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Brief Communication

Mitochondria Activity and CXCR4 Collaboratively Promote the Differentiation of CD11c$^+$ B Cells Induced by TLR9 in Lupus

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Conflict of Interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be

ABSTRACT

Lupus is characterized by the autoantibodies against nuclear Ags, underscoring the importance of identifying the B cell subsets driving autoimmunity. Our research focused on the mitochondrial activity and CXCR4 expression in CD11c$^+$ B cells from lupus patients after *ex vivo* stimulation with a TLR9 agonist, CpG-oligodeoxyribonucleotide (ODN). We also evaluated the response of CD11c$^+$ B cells in ODN-injected mice. Post-*ex vivo* ODN stimulation, we observed an increase in the proportion of CD11c^{hi} cells, with elevated mitochondrial activity and CXCR4 expression in CD11c$^+$ B cells from lupus patients. *In vivo* experiments showed similar patterns, with TLR9 stimulation enhancing mitochondrial and CXCR4 activities in CD11c^{hi} B cells, leading to the generation of anti-dsDNA plasmablasts. The CXCR4 inhibitor AMD3100 and the mitochondrial complex I inhibitor IM156 significantly reduced the proportion of CD11c$^+$ B cells and autoreactive plasmablasts. These results underscore the pivotal roles of mitochondria and CXCR4 in the production of autoreactive plasmablasts.

Keywords: Systemic lupus erythematosus; B-Lymphocyte subsets; Mitochondria; CXCR4 receptor; TLR9 protein

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by hyperactive B cells that produce autoantibodies against nuclear Ags (1,2). CD11c$^+$ B cells, also known as age-associated B cells (ABCs), have received significant attention in the context of autoimmune diseases such as rheumatoid arthritis, Sjogren's syndrome, multiple sclerosis, and lupus (3-5). These cells are predominantly found in the spleen, with few present in lymph nodes or other lymphatic tissues. In older mice, ABCs show a hyper-metabolic state indicated by elevated oxygen consumption rates and extracellular acidification rates (6).

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construed as a potential conflict of interest. J.J.S serves on the scientific advisory board of ImmunoMet Therapeutics. J.J.S and ImmunoMet Therapeutics hold a provisional patent for the use of IM156 for the treatment of autoimmune diseases. All authors except J.J.S declare that there is no competing interest in ImmunoMet Therapeutics and a provisional patent for the use of IM156 for the treatment of autoimmune diseases.

Abbreviations

ABC, age-associated B cell; BCR, B cell receptor; FO, follicular; GOF, gain of function; MFI, mean fluorescence intensity; MTDR, mitotracker deep red; ODN, oligodeoxyribonucleotide; RPMI, Roswell Park Memorial Institute; SDF-1α, stromal cell-derived factor 1α; SLE, systemic lupus erythematosus.

Author Contributions

Conceptualization: Song JJ; Data curation: Jang SH, Kim J, Shin EG, Yoon JH; Formal analysis: Jang SH, Song JJ; Funding acquisition: Kwon HK, Song JJ; Investigation: Jang SH; Methodology: Jang SH, Shim JS; Resources: Kim J, Shin EG, Yoon JH; Software: Lee LE; Supervision: Kwon HK, Song JJ; Validation: Shim JS, Lee LE; Visualization: Jang SH; Writing - original draft: Jang SH; Writing - review & editing: Jang SH, Song JJ.

Unlike follicular (FO) B cells that divide in response to B cell receptor (BCR) cross-linking, ABCs do not respond to BCR stimuli. Instead, they show a robust proliferation response to stimuli engaging TLRs 7 and 9 (7,8). In autoimmune settings, CD11c+ B cells secrete a distinct profile of cytokines and chemokines, contributing to the typical inflammatory milieu (4,5,8). Their abilities to present Ags, secrete cytokines, and interact with T cells are hypothesized to collectively contribute to breaking self-tolerance and promoting autoimmune responses (5,9). Additionally, emerging evidence indicates that CD11c+ B cells have the potential to become plasmablasts (5,10). By transforming into plasmablasts, ABCs can contribute significantly to the body's ability to combat infections quickly, providing an immediate supply of antibodies that are crucial during the early stages of pathogen invasion (11).

In SLE, the number of CD11c+ B cells correlates with disease severity, suggesting their role in pathogenesis and potential as biomarkers for monitoring disease progression (8,12). These cells also display characteristics akin to memory B cells, showing Ag-dependent expansion and rapid differentiation into Ab-secreting cells with an associated increase in energy demands. Mitochondria are essential for ATP production, a critical energy source for the highly demanding process of Ab production by plasmablasts (13). While CXCR5 induces germinal center B cell differentiation in the FO pathway, CXCR4 induces plasmablast differentiation in the extrafollicular pathway (14,15). However, the roles of mitochondria and CXCR4 in the differentiation of CD11c+ B cells in lupus have not been extensively studied.

TLR9 is crucial for generating autoantibodies against DNA-containing Ags in lupus (16). Studies indicate that the expression of TLR9 is increased in SLE patients, particularly in B cells (17-19). However, the response of CD11c+ B cells to TLR9 stimulation in lupus patients remain insufficiently explored. We have recently reported that administering unmethylated CpG oligodeoxyribonucleotide (CpG-ODN), a TLR9 agonist, in mice results in an increase in with CD11c+ B cells along with a significant role of mitochondria (20).

In this study, we examined the proportion of CD11c+ B cells, as well as their mitochondrial activity and CXCR4 expression, in patients with lupus in response to ODN. We also evaluated mitochondrial activity and CXCR4 expression in CD11c+ B cells and plasmablasts of mice with repeated ODN injections. We demonstrated that combination therapy with IM156, a mitochondrial complex I inhibitor, and AMD3100, a CXCR4 inhibitor, additively inhibited the generation of autoreactive plasmablasts and the production of autoantibodies.

