we found that the expression of miR-29 family members was not substantially regulated after stimulation with NOD2 or TLR2 ligands. This was evident with both in vitro-derived BMDCs and splenic $CD11c^+$ cDCs isolated after NOD2 and TLR2 triggering in vivo (Figures S4A and S4B).

To explore the role of miR-29 in control of IL-12p40 in vivo, we used mice with a targeted deletion of the *miR-29a/b-1* locus (hereafter called "miR-29 KO mice") (Papadopoulou et al., 2012) or WT littermate controls with intact miR-29. We found that transcription of the miR-29 target gene, *Il12b*, was substantially enhanced in BMDCs lacking miR-29 after 24 and 48 hr of stimulation, compared to WT controls, with this dysregulation leading to enhanced IL-12p40 protein production by miR-29 KO BMDCs at 72 hr after stimulation (Figures S4C and S4D). To establish whether miR-29 is capable of repressing IL-12p40

Figure 3. miR-29 Regulates IL-12p40 by Directly Targeting the 3′UTR

(A) qPCR analysis of IL-12p40 expression in DCs following miR-29 premiR or antimiR or control transfection, and MDP + Pam_3CSK_4 1 μg/ml stimulation, expressed as relative fold change to control. Statistical analysis by one-way ANOVA with Bonferroni post-test **p = 0.001 to 0.01, and ***p < 0.001.

(B) DCs were transfected with miR-29 premiR or control and stimulated with MDP + Pam_3CSK_4 1 μg/ml for 24 hr prior to IL-12p40 ELISA.

(C) IL-12p40 ELISA of DCs treated as in (B) but stimulated with TLR2, TLR4, or TLR5 ligands at 1 μg/ml for 24 hr (analysis by two-tailed paired t test **p = 0.001 to 0.01).

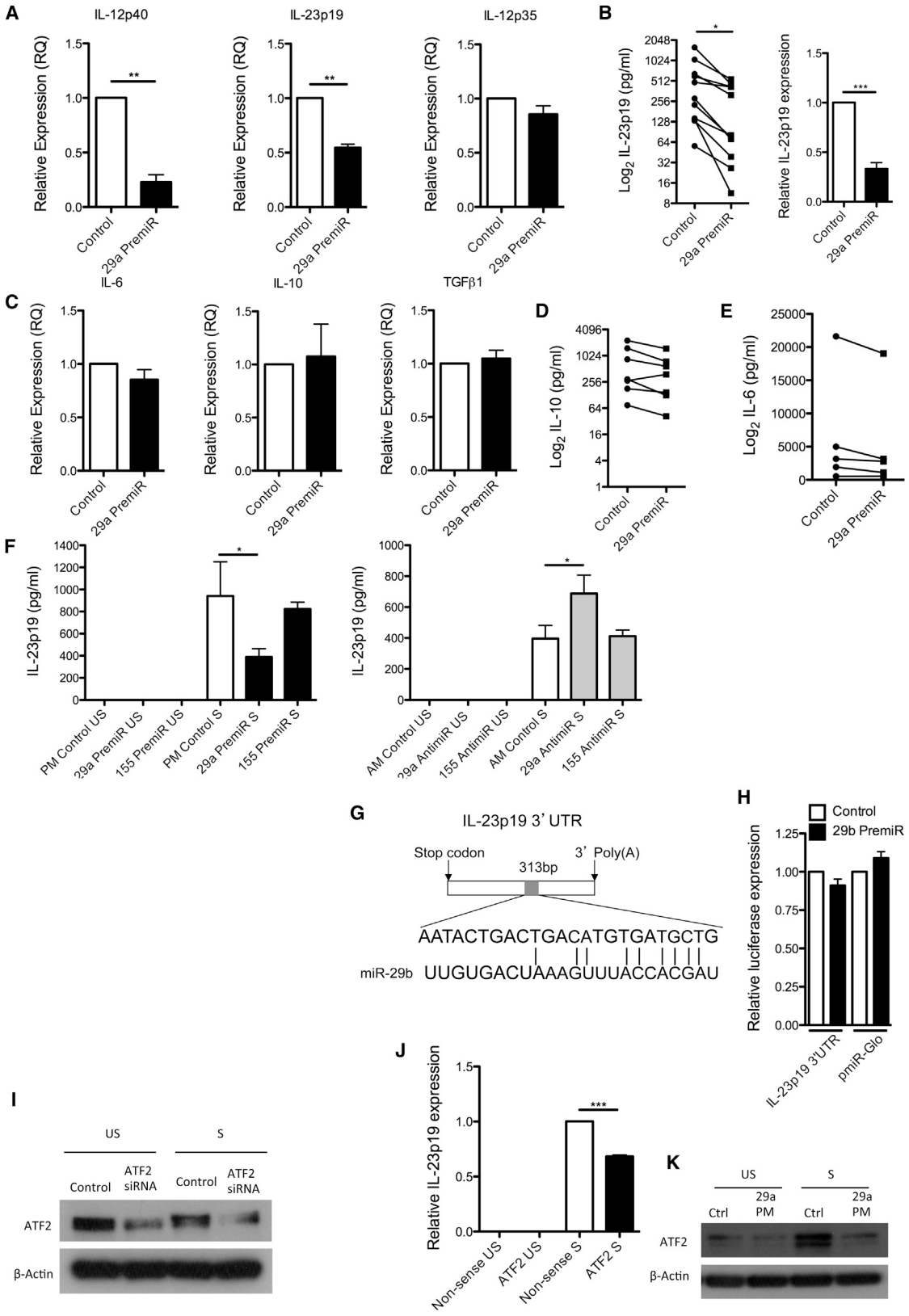
(D) qPCR of IL-12p40 expression in DCs transfected with control, miR-29 premiR, or miR-155 premiR for 24 hr and left unstimulated or stimulated with MDP + Pam_3CSK_4 1 μg/ml for a further 24 hr (left panel); IL-12p40 ELISA in the same DCs (middle panel); IL-12p40 ELISA in DCs transfected with control, miR-29, or miR-155 antimiR for 24 hr and stimulated with MDP + Pam_3CSK_4 1 μg/ml for 24 hr (right panel).

