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# Direct effects of HP Acthar Gel<sup>®</sup> on human B lymphocyte activation in vitro

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## Abstract

**Introduction:** Both clinical experience and experimental evidence have suggested that Adrenocorticotrophic hormone (ACTH) might directly exert immunomodulatory effects not dependent on adrenal steroidogenesis.

**Methods:** The direct effects of H.P. Acthar Gel<sup>®</sup> (Acthar), a repository preparation containing a porcine ACTH analogue, on human B lymphocyte function were studied in vitro using peripheral blood B cells isolated using anti-CD19 coated magnetic beads and activated by interleukin 4 (IL-4) and CD40 ligand (CD40L). Analysis of expression of messenger RNA (mRNA) encoding activation-induced cytidine deaminase (AICDA) was carried out by quantitative real-time polymerase chain reaction (PCR). Cellular proliferation was assessed by a flow cytometric technique using intracellular staining with carboxyfluorescein succinimidyl ester (CFSE). Immunoglobulin G (IgG) production was measured in cell supernatants using an immunoassay.

**Results:** Acthar was found to exert acute, dose-dependent inhibitory effects on IL-4/CD40L-mediated induction of the expression of activation-induced cytidine deaminase (AICDA) after 24 hours, as well as sustained inhibition of B cell proliferation and IgG production during five more days of culture, without deleterious effects on B cell viability.

**Conclusions:** These experiments demonstrate that Acthar can exert direct effects on the humoral immune system independent of any role in the regulation of adrenal steroidogenesis. Although the impact of these findings on clinical disease was not evaluated in this study, these data support the therapeutic potential of Acthar for the management of autoimmune diseases characterized by B cell activation and aberrant humoral immune function.

**Keywords:** Adrenocorticotrophic hormone, ACTH, B lymphocytes, Humoral immunity, Immunoglobulins

## Introduction

Adrenocorticotrophic hormone (ACTH), also known as corticotropin, is a member of a group of peptide hormones (the melanocortins) derived from the proopiomelanocortin (POMC) gene by cell-specific transcription patterns and post-translational processing [1]. The principal source of these gene products is the pituitary and central nervous system, although POMC gene expression occurs at lower levels in a variety of tissues [2]. The melanocortins exert their physiologic effects via binding to one or more of the members of a family of specific G-protein coupled melanocortin receptors (MCRs) and activation of the Jak/STAT pathway [3, 4].

While ACTH exerts its principal physiologic effect to stimulate adrenal growth and to acutely regulate glucocorticoid production by the cells of the adrenal cortex [1], extra-adrenal targets of ACTH action were postulated over 50 years ago [5] and the recognition that MCRs are expressed on a wide variety of cell types, including cells of the immune system, has stimulated renewed interest in the immunomodulatory properties of these peptides [6]. Radioligand binding studies on separated populations of rat lymphoid cells demonstrated the highest levels of specific ACTH binding on B lymphocytes, while T lymphocytes exhibited about half that receptor capacity and thymocytes had low or undetectable levels of ACTH surface binding [7]. At the mRNA level, quantitative PCR experiments have shown lower levels of MCR gene expression in human peripheral B cells compared with CD4<sup>+</sup> T cells, but mRNAs for

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MCR1, MCR3, and MCR5 (all of which bind ACTH) have been identified in the CD19<sup>+</sup> cell population [8].

ACTH may exert effects on physiologic processes either directly (via MCR-mediated action on target cells) or indirectly, through stimulation of adrenal steroidogenesis. Early studies by both Hench et al. [9] and Thorn and Bayles [10] compared the effects of ACTH with those of glucocorticoids on the clinical features of human autoimmune diseases such as rheumatoid arthritis. Both groups concluded that an intact adrenal corticosteroid biosynthetic response was essential for ACTH to mimic the clinical effect of exogenous glucocorticoid therapy [10, 11]. Until it became technically possible to examine hormone action in isolated cells in culture, it was believed that ACTH effects on immune system function could only be indirectly exerted by stimulation of endogenous glucocorticoid production by the adrenals. The first of such studies in vitro reported inhibitory activity of ACTH on peripheral B-lymphocyte function [12]. In that study, ACTH preparations from porcine pituitary glands were found to suppress antibody production by murine spleen cells in vitro in response to both T-cell-dependent and T-cell-independent antigens that had been used as immunogens in vivo. The observed suppressive effects of ACTH occurred at hormone concentrations estimated to be in the micromolar range [12]. Subsequent studies suggested contradictory effects of ACTH on human tonsillar B cells activated in vitro by interleukin (IL)-2 or IL-4, reporting enhanced cellular proliferation and antibody production [13, 14]. Pro-inflammatory activities in T cells and natural killer cells have also been described [15, 16]. Other investigators have reported suppressive effects of synthetic ACTH on immunoglobulin E production in vitro by cultures of human peripheral blood mononuclear cells but such effects were not observed in highly purified B-cell populations [17].

