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Repository corticotropin injection reverses critical elements of the TLR9/B cell receptor activation response in human B cells in vitro

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ABSTRACT

We sought evidence for direct effects of repository corticotropin (RCI; an FDA-approved treatment for selected cases of SLE) on isolated human B lymphocytes activated by engagement of TLR9 and B cell receptors. ODN 2395/αIgM treatment was found to result in induction of 162 distinct mRNAs and suppression of 80 mRNAs at 24 h. RCI treatment resulted in suppression of 14 of the ODN 2395/αIgM -induced mRNAs (mean suppression to 23.6 ± 3.1% of stimulated value). The RCI-suppressed mRNAs included two critical regulators of class switch recombination, AICDA and BATF. RCI treatment also resulted in induction of 5 of the ODN 2395/αIgM -suppressed mRNAs (mean induction by RCI = 7.65 ± 2.34-fold). The RCI-induced mRNAs included SLAMF3, a cell surface receptor capable of inhibiting autoantibody responses. These studies reveal that RCI treatment of human B cells reverses key elements of the early mRNA response to TLR9 and B cell receptor engagement.

1. Introduction

A twenty-five year search for hormonal modulators of "rheumatic" diseases culminated in 1950 in a series of reports by P.S. Hench and colleagues demonstrating the use of adrenal corticosteroids (glucocorticoids) in the management of disorders that we now recognize to be autoimmune in origin [1,2]. These investigators also demonstrated that, as an alternative to administration of exogenous glucocorticoids, it was possible to stimulate increased endogenous glucocorticoid production by the administration of preparations of the pituitary peptide hormone corticotropin (adrenocorticotropin, or ACTH), the principal regulator of production of glucocorticoids from the adrenal cortex [3–6]. It has since been generally assumed that any immunomodulatory effects of corticotropin would be exerted indirectly through its induction of adrenal steroidogenesis. However, subsequent clinical observations have suggested that responses to corticotropin might be, at least in part, independent of glucocorticoid effects [7,8] and a number of in vitro studies have shown evidence for direct actions of corticotropin and related peptides on the function of lymphocytes [9–14].

Repository corticotropin injection (RCI; H.P. Acthar® Gel) is a therapeutic approved by the FDA for the treatment of multiple autoimmune and inflammatory disorders, although its precise mechanism of action is not known. We have recently reported that RCI exerts direct, acute, dose-dependent inhibitory effects on the functions of IL4 + CD40 ligand-stimulated human B lymphocytes in vitro [15] and that these direct effects on human B cells are characterized by changes in gene expression that are largely distinct from those elicited by glucocorticoids under the same IL4/CD40 ligand-activated conditions in vitro [16].

The objective of the present study was to define the effects of RCI on the functions and the patterns of gene expression in human B cells activated by signaling through Toll-like receptor 9 (TLR9), an innate immune signaling pathway that detects unmethylated CpG oligodeoxynucleotides and triggers cellular differentiation, proliferation,

Abbreviations: RCI, Repository Corticotropin Injection; TLR9, Toll-like receptor 9; BCR, B cell receptor; αIgM, anti-IgM antibody

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cytokine production, and immunoglobulin isotype switching and secretion in various human B cell subsets in vitro [17–19]. This pathway of B cell activation is of particular relevance in the context of RCI's use in the treatment of autoimmune diseases such as systemic lupus erythematosus, in which TLR-driven humoral autoreactivity plays an important role [20,21]. We report here evidence that RCI exerts inhibitory effects on IgG secretion from human B cells activated in vitro by TLR9 and B cell receptor engagement. These experiments further show that, well before changes in IgG secretion are evident, RCI reverses ODN 2395/αIgM- induced changes in expression of specific mRNAs encoding proteins that play central roles in the response of human B cells to TLR9/BCR activation signals.

2. Methods

2.1. Human subjects

A total of 21 healthy volunteer subjects (five males and sixteen females) donated peripheral blood samples for these studies. The ages of the subjects ranged from 24 to 63 years and averaged 43.14 ± 2.9 (mean ± SEM) years. Volunteers gave informed consent to participate in the protocol approved by the Penn State/Milton S. Hershey Medical Center Institutional Review Board.

2.2. B lymphocyte preparation and culture

Peripheral blood mononuclear cells were prepared by density gradient centrifugation on Histopaque 1077 (MP Biomedicals, Santa Ana, CA) and peripheral B lymphocytes were then isolated using magnetic CD19 MicroBeads (Miltenyi Biotec, San Diego, CA) and a MidiMACS Separator (Miltenyi Biotec). Recovered B cells were resuspended in complete medium (RPMI 1640 with no phenol red, supplemented with 9% charcoal stripped fetal bovine serum, 1% GlutaMAX-1, and 100 I.U. penicillin/100 μg/ml streptomycin). Cells were plated at a density of 0.5–1.0 × 10^6 cells per ml and stimulated with 1–5 μg/ml ODN 2395 (Invivogen, San Diego, CA) and with 2.0 μg/ml anti-IgM (αIgM; Jackson Immunoresearch, West Grove, PA). Controls included cells that were cultured unstimulated as well as cells that were treated with IL4 and CD40 ligand as previously described [16]. RCI or placebo gel (provided in blinded fashion; Mallinckrodt Pharmaceuticals, St. Louis, MO) was added to replicate cultures at estimated ACTH analog concentrations of 0.124 μM, 1.24 μM, and 2.49 μM (or equal volumes of placebo gel), to replicate the conditions under which RCI exerted biologic effects in our prior studies [15,16]. Placebo gel was identical to RCI, except that it did not contain any active pharmaceutical ingredient. In some experiments, dexamethasone (10 nM final concentration; 0.1% final volume/volume ethanol) or vehicle alone was used. Dexamethasone, a pure glucocorticoid receptor (GR) agonist, was used at concentrations well above the dissociation constant of GR (Kd ~1 nM) to assure receptor saturation. Cells were incubated at 37 °C with 5% CO_2 and cultures were harvested for RNA isolation after 1 day or for IgG measurement in culture supernatants after 4 days. No further additions were made to cultures at any time during the various incubation periods.