MATERIALS AND METHODS

Human blood samples from healthy donors and patients with lupus

Whole blood was collected from healthy donors and lupus patients after obtaining informed consent, with the approval of the Severance Hospital Institutional Review Board program (4-2023-0275, 4-2020-0996). Experiments using human blood were performed in accordance with the Declaration of Helsinki. All the patients fulfilled the American College of Rheumatology SLE classification criteria (21). Disease activity was assessed using the SLE Disease Activity Index (22). The demographic and clinical characteristics of the donors are presented in **Supplementary Table 1**.

Ex vivo TLR9 stimulation on human B cells

PBMCs were isolated using Ficoll-Paque (GE Healthcare, Chicago, IL, USA) and stored in a liquid nitrogen tank. PBMCs were seeded at 1×10^6 cells/well in a 12-well plate with 1 μM ODN2006 (Bioneer, Daejeon, Korea), 25 ng/ml IL-21 (PeproTech, Cranbury, NJ, USA), and 10 ng/ml B cell activating factor (AdipoGen Life Sciences, San Diego, CA, USA) for 48 h. All stimulants were diluted in Roswell Park Memorial Institute (RPMI)-1640 (HyClone, Logan, UT, USA) with 10% FBS (Cytiva, Marlborough, MA, USA) and 1% penicillin-streptomycin (HyClone). The gating strategy for human B cells is presented in **Supplementary Fig. 1**.

Animal experiments

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Yonsei University Health System and in accordance with the relevant guidelines and regulations (2021-0231). All experimental protocols are in accordance with ARRIVE guidelines. Mice were maintained under specific pathogen-free conditions at a controlled temperature (23°C±3°C) and relative humidity (40%–60%). According to American Veterinary Medical Association Guidelines for the Euthanasia of Animals (2020), Mice euthanasia performed by CO_2.

In vivo mouse CD11c+ B cell activation by ODN

An ODN-injected mouse model is described previously (23,24). Seven-week-old B6 mice (Orient Bio, Seoul, Korea) were intraperitoneally injected with 50 μg of ODN1826 (Bioneer) on days 0, 2, 4, 6, and 8. Mice in the treatment group were intraperitoneally injected with IM156 (ImmunoMet Therapeutics, Houston, TX, USA) and AMD3100 (Cayman Chemical, Ann Arbor, MI, USA) on days 1, 3, 5, 7, and 9. Spleen, blood, and bone marrow were collected for analysis on day 10. The gating strategies for mouse B cells are presented in **Supplementary Figs. 2** and **3**.

Ex vivo mouse CD11c+ B cell activation by ODN

Mouse PBMCs were stimulated with 1 μM ODN (ODN1826; Bioneer) and 25 ng/mL IL-21 for 72 h. All stimulants were diluted in RPMI-1640 (Hyclone, Logan, UT, USA) with 10% FBS (Cytiva) and 1% penicillin-streptomycin (Hyclone).

Statistical analysis

The p-values were calculated using a two-tailed Student's *t*-test with Tukey's multiple comparison test as a *post hoc* analysis (GraphPad software version 9; GraphPad Software, Boston, MA, USA).

Detailed descriptions of materials and methods are available in **Supplementary Data 1** and **Supplementary Table 2**.

RESULTS

Ex vivo ODN upregulates mitochondrial activity and CXCR4 expression in CD11c+ B cells from patients with lupus

ABCs are a subset of CD19+ B cells characterized by the expression of CD11c and CD11b (**Fig. 1A**) (25-27). Comparing PBMCs from patients with lupus and healthy donors, the proportion of CD11c+ B cells is higher in lupus (**Fig. 1B**). There was no difference in the proportion of dead cells with or without *ex vivo* ODN stimulation (**Fig. 1C**). Interestingly, ODN increased mitotracker

