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IL-6 targeting compared to TNF targeting in rheumatoid arthritis: studies of olokizumab, sarilumab and sirukumab

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Ann Rheum Dis 2014 73: 1595-1597 originally published online May 15, 2014
doi: 10.1136/annrheumdis-2013-205002

EXTENDED REPORT

The JAK inhibitor, tofacitinib, reduces the T cell stimulatory capacity of human monocyte-derived dendritic cells

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Handling editor Tore K Kvien

▶ Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2013-203756).

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Received 10 April 2013
Revised 6 July 2013
Accepted 20 August 2013
Published Online First 6 September 2013

ABSTRACT

Objective Tofacitinib, which is a Janus kinase (JAK) inhibitor, has shown clinical effects in the treatment of rheumatoid arthritis. JAKs are important kinases in lymphocyte differentiation; however, their function in dendritic cells (DCs) is unknown. In this study, the function of JAKs in DCs was investigated with tofacitinib.

Methods The effects of tofacitinib on the maturation of human monocyte-derived DCs induced by lipopolysaccharide (LPS) stimulation were investigated. In addition, its effects on T cell stimulatory capability was investigated by coculturing with naïve CD45RA-positive T cells.

Results Tofacitinib decreased expression of CD80/CD86 in a concentration-dependent manner in LPS-stimulated DCs; however, it did not affect HLA-DR expression. Tofacitinib suppressed tumour necrosis factor, interleukin (IL)-6 and IL-1β production without affecting transforming growth factor (TGF)-β and IL-10 production. Meanwhile, CD80/CD86 expression in DCs was enhanced by type I interferon (IFN) stimulation, and the LPS-induced CD80/CD86 expression was inhibited by an antibody to type I IFN receptor. Furthermore, tofacitinib suppressed production of type I IFN and activation of interferon regulatory factor (IRF)-7, which is a transcription factor involved in CD80/CD86 and type I IFN expression. Tofacitinib also decreased the T cell stimulatory capability of DCs and increased expression of indoleamine 2,3-dioxygenase (IDO)-1 and IDO-2.

Conclusions Tofacitinib, a JAK1/JAK3 inhibitor, affected the activities of human DCs. It decreased CD80/CD86 expression and T cell stimulatory capability through suppression of type I IFN signalling. These results suggest a novel mode of action for tofacitinib and a pivotal role for JAKs in the differentiation of DCs.

INTRODUCTION

Janus kinase (JAK) family members, constitutively bound to cytokine receptors, play an important role in the biological activation of cytokines through activation of the signal transducer and activator of transcription (STAT), which is a transcription factor. The JAK family consists of JAK1, JAK2, JAK3 and tyrosine kinase (TYK)2. Different JAK family members are activated by different cytokine receptors. JAK1 is activated by the class 1, class 2 and γc cytokines, while JAK3 is activated by γc cytokines. Therefore, JAK family members are not only essential for immune function, but they also play an important role in inflammation response.[1–4] Tofacitinib, which is selective for JAK1 and JAK3,[5 6] is effective for patients with rheumatoid arthritis (RA).[7–10] This finding supports the notion that JAK1 and JAK3 play an important role in autoimmune diseases. Furthermore, it is thought that elucidation of the mode of action of JAK family members in vivo will lead to a better understanding and treatment of autoimmune diseases.

Dendritic cells (DCs) play a key role in bridging natural immunity and acquired immunity. Immature DCs are potent phagocytes, and they mature through toll-like receptor (TLR) signalling. They also show antigen-specific T-cell activation abilities, which are accompanied by induction of expression of major histocompatibility complex (MHC) and costimulus molecules. Moreover, DCs play an important role in autoimmune diseases. They suppress antigen-specific responses and cause induction of immunotolerance relative to the degree of their differentiation and functional modification,[11–15] while suppression of DC apoptosis destroys immunotolerance, resulting in induction of autoimmune diseases.[16] Hence, DCs are potential targets not only for immune responses but also for autoimmune diseases.

We have previously shown that tofacitinib selectively suppresses production of cytokines and proliferation of lymphocytes.[17] These functions can be predicted to some degree by the important role that JAK family members play in the differentiation and proliferation of lymphocytes. We have reported that DCs express JAK1, JAK2 and JAK3; however, DCs derived from a JAK3-deficient mouse have been shown to overproduce interleukin (IL)-10 and exhibit anti-inflammatory activity.[18] However, how the inhibition of JAK signalling affects the phenotype, differentiation and antigen presentation of human DCs, which initiate immune responses, has not been investigated. Elucidation of the effects of tofacitinib on DC function may increase basic scientific knowledge of the clinical efficacy of tofacitinib

An increase in the number of invasive DCs has been observed in the synovitis tissues in RA, and monocyte-derived DCs (MoDCs) from patients with RA produce increased IL-6.[19] In addition, an increase in the number of DCs that express high levels of TLR4 ligands in RA synovial fluid has been reported,[20 21] suggesting activation of DCs and disruption of immunotolerance. Furthermore, JAK expression increases in synovial DCs in active

To cite: Kubo S, Yamaoka K, Kondo M, *et al*. *Ann Rheum Dis* 2014;**73**:2192–2198.

RA,[22–24] indicating its involvement in the regulation of DCs during the pathological processes. This study was conducted in order to investigate the effects of inhibition of JAK1 and JAK3 by tofacitinib and signalling mechanisms in human MoDCs.

METHODS

Inhibitors

Tofacitinib and PF956980[25] were kindly provided by Pfizer (New York, New York, USA). The following inhibitors were purchased; JAK2 kinase inhibitor, G6 (Sigma-Aldrich, St Louis, Missouri, USA), Syk inhibitor I, Syk inhibitor II, PP1, PP2 (Merck, Darmstadt, Germany), anti-IL-6 receptor α antibody, tocilizumab (Chugai Pharmaceutical Co, Tokyo, Japan).

Generation of MoDCs and cell cultures

Peripheral blood mononuclear cells were isolated with lymphocyte separation medium (ICN/Cappel Pharmaceuticals, Aurora, Ohio, USA). Monocytes were obtained by positive magnetic selection using anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). To generate immature MoDCs, we cultured monocytes at 0.5×10^6 cells/mL in the presence of IL-4 (50 ng/mL; R&D systems, Minneapolis, Minnesota, USA) and granulocyte macrophage colony-stimulating factor (50 ng/mL; Peprotech, Rocky Hill, New Jersey, USA) for 6 days. The medium was replaced with one supplemented with cytokines on day 3.

Immature MoDCs were washed and replated in fresh medium at 2.5×10^5 cells/mL and pretreated with tofacitinib (10, 100, 300, 1000 nM), PF956980 (300 nM), G6 (300 nM), PP1 (300 nM), PP2 (300 nM), cycloheximide (5 μg/mL), tocilizumab (5 μg/mL) or interferon (IFN)α/β receptor antibody (10 μg/mL) (Abcam, Cambridge, UK) for 6 h and matured with lipopolysaccharide (LPS) (*Escherichia coli*; Sigma, 100 ng/mL) for 48 h. The concentration of each drug was chosen on the basis of previous studies.[6 26–30] MoDCs were washed twice and used for coculture with T cells. Production of tumour necrosis factor (TNF)α, IL-6, IL-1β and IFNα was determined with the BD Cytometric Bead Array (CBA) human Flex Set (BD Pharmingen, Franklin Lakes, New Jersey, USA), and that of TGFβ and IFNβ was analysed by ELISA (eBioscience, San Diego, California, USA). Apoptosis was analysed with the Apoptosis Detection kit II (BD Pharmingen).

DC–T cell cocultures

CD4 T cells were negatively selected from peripheral blood mononuclear cells with the CD4 T Cell Isolation Kit II (Miltenyi Biotec), and $CD45RA^+$ naive T cells were positively isolated with anti-CD45RA microbeads (Miltenyi Biotec). MoDCs were cocultured with allogeneic human $CD45RA^+$ naive T cells at a 1:10 ratio for 6 days in Roswell Park Memorial Institute medium. IL-10 was analysed by CBA, and T cell proliferation was assessed by $[^3H]$thymidine incorporation in the last 16 h. IFNγ production was analysed after restimulation of T cells with CD3 (1 μg/mL) and CD28 (0.5 μg/mL) monoclonal antibodies (R&D Systems) for 72 h after coculture.

Flow cytometric analysis

MoDCs were incubated in blocking buffer (0.25% human globulin in phosphate-buffered saline) for 15 min and then suspended in 100 μL FACS solution (0.5% human albumin and 0.1% NaN_3 in phosphate-buffered saline) with fluorochrome-conjugated monoclonal antibodies at 4°C for 30 min and then washed with FACS solution and analysed with a FACSVerse (Becton–Dickinson, San Jose, California, USA). The following fluorochrome-conjugated mouse monoclonal antibodies were purchased from BD Pharmingen: fluorescein thiocyanate (FITC)-conjugated anti-CD80, PerCP-conjugated anti-HLA-DR, and antigen presenting cell (APC)-conjugated anti-CD86.

Quantitative real-time PCR

Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Chatsworth, California, USA). First-strand cDNA was synthesised, and quantitative real-time PCR was performed in the Step One Plus instrument (Applied Biosystems, Foster City, California, USA). TaqMan target mixes for tryptophan indoleamine-pyrrole 2,3-dioxygenase (IDO1), IDO paralogue IDO2 (IDO2), CD80 and CD86 were purchased from Applied Biosystems. Expression levels were expressed relative to that of glyceraldehyde-3-phosphate dehydrogenase. The relative quantity was calculated using the quantification-comparative cycle threshold formula–referenced sample of immature DCs.

