NEUROIMMUNOLOGY

Oxidative phosphorylation regulates B cell effector cytokines and promotes inflammation in multiple sclerosis

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Dysregulated B cell cytokine production contributes to pathogenesis of immune-mediated diseases including multiple sclerosis (MS); however, the underlying mechanisms are poorly understood. In this study we investigated how cytokine secretion by pro-inflammatory (GM-CSF–expressing) and anti-inflammatory (IL-10–expressing) B cells is regulated. Pro-inflammatory human B cells required increased oxidative phosphorylation (OXPHOS) compared with anti-inflammatory B cells. OXPHOS reciprocally modulated pro- and anti-inflammatory B cell cytokines through regulation of adenosine triphosphate (ATP) signaling. Partial inhibition of OXPHOS or ATP-signaling including with BTK inhibition resulted in an anti-inflammatory B cell cytokine shift, reversed the B cell cytokine imbalance in patients with MS, and ameliorated neuroinflammation in a myelin oligodendrocyte glycoprotein (MOG)–induced experimental autoimmune encephalitis mouse model. Our study identifies how pro- and anti-inflammatory cytokines are metabolically regulated in B cells and identifies ATP and its metabolites as a "fourth signal" that shapes B cell responses and is a potential target for restoring the B cell cytokine balance in autoimmune diseases.

INTRODUCTION

The capacity of B cells to secrete distinct cytokine profiles that can activate or down-regulate local immune responses has been highlighted in recent years as one of their most important antibody-independent functions in both health and disease states. Pro-inflammatory B cell cytokines [e.g., interleukin-6 (IL-6), tumor necrosis factor–α (TNFα), granulocyte-macrophage colony-stimulating factor (GM-CSF)] can contribute to host protection from a range of pathogens, e.g., by promoting activation of T cells and myeloid cells (*1*, *2*), whereas anti-inflammatory B cells expressing IL-10 or IL-35 are implicated in immune regulation and prevention of autoimmunity (*3*–*5*). B cell cytokine dysregulation is recognized as a contributor to a growing range of immune-mediated diseases, including multiple sclerosis (MS) (*6*–*12*), where an imbalance between pro-inflammatory and anti-inflammatory B cell cytokines has been shown to activate disease-relevant pro-inflammatory T cells and myeloid cells. (*12*–*17*).

Unexpectedly, little is known, however, about the mechanisms involved in the regulation of B cell cytokine expression. Of particular interest is to elucidate the mechanism underlying regulation of the balance between B cell pro-inflammatory GM-CSF and anti-inflammatory IL-10, because the balance between these cytokines affects whether bystander myeloid cells (and consequently T cells) are activated versus down-regulated (*18*). In healthy humans, B cell expression of GM-CSF and IL-10 appears largely mutually exclusive (*18*), suggesting that some mechanism is involved in determining whether a B cell will make GM-CSF or IL-10 (but not both). Compared with healthy controls, patients with MS harbor higher frequencies of GM-CSF$^+$ B cells, and upon activation, B cells of patients with MS secrete exaggerated amounts of GM-CSF (as well as TNFα and IL-6) while they exhibit deficient production of IL-10, an imbalance that contributes to heightened pro-inflammatory myeloid cell responses (*6*, *18*–*20*). The high efficacy of anti-CD20 B cell–depleting therapies in MS has been largely attributed to the removal of pro-inflammatory memory B cells that, when present, drive disease relapses through antigen presentation and bystander activation of myeloid cells and T cells (*18*, *21*). In keeping with this, both myeloid cell and T cell pro-inflammatory responses are diminished after anti-CD20 treatment in MS and other autoimmune conditions (*6*, *18*, *22*, *23*). Although highly effective, concern over potentially increasing risks of long-term broad B cell depletion therapies has generated considerable interest in nondepleting B cell–targeting strategies. Developing such approaches, especially those that may restore the balance between pro-inflammatory and anti-inflammatory B cell cytokines, is predicated on elucidating the mechanisms involved in human B cell cytokine regulation.

Our goal in this study was to investigate the mechanisms involved in the regulation of B cell expression of pro-inflammatory GM-CSF versus anti-inflammatory IL-10. Our findings provide insights into fundamental mechanisms underlying the regulation of human B cell pro- and anti-inflammatory cytokine responses,

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pointing to adenosine triphosphate (ATP) and its metabolites as a potential "fourth signal" that shapes functional B cell responses. We find that abnormal oxidative phosphorylation (OXPHOS) contributes to aberrant B cell pro-inflammatory cytokine responses in patients with MS and point to nondepleting therapeutic strategies that may restore the balance of B cell cytokines in patients with autoimmune conditions by targeting B cell metabolism.

RESULTS

Metabolic pathways regulate cytokine production in human B cells

To better understand the regulation of functionally distinct cytokine-defined human B cell subsets, we applied our recently adapted "cytokine secretion assay" (*24*) to simultaneously isolate IL-10– and GM-CSF–secreting B cells from the same source of peripheral blood B cells for bulk RNA sequencing (RNA-seq) (fig. S1). Principal components analysis (PCA) distinguished GM-CSF$^+$ B cells from IL-10$^+$ B cells at the transcriptomic level (Fig. 1A). Gene set enrichment analysis (GSEA) indicated that, compared with IL-10$^+$ B cells, GM-CSF$^+$ B cells were enriched for several pathways that control cellular metabolic activities, such as OXPHOS, glycolysis, and the phosphatidylinositol 3-kinase (PI3K)–mammalian target of rapamycin (mTOR) pathway (Fig. 1B, fig. S2, and data file S1). GM-CSF$^+$ B cells also exhibited a higher mitochondrial mass as compared with both IL-10$^+$ B cells and B cells expressing neither cytokine (Fig. 1C). We next considered whether in vitro activation of the same pool of B cells, using different modes of stimulation known to induce distinct

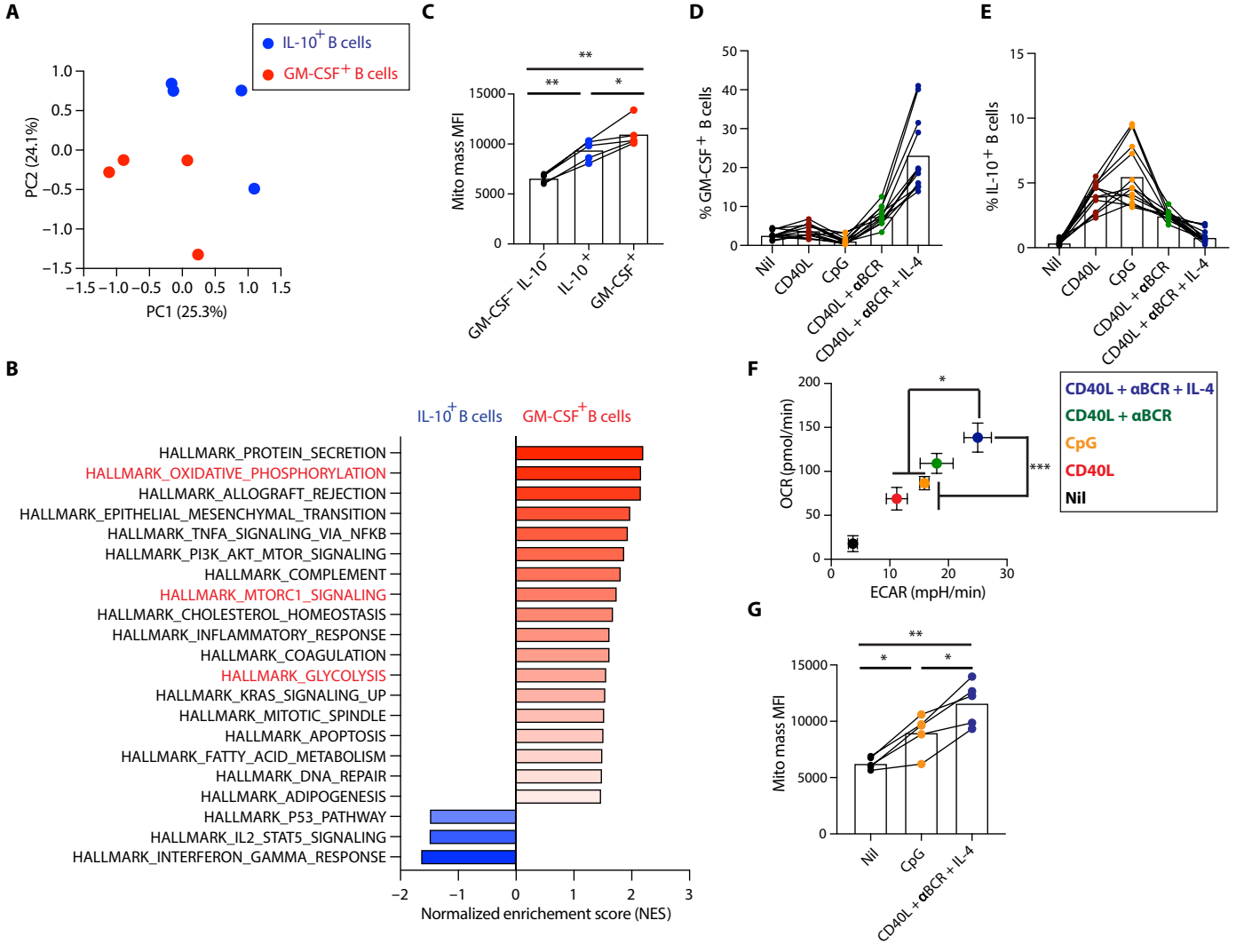

Fig. 1. Transcriptomic profiling of human GM-CSF$^+$ and IL-10$^+$ B cells reveals different metabolic states. (**A**) PCA of transcriptomic profiles of human GM-CSF$^+$ and IL-10$^+$ B cells ($n = 4$). (**B**) GSEA of GM-CSF$^+$ and IL-10$^+$ B cells ($n = 4$). (**C**) MitoTracker mitochondrial staining of GM-CSF$^+$ and IL-10$^+$ B cells ($n = 5$). (**D**) Percentage of GM-CSF$^+$ B cells induced across the different modes of B cell activation [CD40L, CpG, CD40L$^+$ BCR cross-linking antibodies (αBCR), or CD40L + αBCR + IL-4; $n = 11$]. (**E**) Percentage of IL-10$^+$ B cells induced across the different modes of B cell activation ($n = 11$). (**F**) OCR against ECAR across different modes of B cell activation ($n = 4$). (**G**) B cell mitochondrial mass measured by MitoTracker staining after either CpG or CD40L + αBCR + IL-4 stimulation. (C, F, and G): Repeat measure one-way ANOVA with Geisser-Greenhouse and Holm-Šidák correction. MFI, mean fluorescence intensity.

