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IL-10 regulates *Aicda* expression through miR-155

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ABSTRACT

***Aicda* is a critical component of antibody class-switching in B cells. In this work, we study the impact of TLR4 activation and IL-10 stimulation on *Aicda* expression in B cells. Through the global analysis of miRNAs in response to TLR4 activation, in combination with IL-10 stimulation, we identified that IL-10 can suppress TLR4-induced miR-155 expression, an effect that resulted in enhanced *Aicda* expression. Furthermore, when preventing miR-155 control of *Aicda* expression, by genetic mutation of its target site in the *Aicda* mRNA, IL-10 could further potentiate *Aicda* expression. Given that miR-155 expression is lost, and expression levels of both *Aicda* and IL-10 are high in diseases, such as Burkitt's lymphoma, our results suggest a stringent and sophisticated control of *Aicda* by a novel IL-10/miR-155 axis, where the imbalance of IL-10 and/or miR-155 may contribute to disease pathogenesis. *J. Leukoc. Biol.* 97: 71–78; 2015.**

Introduction

IL-10 plays a multifaceted role in regulating immune cell function. In monocyte-derived cells, such as macrophages and dendritic cells, IL-10 acts as a potent anti-inflammatory cytokine, where it inhibits a subset of proinflammatory cytokines induced by pathogen-recognition receptors, such as TLRs [1]. However, in B cells, IL-10 has been shown to promote survival and induce antibody class-switching, processes required to mount a specific antibody response to invading pathogens [2–4].

We have shown previously a role for the miRNA miR-155 in the anti-inflammatory properties mediated by IL-10 in macrophages [5]. miRNAs function to regulate mRNA expression negatively by binding to the 3′UTR of target mRNAs, resulting in mRNA degradation and/or inhibition of translation [6]. We demonstrated that in response to TLR4 stimulation by LPS, IL-10 could potently inhibit miR-155 induction [5]. SHIP1 is a known target of miR-155 regulation, and SHIP1 mRNA expression decreases as a result of increasing miR-155 [7]. In accordance with its inhibition of miR-155, we found that IL-10 could reverse this effect, increasing the expression of SHIP1 [5], which is a negative regulator of TLR-induced responses [8]; thus, an increase in its expression mediated by the IL-10/miR-155 axis can explain some of the anti-inflammatory properties of IL-10. We speculated that IL-10 regulation of miRNAs (in combination with TLR4 activation) could also be a mechanism used by B cells to mediate properties of IL-10 function.

It is established that B cells respond robustly to TLR4 stimulation [9, 10]. Furthermore, there is accumulating evidence that TLRs act in combination with cytokines to direct antibody isotype-switching so that the appropriate immune response to an invading pathogen is mounted [11–14]. For example, LPS, in combination with IL-4, promotes resting B cells to switch from secreting pentameric IgM antibodies to IgG1 and IgE antibodies (these are better able to access tissues as a result of their smaller size, IgG1 activates the classic complement pathway and opsonization, and IgE activates mast cells and basophils), whereas LPS, in combination with IFN-γ, induces switching to IgG2a antibodies that activate phagocytes very efficiently [11, 15]. Although a role for IL-10 and LPS has not yet been investigated, IL-10, in combination with TLR9 and TLR7, was shown to induce rapid B cell proliferation, as well as IgG antibody production [16, 17].

Induction of *Aicda*, which codes for the protein AID, is essential for 2 processes required for the generation of switched

Abbreviations: *Aicda*/AID = activation-induced cytidine deaminase, BM = bone marrow, CD40L = cluster of differentiation 40 ligand, C/ebpβ = CCAAT/enhancer binding protein beta, CSR = class-switch recombination, Ct = comparative threshold, F = forward, GEO = Gene Expression Omnibus, miR-155 = microRNA in humans encoded by the *MIR155* host gene, miRNA = microRNA, NT = nontreated, PMID = PubMed identifier, R = reverse, RMA = robust multichip averaging, SHIP1 = inositol polyphosphate-5-phosphatase, SHM = somatic hypermutation, UTR = untranslated region, WT = wild-type

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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antibodies in B cells: CSR and SHM [18]. Both processes require the deaminase activity of AID to induce single-stranded breaks in DNA that are repaired imperfectly. A potent mutator, such as AID, requires stringent regulation within the cell. Indeed, control of *Aicda*/AID expression is regulated transcriptionally [19–21], post-translationally [22, 23] by intracellular compartmentalization and trafficking [24, 25], and as most recently described, post-transcriptionally by miR-155 [26–28].

Here, we show that a combination of IL-10 and LPS regulates a range of miRNAs in B cells, most notably, miR-155. Specifically, we found that IL-10 represses miR-155 and that this effect acts to boost expression of *Aicda* in B cells. We also show that *Aicda* and miR-155 are inversely correlated in a cohort of Burkitt's lymphoma patients. These data implicate a role for TLR4 and IL-10 in B cell function by fine-tuning *Aicda* expression through the specific regulation of miR-155.

MATERIALS AND METHODS

Mice

WT and Aicda$^{155/155}$ mutant mice [26] on a C57Bl/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Experimentation was conducted with approval from institutional animal ethics committees and in accordance with the Australian and Irish codes of practice for the care and use of animals for scientific purposes.