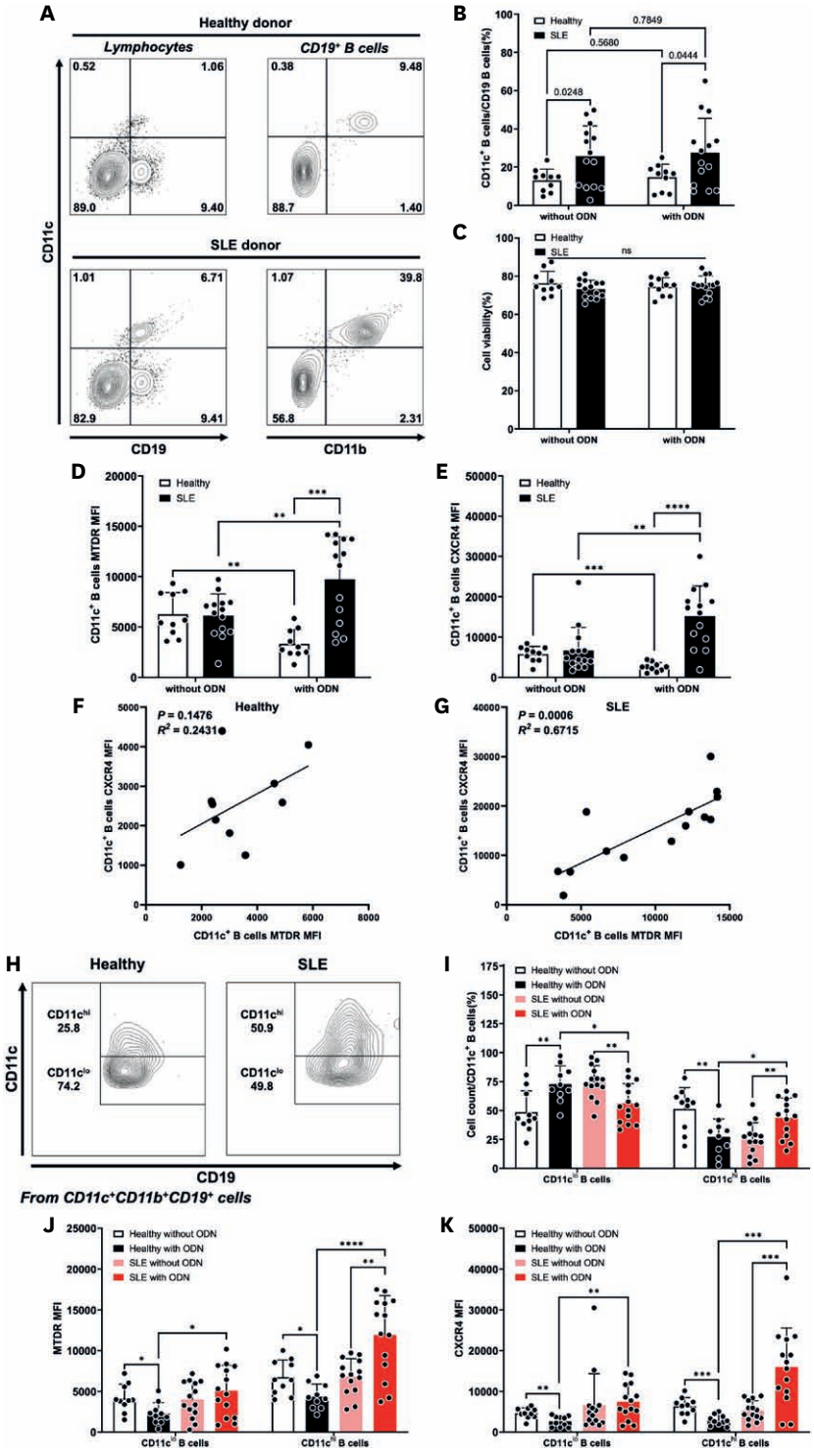

Figure 1. Mitochondrial activity and CXCR4 expression in CD11c⁺ B cells from healthy donors and patients with lupus. (A) Representative flow cytometry plots of human CD11c⁺ B cells. (B) Percentage ratio of CD11c⁺ B cells/CD19 B cells in human PBMCs. (C) Cell viability of human PBMCs measured by 7-AAD. (D) Measurement of MTDR fluorescence in CD11c⁺ B cells. (E) CXCR4 expression in CD11c⁺ B cells. (F) Correlation between MTDR and CXCR4 expression in CD11c⁺ B cells of healthy donors following *ex vivo* ODN stimulation. (G) Correlation between MTDR and CXCR4 expression in CD11c⁺ B cells of patients with lupus following *ex vivo* ODN stimulation. (H) Representative flow cytometry plots of human CD11c^{hi} B cells. (I) Percentage ratio of CD11c^{lo} and CD11c^{hi} B cells/CD11c⁺ B cells. (J) Measurement of MTDR fluorescence in human CD11c^{lo} and CD11c^{hi} B cells. (K) Measurement of CXCR4 expression in human CD11c^{lo} and CD11c^{hi} B cells. Statistical significance was determined by unpaired two-tailed Student's *t*-test and one-way ANOVA.
ns, not significant.
*p<0.05, **p<0.01, ***p<0.005, ****p<0.001.

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deep red (MTDR) fluorescence and the expression of CXCR4 in CD11c$^+$ B cells of patients with lupus but decreased them in those of healthy controls (**Fig. 1D and E**). Notably, we observed a strong positive correlation between CXCR4 expression and MTDR fluorescence in CD11c$^+$ B cells of patients with lupus, but not in controls following ODN stimulation (**Fig. 1F and G**). Our results revealed that CD11c$^+$ B cells derived from patients with lupus exhibited a hyperactive TLR9 response compared with those from healthy donors.

Given the heterogeneity within CD11c$^+$ B cell populations, we evaluated the expression levels of T-bet, CXCR4, and MTDR in CD11c^{hi} and CD11c^{lo} B cells from human PBMCs. The results showed higher expression of T-bet, CXCR4, and MTDR in CD11c^{hi} B cells compared to CD11c^{lo} B cells (**Supplementary Fig. 4**). Subsequent analysis focused on the response of these CD11c$^+$ B cell subsets to *ex vivo* ODN stimulation. In healthy donors, the proportion of CD11c^{hi} cells decreased upon ODN stimulation, whereas an increase was observed in patients with lupus (**Fig. 1H and I**). Additionally, *ex vivo* ODN stimulation resulted in a reduction of CXCR4 and MTDR levels in CD11c^{hi} cells from healthy donors, but an increase in these markers in patients with lupus (**Fig. 1J and K**).

In vivo ODN induces CD11c$^+$ B cells and autoreactive plasmablasts in mice

We evaluated the effect of *in vivo* ODN on CD11c$^+$ B cells and plasmablasts in mice. The ratio of CD11c$^+$ B cells to CD19$^+$ B cells in mouse PBMCs increased following ODN injection (**Fig. 2A and B**). Similar to CD11c$^+$ B cells of patients with lupus, we observed increased mitochondrial activity and CXCR4 expression in CD11c$^+$ B cells following ODN injection (**Fig. 2C and D**). A chemotaxis assay revealed an increased migration of splenocyte from ODN-injected mice toward stromal cell-derived factor 1α (SDF-1α) (**Fig. 2E**). Anti-dsDNA IgG levels increased in the serum of mice injected with ODN on day 10 (**Fig. 2F**). Anti-dsDNA IgG-secreting cells were detected only in the bone marrow and not in the spleen or blood and their numbers were significantly increased in mice injected with ODN (**Fig. 2G and H**). CXCR4 expression increased as CD11c$^+$ B cells migrated from the spleen to the blood and bone marrow following ODN stimulation (**Fig. 2I**). These results suggest that TLR9 induces CD11c$^+$ B cells and autoreactive plasmablasts, accompanied by increased mitochondrial activity and CXCR4 expression.