(E) DCs were transfected with control or miR-155 premiR (top panels) or control and miR-155 antimiR (bottom panels) and immunoblot for SOCS-1 and actin performed.

(F) IL-12p40 3′UTR contains a potential target site for miR-29 (TargetScan).

(G) IL-12p40 3′UTR was cloned into pmiR-Glo dual luciferase vector. The vector was cotransfected with miR-29 premiR into HEK293 cells, and firefly luciferase was quantified at 48 hr (normalized to renilla luciferase activity). The same experiment was performed with the empty vector (pmiR-Glo), or a vector expressing a mutated miR-29 target sequence (IL-12p40 Δ seed).

(H) DCs were transfected with a negative control target protector or IL-12p40 seed-protector sequences and left unstimulated or stimulated with MDP + Pam_3CSK_4 1 μg/ml for 24 hr prior to IL-12p40 qPCR. Statistical analysis by one-way ANOVA with Bonferroni post-test **p = 0.001 to 0.01. Data are from 4 independent experiments (A), 13 independent experiments (B), 2 independent experiments (C), 3 independent experiments (D), 7 independent experiments (F), 8 independent experiments (G), and 3 independent experiments (H). Error bars show SEM. See also Figures S1 and S2.



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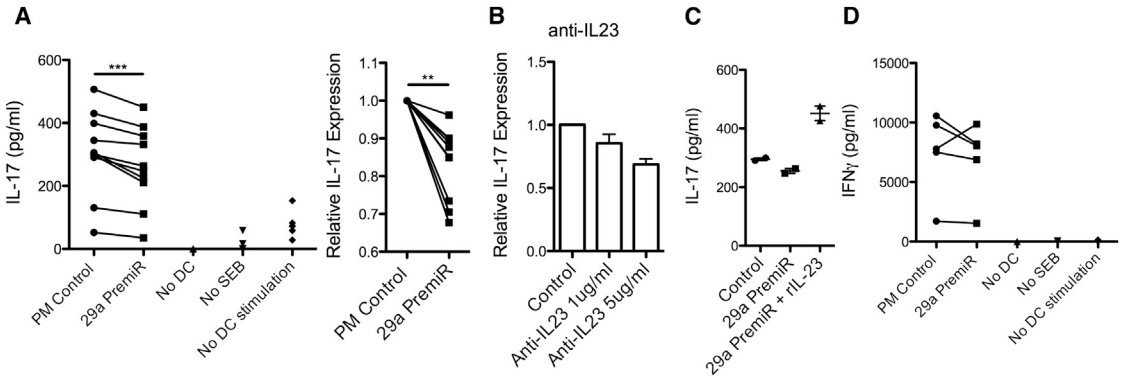


Figure 5. Control of IL-17 Production from T Cells via miR-29

(A) We cocultured 1 × 10^6 $CD4^+$ T cells with 1 × 10^5 DCs, with 100 pg/ml SEB. DCs were transfected with miR-29 premiR or PM control for 8 hr and stimulated with MDP + Pam_3CSK_4 1 μg/ml for 16 hr, before coculture. IL-17 production after 72 hr coculture was measured by ELISA (left panel) and qPCR (right panel). Significant differences by two-tailed paired t test **p = 0.001 to 0.01 and ***p < 0.001.

(B) Anti-IL-23 antibody was added to cocultures at 1 μg/ml or 5 μg/ml and IL-17 production assessed at 72 hr.

(C) DCs were transfected with miR-29 premiR or control prior to $CD4^+$ T cell coculture as before. rIL-23 was added at 0.75 ng/ml to cocultures with DCs expressing the miR-29 premiR.