2.3. Assays for immunoglobulins in cell culture supernatants

Immunoglobulin G and M levels in cell culture supernatants were measured using immunoglobulin heavy chain-specific (IgG) and intact molecule-specific (IgM) microsphere agglutination assays (Pierce Easy-Titer; Thermo-Fisher, Rockford, IL).

2.4. RNA isolation

RNA was isolated from harvested cells using the RNeasy Mini Kit (QIAGEN, Germantown, MD) according to the manufacturer's instructions, with slight modifications to increase column spin and spin-drying times. Contaminating genomic DNA was removed from each RNA prep using DNA-Free DNAse Treatment and Removal Reagents (Ambion/Thermo Fisher Scientific, Waltham MA). RNA concentration was measured with the Qubit RNA HS Assay Kit (Molecular Probes) using a Qubit Fluorometer 2.0 (Invitrogen/Life Technologies/Thermo Fisher Scientific). RNA quality was assessed using an Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA) [16].

2.5. Reverse transcription and real-time PCR quantitation of AICDA and γ1/2-Cμ switch circle transcripts

Equal quantities of RNA from each set of experimental samples were subjected to reverse transcription using components of the High Capacity RNA to cDNA Kit (Applied Biosystems/Thermo Fisher Scientific) in an Applied Biosystems 2720 Thermal Cycler. The resulting cDNA served as template for real-time quantitative polymerase chain reaction (qPCR) using Taqman Gene Expression Master Mix and Taqman gene expression assays for:

AICDA (Hs00221068_ml)
HDAC5 (Hs 00608366_ml)
RORA (Hs00536545_ml)

Assays were performed using a Quant Studio 12K Flex Real Time PCR System (Applied Biosystems/Thermo Fisher Scientific) with the following cycling parameters: 50 °C for 2 min, 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

AICDA mRNA expression was quantitated using the Comparative Ct method with RORA and HDAC5 selected as endogenous control genes for each sample (RORA and HDAC5 mRNA levels were experimentally determined to be more stable than GAPDH between resting and activated B cells; data not shown). Statistical analysis of results from three replicate experiments using cells from unique donors was by ANOVA with Dunnett's multiple comparisons test using Prism 7.0d software for Mac (GraphPad Software, La Jolla, CA). Results are reported as mean ± SEM.

AICDA action (conversion of deoxycytidine to deoxyuridine with consequent base pair mismatch) initiates a process that culminates in DNA strand breakage at the specific “switch regions” of immunoglobulin heavy chain gene locus in activated B cells. The subsequent repair process results in class switch recombination as well as excision from genomic DNA of a double stranded DNA fragment that is capable of circularization and remains transcriptionally active [22]. The transcripts can thus serve as a marker of AICDA's enzymatic activity. In lieu of any other quantitative assay for AICDA protein levels, we measured expression of such γ1/2-Cμ switch circle mRNA transcripts using a real-time PCR-based absolute quantitation assay (Assays by Design, Applied Biosystems). This absolute quantitation assay detects a specific 99 base amplimer corresponding to nucleotides 262–360 of γ1/2-Cμ switch circle mRNA transcript reported in GenBank (AJ617577.1 and AJ617578.1):

GGCCACGCTGCTCGTATCCGACGGGGAATTCTCACAGGAGACGAGGGGGAAAAGGGTTGGGGCGGATGCACTCCCTGCTGTCCTTGGTCCTGGCTGAGA.

This amplified fragment comprises 27 base pairs from the immunoglobulin heavy chain G2 locus (IGHG2) and 72 nucleotides from the heavy chain M locus (IGHM). In genomic DNA these two fragments are separated by > 100 kb.

The 99 base-pair fragment was subcloned into a plasmid vector (pSC-A-amp/kan; Agilent Technologies, La Jolla, CA), and its identity was confirmed by DNA sequencing. The cloned γ1/2-Cμ transcript cDNA plasmid was used as template to generate a standard curve in each assay for absolute quantitation. This standard curve of log γ1/2-Cμ vs Ct is linear between 0.00001 pg and 10 pg and has good reproducibility (mean slope equals −3.36 ± 0.07; r^2 = 0.9945 ± 0.00021; n = 4).

