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Modulating IL-21-driven B cell responses in idiopathic inflammatory myopathies via inhibition of the JAK/STAT pathway

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Abstract

Background Idiopathic inflammatory myopathies (IIM) are autoimmune disorders characterized by muscle inflammation and autoreactive B cell responses. The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway is essential for B cell functions, making it a promising therapeutic target. This study explores the potential of tofacitinib, a JAK1/JAK3 inhibitor, to modulate B cell activity in IIM.

Methods Peripheral B cell populations from dermatomyositis (DM), anti-synthetase syndrome (ASyS) and overlap myositis (OM) patients were analyzed by flow cytometry. Peripheral blood mononuclear cells (PBMC) or sorted memory B cells were cultured with tofacitinib and stimulated with combinations of CD40, IL-21, IL-2, BAFF and CpG. B cell proliferation, differentiation and (auto)antibody, cytokine/chemokine production were assessed by flow cytometry, Luminex, and ELISA/ELiA assays.

Results The IIM peripheral B cell compartment had elevated transitional and naive B cells, with reduced Bmem frequencies compared to healthy donors. Tofacitinib significantly inhibited CD40/IL-21-induced B cell proliferation, plasmablast formation and function in PBMC and B cell-only cultures across all IIM subgroups, predominantly affecting the IL-21-induced differentiation and antibody production. Remarkably, tofacitinib reduced the levels of anti-Jo1 autoantibodies, as well as of CXCL10 and CXCL13 in ASyS memory B cell cultures.

Conclusions These findings highlight the B cell involvement in IIM, evidenced by altered peripheral B cell composition in active disease and the effective inhibition of essential B cell responses, including proliferation, differentiation, and (auto)antibody production, by tofacitinib in vitro. This positions the JAK/STAT pathway as a promising new therapeutic target to modulate B cell activity in IIM.

Keywords B cell, Myositis, JAK, Plasma cell, Tofacitinib

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Background

Idiopathic inflammatory myopathies (IIM), or myositis, are a group of rare systemic autoimmune disorders that encompasses several subtypes, such as dermatomyositis (DM), anti-synthetase syndrome (ASyS) and overlap myositis (OM). The heterogeneous clinical manifestations mainly affect the skeletal muscles, with frequent extramuscular organ involvement, including interstitial lung disease (ILD) [1, 2]. When not treated adequately, chronic inflammation can lead to severe and irreversible tissue damage, including muscle and lung fibrosis, ultimately increasing morbidity and mortality of patients [3].

B and plasma cells (PC) are implicated in the pathogenesis of IIM, as illustrated in active disease by increased numbers of activated peripheral B cells, elevated serum levels of B cell activating factor (BAFF), and the presence of autoantibody-producing B and plasma cells in inflammatory lesions [4–7]. Approximately 70% of IIM patients have myositis-specific autoantibodies (MSAs), which are considered potentially pathogenic and are associated with disease activity, as demonstrated by the accumulation of anti-Jo1 and anti-Mi2, specific for ASyS and DM, respectively, within muscle fibers, possibly contributing to localized muscle damage [1, 4, 8–11]. The effectiveness of B cell-depleting therapies, such as the anti-CD20 monoclonal antibody rituximab, and recent findings with CD19-targeting CAR-T cells in case series of ASyS and DM, further support the pathogenic role of B cells in IIM [12–15]. However, CAR-T cell therapy is costly, burdensome for patients and not widely available, while rituximab does not deplete resident (autoreactive) PC [12], rendering an urgent need to identify additional B cell targets.

One potential target is the Janus kinase (JAK)/signal transduction and activator of transcription (STAT) pathway, an essential intracellular driver for various B lineage cell functions, mainly their differentiation, survival and antibody production [16]. Type I interferons (IFN) play a crucial role in IIM pathogenesis and may contribute to muscle damage [17, 18], and the upregulated IFN signature in DM and ASyS may be downstream of JAK/STAT signaling. This pathway is activated by other relevant cytokines in B cell biology and IIM pathogenesis, particularly IL-6 and IL-21, two potent inducers of antibody production and PC maturation/survival [19, 20]. IL-6-induced activation of JAK/STAT signaling in B cells may promote the activation and phosphorylation of STAT3, an essential phosphoprotein for long-term humoral responses [21] that is elevated in DM muscle biopsies [22], suggesting IL-6-mediated JAK/STAT activation in DM. Unique cytokine-dependent combinations of JAK-STAT proteins convey their signals to the nucleus, triggering specific responses [16]. This specificity may enable more precise modulation of particular B cell responses using JAK inhibitors (JAKi) directed to selected JAK proteins. Tofacitinib, a small-molecule JAKi primarily targeting JAK1/JAK3, is used to treat rheumatoid arthritis and other chronic inflammatory diseases [23]. Moreover, data from small case series and open label studies involving (juvenile) DM [24–26], as well as a retrospective study on refractory ASyS, consistently demonstrated beneficial effects of JAKis [27]. However, the underlying mechanism of action of JAKis accounting for their therapeutic benefits in IIM, and whether this is mediated through effects on B lineage cells, remains unclear.

In this study we aimed to (i) characterize the peripheral B cell populations from patients with DM, ASyS and OM, (ii) to explore the potential modulation of B cell responses, including proliferation, differentiation and antibody production, in a 6-day PBMC culture using tofacitinib in vitro, (iii) to investigate the potential effects of tofacitinib on ASyS B cell responses, including anti-Jo1 autoantibody/cytokine/chemokine production, in a 12-day PBMC culture, and (iv) to confirm the B cell-specific effects of tofacitinib in a 8-day memory B (Bmem) cell culture in ASyS.

Methods

Subjects

Peripheral blood of treatment-naive patients with IIM (i.e. not using any immunosuppressive treatment such as steroids, csDMARDs or biological DMARDs) was collected at Amsterdam UMC (Amsterdam, the Netherlands). Patients were classified as DM, ASyS and OM ($n = 4$–6/subgroup) according to current guidelines [28–30]. Patients were selected from our in/outpatient clinics based on active disease with muscle involvement (including edema and muscle biopsy with signs of inflammatory myopathy) and positive myositis autoantibody profile detected using a line blot immunoassay (Supplementary Table 1). ASyS patients were selected also based on presence of ILD (i.e. compatible clinical signs and symptoms, confirmed radiologically by CT thorax and/or pulmonary function test).

All subjects provided written informed consent in accordance with the Declaration of Helsinki, and the sample collection for our study was approved by the medical ethics committee of Amsterdam UMC. Buffy coats from healthy donors (HD) ($n = 6$) were obtained from Sanquin (Amsterdam, the Netherlands).

