miR-29b directly targets activation-induced cytidine deaminase in human B cells and can limit its inappropriate expression in naïve B cells

Running Title: miR29b regulation of AID

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Abstract

Class-switch recombination (CSR) is an essential B cell process that alters the isotype of antibody produced by the B cell, tailoring the immune response to the nature of the invading pathogen. CSR requires the activity of the mutagenic enzyme AID (encoded by *AICDA*) to generate chromosomal lesions within the immunoglobulin genes that initiate the class switching recombination event. These AID-mediated mutations also participate in somatic-hypermutation of the immunoglobulin variable region, driving affinity maturation. As such, AID poses a significant oncogenic threat if it functions outside of the immunoglobulin locus. We found that expression of the microRNA, miR-29b, was repressed in B cells isolated from tonsil tissue, relative to circulating naïve B cells. Further investigation revealed that while enforced overexpression of miR-29b in human B cells precipitated a reduction in overall AID protein and a corresponding diminution in CSR to IgE, miR-29b knockdown in naïve B cells resulted in elevated AID expression. Similarly, miR-29b was able to directly interact with the AID 5'-UTR and modulate expression in reporter assays. Given miR-29b's ability to potently target AID, a mutagenic molecule that can initiate chromosomal translocations and "off-target" mutations, we propose that miR-29b acts to silence premature AID expression in naïve B cells, thus reducing the likelihood of inappropriate and potentially dangerous deamination activity.

1. Introduction

During the course of the immune response, mature B cells undergo two diversification events at the immunoglobulin loci, somatic hypermutation (SHM) and class-switch recombination (CSR) (Stavnezer, 2011). SHM introduces mutations in the hypervariable loops of the antigen binding site, found within the CDRs of the Immunoglobulin Heavy (*IGH*) and light (*IGL*) chain variable regions. This allows for selection of B cell clones which harbour Igs with increased affinity for antigen, promoting the development of highly specific Ig molecules. In contrast, CSR replaces the Cμ and Cδ heavy chain gene exons (encoding IgM and IgD respectively) with the downstream C-region exons of the α, γ, or ε isotypes (encoding IgA, IgG and IgE) (Stavnezer and Schrader, 2014). This process, which is driven by cytokines released from T helper cell populations, ensures that the effector functions of the antibodies produced by B cells are tailored to the nature of the invading pathogen. Both CSR and SHM require the activity of the mutagenic enzyme, activation-induced cytidine deaminase (AID, encoded by *AICDA*) (Muramatsu et al., 2000; Revy et al., 2000). AID acts to deaminate deoxycytidine residues within the *IG* variable and the *IGH* constant regions, resulting in the production of deoxyuracils(Neuberger et al., 2003). In the case of SHM, the ensuing mismatches initiate low-fidelity DNA repair pathways leading to the incorporation of mutations within the *IGH* and *IGL* variable regions, whereas in CSR the mismatches trigger a deletional-recombination event that replaces the upstream “acceptor” C-region (initially Cμ and Cδ) with a downstream “donor” C region (Cγ1-4, Cε or Cα1-2) (Keim et al., 2013).

The mechanisms targeting AID to the Ig genes are incompletely understood. It has been proposed that transcriptional stalling (Pavri et al., 2010), the formation of RNA:DNA hybrids called R loops (Shinkura et al., 2003), the exosome (Basu et al., 2011), super-enhancers (Qian et al., 2014), germline transcript RNA (Wang et al., 2015), 14-3-3 adaptor proteins (Xu et al., 2010), as well as the sequence location of the Ig genes (Yeap et al., 2015), recruit AID to the DNA and promote deamination. However, AID activity is promiscuous, and deamination-induced mutations are detected at multiple non-Ig sites (M. Liu et al., 2008). This collateral damage has significant oncogenic potential, as demonstrated by AID-induced chromosomal translocations that occur in the context of genomic instability (Robbiani et al., 2008; 2009) and AID-induced mutations of non-Ig genes (Duquette et al., 2005; Pasqualucci et al., 1998; 2001). Therefore, AID expression and activity must be carefully controlled.

One mechanism by which cells are able to fine-tune their protein expression is through microRNAs (miRNAs), short non-coding RNAs that regulate gene expression by promoting mRNA decay and translational repression (Bartel, 2009). In mice miR-155 has been shown to directly repress AID (Teng et al., 2008) and removing the miR-155 binding site within the *Aicda* 3' UTR increases the likelihood of AID-induced *Igh-Myc* chromosomal translocations (Dorsett et al., 2008), a transforming event frequently observed in Burkitt's Lymphoma. Similarly, miR-181 can also directly target *Aicda* and is proposed to prevent inappropriate AID expression in the absence of B cell activation (de Yebenes et al., 2008). Although the miRNA-mediated regulation of AID expression has been well studied in mice, this mode of regulation has not been extensively studied in human B cells.