Antibodies and cell sorting

Single-cell suspensions from BM or spleen were treated to lyse RBCs and subsequently stained with antibody conjugates for flow cytometric analysis by use of FACSCalibur (BD Biosciences, San Jose, CA, USA). Spleens were stained with fluorochrome-conjugated rat mAb to mouse CD93 (AA4.1), CD23 (B3B4), and IgM (11/41; eBioscience, San Diego, CA, USA); B220 (RA3-6B2), CD21/35 (7G6), and IL-10R (1B1.3a; BD PharMingen, San Diego, CA, USA); and LIVE/DEAD fixable stain (Invitrogen, Carlsbad, CA, USA). Transitional 1 B cells (T1) were gated as liveCD93^{+}IgM^{hi}CD23^{lo}, T2 as liveCD93^{+}IgM^{lo}CD23^{hi}, T3 as liveCD93^{+}IgM^{lo}CD23^{hi}, follicular (Fo) as liveCD93^{-}CD21^{lo}CD23^{high}, and marginal zone as liveCD93^{-}CD21^{hi}CD23$^{lo/neg}$. BM cells were stained with rat mAb to mouse IgM, B220, IL-10R, and LIVE/DEAD fixable stain (details as per for spleen), pre and pro B cells were gated as liveIgM^{-}B220^{int}, immature B as liveIgM^{+}B220^{int}, and mature B as liveIgM^{+}B220^{hi} and liveIgM^{hi}B220^{int}. Mean fluorescence intensity was determined by use of (geometric median fluorescence) minus (geometric median isotype) for each antibody on cells from each mouse (n = 6).

Cell culture and reagents

For in vitro B cell assays, B cells were purified from the spleen by use of MACS columns, following the manufacturer's instructions (Miltenyi Biotec, Cologne, Germany), and then plated at 0.5–1 × 10^{6} cells/mL in RPMI 1640, supplemented with 200 μM L-glutamate, 55 μM 2-mercaptoethanol, 10 mM HEPES, 10,000 U penicillin, and 10,000 U streptomycin. LPS (*Escherichia coli* K, 10 μg/mL; Invivogen, San Diego, CA, USA), IL-4 (20 ng/mL; Peprotech, Rocky Hill, NJ, USA), IL-10 (200 ng/mL; R&D Systems, Minneapolis, MN, USA), αIgM (10 μg/mL, F(ab)′$_2$; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and CD40L (kind gift from Margaret Hibbs, Monash University, prepared by use of baculovirus cell line) were used to stimulate cells, as indicated in the figure legends.

RNA purification

Total RNA was extracted by use of the RNeasy kit (Qiagen, Hilden, Germany), modified to obtain small RNA species. In brief, cells were lysed in the appropriate volume of RLT buffer (Qiagen) and frozen immediately at −80°C. Lysates were thawed, and 1.5 vol of 100% ethanol was added. Samples were gently homogenized and added to an RNeasy column, which was centrifuged for 1 min at ≥10,000 g and washed twice with RPE buffer, 500 μL. RNA was eluted in 30 μL RNAse-free water. RNA was diluted to make a stock concentration of 5–20 ng/mL for individual miRNAs and gene expression quantification or 200 ng/mL for low-density miRNA array quantification.

Quantification of miRNA expression

cDNA was made by use of 15 μL reaction volumes with Applied Biosystems High Capacity RNA-to-cDNA Kit by use of 3 μL RNA stock and 0.375 μL TaqMan miRNA primer (Applied Biosystems, Carlsbad, CA, USA) per sample (assaying up to 6 miRNAs simultaneously); dNTPs, buffer, RT, and RNase