(D) IFN-γ production from DC + T cell coculture, experimental conditions as for (B). Data are from more than three independent experiments (A and D) and two independent experiments (B and C). Error bars show SEM.

in murine DCs, miR-29 premiR or control were transfected into BMDCs pre- and post-NOD2 + TLR2 simulation and IL-12p40 mRNA was measured. miR-29 suppressed IL-12p40 expression in murine DCs to a similar extent as observed in human DCs (Figure S4E). We therefore sought to determine the contributions of miR-29 in an experimental model of mucosal pathology.

miR-29 KO mice or WT littermate controls were challenged with DSS. miR-29 KO mice showed an enhanced propensity to develop colitis (1.7-fold higher colitis incidence compared to WT mice, Figure 6A) and exhibited an enhanced pathological score and weight loss (Figures 6B and 6C). The enhanced severity of colitis was associated with a marked "Th17-type" transcriptional signature in distal colonic tissue. This included elevated expression of the miR-29-target genes *Il23a* and *Il12b* (Figures 6D and 6E), as well as mRNA encoding the key Th17 cytokines IL-17A and GM-CSF (Figures 6F and 6G); both of which are associated with Th17-mediated immunopathology (Griseri et al., 2012; Codarri et al., 2011). In addition, mRNA encoding the Th17 subset-determining transcription factor, RORγt, was elevated in distal colonic tissue of colitic miR-29-deficient mice compared to DSS-treated WT mice (Figure 6H), whereas GATA-3 and T-bet mRNA were either lower or unchanged, and Foxp3 mRNA was only slightly enhanced (Figure S5A–S5C). The enhanced colitis in miR-29-deficient mice was not associated with a general increase in inflammatory mediators. *Il1b*, *Tnfa*, and *Il6* mRNAs were elevated in colonic tissue to a similar degree in WT and miR-29 KO mice (Figure 6I), and a lack of miR-29 did not impact colonic expression of IL-10 (Figure S5D). miR-29 targets IFN-γ and influences Th1 bias in other murine models of miR-29 function (Ma et al., 2011; Steiner et al., 2011); however, we found no difference in Th1 cell numbers or IFN-γ in colonic tissue between control and miR-29-deficient mice (Figure 6) In addition, miR-29-deficient mice also demonstrated increased IL-23p19 protein production from intestinal mucosal tissue explants over controls after DSS challenge (Figure 6J). Genes that were modulated by the miR-29 premiR in human DCs, including *Cxcl9*, *Cxcl10*, *Clec7a*, *Cxcl11*, *IL1f9*, and *Ifitm1*, also showed changes in expression in the intestinal mucosa of colitic

Figure 4. miR-29 Preferentially Regulates IL-23 via Attenuation of IL-23p19

(A) DCs were transfected with miR-29 premiR or control and stimulated with MDP + Pam_3CSK_4 1 μg/ml for 24 hr. Graphs show qPCR analysis of IL-12p40, IL-23p19, and IL-12p35.

(B) IL-23p19 ELISA from DCs treated as in (A). Significant differences by two-tailed paired t test **p = 0.001 to 0.01 and ***p < 0.001.

(C) DCs transfected with miR-29 premiR or control and MDP + Pam_3CSK_4 1 μg/ml were stimulated. IL-6, IL-10, and TGFβ were assessed by qPCR and (D) IL-10 and (E) IL-6 by ELISA.

(F) ELISA of IL-23p19 expression from DCs transfected with control, miR-29 premiR, or miR-155 premiR for 24 hr and left unstimulated or stimulated with MDP + Pam_3CSK_4 1 μg/ml for a further 24 hr (left panel). IL-23p19 ELISA in DCs transfected with control, miR-29, or miR-155 antimiR for 24 hr and stimulated with MDP + Pam_3CSK_4 1 μg/ml for 24 hr (right panel).

(G) IL-23p19 3′UTR contains a potential miR-29 target site (MicroCosm).

(H) IL-23p19 3′UTR was cloned into pmiR-Glo dual luciferase vector. The vector was cotransfected with miR-29 premiR into HEK293 cells, and firefly luciferase quantified at 48 hr (normalized to renilla luciferase activity).

(I) DCs were transfected with siRNAs to ATF2 for 24 hr and left unstimulated or stimulated with MDP + Pam_3CSK_4 1 μg/ml for 24 hr prior to immunoblot for ATF2.

(J) qPCR for IL-23p19 expression in DCs treated as in (I).

(K) DCs were transfected with miR-29 premiR (29a PM) or control and subsequently left unstimulated (US) or stimulated with MDP + Pam_3CSK_4 1 μg/ml (S). Immunoblot for ATF2 shown. Statistical analysis by one-way ANOVA and Bonferroni post-test (***p < 0.001). Data are from three or more independent experiments from (A)–(F) and from (H)–(K). Error bars show SEM. See also Figure S3.