We tested the effects of H.P. Acthar Gel<sup>®</sup> (Acthar, Mallinckrodt Pharmaceuticals, Ellicott City, MD, USA), a porcine pituitary-derived ACTH analog approved for the treatment of certain autoimmune disorders in which B-cell dysfunction may contribute to disease pathogenesis. Porcine ACTH differs from human ACTH at a single amino acid (position 31) [18] but no differences have been identified in the steroidogenic, melanotrophic, or lipolytic activities of the ACTH peptides from these two species [19]. We examined the effects of this clinically utilized preparation on human B-lymphocyte function in vitro, using highly purified B-cell populations cultured in the absence of glucocorticoids and stimulated by recombinant IL-4 and CD40 ligand (CD40L) as specific B-cell activating signals.

## Methods

### Human subjects, B-lymphocyte preparation, and culture

Healthy volunteer subjects were recruited for the study and gave informed consent to participate in the protocol

approved by the Penn State/Milton S. Hershey Medical Center Institutional Review Board. The volunteers included 16 females and nine males, with overall mean ( $\pm$  standard error of the mean (SEM)) age of 37 ( $\pm 2$ ) years; each experiment was carried out with a unique individual donor. Peripheral blood mononuclear cells were prepared by density gradient centrifugation on Histopaque 1077 (MP Biomedicals, Santa Ana, CA, USA), and peripheral B lymphocytes were then isolated using magnetic CD19 MicroBeads (Miltenyi Biotec, San Diego, CA, USA) and a MidiMACS Separator (Miltenyi Biotec). B cells prepared using this method were analyzed by flow cytometry after staining with anti-CD20 antibodies and found to be >90 % pure. 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./ml penicillin/100  $\mu$ g/ml streptomycin). Stimulated cultures (using 10 ng/ml IL-4 and 2  $\mu$ g/ml recombinant human CD40L from HEK293 cells; both R&D Systems, Minneapolis, MN, USA) and control cultures were plated at a density of  $0.5 \times 10^6$ – $1.0 \times 10^6$  cells/ml. Acthar or placebo (provided in blinded fashion; Mallinckrodt Pharmaceuticals, St. Louis, MO, USA) was added to cultures to test the effects of varying estimated concentrations of the active compound. Test compounds were freshly prepared in complete medium on the day of the experiment, within 1 hour prior to addition to cell cultures. Placebo was identical to Acthar, except that it did not contain any active compound. Both the Acthar and the placebo preparations were identical to those used in both clinical studies [20] and animal studies in vivo [21]. Cells were incubated at 37 °C with 5 % CO<sub>2</sub> for varying lengths of time from overnight to 6 days. No additions were subsequently made to cultures during the incubation period.

### Assays for immunoglobulins in cell culture supernatants

Immunoglobulin G (IgG) and immunoglobulin M (IgM) 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, USA).

### Quantitation of cellular proliferation in culture

To assess effects of test compounds on B-cell proliferation, cells were stained with carboxyfluorescein succinimidyl ester (CFSE; CellTrace from Molecular Probes, Eugene, OR, USA) using the manufacturer's protocol prior to culture and stimulation in vitro. After completion of the incubation period, cells were harvested, washed, and analyzed by flow cytometry using a BD FACS Canto II instrument (BD Biosciences, Franklin Lakes, NJ, USA) in the Penn State Hershey Flow Cytometry Core Facility. The distribution of peaks of fluorescence intensity was analyzed using FlowJo software

(TreeStar, Ashland, OR, USA) to yield the parameters of percentage of cells that divided, division index (the average number of divisions of all cells), expansion index (the fold-expansion of all cells), proliferation index (the average number of divisions of a responding cell), and replication index (the fold-expansion of responding cells).