Western blot analysis

MoDCs were lysed in Nonidet P-40 buffer containing NaCl, Tris/HCl (pH 8.0), distilled water and protease inhibitor. Lysates were mixed with an equal volume of sample buffer solution (2-mercaptoethanol; Wako Pure Chemical Industries) and boiled for 5 min. Proteins were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, transferred on to nitrocellulose membranes (Whatman, Tokyo, Japan), blocked with 5% skimmed milk, and immunoblotted with antibodies to human phospho-NF-κB p65, human PU.1, human IFN regulatory factor 7 (IRF7), human phospho-STAT1, STAT1, phospho-STAT2 and STAT2 (Cell Signaling Technology, Tokyo, Japan) and horseradish peroxidase-labelled anti-secondary antibodies (NA931V and NA934V; GE Healthcare, Osaka, Japan), using immunoreaction enhancer solution (Can Get Signal, Toyobo, Osaka). Blots were developed with ECL Western Blotting Detection Reagents (GE Healthcare) and visualised with a light-capture instrument (ATTO, Tokyo, Japan).

Statistical analysis

Differences were examined using the Mann–Whitney test. $p<0.05$ denoted the presence of a significant difference.

RESULTS

Tofacitinib inhibited expression of CD80/CD86 without cytotoxicity in human MoDCs stimulated with LPS

The effects of tofacitinib on the expression of costimulators of human MoDCs were investigated. CD80/CD86 and HLA-DR were induced 48 h after LPS stimulation. However, CD80/CD86 expression in MoDCs was suppressed in a concentration-dependent manner by the addition of tofacitinib, whereas expression of HLA-DR was not affected (figure 1A,B). Moreover, induction of CD80/CD86 expression by LPS stimulation was suppressed by PF956980, which is a different JAK1/3 inhibitor, while CD80/CD86 expression was not suppressed by PP1, PP2 or JAK2 inhibitors. These findings suggest that suppression of CD80/CD86 expression was dependent on inhibition of JAK1/3 (figure 1C). Furthermore, cluster formation was observed 24 h after LPS stimulation, which was inhibited in a concentration-dependent manner by the addition of tofacitinib (figure 2A).

In the next set of experiments, cytokine production in DCs was investigated. The production of TNFα, IL-1β and IL-6 was induced by stimulation of MoDCs with LPS for 48 h, while the production of these cytokines was suppressed by tofacitinib in a concentration-dependent manner (figure 2B). However, tofacitinib affected neither the expression of TGFβ mRNA

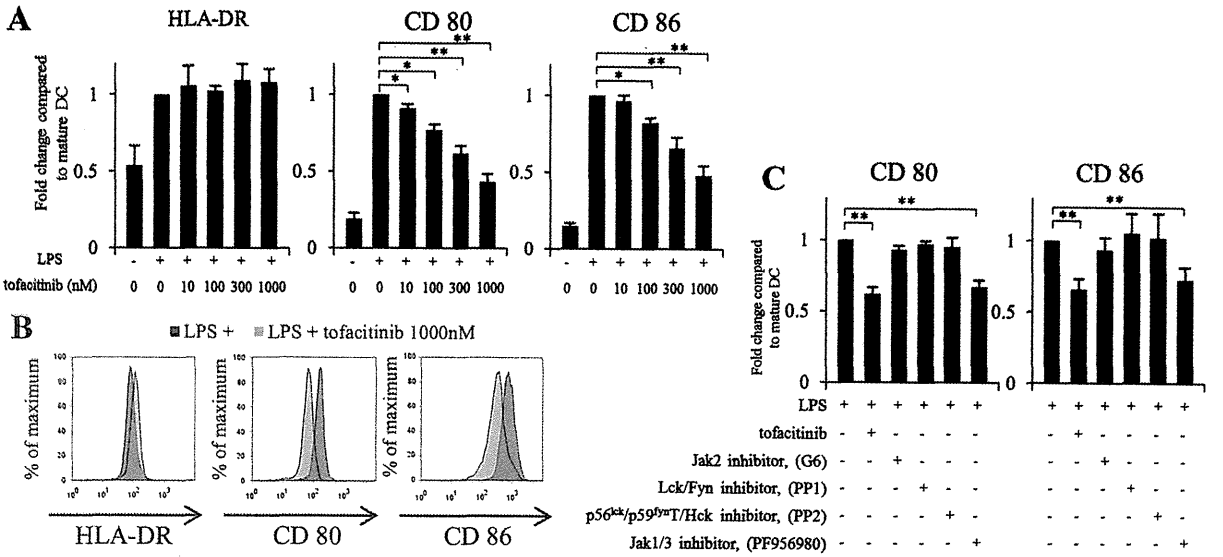

Figure 1 Tofacitinib suppresses CD80/CD86 expression. Immature monocyte-derived dendritic cells (DCs) were washed and cultured with or without tofacitinib or other tyrosine kinases during lipopolysaccharide (LPS; 100 ng/mL) stimulation for 48 h. The DC phenotype was evaluated using flow cytometry. (A) Expression of HLA-DR, CD80 and CD86. (B) Representative histogram data of HLA-DR, CD80 and CD86 expression. (C) Expression of HLA-DR, CD80 and CD86 in the presence of tofacitinib and other tyrosine kinase inhibitors. Representative results of three independent experiments with similar findings. Data are mean±SD. *p<0.05 and **p<0.01 (Mann–Whitney test).

(see online supplementary figure S1A) nor the production of TGFβ (figure 2C), IL-10 and IL-12p70 (see online supplementary figure S1B).

To examine whether these suppressive effects were the result of cytotoxicity of tofacitinib on DCs, the cells were stained with annexin V and propidium iodide. DCs died at a high frequency without stimulation for 48 h, while apoptosis was inhibited by LPS stimulation (figure 3A,B). Tofacitinib did not induce apoptosis, even at concentrations as high as 1000 nM, and it did not cause cytotoxicity. When DCs were pulsed with FITC-labelled albumin, tofacitinib did not affect their capability for micropinocytosis (figure 3C). These results suggest that tofacitinib suppressed cluster formation and changed the phenotype of DCs without causing their cell death.

Expression of CD80 and CD86 was inhibited by antibody to type I IFN receptor

Expression of CD80/CD86 mRNA after LPS stimulation in DCs was suppressed by cycloheximide treatment (figure 4A). After LPS activation, no JAK–STAT pathway involvement was

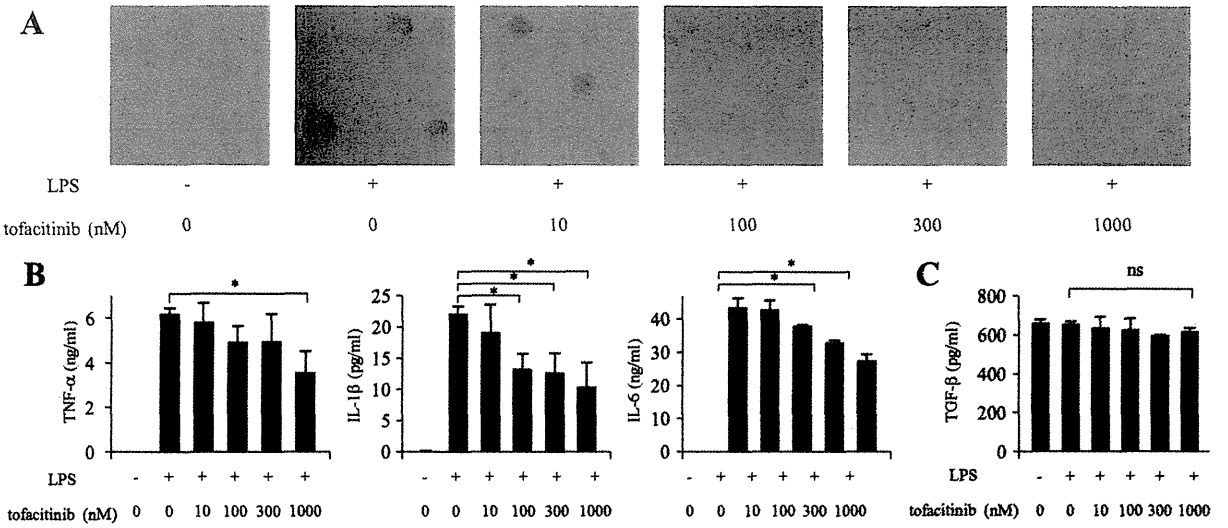

Figure 2 Tofacitinib suppresses cluster formation of dendritic cells (DCs) and inflammatory cytokine production in DCs. Immature monocyte-derived DCs were washed and cultured with or without tofacitinib during lipopolysaccharide (LPS; 100 ng/mL) stimulation for 48 h. (A) Morphology of DC populations as shown by phase-contrast microscopy. Representative results of three independent experiments with similar findings are shown. (B) Tumour necrosis factor (TNF)α, interleukin (IL)-1β and IL-6 concentrations in the supernatants were measured. (C) Transforming growth factor (TGF)β concentration was determined by ELISA. Data of three independent experiments are shown. Data are mean ±SD. *p<0.05 (Mann–Whitney test).