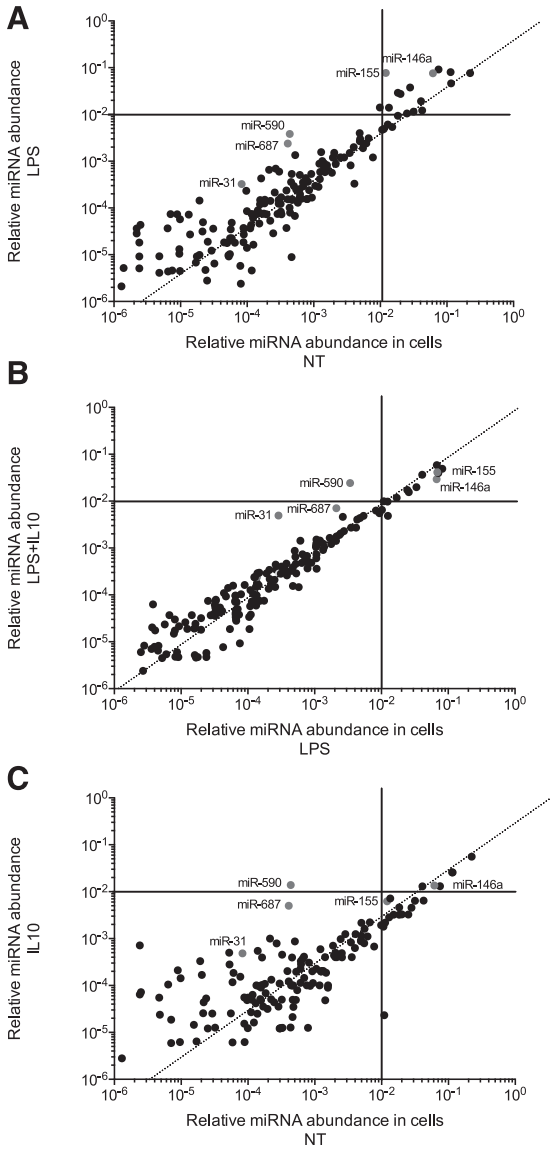

Figure 1. miRNA array in B cells stimulated with LPS and IL-10. Naïve B cells isolated from C57Bl/6 spleens were NT or treated with LPS, LPS + IL-10, or IL-10 alone for 24 h. To determine global miRNA changes, TaqMan MicroRNA arrays were performed for all 4 samples. Data were normalized to housekeeping RNAU6 expression and the relative level of each miRNA compared with the most abundant miRNA (RNAU6). Data were plotted to demonstrate changes in relative abundance, comparing treated samples (A) NT with LPS, (B) LPS + IL-10 with LPS, and (C) NT with IL-10.

inhibitor were added, per the manufacturer's directions. RT-PCR was performed by use of TaqMan Universal PCR Master Mix (Applied Biosystems) and 0.66 μL TaqMan miRNA probe (Applied Biosystems) in a 10 μL reaction volume with 2 μL cDNA added/per well. The real time was performed by use of a 7900 Real-Time PCR system (Applied Biosystems). The fold change in miRNA expression was calculated by use of the formula $2^{-\Delta\Delta Ct}$, comparing the amount of test miRNA with snoRNA202 as the housekeeping miRNA and comparing expression changes with a reference sample, e.g., NT cells.

Quantification of gene expression

cDNA was made with 20 μL reaction volumes by use of the Applied Biosystems High Capacity RNA-to-cDNA Kit and adding 8 μL of the RNA stock. RT-PCR was performed by use of in-house SYBR Green Mix with a 10 μL reaction volume. cDNA (2 μL) was added/well. The fold change was calculated as above, but the housekeeping gene used was GAPDH or hypoxanthine phosphoribosyltransferase, as indicated. Primer sequences included the following: *Aicda* F_ggacagccttctgatgaagc, R_gcgtaggaacaacaattcca; PU.1 F_atgcacgtcctcgatactcc, R_ctccaagccatcagcttctc; SHIP1 F_ggtggtacggtttgga-gaga, R_atgctgagcctctgtggtct; C/ebpβ F_atcgacttcagcccctacct, R_ggctcacg-taaccgtagtcg; Noxa F_cccagattggggaccttagt, R_agttatgtccggtgcactcc; IL-10R F_tcattgcatacgggacagaa, R_tggatgtcattccaggttga.

Low-density miRNA arrays

Naive B cells, isolated from C57Bl/6 spleens, were prepared by use of the following conditions: NT, LPS (100 ng/mL), LPS + IL-10 (200 ng/mL), and IL-10 alone. RNA was extracted and prepared for global detection of miRNAs by use of TaqMan Array Rodent MicroRNA (A Cards v2.0; Applied Biosystems). In brief, 600 ng total RNA containing small RNAs was reverse transcribed by use of the Megaplex RT Primers, Rodent Pool A with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Each plate was run consecutively by use of TaqMan Universal Master Mix II on the 7900 Real-Time PCR system, according to the manufacturer's instructions. Simultaneous analysis of the 4 different plates (NT, LPS, LPS + IL-10, and IL-10) was carried out by use of RQ Manager software (Applied Biosystems). To determine significant miRNA expression, the following filtering was applied: 1) miRNAs that had Ct values >38 for any of the 4 conditions were not analyzed. 2) Data were normalized to the snoRNA202 probe, present on each array (giving ΔCt values). 3) The relative level of each miRNA compared with the most-abundant miRNA (RNAU6; $\Delta\Delta$Ct values) was inferred by use of $2^{-\Delta\Delta Ct}$ and plotted accordingly, as reported previously [29].

Microarray analysis of GSE26673

Whole genome expression Affymetrix microarray data from Burkitt's lymphoma cases were downloaded from GEO Accession Number GSE26673 (PMID: 21245480). RMA probe summarization (RMA background correction, quantile normalization, and RMA probe-set summarization [30]) was performed on the microarrays with GeneSpring 12.6 (Agilent Technologies, Santa Clara, CA, USA). For each probe, the "baseline to median of all samples" was then applied on the log2-summarized values, resulting in the median value from all of the samples to be subtracted from the log2 value for each sample. Two Burkitt's lymphoma patients who were concurrently infected with HIV were excluded from these analyses (samples GSM656465.CEL and GSM656466.CEL). The following probes were used for the analyses (see Fig. 5): AICDA, 224499_s_at; MIR155HG, 229437_at. A one-tailed correlation was used to test the hypothesis that high miR-155 correlates with low *Aicda*.