#### Flow cytometric assessment of cell viability

Cultured B cells were assessed for viability using the LIVE/DEAD Fixable Dead Cell Stain kit with green fluorescent reactive dye (Molecular Probes). Cells were harvested from culture plates at the end of various treatments, pelleted by centrifugation at  $300 \times g$  for 10 minutes, washed, and resuspended in phosphate-buffered saline (PBS). Cells were stained with green fluorescent amine reactive dye reconstituted in dimethylsulfoxide (DMSO; 1  $\mu$ l per million cells), stored on ice in the dark for 30 minutes, and then washed once with PBS. Cells were analyzed for dye exclusion (live cells) or inclusion (dead cells) by flow cytometry on a BD FACS Canto II instrument in the Penn State Hershey Flow Cytometry Core Facility using the 488 nm excitation laser and 530 nm detection band. Parallel samples were analyzed by visual microscopic inspection for trypan blue dye exclusion.

#### RNA isolation and RT-PCR quantitation of activation-induced cytidine deaminase

RNA was isolated from harvested cells using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. The RNA concentration was quantitated using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific - Nanodrop Products, Wilmington, DE, USA). Reverse transcription of RNA was performed using the High Capacity RNA to cDNA Kit (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA).

Activation-induced cytidine deaminase (AICDA) mRNA expression was quantitated using real-time PCR technique with Taqman<sup>®</sup> reagents from Applied Biosystems (Gene Expression Assay Hs 00221068), with GAPDH (Gene Expression Assay Hs 03929097) as a control mRNA for calculation of induction using the  $\Delta\Delta C_t$  method. Assays were run on an Applied Biosystems Quant Studio 12 K Flex real-time PCR system in the Penn State Hershey Genome Sciences facility.

#### Statistical analyses

Statistical analyses were carried out using Prism software (version 6.0; Graph Pad, San Diego, CA, USA) or SAS (SAS Institute Inc., Cary, NC, USA). Results are presented as mean and SEM values. Multiple measurements were analyzed for statistical significance using analysis of variance (ANOVA) with Tukey's multiple comparison post test or with the Kruskal–Wallis test with Dunn's

multiple comparison post test for experiments in which datasets were not normally distributed.  $p < 0.05$  was considered significant.