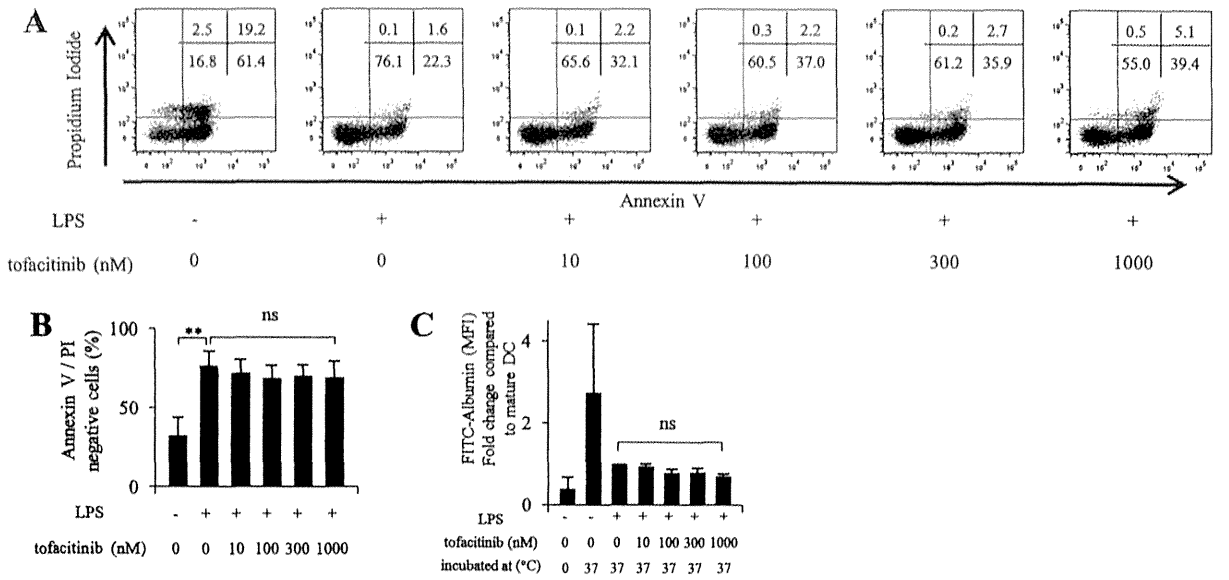

Figure 3 Tofacitinib does not induce cell death. Immature monocyte-derived dendritic cells (MoDCs) were washed and cultured with or without tofacitinib during lipopolysaccharide (LPS; 100 ng/mL) stimulation for 48 h. Early and late apoptosis of DCs were evaluated with flow cytometry. (A) Representative histogram data of annexin V/propidium iodide (PI) staining. (B) Rate of viable cells (annexin V_{neg}/PI_{neg}). (C) MoDCs were pulsed with 5 μg/mL fluorescein isothiocyanate (FITC)-conjugated albumin for 60 min. Cells were incubated at 0°C for background uptake and at 37°C for albumin uptake. Data indicate the ratio compared with MoDCs stimulated with LPS. Data of three independent experiments are shown. Data are mean±SD. **p<0.01 (Mann–Whitney test). MFI, mean fluorescence intensity.

indicated in the signalling pathway downstream of TLR4; therefore, an indirect mechanism was considered in which the suppression of CD80/CD86 expression by tofacitinib occurred through a proteinogenic mechanism.

We next assessed if CD80/CD86 is induced by cytokines in MoDCs. CD80/CD86 expression was not induced by IL-6, whereas it was induced by LPS stimulation. Furthermore, the CD80/CD86 expression that was induced by LPS was not affected by tocilizumab, which is an IL-6 receptor antibody (figure 4B). In contrast with IL-6, expression of CD80/CD86 was induced by type I IFN stimulation and was completely inhibited by tofacitinib. Expression of CD80/CD86, which was induced by LPS, was suppressed by a type I IFN receptor antibody (figure 4C). These results suggest that the inhibition of JAK1/3 in MoDCs partially suppressed the expression of CD80/CD86 by suppressing type I IFN signalling.

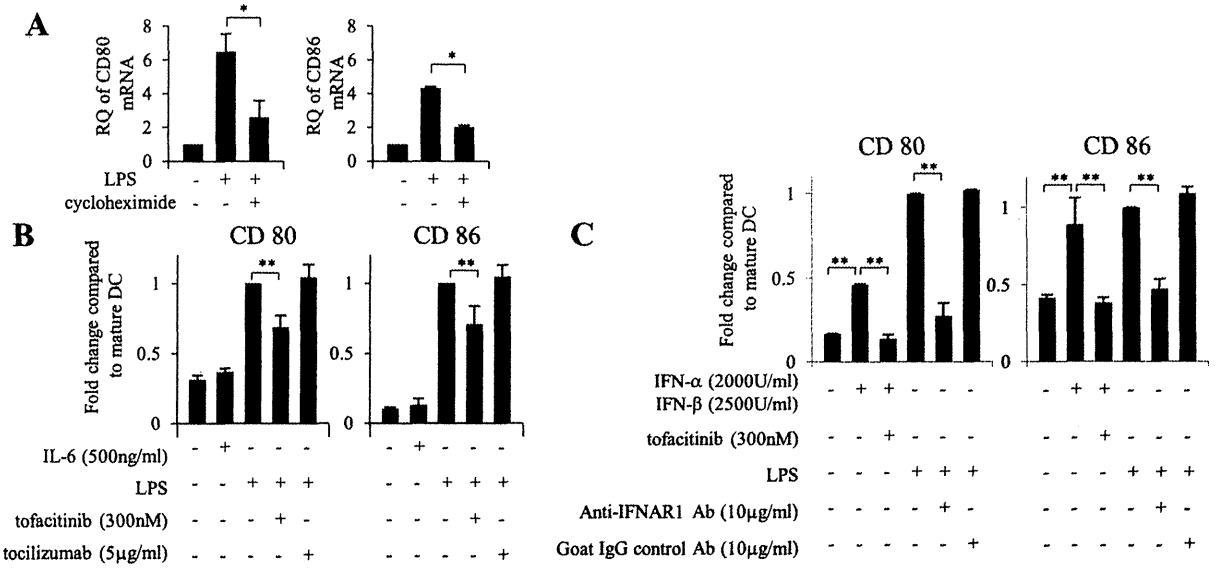

Figure 4 Lipopolysaccharide (LPS) induces the expression of CD80 and CD86 through type I interferon (IFN). Immature monocyte-derived dendritic cells (DCs) were washed and cultured with or without cycloheximide, an antibody to interleukin (IL)-6 receptor (tocilizumab) or to type I IFN receptor (IFNAR1) during LPS (100 ng/mL) stimulation for 48 h. (A) CD80 and CD86 mRNA expression were measured by quantitative real-time PCR. (B and C) Expression of HLA-DR, CD80 and CD86 determined by flow cytometry. Data are mean±SD. *p<0.05 and **p<0.01 (Mann–Whitney test). RQ, relative quantity.

Tofacitinib inhibited expression of CD80 and CD86 through reduction of IRF7 signalling

The involvement of NF-κB,[31] PU.1[32] and IRF7,[33] which are transcription factors that regulate CD80/CD86 expression, was investigated. NF-κB and PU.1 were activated within 5 min, while IRF7 was activated within 3 h in DCs after LPS stimulation. The activation of NF-κB and PU.1 by LPS stimulation was not suppressed by tofacitinib, while the activation of IRF7 was suppressed by tofacitinib (figure 5A). Consistent results were achieved on analysis of nuclear and cytoplasmic fractions. LPS stimulation for 5 min induced phospho-IκBα and concurrently induced phospho-NF-κB and the subsequent translocation of phospho-NF-κB and PU.1 into the nucleus. However, tofacitinib affected the translocation of neither phospho-NF-κB nor PU.1 induced by LPS (see online supplementary figure S2). On the other hand, expression and nuclear translocation of IRF7 was induced after 3 h stimulation with LPS, and the induced translocation of IRF7 was suppressed by tofacitinib (see online supplementary figure S2). In addition, tofacitinib decreased IFNβ production (figure 5B), while IFNα production was undetectable (data not shown). Tofacitinib also suppressed phospho-STAT1/STAT2 induced by exogenous type I IFN (figure 5C). Furthermore, IFNα/β receptor was constitutively expressed and not affected by tofacitinib (data not shown). These results indicate that tofacitinib suppressed the phosphorylation of STAT1/STAT2 induced by autocrine stimulation with type I IFN, continuously suppressing IRF7 expression and the production of type I IFN, which decreased CD80/CD86 expression in MoDCs.

Tofacitinib reduced T cell stimulatory ability and induced expression of IDO in MoDCs

Finally, the T cell stimulation capability of MoDCs treated with tofacitinib was examined. MoDCs were cultured for 48 h in the presence of tofacitinib and LPS, washed, and then cocultured with allogeneic $CD4^+CD45RA^+$-naïve T cells for 6 days. MoDCs that were treated with LPS exhibited increased T cell growth capability, and IFNγ production capability was induced. However, MoDCs that were pretreated with tofacitinib exhibited decreased T cell stimulatory capability and demonstrated a concentration-dependent decrease in IFNγ production (figure 6A–C), while IL-10 production was increased (figure 6C) without any effects on regulatory T cell population (see online supplementary figure S3).

It has been reported that DCs that express IDO, which is an enzyme with catalytic activity on tryptophan, show decreased T cell stimulatory capability.[34] Therefore, the IDO mRNA in DCs was measured. Both IDO1 and IDO2 were significantly induced by tofacitinib (figure 6D). These results suggest that inhibition of JAK1/3 in DCs with tofacitinib suppressed cell maturation and induced DCs with decreased T cell stimulatory capability.

DISCUSSION

It is shown here that tofacitinib, a JAK inhibitor, promoted a tolerogenic phenotype in human DCs. The data indicate that inhibition of JAK1/JAK3 by tofacitinib regulated transcription of IRF7 by suppressing type I IFN signalling and CD80/CD86 expression. Tofacitinib was approved for treatment of RA in the USA and Japan in 2012 and 2013, respectively. The therapeutic efficacy of tofacitinib has been shown to be equivalent to TNF inhibitors,[35] and it was also found to be effective in patients who did not respond to TNF inhibitors.[36] These clinical study results indicate that JAK1/JAK3 plays an important role in inflammatory immune diseases such as RA. However, the direct suppressive effect of tofacitinib on T cells alone does not completely explain the mechanism. The results in this report suggest a novel mechanism of tofacitinib involving the induction of immunotolerance in DCs.