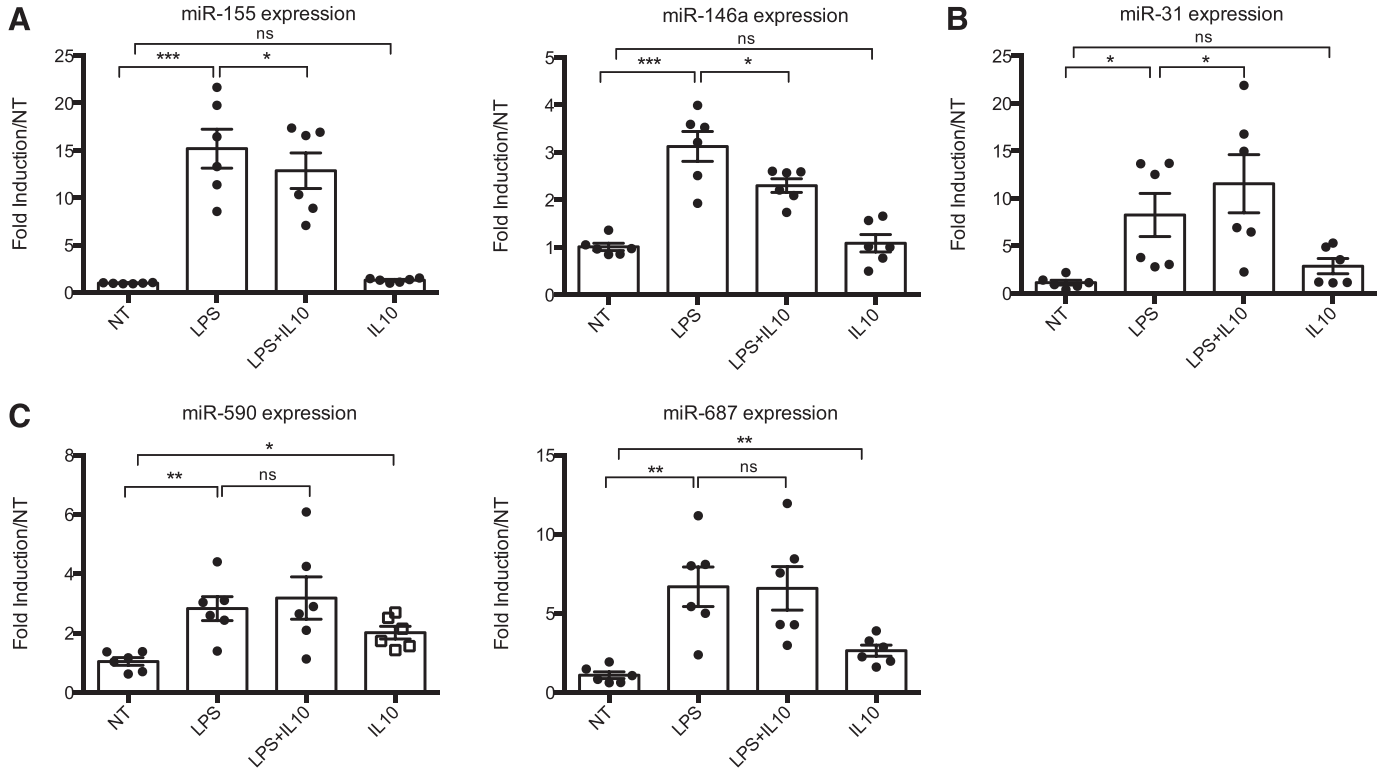

Figure 2. Validation of IL-10-modulated miRNAs. Naïve B cells isolated from C57Bl/6 spleens were NT or treated with LPS, LPS + IL-10, or IL-10 alone for 24 h. miRNA expression was measured by TaqMan RT-PCR for miR-155, miR-146a, miR-31, miR-590, and miR-687. Data were normalized to housekeeping snoRNA202 and represented as fold induction relative to NT by use of the formula $2^{-\Delta\Delta Ct}$. Data represent mean ± SEM of 2 combined experiments, each experiment contained 3 biologic replicates ($n = 6$; technical duplicates within the biologic replicate were pooled before analysis), and paired *t*-tests were conducted for significance testing.

Statistical analyses

Paired and unpaired two-tailed *t*-tests were performed by use of Microsoft Excel (Microsoft, Redmond, WA, USA) software or GraphPad Prism software (GraphPad Software, La Jolla, CA, USA), as appropriate. Differences were deemed significant where $*P \leq 0.05$, $**P < 0.01$, or $***P < 0.001$ or not significant.

Online Supplemental material

Supplemental Fig. 1 shows IL-10R expression on B cell subsets in the BM and periphery by protein and mRNA.

RESULTS

Global detection of miRNAs in B cells stimulated with LPS and IL-10

We confirmed that IL-10R could be detected in mature B cell populations in the mouse and showed the relative expression of IL-10R in all B cell subsets (Supplemental Fig. 1). Furthermore, we could influence isotype switching in vitro by use of IL-10 (data not shown). To determine whether miRNAs play a role in IL-10 function in B cells, naive B cells isolated from spleen were NT or stimulated with LPS, LPS + IL-10, or IL-10 alone (**Fig. 1**). RNA was extracted, and a low-density TaqMan miRNA array was performed. Data were normalized and plotted according to relative abundance in the cell, where the upper-right quadrants illustrate changes in the most abundant miRNAs. Any changes in highly abundant miRNAs would thus add weight to their overall contribution to cellular function compared with less-abundant miRNAs. Furthermore, the plotting of data in such a way enabled the quick identification of miRNAs that deviated from any unchanged miRNAs located on the transverse line.