We also analyzed plasmablasts and plasma cells in ODN-injected mice. Almost all plasmablasts expressed CD11c and CD11b, but about half of plasma cells did not express CD11c and CD11b (**Fig. 2J**). The rise in CD11c levels following ODN administration was notably more pronounced in plasmablasts (**Fig. 2K**). Plasmablasts exhibited significantly elevated mitochondrial activity, and increased expression levels of CXCR4 and TLR9 compared to plasma cells (**Fig. 2L-N**).

In vivo ODN increases the number of CD11c^{hi} B cells, as well as their CXCR4 and MTDR levels in mice

Using the CD11b$^+$CD11c$^+$ B cells obtained on day 0, we classified CD11b$^+$ B cells into three groups based on CD11c mean fluorescence intensity (MFI). Cells with lower CD11c MFI levels than CD11b$^-$ B cells were defined as CD11c^{lo} B cells. Among the remaining cells except CD11c^{lo} B cells, the bottom 50% MFI were defined as CD11c^{int} B cells, and those with higher MFI levels were defined as CD11c^{hi} B cells (**Fig. 3A**). Similar to observation in human PBMC, the proportion of CD11c^{hi} B cells was elevated in mice with *in vivo* ODN injection compared to control mice (**Fig. 3B**). Additionally, levels of MTDR and CXCR4 were also elevated in CD11c^{hi} B cells following ODN injection (**Fig. 3C and D**). However, no such increase was observed

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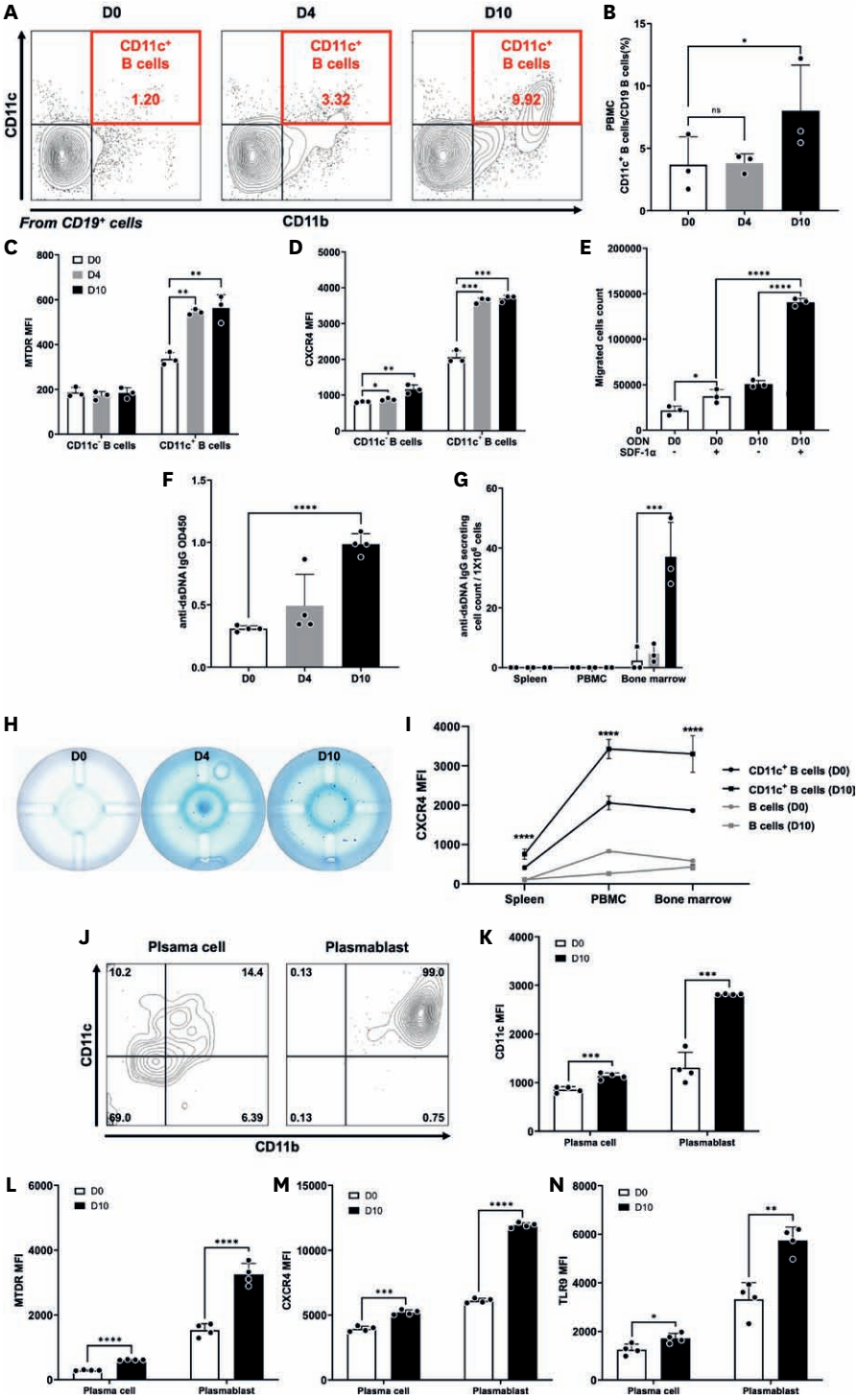