Tofacitinib did not affect the expression of MHC class II molecules, whereas it did suppress CD80/CD86 expression. Tofacitinib has been shown to exhibit a suppressive effect on JAK1/JAK3, while its suppressive effects on JAK2/Tyk2 are

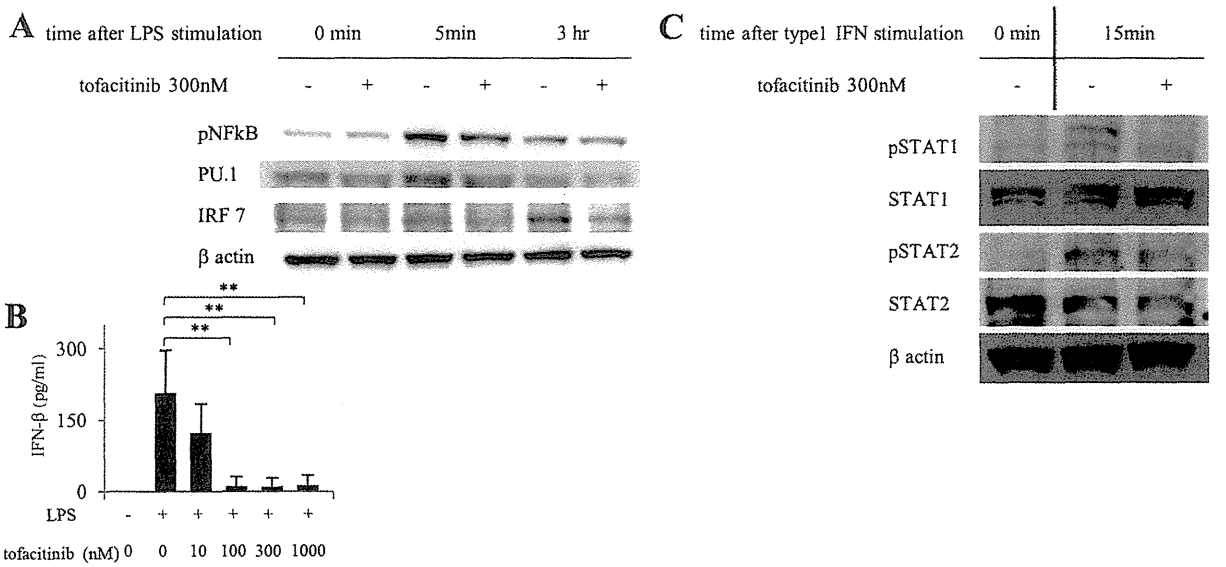

Figure 5 Tofacitinib suppresses interferon regulatory factor (IRF) activation. Immature monocyte-derived dendritic cells (MoDCs) were washed and cultured with or without tofacitinib during lipopolysaccharide (LPS; 100 ng/mL) stimulation for 48 h. The time course of suppression was evaluated as shown. (A) Phospho-NF-κB, PU.1, IRF7 and β-actin were detected by western blotting. (B) Interferon (IFN)β concentration in the supernatant from MoDCs cultured for 48 h was measured. (C) Phospho-signal transducer and activator of transcription 1 (pSTAT1), STAT1, phospho-STAT2 (pSTAT2), STAT2 and β-actin were detected by western blotting after stimulation by type-1 IFN for 15 min.

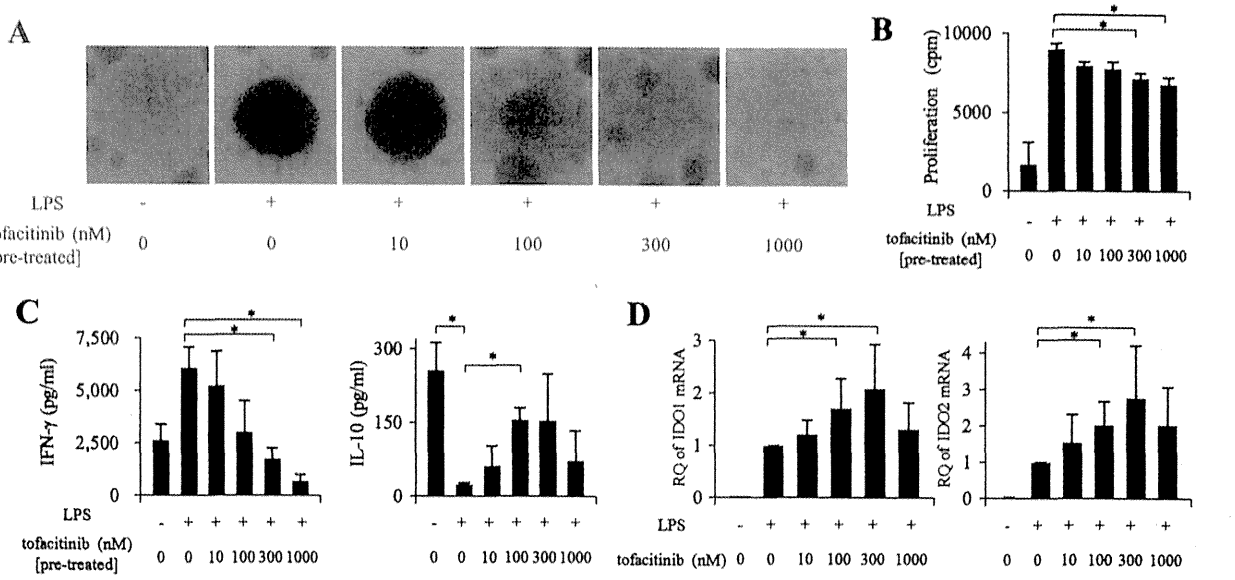

Figure 6 Tofacitinib decreased T cell stimulatory capability of dendritic cells (DCs) and increased indoleamine 2,3-dioxygenase (IDO) expression. Immature monocyte-derived DCs were cultured with or without tofacitinib during lipopolysaccharide (LPS; 100 ng/mL) stimulation for 48 h and washed. They were then cocultured with allogeneic $CD4^+CD45RA^+$-naïve T cells for 6 days. (A) Clustering of T cells as shown by phase-contrast microscopy. (B) [^{3}H]Thymidine incorporation was measured during the last 18 h of the 72 h culture. (C) Interferon (IFN)γ and interleukin (IL)-10 concentrations in the supernatant. (D) Indoleamine 2,3-dioxygenase (IDO)1 and IDO2 mRNA expression in DCs measured by quantitative real-time PCR. Data of three independent experiments are shown. Data are mean±SD. *p<0.05 (Mann–Whitney test). RQ, relative quantity.

limited.[5] Furthermore, we found that the JAK2 inhibitor (G6) did not show any effects on CD80/86 expression (figure 1), indicating that JAK1 is involved in CD80/86 induction. Recent clinical trials proved that a JAK1/JAK2 inhibitor possesses similar clinical efficacy to tofacitinib,[37] and there could be a similar inhibitory action of JAK1-mediated signalling on DCs by this JAK1/JAK2 inhibitor. Furthermore, therapies targeting suppression of CD80/CD86-mediated T cell stimulation, such as abatacept, have been successful in the treatment of autoimmune diseases, and TNF inhibitors are also able to suppress CD80/86 expression.[38] Therefore, suppression of costimulators is considered an important mechanism of action of tofacitinib.

CD80/86 expression is regulated by three transcription factors, NF-κB,[31] PU.1[32] and IRF7.[33] NF-κB and PU.1 are directly induced by TLR4 stimulation,[39 40] while IRF7 is induced through JAK1/Tyk2, which are activated by type I IFN and its downstream signals, STAT1/STAT2.[41] Furthermore, IRF7 promotes type I IFN production, which results in the formation of a positive feedback pathway.[42] The results of our study indicate that tofacitinib did not affect activation of NF-κB and PU.1, whereas it did suppress IRF7 expression. Moreover, CD80/CD86 expression was suppressed in the presence of an antibody to type I IFN receptor. According to a report by Lim *et al*,[33] IRF7 bound to the promoter lesion of CD80 and regulated its expression. Although the regulation of CD80/86 remains unclear, there may be coordinated regulatory mechanisms among NF-κB, PU.1 and IRF7, and tofacitinib may inhibit CD80/86 expression partly through IRF7.

The most significant finding of this study is that JAK1/JAK3 inhibition by tofacitinib in human DCs suppressed induction of their T cell stimulatory capability. A decrease in costimulator expression, as well as an increase in IDO expression, was observed after tofacitinib treatment. IDO is a rate-limiting enzyme in tryptophan metabolism; however, it has a strong immunomodulation effect and plays an important role in the expression of tolerogenic DC function.[34 43 44] Expression of costimulators and cytokine production capability were suppressed, and expression of IDO was increased, in MoDCs in the presence of tofacitinib. Although the mechanisms of IDO induction remain unclear, we assume that the inhibition of IL-4 played a role in IDO induction by tofacitinib for the following reasons: IL-4 is produced by DCs[45]; IL-4 activates JAK1/JAK3; tofacitinib inhibits IL-4-mediated signalling; IL-4 is known to inhibit IDO expression.[46]

To clarify the functions of IDO in DCs, MoDCs were pretreated with tofacitinib and cocultured with allogeneic CD4 T cells in the presence of 1-methyltryptophan (1-MT), an IDO inhibitor. However, the treatment of MoDCs with 1-MT did not cancel the tofacitinib-mediated suppressive effects on T cell stimulation (data not shown). Furthermore, other molecules involved in immune tolerance such as programmed death ligand (PDL)-1 and PDL-2 were not induced by tofacitinib (see online supplementary figure S4). Thus, the functional significance of IDO expression in DCs remains unclear in our studies, and we suppose that the suppressive effects of tofacitinib-treated DCs on T cell stimulation mainly depend on the inhibition of CD80/86 expression in DCs.

On oral administration of tofacitinib 5 or 10 mg twice a day, serum levels of approximately 100–300 nM are achieved, and such therapeutic levels are known to last for 4–6 h. The in vitro levels of tofacitinib used in our studies were almost comparable to the therapeutic levels achieved. Although the in vivo half-life of tofacitinib is 2–3 h, an effective concentration could be obtained in vitro by administration twice a day.

These findings suggest that the inhibition of JAK1 and JAK3 responses after LPS stimulation in human DCs was involved in the regulation of disease states through a novel mechanism. In addition to the known effects of tofacitinib on lymphocytes, we discovered novel effects on human MoDCs: tofacitinib suppressed a production and stimulation loop of type I IFN

through JAK1/JAK3, decreased CD80/CD86 expression, induced IDO expression, and suppressed T cell stimulatory capabilities. Thus, tofacitinib not only suppressed cytokine production, but also suppressed expression of costimulators by inhibiting the positive loop of type I IFN–IRF7 in DCs, which leads to immunomodulatory effects.