Figure 1A illustrates miRNA expression changes in LPS-stimulated B cells compared with NT B cells. Two abundant miRNAs (miR-155 and miR-146a) and 3 less-abundant miRNAs (miR-31, miR-590, and miR-687) were induced by LPS treatment of B cells. In the presence of IL-10 (Fig. 1B), LPS-induced miR-155 and miR-146a levels were reduced (as observed by their relocation to the transverse line), whereas the expression of miR-31, miR-590, and miR-687 remained high. IL-10 alone had little effect on miRNA levels (Fig. 1C), with the exception of miR-31, miR-590, and miR-687.

To confirm these results, individual miRNA analyses on miR-31, miR-146a, miR-155, miR-590, and miR-687 were performed. B cells obtained from independent mice were treated with LPS, LPS + IL-10, and IL-10 (**Fig. 2**). The miRNAs whose expression changed in response to IL-10 could be differentiated into three different profiles. The first profile identified miRNAs (miR-155 and miR-146a) that were induced by LPS but could be significantly inhibited by IL-10 (Fig. 2A). The second profile, composed only of miR-31, was also significantly induced upon LPS stimulation; however, in the presence of IL-10, its expression increased (Fig. 2B). The third profile of miRNAs was that which IL-10 alone could increase (miR-590 and miR-687; Fig. 2C). We decided to focus our attention on miR-155, a miRNA well-known to play a prominent role in regulating the immune response [31]. Furthermore, we have shown previously that IL-10 inhibits miR-155 in macrophages [5], suggesting that the regulation of IL-10 on miR-155 could also be an important mechanism in B cells.

IL-10 increases the expression of the miR-155 target, *Aicda*, in response to LPS

TLR4 activation is achieved through the binding of its ligand, LPS, in multiple cell types, including B cells. It is well established that LPS potently induces the expression of miR-155 [5, 32]. As shown in Figs. 1 and 2, IL-10 can significantly reduce the expression of miR-155 in response to LPS. However, the impact on miR-155 expression in response to traditional ligands for B cell activation, such as αIgM (stimulating the BCR) and CD40L (mimicking T cell help), in the presence of IL-10, has not been

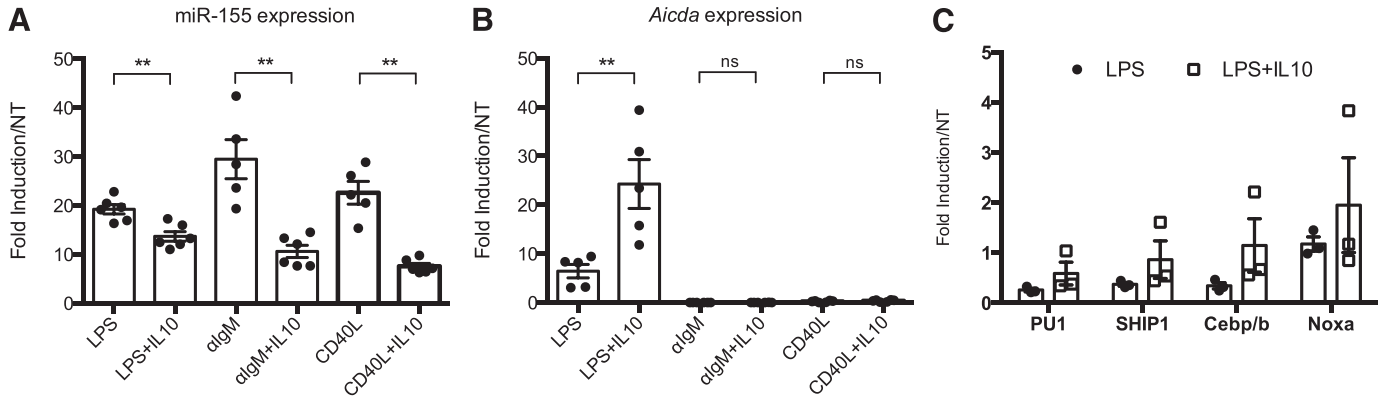

Figure 3. IL-10 increases the expression of *Aicda* in response to LPS. (A and B) Naïve B cells isolated from C57Bl/6 spleens were treated with LPS or LPS + IL-10, αIgM or αIgM + IL-10, CD40L or CD40L + IL-10 for 24 h. miR-155 expression (A) and mRNA expression for *Aicda* (B) were measured. Data were normalized to housekeeping genes (snoRNA202 and GAPDH) and are represented as fold induction relative to NT by use of the formula $2^{-\Delta\Delta Ct}$. Data represent mean ± SEM of 2 combined experiments, each performed on 3 biologic replicates ($n = 6$). (C) Naïve B cells isolated from C57Bl/6 spleens were treated with LPS or LPS + IL-10. mRNA expression for the following miR-155 targets was measured by RT-PCR: *Aicda*, PU.1, SHIP1, C/ebpβ, and Noxa. Data were normalized to the GAPDH housekeeping gene and represented as fold induction relative to NT by use of the formula $2^{-\Delta\Delta Ct}$. Data are representative mean ± SEM of 6 biologic replicates from 2 independent experiments ($n = 6$; technical duplicates were pooled before analysis).

investigated. Accordingly, we show in **Fig. 3A** that similar to LPS, αIgM and CD40L could potently induce miR-155, and this expression was inhibited significantly in the presence of IL-10.