To identify miRNAs involved in coordinating the B cell response in humans, we analysed the published literature that had profiled the miRNA pool of distinct human B cell subpopulations (Basso et al., 2009; Malumbres et al., 2009; Tan et al., 2009; J. Zhang et al., 2009). These studies consistently reported that the conserved miRNA miR-29b was less abundant in germinal centre B cells relative to naïve and memory cell compartments. Similarly, we show that miR-29b has diminished expression in B cells isolated from tonsil tissue relative to naïve B cells isolated from peripheral blood. It is of interest to note that miR29b expression has also been reported to be reduced in mantle cell lymphoma, a lymphoma thought to originate from Pre-GC mature B cells (Zhao et al., 2010). In this report we investigate potential miR29b targets and demonstrate that it is able to directly target *AICDA* mRNA. Enforced overexpression of miR-29b in human B cells reduces overall AID protein and causes a corresponding diminution of CSR to IgE *in vitro*. Together, these results suggest that miR-29b is able to silence "leaky" expression of AID, limiting its expression to appropriately activated B cells and thus helping to maintain chromosomal integrity.

2. Materials and Methods

2.1 Ethics

Ethical approval was granted by London Bridge Research Ethics Committee for both peripheral blood donations (09/H0804/77 and 14/LO/1699) and for tonsil donations (08/H0804/94). Full written informed consent was obtained from all donors or the donors' parents or legal guardian.

2.2 B cell isolation, transfection and culture

Naïve B cells were isolated from peripheral blood using the Naive B Cell Isolation Kit II (Miltenyi Biotec). B cells were isolated from tonsil tissue as previously described (Cooper et al., 2012). Briefly, mononuclear cells were isolated from dissected tonsil tissue on a density gradient (Lymphoprep, Axis-Shield PoC AS) followed by incubation with aminoethyl isothiouronium bromide-treated sheep red blood cells to rosette T cells. To overexpress miR-29b, 800 nM miR-29b Pre-miR miRNA Precursor (Thermo Fisher Scientific) or the equivalent non-targeting negative control molecules was delivered into 5×10^6 freshly isolated primary B cells using the Amaxa Human B cell Nucleofector Kit (LONZA). The Pre-miR miRNA Precursors used were proprietary short (16-28bp) double stranded RNA molecules chemically modified to ensure the desired strand is loaded into the RISC complex(Barnes et al., 2012). To inhibit miR-29b, 800 nM miR-29b of mirVana miRNA Inhibitor (Thermo Fisher Scientific) or the equivalent non-targeting negative control molecule was delivered into 5×10^6 freshly isolated primary B cells using the Amaxa Human B cell Nucleofector Kit (LONZA). The mirVana miRNA Inhibitors used were single stranded, chemically modified RNA molecules designed to irreversibly bind and inhibit endogenous

miRNAs (Barnes et al., 2012). Samples were nucleofected using program U-15 on the Nucleofector Device and cultured at a concentration 5×10^5 mL^{-1} in RPMI 1640, 10% HyClone Fetal Bovine Serum, 100 μg/mL Streptomycin, 100 U/ml Penicillin, 2 mM L-Glutamine. To stimulate CSR, the medium was supplemented with 1 mg/mL of anti-CD40 antibody, 200 IU/mL of recombinant human IL-4, 5 mg/mL of insulin and 35 mg/mL of transferrin. The human monoclonal IgM^+ IgD^+ CL01 B cell line (Cerutti et al., 1998) was cultured in RPMI 1640, 10% HyClone Fetal Bovine Serum, 100 μg/mL Streptomycin, 100 U/mL Penicillin, 2 mM L-Glutamine and kept at a concentration of between 1-5 × 10^5 cells/mL. To induce expression of AID, the culture medium was supplemented with 1 mg/mL of anti-CD40 antibody and 200 IU/mL of recombinant human IL-4. The CL01 cell line was nucleofected using the Amaxa Cell Line Nucleofector Kit V along with program C-09 on the Nucleofector Device. All cells were cultured in a humidified incubator at 37°C, 5% CO2.

2.3 RNA Isolation and quantitative (q) RT-PCR analysis

Total RNA was extracted from cultured cells using the miRNeasy Mini Kit with QIAzol Lysis Reagent (Qiagen). Residual gDNA was removed by incubating the extracted sample with 20 units of TURBO DNase enzyme (Thermo Fisher Scientific) at 37°C for one hour followed by a second phenol-chloroform cleanup. The integrity and yield of isolated RNA was checked on a 2100 Bioanalyzer (Agilent) using the RNA6000 Pico Assay. For analysis of mRNA expression, cDNA was generated from total RNA using random hexamers with RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific) and all genes (with the exception of *AICDA*, *εGLT, IgG* and *IgE*) were detected expression using TaqMan MGB Gene Expression Assays (Thermo Fisher Scientific). The primer and probe set for *AICDA* was designed using the Universal