Isolation of peripheral blood mononuclear cells (PBMC) and processing for flow cytometry

The isolation, cryopreservation and thawing of PBMC from HD-buffy coats and peripheral blood from patients have been previously described [31, 32]. For direct flow cytometry analysis of cryopreserved PBMC, washing

steps were performed using PBA buffer (PBS [Fresenius Kabi, Bad Homburg, Germany] supplemented with 2 mM EDTA [MilliporeSigma, Burlington, MA, USA], 0.5% BSA [Roche Holding AG, Basel, Switzerland] and 0.02% NaN_3). We stained 2.0×10^6 PBMC for 30 min on ice with an antibody cocktail to identify extracellular B cell/PC markers: anti-human CD20 (L27), CD19 (HIB19), IgD (IA6-2), CD38 (HB7), CD27 (L128) (BD Biosciences, Franklin Lakes, NJ, USA); CD3 (UCHT1), CD138 (MI15), IgM (MHM-88), CD24 (ML5) (Biolegend, San Diego, CA, USA); CD20 (2H7) and Fixable Viability Dye eFluor™ 780 (L/D marker) (eBiosciences|Thermo Fisher Scientific, Waltham, MA, USA). Single-stained beads (OneComp eBeads™, Invitrogen|Thermo Fisher Scientific) and unstained cells were used for controls/compensation. Cells were washed and acquired using either a FACSCanto II or FACSymphony A1 device (BD Biosciences), performing the analysis using FlowJo software v10.10.0 (BD, Ashland, OR, USA).

Functional PBMC assay: 6- and 12-day in vitro proliferation and differentiation

PBMC were resuspended in PBS (6-day culture only) or IMDM (Gibco|Thermo Fisher Scientific) supplemented with 10% FCS (Capricorn Scientific, Ebsdorfergrund, Germany), 1:200 gentamicin (Gibco|Thermo Fisher Scientific), 0.00036 (v/v)% 2-Mercaptoehanol (Merck, Burlington, MA, USA) (hereafter named culture medium). Proliferation was assessed staining 5-10.0×10^6 cells/mL with 0.5 µM CFSE (carboxyfluorescein succinimidyl ester, Molecular Probes|Thermo Fisher Scientific) as described in [33]. Subsequently, 2.0×10^4 culture medium-resuspended B cells/well were seeded in a 96-well plate. Tofacitinib citrate was reconstituted in DMSO (Sigma|Saint Louis, MO, USA), and serial dilutions (1.0, 2.5 and 5.0 µM) were prepared. Cells were treated with tofacitinib or DMSO, and after 2 h at 37ºC, they were stimulated for 6 days with 2.5 µg/mL anti-CD40 (G28.5) and 20 ng/mL IL-21 (R&D systems, Minneapolis, MN, USA), or 1.0 µg/mL CpG-ODN (InvivoGen, San Diego, CA, US) and 100 U IL-2 (R&D systems), to mimic T cell-dependent or independent activation, respectively. To ensure PC formation, 1.0×10^6 PBMC/well were stimulated with 100 ng/mL BAFF (PeproTech|Thermo Fisher Scientific), 3.2 µg/mL CpG and 100 ng/mL IL-21 in 24-well plates. At days 6 and 12, supernatants were stored for (auto)antibody/cytokine/chemokine detection, and the cells were stained (CD19, CD20, IgD, CD27, CD38, CD138, L/D marker) for flow cytometric analysis as described above.

Memory B cell expansion: sorting and 8-day expansion

Cryopreserved ASyS-PBMC ($n = 4$–5) were resuspended in sterile PBA and stained for CD20, CD19 (HIB19; Biolegend), CD27, CD38, IgD, CD3, L/D marker. 250 live $CD3^-CD20^+CD19^+CD27^+$ Bmem cells per condition were sorted in culture medium using a FACSAria II (BD Biosciences) (sorting purity of 97%), and seeded with 1.0×10^4 CD40L-expressing irradiated L-cells/well (96-well plate). We added tofacitinib 1.0 or 2.5 µM, or DMSO, at days 0, 2 and 4 after stimulation with 10 ng/mL BAFF, 10 ng/mL IL-21 and 50 ng/mL IL-2. At days 4 and 6, half of the medium was refreshed. After 8 days, the supernatants were stored for downstream analysis, and the cells were washed and stained with CD19, CD20, IgD, CD27, CD38, CD138, L/D marker to assess expansion/differentiation.

(Auto)antibody and cytokine/chemokine detection by Enzyme-linked immunoassay (ELISA), ELiA and Luminex

Culture supernatants were tested for total IgG, IgM and IgA levels using ELISA as described previously [32], using polyclonal rabbit anti-human IgG, IgM and IgA, and a serum protein calibrator (Dako, Heverlee, Belgium) [33]. HRP-binding 1-Step Ultra TMB-ELISA substrate was used (ThermoScientific) and Stop Solution (14.9% H_2SO_4 in dH_2O) was prepared in-house. Plates were analyzed at 450 nm using an ELISA plate-reader (VersaMax microplate reader, Molecular Devices, CA, USA). Anti-Jo1 titers in the 12-day supernatants were assessed with ELiA (Phadia 250 machine, Thermo Fisher Scientific), and cytokine/chemokine profiles were measured by Luminex according to the manufacturer instructions (R&D; Cat. LXSAHM-12) and using Luminex®200™ (R&D) [34].

Statistical analysis

The significance when $n > 6$ was determined applying a Wilcoxon signed-rank test, assuming non-normal distribution and paired data, using GraphPad Prism (v10; GraphPad Software Inc.). In Fig. 1, one-way ANOVA test with multiple comparisons was performed. For the 12 and 8-day cultures, a t-test was performed after testing for normality with the Shapiro-Wilk test. p values < 0.05 were considered significant, assigned as follows: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Results

Altered proportions of peripheral B cell populations in the IIM subgroups DM, ASyS and OM

We first studied potential differences in peripheral B cell populations among three of the most prevalent IIM subgroups: DM, ASyS and OM. The $CD19^+CD20^+$ B cell populations in PBMC from treatment-naive patients with active disease contained $CD27^+IgD^-$ switched memory (SWM), $CD27^+IgD^+$ non-switched memory (NSM), $CD27^-IgD^-$ double negative (DN) and $CD27^-IgD^+$ naive B cells (Fig. 1A). The proportion of these populations was similar between the three subgroups, and we also compared them with those of HD-PBMC (Supplementary