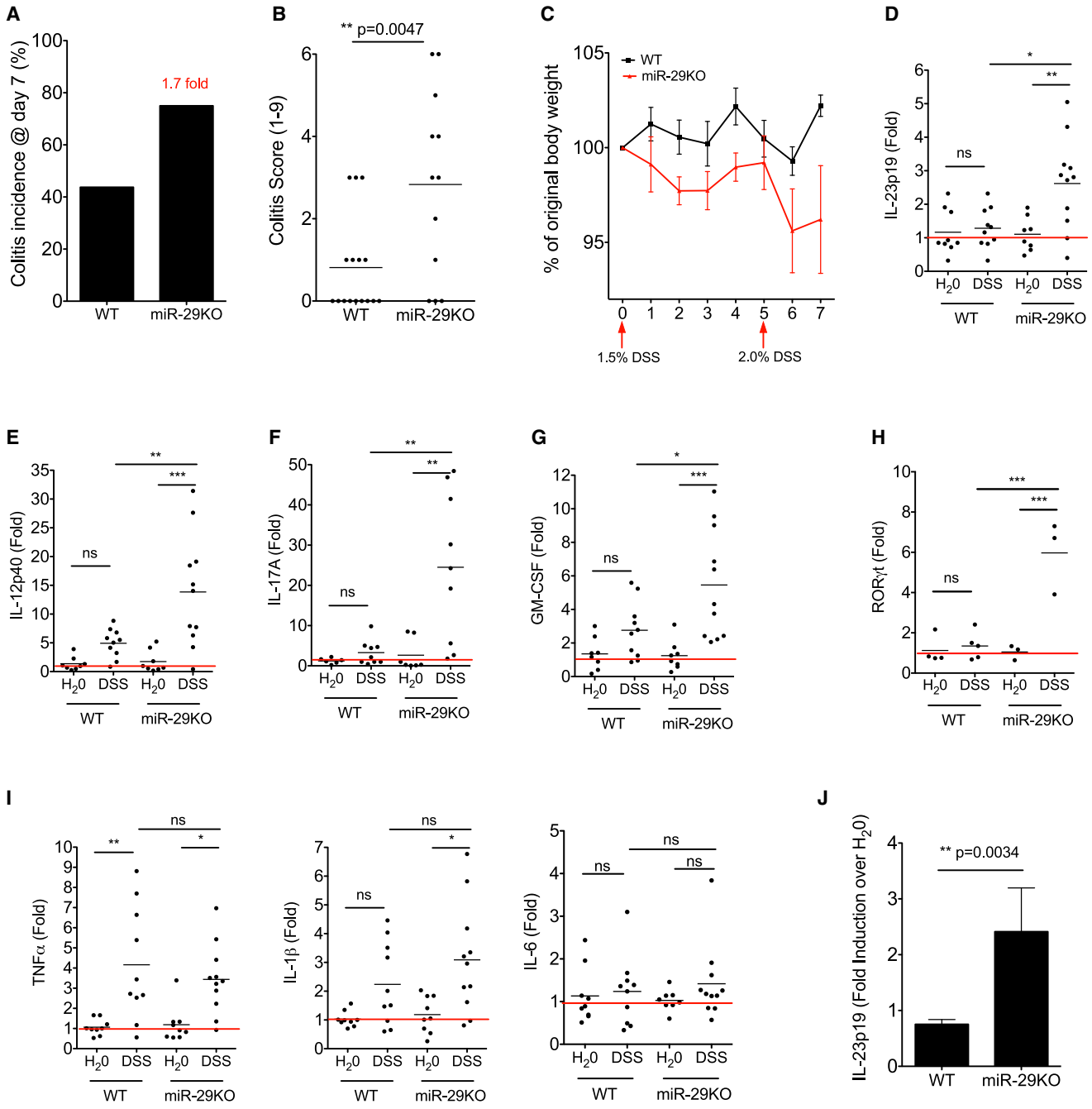


Figure 6. miR-29 Deficient Mice Develop Exacerbated Intestinal Inflammation Associated with an Enhanced Th17 Transcriptional Signature in Colonic Tissue

(A–H) miR-29 KO mice and WT littermates received low-dose DSS in drinking water for 7 days. Weight loss was monitored, and on day 7 mice were sacrificed, colitis scored, and distal colonic tissue assessed for mRNA abundance of indicated genes. (A) and (B) show colitis incidence and severity of DSS-treated WT or miR-29-deficient mice, and (C) shows weight loss curves. Expression of IL-23p19 (D), IL-12p40 (E), IL-17a (F), GM-CSF (G), and RORγt (H) mRNA in distal colonic tissue was determined as the fold change in transcript abundance in colitic mice relative to mice of each genotype receiving H_20.

(I) Shows expression of the indicated mRNA in distal colonic tissue determined as the fold change in transcript abundance in colitic mice relative to mice of each genotype receiving H_20.

(J) Shows IL-23p19 protein in distal colonic explant cultures from mice of each genotype, expressed as the fold change in expression in from colitic mice compared to those receiving H_20. Data are pooled from two independent experiments with n = 3–12 mice per group; data in (H) is from one experiment with n = 3–5 mice per group. Data are represented as mean ± SEM. See also Figures S4–S7.

miR-29 KO mice, providing further parallels between miR-29 targeting in the murine model and human DCs (Figure S7).