Contributors All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. YT had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Competing interests Y Tanaka received consulting fees, lecture fees, and/or honoraria from Mitsubishi-Tanabe Pharma, Eisai, Chugai Pharma, Abbott Japan, Astellas Pharma, Daiichi-Sankyo, Abbvie, Janssen Pharma, Pfizer, Takeda Pharma, Astra-Zeneca, Eli Lilly Japan, GlaxoSmithKline, Quintiles, MSD, Asahi-Kasei Pharma, and received research grants from Bristol-Myers, Mitsubishi-Tanabe Pharma, Abbvie, MSD, Chugai Pharma, Astellas Pharma, Daiichi-Sankyo. M Kondo is an employee of the Mitsubishi Tanabe Pharma Corporation. K Yamaoka received consulting fees from Pfizer.

Ethics approval The institutional review board of the University of Occupational and Environmental Health Japan.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1 Ghoreschi K, Laurence A, O'Shea JJ. Janus kinases in immune cell signaling. *Immunol Rev* 2009;228:273–87.
2 Müller-Ladner U, Judex M, Ballhorn W, *et al*. Activation of the IL-4 STAT pathway in rheumatoid synovium. *J Immunol* 2000;164:3894–901.
3 Li WQ, Dehnade F, Zafarullah M. Oncostatin M-induced matrix metalloproteinase and tissue inhibitor of metalloproteinase-3 genes expression in chondrocytes requires Janus kinase/STAT signaling pathway. *J Immunol* 2001;166:3491–8.
4 Frucht DM, Aringer M, Galon J, *et al*. Stat4 is expressed in activated peripheral blood monocytes, dendritic cells, and macrophages at sites of Th1-mediated inflammation. *J Immunol* 2000;164:4659–64.
5 Karaman MW, Herrgard S, Treiber DK, *et al*. A quantitative analysis of kinase inhibitor selectivity. *Nat Biotechnol* 2008;26:127–32.
6 Ghoreschi K, Jesson MI, Li X, *et al*. Modulation of innate and adaptive immune responses by Tofacitinib (CP-690,550). *J Immunol* 2011;186:4234–43.
7 Kremer JM, Cohen S, Wilkinson BE, *et al*. A Phase 2B dose-ranging study of the oral JAK inhibitor tofacitinib (CP-690,550) versus placebo in combination with background methotrexate in patients with active rheumatoid arthritis and inadequate response to methotrexate alone. *Arthritis Rheum* 2011;64:970–8.
8 Tanaka Y, Suzuki M, Nakamura H, *et al*. Phase II study of tofacitinib (CP-690,550) combined with methotrexate in patients with rheumatoid arthritis and an inadequate response to methotrexate. *Arthritis Care Res (Hoboken)* 2011;63:1150–8.
9 Kremer JM, Bloom BJ, Breedveld FC, *et al*. The safety and efficacy of a JAK inhibitor in patients with active rheumatoid arthritis: results of a double-blind, placebo-controlled phase IIa trial of three dosage levels of CP-690,550 versus placebo. *Arthritis Rheum* 2009;60:1895–905.
10 Coombs JH, Bloom BJ, Breedveld FC, *et al*. Improved pain, physical functioning and health status in patients with rheumatoid arthritis treated with CP-690,550, an orally active Janus kinase (JAK) inhibitor: results from a randomised, double-blind, placebo-controlled trial. *Ann Rheum Dis* 2010;69:413–16.
11 Kuwana M. Induction of anergic and regulatory T cells by plasmacytoid dendritic cells and other dendritic cell subsets. *Hum Immunol* 2002;63:1156–63.
12 Kretschmer K, Apostolou I, Hawiger D, *et al*. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 2005;6:1219–27.
13 Chen M, Wang Y, Wang Y, *et al*. Dendritic cell apoptosis in the maintenance of immune tolerance. *Science* 2006;311:1160–4.
14 Jung S, Unutmaz D, Wong P, *et al*. In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity* 2002;17:211–20.
15 Whartenby K, Calabresi P, McCadden E, *et al*. Inhibition of FLT3 signaling targets DCs to ameliorate autoimmune disease. *Proc Natl Acad Sci USA* 2005;102:16741–6.
16 Teichmann LL, Ols ML, Kashgarian M, *et al*. Dendritic cells in lupus are not required for activation of T and B cells but promote their expansion, resulting in tissue damage. *Immunity* 2010;33:967–78.
17 Maeshima K, Yamaoka K, Kubo S, *et al*. The JAK inhibitor tofacitinib regulates synovitis through inhibition of interferon-γ and interleukin-17 production by human CD4+ T cells. *Arthritis Rheum* 2012;64:1790–8.
18 Yamaoka K, Min B, Zhou Y, *et al*. Jak3 negatively regulates dendritic-cell cytokine production and survival. *Blood* 2005;106:3227–33.
19 Wenink MH, Santegoets KC, Butcher J, *et al*. Impaired dendritic cell proinflammatory cytokine production in psoriatic arthritis. *Arthritis Rheum* 2011;63:3313–22.
20 Roelofs MF, Joosten LA, Abdollahi-Roodsaz S, *et al*. The expression of toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and costimulation of toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. *Arthritis Rheum* 2005;52:2313–22.
21 Martin CA, Carsons SE, Kowalewski R, *et al*. Aberrant extracellular and dendritic cell (DC) surface expression of heat shock protein (hsp)70 in the rheumatoid joint: possible mechanisms of hsp/DC-mediated cross-priming. *J Immunol* 2003;171:5736–42.
22 Walker J, Ahern M, Coleman M, *et al*. Characterisation of a dendritic cell subset in synovial tissue which strongly expresses Jak/STAT transcription factors from patients with rheumatoid arthritis. *Ann Rheum Dis* 2007;66:992–9.
23 Walker J, Ahern M, Coleman M, *et al*. Changes in synovial tissue Jak-STAT expression in rheumatoid arthritis in response to successful DMARD treatment. *Ann Rheum Dis* 2006;65:1558–64.
24 Walker J, Ahern M, Coleman M, *et al*. Expression of Jak3, STAT1, STAT4, and STAT6 in inflammatory arthritis: unique Jak3 and STAT4 expression in dendritic cells in seropositive rheumatoid arthritis. *Ann Rheum Dis* 2006;65:149–56.
25 Changelian PS, Moshinsky D, Kuhn CF, *et al*. The specificity of JAK3 kinase inhibitors. *Blood* 2008;111:2155–7.
26 Kiss R, Polgár T, Kirabo A, *et al*. Identification of a novel inhibitor of JAK2 tyrosine kinase by structure-based virtual screening. *Bioorg Med Chem Lett* 2009;19:3598–601.
27 Steele AJ, Prentice AG, Cwynarski K, *et al*. The JAK3-selective inhibitor PF-956980 reverses the resistance to cytotoxic agents induced by interleukin-4 treatment of chronic lymphocytic leukemia cells: potential for reversal of cytoprotection by the microenvironment. *Blood* 2010;116:4569–77.
28 Hanke JH, Gardner JP, Dow RL, *et al*. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J Biol Chem* 1996;271:695–701.
29 Yamaoka K, Otsuka T, Niiro H, *et al*. Activation of STAT5 by lipopolysaccharide through granulocyte-macrophage colony-stimulating factor production in human monocytes. *J Immunol* 1998;160:838–45.
30 Betts BC, St Angelo ET, Kennedy M, *et al*. Anti-IL6-receptor-alpha (tocilizumab) does not inhibit human monocyte-derived dendritic cell maturation or alloreactive T-cell responses. *Blood* 2011;118:5340–3.
31 Zou GM, Hu WY. LIGHT regulates CD86 expression on dendritic cells through NF-kappaB, but not JNK/AP-1 signal transduction pathway. *J Cell Physiol* 2005;205:437–43.
32 Kanada S, Nishiyama C, Nakano N, *et al*. Critical role of transcription factor PU.1 in the expression of CD80 and CD86 on dendritic cells. *Blood* 2011;117:2211–22.
33 Lim W, Gee K, Mishra S, *et al*. Regulation of B7.1 costimulatory molecule is mediated by the IFN regulatory factor-7 through the activation of JNK in lipopolysaccharide-stimulated human monocytic cells. *J Immunol* 2005;175:5690–700.
34 Mellor AL, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* 2004;4:762–74.
35 van Vollenhoven RF, Fleischmann R, Cohen S, *et al*. Tofacitinib or adalimumab versus placebo in rheumatoid arthritis. *N Engl J Med* 2012;367:508–19.
36 Burmester GR, Blanco R, Charles-Schoeman C, *et al*. Tofacitinib (CP-690,550) in combination with methotrexate in patients with active rheumatoid arthritis with an inadequate response to tumour necrosis factor inhibitors: a randomised phase 3 trial. *Lancet* 2013;381:451–60.
37 van Vollenhoven RF. Small molecular compounds in development for rheumatoid arthritis. *Curr Opin Rheumatol* 2013;25:391–7.
38 Baldwin H, Ito-Ihara T, Isaacs J, *et al*. Tumour necrosis factor alpha blockade impairs dendritic cell survival and function in rheumatoid arthritis. *Ann Rheum Dis* 2010;69:1200–7.
39 Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2001;2:675–80.
40 Lodie TA, Savedra R, Golenbock DT, *et al*. Stimulation of macrophages by lipopolysaccharide alters the phosphorylation state, conformation, and function of PU.1 via activation of casein kinase II. *J Immunol* 1997;158:1848–56.
41 Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004;4:499–511.
42 Sato M, Suemori H, Hata N, *et al*. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. *Immunity* 2000;13:539–48.
43 Mellor AL, Munn DH. Tryptophan catabolism and T-cell tolerance: immunosuppression by starvation? *Immunol Today* 1999;20:469–73.
44 Munn DH, Zhou M, Attwood JT, *et al*. Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science* 1998;281:1191–3.
45 Maroof A, Penny M, Kingston R, *et al*. Interleukin-4 can induce interleukin-4 production in dendritic cells. *Immunology* 2006;117:271–9.
46 Musso T, Gusella GL, Brooks A, *et al*. Interleukin-4 inhibits indoleamine 2,3-dioxygenase expression in human monocytes. *Blood* 1994;83:1408–11.