B cell cytokine responses, could result in differential B cell metabolic activity and mitochondrial mass. Prior work has shown that stimulation by either CD40 ligand (CD40L) or CpG alone induces human B cells with regulatory functions mediated through their secretion of IL-10 (*9*, *25*, *26*), whereas signaling through the B cell receptor (BCR) together with stimulation by CD40L (and particularly with further addition of IL-4) induces B cells with pro-inflammatory properties that are mediated partly by their secretion of GM-CSF (*18*, *27*). We therefore activated B cells across a range of known IL-10– or GM-CSF–promoting conditions (Fig. 1, D and E) and measured their mitochondrial respiration [indicated by oxygen consumption rate (OCR)] and glycolysis [indicated by extracellular acidification rate (ECAR)] using Seahorse assays as well as assessed their mitochondrial mass using the MitoTracker. Although all modes of B cell activation induced both mitochondrial respiration and glycolysis (Fig. 1F), the magnitude of these responses varied, with a correlation between the induction of B cell mitochondrial respiration and glycolysis across activating conditions. Stimuli that promoted B cell GM-CSF production induced considerably higher metabolic activity as compared with the IL-10–promoting conditions (Fig. 1F). GM-CSF–promoting activation also resulted in higher B cell mitochondrial mass than IL-10–promoting activation (Fig. 1G). Together, these findings demonstrate that pro-inflammatory B cells are more metabolically active, suggesting that different modes of activation are associated with distinct B cell metabolic states and implicating metabolic pathways in human B cell cytokine regulation.

Partial inhibition of OXPHOS in B cells reciprocally regulates pro- and anti-inflammatory cytokine production

Our RNA-seq data indicate that mTOR signaling is enriched in GM-CSF$^+$ B cells (Fig. 1B), and because mTOR signaling has previously been implicated in control of B cell metabolic activities, we tested whether blockade of mTOR signaling would influence B cell cytokine responses. Human B cells were pretreated with mTOR inhibitors (Torin2 or AZD-3147) followed by stimulation under GM-CSF–promoting conditions [CD40L$^+$ BCR cross-linking antibodies (αBCR) + IL-4]. Inhibition of mTOR reduced GM-CSF expression and increased IL-10 expression (fig. S3, A to C), which shifted the balance of B cell cytokines toward a more anti-inflammatory phenotype (fig. S3D). Given the apparent relationship between differing B cell metabolic states and distinct cytokine-expression profiles, and particularly the association of increased metabolic activity with pro-inflammatory B cell cytokine responses, we examined whether pro-inflammatory activation of B cells in the presence of either rotenone (complex I inhibitor) or 2-deoxy-D-glucose (2-DG, inhibitor of glycolysis) would affect B cell cytokine expression. We found that partial inhibition of OXPHOS (but not of glycolysis) decreased intracellular expression and secretion of GM-CSF while reciprocally increasing the production of IL-10 without affecting B cell viability (Fig. 2, A to G, and fig. S4). Similar results were observed when we blocked complex III and complex V using antimycin and oligomycin, respectively (Fig. 2, H and I). These findings suggested that limiting mitochondrial respiration during pro-inflammatory B cell activation might mediate an anti-inflammatory shift in their functional response. To assess the consequences of such modulation, we considered prior work demonstrating that shifting the balance between GM-CSF and IL-10 secreted by B cells affects the outcome of B cell interactions with myeloid cells (*18*). We therefore generated culture supernatants from activated B cells that were preexposed to either rotenone or vehicle control, applied these supernatants to monocyte-derived macrophage, and subsequently assessed the cytokine responses of the myeloid cells. Whereas supernatants of activated B cells preexposed to only the vehicle induced robust macrophage secretion of IL-12 and IL-6 as expected (*18*), partial inhibition of B cell OXPHOS substantially limited the B cell capacity to induce pro-inflammatory macrophage responses (Fig. 2, J and K). These results are unlikely to merely reflect "carryover" of rotenone directly acting on the myeloid cell cytokine responses because the preexposed B cells were thoroughly washed after rotenone exposure and, moreover, addition of mock culture supernatant to the myeloid cells did not affect the myeloid-cytokine responses (fig. S5).

We next assessed whether deficient mitochondrial respiration affects human B cell cytokine responses in vivo. We compared cytokine profiles of B cells isolated from patients with rare mitochondrial respiratory chain mutations [table S1; $n = 11$, age (mean ± SD): 23.18 ± 17.2, female/male (F/M): 4/7] and a cohort of sex- and age-balanced healthy controls (table S2; $n = 13$, age (mean ± SD): 25.77 ± 5.9, F/M: 6/7]. Total circulating B cell counts and proportions of major B cell subsets were similar between patients and controls (fig. S6, A to F). B cells from patients with mitochondrial respiratory chain mutations exhibited substantially diminished cytokine responses (Fig. 2, L to O), providing in vivo proof of principle that mitochondrial respiration is relevant to B cell cytokine expression. Given that B cells from these patients were deficient in both pro- and anti-inflammatory cytokine production, we next considered whether this reflected the relatively severe respiratory chain deficiency in B cells of these patients, resulting in broader abrogation (rather than reciprocal modulation) of cytokine responses. Titrating the concentration of OXPHOS inhibitors (fig. S7, A to D) to vary the extent of inhibition of cellular respiration resulted in a dose-dependent inhibition of pro-inflammatory cytokine secretion (GM-CSF, TNFα, and IL-6) from healthy B cells across a broad range of rotenone concentrations. Conversely, IL-10 secretion was induced by lower to intermediate concentrations of rotenone and then inhibited at higher rotenone concentrations (Fig. 2P and fig. S7, E to H). We confirmed the differential effects of titrated respiratory chain inhibition on pro-inflammatory versus anti-inflammatory B cell cytokine profiles using other mitochondrial respiration inhibitors including oligomycin (Fig. 2Q and fig. S7, I to L) and antimycin A (fig. S8). Together, these data highlight a role for OXPHOS, but not glycolysis, in the expression and regulation of human B cell functional cytokine responses, indicating that a higher metabolic demand is required for the generation of pro-inflammatory B cells and that partially limiting OXPHOS during B cell activation results in an anti-inflammatory modulation of their responses.

BTK inhibition limits BCR-induced respiration and mediates an anti-inflammatory B cell cytokine shift

To identify the mechanisms that underlie the metabolic regulation of B cell cytokine responses, we first considered our observation that the addition of BCR engagement to CD40L-stimulated B cells resulted in their decreased IL-10 and increased GM-CSF production compared with B cells stimulated with CD40L alone (Fig. 1, D and E). Given the known role of Bruton's tyrosine kinase (BTK) as a signaling molecule downstream of the BCR and its previous implication in OXPHOS (*28*), we carried out a series of experiments using a highly selective BTK inhibitor (BTKi) (*29*, *30*) to test whether

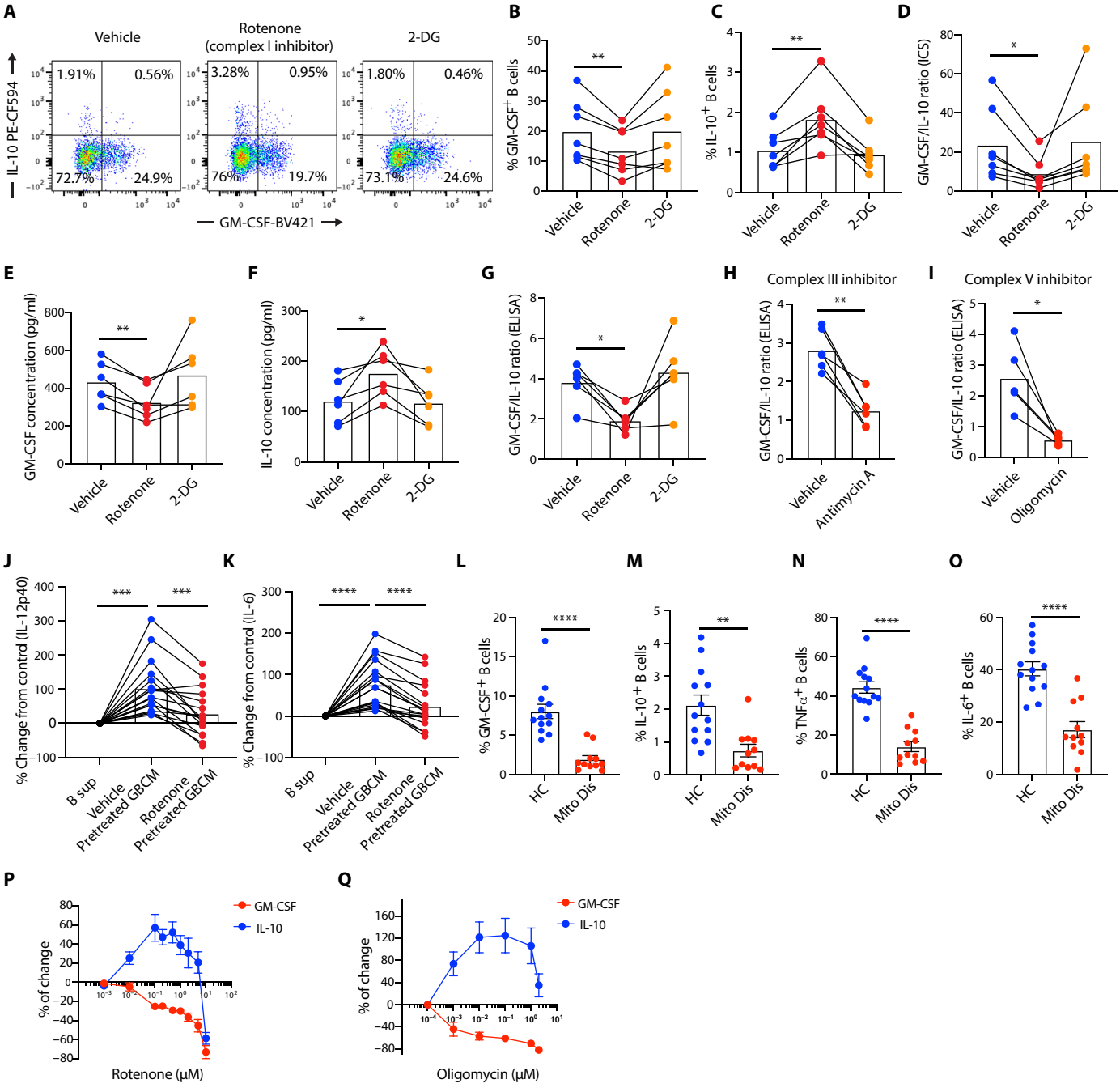

Fig. 2. Mitochondrial respiration regulates the balance of human B cell cytokine responses. (**A**) Representative flow cytometry dot plot showing the percentage of GM-CSF$^+$ and IL-10$^+$ B cells under CD40L + αBCR + IL-4 stimulation in the presence of either vehicle, rotenone (complex I inhibitor, 0.1 μM), or 2-DG (0.5 mM). (**B** to **G**) B cell cytokines were assessed both by ICS (B to D) and by ELISA (E to G; $n = 6$ to 7). (**H** and **I**) B cells were stimulated with CD40L + αBCR + IL-4 in the presence of vehicle, antimycin A (complex III inhibitor, 0.1 μM; $n = 6$), or oligomycin (complex V inhibitor, 0.01 μM; $n = 5$). GM-CSF and IL-10 from B cells were measured by ELISA. (**J** and **K**) B cell culture supernatants were generated (for details, see Materials and Methods) and applied to human monocyte-derived macrophages. Cells were then treated with LPS. IL-12 (J, $n = 17$) and IL-6 (K, $n = 18$) from macrophages were measured in the myeloid cell supernatants by ELISA and are depicted relative to the cytokine levels expressed by LPS-stimulated myeloid cells that were exposed to unstimulated B cell supernatants. (**L** to **O**) Cytokine profiles of B cells isolated from patients with mitochondrial respiratory chain mutations (table S1; $n = 11$) versus controls ($n = 13$). (**P** and **Q**) B cells were pretreated with either vehicle or various doses of inhibitors before stimulation. Percent change of B cell GM-CSF and IL-10 secretion, as affected by different doses of rotenone (P, $n = 3$) and oligomycin (Q, $n = 4$). (B to G and J and K) Repeat measure one-way ANOVA with Geisser-Greenhouse and Holm-Šidák correction. (H and I) Two-tailed paired *t* test. (L to O) Two-tailed Mann-Whitney test. Vehicle: dimethyl sulfoxide (DMSO). PE, phycoerythin; HC, healthy control; GBCM, GM-CSF+ B cell conditional medium.