Defective miR-29 Expression and Increased IL-12p40 Release in Human DCs Expressing NOD2 Variants

We next investigated whether Crohn's patient DCs expressing variants of NOD2 associated with the disease exhibited defects in miR-29 upregulation on either NOD2 or NOD2 + TLR triggering. Patient DCs either homozygous for 1007fsinsC NOD2 expression or compound heterozygous for any of the Crohn's-associated NOD2 polymorphisms fail to induce miR-29 on stimulation of NOD2, NOD2 + TLR2, or NOD2 + TLR5 combined stimulation (Figures 7A and 7B). In contrast, these Crohn's donor DCs induced miR-155 similarly to WT NOD2 expressing DCs following PRR triggering (Figure 7C). Restoring miR-29 expression in Crohn's donor DCs expressing associated NOD2 polymorphisms effectively downregulated IL-12p40 (Figure 7D) as previously observed in healthy donors. We explored whether loss of miR-29 induction by CD DCs expressing NOD2 polymorphisms might contribute to dysregulated IL-12p40 release from these cells on exposure to intestinal bacteria. We challenged healthy or CD DCs with adherent invasive *E. coli* (AIEC) and measured release of IL-12p40 at day 3, day 5, and day 7 after exposure. We found enhanced release of IL-12p40 in CD donor cells at days 5 and 7 after challenge (Figure 7E). AIEC treatment of healthy donor or CD donor DCs showed no change in expression of miR-29 in the CD donor cells over a week after exposure to these bacteria (Figure 7F). Finally we assessed whether introduction of miR-29 premiR into CD donor DCs could also reduce the amount of IL-12p40 release after bacterial exposure and again found efficient reduction of IL-2p40 after transfection of miR-29 mimic, but not premiR control, into either healthy donor or CD donor cells exposed to AIEC (Figure 7G). In contrast, introduction of miR-29 antimiR exaggerated IL-12p40 release from healthy donor cells but had no effect on IL-12p40 levels expressed in CD donor cells. These observations demonstrate that Crohn's donor DCs show defects in induction of miR-29 that correlate with enhanced IL-12p40 release following DC exposure to AIEC, suggesting that this might contribute to increased IL-23 production observed in the intestinal mucosa during this disease.