Figure 2. *In vivo* CpG-ODN induces autoimmune CD11c⁺ B cells. Mice were intraperitoneally injected with 50 μg of ODN1826 five times over 10 days. (A) Representative flow cytometry plots of mouse CD11c⁺ B cells. (B) Percentage ratio of CD11c⁺ B cells/CD19 B cells in mouse PBMCs after ODN *in vivo* stimulation. (C) Measurement of MTDR in CD11c⁻ B cells and CD11c⁺ B cells. (D) CXCR4 expression in CD11c⁻ B cells and CD11c⁺ B cells. (E) Chemotaxis assay of SDF-1α using mouse splenocytes. (F) Serum anti-dsDNA IgG levels. (G) Counts of anti-dsDNA IgG-secreting cells. (H) Representative ELISPOT figures of anti-dsDNA IgG-secreting cells in bone marrow. (I) CXCR4 expression in B cells and CD11c⁺ B cells of the spleen, PBMCs, and bone marrow. (J) Representative flow cytometry plots of plasmablasts and plasma cells. (K) Measurement of CD11c expression in plasmablasts and plasma cells. (L) Measurement of MTDR fluorescence in plasmablasts and plasma cells. (M) Measurement of CXCR4 expression in plasmablasts and plasma cells. (N) Measurement of TLR9 expression in plasmablasts and plasma cells. Statistical significance was determined by unpaired two-tailed Student's *t*-test and one-way ANOVA (n=3–4). D10 refers to the 10th day after the start of the experiment. Each experiment was repeated three times independently to ensure reproducibility.
ns, not significant.
*p<0.05, **p<0.01, ***p<0.005, ****p<0.001.

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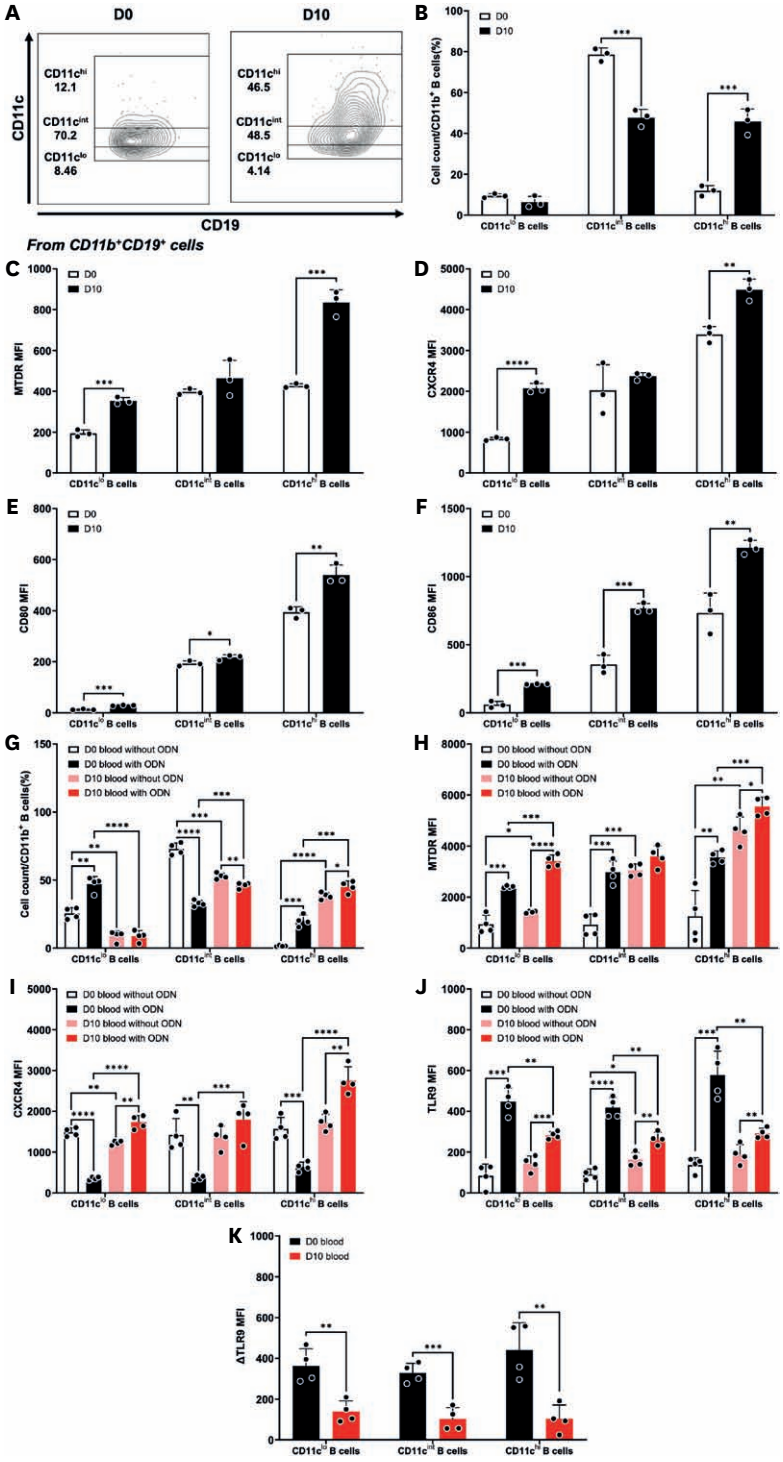

Figure 3. Comparison between CD11c^{lo}, CD11c^{int} and CD11c^{hi} B cells in mouse blood following *in vivo* ODN injection and *ex vivo* ODN treatment. (A) Representative flow cytometry plots of mouse CD11c^{lo}, CD11c^{int} and CD11c^{hi} B cells *in vivo*. (B) Percentage ratio of CD11c^{lo}, CD11c^{int} and CD11c^{hi} B cells/CD11b^{+} B cells *in vivo*. (C) Measurement of MTDR fluorescence in CD11c^{lo}, CD11c^{int} and CD11c^{hi} B cells *in vivo*. (D) Measurement of CXCR4 expression in CD11c^{lo}, CD11c^{int} and CD11c^{hi} B cells *in vivo*. (E) Measurement of CD80 expression in CD11c^{lo}, CD11c^{int} and CD11c^{hi} B cells *in vivo*. (F) Measurement of CD86 expression in CD11c^{lo}, CD11c^{int} and CD11c^{hi} B cells *in vivo*. (G) Percentage ratio of CD11c^{lo}, CD11c^{int} and CD11c^{hi} B cells/CD11b^{+} B cells in mouse PBMCs in CD11c^{lo}, CD11c^{int} and CD11c^{hi} B cells *ex vivo*. (H) Measurement of MTDR fluorescence in CD11c^{lo}, CD11c^{int} and CD11c^{hi} B cells *ex vivo*. (I) Measurement of CXCR4 expression in CD11c^{lo}, CD11c^{int} and CD11c^{hi} B cells *ex vivo*. (J) Measurement of TLR9 expression in CD11c^{lo}, CD11c^{int} and CD11c^{hi} B cells *ex vivo*. (K) ΔTLR9 expression calculated by subtracting the TLR9 MFI value without ODN *ex vivo* from the TLR9 MFI value with ODN *ex vivo*. Statistical significance was determined by unpaired two-tailed Student's *t*-test (n=3). Each experiment was repeated three times independently to ensure reproducibility.
*p<0.05, **p<0.01, ***p<0.005, ****p<0.001.