blocking BCR signaling during B cell activation would modulate their cytokine responses through regulation of cellular metabolism (Fig. 3). We applied transcriptomic analysis to first confirm that the BTKi reverses BCR-induced B cell responses (fig. S9, A and B). GSEA further revealed that many of the major changes resulting from BTKi can be linked with pathways involved in cellular metabolism (fig. S9C). Using Seahorse assays, we confirmed that the BTKi decreased the mitochondrial respiration induced by BCR-mediated activation of the B cells (Fig. 3, A to D). The BTKi also preferentially decreased GM-CSF expression by the B cells, resulting in a significantly decreased GM-CSF/IL-10 ratio (Fig. 3, E to I). We next generated culture supernatants from B cells that were preexposed to either BTKi or vehicle and then activated under pro-inflammatory GM-CSF–promoting conditions and applied the B cell supernatants to macrophages, as described above. Whereas supernatants of activated B cells pretreated with the vehicle induced the expected robust macrophage secretion of IL-12 and IL-6, supernatants of B cells pretreated with BTKi could not promote the macrophage pro-inflammatory cytokine responses (Fig. 3, J and K). Last, leveraging access to peripheral blood mononuclear cells (PBMCs) isolated from a small cohort of healthy controls participating in a phase 1 study of BTKi (*29*), we demonstrated that in vivo BTK inhibition results in a preferential decrease of B cell GM-CSF expression (Fig. 3, L to N). Together, these data indicate that BCR-mediated activation of BTK induces B cell OXPHOS and mediates a pro-inflammatory cytokine shift both in vitro and in vivo and that BTK could be a potential therapeutic target that can specifically modulate B cell cytokine responses by modulating B cell metabolism.

Inhibition of GSK3 leads to a pro-inflammatory shift of B cell cytokine responses

The data above indicated that partial inhibition of B cell OXPHOS has the capacity to down-regulate B cell pro-inflammatory cytokines production while reciprocally enhancing their production of IL-10. We next wished to test whether driving B cell metabolic activity may inversely modulate these B cell cytokine responses. We observed that glycogen synthase kinase 3 (GSK3), which was previously identified as a metabolic checkpoint of murine B cell metabolism (*31*), exhibited a highly up-regulated gene signature in our RNA-seq BTKi condition versus a highly down-regulated gene signature in the vehicle control condition (fig. S10, A and B). In keeping with this, we observed that GSK3 inhibition (GSK3i) during B cell activation boosted their metabolic activity (Fig. 3O and fig. S11) and induced a pro-inflammatory cytokine shift, such that B cells secreted higher levels of GM-CSF while reciprocally decreasing their IL-10 secretion, together resulting in substantially increased GM-CSF/IL-10 ratios (Fig. 3, P to S). This pro-inflammatory cytokine shift mediated by GSKi was observed whether the B cells were activated under pro-inflammatory–promoting (CD40L + αBCR + IL-4) conditions (Fig. 3, P to S) or anti-inflammatory–promoting (CpG) conditions (fig. S12, A to C), pointing to GSK3 as a "master switch" involved in reciprocal regulation of B cell pro- and anti-inflammatory cytokines. This effect of GSK3i appeared partially mediated through enhancing B cell OXPHOS, given that the blockade of OXPHOS (but not of glycolysis) partially limited the GSK3i-mediated changes in B cell cytokine responses (Fig. 3R and fig. S12D). Together, these data suggest that promoting B cell OXPHOS by blocking the B cell metabolic check point (GSK3) leads to a pro-inflammatory shift of the B cell cytokine profile.

Increased OXPHOS underlies the imbalanced B cell cytokine profile in MS, which is restored with inhibition of either OXPHOS or BTK

Because patients with MS harbor increased frequencies and greater response propensities of circulating GM-CSF–expressing B cells yet reduced IL-10–producing B cells (*12*, *17*, *32*), and given our current observations with healthy donor B cells that the balance between GM-CSF and IL-10 expression can be metabolically regulated (Fig. 2) and that BTKi can limit both B cell respiration (Fig. 3, A to D) and pro-inflammatory cytokine responses (Fig. 3, E to L), we assessed whether the B cell cytokine imbalance in MS was associated with a dysregulated B cell metabolic state and how BTKi would affect their responses. Comparing B cells isolated from untreated patients with MS (table S3, $n = 15$) and age- and sex-balanced healthy controls (table S2; $n = 14$), we observed that both basal and maximal mitochondrial respiration are increased in B cells of patients with MS and that treatment with BTKi essentially reverses these abnormalities so that respiration of BTKi-treated B cells from patients with MS and controls was comparable (Fig. 4, A and B). We replicated prior observations of increased frequencies of GM-CSF$^+$ B cells (Fig. 4C) and diminished frequencies of IL-10$^+$ B cells (Fig. 4D) in untreated patients with MS compared with controls and further observed that both BTK inhibition and partial inhibition of OXPHOS could reverse and essentially normalize both the GM-CSF and IL-10 of the MS B cells (Fig. 4, C to E). The functional consequences of inhibiting BTK or limiting respiration of the patient with MS B cells were then assessed by transiently applying soluble products of the different B cells to macrophages, with subsequent assessment of the macrophage cytokine expression (similar to experiments in Figs. 2, J and K, and 3, J and K). Compared with healthy control B cells, the capacity of untreated MS B cells to induce exaggerated pro-inflammatory myeloid-cytokine (IL-12 and IL-6) responses was abrogated when MS B cells were pre-exposed to either BTKi or rotenone (Fig. 4, F and G). These data suggest that abnormal B cell OXPHOS activity may be responsible for the imbalanced cytokine profile and the enhanced pro-inflammatory function of B cells in patients with MS.

B cell OXPHOS contributes to the pathogenesis of neuroinflammation in vivo

We next considered whether inhibiting B cell OXPHOS could reduce neuroinflammation in vivo. We generated B cell–specific *Ndufs4*$^{+/-}$ mice, which have impaired mitochondrial respiration using mixed bone marrow chimeras. *Ndufs4* encodes a nuclear-encoded accessory subunit of the mitochondrial membrane respiratory chain reduced form of nicotinamide adenine dinucleotide dehydrogenase, complex I. Specifically, we mixed bone marrow from *Ndufs4*$^{+/-}$ mice with bone marrow from either wild-type (WT) or IgM$^-$IgD$^-$ (B cell-deficient) mice at a ratio of 1:4 and transplanted these into lethally irradiated CD45.1 WT recipients (Fig. 4H). After allowing 6 to 8 weeks of bone marrow reconstitution, we measured *Ndufs4* expression in different types of immune cells by real-time polymerase chain reaction (PCR) (fig. S12). As expected, about 50% decrease of *Ndufs4* expression was observed in B cells isolated from the experimental group, whereas the expression of *Ndufs4* was not altered in T cells or myeloid cells (fig. S13). Using these mice, we induced experimental autoimmune encephalomyelitis (EAE) with recombinant myelin oligodendrocyte glycoprotein (MOG). We observed that, compared with the control mice, lowering B cell