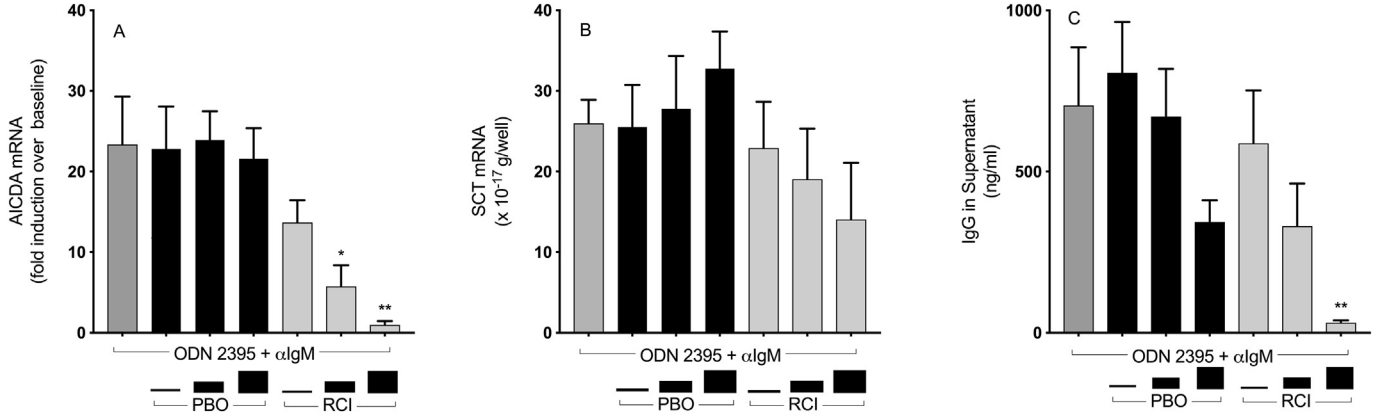

Fig. 1. RCI suppresses human B lymphocyte function under conditions of activation in vitro by TLR9/BCR engagement. Expression of AICDA mRNA (Panel A) or γ1/2-Cμ switch circle transcripts (Panel B) was assessed in human peripheral B cells cultured for 1 day under control conditions (dark gray bar), with placebo gel (PBO; black bars) or with RCI (light gray bars) at estimated ACTH analogue concentrations of approximately 0.124 μM, 1.24 μM, or 2.49 μM (denoted by stepped bars). Cellular mRNA was isolated and quantitated by RT/PCR as described in the methods section. Data were analyzed by ANOVA with Dunnet's multiple comparison test. AICDA mRNA levels on day one were reduced by medium dose RCI (*; adjusted $p = .0140$), and by high dose RCI (**; adjusted $p = .0018$). RCI treatment also resulted in an apparent dose-related trend downward for γ1/2-Cμ switch circle transcripts on day one, but the differences did not reach statistical significance. Results shown are mean ± SEM of three independent experiments with cells from unique donors.
Immunoglobulin G (Panel C) was measured in supernatants from human peripheral B cells cultured for 4 days under stimulation with the TLR9 ligand ODN 2395 and anti IgM under control conditions (dark gray bar), with Placebo control gel (black bars) or with RCI (light gray bars) at estimated ACTH analogue concentrations of approximately 0.124 μM, 1.24 μM, or 2.49 μM (denoted by the stepped bars). Supernatants were harvested and assayed for IgG and results analyzed by one-way ANOVA ($p = .0104$); the bar marked with the asterisk (*) was found significantly different from the ODN 2395 + αIgM-stimulated control by Dunn's post-test at $p = .011$. Results shown are mean ± SEM for six independent experiments using cells from unique healthy donors.

2.6. cDNA library preparation and multiplexed high-throughput sequencing

cDNA libraries were prepared using the TruSeq Stranded Total RNA with Ribo-Zero Gold Library Prep Kit (Illumina) as previously described [16]. Total RNA was depleted of ribosomal RNA (rRNA) using proprietary rRNA-depletion oligos. rRNA-depleted RNA was subjected to fragmentation, reverse transcription, end repair, 3′-end adenylation, adaptor ligation and subsequent PCR amplification. The unique barcode sequences were incorporated in the adaptors for multiplexed high-throughput sequencing. The final product was assessed for its size distribution and concentration using BioAnalyzer High Sensitivity DNA Kit (Agilent) and Kapa Library Quantification Kit (Kapa Biosystems). The libraries were pooled and diluted to 2 nM in EB buffer (Qiagen) and then denatured using the Illumina protocol. The denatured libraries were diluted to 10 pM with pre-chilled hybridization buffer and loaded onto TruSeq SR v3 flow cells on an Illumina HiSeq 2500 (Illumina) and run for 50 cycles using a single-read recipe (TruSeq SBS Kit v3, Illumina) [16].

2.7. Quality control, mapping, and quantification of RNA-Seq reads

Illumina CASAVA pipeline Version 1.8 was used to extract demultiplexed sequencing reads. FastQC (version 0.11.2) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to validate the quality of the raw sequence data. Additional quality filtering used FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) using a quality score cutoff of 20. Next, alignment of the filtered reads to the human reference genome (hg38) was done using Tophat (version 2.0.9) [23] allowing two mismatches. Picard (version1.102) (https://github.com/broadinstitute/picard) was used to assess proportion of mapped bases to coding, UTR, intronic, and intergenic regions, respectively and correctness of strand specificity. Picard was also used to find coverage across gene body to determine 5′- or 3′- bias. Read counts were calculated using HTSeq [24] as provided with the Ensembl gene annotation (release 78).