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in CD11c^{int} B cells. Conversely, surface markers of B cell activation, CD80 and CD86, were increased in both CD11c^{hi} and CD11c^{int} B cells (**Fig. 3E and F**). CD11c^{lo} B cells display different characteristics from CD11c^{int} B cells in several markers, including MTDR, CXCR4, CD80, and CD86. This demonstrates the heterogeneity of TLR9 sensitivity within CD11c^{+} B cells.

Ex vivo ODN induces activation of CXCR4 in CD11c^{+} B cells from ODN-injected mice

To investigate the opposite results of CXCR4 following *ex vivo* ODN stimulation in CD11c^{+} B cells from healthy donors and patients with lupus, we collected PBMCs at baseline (D0) and after 10 days (D10) from ODN-injected mice. In addition to *in vivo* ODN injection, *ex vivo* ODN stimulation was conducted over three days on PBMCs. This additional *ex vivo* ODN stimulation increased the proportion of CD11c^{hi} B cells in both D0 and D10 samples (**Fig. 3G**). *Ex vivo* ODN stimulation resulted in elevated MTDR levels in all CD11c^{+} B cell subsets from both D0 and D10 samples (**Fig 3H**). Remarkably, while CXCR4 expression decreased in CD11c^{+} B cells from D0 samples, it paradoxically increased in CD11c^{+} B cells from D10 samples (**Fig. 3I**), mirroring the response observed in human CD11c^{+} B cells under similar experimental conditions. This pattern highlights an inability of these cells to downregulate CXCR4 in response to additional TLR9 stimulation, indicating a complex regulatory mechanism within TLR9 signaling pathways. Additionally, *ex vivo* ODN stimulation increased TLR9 expression in CD11c^{+} cells from D0 samples; however, this elevated expression was diminished in CD11c^{+} cells from D10 samples (**Fig. 3J and K**).

IM156 and AMD3100 additively downregulate CD11c^{+} B cells and autoreactive plasmablasts

Because CD11c^{+} B cells in lupus have the distinct characteristics of high MTDR and CXCR4 expression, we investigated the capacity of combination therapy with low doses of IM156 and AMD3100 to suppress CD11c^{+} B cells in ODN-injected mice. The dose of IM156 was selected based on our previous experiments (20), while the dose of AMD3100 was determined from preliminary data (**Supplementary Fig. 5**). Additive effects were observed in reducing the ratio of CD11c^{+} B cells to CD19^{+} B cells in PBMCs (**Fig. 4A**), and the ratio of plasmablasts to CD19^{+} B cells in the bone marrow (**Fig. 4B**). Moreover, our results revealed an additive effect in reducing both CXCR4 expression (**Fig. 4C**) and MTDR levels (**Fig. 4D**) in bone marrow plasmablasts.

A chemotaxis assay demonstrated that IM156 and AMD3100 additively inhibited splenocyte migration toward SDF-1α (**Fig. 4E**). Furthermore, combination therapy with IM156 and AMD3100 reduced anti-dsDNA IgG levels and the number of anti-dsDNA IgG-secreting cells (**Fig. 4F and G**). Subset analysis on CD11c^{+} B cells demonstrated that combination therapy with low doses of IM156 and AMD3100 notably decreased the proportion of CD11c^{hi} B cells in the blood by additive inhibition of their metabolism and migration more effectively than either agent alone (**Fig. 4H**). While AMD3100 effectively reduced MTDR and CXCR4 expression in CD11c^{hi} B cells (**Fig. 4I**), IM156 mainly decreased MTDR levels in CD11c^{+} B cells without significantly impacting CXCR4 expression (**Fig. 4J**). Overall, these findings suggest that combination therapy with low doses of IM156 and AMD3100 significantly downregulated CD11c^{+} B cells and autoreactive plasmablasts.