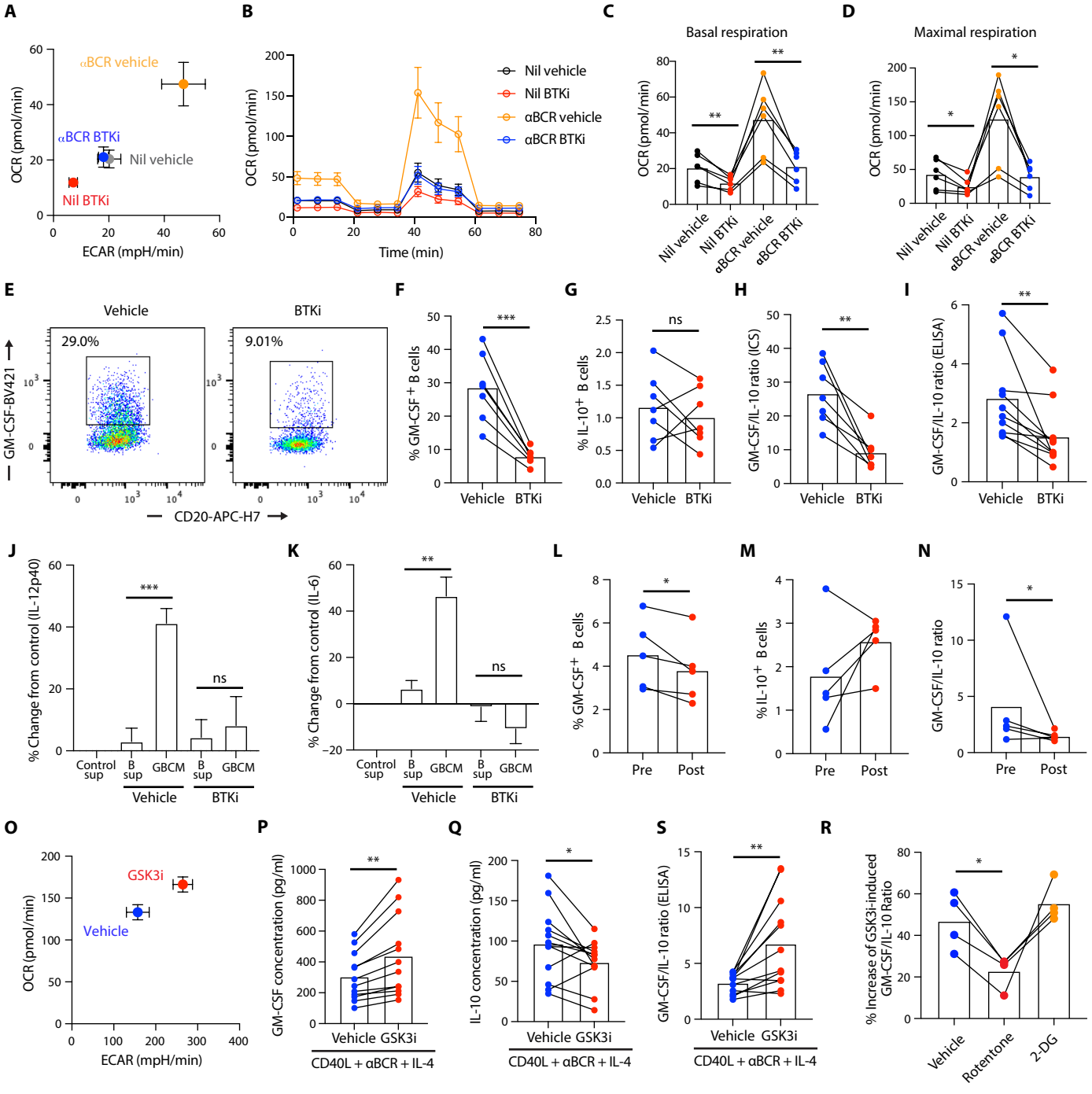

Fig. 3. Modulation of B cell mitochondrial respiration by BTK or GSK3 inhibitors influences B cell cytokine profiles. (**A** to **D**) Human B cells were stimulated with αBCR in presence of vehicle or BTKi. Seahorse was used to analyze B cell mitochondrial respiration ($n = 6$). (**E** to **I**) B cells were either left unstimulated or stimulated with CD40L + αBCR + IL-4 in the presence of vehicle or BTKi for 48 hours, at which time B cell cytokine expression was measured using flow cytometry ($n = 6$ or 7) and ELISA ($n = 10$). (**J** and **K**) B cell culture supernatants were generated and applied to human monocyte-derived macrophages. Myeloid cell cytokine responses were measured by ELISA and depicted relative to the cytokine levels of control supernatants (sup) (J, IL-12, $n = 7$; K, IL-6, $n = 8$). (**L** to **N**) Healthy volunteers participating in a phase 1 clinical trial of BTKi were treated with BTKi 300 mg BID for 7 days. Ex vivo cytokine expression of the B cells before or after in vivo BTKi exposure was measured by ICS ($n = 5$). (**O**) B cells were stimulated with CD40L + αBCR + IL-4 in the presence of vehicle or GSK3i. B cell mitochondrial respiration was measured by a Seahorse analyzer ($n = 3$). (**P** to **R**) B cells were pretreated with either vehicle or GSK3i and then stimulated with CD40L + αBCR + IL-4. GM-CSF and IL-10 were measured by ELISA ($n = 11$). (**S**) B cells were pretreated with rotenone or 2-DG and then stimulated with CD40L + αBCR + IL-4, in the presence of GSK3i or vehicle ($n = 4$). (C and D and J and K) Repeat measure two-way ANOVA with Geisser-Greenhouse correction. (R) Repeat measure one-way ANOVA with Geisser-Greenhouse and Holm-Šidák correction. (F to I, L to N, and P to S) Two-tailed paired *t* test; each line represents results from a single donor. Vehicle: DMSO. APC, allophycocyanine.

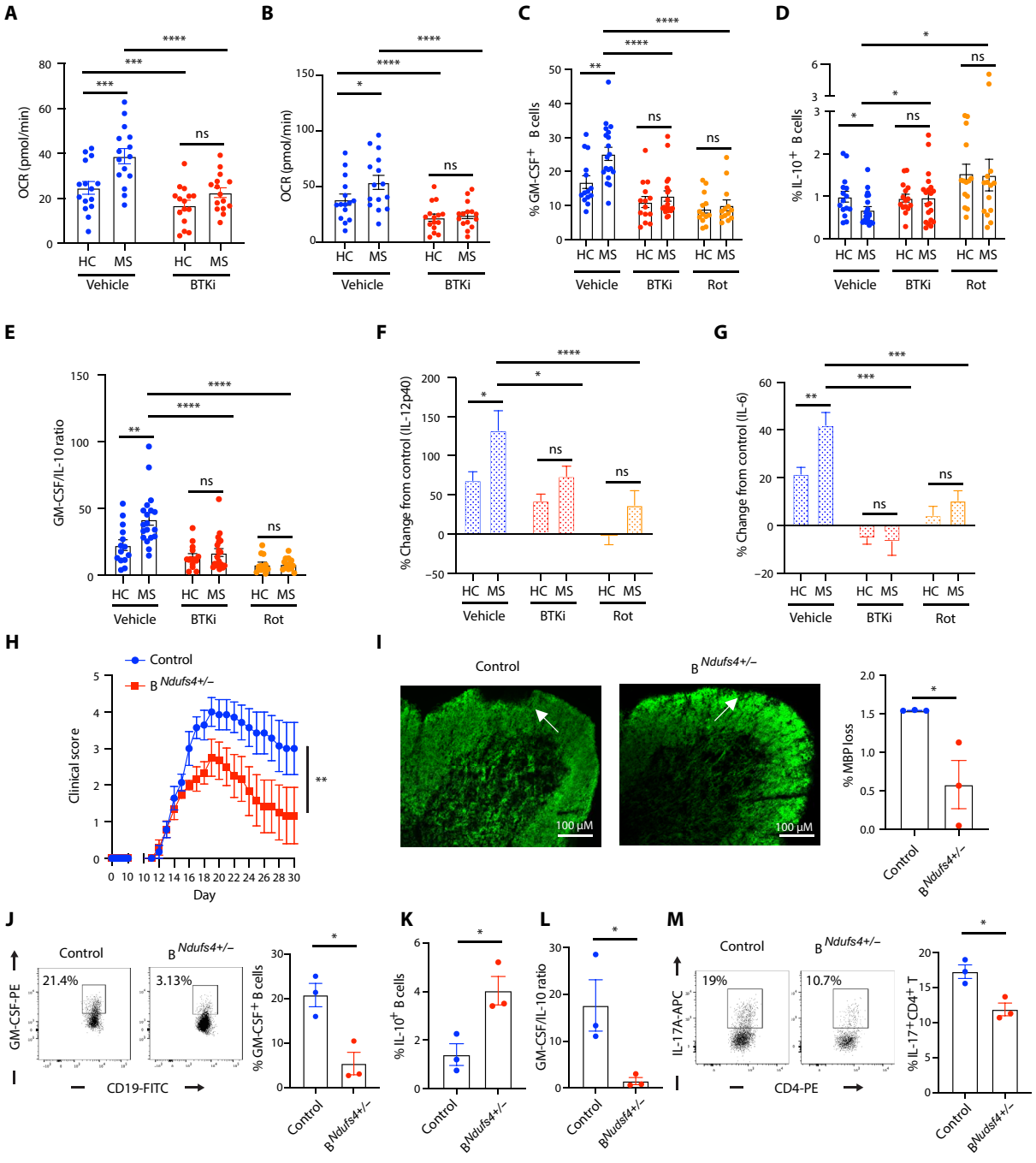

Fig. 4. Implication of B cell OXPHOS in the pathogenesis of MS and EAE. (**A** and **B**) B cells from either healthy controls ($n = 14$, table S2) or untreated patients with MS (MS, $n = 15$; table S3) were pretreated with either vehicle or BTKi and then stimulated with CD40L + αBCR + IL-4. Seahorse analysis of B cell mitochondrial respiration was performed using standard Mito stress test. (**C** to **E**) B cells from either untreated patients with MS ($n = 19$) or healthy controls ($n = 14$) balanced for age and sex were pretreated with either vehicle, BTKi, or rotenone and then stimulated with CD40L + αBCR + IL-4. GM-CSF and IL-10 were measured by ICS and flow cytometry. (**F** and **G**) Cell culture supernatant from B cells of either untreated patients with MS ($n = 8$) or healthy controls ($n = 12$) was generated (for details, see Materials and Methods) and applied to monocyte-derived macrophages of healthy control donors. Subsequent secretion of IL-12 and IL-6 by the macrophage was quantified by ELISA. (**H** to **N**) EAE was induced in mixed bone marrow chimeras with recombinant human MOG. (H) Clinical score of EAE ($n = 12$ per group). (I) MBP staining of the spinal cord (day 18 after EAE induction, $n = 5$ per group; white arrows point to loss of MBP-stained myelin). (J to L) B cells were isolated from the spleens of either control mice or B$^{Ndufs4+/-}$ mice at day 7 after EAE induction and stimulated with CD40L + αBCR + IL-4 for 16 hours. GM-CSF and IL-10 were detected by ICS ($n = 4$ per group; B cells were pregated with CD45.2 as shown in fig. S25). (M) CNS infiltrating cells were isolated at day 16 after EAE induction. IL-17 was measured by ICS ex vivo ($n = 4$ per group). (A to G and H) Mixed-effect two-way ANOVA with Geisser-Greenhouse and Holm-Šidák correction. (I to M) Two-tailed Mann-Whitney test. Vehicle: DMSO. ns, not significant; FITC, fluorescein isothiocyanate.

mitochondrial respiration decreased clinical EAE severity and histological damage (Fig. 4, H and I). In keeping with both our in vitro and ex vivo human findings, we observed substantially diminished frequencies of GM-CSF–expressing B cells (Fig. 4J and fig. S14A) and increased frequencies of IL-10–expressing B cells (Fig. 4K and fig. S14B) isolated from the mice with in vivo reduced B cell OXPHOS activity, together resulting in a much-diminished B cell GM-CSF/IL-10 ratio (Fig. 4L). The reduced in vivo B cell mitochondrial respiration also resulted in decreased infiltration of pathogenic T helper 17 (T_H17) cells into the central nervous system (CNS) of the EAE mice (Fig. 4M). Together, these data provide in vivo proof of principle that the OXPHOS status of B cells can shape the balance of their pro-inflammatory versus anti-inflammatory cytokine expression and affect the state of CNS inflammation through modulation of disease-implicated immune responses.

Extracellular ATP shapes the balance between pro- and anti-inflammatory B cell cytokine responses in MS and EAE

Prior studies linking cellular metabolism to immune responses identified that ATP generated by OXPHOS can act as an immune modulator and that extracellular ATP (eATP) released from injured cells can initiate and amplify immune responses through both paracrine and autocrine mechanisms (*33*–*37*). We therefore wished to assess the impact of eATP and its metabolites on B cell cytokine responses. We first measured eATP levels after stimulating human B cells under the different activating conditions and found that the activating stimuli that induced increasing B cell GM-CSF production induced correspondingly higher ATP production and release (Fig. 5A) and that blocking either BTK or OXPHOS substantially decreased ATP released by the activated B cells (fig. S15).

To next investigate the mechanism by which exogenous ATP might influence cytokine production, we assessed B cell expression of purinergic receptors (known to be involved in eATP signaling) and observed that when activated under the GM-CSF–promoting conditions, B cells predominantly expressed the *P2RX4* and *P2RX7* (Fig. 5B). Whereas inhibition of P2RX4 (P2RX4i) during B cell activation did not appreciably influence GM-CSF or IL-10 secretion (fig. S16), inhibiting P2RX7 (P2RX7i) reciprocally decreased B cell secretion of GM-CSF while increasing IL-10 secretion, which resulted in a substantially diminished GM-CSF/IL-10 ratio (Fig. 5, C to E).