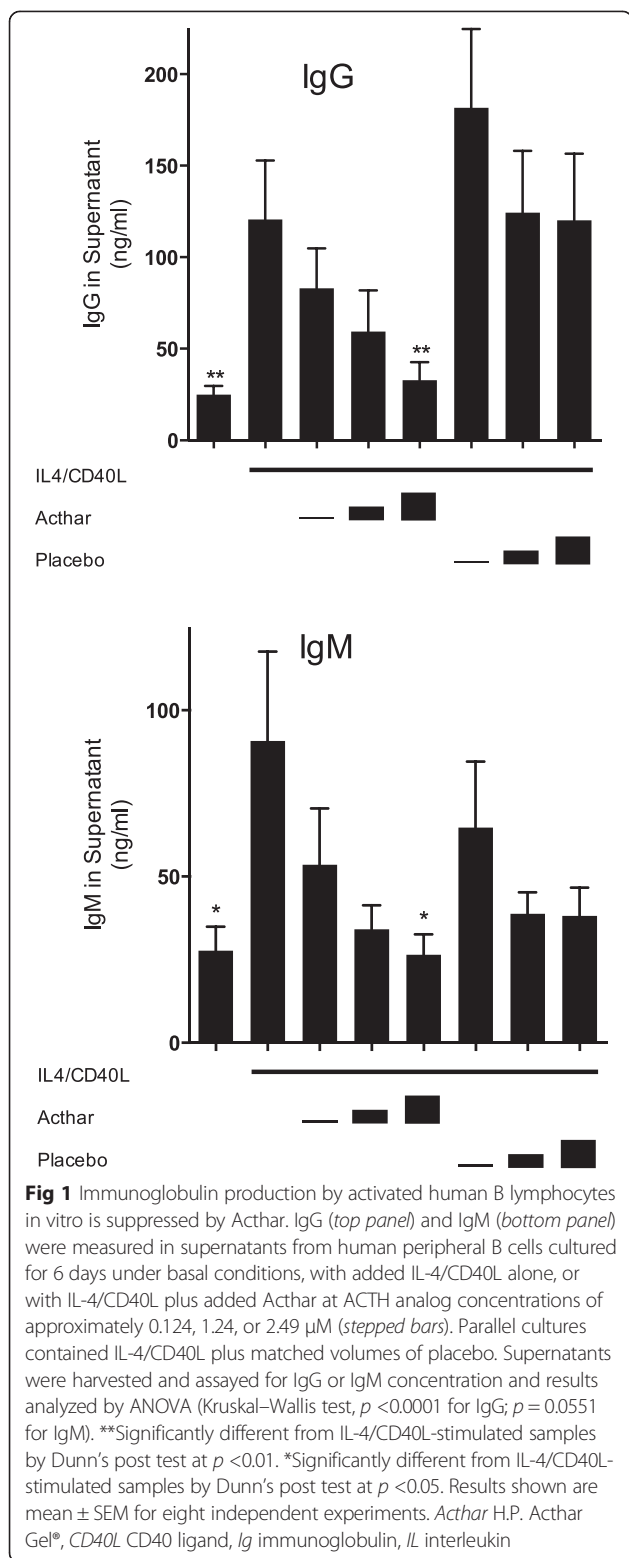
#### Results

Treatment of IL-4/CD40L-activated human B cells in vitro with Acthar resulted in a dose-dependent reduction of IgG accumulation in culture supernatants after 6 days (Fig. 1, top panel). Maximal inhibition of IgG production (72.7 %) was noted at an estimated ACTH analog concentration of approximately 2.49  $\mu$ M ( $p < 0.01$ ). Treatment with placebo at identical volumes did not alter IgG levels compared with IL-4/CD40L-stimulated controls. Effects of the Acthar on IgM accumulation in culture supernatants were also observed—with maximal suppression of 70.8 % at the highest concentration of Acthar ( $p < 0.05$ ; Fig. 1, bottom panel). Treatment with the placebo preparation did not result in significant effects on IgM.

During B-lymphocyte activation, the process of immunoglobulin class switching is known to be dependent on cellular proliferation [22]. We therefore examined whether Acthar altered IL-4/CD40L-induced proliferation of human B cells in vitro using CFSE staining to define successive waves of cellular proliferation in culture. We noted a dose-dependent effect of Acthar to inhibit proliferation of activated B cells—with significant reductions in the percentage of cells that divided in culture (Fig. 2a), the average number of divisions of all cells (Fig. 2b), and the fold-expansion of all cells (Fig. 2c). Placebo treatment did not significantly alter any of these parameters of cell proliferation. No significant differences were observed between unstimulated and IL-4/CD40L-stimulated cells with respect to either the number of divisions undertaken by responding cells ("proliferation index"; not shown) or the fold-expansion of responding cells ("replication index"; not shown).

The effects of Acthar on cell proliferation were not explained by toxic or lytic effects on human B cells. Neither intracellular staining with Live/Dead fluorescent amine reactive dye (Fig. 3) or trypan blue exclusion (not shown) revealed any significant effect of Acthar on these assessments of cell integrity.

The ability of B cells to switch the class of immunoglobulin produced from IgM to IgG is dependent not only on cellular proliferation, but also on the action of the DNA-modifying enzyme AICDA that initiates the genomic changes resulting in immunoglobulin heavy chain gene class switch recombination as well as somatic hypermutation (SHM) of the variable regions of immunoglobulin heavy and light chain genes [23, 24]. Examination of AICDA expression in IL-4/CD40L-activated human B cells revealed an acute, concentration-dependent inhibitory action of the ACTH analog, reducing the levels of AICDA ordinarily seen in the first 24 hours after such