Probe Library Assay Design Centre (Roche) while the *εGLT*, *IgG* and *IgE* primer and probe sets were designed in house. Individual samples were subjected to qPCR and run in triplicate with TaqMan Universal Master Mix II on the ViiA 7 Real-Time PCR System using 18S rRNA as a normalization control and gene expression was determined using the $2^{-\Delta\Delta Ct}$ method. For qRT-PCR analysis of miRNA expression, the TaqMan Small RNA Assay for miRNA quantification (Thermo Fisher Scientific) was used. This involved separate cDNA generation for each miRNA, utilising a miRNA-specific, stem-loop primer to facilitate reverse transcription. The small nucleolar RNA RNU6B was used as a normalization control and gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

2.4 Gene expression arrays

Fresh tonsil B cells were transfected with a miR-29b mimic or its associated negative control and cultured in class switching stimuli for 24 hours. RNA was isolated using the miRNeasy Mini Kit with QIAzol Lysis Reagent (Qiagen) and then DNase-treated as described. 10 ng RNA was amplified using the Ovation Pico WTA System V2 (Nugen). 4 μg of amplified single-stranded cDNA was biotin-labeled using the Encore BiotinIL Module (Nugen). Finally, transcriptomic analysis was performed by hybridizing 750 ng of biotin-labeled single-stranded cDNA onto a HumanHT-12 v4 Expression BeadChip (Illumina) and scanned using the Illumina iScan System. QC analysis and RMA normalization was performed in Illumina's Genome Studio Suite v1.0. Assessment of differential gene expression and statistical analysis was performed in Partek Genomics Suite version 6.6. Data from this study have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO

Series accession number GSE100735 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100735).

Immunoblotting

B cells were stimulated for 60 hours in class-switching stimuli following transfection. Cells were harvested in protein lysis buffer (pH 7.4, 25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP40, 5% Glycerol, 10% Roche Protease Inhibitor). Total protein levels were quantified using the bicinchoninic acid assay and approximately 50 ug protein lysate were run on SDS-polyacridimide gels. Following wet transfer onto nitrocellulose membranes, the membranes were probed with the following antibodies for protein expression (AID - EK2 5G9, STAT6 - D3H4, p-STAT6^{Tyr641} - C11A12, p38 MAPK - D13E1, p-p38$^{Thr180/Tyr182}$ - D3F9, AKT - 40D4, p-AKT$^{ser\ 473}$ - D9E, GAPDH - 6C5), and images were developed using the Molecular Imager® ChemiDoc^{TM} XRS System (Bio-Rad). GAPDH (clone 6C5, Abcam) was used as a loading control.

2.5 Luciferase Assays

The region of the *AICDA* 3' UTR encompassing the miR-29 binding site was subcloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega), which harbours both a firefly luciferase reporter gene, for analysis of miRNA-mRNA binding site interactions, and a renilla luciferase reporter gene, which acts as a transfection efficiency control. For the mismatched construct, the miR-29 binding site in the AID 3' UTR was replaced with a BamHI restriction site using sequential rounds of mutation cloning. HEK293T cells were seeded onto white 96-well tissue culture plates and having reached 70% confluence were transfected with 50 ng of one of the luciferase constructs and 10 nM of miR-29 mimic using Lipofectamine 2000 (Thermo Fisher Scientific). Reactions were performed in triplicate. Luciferase activity was

quantified after 24 hours on a GloMax-Multi Microplate Luminometer (Promega, 2 seconds integration time) using the Dual-Glo Luciferase Assay System (Promega). The luciferase signal ratio was calculated by dividing firefly luciferase activity by renilla luciferase activity and normalized to cells transfected with an insert-free pmirGLO vector.

2.6 Flow Cytometry

Apoptosis staining was performed using the Annexin V Apoptosis Detection Kit (eBioscience). For cell proliferation analysis, 10^7 transfected CL01 cells were washed with PBS, resuspended in 1 mL PBS containing 1 μM CellTrace Violet dye (Thermo Fisher Scientific), and left in the dark at 37°C for 10 minutes. The reaction was quenched by the addition of cell culture media. The cells were washed, resuspended and cultured in a 37°C incubator until analysis via flow cytometry. Intracellular antibody staining of IgG and IgE was performed as previously described[16] using the a fixable viability stain (Zombie Aqua Thermo Fisher Scientific).

3. Results

3.1 miR-29b is repressed in tonsil B cells relative to peripheral naïve B cells

In order to identify dynamically regulated miRNAs within the context of mature human B cells, we analysed the available literature profiling the miRNA pool in different B cell subpopulations (Basso et al., 2009; Malumbres et al., 2009; Tan et al., 2009; J. Zhang et al., 2009). We observed that the evolutionarily conserved miRNA miR-29b was less abundant in germinal centre B cells relative to naïve and memory cell compartments (supplementary Fig. 1A & 1B). miR-29b is a member of the miR-29 family, also including miR-29a and miR-29c, which share the same seed region and thus overlap in their predicted targets (Liston et al., 2012). Interestingly miR29c was also less abundant in GC B cells compared to naïve or memory compartments, potentially reinforcing the effects of this miRNA family (supplementary Fig. 1A & 1B). We analysed the abundance of miR-29b in B cells isolated from tonsils, a large proportion of which have a germinal centre phenotype ($CD19^+$ $CD38^+$ $CD27^-$ or $CD19^+$ $CD38^+$ $CD27^+$) (supplementary Fig. 2A), and compared its prevalence to naïve B cells ($CD19^+$ IgD^+ $CD27^-$) (supplementary Fig. 2B) isolated from peripheral blood. In line with previous reports(Malumbres et al., 2009), miR-29b was nearly 3 times more abundant in the circulating naïve B cells than in tonsil-derived B cells (Fig. 1).