We then assessed whether the ATP-P2RX7 axis (which we showed can mediate pro-inflammatory cytokine production by healthy B cells; Fig. 5, C to E) might also be a target for restoring the balance of MS B cell cytokine responses. We blocked P2RX7 in B cells isolated from a new cohort of untreated patients with MS ($n = 12$) and age- and sex-balanced healthy controls ($n = 10$) and observed that inhibition of P2RX7 in essence restored the balance of MS B cell pro-inflammatory and anti-inflammatory cytokine responses (Fig 5, F and G). Whereas B cell surface expression of P2RX7 did not appreciably differ between MS and healthy controls (fig. S17), activated B cells (but not resting B cells) of patients with MS produced higher levels of ATP (Fig. 5H), suggesting that the pro-inflammatory shift observed for MS B cell cytokine responses is mainly due to the higher production of ATP when these cells are activated rather than higher expression of P2RX7.

To test the role of B cell P2XR7 in vivo, we generated B cell–specific *P2rx7* knockout mice using mixed bone marrow chimeras (fig S18). We mixed bone marrow from $P2rx7^{-/-}$ mice with bone marrow from either WT or $IgM^{-}IgD^{-}$ (B cell–deficient) mice at a ratio of 1:4 and transplanted them into lethally irradiated CD45.1 WT recipients. EAE was induced with recombinant MOG after 6 to 8 weeks of bone marrow reconstitution. $B^{P2rx7-/-}$ mice exhibited slightly reduced clinical severity and decreased histological damage compared with control animals (Fig. 5, I and J). In keeping with our human findings, we observed that B cell expression of GM-CSF was decreased in the B cell P2rx7-deficient EAE mice (Fig. 5K), whereas their IL-10 expression was increased (Fig. 5L), together resulting in a diminished B cell GM-CSF/IL-10 profile (Fig. 5M). The reduced in vivo B cell *P2rx7* expression also resulted in decreased infiltration of pathogenic T_H17 cells into the CNS of the EAE mice (Fig. 5N). Together, these data provide in vivo support that ATP can modulates B cell responses through its surface receptor P2RX7 and that the B cell ATP-P2RX7 axis is involved in shaping B cell pro-inflammatory versus anti-inflammatory cytokine expression, with the potential to impact the state of CNS inflammation through modulation of disease-implicated immune responses.

Adenosine preferentially induces anti-inflammatory B cell cytokine responses through its surface receptor A2a receptor (A2aR)

Because our findings above suggested a role of OXPHOS-derived ATP in promoting pro-inflammatory cytokine responses in B cells, we tested whether exogenous ATP could rescue the diminished B cell cytokine responses that we observed in patients with mitochondrial respiratory chain mutations (Fig. 2, L to O). We found that ATP added exogenously to ex vivo activated B cells of patients with mitochondrial respiratory chain mutations could rescue their diminished cytokine expression to levels similar to those seen in control B cells (Fig. 6, A and B). We then assessed the effect of adding exogenous ATP to the normal B cells, expecting the ATP to induce a pro-inflammatory cytokine response profile (i.e., opposite to the effect observed with the P2RX7i). To our initial surprise, the addition of ATP increased B cell intracellular expression and secretion of both GM-CSF and IL-10 (fig. S19), which in the balance resulted in a decrease rather than increase in the GM-CSF/IL-10 ratio (Fig. 6C). We further noted that inhibition of P2RX7 did not block the ability of ATP to induce B cell IL-10 (Fig. 6D) and only partially blocked the ATP effect on GM-CSF (fig. S20). This suggested that the ability of exogenous ATP to induce both GM-CSF and IL-10 was not mediated exclusively through P2RX7. Because B cells are known to express functional levels of CD39 and CD73, two key molecules that convert eATP through adenosine diphosphate (ADP) and adenosine monophosphate (AMP) into adenosine (*38*–*42*), we hypothesized that the effect of eATP on B cell cytokine regulation may reflect the partial conversion of ATP to adenosine and the consequent signaling of adenosine through adenosine receptors. Analysis of the expression profile of adenosine receptors demonstrated that the A2aR is the major adenosine receptor expressed by resting human B cells (Fig. 6E). Blockade of adenosine signaling by selective A2aR inhibition [A2a receptor inhibitor (A2aRi)] or blockade of the conversion of ATP to adenosine using CD39 inhibitor (CD39i) could both reverse the ability of ATP to induce B cell IL-10 (Fig. 6, F and G). Furthermore, direct addition of either adenosine (Fig. 6H) or of AMP (fig. S21) induced substantial B cell IL-10 secretion, resulting in anti-inflammatory cytokine shifts that were reflected in diminished GM-CSF/IL-10 ratios (Fig. 6I and fig. S21B, respectively), and these effects of adenosine or AMP were

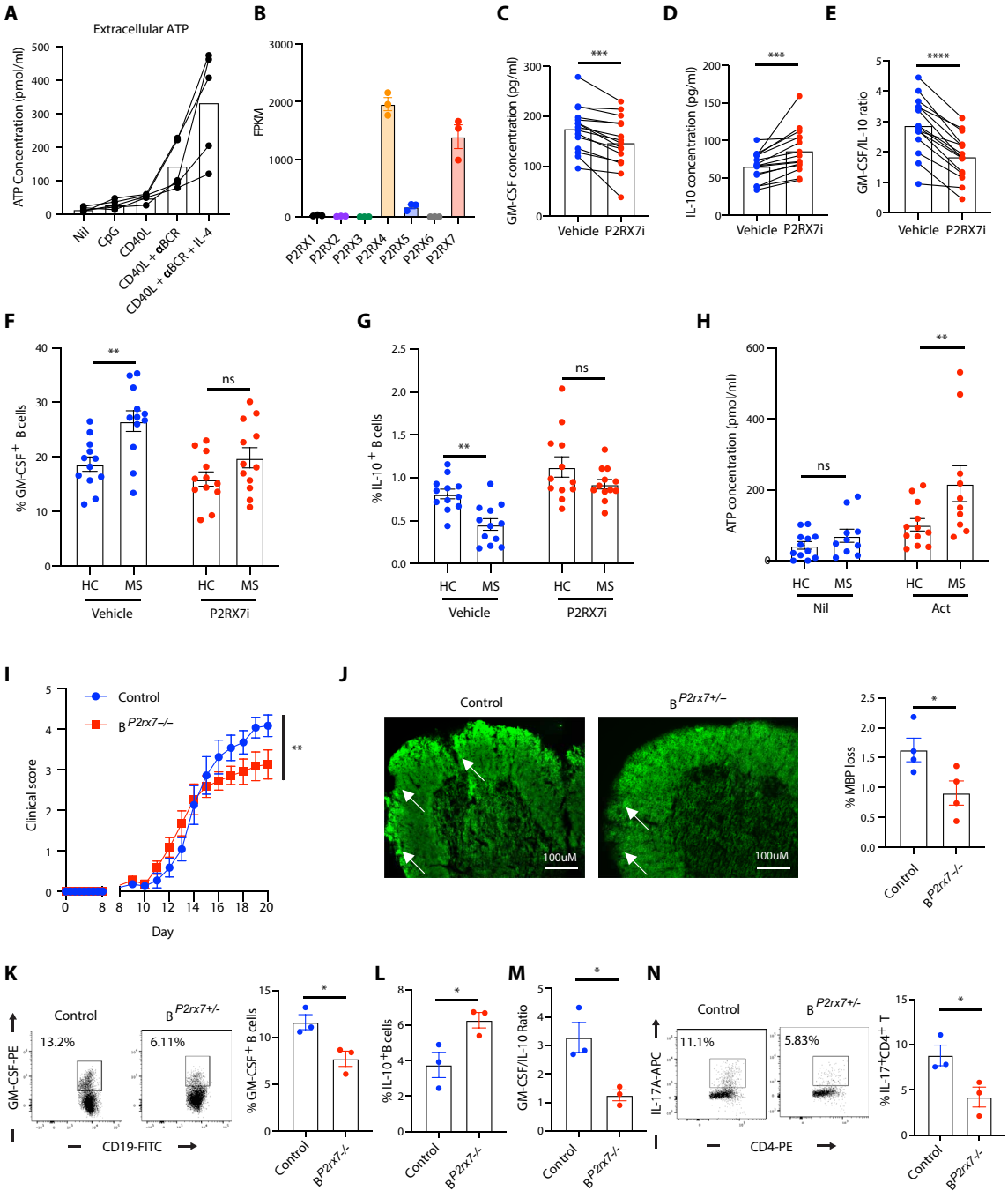

Fig. 5. The ATP-P2RX7 axis modulates B cell cytokine response profiles. (**A**) ATP bioluminescence assay was used to measure ATP produced by B cells under different stimulation conditions (CD40L, CpG, CD40L + αBCR, or CD40L + αBCR + IL-4; $n = 5$). (**B**) Expression of P2X purinergic receptors detected by bulk RNA-sequencing of normal human B cells under CD40L + αBCR + IL-4 stimulation. (**C** to **E**) B cells were pretreated with either vehicle (DMSO) or P2RX7 inhibitor (P2RX7i) and then stimulated with CD40L + αBCR + IL-4. GM-CSF and IL-10 secretion was measured by ELISA ($n = 12$). (**F** and **G**) B cells from either patients with MS ($n = 12$) or age- and sex-matched healthy controls ($n = 12$) were pretreated with P2RX7i or vehicle (DMSO) and then stimulated with CD40L + αBCR + IL-4 for 48 hours. Intracellular GM-CSF and IL-10 were measured by flow cytometry. (**H**) ATP from B cells of either patients with MS ($n = 12$) or healthy controls ($n = 10$) was measured by ATP bioluminescence assay. (**I** to **N**) EAE was induced in mixed bone marrow chimeras with recombinant human MOG. (I) Clinical score of EAE ($n = 12$ per group). (J) MBP staining of the spinal cord (day 18 after EAE induction, $n = 5$ per group; white arrows point to loss of MBP-stained myelin). (K to M) B cells were isolated from the spleens of either control mice or $B^{P2rx7-/-}$ mice at day 7 after EAE induction and stimulated with CD40L + αBCR + IL-4 for 16 hours ($n = 4$ per group). GM-CSF and IL-10 were detected by ICS. (**N**) CNS infiltrating cells were isolated at day 16 after EAE induction. IL-17 was measured by ICS ex vivo ($n = 4$ per group). (C to E) Two-tailed paired *t* tests. (F to H and I) Mixed-effect two-way ANOVA with Geisser-Greenhouse and Holm-Šidák correction. (J to N) Two-tailed Mann-Whitney test. Vehicle: DMSO. FPKM, fragments per kilobase of transcript per million mapped reads.