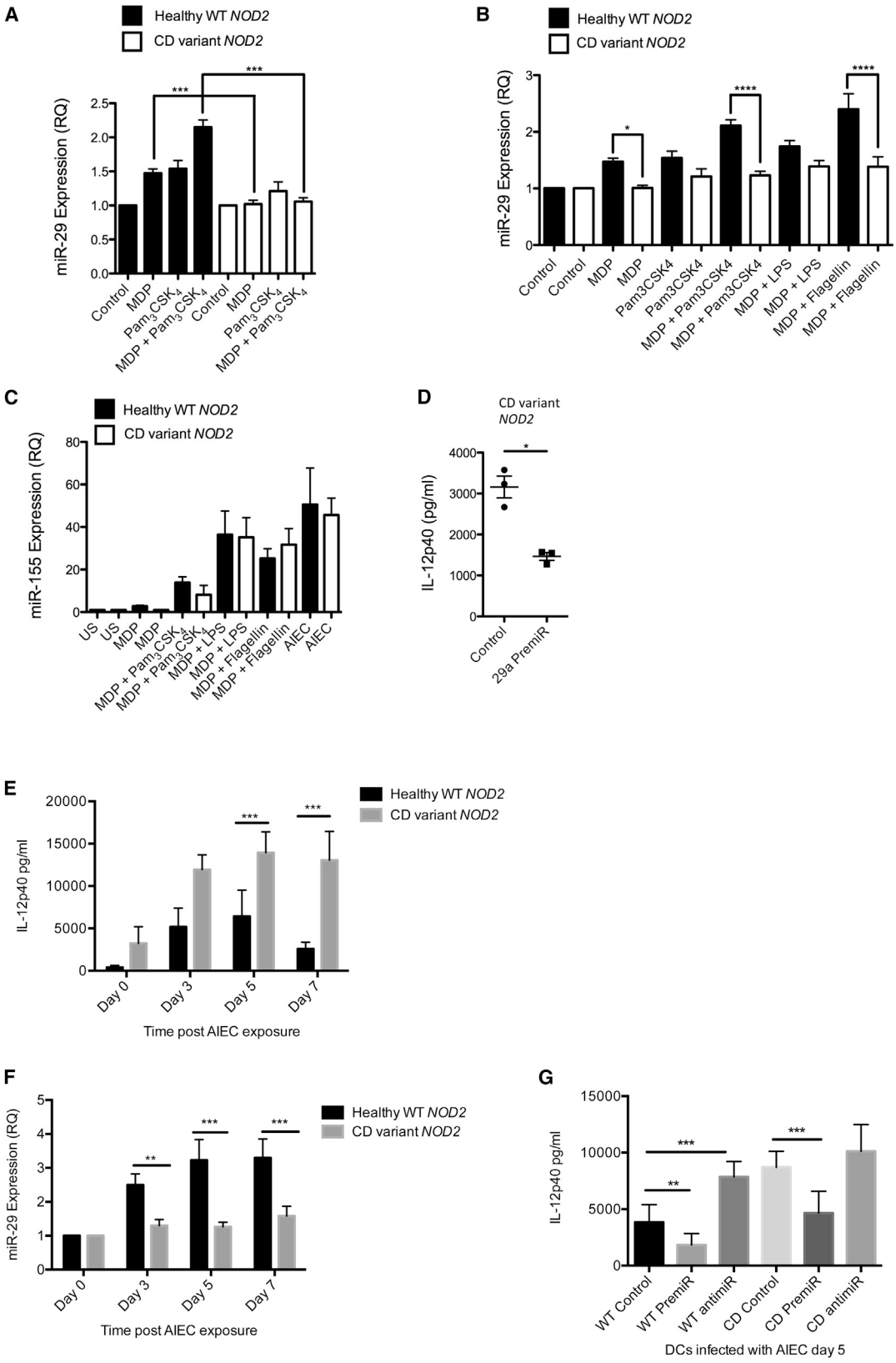
DISCUSSION

In this work, we demonstrate that NOD2 can control miRNA expression in human cells. Similar to its effects on mRNA expression, NOD2 plays a role in synergizing with other PRRs in inducing key miRNAs, such as miR-155 in DCs. In addition to this effect, NOD2 plays an exclusive role in directing expression of the miR-29 family, in contrast to other PRRs expressed in these cells. This ability of NOD2 requires the NOD2 signal transducer RIPK-2, but not the TLR signal transducer MyD88, indicating that RIPK-2 plays a key role in mediating NOD2 crosstalk with other PRRs to induce miR-29 expression. By using both computational and biological approaches, we define a series of new genes regulated by miR-29 in human DCs including CCL8, involved in neuroinflammation (Banisor et al., 2005), Bradykinin receptor 2 (BDKR2), implicated in IL-12 induction from dendritic cells (Aliberti et al., 2003), Claudin-1 a tight junction protein required for DCs to penetrate gut epithelial monolayers to sample bacteria (Rescigno et al., 2001), Indoleamine 2,3-dioxygenase (IDO), a rate-limiting enzyme of tryptophan catabolism through the kynurenine pathway that is involved in innate immune responses (Matteoli et al., 2010), and IL-2 receptor alpha chain (IL2RA), an immune regulatory molecule expressed on myeloid DCs (Driesen et al., 2008). mir-29 also upregulates Aldehyde oxidase 1 (AOX1), induced as a stress response in plants (Polidoros et al., 2009) and CARD9 that is important for innate responses against yeast (Vautier et al., 2010).

The gene most strongly regulated by miR-29 was IL-12p40. 3′UTR luciferase assays and seed-protector experiments provide evidence for miR-29 directly targeting IL-12p40. In contrast, miR-29 appears to indirectly target IL-23p19, at least in part by suppressing its transcriptional activator ATF2, to control IL-23 released from DCs. NOD2 and TLRs are key regulators of IL-23 production by cells of the innate immune system such as DCs and macrophages (Napolitani et al., 2005), so induction of miR-29 controlled by NOD2 represents a key intrinsic homeostatic mechanism to switch off this critical driver of Th17 responses.

IL-23 plays a key role in driving colitis, and we find that miR-29-deficient mice develop worsened colitis on DSS challenge associated with increased expression of Th17-associated genes and IL-23, as opposed to general inflammatory signature genes, in the intestinal mucosa, consistent with a physiological role for miR-29 in controlling Th17-mediated intestinal inflammation in vivo. We found that Crohn's patient DCs homozygous or compound heterozygous for NOD2 variants associated with the disease failed to induce miR-29 to any great degree upon NOD2 or NOD2 + TLR triggering. This was associated with increased release of IL-12p40 at later time points after infection with AIEC compared to controls. Loss of this immunoregulatory pathway in small bowel intestinal DCs responsible for producing copious amounts of IL-23 in response to microbes might contribute to the increased IL-23 observed in the intestinal mucosa in Crohn's patients. Increased basal IL-23 production could be important in driving inflammation in combination with other defects in NOD2 function observed in Crohn's, such as defects in autophagy induction (Cooney et al., 2010; Travassos et al., 2010) where bacterial persistence could provide an ongoing trigger for IL-23 production in the absence of intrinsic control mediated by miR-29. Indeed, increased inflammatory cytokine release from human antigen-presenting cells with defects in autophagy, including Crohn's donor cells, after exposure to bacteria has been observed (Lapaquette et al., 2012; Plantinga et al., 2011). It is also possible these effects would be increased in patients with coexisting *IL23R* polymorphisms that might require lower amounts of IL-23 to result in proinflammatory signaling. Furthermore, these effects could be compounded via Crohn's-associated NOD2 variants' active suppression of immunoregulatory IL-10 production via inhibition of hnRNP-A1 activity (Noguchi et al., 2009).

In the murine immune system, miR-29 exerts a number of immunoregulatory roles. miR-29 expressed in NK cells, $CD4^+$ T cells, and $CD8^+$ T cells downregulates IFN-γ expression, and suppression of miR-29 expression by transgenic expression of a sponge target reduces bacterial burden in mice exposed to *Listeria monocytogenes* or *Mycobacterium bovis* bacillus



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Calmette-Guerin (BCG) (Ma et al., 2011). Furthermore, miR-29 represses T-bet and Eomes in T cells to regulate helper T cell differentiation (Steiner et al., 2011). In addition, miR-29 targets IFN-α expression by thymic epithelium to increase the threshold for infection-associated thymic involution (Papadopoulou et al., 2012). Our work adds an immunoregulatory function for miR-29 to this repertoire in human antigen-presenting cells mediated by NOD2.