3.2 Identification of AICDA as a miR-29b target gene in human tonsil B cells

To gain insight into the role of miR-29b in the context of B cell activation we overexpressed miR-29b in tonsil B cells and investigated global gene expression changes following 24 hours culture with IL-4 and anti-CD40 stimulation using Illumina BeadChip Microarrays (HumanHT-12 v4). Transfection of a miR-29b mimic into tonsil

B cells increased miR-29b abundance by approximately 15-fold 24 hours after transfection, compared to cells transfected with a negative control mimic molecule (supplementary Fig. 3A). No detectable difference was seen in cell viability between the miR29b mimic or negative control as judged by flow cytometry FSC/SSC although cell viability was reduced approximately 2 fold compared to un-transfected cells. Analysis of the array data revealed only a moderate effect on overall gene expression, with just 29 genes being modulated by more than 1.2 fold (supplementary table I and Fig. 2A). However, of these genes, 20 (69%) contained miR-29 binding sites within their 3' UTRs, suggesting potential for direct targeting by miR-29 (Fig. 2B). Further analysis of the genes with miR29 binding sites revealed that 16 (80%) of the seed regions contained either 8mers (an exact match to positions 1-8 of the mature miRNA including an A opposite position 1 of the miRNA) or 7mers (an exact match to positions 2-8 of the mature miRNA) (Fig. 2B).

Although, a 15 fold overexpression of miR-29 is non-physiological, the incorporation of miR into "active" RISC complexes is known to be far lower than cytoplasmic levels (Flores et al., 2014). Despite this, these data demonstrate the potential for miR-29 to regulate a number of genes of interest to B cell regulation. We observed that *AICDA* was one of the top target genes repressed by miR-29 overexpression, while the previously validated miR-29b target, *CDK6* (Zhao et al., 2010), was also robustly repressed (Fig. 2A, C). Other validated miR-29 targets repressed following miR-29b overexpression in tonsil B cells included the guanine exchange factor *RCC2* (Matsuo et al., 2013), the thymine glycosylase *TDG* (Morita et al., 2013; P. Zhang et al., 2013) and the trafficking protein *TRAK2* (Kato et al., 2016) (Fig. 2C).

Given its indispensable role in promoting B cell antibody diversification, the repression of *AICDA* following miR-29b overexpression was of particular interest. To further explore the potential regulation of *AICDA* by miR-29b, tonsil B cells were transfected with a miR-29b mimic and cultured in the presence of IL-4 and anti-CD40 antibody to induce *AICDA* expression CSR and plasmablast differentiation; as previously demonstrated this resulted in an 15 fold increase in miR29b levels, supplementary figure 3A). RNA was isolated 24 hours after transfection and the expression of a series of miR-29 targets associated with either CSR-, GC- and plasma cell differentiation and function, including *AICDA,* were assessed via qRT-PCR.

In line with the array data, *AICDA* was consistently repressed by over 2-fold following miR-29b overexpression (Fig. 3A). Similarly, the bona fide miR-29 target, *CDK6* (Zhao et al., 2010), was also robustly reduced following miR-29b overexpression, demonstrating the biological validity of these experiments (Fig. 3A). However, two previously reported miR-29 targets, *AKT3* (Wei et al., 2013) and *SP1* (Jia et al., 2014), remained unperturbed (Fig. 3A), suggesting that in the context of human B cells, they are not subject to miR-29b repression at the transcript level. The expression levels of BATF (Ise et al., 2011), HOXC4 (Park et al., 2009), MYC (Fernandez et al., 2013) and IRF4 ((Luo and Tian, 2010), previously reported to directly regulate AID transcription, and the expression of factors critical to the GC/PB phenotype (BCL6, PAX5, XBP1 and BLIMP1) were all perturbed by >20%, although *BATF*, XBP1 and *MYC* did show small but reproducible decreases.

298 The repression of *AICDA* mRNA following miR-29b overexpression was also mirrored
299 at the protein level. Immunoblotting 60 hours post transfection using the EK2 5G9 anti-
300 AID monoclonal antibody showed a clear reduction in AID protein (Fig. 3B).
301 Densitometry revealed this to be an approximate 60% decrease, indicating a strong
302 correlation between mRNA and protein expression changes (Fig. 3B). Overall, the data
303 show that the overexpression of miR-29 in tonsil B cells activated to express AID was
304 indeed capable of repressing AID expression at both the protein and mRNA level.
305
306 *3.3 Knockdown of miR-29b in activated naïve B cells augments AID expression*