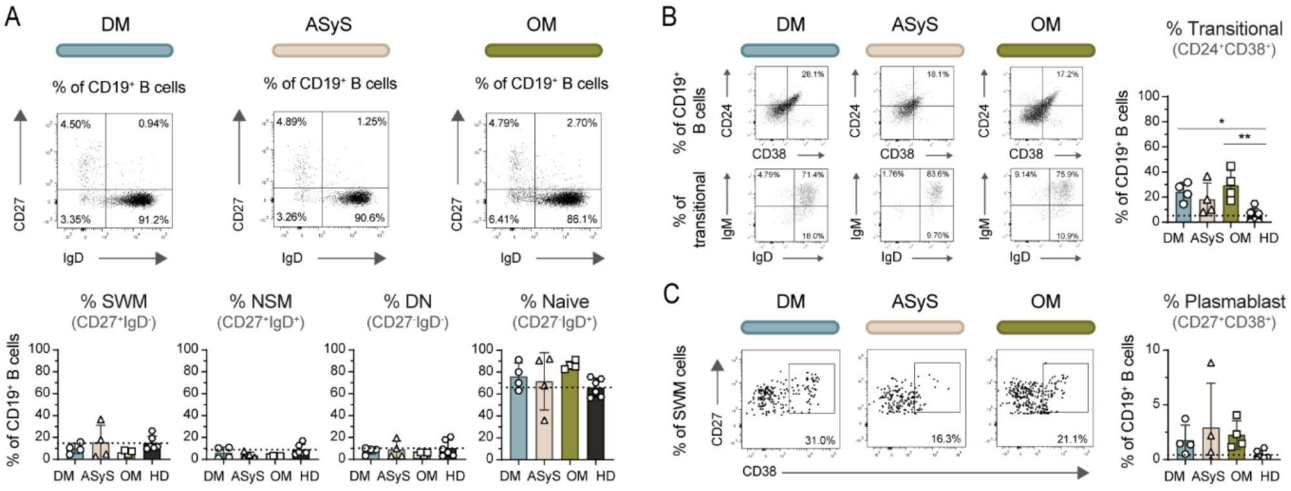

Fig. 1 Peripheral B cell composition of IIM patients with subgroups DM, ASyS and OM. (**A**) Dot plots showing the gating strategy used to identify peripheral B cell populations in one representative patient per phenotype, including $CD27^+IgD^-$ switched memory cells (SWM), $CD27^+IgD^+$ non-switched memory (NSM) cells, $CD27^-IgD^-$ double negative (DN) cells and $CD27^-IgD^+$ naive B cells. (**B**) $CD24^+CD38^+$ transitional B cells were gated within total $CD19^+$ B cells, with subtypes 1 (IgM^+IgD^-), 2 (IgM^+IgD^+) and 3 (IgM^-IgD^+) identified as shown. The percentages of the identified subtypes from the dot plots are included. (**C**) Within the SWM cells, $CD27^+CD38^+$ plasmablasts were identified as shown in the dot plots, and graph shows the percentage within total B cells. Dotted line in A-C represents HD reference values. DM: dermatomyositis, ASyS: anti-synthetase syndrome, OM: overlap myositis, HD: healthy donor. Graphs display the mean, SD and individual values (*$p < 0.05$, **$p < 0.01$, $n = 4$/IIM subgroup; $n = 6$ HD)

Fig. 1) as reference (dotted line). A higher proportion of naive B cells in 11 out of 12 patients was observed compared to HD, while the proportions of NSM, and particularly SWM, were smaller. Notably, the NSM population when pooling the IIM samples was significant compared to the HD (data not shown). The increased naive population raised our interest for the $CD24^+CD38^+$ transitional B cells, a subset enriched in IMIDs [35, 36]. Although no significant differences were observed between subgroups, the transitional B cell fraction was increased in all patients compared to HD, being significant in DM and OM (and overall in the pooled IIM samples compared to the HD; data not shown) (Fig. 1B). Among the transitional B cell subtypes particularly the T2 (IgM^+IgD^+) subtype, rather than the T1 (IgM^+IgD^-) and T3 (IgM^-IgD^+) subtypes, was enriched. Furthermore, the proportion of $CD27^+CD38^+$ plasmablasts was increased in all patients compared to HD despite their typically low frequency in peripheral blood (Fig. 1C). These data indicate an enrichment of naive and transitional B cells, along with plasmablasts, in the peripheral blood of DM, ASyS and OM, accompanied by a notable reduction in Bmem cells.

JAK inhibition by tofacitinib dampens B cell responses in a dose-dependent manner

Supported by the clinical benefits of JAKi treatment in IIM [24, 26, 27], the central role of B cells in IIM, the observed skewed myositis peripheral B cell compartment, and the effects of tofacitinib on B cell responses in vitro [37], we investigated the effects of tofacitinib on myositis B cells. Given the limited availability of patient samples, we initially optimized a 6-day functional assay to evaluate the dose-response of tofacitinib application (ranging from 1.0 to 5.0 μM) using HD-PBMC. CFSE stainings showed a significant, dose-dependent reduction in B cell proliferation in tofacitinib-treated cells, particularly under anti-CD40/IL-21 stimulation (Fig. 2A). Tofacitinib also significantly inhibited the B cell differentiation into $CD27^+CD38^+$ plasmablasts (Fig. 2B), which despite occurring under both stimulatory conditions, was more pronounced upon anti-CD40/IL-21 activation. Consistent with the impaired plasmablast formation, we found significantly reduced IgG titers upon treatment under both stimulatory conditions (Fig. 2C). However, IgM and IgA levels were significantly reduced only following anti-CD40/IL-21 stimulation. Taken together, this 6-day in vitro HD-PBMC assay demonstrates that JAK1/3 inhibition reduces B cell proliferation, differentiation and antibody production, most significantly after anti-CD40/IL-21 stimulation.

Tofacitinib primarily affects IL-21-induced B cell differentiation and antibody production

Tofacitinib most prominently affected anti-CD40/IL-21-induced B cell responses in the 6-day HD-PBMC culture. Thus, we further explored the primary target of tofacitinib responsible for proliferation, differentiation and antibody production in this condition. Based on the previous results, we used tofacitinib at concentrations 2.5 and 5.0 μM prior to stimulation by either IL-21 or anti-CD40. Tofacitinib significantly reduced the B cell proliferation after IL-21 stimulation, which was not observed