Aicda has previously been identified as an important target for miR-155 in B cells [28]. miR-155 acts to repress the expression of *Aicda* by binding to a miR-155-binding site located in the 3′UTR of *Aicda* mRNA, resulting in mRNA degradation. The inhibition of miR-155 by IL-10 suggested that the repression of *Aicda* would be alleviated in the presence of IL-10, resulting in increased mRNA expression. Accordingly, IL-10 treatment of B cells enhanced *Aicda* expression in response to LPS only (Fig. 3B), whereas no effect was observed in response to αIgM and CD40L. This suggested that TLR4, together with IL-10, modulated the expression of the miR-155 target, *Aicda*.

To date, several miR-155 targets have been identified and validated in B cells, including *Aicda*, PU1, and SHIP1 [7, 27, 28]. Noxa and C/ebpβ are also predicted to have 8-mer seed matches and are expressed in B cells (TargetScanMouse 6.2 [33] and ImmGen database, detectable expression above background by use of microarray). We thus wanted to examine the effects of LPS + IL-10 on these miR-155 targets. Figure 3C demonstrated that whereas there was a trend toward IL-10 increasing the expression of all miR-155 targets tested, there failed to be a significant change compared with that observed with *Aicda* (Fig. 3B).

IL-10 regulates *Aicda* expression through miR-155

As miR-155 is a known post-transcriptional repressor of *Aicda*, we investigated whether IL-10 was boosting *Aicda* through the regulation of miR-155. To do this, we used an animal model, where the miR-155-binding site in the 3′UTR of *Aicda* was mutated (Aicda$^{155/155}$) [26]. In B cells from these mice, miR-155 is expressed normally; however, it is unable to bind and repress *Aicda* through its 3′UTR. As expected, Aicda$^{155/155}$ mutant mice displayed normal miR-155 expression in response to LPS, and this expression was inhibited by IL-10 (**Fig. 4A**).

In both WT and Aicda$^{155/155}$ mutant mice, LPS induced *Aicda*, but the increase was greater in Aicda$^{155/155}$ mutant mice, confirming the fact that miR-155 directly represses LPS-driven *Aicda* induction (Fig. 4B). Furthermore, IL-10 resulted in greater expression of *Aicda* with levels significantly and substantially greater in Aicda$^{155/155}$ mutant mice (Fig. 4B). This demonstrates that IL-10 could potentiate the expression of *Aicda* when there was an absence of miR-155 control.

miR-155 and *Aicda* are inversely correlated in Burkitt's lymphoma samples

As miR-155 has been shown to be down-regulated in some Burkitt's lymphoma samples [34–36], we sought to determine whether loss of miR-155 would lead to increased *Aicda* expression in published lymphoma samples. We interrogated deposited data in the GEO and found a dataset of Burkitt's lymphoma samples that had probes to both miR-155 and *Aicda* (GSE26673). When we analyzed these data, we observed an inverse correlation ($P = 0.05$) between the levels of the probes for AICDA and MIR155HG (pri-miRNA; **Fig. 5**). This correlation exists from a cohort of 14 patients (the entire cohort