307 To gain further insight into the role of miR-29b in the context of B cell activation, naive
308 B cells, which express endogenous miR-29b, were transfected with a commercial miR-
309 29 inhibitor (see methods for details) or non-silencing control and cultured with IL-4
310 and anti-CD40 stimulation. Delivery of the inhibitor repressed endogenous miR-29b by
311 approximately 2 fold (as assessed by qPCR) when compared to B cells transfected with
312 a negative control, and lasted for approximately 48 hours before beginning to recover
313 (supplementary Fig. 3B). This repression of miR-29 represents a far more
314 physiological, and therefore biologically relevant, manipulation; bringing miR-29
315 expression down to a similar level to that seen in tonsil B cells (3-fold lower than naïve
316 B cells). As in total B cell experiments, no detectable difference was seen in cell
317 viability between the miR29b inhibitor or non-silencing control as judged by flow
318 cytometry FSC/SSC although cell viability was reduced approximately 2 fold
319 compared to un-transfected cells. The biological consequences of this inhibition were
320 assessed by monitoring the expression of *AICDA* as well as CSR, GC and plasma cell
321 associated genes and three previously validated miR-29 targets, by qRT-PCR.

In a reciprocal manner to the miR-29b overexpression results, we observed that *AICDA* was significantly increased following 60% miR-29b inhibition (supplementary Figure 3B & Fig. 4A). Similarly, expression of the previously validated miR-29 target, *CDK6* (Zhao et al., 2010) and *MYC,* previously indirectly linked to miR-29 (S. Liu et al., 2010), were elevated. Although a modest increase in εGLT expression was detected following miR29 knockdown this was not found to be significant compared to the control. Also in line with our overexpression analysis, the reported miR-29 targets, *AKT3* (Wei et al., 2013) *a*nd *SP1* (Garzon et al., 2009), were unaltered following miR-29b inhibition (Fig. 4A).

To determine whether the elevated *AICDA* mRNA expression was reflected at the protein level, we performed immunoblots 60 hours post transfection. Further confirming the effect at the mRNA level, we observed that AID protein expression was increased by 1.6-fold following miR-29b inhibition, as determined via densitometry (Fig. 4B). Together, these data demonstrate that even reducing the physiological levels of miR-29 expression found in naïve B cells less than that seen in tonsil B cells (which express 3 fold less miR-29 than naïve B cells rather than the 2 fold reduction induced) is sufficient to de-repress AID in circulating naïve B cells.

3.4 miR-29b directly targets the miR-29 binding site within the AICDA 3' UTR

To investigate the possibility that AID may be indirectly regulated by miR-29b, the expression of a number of signalling components of the B cell activation pathway were investigated to determine the impact of potential indirect modes of regulation. Following miR-29b manipulation, the expression and phosphorylation of members of

346 the PI3K, MAPK and STAT6 pathway were found to be unperturbed (supplementary
347 Fig. 3C). This further confirmed that miR-29b is likely directly targeting *AICDA*.
348 Bioinformatic analysis of the human *AICDA* 3' UTR revealed the presence of a
349 pronounced miR-29 binding site **(**Fig. 5A). This site is found in the *AICDA* 3' UTR of
350 other primate species, such as chimpanzees and rhesus macaques, but not in more
351 distantly related mammals, such as mice, rats and rabbits. The miR-29 binding site in
352 the *AICDA* 3' UTR shows complementarity from nucleotides 1-10 at the 5' end of the
353 miRNA, and includes an adenosine opposite position 1 **(**Fig. 5A). Seed region
354 complementarity and an adenosine opposite position 1 are both strong indicators of
355 miRNA targeting (Agarwal et al., 2015). In addition, the site is located in a favourable
356 genomic context, it is under 200 nucleotides away from the 3' end of the 3' UTR, and
357 embedded in an AU-rich environment. The miRNA targeting prediction tool
358 TargetScan reported *AICDA* to be in the 4th percentile in terms of favourability for all
359 miR-29 binding sites (not shown).
360
361 Since *AICDA* possessed a strong miR-29b binding site in its 3'UTR, the direct targeting
362 of AID by miR-29b was assessed. The region encapsulating the miR-29 binding site of
363 the AICDA 3'UTR was sub-cloned into a luciferase reporter vector. Constructs in
364 which the miR-29 binding site was mutated and replaced with a BamHI restriction site
365 **(**Fig. 5A**)** and constructs containing no insert were used as negative controls. Each
366 vector was separately cotransfected into HEK293 cells along with a miR-29b mimic
367 molecule. Following 24 hours in culture, miR-29b was shown to be capable of
368 repressing the luciferase activity of the test vector, relative to both negative control
369 vectors **(**Fig. 5B**)**. This demonstrated that miR-29b was indeed capable of directly
370 interacting with the *AICDA* 3' UTR and suppressing protein expression.

3.5 miR-29 dampens CSR to IgE in stimulated tonsil B cells

In order to mount a diversified humoral immune response, B cells not only undergo CSR and SHM but must also coordinate their proliferative and apoptotic potential (Recaldin and Fear, 2016). As such, the phenotypic consequences of miR-29b overexpression were assessed in both activated tonsil B cells and the CL01 germinal centre cell line. Following anti-CD40 and IL-4 stimulation, overexpression of miR-29b had no detectable effect on apoptosis or cell proliferation (supplementary Fig. 3D-F).