2.8. Differential gene expression analysis

RUVSeq R package v3.1 [25] along with edgeR [26] was used to identify genes differentially expressed between control and RCI-treated samples. First we normalized the raw read counts by selecting a set of "in-silico empirical" negative controls, i.e., least significantly differentially expressed genes based on a first-pass differentially expressed analysis performed prior to normalization. Normalized read counts were applied to differentially expressed genes, using the negative binomial GLM approach implemented in edgeR. Significantly differentially expressed genes were defined to be those with corrected p-value $< .05$ in each of the three independent experiments based on unique cell donors. Degree of upregulation for individual mRNAs is reported as fold increase above control (mean for three independent experiments); downregulation is reported as fraction of control (mean for three independent experiments).

2.9. Other data analyses

Data analysis by ANOVA and multiple comparison tests was performed using Prism 7.0d for Mac software (GraphPad Software, LA Jolla, California). Heatmaps of gene expression data were generated using Heatmapper [27]. Proportional graphical representations of overlapping regulated mRNA sets were prepared using EulerAPE [28]. The probability of observed overlaps between regulated mRNA sets was estimated using a web-based hypergeometric distribution calculator (http://nemates.org/MA/progs/overlap_stats.html).

3. Results

3.1. RCI suppresses AICDA mRNA expression and IgG secretion in ODN 2395/αIgM-activated human B cells in vitro

We have previously reported a direct effect of RCI to inhibit induction of expression of the DNA-modifying enzyme activation induced cytidine deaminase (AICDA) in IL4/CD40L-activated human B cells [15]. This enzyme initiates the genomic changes (described above) that result in immunoglobulin heavy chain gene class switch recombination

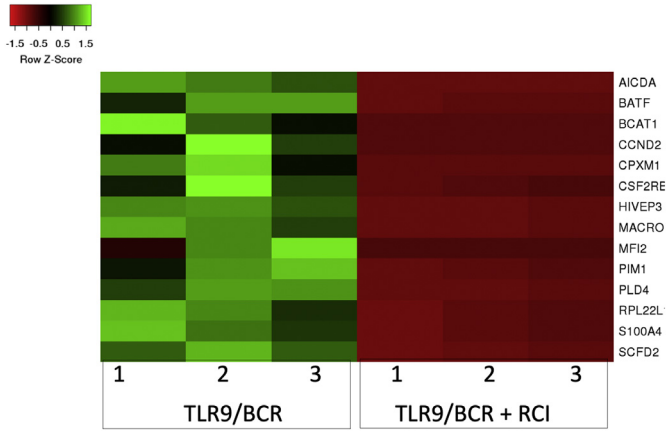

Fig. 2. Heat map of relative expression values for 14 mRNAs that were upregulated by TLR9/BCR-mediated activation of isolated human B cells and were downregulated by RCI treatment. Each column represents data from one of three unique donors. Green colors represent degrees of upregulation over control (unstimulated) cells. Red colors represent degrees of downregulation compared to TLR9/BCR-stimulated cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(CSR) as well as somatic hypermutation (SHM) of the variable regions of immunoglobulin heavy and light chain genes and its action is required for the production of IgG by differentiated B cells. In ODN 2395/αIgM-activated B cells we found that RCI treatment resulted in a dose-dependent reduction in AICDA expression after 1 day in culture (Fig. 1A). This time point was chosen to assess immediate/early effects of RCI on B cell gene expression before effects on cellular proliferation or cell death become apparent in culture. ODN 2395/αIgM-stimulated cells exhibited increases in AICDA expression (23.33 ± 5.97-fold above control); this induction was partially reversed (to 5.77 ± 2.60-fold above control; adjusted $p = .029$) by the medium dose of RCI and was completely reversed to control levels (0.97 ± 0.46; adjusted $p = .006$) by the highest dose RCI. Placebo gel had no demonstrable effect.

The observed inhibition of AICDA expression by RCI was accompanied by an apparent reduction in levels of γ1/2-Cμ switch circle transcripts, mRNAs transcribed from excised DNA fragments produced during active class switch recombination (Fig. 1B). These switch circle transcripts were reduced to about 54% of ODN 2395/αIgM-stimulated values by the highest dose of RCI, although these changes did not reach statistical significance.

As a definitive readout of RCI effects on B cell function we assessed IgG secretion into culture supernatants of ODN 2395/αIgM-activated human B cells. RCI treatment resulted in a dose-dependent reduction of IgG accumulation in culture supernatants at 4 days (Fig. 1C). The 4-day time point was chosen because preliminary experiments revealed significant rates of cell death in TLR9-activated cells after 4 days. Maximal inhibition of IgG production (to 4.4% of the values observed in control cells) was noted in ODN 2395/αIgM-stimulated cells treated with the highest dose of RCI ($p < .01$). Changes in IgG production observed in cells treated with the highest dose of placebo gel did not reach statistical significance.

As we previously reported for human B cells activated with IL4 and CD40 ligand [15], treatment with 100 nM dexamethasone (not shown) also resulted in inhibitory effects on ODN 2395/anti-IgM-stimulated cells. In such glucocorticoid-treated cells AICDA mRNA expression to about fell to 28.6% of the level seen in ODN 2395/anti-IgM-stimulated control cells. Switch circle transcripts fell to about 66.1% of stimulated controls, and IgG levels in supernatants (at day 4) fell to 69.7% of those seen in stimulated control cells.