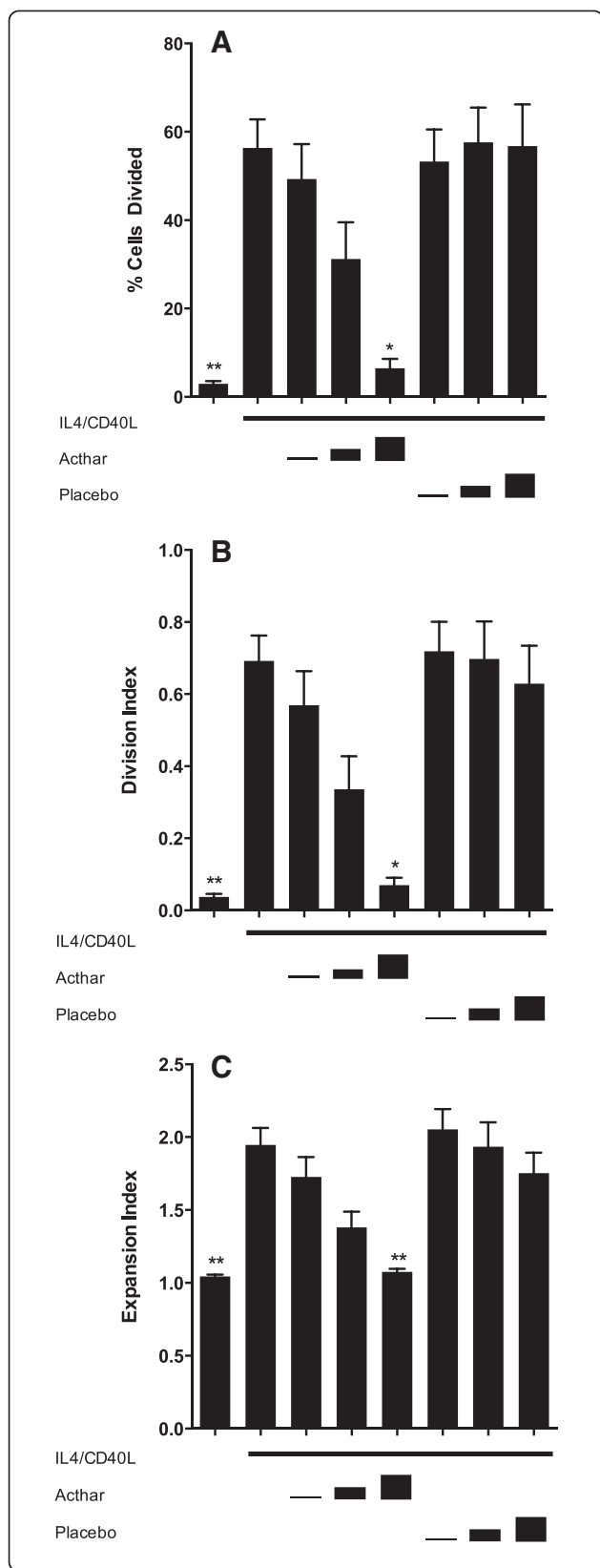
stimulation. At the highest concentration of Acthar, AICDA expression was suppressed on average to  $2.67 \pm 0.92$  % of IL-4/CD40L-stimulated values ( $p < 0.05$ ), comparable with the level of expression seen in unstimulated

cells (Fig. 4a). After 6 days in culture, AICDA mRNA levels in Acthar-treated cells were comparable with those observed in placebo-treated control cells, consistent with the prediction that active peptide would not still be present in cultures at this time point (Fig. 4b). Placebo-treated cells did not show statistically significant changes in AICDA expression at either time point. These data confirm that Acthar exerted an inhibitory effect on the expression of the key regulator of class switch recombination and SHM in activated human B cells, and that this inhibitory effect is exerted promptly during early phases of exposure to the peptide.