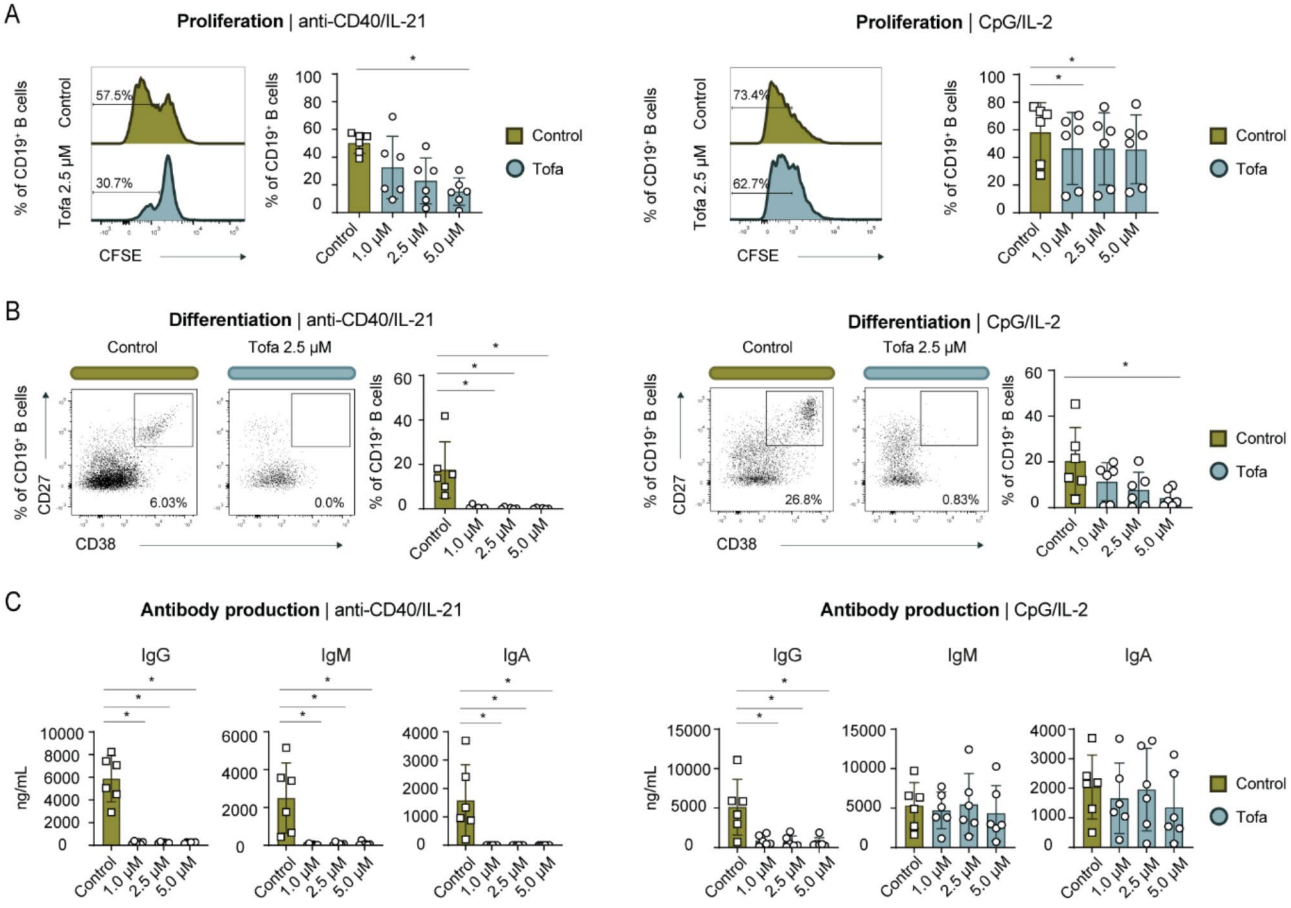

Fig. 2 Dose-dependent effects of tofacitinib on B cell responses in a 6-day HD-PBMC assay. (**A**) Representative histograms showing CFSE staining of PBMC to assess proliferation of $CD19^+CD20^+$ cells for conditions with tofacitinib (Tofa) or control under anti-CD40/IL-21 (left) and CpG/IL-2 stimulation (right). Gates were set using unstimulated cells as control, and percentages of proliferated $CD19^+CD20^+$ cells are shown. (**B**) Representative dot plots showing the percentage of gated plasmablasts ($CD27^+CD38^+$) within $CD19^+CD20^+$ B cells. Quantification of plasmablast proportions is shown for anti-CD40/IL-21 (left) and CpG/IL-2 stimulation (right). (**C**) Concentrations of IgG, IgM and IgA in the culture supernatants collected under anti-CD40/IL-21 (left) and CpG/IL-2 (right) stimulation. Graphs display the mean, SD and individual values (*$p < 0.05$, $n = 6$ HD)

after anti-CD40 activation (Fig. 3A). Additionally, JAK1/3 inhibition significantly affected the IL-21-induced $CD27^+CD38^+$ plasmablast formation, which was a more effective differentiation-inducer than anti-CD40. Nevertheless, while IL-21-induced differentiation was blocked by tofacitinib at 2.5 µM, tofacitinib at 5.0 µM also significantly impaired this process downstream of anti-CD40 (Fig. 3B). IL-21 stimulation triggered IgG, IgM and IgA production, which was abrogated by tofacitinib (Fig. 3C), whereas the lower titers of these immunoglobulins induced by anti-CD40 showed no significant reduction. These results from the HD-PBMC assay demonstrate the preferential effects of tofacitinib on IL-21 B cell-mediated responses, rather than CD40-induced signaling events.

Tofacitinib preferentially inhibits the differentiation and antibody production of IIM peripheral B cells

After optimization, we explored the potential modulation of anti-CD40/IL-21-induced B cell responses from IIM subgroups DM, ASyS and OM using tofacitinib. JAK1/3 inhibition resulted in a significant reduction in proliferated cells, regardless of the IIM subgroup (Fig. 4A). Additionally, the $CD27^+CD38^+$ plasmablast formation was significantly decreased, accompanied by reduced proportions of $CD138^+$ PC (Fig. 4B, Supplementary Fig. 2). Furthermore, we observed an increase in the percentage of naive cells and a simultaneous reduction of the SWM population in the presence of tofacitinib (Fig. 4C). Consistent with the reduced plasmablast and PC proportions, the levels of IgG, IgM and IgA were significantly lower after treatment (Fig. 4D). Altogether, these results demonstrate that tofacitinib predominantly and significantly abrogates the plasmablast/PC formation, as well as their antibody production, irrespective of the IIM subgroup.