3.2. RCI treatment in vitro reverses critical elements of the gene expression response to TLR9/BCR-mediated activation of human B cells

Given these observations of RCI's impact on ODN 2395/αIgM -activated B cell gene function we sought first to define the early response of isolated human B cells to TLR9 and BCR engagement in vitro at the level of mRNA expression and then to assess the impact of RCI on that response. Treatment of isolated human B cells in steroid-free medium with ODN 2395/αIgM resulted in significant, reproducible induction of 162 distinct mRNAs (Supplementary Table 1A). On average, these upregulated mRNAs rose by 8.87 ± 0.95-fold over the level observed in unstimulated control cells (range = 2.5 to 118.3-fold). ODN 2395/αIgM treatment resulted in suppression of 80 mRNAs (Supplementary Table 1B). On average, these downregulated mRNAs fell to 21.8 ± 0.8% of baseline expression observed in unstimulated cells (range = 6% to 39%).

RCI treatment at the highest dose resulted in significant, reproducible suppression of 14 of the ODN 2395/αIgM-induced mRNAs (Fig. 2, Table 1). For each of these mRNAs, RCI treatment resulted in reversal of the ODN 2395/αIgM effect by > 50%. On average, mRNAs were suppressed to 23.3 ± 3.1% of the ODN 2395/anti IgM-stimulated value (range 9.9 to 41.2%). Conversely, RCI treatment resulted in significant, reproducible induction of 5 of the ODN 2395/anti-IgM -suppressed mRNAs (Fig. 3, Table 2). Mean induction by RCI was 7.65 ± 2.34-fold; range = 4.7 to 16.9-fold). No ODN 2395/anti IgM-induced mRNAs exhibited further increase in expression levels in response to RCI treatment. Neither did RCI treatment result in any apparent further reduction in levels of mRNAs that were suppressed by ODN 2395/anti-IgM. Furthermore, hierarchical clustering of RNA-Seq expression data from ODN 2395/anti-IgM treated cells (each significant mRNA modulation replicated across the three independent experiments) with mRNA expression changes induced by RCI suggests a broad reversal of the TLR9/B cell receptor-initiated program of gene expression by RCI (Supplementary Fig. 1).

The specific mRNAs whose induction in ODN 2395/anti-IgM-activated B cells was found to be reversed by RCI treatment are detailed in Table 1. These include mRNAs that encode proteins known to play roles in a variety of essential B cell functions including class switch recombination [29–33], somatic hypermutation [34,35] proliferation [36,37], and cellular differentiation [38–43].

The specific mRNAs whose suppressed expression in ODN 2395/anti-IgM-activated B cells was found to be reversed by RCI treatment are detailed in Table 2. These include mRNAs that encode proteins known to play roles in B cell survival and proliferation [44], antibody production [45–47], and cellular migration [48–50].

RCI treatment also resulted in modulation of levels of a number of mRNAs with previously recognized functions in B cells, but whose expression was not significantly altered by ODN 2395/anti-IgM-mediated

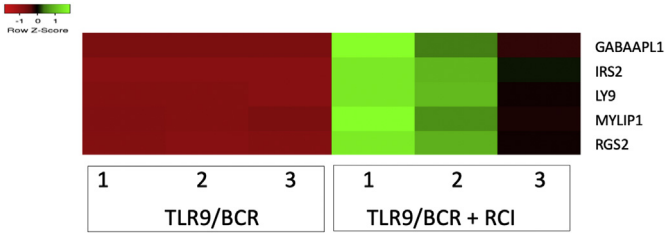

Fig. 3. Heat map of relative expression values for 5 mRNAs that were downregulated by TLR9/BCR-mediated activation of isolated human B cells and were upregulated by RCI treatment. Each column represents data from one of three unique donors. Red colors represent degrees of downregulation compared to control (unstimulated) cells. Green colors represent degrees of upregulation compared to TLR9/BCR-stimulated cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
TLR9/BCR-Induced, RCI-Downregulated mRNAs In Isolated Human B Cells.

mRNA	Description	Up Regulation by TLR/αIgM (Fold above control)	Down Regulation by RCI (Fraction of TLR/αIgM- stimulated level)	Down Regulation By RCI (normalized to original baseline level)	Literature Citations on known B cell functions
AICDA	Activation-Induced Cytidine Deaminase	9.98	0.099	0.898	[29–31,34,35]
BATF	B Cell Activating Transcription Factor	9.66	0.320	3.090	[32,33]
BCAT1	Branched Chain Amino Acid Transaminase 1	9.14	0.302	3.090	
CCND2	Cyclin D2	16.98	0.172	2.920	[36,37,63]
CPXM1	Carboxypeptid-ase X, M14 family member 1	16.37	0.133	2.177	
CSF2RB	Colony Stimulating Factor 2 Receptor β subunit	4.70	0.406	1.908	
HIVEP3	HIV enhancer binding protein 3	3.30	0.193	0.637	[39–41]
MACROD2	MACRO domain containing-2	12.98	0.174	2.258	
MFI2	Melanotransferrin	30.37	0.140	4.252	
PIM1	PIM-1 proto-oncogene	7.53	0.367	2.763	[42,43,64]
PLD4	Phospholipase D-4	11.45	0.248	2.836	
RPL22L1	Ribosomal Protein L22 like 1	2.93	0.360	1.055	
S100A4	S100 calcium binding protein A4	3.22	0.100	0.322	
SCFD2	Sec Family Domain-containing 2	4.52	0.412	1.862	

activation. Such mRNAs that were upregulated by RCI treatment are listed in Supplementary Table 2A along with literature references to any identified role in B cell function. RCI- downregulated mRNAs whose levels had not been affected by cellular activation are summarized in Supplementary Table 2B.