ARD

The JAK inhibitor, tofacitinib, reduces the T cell stimulatory capacity of human monocyte-derived dendritic cells

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Ann Rheum Dis 2014 73: 2192-2198 originally published online September 6, 2013
doi: 10.1136/annrheumdis-2013-203756

Research Paper

MULTIPLE SCLEROSIS JOURNAL | MSJ

Multiple Sclerosis Journal
2014, Vol. 20(10) 1371–1380

DOI: 10.1177/1352458514523496
msj.sagepub.com
SAGE

Differential effects of fingolimod on B-cell populations in multiple sclerosis

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Abstract

Background: Fingolimod is an oral drug approved for multiple sclerosis (MS) with an ability to trap central memory T cells in secondary lymphoid tissues; however, its variable effectiveness in individual patients indicates the need to evaluate its effects on other lymphoid cells.

Objective: To clarify the effects of fingolimod on B-cell populations in patients with MS.

Methods: We analysed blood samples from 9 fingolimod-treated and 19 control patients with MS by flow cytometry, to determine the frequencies and activation states of naive B cells, memory B cells, and plasmablasts.

Results: The frequencies of each B-cell population in peripheral blood mononuclear cells (PBMC) were greatly reduced 2 weeks after starting fingolimod treatment. Detailed analysis revealed a significant reduction in activated memory B cells ($CD38^{int\text{-}high}$), particularly those expressing Ki-67, a marker of cell proliferation. Also, we noted an increased proportion of activated plasmablasts ($CD138^{+}$) among whole plasmablasts, in the patients treated with fingolimod.

Conclusions: The marked reduction of Ki-67^{+} memory B cells may be directly linked with the effectiveness of fingolimod in treating MS. In contrast, the relative resistance of $CD138^{+}$ plasmablasts to fingolimod may be of relevance for understanding the differential effectiveness of fingolimod in individual patients.

Keywords

B cells, CD38, CD138, fingolimod, memory B cell, multiple sclerosis, plasmablast, proliferation, resistance, sphingosine 1-phosphate receptor 1

Date received: 5 September 2013; accepted: 16 January 2014

Introduction

It is currently assumed that a large proportion of autoreactive T cells in multiple sclerosis (MS) is derived from a pool of $CCR7^{+}$ central memory T cells that are passing through the secondary lymphoid tissues (SLT).[1] Accordingly, egress of the T cells from the SLT represents a key process in MS pathogenesis. This process follows a rule of chemotaxis, in which the sphingosine 1-phosphate (S1P) receptor 1 (S1P1) expressed by lymphocytes is critically involved.[2] Fingolimod, an oral drug for treating relapsing–remitting MS (RRMS), serves as a functional antagonist for S1P1: Fingolimod induces internalisation and degradation of S1P1 in lymphocytes, causing the lymphocytes to lose the ability to respond to S1P and consequently, to become trapped in the SLT.[3] Analysis of large cohorts of patients with RRMS demonstrate the overall effectiveness of fingolimod in reducing the annualised relapse rate (ARR), as well as the appearance of new brain lesions in the patients' magnetic resonance imaging (MRI) scans.[4,5]

The number of central memory interleukin 17-producing $CD4^{+}$ T cells (Th17 cells) is reduced in the peripheral blood of fingolimod-treated patients. This is now being interpreted as a major mechanism of drug action;[6] however, fingolimod is not able to prevent relapses nor exhibit

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Table 1. Clinical data of the patients in this study.

Patient	Gender	Age (years)	Duration (years)	Relapse frequency (last 2 yrs)	EDSS	DMT before initiation of fingolimod	Complications
1	M	34	7	5	1.5	IFNβ1a + PSL	Asthma
2	M	43	6	2	2.5	PSL	Graves' disease
3	M	39	5	1	3.5	None	Depression
4	M	41	13	1	3.5	IFNβ1b	None
5	M	29	2	3	2.0	IFNβ1b	Pectus excavatum
6	F	41	24	6	3.5	IFNβ1b → GA → Dex	Depression
7	M	56	16	2	5.5	IFNβ1b → IFNβ1b + PSL → IFNβ1a + AZP	Osteoporosis
8	M	41	9	2	4.0	IFNβ1b → IFNβ1a	Depression
9	M	60	20	1	3.5	AZP → MZR → IFNβ1b	None
mean ± SD		42.7 ± 9.8	11.3 ± 7.4	2.5 ± 1.8	3.3 ± 1.2		

AZP: Azathioprine; Dex: dexamethasone; DMT: disease-modifying treatment; EDSS: Expanded Disability Status Scale; F: female; GA: glatiramer acetate; IFN: interferon; M: male; MZR: mizoribine; PSL: prednisolone.

appreciable effectiveness in all patients. In fact, recent case reports document the presence of fingolimod-treated MS patients who have developed tumefactive brain lesions, after receiving fingolimod.[7–10] Moreover, clinical worsening accompanied by large brain lesions is described in patients with neuromyelitis optica (NMO), within months of starting fingolimod.[11,12] Our current understanding of fingolimod-related biology therefore remains incomplete, particularly regarding differential effectiveness in individual patients.

Not only the presence of clonally-expanded B cells in the central nervous system (CNS),[13,14] but the efficacy of the anti-CD20 monoclonal antibody (mAb) rituximab[15] rationally indicates the involvement of B cells in the pathogenesis of MS. Therefore, B-cell migration can serve as a therapeutic target in MS, so we were prompted to investigate whether inhibition of B-cell migration may explain the differential effectiveness of fingolimod. Because the effects of fingolimod on B cells in MS have not been fully characterised,[16] we analysed the alterations of B-cell populations in fingolimod-treated RRMS patients by flow cytometry, measuring the frequencies and activation states of their peripheral blood B-cell populations.

Materials and methods

Patients and sample collection

The following subjects were enrolled in the Multiple Sclerosis Clinic of the National Centre of Neurology and Psychiatry (NCNP) in Japan:

(a) Fingolimod-naïve patients with RRMS ($n = 9$);
(b) RRMS patients who were treated with other disease-modifying treatments (DMTs) or corticosteroids ($n = 19$); and
(c) Healthy donors ($n = 3$).

All MS patients fulfilled the revised McDonald criteria.[17] Fingolimod (0.5 mg once/day) was administered to nine fingolimod-naïve patients. These patient's blood samples were collected before and 2 weeks after initiating fingolimod therapy. Most of these patients discontinued other DMTs at least 2 weeks before entry into the study, due to non-responsiveness to their DMT treatment or due to adverse events. The absence of serum anti-aquaporin 4 (AQP4)-Ab was confirmed by cell-based assays.[18,19] Upon MRI, no patient showed longitudinally-extensive spinal cord lesions extending over three or more vertebrae. The clinical data of these nine patients are summarised in Table 1.

Control blood samples were collected from 19 patients with RRMS (mean age ± SD: 41.8 ± 13.8 years; female:male ratio: 15:4) who had not been exposed to fingolimod before nor during the study. The three healthy donors were males (mean age ± SD: 40.0 ± 3.6 years). This study was approved by the Ethics Committee of the NCNP. We obtained written informed consent from all subjects.

Reagents

The following fluorescence- or biotin-labelled mAbs were used: anti-CD19-allophycocyanin (APC)-cyanine 7 (Cy7), anti-CD27-V500 and anti-CD27-phycoerythrin (PE)-Cy7 (BD Biosciences, San Jose, CA, USA); anti-CD180-PE and anti-CCR7-fluorescein isothiocyanate (FITC) (BD Pharmingen, San Jose, CA, USA); anti-CD38-FITC, anti-CD3-FITC and mouse IgG1-FITC (Beckman Coulter, Brea, CA, USA); anti-CD138-APC, mouse IgG1κ-APC, anti-HLA-DR-Pacific Blue, mouse IgG2Aκ-Pacific Blue, anti-CD183 (CXCR3)-peridinin-chlorophyll-protein (PerCp)-cyanine 5.5 (Cy5.5), mouse IgG1κ-PerCp-Cy5.5, anti-CD38-APC, anti-CD38-PerCp-Cy5.5, anti-CD14-Pacific Blue, anti-Ki-67-Brilliant Violet, mouse IgG1κ-Brilliant Violet and streptavidin-PE-Cy7 (BioLegend, San

Diego, CA, USA); and anti-CXCR4-biotin and mouse IgG2A-biotin (R&D Systems, Minneapolis, MN, USA).

Cell preparation and flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation, using Ficoll–Paque Plus (GE Healthcare Bioscience, Oakville, ON, Canada). B-cell populations were defined in reference to our previous paper,[19] as follows: total B cells, $CD19^+$; naïve B cells (nBs), $CD19^+CD27^-$; memory B cells (mBs), $CD19^+CD27^+CD180^+$; and plasmablasts (PBs), $CD19^+CD27^+CD180^-CD38^{high}$.

To evaluate the frequency and activation state of each B-cell population, PBMC were stained with anti-CD19-APC-Cy7, anti-CD27-V500, anti-CD38-FITC, anti-CD180-PE, anti-CD138-APC, anti-CXCR3-PerCp-Cy5.5, anti-CXCR4-biotin, streptavidin-PE-Cy7 and anti-HLA-DR-Pacific Blue. To assess the expression of CCR7 in each B cell population, PBMC were stained with anti-CD19-APC-Cy7, anti-CD27-PE-Cy7, anti-CD38-APC, anti-CD180-PE and anti-CCR7-FITC.

For examining Ki-67 expression in each B-cell population, PBMC were stained with anti-CD19-APC-Cy7, anti-CD27-PE-Cy7, anti-CD38-PerCp-Cy5.5, anti-CD180-PE and anti-CD138-APC, then fixed in phosphate-buffered saline (PBS) containing 2% paraformaldehyde and permeabilised with 0.1% saponin. Subsequently, these cells were stained with anti-Ki-67-Brilliant Violet. We used the appropriate isotype control antibodies as negative controls for each staining. At the end of the incubation, the cells were washed and resuspended in PBS supplemented with 0.5% bovine serum albumin (BSA) and analysed by FACS Canto II (BD Biosciences), according to the manufacturer's instructions.