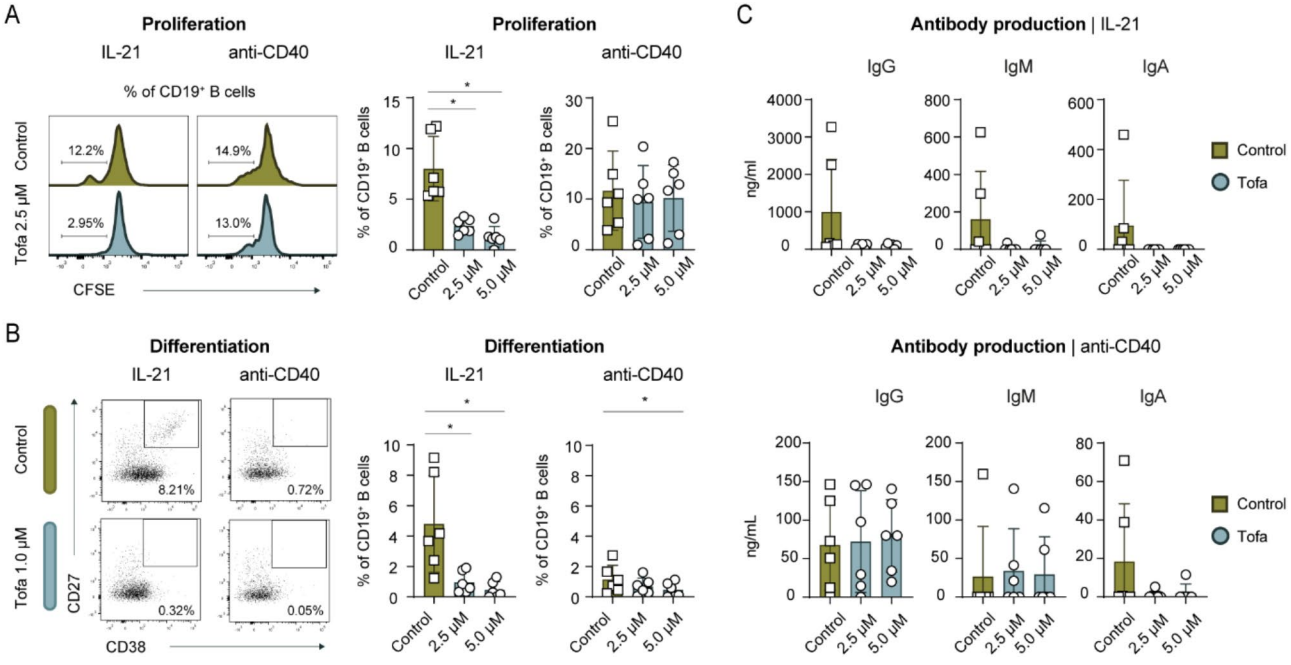

Fig. 3 Tofacitinib dampens IL-21-induced B cell responses in a 6-day HD-PBMC culture. (**A**) Representative histograms (left) showing the effect of tofacitinib (Tofa) or control on the percentage of proliferating B cells, as measured by CFSE staining, under IL-21 or anti-CD40 stimulation. Gates were set using unstimulated cells as control. Quantification (right) of the percentage of proliferated B cells. (**B**) Representative dot plots (left) showing the gating of $CD27^+CD38^+$ plasmablasts within $CD19^+CD20^+$ B cells. Quantification (right) of the plasmablast proportions. (**C**) Concentration of IgG, IgM and IgA in culture supernatants from IL-21 (top) or anti-CD40 (bottom)-stimulated cells. Graphs show the mean, SD and individual values (*$p < 0.05$, $n = 6$ HD)

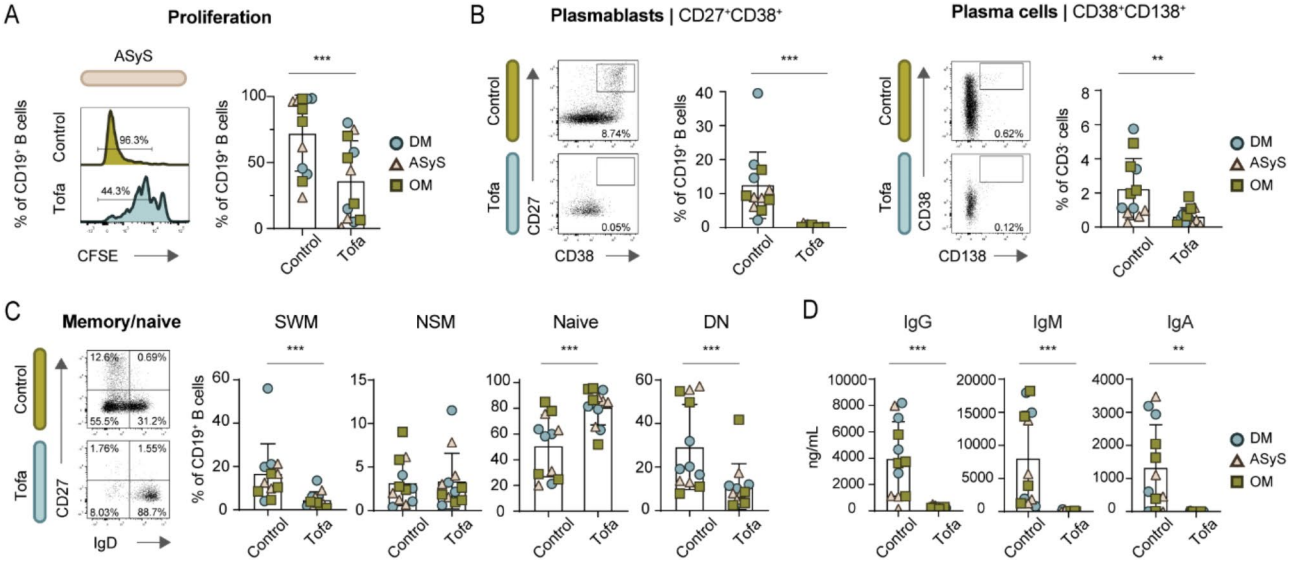

Fig. 4 Tofacitinib treatment of PBMC from DM, ASyS and OM significantly reduces B cell proliferation, but mainly affects their differentiation and antibody production in a 6-day culture. (**A**) Histograms show the percentage of proliferation based on CFSE staining from a representative ASyS patient within $CD19^+CD20^+$ B cells. Graphs include analysis from all samples. (**B**) Percentage of $CD27^+CD38^+$ plasmablasts (of $CD19^+CD20^+$ B cells) and of $CD138^+$ PC (of $CD3^-$ cells). (**C**) Representative dot plots and analysis of the percentage of naive and memory populations (within $CD19^+CD20^+$ B cells): switched memory (SWM) ($CD27^+IgD^-$), non-switched memory (NSM) ($CD27^+IgD^+$), naive ($CD27^-IgD^+$) and double negative (DN) ($CD27^-IgD^-$). (**D**) Analysis of the IgG, IgM and IgA levels in the culture supernatants. DM: dermatomyositis, ASyS: anti-synthetase syndrome, OM: overlap myositis. Tofa: tofacitinib (2.5 µM). Graphs show the mean, SD and individual values (**$p < 0.01$; ***$p < 0.001$, $n = 4$/IIM subgroup)

JAK1/3 inhibition suppresses plasmablast and PC formation, as well as antibody production in an ASyS PBMC culture

The 6-day IIM CD138$^+$ PC formation was impaired by tofacitinib, yet their percentages were relatively low in the control cultures (~2%). ASyS has a prevalent anti-Jo1 autoantibody signature, but, unlike DM, the reports of JAKi treatment in ASyS are more scarce. Consequently, we addressed the modulation of B cell responses, including autoantibody production, in ASyS. We optimized the plasmablast/PC formation and (auto)antibody production stimulating PBMC with BAFF/CpG/IL-21 over 12 days [32, 38]. In the control condition, the major population was CD27$^+$IgD$^-$ SWM cells (56.5%), which decreased to 10% upon inhibition (Fig. 5A). Additionally, treatment with tofacitinib increased the percentage of CD27$^-$IgD$^+$ naive B cells from 14 to 81%, further suggesting impaired differentiation. Consistent with this, plasmablast and CD138$^+$ PC formation was reduced after treatment, followed by decreased IgG levels, and to a lesser extent, of IgM and IgA (Fig. 5B-C). Importantly, in 3 out of 4 patients, anti-Jo1 titers were reduced (Fig. 5D). CXCL10, a biomarker of active disease in IIM, showed reduced levels after treatment (Fig. 5E) [39]. Moreover, the germinal-center-associated chemokine CXCL13 was significantly reduced by tofacitinib, consistent with the impaired memory formation and class-switching (Fig. 5E). Overall, these findings demonstrate the potential of JAK1/3 inhibition to modulate the plasmablast/PC formation and (auto)antibody production in ASyS-PBMC.