Given that miR-29b modulates AID levels, we also investigated its effect on *in vitro* stimulated CSR. Although we show that transient transfection of naïve B cells could be successfully accomplished and that mir29b over-expression resulted in a robust diminution of induced AID expression, cells transfected with either the miRNA mimic or non-silencing control produced very little isotype switched immunoglobulin following 14 days culture. In order to demonstrate a biological consequence of miRNA29b diminished AID expression we overexpressed miR29b in total tonsillar B cells. CSR to IgE is most robustly stimulated in tonsil B cells following activation with IL-4 and anti-CD40(Ramadani et al., 2017; 2015) and we reasoned that although miR-29b repression of AID is only transient (approx. 48 hours) in this system, this is the timepoint at which most AID is functionally active, and thus might affect CSR. When provided with anti-CD40 and IL-4 stimulation, tonsil B cells maintain their IgG expression, with switching to and from IgG being balanced, over the course of 7 days, but significantly increase switching to IgE from negligible to detectable levels, and thus can be tracked via flow cytometry (Ramadani et al., 2015). Assessment of Ig gene

395 expression in B cell cultures by qRT-PCR, 5 days after transfection with a miR-29b
396 mimic, revealed a moderate but reproducible, 30% decrease in IgE mRNA **(**Fig. 6A**)**.
397 After 7 days in culture, IL-4 and anti-CD40 stimulated B cells transfected with either
398 the miR29b mimic or non-silencing control were similarly viable (cell viability 10.3%
399 $^{+}$/- 2.2 versus 11.2% $^{+}$/- 2.0), although viability was reduced 2.5 fold compared to
400 similarly stimulated un-transfected cells(Ramadani et al., 2015). Intracellular staining
401 of immunoglobulin protein at day 7 reproduced a similar moderate but statistically
402 significant reduction in the percentage of IgE-expressing cells **(**Fig. 6B). The
403 percentage of IgG-expressing cells was not significantly altered **(**Fig. 6B**)**. Knockdown
404 of AID through use of a short-interfering RNA to *AICDA* resulted in a similar pattern
405 of Ig expression; giving a small but reproducible reduction in the percentage of IgE-
406 expressing cells, but no significant alteration in IgG-expressing cells **(**Fig. 6C**)**.
407 Demonstrating that miR-29 OE not only modulates AID expression but is able to illicit
408 a biologically relevant functional effect. Together these results demonstrated that miR-
409 29b, a miRNA downregulated in activated B cells, is capable of directly targeting
410 *AICDA* mRNA, and decreases CSR to IgE following stimulation with IL-4 and anti-
411 CD40 antibody. We posit that miR 29 represents a previously unrecognised player in
412 human B cell biology that warrants further investigation.

4. Discussion

miRNAs play an important role in fine tuning cells' protein output and have previously been shown to regulate important aspects of B cell biology (Taganov et al., 2007). However, the functional role of miRNAs have not been extensively studied in the activation of human B cells. This study sought to identify miRNAs that were dynamically and temporally regulated between different mature B cell subpopulations, and identify their functional consequences in the development of the humoral response. Previous studies have used PCR, microarray or RNA-seq to monitor the miRNA expression changes that occur during the course of a B cell response, predominantly focusing on the differences between naïve, GC and memory B cells found within the same secondary lymphoid organs (Basso et al., 2009; Malumbres et al., 2009; Tan et al., 2009; J. Zhang et al., 2009). The original data from Malumbres et al., (Malumbres et al., 2009) indicated that miR-29b, a member of the miR-29 family that is important in coordinating the lymphocyte response to intracellular pathogens such as *Listeria monocytogenes* (Ma et al., 2011), was repressed in the GC compartment. Interrogation of data from other papers that analysed the human B cell miRNome (Basso et al., 2009; Tan et al., 2009; J. Zhang et al., 2009) confirmed this observation. The miR-29 family of miRNAs are known to be particularly important in T cells, where they control aspects of development and effector function (Ma et al., 2011; Papadopoulou et al., 2011; Steiner et al., 2011), and in B cells have recently been shown to regulate germinal centre dynamics in a murine model of collagen-induced arthritis (Nieuwenhuijze, 2017). However, the functional consequences of this miRNA in naïve human B cells and B cell activation have not previously been studied.

Here, we found that mature miR-29b was approximately 3-fold less abundant in B cells isolated from tonsil lymphoid tissue than in circulating naïve B cells isolated from blood, confirming that miR-29b was dynamically regulated upon exit from the circulation and entry into the lymphoid tissue. In order to identify miR-29b's function in this context and identify potential target molecules, it was overexpressed through the use of miRNA mimic molecules and global gene expression changes monitored by microarray analysis. This revealed that the second most dynamically regulated gene following miR-29b overexpression, after the eukaryotic initiation factor *EIF4E2*, was found to be *AICDA*, which plays a critical role in B cell biology (Fig. 2A, B). Considering that AID is also a potent genome mutator and has oncogenic potential (Robbiani et al., 2009; 2008) its expression and activity must be carefully regulated. In mice, *Aicda* is post-transcriptionally regulated by miR-155 and miR-181b (de Yebenes et al., 2008; Dorsett et al., 2008; Teng et al., 2008). However, regulation of *AICDA* by different miRNA members has not been fully investigated in human B cells.