Cell sorting

PBMC were labelled with CD3 and CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and then separated into positive and negative fractions by AutoMACS (Miltenyi Biotec). The positive fraction was stained with anti-CD3-FITC and anti-CD14-Pacific Blue, whereas the negative fraction was stained with anti-CD19-APC-Cy7, anti-CD27-PE-Cy7, anti-CD38-APC and anti-CD180-PE. Each positive and negative fraction was sorted into $CD3^+$ T cells and $CD14^+$ monocytes, or into nBs, mBs and PBs by a FACS Aria II cell sorter (BD Biosciences). The purity of the sorted cells was > 95%.

Quantitative real-time PCR

Messenger ribonucleic acid (mRNA) was prepared from the sorted cells using the RNeasy Kit (Qiagen, Tokyo, Japan), further treated with DNase using the RNase-Free DNase Set (Qiagen), and reverse-transcribed to complementary DNA (cDNA) using the cDNA Synthesis Kit (Takara Bio, Shiga, Japan). We performed polymerase chain reaction (PCR) using iQ SYBR Green Supermix (Takara Bio) on a LightCycler (Roche Diagnostics, Indianapolis, IN, USA). RNA levels were normalised to endogenous β-actin (ACTB) for each sample. The following primers were used: S1P1 forward, CGAGAGCACTACGCAGTCAG; and S1P1 reverse, AGAGCCTTCACTGGCTTCAG.

Data analysis and statistics

We used Diva software (BD Biosciences) to analyse our flow cytometry data. We performed the statistical analysis with Prism software (GraphPad Software, San Diego, CA, USA). Paired or unpaired *t*-tests were used once the normality of the data was confirmed by the Kolmogorov-Smirnov test. Otherwise, the Wilcoxon signed-rank test or the Mann-Whitney *U*-test was used, as appropriate. One-way analysis of variance (ANOVA) was used to compare data from more than two groups. If the one-way ANOVA was significant, we performed *post hoc* pairwise comparisons using Tukey's test. A p value < 0.05 was considered statistically significant.

Results

B-cell populations express S1P1 mRNA

First, we used flow cytometry to examine S1P1 expression on the surfaces of the B-cell populations; however, surface S1P1 was hardly detected (data not shown). This is probably because of its internalisation following S1P binding. In support of this, it is known that S1P is abundantly present in peripheral blood.[2] Thus, we measured S1P1 mRNA in purified lymphocyte populations from the PBMCs of three healthy donors. Each B-cell population was identified by flow cytometry, as shown in Figure 1(a). We found that comparable levels of S1P1 mRNA were expressed in T cells, nBs and mBs. In comparison, PBs expressed a significantly lower level of S1P1, and S1P1 expression in monocytes was virtually absent (Figure 1(b)). Of note, a lower S1P1 expression by PBs, as compared with other B cell populations, is also described in mice.[20,21] These S1P1 mRNA expression profiles suggested that not only T cells, but B-cell migration, could also be influenced by fingolimod.

Next, we measured the frequencies of the B-cell populations in the PBMCs from nine patients with RRMS, before and 2 weeks after starting fingolimod. Results of flow cytometry showed that the frequencies of nBs, mBs and PBs among PBMCs were significantly decreased after initiating fingolimod treatment (Figure 1(c)). We confirmed that the absolute numbers of each population in the peripheral blood were also significantly decreased after starting fingolimod (Figure 1(d)). The mean decrease rate ± SD of each cell population was calculated based on the absolute cell number, giving the following results: total B cells, 87.6 ± 5.8%; nBs, 88.1 ± 6.0%; mBs, 85.4 ± 9.1% and PBs, 89.8

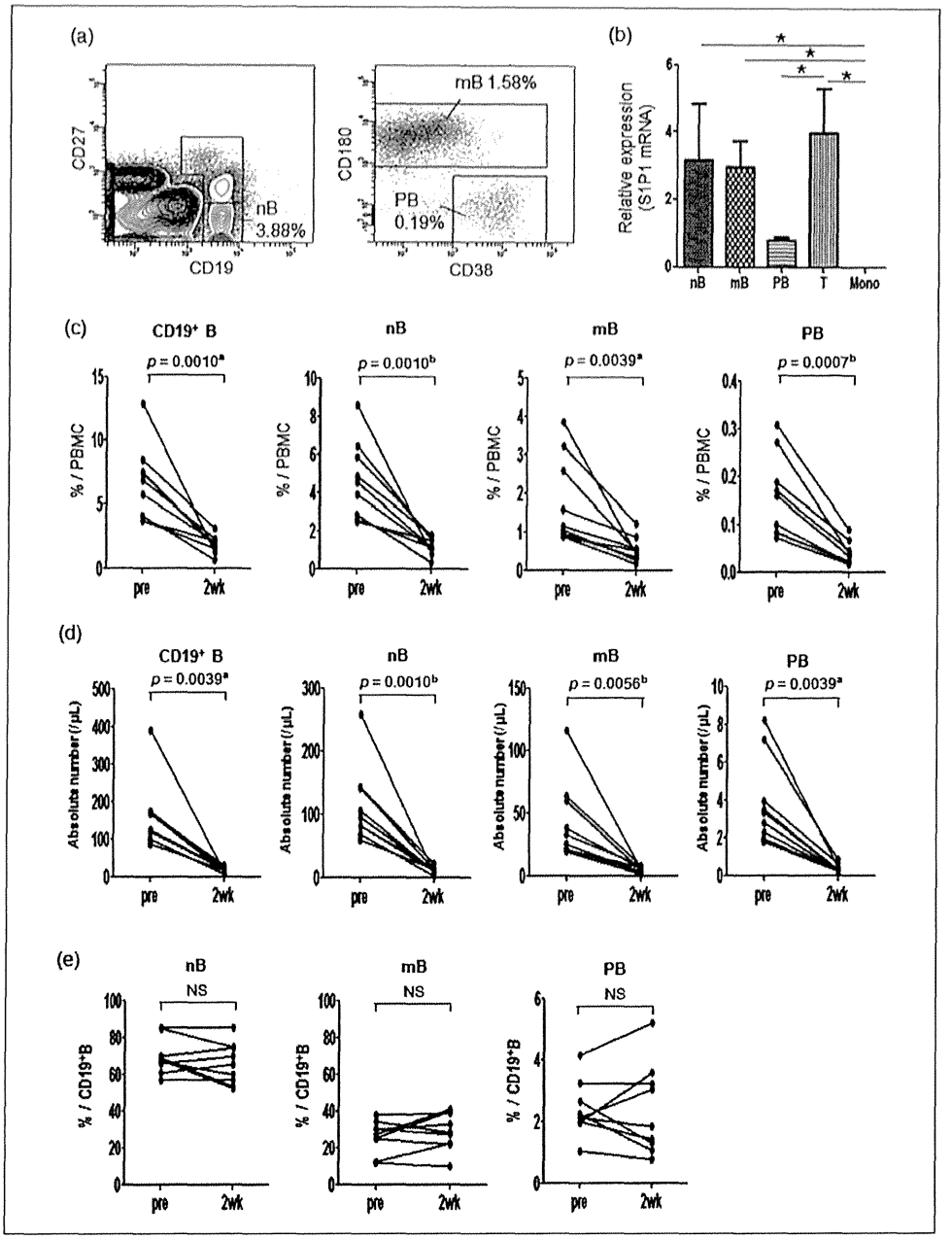

Figure 1. Frequency and absolute number of each B-cell population found in peripheral blood from MS patients.
(a)Representative flow cytometry scheme to analyse B-cell populations in PBMC. The PBMC were simultaneously stained with fluorescence-conjugated anti-CD19, -CD27, -CD38 and -CD180 mAbs. The gate for $CD19^+CD27^-$ nBs is shown in the left panel. The $CD19^+CD27^+$ fraction partitioned in the left panel was analysed for CD180 and CD38 expression to specify $CD180^+$ cells (mBs), and for $CD180^-CD38^{high}$ cells (PBs) in the right panel. Values represent frequencies of B-cell populations in PBMC. Total $CD19^+$ B cell counts were calculated by summing the frequencies of the partitioned populations in the left panel. (b) Each B-cell population, $CD3^+$ T cells and $CD14^+$ monocytes in PBMCs from three healthy donors were sorted by FACS, and S1P1 mRNA expression levels were determined by quantitative RT-PCR. Data were normalised to the amount of ACTB for each sample. Data are represented as mean relative expression ± SD. *$p < 0.05$ by one-way ANOVA and *post hoc* Tukey's test. (c), (d), and (e) Data shown are the frequencies of B-cell populations in PBMC (c), the absolute numbers of B cell populations in peripheral blood (d) and the frequencies of B-cell populations in $CD19^+$ B cells (e) from nine patients with MS before (pre) and 2 weeks after (2 wk) initiating fingolimod. Data from the same patients are connected with lines.
$p^a < 0.05$ by Wilcoxon signed-rank test.
$p^b < 0.05$ by paired *t*-test.
ACTB: endogenous beta actin; ANOVA: analysis of variance; FACS: Fluorescence-activated cell sorting; mAbs: monoclonal antibodies; mBs: memory B cells; mono: monocytes; mRNA: messenger ribonucleic acid; MS: multiple sclerosis; nBs: naïve B cells; NS: not statistically significant; PBMC: peripheral blood mononuclear cells; PBs: plasmablasts; pre: before treatment; RT-PCR: reverse transcriptase - polymer chain reaction; S1P1: sphingosine 1 phosphate receptor 1; T: T cells; 2 wk: 2 weeks after treatment initiation.

± 3.3%. Thus, all B-cell populations decreased at similar rates, regardless of their S1P1 expression levels. We also noticed that reduction of the B-cell populations did not correlate with CCR7 expression (a large proportion of nBs and mBs expresses CCR7, whereas only a small percentage of PBs expresses CCR7 (Supplementary Figure 1)). Consistently, the frequency of each B-cell population within $CD19^+$ B cells was not significantly altered in the fingolimod-treated patients (Figure 1(e)).