Tofacitinib inhibits ASyS Bmem differentiation and antibody production, as well as CXCL10 and CXCL13 production

The myositis PBMC cultures demonstrated specific dampening of B cell responses by tofacitinib. However, the multiple cell types within PBMC, including T cells and monocytes, may indirectly influence the observed effects. Therefore, we validated our results in an 8-day B cell-only in vitro culture. Sorted CD27$^+$ Bmem cells from ASyS patients were cultured with irradiated CD40L-expressing L-cells and BAFF/IL-21/IL-2 to support their expansion/differentiation, and tofacitinib was added at days 0, 2 and 4 after stimulation. In the control condition, approximately 80% of B cells were CD27$^+$IgD$^-$ SWM cells, compared to ~24% after treatment (Fig. 6A). This was irrespective of time point of inhibition and further suggests that tofacitinib impairs the differentiation of ASyS Bmem cells. The plasmablast formation in the control condition was somewhat higher at days 2 and 4, being reduced in percentage and count after treatment, even at a concentration as low as 1.0 µM (Fig. 6B). Compared with the 12-day ASyS PBMC culture, PC formation was enhanced, indicated by a higher CD138 expression over time (Fig. 6C). Interestingly, tofacitinib not only modulated PC generation when added at days 0, 2 and

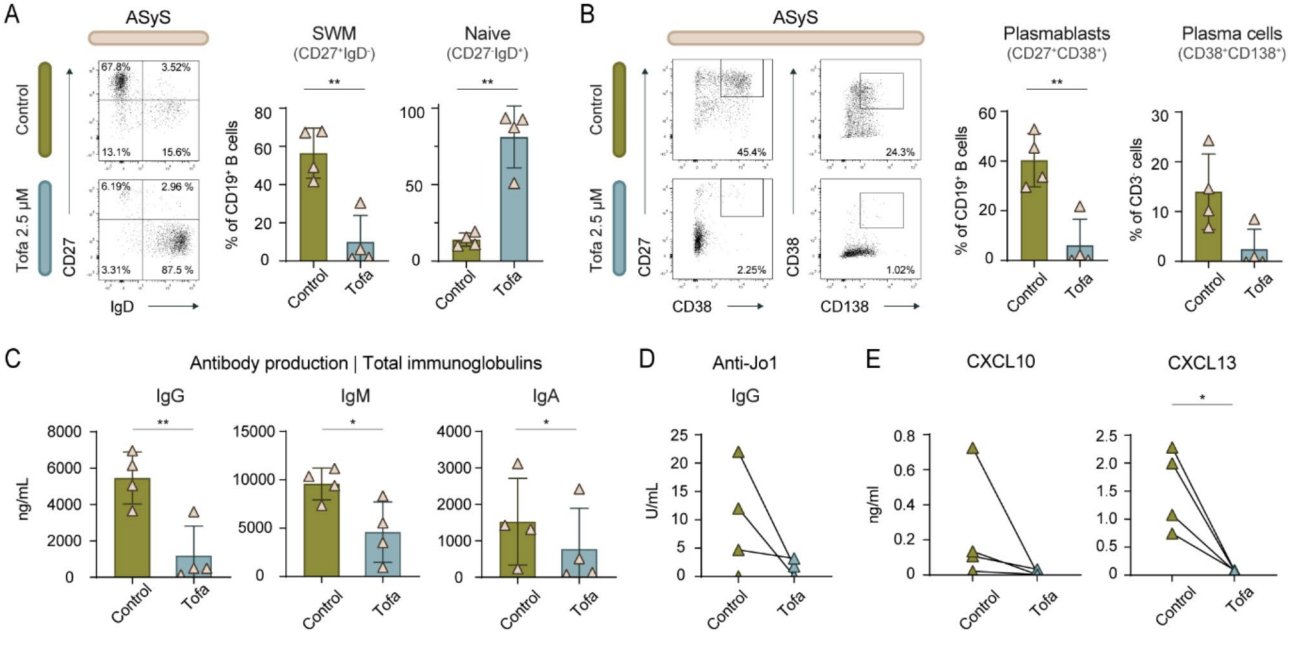

Fig. 5 Effects of tofacitinib on B cell differentiation from ASyS patients in a 12-day PBMC culture. PBMC were cultured with BAFF/CpG/IL-21, with or without tofacitinib, and DMSO was used as control. (**A**) Representative dot plots and analysis of the percentage of naive (CD27$^-$IgD$^+$), switched memory (SWM, CD27$^+$IgD$^-$), double negative (CD27$^-$IgD$^-$), non-switched memory (NSM, CD27$^+$IgD$^+$) cells. (**B**) Representative dot plots and analysis of the percentage of CD27$^+$CD38$^+$ plasmablasts (within CD19$^+$CD20$^+$ B cells) and of CD138$^+$ PC (within CD3$^-$ cells). (**C**) Total levels of IgG, IgM and IgA detected by ELISA and (**D**) anti-Jo1 detected with ELiA. (**E**) Levels of CXCL10 and CXCL13 in supernatants measured by Luminex. ASyS: anti-synthetase syndrome. Tofa: tofacitinib (2.5 µM). Graphs show the mean, SD and individual values (*$p < 0.05$, **$p < 0.01$, $n = 4$ ASyS patients)