The human *AICDA* 3' UTR contains a pronounced miR-29 binding site with extensive complementarity, indicating the possibility of a direct interaction. This is confirmed by our results which show that over expression of a miR-29b mimic represses luciferase activity in HEK293 cells transfected with a luciferase reporter construct containing the region of *AICDA* 3' UTR bearing the miR-29 binding site (Fig. 5B) while miR-29b overexpression in tonsil B cells significantly reduced *AICDA* expression, AID protein levels (Fig. 3) and CSR to IgE (Fig. 6). Although we cannot rule out additional indirect mechanisms of miR-29b eliciting a response on both AID expression and CSR to IgE, miR-29b OE did not affect the expression of apoptotic markers in either tonsil b cells (as evidenced by the microarray study) or a B cell line (Supplementary figure 3E) and

462 did not affect cell proliferation (Supplementary figure 3F). This raises the question as
463 to the purpose of such an interaction. AID and miR-29b expression are inversely
464 correlated. *AICDA* is detectable in the lymphoid follicles and most abundant in GC B
465 cells, but absent in circulating naïve B cells. Conversely, miR-29b is reduced in the
466 follicles but prominent in circulating naïve B cells. This indicates that the function of
467 miR-29b may be to suppress premature AID at the early stages of a mature B cell's life
468 cycle, or following inappropriate activation in the absence of suitable T cell help or T-
469 independent "danger" signals.
470
471 Upon entry into the lymphoid tissue and subsequently the GC, where AID expression
472 is appropriate, the down regulation of miR-29b would relieve the block on *AICDA* and
473 allow its full regulated expression. Indeed, the potential of endogenous miR-29b to
474 regulate AID in this context was demonstrated by the increase of AID mRNA and
475 protein expression levels when miR-29b was inhibited in IL4 and antiCD40 stimulated
476 naïve B cells (Fig. 4A and 2B). We believe this to be a far more compelling result
477 (being carried out at a physiologically relevant level of miR-29b) than the
478 overexpression study and evidence of the potential for miR-29b to regulate AID
479 expression. In contrast, in tonsil B cells, where miR-29b is less abundant, miR29b
480 inhibitors did not elevate *AICDA* (data not shown). This strongly suggests that while
481 endogenous miR-29b expression in naïve B cells is sufficient to suppress *AICDA*
482 expression, the low levels of miR-29b in GC B cells is insufficient to do so (although
483 overexpression of miR29b above physiological levels does indeed strongly repressing
484 AID and partially blocks CSR). As such, it is likely that miR-29b is able to repress
485 leaky or inappropriate expression of AID prior to B cell activation. In relation to
486 miR29s potential role in preventing inappropriate AID expression, and therefore

function, in naïve B cells, it of interest to note that miR29 (a, b and c) was found to be decreased in Mantle cell lymphoma (MCL) (Zhao et al., 2010), a lymphoma with characteristics of $CD5^+$, antigen-naive pregerminal center B-cells. Although Cyclin D1 translocation and the up-regulation of CDK6 (a direct miR29 target) is thought to account for the transformation of MCL cells, they were also found to frequently express AID possibly accounting for at least some of wide range of chromosomal abnormalities present in this disease(Babbage et al., 2004).

Following B cell exit from the circulation, miR-29b silencing in GC B cells leads to a de-repression of *AICDA* transcription. Such a model has been previously ascribed to miR-181b regulation of *aicda* in mice (de Yebenes et al., 2008). Interestingly, miR29c shares a similar expression pattern to miR-29b, being most highly expressed in naïve B cells and low in GC B cells (Supplementary Figure 1). Since miR29 family members share seed region homology and therefore predicted targets, the expression of these miRs would be expected to reinforce their functional effects. It should be noted that the miR-29 site in the *AICDA* 3' UTR is conserved amongst other primate species, but not in more distantly related mammals, such as mice, rats and rabbits. Thus, it is not an interaction that has been strongly conserved throughout mammalian evolution and may have arisen more recently.

In conclusion, the data in this paper have shown that miR-29b, a member of the conserved miR-29 family, is repressed in tonsil B cells relative to circulating naïve B cells. Enforced overexpression of miR-29b in tonsil B cells led to the direct targeting of *AICDA*, through a pronounced binding site in the *AICDA* 3' UTR and this interaction was sufficient to reduce CSR to IgE. Similarly, inhibition of endogenous miR-29b in naïve B cells resulted in elevated AID expression. We hypothesise that one of the

endogenous functions of miR-29 is to silence leaky expression of AID, a mutagenic protein whose expression must be controlled in order to maintain chromosomal integrity.

