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

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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 miR-29b was able to directly initiate the degradation of AID mRNA. Enforced overexpression of miR-29b in human B cells precipitated a reduction in overall AID protein and a corresponding diminution in CSR to IgE. 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

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the mismatches trigger a deletional-recombination event that replaces the upstream "acceptor" C-region (initially Cμ and C*δ*) 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 (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; 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 $5 \times 10^5\,mL^{-1}$ 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 $\times 10^5$ 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 h. 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