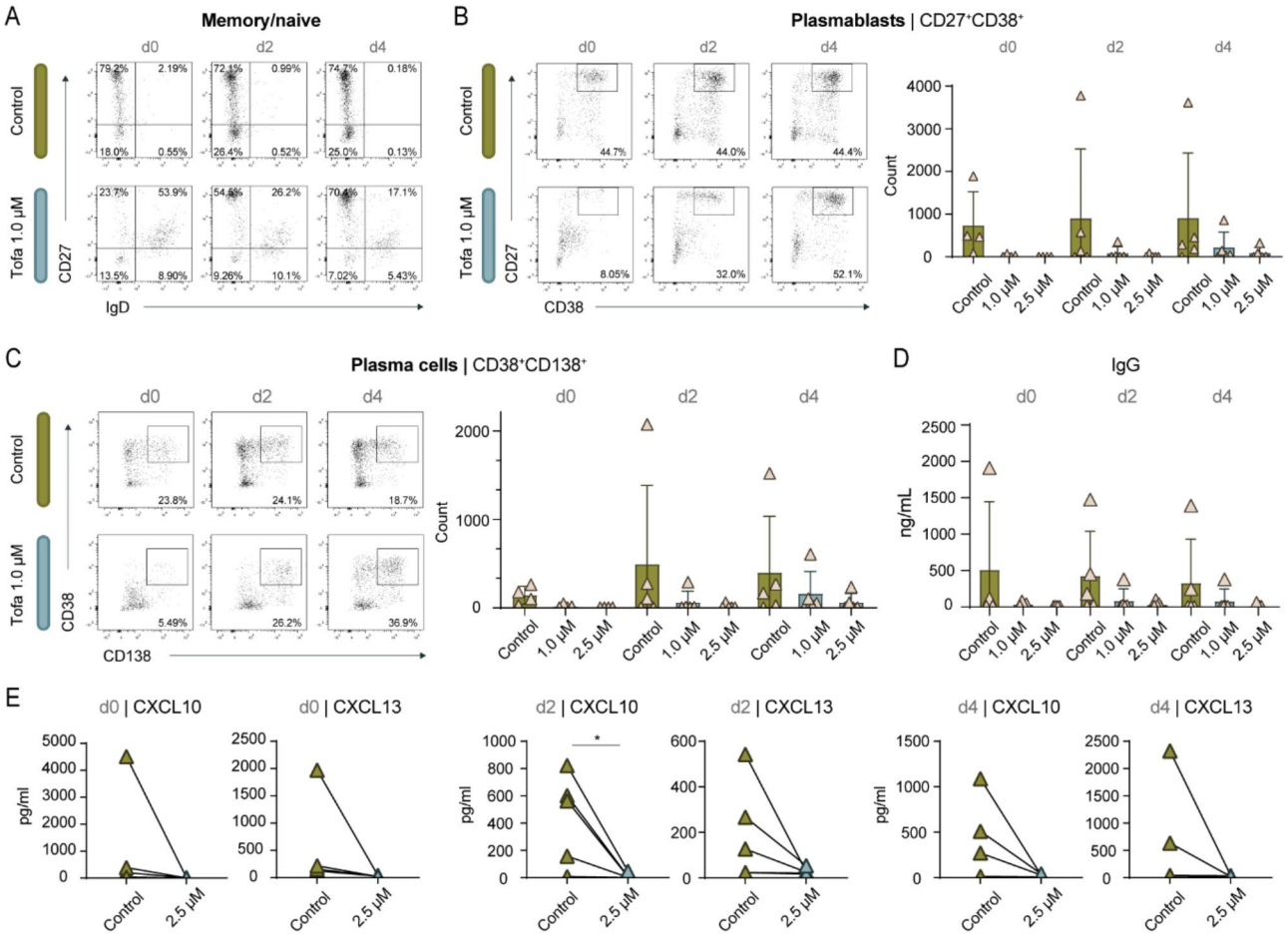

Fig. 6 Effects of JAK inhibition using tofacitinib in an 8-day memory B cell expansion and differentiation assay from ASyS patients. (**A**) Dotplots from one representative ASyS patient (also in **B**-**C**) showing the identification of memory and naive populations within $CD19^+CD20^+$ B cells: switched memory (SWM, $CD27^+IgD^-$), non-switched memory (NSM, $CD27^+IgD^+$), $CD27^-IgD^+$ and double negative (DN, $CD27^-IgD^-$) cells after an 8-day in vitro culture. Previously sorted memory B cells were cultured for 8 days with irradiated CD40L-expressing L-cells, and stimulated with BAFF/IL-21/IL-2 in the presence/absence of tofacitinib at days 0, 2 and 4. (**B**) Dotplots showing the percentage of $CD27^+CD38^+$ plasmablasts, showing the count in graphs. (**C**) Dotplots showing the $CD138^+$ PC percentage (of B cells), representing the count in graphs. (**D**) IgG levels measured in the culture supernatants are shown as ng/mL. (**E**) Chemokine levels measured in the culture supernatants are shown as pg/mL. Tofa: tofacitinib (1.0 and 2.5 µM). Graphs show the mean, SD and individual values (*$p < 0.05$, $n = 4$ ASyS patients at day 0 and $n = 5$ ASyS patients at days 2–4)

4, but also affected their secretory function, observing reduced IgG titers even with the lowest concentration (Fig. 6D). Similar results were obtained for IgM and IgA (Supplementary Fig. 3). Moreover, CXCL10 and CXCL13 levels were consistently lower at all time points (Fig. 6E). Altogether, the ASyS Bmem-only culture findings demonstrate the direct B cell-specific effects of tofacitinib on the differentiation of Bmem towards plasmablast/PC, and on their (auto)antibody, CXLCL10 and CXCL13 production.

Discussion

We investigated the potential of JAK/STAT signaling inhibition to modulate IIM B cell responses using tofacitinib, as we identified a skewed peripheral B cell compartment in active DM, ASyS and OM towards naive/transitional B cells and plasmablasts, supporting their role in the disease pathogenesis. We demonstrated that tofacitinib primarily reduced IL-21-induced plasmablast/PC formation and (auto)antibody production in PBMC cultures from all IIM subgroups. Remarkably, tofacitinib application in ASyS Bmem-only cultures revealed the potential to directly modulate autoreactive B cells. Altogether, our data provide evidence for IIM B cell-directed effects of tofacitinib in vitro, which may to some extent account for the clinical benefits of JAKi treatment in IIM.

Tofacitinib, a JAK1/3 inhibitor approved for several chronic inflammatory diseases, has recently been successfully tested in open-label studies for DM [24, 40], and in case reports for ASyS [41, 42]. The hitherto largely unclear underlying molecular mechanisms contributing to these benefits in DM, ASyS, and potentially OM,

combined with recent in vitro data from HD-PBMC studies [37], prompted the hypothesis that direct modulation of pathogenic B cell responses may be responsible. Consistently, tofacitinib in our 6-day HD-PBMC culture reduced B cell proliferation, differentiation and antibody production, the latter two being most prominently impaired when induced by CD40/IL-21 rather than by CpG/IL-2. CpG is required for Bmem proliferation and may indirectly activate the JAK/STAT pathway via TLR9 [37, 43]. However, the observed inhibitory effects in this context may be explained by other cells within PBMC, potentially providing additional B cell stimulatory mechanisms/factors when activated. Moreover, the preferential modulation of IL-21-driven responses over CD40 confirms the specificity of tofacitinib for JAK/STAT-mediated functions, as CD40 regulates B cell responses primarily via the NF-κB pathway [32, 44].

Similar to other autoimmune diseases, the pathogenic B cell signature in IIM appears to be specific, marked by enriched naive/transitional B cells in the peripheral blood of active DM, ASyS and OM, and reduced SWM and NSM populations compared to HD. The increase in transitional B cells may indicate an enhanced bone marrow output, increased proliferative capacity, or a response to IFN stimulation required for their development [35]. Elevated transitional B cells are found in the autoimmune diseases systemic lupus erythematosus [36], systemic sclerosis (SSc) [45] and primary Sjögren's syndrome [46], and notably in juvenile DM [35], aligning with our findings in the peripheral blood of adult DM, ASyS and OM. The dual pro-inflammatory (IL-6) and anti-inflammatory (IL-10) role of transitional B cells may become unbalanced in autoimmunity. Specially, increased IL-6-producing transitional B cells in SSc promoted lung fibrosis [45], and given the association of IL-6 with active IIM [47] and ILD development, future studies may assess the potential pro-inflammatory characteristics of the observed increased transitional population. Higher proportions of naive cells and decreased Bmem cells have also been reported in DM and ASyS [48, 49]. The identified low peripheral Bmem content could be attributed to enhanced differentiation, impaired maturation/survival [50], or migration to target tissues, as observed in DM [5].

Given the increased proportions of transitional/naive B cells observed in DM, ASyS and OM, we first evaluated the effects of tofacitinib on the total B cells within PBMC under anti-CD40/IL-21 activation. Upon stimulation, B cells differentiated into SWM and DN cells, a transition that was inhibited by tofacitinib, leaving ~ 80% of B cells at the naive stage. Interestingly, antibody production was abrogated, demonstrating the affected function of Bmem. This may have important clinical implications, as treatment with tofacitinib in IIM patients could affect humoral responses to new antigens or vaccines.