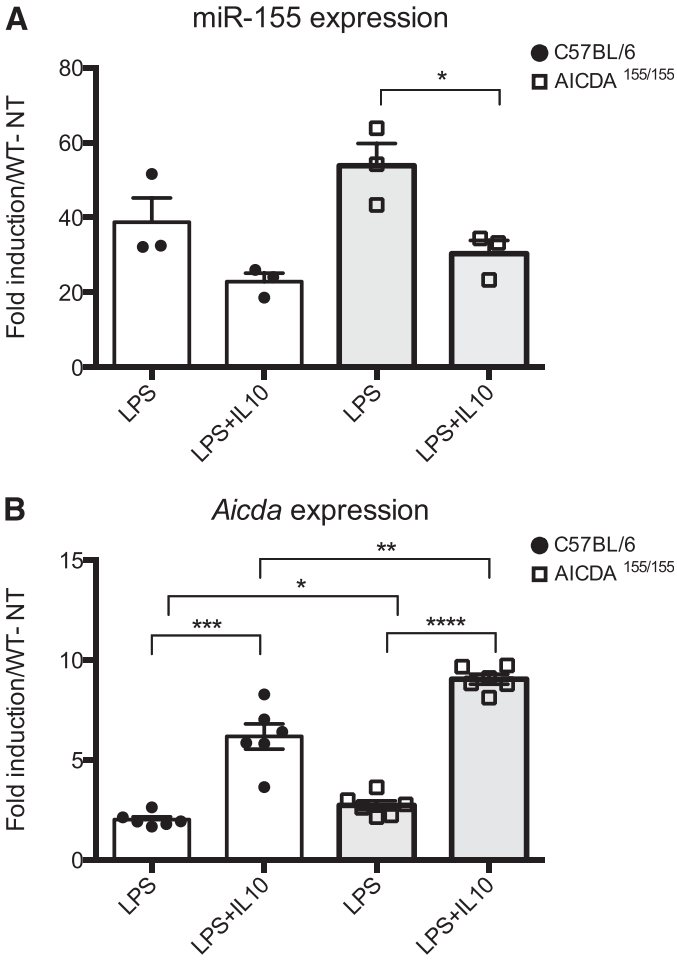

Figure 4. IL-10 potentiates *Aicda* expression in Aicda$^{155/155}$ mutant mice. Naïve B cells, isolated from C57Bl/6 and Aicda$^{155/155}$ spleens, were treated with LPS or LPS + IL-10 for 24 h. miR-155 expression (A) and *Aicda* mRNA expression (B) were measured by RT-PCR. Data were normalized to housekeeping genes (snoRNA202 and GAPDH) and are represented as fold induction relative to NT by use of the formula $2^{-\Delta\Delta Ct}$. Data show the representative mean ± SEM of 2 independent experiments (A) or representative mean ± SEM of 2 combined experiments (B), each performed on 3 biologic replicates (technical duplicates were pooled before analysis).

contained 16 patients, but 2 were excluded for this analysis, as they were HIV positive). The correlation of AICDA versus MIR155HG is −0.4571, reflecting the fine-tuning that miR-155 would exert on *Aicda*.

Together, our data highlight a stringent and sophisticated mechanism of control on *Aicda* expression by IL-10 through its ability to fine-tune and concisely modulate miR-155 expression levels (**Fig. 6**).

DISCUSSION

Here, we identified that IL-10, in the presence of TLR4 stimulation, is a potent inducer of *Aicda* mRNA expression. Our data establish for the first time that IL-10 can act in concordance with TLR4 activation to provide a positive signal

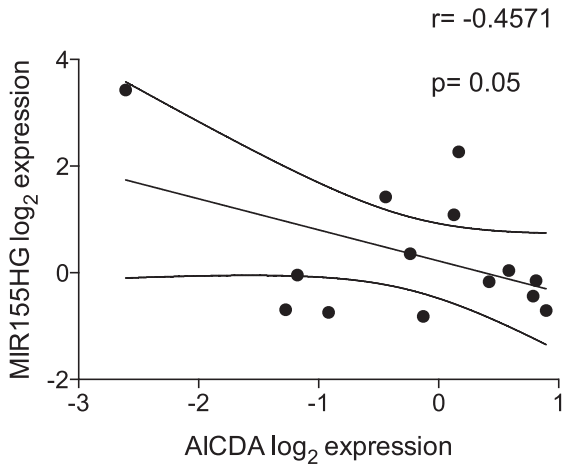

Figure 5. AICDA and miR-155 are inversely correlated in Burkitt's lymphoma samples. Whole genome expression Affymetrix microarray data from Burkitt's lymphoma cases were downloaded from GEO Accession Number GSE26673 (PMID: 21245480) and RMA normalized. Probes to AICDA: 224499_s_at and MIR155HG: 229437_at were plotted on an *x,y* scatter plot. A one-tailed interpolation of correlation showing error was used to test the hypothesis that high miR-155 correlates with low AICDA.

for *Aicda* mRNA expression. Furthermore, we established that TLR4 and IL-10 play a crucial role in modulating post-transcriptional regulation of *Aicda* via miR-155, as well as establishing that the IL-10/miR-155 axis fine-tunes an important regulator of B cell function. These data confirm a role for the cooperation of cytokines and TLRs in the propagation of an appropriate B cell response to invading pathogens [11].

Control of *Aicda*/AID expression is regulated transcriptionally [19–21], post-translationally [22, 23], by intracellular compartmentalization and trafficking [24, 25], and most recently, post-transcriptionally, by miR-155 [26–28]. This was first demonstrated in microarray analysis performed in B cells deficient in miR-155. These cells were shown to have an increase in *Aicda* expression, in addition to deregulation of at least 60 other genes in response to LPS + IL-4 [28]. A more specific method for delineating the exact role of miR-155 on *Aicda* expression and function was undertaken by two independent groups. They individually created mouse models where the miR-155 seed region in the 3′UTR of *Aicda* was mutated, thereby resulting in ablation of any miR-155:*Aicda* interaction [26, 27]. Both of these studies confirmed that in the absence of miR-155 regulation, there was a dramatic increase in the expression of *Aicda* mRNA and protein in response to LPS + IL-4. Our data consolidate these results with additional evidence that IL-10 cooperates with TLR4 activation to increase the expression of *Aicda*, with a potentiated increase in Aicda$^{155/155}$ mutant mice.

miR-155 is potently induced by TLR stimulation in macrophages, dendritic cells, and B cells [5, 7, 27, 31]. In B cells, miR-155 expression, together with *Aicda* expression, is up-regulated in cells stimulated to undergo CSR [27]. Up-regulation of miR-155 acts to curtail *Aicda* overexpression. However, we have shown that miR-155 expression is dampened in the presence of IL-10, resulting in enhanced *Aicda* expression, thereby creating a positive signal for increased Ig class-switching in response to invading pathogens. This is interesting, considering that IL-10 has been known as a positive regulator of B cell function, whereas IL-10 acts as an anti-inflammatory molecule in monocyte-derived cells [37]. We believe that we have unlocked a mechanism whereby IL-10 can fine-tune the expression levels of miR-155 to administer the appropriate IL-10 function in any particular cell type. With the consideration that miR-155 is predicted to have multiple targets based on its "seed matching" to the 3′UTR of mRNA target molecules, it will