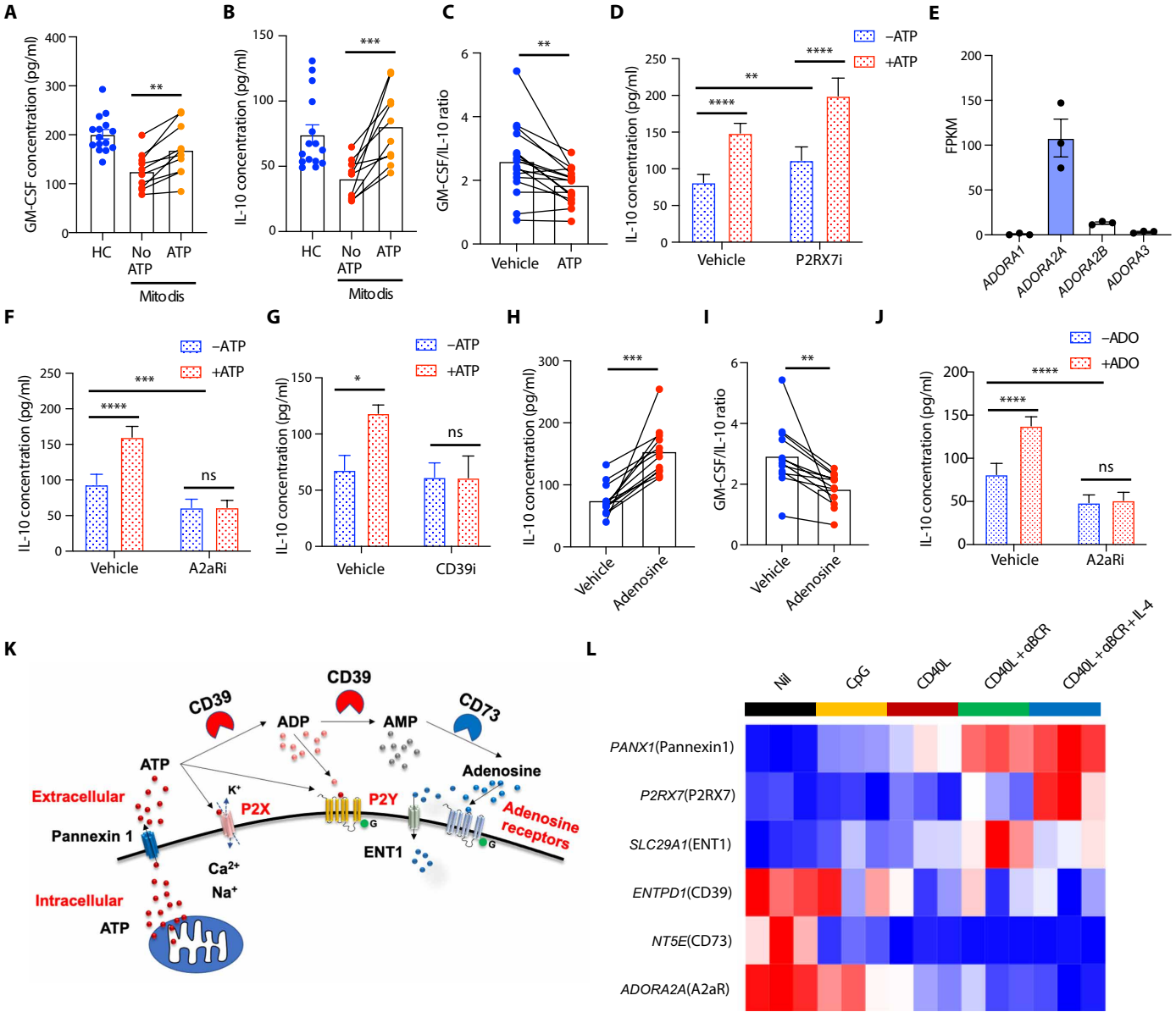

Fig. 6. Adenosine preferentially induces anti-inflammatory cytokine production in B cells. (**A** and **B**) B cells from patients with mitochondrial complex deficiency (mito dis, $n = 10$) were pretreated with ATP and then stimulated with CD40L + αBCR + IL-4. GM-CSF and IL-10 were measured by ELISA. (**C**) B cells were pretreated with vehicle or ATP and then stimulated with CD40L + αBCR + IL-4. B cell cytokine expression was measured using ELISA ($n = 16$). (**D**) B cells were pretreated with either vehicle or P2RX7i before ATP treatment. B cells were then stimulated with CD40L + αBCR + IL-4, and IL-10 was measured by ELISA ($n = 7$). (**E**) Bulk RNA-seq revealed that the adenosine receptor A2a is the predominant adenosine receptor expressed by resting B cells ($n = 3$). (**F**) B cells were pretreated with either vehicle or A2aRi in the presence or absence of ATP before stimulation. IL-10 secretion was measured by ELISA ($n = 6$). (**G**) B cells were pretreated with either vehicle or CD39i in the presence or absence of ATP before stimulation. IL-10 was measured by ELISA ($n = 3$). (**H** and **I**) B cells were pretreated with vehicle or adenosine (ADO) and then stimulated with CD40L + αBCR + IL-4. B cell cytokine was measured by ELISA ($n = 12$). (**J**) B cells were pretreated with either vehicle or A2aRi, with or without subsequent addition of adenosine before stimulation. B cell cytokine secretion was measured by ELISA ($n = 6$). (**K**) Schematic of B cell ATP molecular signaling machinery. (**L**) Distinct modes of activation that induce pro-inflammatory or anti-inflammatory cytokine-defined B cells induce coordinated regulation of a molecular network involved in ATP metabolism ($n = 3$). (A to C and H and I) Two-tailed paired *t* test. (D, F, G, and J) Repeat measure two-way ANOVA with Geisser-Greenhouse correction. Vehicle: DMSO/H_2O.

entirely blocked by A2aRi (Fig. 6J and fig. S21C, respectively). These data indicate that signaling by ATP and its metabolites through surface purinergic receptors can modulate the balance between B cell pro-inflammatory and anti-inflammatory cytokine expression, thereby shaping functional responses of activated B cells and providing a form of "signal 4" that complements traditional signals 1, 2, and 3 [mediated through the BCR, costimulatory receptors, and cytokine receptors, respectively (*43*, *44*)].

Distinct modes of B cell activation regulate cytokine responses through coordinated expression of ATP pathway molecular machinery

Using RNA-seq, we further noted that when human B cells are stimulated across the range of activating conditions that progressively alters their metabolic state to mediate an anti-inflammatory to pro-inflammatory cytokine shift (Fig. 1G) while also inducing graded increases in their release of ATP (Fig. 5A), the same B cells exhibit differential and coordinated regulation of the expression of multiple molecules involved in ATP signaling (Fig. 6, A and B). In particular, activation that promotes B cell GM-CSF expression and inhibits IL-10 expression (i.e., stimulation with CD40L + αBCR, with or without IL-4) induced increased expression of the ATP transporter pannexin 1 (*PANX1*) in addition to *P2RX7* (fig. S22, A and B), which would both tend to promote ATP signaling while also inducing increased expression of *ENT1* (fig. S22C) that would serve to limit adenosine signaling. At the same time, levels of expression of *CD39*, *CD73*, and *ADORA2A* that tend to promote adenosine signaling were either decreased or remained unchanged (fig. S22, D to F). Together, this activation-induced expression profile serves to enhance B cell ATP signaling while inhibiting adenosine signaling, explaining the observed IL-10 to GM-CSF cytokine shift. Last, in keeping with our observations that BTKi substantially decreases B cell release of ATP (fig. S15) and diminishes B cell expression of GM-CSF (Fig. 3, E and F), we found that the modular regulation of ATP signaling molecules induced under the pro-inflammatory–activating condition was largely abrogated by BTKi (fig. S23). Only *P2RX7* expression was not consistently affected by the BTKi, reflecting its lesser dependence on BCR signaling and its greater dependence on IL-4 signaling (in keeping with Fig. 6L). Overall, these findings underscore a mechanism involving the coordinated and "modular" context-dependent regulation of multiple ATP signaling molecules that shapes the balance between human B cell pro-inflammatory and anti-inflammatory cytokine responses (fig. S24).

DISCUSSION

Although B cells are often considered primarily for their capacity to differentiate into antibody-secreting cells (whether to produce host-protective antibodies or potentially pathogenic autoantibodies), they are increasingly well recognized for a range of "antibody-independent" immune functions. Key among these is their capacity to elaborate distinct cytokine profiles that can contribute to either activating or down-regulating adaptive and innate immune responses, with relevance in both health and disease. Abnormal B cell cytokine responses have now been implicated in the pathophysiology of a number of human immune-mediated conditions, including MS (*3*, *4*, *17*). Whether activated B cells produce pro-inflammatory or anti-inflammatory cytokines appears to be influenced in part by the type of B cell being activated (e.g., naïve versus memory), its prior exposure history, and the context in which it is stimulated (i.e., the combination of particular signals it integrates during activation, including stimulation through the BCR, CD40, cytokine receptors, Toll-like receptors, etc). Despite the major importance of B cell derived cytokines, relatively little is known about both the cell-extrinsic and cell-intrinsic mechanisms that regulate cytokine expression, particularly those involved in the balance between pro-inflammatory and anti-inflammatory cytokines. We focused here on the balance between human B cell GM-CSF and IL-10 expression because of their abnormal implication in many autoimmune diseases, including MS, and because their expression in normal B cells appears to be largely mutually exclusive, suggesting that some mechanism must be involved in the "choice" of whether a B cell will produce one or the other. Our results also have broader implications as to how functional human B cell responses may be regulated in health and disease. We find that B cell cytokine production is metabolically regulated both in vitro and in vivo, with particular involvement of mitochondrial respiration, and that distinct modes of B cell activation shift their metabolic state, regulate the extent of ATP release by the B cells, and trigger the coordinated and modular regulation of ATP pathway molecular machinery that enables ATP and its metabolites to function as a fourth signal, reciprocally modulating the balance between pro- and anti-inflammatory cytokine expression. We further implicate both B cell metabolism and B cell ATP signaling in shaping CNS inflammation in the EAE animal model in vivo and implicate abnormal B cell metabolism and ATP signaling as contributors to disease-implicated aberrant B cell cytokine responses in patients with MS, demonstrating the potential to normalize both their metabolic state and cytokine responses with the emerging autoimmune disease therapeutic approach of BTK inhibition.

Cellular metabolism plays key roles in modulating cellular immune responses (*45*–*47*). In the case of B cells, stimulation through the BCR, CD40, or IL-4R has been shown to rapidly enhance B cell metabolic activity, particularly glycolysis, through the PI3K-AKT-mTORC1 pathways (*48*–*52*). Metabolic changes induced by various stimuli can prime B cells to proliferate and differentiate during the germinal center reaction (*31*, *53*), and different substrates that fuel mitochondrial machinery support B cell survival, as shown for long-lived plasma cells in the bone marrow (*54*). Our findings extend these prior studies that considered the effects of metabolism on B cell activation, differentiation, and survival, with the demonstration that metabolic regulation of B cell effector and regulatory cytokines is an added dimension of interest within the growing field of immunometabolism.

Our unbiased bulk RNA-seq of cytokine-defined human B cell subpopulations pointed to different expression profiles of cellular metabolism pathways, including OXPHOS and mTOR signaling. We demonstrate that higher metabolic activity is required for the generation of pro-inflammatory B cells as compared with IL-10–expressing B cells. Titration of cellular metabolic activity results in hierarchical modulation of pro- and anti-inflammatory B cell cytokines, and partial inhibition of OXPHOS (but not of glycolysis) can reciprocally regulate secretion of GM-CSF and IL-10 by the B cells, which, in turn, exhibit a much-diminished functional capacity to induce pro-inflammatory myeloid cell responses. Titration of rotenone, oligomycin, and antimycin A recapitulated the reciprocal modulation of B cell GM-CSF and IL-10 expression with partial OXPHOS inhibition and also indicated that this consistent observation is unlikely to reflect "off-target" effects of these three reagents.