3.3. Glucocorticoids modulate a distinct set of mRNAs in ODN 2395/αIgM-activated B cells, but with significant overlap of the RCI response

Additional experiments were undertaken to compare the effects of RCI treatment of TLR9-activated B cells with the effects of glucocorticoids under the same conditions. Our previous experiments in isolated human B cells activated with IL4 and CD40 ligand in vitro had revealed minimal overlap between the response to RCI and the response to dexamethasone, a synthetic glucocorticoid [16]. We examined the overlap between the responses to these two agents in ODN 2395/anti-IgM-activated human B cells from the same subjects. The changes in mRNA expression induced by dexamethasone in human B cells activated by ODN 2395/anti-IgM are presented in Supplementary Table 3A (mRNAs upregulated by glucocorticoid) and Supplementary Table 3B (mRNAs downregulated by glucocorticoid). The overlaps among the sets of mRNAs upregulated by TLR9 activation and those downregulated by RCI or dexamethasone are shown in Fig. 4. Three mRNAs (BCAT1, CSF2RB, and MFI2) were found to be downregulated by both RCI and dexamethasone in ODN 2395/anti IgM-activated B cells. The overlaps among the sets of mRNAs downregulated in TLR9/BCR-activated cells and those upregulated by RCI or dexamethasone are shown in Fig. 5. Two mRNAs (GABARAPL1 and IRS2) were found to upregulated by both RCI and dexamethasone treatment in the context of TLR9/BCR activation.

4. Discussion

In these experiments, we sought evidence for direct effects of RCI on isolated human B lymphocytes activated in vitro by engagement of TLR9 and of the B cell receptor. We further sought to characterize the molecular signature of the drug's early effects on B cells at the mRNA level and to compare that signature with that elicited by a synthetic glucocorticoid. We found evidence for direct, dose-dependent suppressive effects on IgG production by TLR9/αIgM-activated B cells and for RCI-mediated downregulation of mRNA encoding AICDA, the principal regulator of immunoglobulin gene somatic hypermutation and class switch recombination. RNA-Seq analyses revealed that RCI treatment results in reversal of key TLR9/BCR-mediated effects on gene expression evident within the first 24 h of B cell activation by this pathway.

Our experiments were designed to test the direct effects of RCI on human B cells by excluding glucocorticoids from the test system. In vitro analyses with isolated B cells exclude the possibility of indirect effects resulting from stimulation of adrenal steroidogenesis. Potential extra-adrenal sources of glucocorticoids have previously been identified by their expression of steroidogenic enzyme pathways capable of de novo glucocorticoid synthesis, including cells of the thymus, brain, intestine, and skin [51,52]. We used purified peripheral blood B cells, which have not been recognized to express such steroidogenic capacity. Furthermore, at least one study has reported that ACTH acts on immune system cells (murine thymocytes) to downregulate rather than to stimulate steroidogenic activity [53]. Thus, while we have not formally excluded an effect of RCI to elicit a steroidogenic response from our test cells, prior reports make it seem unlikely that such a steroidogenic response could occur in human B cells, the short time course of our experiments makes it unlikely that any of our observed effects represent such secondary responses, and our RNA-Seq analyses did not identify upregulation of mRNAs encoding any steroidogenic enzymes or key steroidogenic regulators.

The mRNAs found to be modulated by the action of RCI include 14 mRNAs that were upregulated in the context of activation of B cells by TLR9 and BCR engagement and were found to be suppressed by RCI treatment under the same activating conditions. Among the RCI-downregulated mRNA transcripts with identified roles in B cell function, the most profoundly suppressed was AICDA (suppressed to < 10% of levels observed ODN 2395/anti IgM-activated control cells). This gene product plays an essential role in immunoglobulin gene somatic hypermutation and class switch recombination [29,54] and its suppression would, even in the absence of any RCI-induced regulatory effects on other mRNAs, be expected to have significant inhibitory effects on B cell activation. Likewise, the observed suppression of BATF by RCI would be expected to have significant inhibitory effects on the B cell. BATF was the first identified member of a family of basic leucine zipper transcription factors [55]. In single gene “knockout” experiments BATF has been found to have an essential role in development of IL17-producing helper T cells [32], but also to have complex B cell-intrinsic functions in the regulation of AICDA [32,33] as well as in expression of the germline transcripts of immunoglobulin heavy chain C region, an

Table 2
TLR9/BCR-Suppressed, RCI-Upregulated mRNAs In Isolated Human B Cells.

mRNA	Description	Down Regulation by TLR/αIgM (Fraction of control level)	Up Regulation by RCI (Fold induction compared to TLR/αIgM-stimulated)	Up Regulation By RCI (normalized to original baseline)	Literature Citations on Known B cell functions
GABARPL1	GABA Type A Receptor Associated Protein Like 1	.26	16.90	4.39	
IRS2	Insulin Receptor Substrate 2	.27	4.68	1.26	[44,45]
LY9 (SLAMF3)	Signaling Lymphocytic Activation Molecule 3	.27	5.15	1.39	[46,47,56,57,65]
MYLIP1	Myosin Regulatory Light Chain Interacting Protein	.17	4.87	0.83	
RGS2	Regulator of G Protein Signaling 2	.25	6.65	1.66	[48–50]

obligatory step in class switch recombination [33]. Additional experiments (not shown) have indicated a marked suppression of germline IgH transcripts in response to RCI treatment of human B cells in the context of IL4/CD40 ligand-induced activation. Whether the observed reduction of AICDA expression in the present experiments might be secondary to RCI's effect to reduce BATF expression levels remains to be investigated.