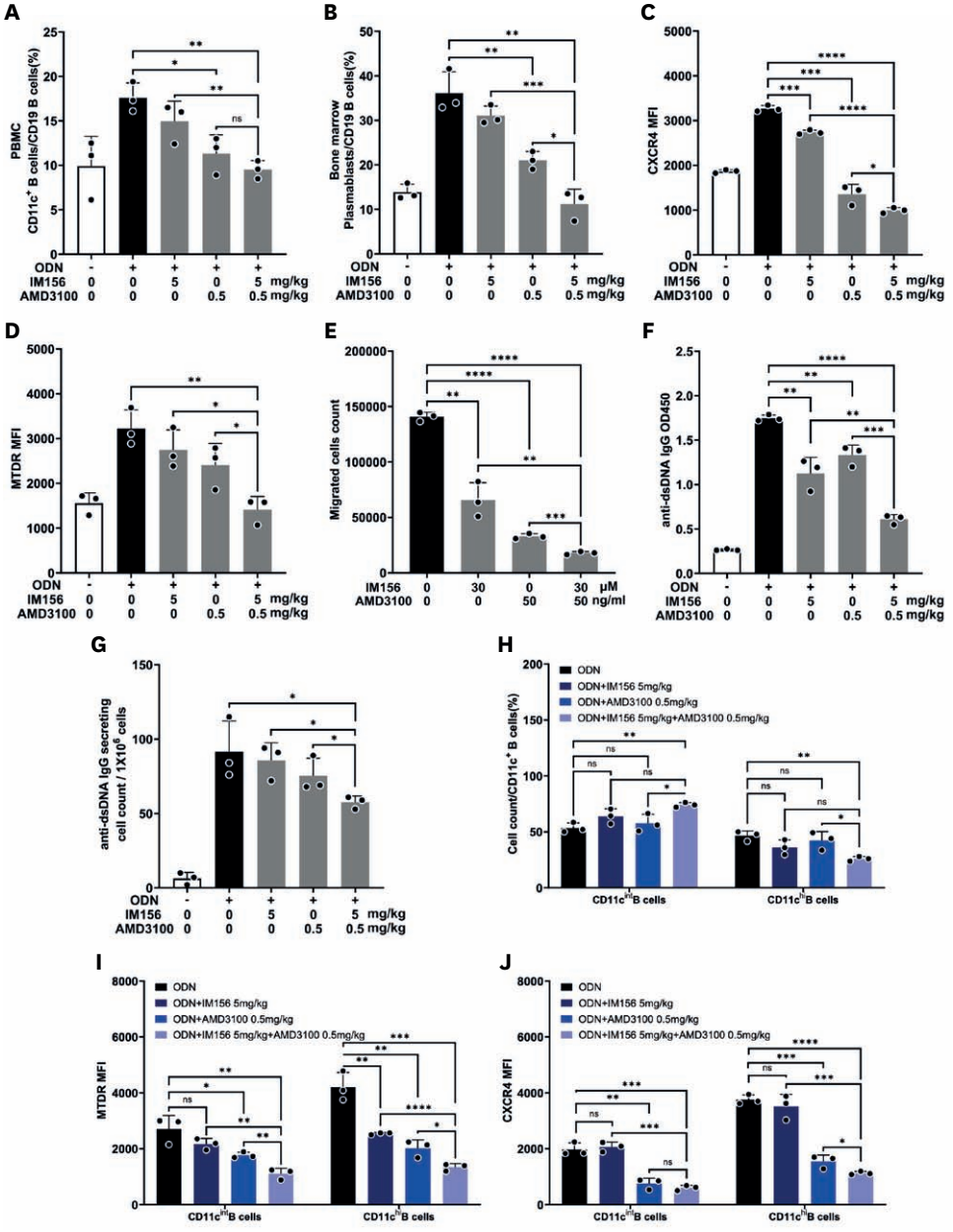

Figure 4. Combination therapy with IM156 and AMD3100 downregulates autoreactive CD11c⁺ B cells and plasmablasts in ODN-injected mice. Mice were intraperitoneally injected with 50 μg of ODN1826 five times over 10 days with AMD3100 and/or IM156. (A) Percentage ratio of CD11c⁺ B cells/CD19 B cells in mouse PBMCs treated with IM156 and AMD3100. (B) Percentage ratio of plasmablasts in the bone marrow treated with IM156 and AMD3100. (C) CXCR4 expression in bone marrow plasmablasts treated with IM156 and AMD3100. (D) Measurement of MTDR in bone marrow plasmablasts treated with IM156 and AMD3100. (E) Chemotaxis assay of SDF-1α using mouse splenocytes treated with IM156 and AMD3100. (F) Serum anti-dsDNA IgG levels treated with IM156 and AMD3100. (G) Counts of anti-dsDNA IgG-secreting cells in the bone marrow treated with IM156 and AMD3100. (H) Percentage ratio of $CD11c^{int}$ and $CD11c^{hi}$ B cells/$CD11c^{+}$ B cells with IM156 and AMD3100. (I) Measurement of MTDR fluorescence in $CD11c^{int}$ and $CD11c^{hi}$ B cells with IM156 and AMD3100. (J) Measurement of CXCR4 expression in $CD11c^{int}$ and $CD11c^{hi}$ B cells with IM156 and AMD3100. Statistical significance was determined by unpaired two-tailed Student's *t*-test (n=3). Each experiment was repeated three times independently to ensure reproducibility.
ns, not significant.
*p<0.05, **p<0.01, ***p<0.005, ****p<0.001.

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DISCUSSION

Lupus is a relapsing autoimmune disease in which B cells play a central role in pathogenesis through autoantibody production, cytokine secretion, and Ag presentation (28). In our study, we focused on CD11c$^+$ B cells in lupus and investigated their mitochondrial activity and CXCR4 expression following *ex vivo* TLR9 stimulation with ODN. Indeed, CD11c$^+$ B cells were increased in patients with lupus, which is consistent with previous reports (4,10). Furthermore, we observed that CD11c$^+$ B cells from patients with lupus exhibited enhanced mitochondrial activity and CXCR4 expression compared with those from healthy controls after *ex vivo* ODN stimulation. This upregulation was critical for plasmablast differentiation in our mouse model, in which ODN administration led to an increase in the number of CD11c$^+$ B cells with increased mitochondrial activity and CXCR4 expression. This was accompanied by an increase in serum anti-dsDNA Abs and autoreactive plasmablasts in the bone marrow.

The identification of ABCs has deepened our understanding of SLE (8,29). Although heterogeneity exists within the ABC population (29), it is largely an Ag-experienced pool that arises under specific signaling circumstances (8). ABCs, which serve as precursors to plasmablasts, are intricately associated with SLE pathogenesis, especially during disease flares (30,31).