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Figure legends

Figure 1: miR-29 is less abundant in B cells isolated from tonsillar tissue than in naïve B cells isolated from peripheral blood.

The expression level of miR-29 in IgD^+ $CD27^-$ naive B cells isolated from peripheral blood compared to $CD19^+$ tonsil B cells, as assessed via qRT-PCR. Values are plotted relative to the small nucleolar RNA RNU6B. ** $P < 0.01$(unpaired t-test).

Figure 2: Genome-wide analysis of miR-29b targeting in tonsillar B cells shows an enrichment in miR-29 binding sites within the 3' UTR of downregulated genes.

(A) Heatmap representing expression changes in the most downregulated genes in tonsillar B cells transfected with a miR-29 mimic and stimulated with anti-CD40 antibody and IL-4 cytokine for 24 hours, as detected by Illumina BeadChip Array. Genes containing predicted miR-29 binding sites in their 3' UTR are listed in red. P values (two-way ANOVA) are also included.

(B) Pie chart displaying the prevalence of miR-29 binding sites within the 3' UTR of genes downregulated following miR-29b overexpression. The percentage of 8mers (an exact match to positions 2-8 of the mature miRNA with an A opposite position 1 of the miRNA), 7mer-m8s (an exact match to positions 2-8 of the mature miRNA) and 6mers (an exact match to positions 2-7 of the mature miRNA) within the miR-29 binding sites is also shown.

(C) Dot plot representations of the change in fluorescence intensity for *AICDA*, as well as previously validated targets including *CDK6*, *RCC1*, *TDG* and *TRAK2*, in tonsil B cells transfected with a miR-29 mimic and stimulated with anti-CD40 antibody and IL-4 cytokine for 24 hours, as detected by Illumina BeadChip Array.

Figure 3: Overexpression of miR-29 reduces AID abundance in tonsillar B cells.

tonsil B cells were transfected with a miR-29b mimic or its associated negative control molecule and subsequently activated with IL-4 and anti-CD40.

(A) Gene expression of key B cell molecules after 24 hours of stimulation as assessed via qRT-PCR. Values are normalized to 18S ribosomal RNA and plotted relative to the abundance of each gene in the control transfected cells, which were arbitrarily assigned a value of 1. N = 3 - 12, mean and s.e.m. * P <0.05, ** P < 0.01, **** P < 0.0001 (paired t-test).

(B) Representative immunoblot and cumulative densitometry analysis (n = 3) of AID protein in follicular B cells following miR-29b overexpression and 60h of culture in IL-4 and anti-CD40 stimulus, compared to AID protein in the control transfected cells. GAPDH served as a loading control.

Figure 4: Knockdown of miR-29 increases AID abundance in naïve B cells.

Naive B cells were transfected with a miR-29 inhibitor or its associated negative control molecule and subsequently activated with IL-4 and anti-CD40.

(A) Gene expression of key B cell molecules after 48 hours of stimulation as assessed via qRT-PCR. Values were normalized to 18S ribosomal RNA and plotted relative to the abundance of each gene in the control transfected cells, which were arbitrarily assigned a value of 1. N = 6 - 12. Mean and s.e.m. ** P < 0.01, *** P < 0.001, **** P < 0.0001 (paired t-test).

(B) Representative immunoblot and cumulative densitometry analysis (n = 3) of AID protein in naïve B cells following miR-29b knockdown and 60h of culture in IL-4 and anti-CD40 stimulus, compared to AID protein in the control transfected cells. GAPDH served as a loading control.

Figure 5: miR-29 is capable of directly targeting the miR-29 binding site within the AICDA 3' UTR.

(A) The miR-29 binding site within the human, chimpanzee and rhesus macaque *AICDA* 3' UTR, as well as the mismatched sequence used for luciferase analysis.

(B) HEK293T cells were contransfected with one of the dual luciferase plasmids (endogenous *AICDA* 3' UTR, mismatched 3' UTR or no insert) and a miR-29 mimic. Firefly luciferase activity was quantified after 24 hours, normalized to renilla luciferase and plotted relative to the no insert plasmid. * $P < 0.05$ (one-way ANOVA). N = 3, mean and s.e.m.

Figure 6: miR-29 overexpression dampens induction of class switching to IgE.

Tonsil B cells were transfected with a miR-29 mimic or negative control molecules and subsequently cultured in class-switching conditions (IL-4 and ati-CD40 antibody).

(A) IgE and IgG mRNA were detected via qRT-PCR following 5 days of culture. Values are plotted relative to 18S ribosomal RNA. N = 7 for IgE and 5 for IgG, mean and s.e.m.

(B) Intracellular IgE and IgG was analysed following 7 days of culture via flow cytometry. * $P < 0.05$ (paired t test).

(C) Intracellular IgE and IgG was analysed following 7 days of culture via flow cytometry. Values were plotted relative to the control transfected cells, which were arbitrarily assigned a value of 1. N = 3 for siAID, and 10 for miR-29 versus the negative control mimic. * $P < 0.05$ (paired t test). Mean and s.e.m.