Five mRNAs that were downregulated in B cells by TLR9 and BCR engagement were found to be induced by RCI treatment under those activating conditions. Of considerable interest was the observation of the robust restoration of expression of Ly9 in RCI treated cells. Ly9 (CD229, SLAMF3) is a member of the Signaling Lymphocyte Activation Molecule family of cell surface receptors. Humoral autoimmunity in B6.SLE1b mice has been found to be associated with different isoform usage of SLAMF3 compared to congenic normal B6 mice [56]. In humans, polymorphism at the SLAMF3 locus is associated in some populations with systemic lupus erythematosus (SLE) [57]. Evidence from both genetic manipulation of Ly9 and induced signaling through Ly9 suggests that it functions to inhibit innate-like B cell development and function [46,47]. RCI mediated up-regulation of Ly9 would be consistent with such an inhibitory effect.

Most of the mRNAs that were found to be upregulated by RCI treatment of B cells during TLR9/BCR-mediated activation had not exhibited changes in expression as a consequence of the activation process itself (Supplementary Table 2A). Nevertheless, a number of these gene products have been previously reported to play key roles in B cell differentiation or function. Among such RCI up-regulated mRNAs is GADD45A, a p53-regulated gene product that functions in suppression of cell proliferation. GADD45A “knockout” mice develop autoimmunity with characteristics of human Systemic Lupus Erythematosus, such as autoantibodies directed against double-stranded DNA, and autoimmune glomerulonephritis [58]. A second RCI-upregulated mRNA, TSC22D3, encodes the glucocorticoid-inducible leucine zipper protein (GILZ) that has been reported to function as an inhibitor of B cell activation and proliferation [59–61]. Manipulation of cellular levels of either of these mRNAs and their encoded proteins in isolation has previously been found sufficient to elicit changes in B cell function, and our present observation of their RCI-induced upregulation, in association with suppressive effects on B cell function, is consistent with their known functional properties.

These experiments also revealed that some changes in mRNA expression elicited by RCI treatment of human B cells during TLR9/BCR-mediated activation could also be induced by glucocorticoid treatment in the same context. RCI and DEX treatment both resulted in increased levels of eleven unique mRNAs (Fig. 5), two of which (GABARAPL1, IRS2) were suppressed by the TLR9/BCR-mediated activation process. Both RCI and Dex treatment resulted in suppressed levels of six unique mRNAs (Fig. 4), three of which (BCAT1, CSF2RB, and MFI2) were noted to be induced by TLR9/BCR -mediated activation. In the current RNA-Seq experiments, AICDA did not meet our stringent criteria for inclusion as a glucocorticoid downregulated gene (since its downregulation did not reach significance in one of three RNA-Seq samples). However, we had previously observed such down-regulation of AICDA by glucocorticoid treatment using RT-PCR methods [62], and it seems likely that this critical component of the activation response can be modulated by both RCI and glucocorticoids. The findings of these overlaps in RCI and Dex effects on B cell gene expression in TLR9/BCR treated cells differ from our previous findings in the setting of IL4/CD40 ligand-mediated activation [16]. The relative importance of the various independently regulated or co-regulated gene products is a question of interest for future investigation.

While these experimental results add significantly to the evidence for direct actions of RCI on human B cells, a number of questions remain for future investigation. First, the relationship of these in vitro findings to the actions of corticotropin preparations administered in vivo is unresolved. In the latter setting, additional “indirect” effects