The consistent effects of tofacitinib on B cell responses across DM, ASyS and OM in vitro emphasize that, despite the diverse clinical manifestations and autoantibody profiles of these IIM subgroups, they share underlying (molecular) mechanisms that drive disease pathology. Notably, type 1 and 2 IFN pathways are activated across IIM subgroups, with high IFN1 activation in DM and moderate IFN1 activation in ASyS, and IFN2 activation being shared by both [18]. This may account for the similar modulation of B cell responses by tofacitinib across the IIM subgroups in vitro. To confirm this, techniques such as (single-cell) RNA-sequencing (RNA-seq) may clarify whether dysregulated gene signatures of B cell populations in peripheral blood and inflamed tissue, including JAK/STAT signaling, are shared, revealing potential therapeutic targets.

Of the three subgroups, preliminary studies in (juvenile) DM have demonstrated the therapeutic potential of JAKi. However, the pronounced anti-Jo1 autoantibody signature and thereby the probably more clonally-restricted autoreactive B cell populations in ASyS, as well as its shared IFN2 signature with DM, suggest that these patients may particularly benefit from JAKi treatment, as supported by two recent case reports [41, 42]. These factors together with the relatively scarce reports on JAKi treatment of ASyS compared to DM further propelled our focus on this subgroup.

To build on the potential benefits of JAK1/3 targeting in ASyS, we performed a modified 12-day PBMC assay to enhance plasmablast/PC formation and thereby increase (auto)antibody production. Consistently, tofacitinib inhibited differentiation, rendering ~ 80% of B cells in the naive stage compared to ~ 2% in the control condition. Additionally, a near-complete reduction in plasmablast/PC generation was observed, whereas the control culture yielded ~ 40% plasmablasts and ~ 14% PC. Notably, PCs were enriched to 11% compared to the 6-day cultures. Tofacitinib also affected the plasmablast/PC function, significantly reducing IgG levels and, to a lesser extent, those of IgM and IgA. The incomplete abrogation of plasmablast formation, and particularly of the IgM/IgA secretion compared to the 6-day cultures, may be explained by the survival of pre-existing IgM/IgA-Bmem cells within PBMC. The significant decrease in CXCL13 may support this, indicating impaired Ig-class-switching. This PC-enriched culture allowed the production and detection of anti-Jo1 in 3 of 4 samples using a specific/sensitive ELiA assay, observing a strong reduction following tofacitinib treatment. A line blot immunoassay designed for detecting DM-specific autoantibodies (anti-SAE1, anti-TIFg, and anti-Mi-2b) and OM-associated autoantibodies (anti-Ro52, anti-PM75, and anti-PM100) in blood

samples for diagnostic purposes did not generate reliable results due to limited sensitivity (data not shown). However, we envision that more sensitive ELiA assays designed to detect DM- and OM-specific autoantibodies in culture supernatants before and after tofacitinib treatment may reveal a similar reduction as observed for anti-Jo1 levels in ASyS.

Our assays with PBMC from HD and IIM patients demonstrated the potential of JAK/STAT inhibition to dampen IL-21-driven B cell responses implicated in autoimmune processes, specifically the differentiation of transitional/naive B cells. Given the fact that PBMC include T cells, monocytes and other cell types that can interact with B cells via CD40-CD40L interactions and soluble factors such as BAFF and IL-21, we confirmed the PBMC assay results in vitro by isolating $CD27^+$ Bmem from ASyS patients, and thus removing other (co-stimulatory) cell types. This approach ensured that the observed effects of tofacitinib were directly dependent on the B cells. We observed increased proportions of $CD138^+$ PC compared to the PBMC cultures, which were reduced with tofacitinib. Additionally, IgG, IgM and IgA levels were almost completely abrogated, similar to the 6-day PBMC assay findings. Notably, the percentage of plasmablasts also decreased when cells were treated at later time points (day 2 and 4), with the most pronounced effects on SWM and plasmablast formation when treated at day 2. Consistent with the PBMC cultures, the majority of sorted Bmem cells underwent Ig-class-switching, becoming $CD27^+IgD^-$ without inhibitor. However, the proportion of IgD^+ non-switched Bmem increased after treatment, while CXCL13 levels decreased, suggesting that tofacitinib impairs Ig-class-switch. This may indicate that the sorted $CD27^+$ Bmem contained IgD^+ cells that became SWM cells of IgG/IgM/IgA isotypes after stimulation, rather than pre-existing SWM cells, as suggested in the 12-day PBMC assay, with less affected IgM/IgA levels. We propose that $CD27^+IgD^+$ cells require JAK/STAT signaling, similar to naive/transitional B cells, to differentiate into SWM and plasmablasts. Of note, since treatment-naive patients already present with (auto) antibody-secreting switched plasmablast/PCs, based on our results we speculate that tofacitinib mainly affects the *de novo* differentiation from naive/transitional and NSM cells, a process active in autoimmunity, as shown by the increased naive/transitional cells in blood and lower Bmem cells.

Our study has some limitations, including the limited number of patients per myositis subtype, as we focused on treatment-naive patients. Future studies should increase the sample size and perhaps compare active disease and remission. In addition, RNAseq data may complement our results, assessing the impact of JAKi on the JAK/STAT signature and other pathways in the B cell populations of the studied IIM subtypes.

Conclusions

In conclusion, targeting the JAK/STAT pathway could provide a novel therapeutic approach for IIM. Compared to B cell-depleting therapies like rituximab and CAR-T cells, this approach may offer advantages, such as fewer side effects and reversibility due to the short half-life of the JAKi. Collectively, our findings support the rationale for further testing JAK inhibition as a strategy to target B lineage cells in IIM.

Abbreviations

ASyS	Anti-synthetase syndrome
BAFF	B cell activating factor
Bmem	Memory B cell
CFSE	Carboxyfluorescein succinimidyl ester
DM	Dermatomyositis
DN	Double negative
ELiA	Enzyme-linked immunoassay
ELISA	Enzyme-linked immunosorbent assay
HD	Healthy donor
IFN	Interferon
IIM	Idiopathic inflammatory myopathies
ILD	Interstitial lung disease
JAK	Janus kinase
JAKi	JAK inhibitor
MSAs	Myositis-specific autoantibodies
NSM	Non-switched memory
OM	Overlap myositis
PBMC	Peripheral blood mononuclear cells
PC	Plasma cell
RNAseq	RNA sequencing
SD	Standard deviation
SSc	Systemic sclerosis
STAT	Signal transducer and activator of transcription
SWM	Switched memory
Tofa	Tofacitinib

Supplementary Information

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Supplementary Material 1

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Author contributions

A.vdK., M.K. and J.R. performed the inclusion of patients at Amsterdam UMC. L.L., A.M-V., M.K., E.M.vL. and G.F. participated in performing the experiments. S.W.T., JP.vH., J.R. and A.vdK. conceived the general idea, provided expert opinion/knowledge input, and co-wrote the manuscript. A.M-V. and M.K. conceived the general idea and wrote the manuscript. A.M-V. created the figures. E.M.vL., I.L. and L.B. provided expert opinion/knowledge input and co-wrote the manuscript.

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Data availability
All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate
All subjects included in this study provided written informed consent in accordance with the Declaration of Helsinki, and the sample collection for our study was approved by the medical ethics committee of Amsterdam UMC.

Competing interests
The authors declare no competing interests.

Consent for publication
Not applicable.

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