In the context of autoimmune disease, our observation of abnormally increased respiration in B cells of untreated patients with MS, together with the demonstration that partial blockade of mitochondrial respiration (whether using rotenone or inhibiting the P2RX7-ATP pathway) restores the balance of MS B cell cytokine responses and limits their capacity to induce pro-inflammatory myeloid cell responses, suggests that enhanced mitochondrial respiration underlies at least some of the previously reported abnormalities of MS B

cell cytokine expression, including their exaggerated propensity to secrete GM-CSF and deficient expression of IL-10 (*6*, *18*–*20*, *55*). This raises the prospect of therapeutically targeting aberrant B cell contribution to autoimmune disease by modulating their metabolism.

How to target metabolism, however, in a cell type–specific (or selective) manner is an important consideration (*47*). By assessing the balance between pro-inflammatory and anti-inflammatory B cell cytokine expression across a range of stimulation conditions in healthy B cells, we identified that BCR can drive the transition from B cell production of IL-10 to production of GM-CSF. This suggested that blocking BCR signaling during B cell activation may mediate an anti-inflammatory cytokine shift. We could then demonstrate that targeting BTK, which functions downstream of the BCR with a BTKi, limits B cell OXPHOS and preferentially reduces their pro-inflammatory cytokine expression. These observations extend prior work showing that BTKi can limit B cell activation through inhibition of OXPHOS (*28*) by demonstrating that inhibiting metabolism through BTKi does not merely quantitatively reduce cellular activation but rather can regulate the reciprocal modulation of cellular functions that contribute to shifting the balance between pro- and anti-inflammatory immune responses (i.e., a "qualitative," not just "quantitative" effect). In particular, inhibition of BTK signaling during activation results in an anti-inflammatory shift in the GM-CSF/IL-10 profile of B cells both in vitro and in vivo without substantially affecting their survival. This effect of BTKi on B cell metabolism appeared to be mediated partly through disinhibition of GSK3 signaling, linking prior work showing that BCR engagement suppresses GSK3 activity (*56*) with the more recent implication of GSK3 in sensing and regulating B cell metabolism during CD40L-mediated stimulation (*31*). The selective expression of BTK mainly by B cells (and myeloid cells) has fueled interest in BTKi as a nondepleting B cell–targeting approach in patients with autoimmune diseases including MS, where it has been found to decrease new disease activity in early phase clinical trials (*57*, *58*). Unexpectedly, little is known about how BTKi affects MS disease-relevant functions of human B cells. Most prior human work has considered the effects of BTKi on malignant cells, and studies into the therapeutic mode of action of BTKi on nonmalignant cells in autoimmune diseases including MS have largely focused on murine models. Our findings that BTKi reverses the abnormally enhanced respiratory state of MS B cells and restores the balance of patients' B cell cytokine responses extend our understanding of the therapeutic mode of action of BTKi in MS and potentially other autoimmune diseases.

We further show that patients with mitochondrial respiratory chain deficiencies exhibit diminished B cell cytokine responses that can be corrected by eATP supplementation and that eATP and its metabolite, adenosine, can shape the balance of human B cell pro-inflammatory and anti-inflammatory cytokine responses through the cell surface purinergic receptors P2RX7 and A2aR. Our results parallel and extend prior findings (*34*, *35*, *37*, *59*) that eATP and its metabolites can serve as important immune-response modulators, particularly in the context of innate immunity. During the acute phase of an inflammatory event, local release of ATP by injured or apoptotic cells has been shown to promote myeloid cell and neutrophil recruitment and activation through multiple purinergic receptors, including P2RXs and P2RYs (*60*–*66*). In addition, ATP can also directly increase T cell pro-inflammatory responses in an autocrine manner (*36*, *67*–*72*). In contrast to ATP, adenosine can dampen inflammation, induce type 2 immune responses, and promote wound healing (*34*). Both myeloid and T cells express adenosine receptors and could receive signals from extracellular adenosine (*73*, *74*). It has also been shown that adenosine may promote lipopolysaccharide (LPS)–induced macrophage IL-10 production through an A2bR-mediated posttranscriptional mechanism (*75*). Whether adenosine modulates B cell cytokine responses through similar mechanisms requires further investigation. Compared with other immune cells, B cells express higher level of surface CD39 and CD73 (*38*, *41*) that are known to convert eATP into adenosine, and adenosine generated by B cells has been identified as a potential mechanism by which B cells can down-regulate T cell responses (*38*). In addition to modulating T cells, adenosine is also involved in regulating B cell responses (*76*). For example, adenosine can directly deplete $CD11c^+$ T-bet^+ pathogenic B cells in animal models of SLE (*77*). In patients with SLE, the activity of CD73 was found to be silenced in B cells, further suggesting that a defect in adenosine-mediated immune regulation may link with autoimmune diseases (*78*). Our findings support a model wherein eATP released by the B cells themselves or otherwise available in the external environment can promote pro-inflammatory B cell cytokine responses, BCR signaling, Ca^{2+} influx, and immunoglobulin production through the P2RX7 receptor (*79*–*83*), whereas the ATP metabolite adenosine can enhance B cell anti-inflammatory cytokine responses through the A2a receptor. Similar to T cells, B cells are known to integrate multiple signals that determine their activation, proliferation, and differentiation states (*43*, *44*), including stimulation through the BCR, CD40, and cytokine receptors, referred to as signals 1, 2, and 3, respectively. We demonstrate that distinct modes of B cell activation induce coordinated modulation of a molecular network involved in ATP metabolism that contributes as "signal" 4, shaping the balance of B cell pro-inflammatory and anti-inflammatory cytokine responses.

There are several limitations to this study. The specific molecular mechanisms underlying the downstream modulatory effect of ATP and its metabolites on B cell cytokine responses remain unknown and warrant further investigation. Although our study focused on how ATP and its metabolites released by B cells could modulate B cell cytokine responses (i.e., potentially in an autocrine or paracrine fashion), there are likely other sources of ATP and its metabolites (such as other metabolically active or dying cells) that may confer context-dependent modulation of B cells. Our observation that OXPHOS blockade has a bigger effect on B cell cytokine responses as compared with P2rx7 blockade suggests that OXPHOS modulation of B cell cytokine responses may be mediated through multiple pathways, underscoring the complexity of metabolic regulation of immune responses.

Overall, our study reveals a mechanism involving metabolic regulation of human B cell cytokine responses with relevance to both health and disease. We report that regulation of mitochondrial respiration and ATP and its metabolite adenosine can reciprocally modulate the balance between human B cell pro- and anti-inflammatory cytokine expression and propose that integration of input from a coordinated network of molecules involved in ATP pathway signaling including surface purinergic receptors can provide a fourth signal that shapes B cell responses. The opposing effect of partial OXPHOS inhibition on the B cell cytokines suggests to us that production of the pro-inflammatory cytokines by B cells has greater reliance on higher levels of OXPHOS as compared with IL-10. Inhibition of BTK limits OXPHOS by disinhibiting GSK3,

mediates an anti-inflammatory cytokine shift in B cells both in vitro and in vivo, and reverses the abnormal respiratory state and aberrant pro-inflammatory cytokine responses of B cells of patients with MS. Our complementary studies in EAE provide proof of principle that both B cell OXPHOS and B cell ATP signaling can shape the expression of CNS inflammation in vivo. In addition to advancing the understanding of fundamental mechanisms involved in human B cell cytokine regulation and abnormalities in patients with MS, our findings provide insights into the therapeutic mode of action of BTKi as an attractive nondepleting B cell–targeting approach and underscore the potential to modulate cellular metabolism as a therapeutic strategy for conditions involving B cell cytokine dysregulation.

MATERIALS AND METHODS

Study design

The overall aim of this study was to elucidate fundamental mechanisms underlying the regulation of human B cell pro-inflammatory and anti-inflammatory cytokine expression. Cytokine capture assays were used initially to purify cytokine-defined B cell populations ex vivo from healthy individuals, followed by unbiased RNA-seq that revealed transcriptomic differences between IL-10$^+$ and GM-CSF$^+$ human B cells attributable to metabolic pathways. A series of in vitro experiments identified a fundamental mechanism involving OXPHOS regulation of B cell cytokine profiles, which was supported by in vivo proof of principle from patients with mitochondrial respiratory chain mutations. B cells of untreated patients with MS exhibited abnormal OXPHOS that was associated with their aberrant pro-inflammatory cytokine responses, and both abnormalities were reversed in vitro with either selective inhibition of cell respiration or with BTKi. Furthermore, access to samples from a phase I BTKi study in healthy controls provided in vivo confirmation that BTK inhibition could limit B cell pro-inflammatory cytokine responses. Last, using animal models of neuroinflammation, we could confirm that interference with B cell OXPHOS and P2RX7 signaling could modulate neuroinflammation in vivo. Study components were not predefined. The number of replicates for each experiment is indicated in Results and figure legend sections. All experiments related to comparisons between MS and healthy controls were performed blindly. The clinical scores of EAE were assessed by independent examiners in a blinded manner. All mechanistic studies using immune cells exclusively from healthy donors were performed without randomization or blinding.

Human participants

Healthy volunteers, patients with MS, and patients with mitochondrial complex deficiency were recruited and provided written informed consent as approved by the University of Pennsylvania and Children Hospital of Philadelphia (CHOP) Institutional Review Boards (IRBs; IRB numbers: 828283 and 816805). The phase 1 clinical trial of BTKi was performed in accordance with Title 21, US CFR Parts 50, 54, 56, and 312 Subpart D; the ICH guideline on GCP (E6); the European Union Clinical Trial Directive 2001/20/EC; and the ethical principles outlined in the Declaration of Helsinki. The IRB approving the phase 1 clinical trial was Midlands IRB (8417 Santa Fe Drive, Suite 100, Overland Park, KS 66212, USA). Tables S1 to S3 summarize the demographics of donors involved in this study.

Animals

Ndufs4$^{+/-}$(NM-KO-201311), *P2rx7*$^{-/-}$(NM-KO-200404), *Ighm*$^{-/-}$*Ighd*$^{-/-}$(NM-KO-200610), CD45.1 (NM-KO-210226), and WT C57BL/6 (SM-001) mice were purchased from MODEL ORGANISMS (Shanghai) and housed under specific pathogen–free conditions in an animal facility with a 12-hour light/12-hour dark cycle. All animals had free access to standard rodent diet and autoclaved tap water. Room temperature was kept between 21° and 23°C, and humidity was 65%. All experimental procedures were conducted in accordance with the principles outlined in the Care and Use of Laboratory Animals document published by the China National Institute of Health. All studies were ratified by the Institutional Animal Care and Use Committee of Harbin Medical University (IRB reference number: SYXK2022-014; 81430035).