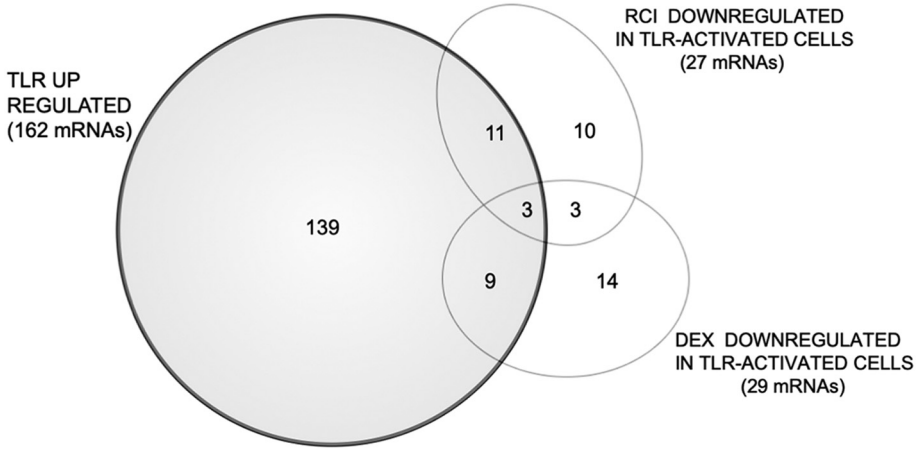

Fig. 4. Intersections among the sets of mRNAs upregulated by TLR9/BCR engagement in isolated human B cells and the sets of mRNAs downregulated by RCI or dexamethasone (DEX) in TLR9/BCR-activated B cells. The universe of mRNA transcripts evaluated was 22,473 ± 184 in fifteen independent paired comparisons. 162 unique mRNAs were increased in expression level in ODN 2395/anti IgM-stimulated B cells compared to control cells from the same donors (shaded circle). Of these, 14 were downregulated by treatment of TLR9/BCR-activated cells with RCI, and 12 were downregulated in cells treated with DEX. Three of the ODN 2395/anti IgM-induced mRNAs were downregulated by either RCI or DEX. Hypergeometric probability calculation (probability of the observed overlap occurring by chance alone) for the set of 14 genes from the TLR9/BCR-upregulated set that were downregulated by RCI revealed $p < 1.06 \times 10^{-23}$. The representation factor (number of genes in observed overlap group / number expected) was 71.9. For the set of 12 genes from the TLR9/BCR-upregulated set that were downregulated by DEX: $p < 6.07 \times 10^{-19}$; representation factor = 57.4. For the set of 6 genes from the RCI-down regulated set that were also downregulated by Dex: $p < 7.27 \times 10^{-13}$; representation factor = 172.2.

mediated through other cellular targets (e.g., adrenocortical cells or T lymphocytes) with consequent induction of other immunomodulatory mediators (e.g., glucocorticoids or cytokines) could be operative. Elucidation of how these various direct and indirect effects sum to a biologic response will be a considerable experimental challenge. Second, since RCI is a preparation of corticotropin derived from biological tissue (porcine pituitary), the active components mediating these results have not been defined. Third, the signaling system through which RCI's direct effects are exerted on B cells remains to be determined. Lastly, the finding of multiple RCI-modulated mRNAs whose known roles in B cell function have been found to be independently critical for processes of class switch recombination, somatic hypermutation, or antibody production raises the question of whether RCI's modulation of any single gene product is the basis of its direct effect on B cells in vitro, or whether regulation of expression of multiple RNAs is required.

Author contributions

Nancy J. Olsen participated in the design of the experiments, the analysis of the data, and the writing of the manuscript.

Ann L. Benko participated in the design and performance of the experiments, the analysis of the data, and the writing of the manuscript.

Carl A. McAloose participated in the design and performance of the experiments, the analysis of the data, and the writing of the manuscript.

Patrice M. Becker participated in the design of the experiments, the analysis of the data, and the writing of the manuscript.

Dale Wright participated in the design of the experiments, the analysis of the data, and the writing of the manuscript.

Teresa Sunyer participated in the execution of the project, the analysis of the data, and the writing of the manuscript.

Yuka Imamura Kawasawa participated in the design and performance of the experiments, the analysis of the data, and the writing of the manuscript.

William J. Kovacs participated in the design of the experiments, the analysis of the data, and the writing of the manuscript.

Competing interests

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Dale Wright, Teresa Sunyer and Patrice Becker are employees of Mallinckrodt Pharmaceuticals.

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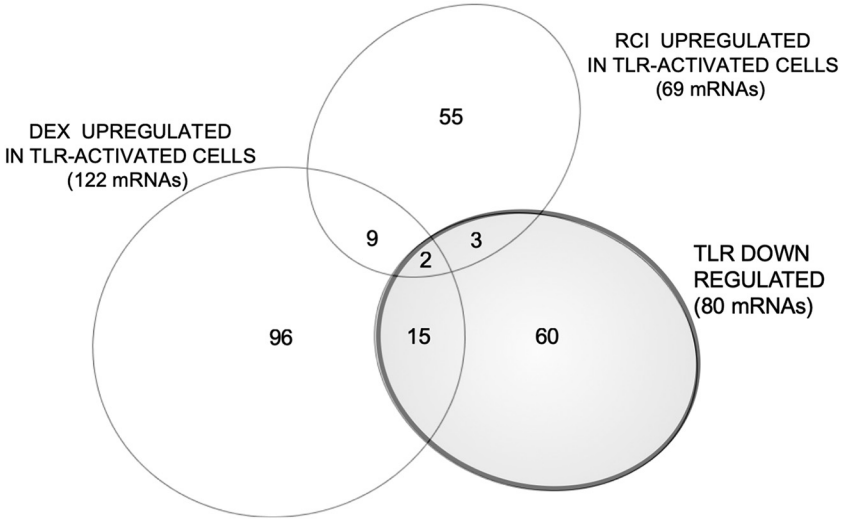

Fig. 5. Intersections among the sets of mRNAs downregulated by TLR9/BCR engagement in isolated human B cells and the sets of mRNAs upregulated by RCI or dexamethasone (DEX) in TLR9/BCR-activated B cells. 80 unique mRNAs were decreased in expression level in ODN 2395/anti IgM-stimulated B cells compared to control cells from the same donors (shaded oval). Of these, 5 were up-regulated by treatment of TLR9/BCR-activated cells with RCI, and 17 were upregulated in cells treated with DEX. Two of the ODN 2395/anti IgM-suppressed mRNAs were upregulated by either RCI or DEX. Hypergeometric probability calculation (probability of the observed overlap occurring by chance alone) for the set of 5 genes from the TLR9/BCR-downregulated set that were upregulated by RCI revealed $p < 4.09 \times 10^{-6}$. The representation factor (number of genes in observed overlap group / number expected) was 21. For the set of 17 genes from the TLR9/BCR-downregulated set that were upregulated by Dex: $p < 7.29 \times 10^{-23}$; representation factor = 39.1. For the set of 11 genes from the RCI- upregulated set that were also upregulated by Dex: $p < 7.57 \times 10^{-14}$; representation factor = 30.2.

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