miR-29 is a key miRNA in a number of other cellular processes, and it is possible that NOD2 may function via modulation of miR-29 in other settings. For example, miR-29 regulates methylation of target genes by controlling expression of DNMT3A (Fabbri et al., 2007), which is also a recently described Crohn's-susceptibility gene (Franke et al., 2010). Altered DNA methylation via this pathway could modulate epigenetic pathways, maturation of DCs, and thus influence the nature of the adaptive immune response. miR-29 also associates with fibrosis via control of collagen expression (Maurer et al., 2010; Roderburg et al., 2011). NOD2 polymorphisms in Crohn's are associated with a subphenotype of the disease—stricturing disease (Seiderer et al., 2006)—where fibrosis occurs in the small bowel causing obstructive symptoms and requiring surgical intervention. It is possible that the inability of Crohn's NOD2 to induce miR-29 might contribute to this phenotype. NOD2 mutations are also strongly associated with disease of the terminal ileum (Cuthbert et al., 2002), and our data is compatible with this phenotype as IL-12p40 and IL-23p19 are most highly constitutively expressed in the terminal ileum (Becker et al., 2003), increasing the need for effective regulatory mechanisms in this mucosa.

NOD2 also influences the composition of the microbiome (Rehman et al., 2011). Although some of this effect might be explained by reduced expression of Paneth cell defensins (Wehkamp et al., 2004) observed in the presence of Crohn's variant NOD2, it is possible that increased basal IL-23 in the mucosa mediated by loss of miR-29 might facilitate this effect. It will be interesting to see the effect of NOD2 expression on miRNAs in epithelial cells and Paneth cells, in which expression of factors affecting barrier function might be controlled.

In summary, we have shown that NOD2 is critical for induction of miR-29 in DCs and defined a number of new miR-29 regulated genes in these cells, including those affecting IL-23 expression. Simultaneous expression by NOD2 of the key immune effector IL-23 and a molecular switch to arrest its induction illustrates the elegant ability of innate immune receptors to activate timely defense mechanisms while shutting off harmful immune responses. It is likely that other PRRs will operate such paradigms and that these may be disrupted in inflammatory diseases, an important area for future study.

EXPERIMENTAL PROCEDURES

Preparation of Human Monocyte-Derived Dendritic Cells and NOD2 Genotyping

$CD14^+$ monocytes were positively selected (anti-CD14 microbeads; Miltenyi Biotech) from peripheral blood mononuclear cells (PBMCs), from either WT NOD2 donors or homozygous mutant NOD2 Crohn's patients (Research Ethics Committee Reference: 07/H0603/43). For details of Crohn's patients, see Supplemental Information. Monocytes were cultured together with IL-4 and GM-CSF (Peprotech). Immature dendritic cells were harvested on day 5 of culture. For NOD2 genotyping, PCR of NOD2 polymorphisms (R702W, G908R, FS1007insC) was performed prior to sequencing. For details of oligonucleotides used, see Supplemental Information.

Cell Stimulations, miRNA Microarrays, and qPCR of miRNAs

We left 5×10^6 DCs unstimulated, stimulated with 1 μg/ml MDP or 1 μg/ml Pam_3CSK_4 (Invivogen), or both for 24 hr. In some experiments, a PRR ligand panel was used and consisted of lipopolysaccharide (LPS) 1 μg/ml, Poly I:C 10 μg/ml, ssRNA 1 μg/ml, CpG type A ODN2216 1 μM, HKLM 10^8 cells/ ml, FSL-1 1 μg/ml, and flagellin 1 μg/ml (Invivogen). For miRNA microarrays, four biological replicates were used. RNA was extracted (miRNeasy; QIAGEN), and RNA quality checked with RNA 6000 Nano Assay on Agilent bioanalyzer 2100. Total RNA was hybridized to Agilent human single color miRNA arrays. miRNA microarrays represented 866 human and 89 human viral miRNAs sourced from Sanger miRBase (release 12.0). Results were analyzed with Genespring. Raw microarray data for this experiment has been submitted to GEO (NCBI) (GSE numbers GSE44784 and GSE44785). For qPCR of miRNAs, RNA was prepared as before. Reverse transcription to cDNA was achieved with miRNA-specific primers (Applied Biosystems) prior to qPCR (TaqMan; Applied Biosystems). Noncoding small RNA control RNU44 (Applied Biosystems) served as an endogenous reference gene, with changes in expression calculated by the change in threshold ($\Delta\Delta C_T$) method. For miRNA knockdown or overexpression and miRNA Target Identification, see Supplemental Information.

Immunoblots, Antibodies, ELISAs

We transfected 3×10^6 DCs with siRNAs, final concentration 5 nM (RIPK-2 SI02758833; MyD88 SI00300909; QIAGEN) or non-sense control (AllStar Negative Control, QIAGEN) by nucleofection (Lonza). Immunoblot was used to confirm knockdown with anti-human antibodies: anti-RIPK-2, 1:1000 (4982; Cell Signaling); anti-MyD88, 1:1000 (4283; Cell Signaling). For miRNA target confirmation, 3×10^6 DCs were transfected with miR-29 premiR or negative control, as described, and where indicated were stimulated with NOD2 ± TLR ligands. Anti-ATF2, 1:1000 (20F1; Cell Signaling); anti-SOCS-1 (A156), 1:1000 (3950; Cell Signaling) antibodies were used. For ELISAs, DCs

Figure 7. Crohn's Patients DCs Fail to Both Upregulate miR-29 and Control IL-12p40

DCs were derived from CD patients homozygous for FS1007insC NOD2 (10 patients) or R702W + G908R NOD2 compound heterozygotes (5 patients). All patients have terminal ileal disease, were in clinical remission, and were off all immunomodulators.