### Discussion

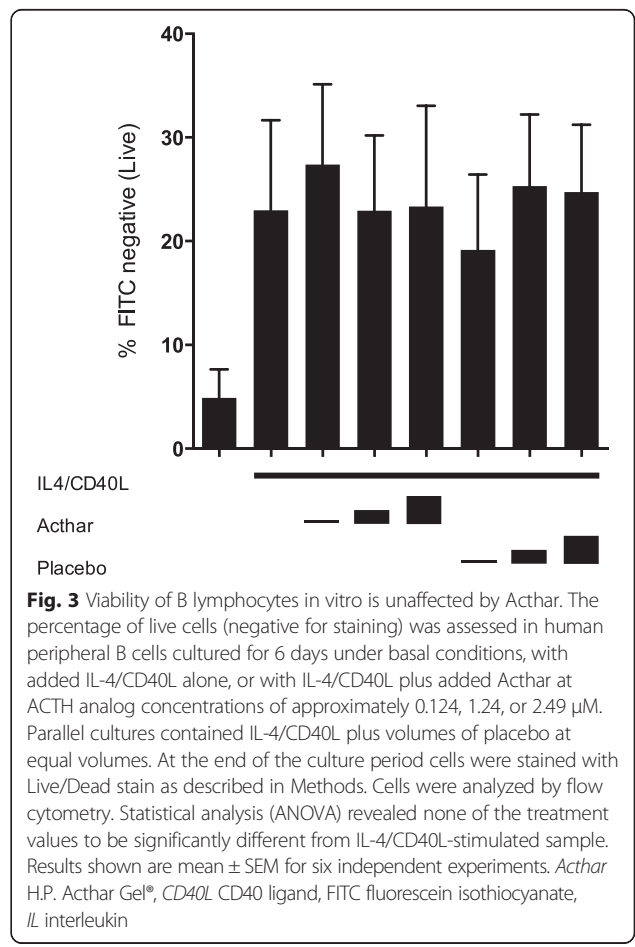
Acthar has US Food and Drug Administration approval for treatment of a number of autoimmune conditions, including the treatment of acute exacerbations of multiple sclerosis, during an exacerbation or as maintenance therapy in selected cases of systemic lupus erythematosus, dermatomyositis, and polymyositis, and as adjunctive therapy for short-term administration in psoriatic arthritis, rheumatoid arthritis, and ankylosing spondylitis (Acthar prescribing information). The therapeutic effects of ACTH preparations in human autoimmune disorders have historically been believed to be the consequence of the stimulatory effect of the hormone on adrenal cortisol production and secretion [10, 11, 25]. However, clinical observation of therapeutic effects of Acthar in glucocorticoid-resistant patients with multiple sclerosis [26], prior in vitro observations of direct effects of ACTH preparations on cultured human B cells [13], and the recognition that human monocytes, macrophages, and T and B lymphocytes express specific MCRs [6, 8, 27] have all suggested that some extra-adrenal effects of this corticotropin preparation might be immunomodulatory. While we have found previously that glucocorticoids acutely downregulate AICDA expression in cultured human B cells [28], the present re-examination of the effects of Acthar on purified human B lymphocytes supports direct effects of a clinically utilized corticotropin preparation on the processes of B-cell activation and immunoglobulin G production, since our experiments were performed in vitro under glucocorticoid-free conditions.

The experiments presented here revealed an acute effect of Acthar to suppress AICDA mRNA expression during the first 24 hours of exposure to IL-4 and CD40L stimulation. As we observed previously with glucocorticoid exposure of human B cells, this effect on AICDA mRNA expression was not sustained, but with Acthar we also found significant suppression of cellular proliferation and IgG production observable 5 days later, an effect not seen with glucocorticoid treatment (glucocorticoid data not shown). Since cellular proliferation is required for initiation of IgG production in activated B cells [22], this effect