In SLE, TLR7 is recognized for its pathogenic influence, whereas the role of TLR9 remains a subject of debate. TLR9 plays a key role in recognizing DNA-containing Ags, which are central to SLE pathology. Despite its involvement in generating autoantibodies against DNA, TLR9 generally exerts a protective impact on the disease. Research indicates that mice lacking TLR9 display heightened autoimmune responses and more severe symptoms, suggesting TLR9's crucial regulatory function in mitigating SLE (32). This protective effect of TLR9 is at least partially mediated by its ability to limit the stimulatory activity of TLR7 (33).

We observed that CD11c^{hi} B cells from patients with lupus increase CXCR4 expression following TLR9 stimulation, contrasting with CD11c^{hi} B cells from healthy donors where CXCR4 is downregulated. For the proper regulation of plasmablast trafficking, it is important that CXCR4 becomes temporarily unresponsive or less sensitive to its ligand SDF-1α (34). Therefore, this aberrant response might contribute to the pathogenesis of lupus, as seen in CXCR4 gain of function (GOF) mice, where improper CXCR4 regulation leads to exacerbated B cell responses and increased IgM levels (35). These CXCR4 GOF mice also demonstrate significant increases in bone marrow plasmablasts. Furthermore, this hyperactive CXCR4 expression in lupus B cells aligns with enhanced mitochondrial activity. Following ODN injections, similar increases in CXCR4 and mitochondrial activity were noted in mice, suggesting complex TLR9-CXCR4 interactions potentially influenced by mitochondrial dynamics.

TLR9 stimulation, specifically through CpG treatment, differentially affects B cell subsets, particularly in relation to mitochondrial activity. Our *in vivo* data highlight that CD11c$^+$ B cells, which typically upregulate CD11c in response to TLR9 stimulation, exhibit increased mitochondrial activity, whereas CD11c$^-$ B cells maintain unchanged mitochondrial activity levels.

Short-lived plasmablasts, which function as transient effector cells during the initial Ab response, often escape immune tolerance mechanisms in autoimmune diseases such as lupus (36-38). Furthermore, we and others reported that the upregulation of mitochondrial oxidative phosphorylation genes is contingent on B cells functioning as plasmablasts to

fulfill high energy demands (13,20). Our study highlights the pivotal role of mitochondrial metabolism in the development of CD11c+ B cells and autoreactive plasmablasts. This enhanced mitochondrial respiration opens new therapeutic avenues for the metabolic regulation of CD11c+ B cells in lupus. IM156, a novel biguanide derivative, has demonstrated 60-fold higher potency than metformin *in vivo*, effectively modulating oxidative phosphorylation by complex I inhibition (39-41).

However, IM156 could not fully suppress abnormal CXCR4 expression, leading us to explore a combination strategy employing IM156 and AMD3100 for more effective inhibition of autoimmune plasmablasts. CXCR4 reportedly facilitates the migration of plasma cells to the bone marrow (42,43). Our study extends this understanding by demonstrating that autoreactive plasmablasts derived from CD11c+ B cells migrate to the bone marrow via CXCR4. Treatment with IM156 reduces mitochondrial activity and inhibits the differentiation of CD11c+ B cells into plasmablasts. Concurrently, AMD3100 suppresses the migration of CD11c+ B cells to the bone marrow, thereby reducing autoantibody production. Combination therapy using low doses of IM156 and AMD3100 has demonstrated additive effects in suppressing autoreactive plasmablasts and reducing anti-dsDNA Ab levels.

Our study had limitations. First, the gender ratios between groups of SLE patients and healthy donors are significantly different. Regarding our study's primary variable—the proportions of CD11c+ B cells—results from several studies on human subjects and animal models indicate no significant gender-based differences in these proportions (5,44). Additionally, the small sample sizes of our study groups may limit the generalizability of our results. An increased sample size in future studies would not only enhance the statistical power but also enable a more detailed analysis of the correlations between CD11c+ B cells and clinical manifestations.

Various B cell-targeted therapies are being developed, but there are still no treatments for lupus that directly target mitochondria activity (45,46). The critical roles of mitochondrial activity and CXCR4 expression in generating anti-DNA Abs by these cells underscore the key involvement of CD11c^{hi} B cells in lupus pathogenesis and warrant further investigation into the autoimmune mechanisms.

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SUPPLEMENTARY MATERIALS

Supplementary Data 1

Supplementary materials and methods

Supplementary Table 1

Demographic and clinical characteristics of healthy donors and patients with lupus

Supplementary Table 2
List of Abs and chemicals used for flow cytometry

Supplementary Figure 1
Gating strategy of human CD11c+ B cells using peripheral blood mononuclear cells.

Supplementary Figure 2
Gating strategy of mouse CD11c+ B cells using peripheral blood mononuclear cells.

Supplementary Figure 3
Gating strategy of mouse plasmablasts using bone marrow cells.

Supplementary Figure 4
Measurement of T-bet, CXCR4 expression and MTDR fluorescence in human CD11c^{lo} B cells and CD11c^{hi} B cells. (A) Representative flow cytometry plots of T-bet expression in human CD11c^{lo} and CD11c^{hi} B cells. (B) Representative flow cytometry plots of CXCR4 expression and MTDR fluorescence in human CD11c^{lo} and CD11c^{hi} B cells.

Supplementary Figure 5
AMD3100 downregulates CXCR4 expression. Mice were intraperitoneally injected with 50 μg of ODN1826 five times over 10 days with or without AMD3100. (A) Percentage ratio of CD11c+ B cells/CD19 B cells in mouse PBMCs treated with AMD3100. (B) Percentage ratio of plasmablasts in the bone marrow treated with AMD3100. (C) CXCR4 expression in bone marrow plasmablasts treated with AMD3100. (D) Measurement of MTDR in bone marrow plasmablasts treated with AMD3100. (E) Serum anti-dsDNA IgG levels after treatment with AMD3100. (F) Counts of anti-dsDNA IgG-secreting cells in the bone marrow treated with AMD3100. Statistical significance was determined by unpaired two-tailed Student's *t*-test (n=3). Each experiment was repeated three times independently to ensure reproducibility.

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