(A and B) qPCR analysis of miR-29 expression in DCs expressing WT NOD2 or Crohn's variant NOD2, after MDP and/or Pam_3CSK_4, or MDP + Flagellin, or MDP + LPS stimulation (1 μg/ml). Statistical analysis was performed by by one-way ANOVA and Bonferroni post-test (*p = 0.01 to 0.05, ***p < 0.001, and ****p < 0.0001).

(C) qPCR of miR-155 expression in DCs from healthy WT NOD2 expressing DCs versus CD donors expressing NOD2 polymorphisms pre- and poststimulation with panels of PRR ligands as above.

(D) IL-12p40 ELISA following MDP + Pam_3CSK_4 1 μg/ml stimulation in FS1007insC NOD2 DCs, after transfection of miR-29 premiR or control. Significant differences by two-tailed paired t test, *p = 0.01 to 0.05.

(E) IL-12p40 release in response to adherent invasive *E. coli* from healthy donor DCs versus CD DCs at days 0, 3, 5, and 7 after bacterial exposure.

(F) miR-29 expression in healthy donor DCs or CD donor DCs at day 0, 3, 5, and 7 following exposure to adherent invasive *E. coli*.

(G) IL-12p40 release from healthy donor DCs or CD donor DCs transfected with premiR control, miR-29 premiR, or miR-29 antimiR for 24 hr at day 5 after exposure to adherent invasive *E. coli*. Statistical analysis was performed by one-way ANOVA and Bonferroni post-test, **p = 0.001 to 0.01, and ***p < 0.001. Data are from 8 independent experiments (A), 14 independent experiments (B), 3 independent experiments (C–E) 2 independent experiments (F and G); error bars show SEM.

were stimulated with NOD2 ± TLR ligands as indicated, ±transfection of miRNA premiRs or antimiRs with appropriate negative controls, before supernatants were harvested after 48 hr unless otherwise indicated, and stored at −80°C. Human IL-12/IL-23p40, IL-6, IL-10, IL-12p70, IL-17 and IFN-γ Duosets, and Human IL-23 Quantikine ELISA Kit, (R&D) were used following standard protocols. For 3′ UTR luciferase reporter assays, see Supplemental Information.

DC + T Cell Coculture, Bacterial Infections, Colitis Challenge

DCs were prepared as described above. $CD4^+$ T cells were negatively selected from the remaining PBMCs with $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec). DCs were transfected with miRNA premiR or negative control for 8 hr before 16 hr stimulation with 1 μg/ml MDP and Pam_3CSK_4. We cocultured 1×10^5 DCs with 1×10^6 $CD4^+$ T cells in 12 well plates, with 100 pg/ml SEB. Recombinant IL-23 (1290-IL/CF; R&D, at 0.75 ng/ml or 5 ng/ml) or anti-IL23 antibody (AF1716; R&D, at 1 μg/ml or 5 μg/ml) was added to selected wells. Culture media and cells were collected for ELISA and qPCR. AIEC-GFP was a kind gift from Dr. Barry Campbell (Liverpool). For DC bacterial infections, we exposed 1×10^5 DCs to *E. coli* at an MOI of 25 for 1 hr and then in gentamicin (50 μg ml^{-1})-containing medium for the remaining time periods. For miR-29 KO mice, DSS challenge, colitis scoring, and murine qPCRs, see Supplemental Information.

Statistical Analyses

We used Prism software (GraphPad) to determine the statistical significance in the means of experimental groups. When making multiple comparisons on a data set, analysis was by one-way ANOVA with Bonferroni post-test. For experiments with two sample groups (one condition, one control) and a single comparison, analysis was by paired, two-tailed Student's t test.

ACCESSION NUMBERS

Raw microarray data have been submitted to GEO (NCBI) under the GSE accession numbers GSE44784 and GSE44785.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.08.035.

ACKNOWLEDGMENTS

We would like to thank Fiona Powrie and Holm Uhlig for helpful discussions and our Crohn's patients who generously gave blood samples for this study. The authors are supported by the UK Medical Research Council (T.P., A.S.), the UK Higher Education Funding Council, Oxford NIHR Biomedical Research Centre, and the Sir Jules Thorn Trust (A.S.), the Wellcome Trust (B.M.J.O., S.S., P.A., E.K., A.S., K.M.), Marie Curie Fellowship (T.S.), Digestive Disorders Foundation and National Association for Crohn's and Colitis and The Barbour Trust in Memory of Simon Ash (O.B.), the VIB and the European Research Council (A.L.).

Received: June 27, 2012
Accepted: May 9, 2013
Published: September 19, 2013

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