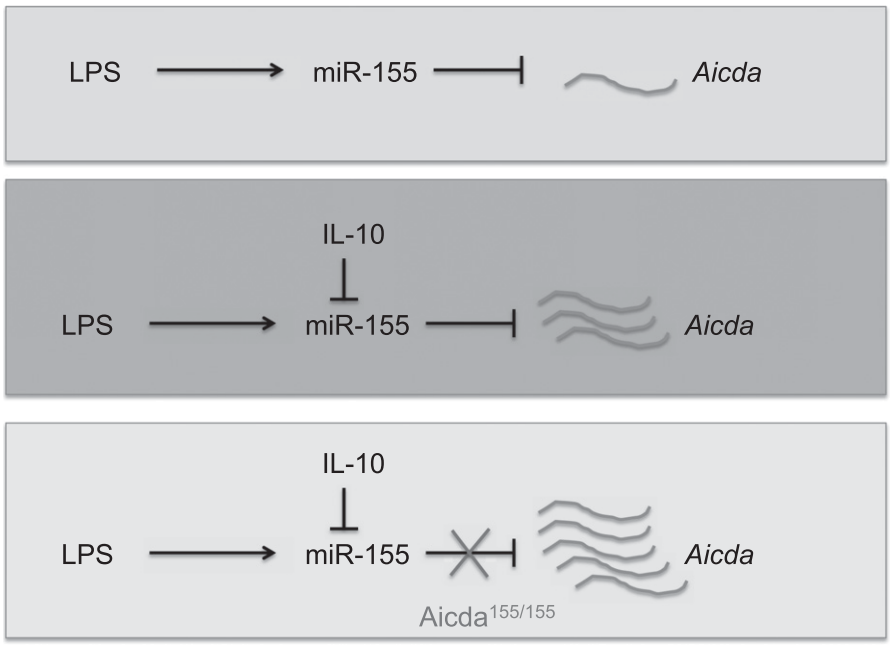

Figure 6. Schematic illustrating the role of IL-10 on *Aicda* expression. LPS induces high expression of miR-155, which acts as a negative regulator of *Aicda* mRNA and thus, acts to repress overproduction of *Aicda* (top). In the presence of IL-10, which limits miR-155 expression, *Aicda* expression is boosted (middle). In this way, IL-10 acts to enhance and boost *Aicda* expression synergistically upon TLR4 stimulation. *Aicda* expression is potentiated further in response to IL-10 when miR-155 can no longer bind and repress *Aicda* expression, as demonstrated in Aicda$^{155/155}$ mutant mice (bottom). This suggests that complete ablation of miR-155 in response to IL-10 may have a negative impact on the cell, producing *Aicda* over and beyond what is required. Together, this highlights a tight and sophisticated mechanism of control on *Aicda* expression by IL-10 through its ability to fine-tune and succinctly modulate miR-155 expression levels.

be intriguing to investigate further the role of the IL-10/miR-155 axis on other miR-155 target genes.

The fact that IL-10 can further potentiate *Aicda* expression in Aicda$^{155/155}$ mutant mice suggests that complete ablation of miR-155 or a lack of miR-155 control may have a negative impact on the cell, producing more *Aicda* than is required and leaving open the potential for excess mutation. AID induces SHM by inducing double-strand breaks in antibody-variable regions; however, AID can also produce off-target lesions in other genes, including oncogenes [38–40]. As a result, overexpression of AID favors tumor development. For example, overexpression has been shown to result in c-myc/IgH translocations, a characteristic of Burkitt's lymphoma, as well as leading to T cell lymphomas [39, 41, 42]. Given that miR-155 expression is lost in Burkitt's lymphoma, this suggests a mechanism contributing to enhanced AID expression [35]. To confirm this, we demonstrated an inverse correlation between miR-155 and *Aicda* in a cohort of 14 Burkitt's lymphoma patients. The fact that we have found IL-10 to inhibit miR-155 adds more complexity, suggesting that the IL-10 modulation of miR-155 is another mechanism used by B cells to enhance *Aicda* mRNA levels. This is more intriguing considering that IL-10 is constitutively expressed in EBV-positive Burkitt's lymphoma cells, and often, IL-10 serum levels are used as a diagnostic tool to predict prognosis [37, 43, 44]. Together, our data highlight that there is a tight and sophisticated control of *Aicda* expression mediated by the IL-10/miR-155 axis, where an imbalance of each of these molecules, individually or combined, is strongly correlated to diseases, such as Burkitt's lymphoma.

AUTHORSHIP

K.A.F. designed, performed, and analyzed experiments and wrote the manuscript. M.P.G. designed and analyzed experiments, provided reagents, and edited the manuscript. F.M. provided resources, reagents, and discussion. B.R.G.W. provided resources and reagents and edited the manuscript. C.E.M. conceived of the project; designed, performed and analyzed experiments; and wrote the manuscript.

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DISCLOSURES

The authors declare no conflict of interest.

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