Mixed bone marrow chimeras

B$^{Ndufs4+/-}$ and B$^{P2rx7-/-}$ mice were generated as previously described (*84*) using lethally irradiated CD45.1 C57BL/6 mice (8 weeks old) as recipients. Briefly, B$^{Ndufs4+/-}$ or B$^{P2rx7-/-}$ mice were generated by reconstituting recipient mice with a mixture of bone marrow cells from B cell–deficient IgM$^{-/-}$ IgD$^{-/-}$ mice (8×10^6 per mice, 80%, 8 weeks old) and *Ndufs4*$^{+/-}$ or *P2rx7*$^{-/-}$ mice (2×10^6 per mice, 20%, 8 weeks old). Control B^{WT} chimera were generated using a mixture of bone marrow cells from WT mice (8×10^6 per mice, 80%, 8 weeks old) and *Ndufs4*$^{+/-}$ or *P2rx7*$^{-/-}$ mice (2×10^6 per mice, 20%, 8 weeks old). Bone marrows were reconstituted over 6 to 8 weeks. Real-time PCR was used to validate expression of *Ndufs4* and *P2rx7*, with *Gapdh* used as the housekeeping gene. Briefly, immune cell subsets (B cells, T cells, and myeloid cells) were sorted (subset purities shown in fig. S24), and RNA was then extracted from each immune subset (1×10^6 cells per subset) using the RNeasy Plus Micro Kit (QIAGEN, catalog no. 74034). A high-capacity RNA-to-cDNA kit (Thermo Fisher Scientific, 4387406) was used to generate cDNA from mRNA. Real-time PCR was performed using 7500 real-time PCR system with TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, 4444557). The primers used are listed in table S4.

Experimental autoimmune encephalomyelitis

Mice were injected subcutaneously with 100 μg of recombinant human MOG (synthesized by ChinaPeptides) in incomplete Freund's adjuvant supplemented with heat-inactivated *Mycobacterium tuberculosis* (5 mg/ml; Sigma-Aldrich). Pertussis toxin (List Biological Laboratories), 300 ng per mouse, was injected intraperitoneally twice at day 1 and day 3. Mice were examined daily and scored using the following scoring system: (0) no disease, (1) floppy tail, (2) hind limb weakness, (3) full hind limb paralysis, (4) quadriplegia, and (5) death. Mice with intermediate-level presentations were scored in increments of 0.5.

Primary cell isolation and culture

Human PBMCs from patients and healthy controls were separated from antecubital venous blood by density centrifugation using Ficoll (GE health care). Human B cells and monocytes were isolated by CD19 or CD14 magnetic bead following standard manufacturers' protocols (Miltenyi Biotec, Auburn, CA). The purity of each isolated immune cell population was routinely confirmed as >98%. All cells were cultured in serum-free X-VIVO 10 Media (Lonza). In experiments studying total B cells, the B cells were plated in U-bottom 96-well plates at between 1×10^5 to 3×10^5 per well

(depending on the assay) in a total volume of 200 μl per well. Macrophage CSF (20 ng/ml; R&D Systems) was used to induce macrophage differentiation from $CD14^+$ monocytes cultured in flat-bottom 96-well plates at 1×10^5 per well in a total volume of 200 μl. Reagents that are used to stimulate/modulate immune cells are listed in table S4. For experiments where inhibitors were used, cells were pretreated with inhibitors for 30 min before stimulation. Following extensive dose titrations of reagents used to modulate metabolism (e.g., figs. S6 and S7), reagent concentrations for subsequent experiments were selected with a goal of achieving partial inhibition of OXPHOS or glycolysis. For B cell–macrophage functional assays, B cells were either left unstimulated or activated with CD40L + αBCR + IL-4, with addition of rotenone, BTKi, or vehicle for 18 hours. The B cells were then thoroughly washed and replated in fresh medium for an additional 48 hours, at which time their culture supernatants were collected and applied for 24 hours to monocyte-derived macrophage isolated from the same donors. The macrophage were then carefully washed and stimulated with LPS for 24 hours, at which time the culture supernatants were assayed for myeloid cell–secreted cytokine by enzyme-linked immunosorbent assay (ELISA), with results depicted relative to the cytokine levels expressed by LPS-stimulated myeloid cells that were exposed to unstimulated B cell supernatants.

Isolation of cytokine-secreting B cell subsets (CSA-Flow)

The cytokine secretion assay (CSA-Flow; Miltenyi biotec, Auburn, CA) was used to capture distinct cytokine-producing B cell populations as we previously described (*24*). Briefly, B cells purified as described above were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 hours. Cells were then washed once with cold X-VIVO 10 medium and labeled with capture antibodies for GM-CSF and IL-10 on ice for 10 min. Warm X-VIVO 10 was then added to the cells that were incubated at 37°C on a rotator for 1 hour. After two washes, cells were incubated with surface antibodies for CD20 and CD27 and detecting regents for GM-CSF and IL-10 on ice for 20 min. Cells were lastly washed twice before GM-CSF– or IL-10–secreting B cells were sorted by a BD FACSAria III sorter (BD Bioscience; stringently maintained by the flow cytometry core facility at the University of Pennsylvania). Purities of the sorted cytokine-defined B cell populations were confirmed by both flow cytometry and real-time PCR. Antibodies used are listed in table S4.

Surface immunophenotyping

Isolated cells were washed once with phosphate-buffered saline (PBS) before adding LIVE/DEAD Aqua staining reagent (1 μl/1 × 10^6 cells) at room temperature, following the manufacturer's protocol (Thermo Fisher Scientific) to exclude dying/dead cells. After two washes, cells were incubated with surface antibody cocktails for 20 min. Cells were then washed and fixed before being analyzed by flow cytometry (FACSFortessa, BD Bioscience; stringently maintained by the flow cytometry core facility at the University of Pennsylvania). Antibodies used for flow cytometry staining are listed in table S4.

Intracellular cytokine staining

To assess intracellular cytokine staining (ICS), PMA (20 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), and GolgiStop (Monensin, BD Bioscience) were added to cells 4 to 5 hours before staining. LIVE/DEAD reagent was used routinely, after which cell surface staining was performed, as described above. Cells were then fixed and permeabilized using fixation/permeabilization buffer (BD Bioscience). ICS antibodies were added and incubated for 30 min on ice. Samples were then washed twice and analyzed by the FACS Fortessa (BD Bioscience).

Mitochondrial staining

Cells were first stained with LIVE/DEAD reagent as described above, then resuspended in prewarmed X-VIVO 10 medium and incubated with 1 nM MitoTracker Deep Red FM dye (Thermo Fisher Scientific) at 37°C for 15 min. After two washes, cell surface staining was performed, and cells were then fixed before FACS analysis.

Cell survival assays

Annexin V and propidium iodide (PI) (BD Bioscience) were used to measure B cell survival according to the manufacturer's protocol. Briefly, cultured B cells were washed once with PBS at room temperature. Cell surface antibodies were then added to the cells and incubated on ice for 20 min and then washed once with PBS. Cells were then incubated with annexin V and PI for 15 min at room temperature before FACS analysis. All incubation steps were done in the dark, and all the procedures were performed with limited exposure of samples to light.

Enzyme-linked immunosorbent assay

Cytokine levels in culture supernatants were measured by OptEIA ELISA kit (GM-CSF, TNFα, IL-10, IL-6, and IL-12, BD Bioscience) following the manufacturers' protocols. Briefly, ELISA plates were coated with capture antibody at least 12 hours in advance. After another hour of blocking with blocking buffer (10% fetal bovine serum in PBS), samples were added to the plate and incubated for 2 hours at room temperature. Detection antibodies were then added and incubated for 1 hour at room temperature. The plates were carefully washed with ELISA washing buffer (0.05% Tween 20 and PBS) between each step. The color of each plate was developed by tetramethylbenzidine (BD Bioscience) with the reaction stopped by 0.01 N H_2SO_4, after which the plate was read by a microplate reader (Varioskan, Thermo Fisher Scientific).

MBP staining

Frozen sections with a thickness of 10 μm were prepared from the thick lumbar segment of spinal cord. FluoroMyelin Green staining was used (Thermo Fisher Scientific kit) to stain myelin following the manufacturer's protocol. Briefly, tissue sections on slides were first brought to room temperature. After rehydration with PBS (20 min) and permeabilization with Triton X-100 (20 min), the sections were stained with the kit staining solution for 20 min at room temperature. Slides were then washed three times with PBS (10 min each time) and mounted with antifade mounting medium. Confocal microscopy was used to capture the image. The regions of interest (ROIs) were selected at the same location across different spinal cord slices. Myelin staining was measured by positive pixel identification using the same threshold cutoff in ImageJ software across animals. The percentages of myelin loss were defined as the MBP-negative stained area of the total area of the ROI.

RNA sequencing

Total RNA was isolated from the differentially stimulated and treated B cells (9×10^5 per condition) using the RNeasy Plus Micro Kit

(QIAGEN) according to the manufacturer's protocol. RNA quantity and quality were assessed by TapeStation and NanoDrop. To calculate RNA integrity, samples were run on a Bioanalyzer (Agilent) with the RNA 6000 Pico Kit (Agilent). RNA-seq was performed using a NovaSeq6000 instrument (60 million reads per sample, 2 × 100 base pair; Illumina). Raw reads were aligned to reference genome hg19 using STAR (software: BasePair). Differentially expressed genes were calculated using DEseq2 (software: BasePair). PCA analysis was performed using BioVinci V1. GSEA was performed using GSEA software (a joint project by UC San Diego and Broad Institute).

Seahorse assays

B cell mitochondrial respiration was detected by XF Mitochondria stress test kit (Agilent) following the manufacturers' protocols. Briefly, B cells were cultured under various conditions (as noted in figure legends) for 36 hours. Cells were then harvested, counted, and then replated in a Cell-Tak (22.4 μg/ml; Corning)–coated XF96 microplate with the same density of live cells (4×10^5 per well) across different conditions. To enhance the attachment of cells to the plate, the plate was centrifuged at 200*g* for 1 min. The plate was then transferred to a 37°C incubator not supplemented with CO_2 for 50 to 60 min before analysis by a Seahorse XF96 analyzer. Key components for the assay included XF medium (non-buffered Dulbecco's modified Eagle's medium + 10 mM glycose, 4 mM L-glutamine, and 2 mM sodium pyruvate), 1 μM oligomycin, 1 μM trifluoromethoxy carbonylcyanide phenylhydrazone, and 1 μM rotenone/antimycin A.

Statistical analysis

All values are expressed as means ± SEM or as individual data points, and *P* values were assessed as appropriate by Student's *t* test, one-way analysis of variance (ANOVA), or two-way ANOVA, with post hoc test (adjusted for multiple comparison) using Graphpad Prism version 9. Statistical significances were as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$; individual *P* values are provided in data file S2. Flow cytometry data were analyzed by FlowJo V10. Morpheus (Broad Institute; https://software.broadinstitute.org/morpheus) was used for heatmap generation and clustering analysis.

Supplementary Materials

The PDF file includes:
Figs. S1 to S25
Tables S1 to S4

Other Supplementary Material for this manuscript includes the following:
Data files S1 and S2
MDAR Reproducibility Checklist

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