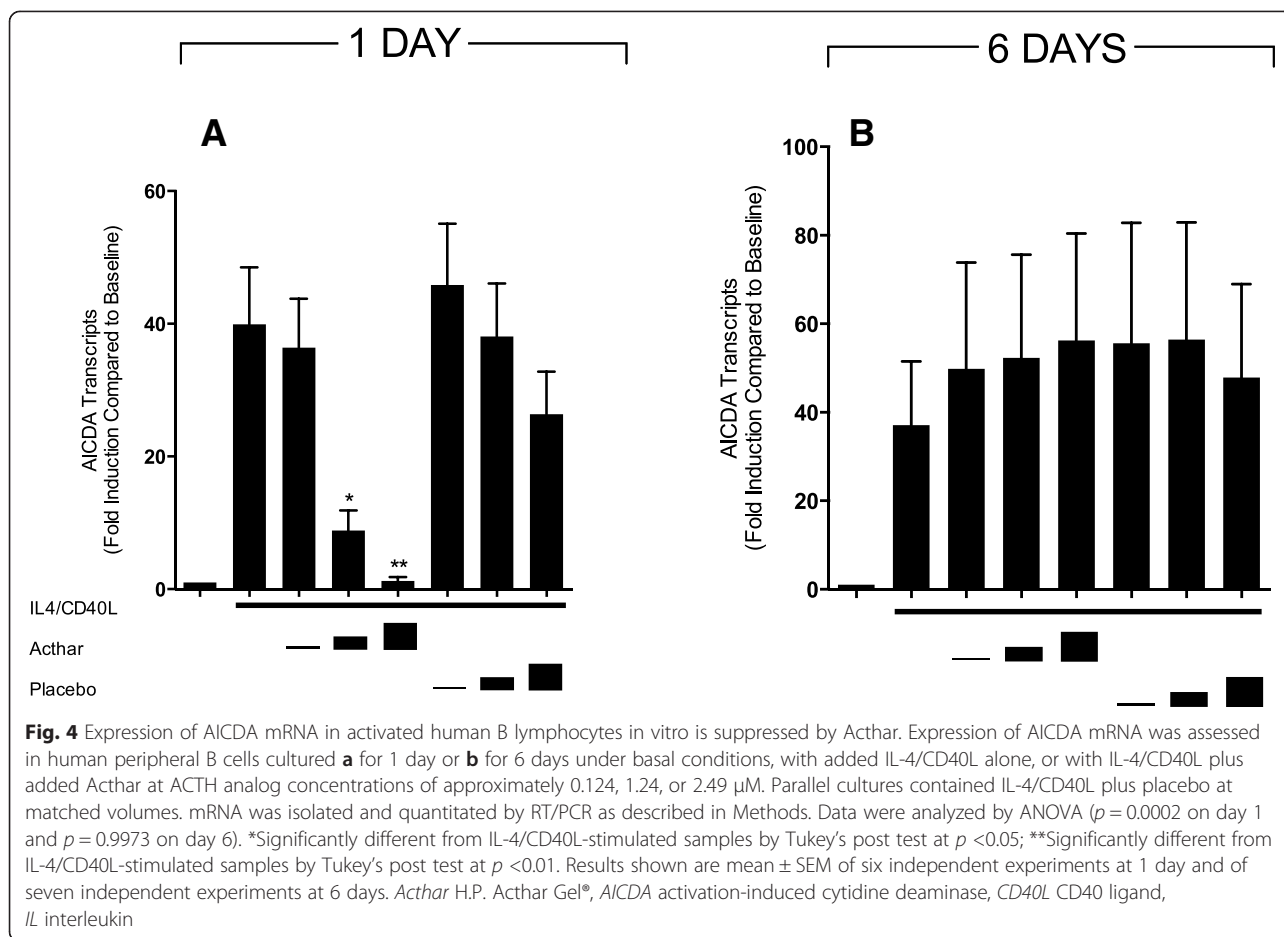
**Fig 2** Proliferation of activated human B lymphocytes in vitro is suppressed by Acthar. **a** The percentage of cells that divided, **b** the average number of divisions of all cells (division index), and **c** the fold-expansion of all cells (expansion index) were assessed in CFSE-loaded human peripheral B cells cultured for 6 days under basal conditions, with added IL-4/CD40L alone, or with IL-4/CD40L plus added Acthar at ACTH analog concentrations of approximately 0.124, 1.24, or 2.49 μM. Parallel cultures contained IL-4/CD40L plus matched volumes of placebo. Cells were examined by flow cytometry and data analyzed by ANOVA (Kruskal–Wallis test,  $p < 0.0001$  for percentage of cells divided,  $p < 0.0001$  for division index, and  $p < 0.0001$  for expansion index). \*\*Significantly different from IL-4/CD40L-stimulated samples by Dunn’s post test at  $p < 0.01$ . \*Significantly different from IL-4/CD40L-stimulated samples at  $p < 0.05$ . Results shown are mean ± SEM for six independent experiments. Acthar H.P. Acthar Gel®, CD40L CD40 ligand, IL interleukin

could be interpreted as antiproliferative. The sustained effects of Acthar exposure on cellular proliferation and IgG production, however mediated, do seem likely to depend on early events, since it seems unlikely that active corticotropin or related peptides remained in the cultures for any sustained period of time. Whether our observations could be the consequence of antagonism of IL-4 action, CD40L



**Fig. 3** Viability of B lymphocytes in vitro is unaffected by Acthar. The percentage of live cells (negative for staining) was assessed in human peripheral B cells cultured for 6 days under basal conditions, with added IL-4/CD40L alone, or with IL-4/CD40L plus added Acthar at ACTH analog concentrations of approximately 0.124, 1.24, or 2.49 μM. Parallel cultures contained IL-4/CD40L plus volumes of placebo at equal volumes. At the end of the culture period cells were stained with Live/Dead stain as described in Methods. Cells were analyzed by flow cytometry. Statistical analysis (ANOVA) revealed none of the treatment values to be significantly different from IL-4/CD40L-stimulated sample. Results shown are mean ± SEM for six independent experiments. Acthar H.P. Acthar Gel®, CD40L CD40 ligand, FITC fluorescein isothiocyanate, IL interleukin





action, or other processes operative in B-cell activation remains to be explored.

Recently published studies have identified suppressive effects of Acthar on humoral autoimmunity in the NZB/W F1 mouse model of lupus [21]. The in vivo treatment paradigm utilized in that study included glucocorticoid-treated control animals, but did not explicitly address the question of extra-adrenal versus adrenal-dependent actions of the hormonal preparation (all animal subjects had intact adrenals), so that corticotropin-stimulated production of dehydroepiandrosterone (DHEA) or other potentially immunomodulatory adrenal steroid products could not be formally excluded. The direct effects of Acthar observed on human B cells in vitro in the present work support the contention that adrenal-independent actions directed at B cells themselves were operative in vivo. The in vivo treatment of NZB/W F1 mice with Acthar was associated with expanded populations of immature cells in transitional type 1 and marginal zone B cells in the spleen, reductions in germinal center cells, and reductions in circulating IgG and IgM autoantibody titers [21]. The present studies in human cells in vitro corroborate this finding of inhibitory effects of Acthar

on immunoglobulin production by circulating B cells that have passed tolerance checkpoints.

Other melanocortin peptides have previously been found to modulate immune function in models of inflammatory bowel disease [29, 30] and in models of T-cell-mediated diseases including autoimmune uveitis [31, 32] and experimental autoimmune encephalomyelitis [33]. Recent studies of melanocortin peptide effects on murine models of systemic lupus erythematosus [21, 34] have now also revealed potential therapeutic roles for these peptides in B-cell-mediated autoimmunity. Our current studies in human cells, cultured in vitro under glucocorticoid-free conditions, suggest that direct effects of one or more components of the Acthar preparation on B lymphocytes may contribute to the disease modulation observed in such models in vivo.

While clearly independent of ACTH effects on glucocorticoid biosynthesis, the molecular mechanisms underlying the observed effects of Acthar on B-lymphocyte function remain to be explored. B lymphocytes have been reported to express MCRs that could mediate these hormonal effects. Human B cells have been found to express MCR1 and MCR3 mRNA [27] and a mouse pro-B

cell line has been reported to express MCR5 [4]. Intact corticotropin (as well as alpha MSH, which corresponds to the first 13 amino acids of ACTH) binds to these receptors [6, 27]. Our present studies have not identified the molecular target or the receptor-signaling pathway by which the observed effects on B cells are exerted.

Although it is difficult to directly extrapolate clinically effective dose ranges from *in vitro* studies, these findings of direct Acthar effects on the function of activated human B lymphocytes have implications for the treatment of autoimmune diseases characterized by B-cell activation and humoral autoimmunity. Abnormalities of the hypothalamic–pituitary–adrenal axis are associated with many autoimmune disorders, and glucocorticoid resistance of immune tissues in many of these patients has been also described [35–37]. The observed direct effects of Acthar on cellular targets in the humoral immune system might thus be exploitable as an additional therapeutic approach.

## Conclusions

These experiments are the first to demonstrate that a clinically utilized ACTH preparation (H.P. Acthar® gel) exerts direct immunomodulatory effects on human B cells activated *in vitro*. The findings suggest potential for such agents in the treatment of autoimmune diseases characterized by abnormal B-cell activation.

## Abbreviations

AICDA: Activation-induced cytidine deaminase; ACTH: Adrenocorticotropic hormone; Acthar: H.P. Acthar Gel®; ANOVA: Analysis of variance; CD40L: CD40 ligand; CFSE: Carboxyfluorescein succinimidyl ester; DMSO: Dimethylsulfoxide; IgG: Immunoglobulin G; IgM: Immunoglobulin M; IL: Interleukin; MCR: Melanocortin receptor; NK: Natural killer; PBS: Phosphate-buffered saline; POMC: Proopiomelanocortin; SEM: standard error of the mean; SHM: Somatic hypermutation.

## Competing interests

NJO received research funding from Mallinckrodt Pharmaceuticals supporting the conduct of these studies. DAD, PH, and PMB are full-time employees of Mallinckrodt Pharmaceuticals. The remaining authors declare that they have no competing interests.

## Authors' contributions

NJO participated in the design of the experiments, reviewed the data, and revised the manuscript. DAD participated in the design of the experiments, reviewed the data, and revised the manuscript. PH analyzed the data and revised the manuscript. PMB participated in the design of the experiments, reviewed the data, and revised the manuscript. CAMcA carried out cell isolations, flow cytometry, and immunoglobulin assays, analyzed the data, and revised the manuscript. ALB carried out mRNA preparations, conducted real-time PCR experiments, analyzed the data, and revised the manuscript. WJK participated in the design of the experiments and oversaw their conduct, analyzed the data, drafted the manuscript, and performed final revisions. All authors read and approved the final manuscript.

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