$CD38^{int}$- and $CD38^{high}$-activated memory B cells are preferentially decreased in fingolimod-treated patients

We next assessed mBs, which are assumed to play an important role in MS.[22,23] To evaluate the effects of fingolimod on the activation state of mBs, we first analysed CD38 expression of mBs in the nine patients, before and after initiating fingolimod. CD38 is a marker that is upregulated upon B-cell activation.[24] We found that mBs could be classified into three subpopulations according to CD38 expression levels ($CD38^{low}$, $CD38^{int}$ and $CD38^{high}$). Notably, frequencies of $CD38^{int}$ and $CD38^{high}$ mBs were significantly decreased 2 weeks after initiating fingolimod, whereas the frequency of the $CD38^{low}$ subpopulation became significantly increased (Figure 2(a) and (b)).

We further examined the expression of another activation marker, HLA-DR, within the $CD38^{low}$, $CD38^{int}$ and $CD38^{high}$ mB subpopulations. We found that the $CD38^{high}$ subpopulation expressed a significantly higher level of HLA-DR, compared with the $CD38^{low}$ mB population, as assessed by mean fluorescence intensities (MFIs) (Figure 2(c) and (d)). Although not statistically significant, HLA-DR expression in the $CD38^{int}$ subpopulation was intermediate, compared with that in the $CD38^{low}$ mB subpopulation. We also found that the MFIs of forward scatter (FSC), which reflects cell size, were significantly higher in the $CD38^{high}$ subpopulation, compared with the $CD38^{low}$ and $CD38^{int}$ subpopulations (Figure 2(c) and (d)). These findings suggest that $CD38^{high}$ mBs may contain a larger number of recently-activated blastic cells.

Fingolimod reduced Ki-67^+ recently-activated memory B cells in peripheral blood

The nuclear antigen Ki-67 is exclusively expressed in the active stages of the cell cycle (G1, S, G2 and M phases),[25] and Ki-67^+ circulating immune cells are considered to be recently activated cells that have just egressed from the SLT. To clarify whether $CD38^{high}$ and $CD38^{int}$ mB subpopulations are enriched for recently-activated cells, we examined the frequency of Ki-67^+ cells in each mB subpopulation, in the six MS patients who were not treated with fingolimod. This analysis revealed that $CD38^{high}$ mBs contained a significantly higher frequency of Ki-67^+ cells than did $CD38^{low}$ and $CD38^{int}$ mBs, and that $CD38^{int}$ mBs were likely to contain a higher frequency of Ki-67^+ cells than the $CD38^{low}$ mBs (Figure 3(a) and (b)). In addition, we compared the frequency of Ki-67^+ cells in each mB subpopulation, between fingolimod-treated (n = 5) and -untreated control patients (n = 6), and found that $CD38^{int}$ and $CD38^{high}$ mBs of the fingolimod-treated patients contained a significantly lower percentage of Ki-67^+ cells compared with those of the untreated patients (Figure 3(c)). These findings suggest that recently activated mBs are enriched in $CD38^{int}$ and $CD38^{high}$ subpopulations and that fingolimod efficiently blocks the egress of these cells from the SLT into the peripheral circulation.

The $CD138^+$ subpopulation in plasmablasts is relatively resistant to fingolimod

Finally, we analysed alterations of PBs by fingolimod in more detail. As PBs serve as migratory B cells that produce pathogenic autoantibody directed against AQP4,[19] their role in the antibody-mediated pathology is being considered also in the pathogenesis of MS. Notably, CD138 expression appears to separate PB subpopulations that could become differentially altered during the inflammatory process. In fact, $CD138^+$ PBs have a higher potential to migrate to inflamed tissues than $CD138^-$ PBs.[26] Moreover, as has recently been reported by us, $CD138^+HLA\text{-}DR^+$ PBs are selectively enriched in the cerebrospinal fluid (CSF) during relapse of NMO, and the $CD138^+HLA\text{-}DR^+$ PBs migrating to the CSF express CXCR3.[27] Therefore, we compared the frequencies of $CD138^+$ cells in PBs, as well as their expression of HLA-DR and CXCR3, before and after fingolimod treatment.

We found that the frequencies of $CD138^+$ PBs among total PBs were significantly increased after fingolimod initiation (Figure 4(a) and (b)); however, the absolute numbers of both subpopulations decreased, implying that $CD138^+$ PBs are relatively resistant to fingolimod, compared with $CD138^-$ PBs (Supplementary Figure 2(a) and (b)). After initiating fingolimod, $CD138^-$ PBs showed lower expression of HLA-DR, whereas the percentages of $CXCR3^+$ cells remained unchanged (Figure 4(c) – (e)). In contrast, fingolimod treatment did not significantly reduce the expression level of HLA-DR among $CD138^+$ PBs. More interestingly, $CD138^+$ PBs became more enriched with $CXCR3^+$ cells after initiating fingolimod (Figure 4(c) – (e)). The definition of PBs as $CD19^+CD27^+CD180^-CD38^{high}$ cells in this study was modified to efficiently specify autoantibody-producing cells;[19] however, adopting a more commonly used definition of PBs as $CD19^+CD27^+CD38^{high}$ cells did not alter the results (Supplementary Figure 3(a) – (e)).

Discussion

Previous studies show that fingolimod markedly decreases the number of T and B cells in the peripheral blood, without

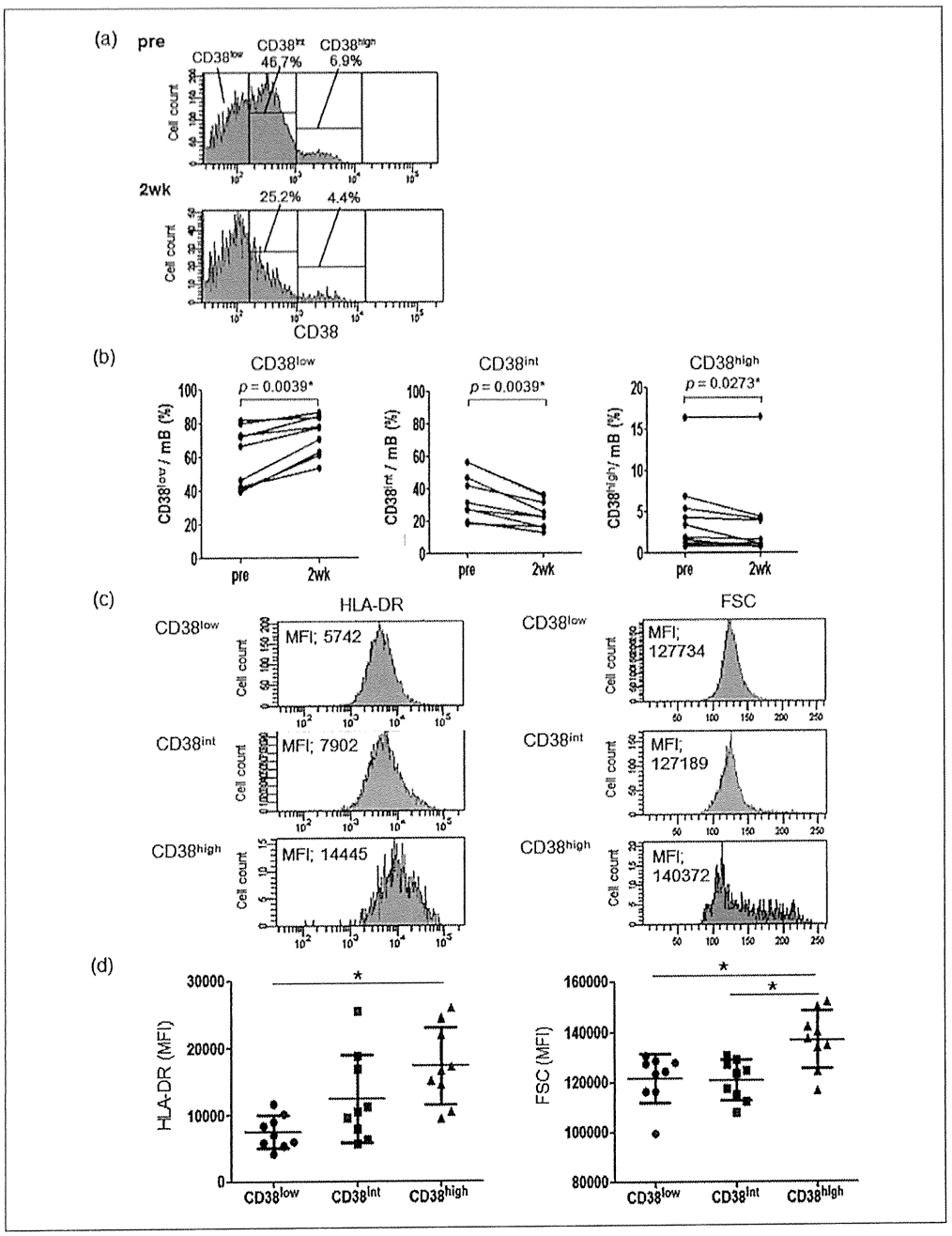

Figure 2. Frequency and activation state of each mB subpopulation in the peripheral blood of MS patients.
(a)Representative histograms of CD38 expression in mB of peripheral blood from a fingolimod-treated patient. Upper (pre) and lower (2wk) panels show the histograms before and 2 weeks after fingolimod initiation, respectively. The two values above each histogram indicate frequencies of the mB subpopulations with intermediate ($CD38^{int}$, left) and high ($CD38^{high}$, right) CD38 expression. (b) Data shown are frequencies of mB subpopulations, classified by CD38 expression levels ($CD38^{low}$ (left panel), $CD38^{int}$ (middle panel) and $CD38^{high}$ (right panel)), in the peripheral blood from nine patients with MS, before (pre) and 2 weeks after (2wk) fingolimod initiation. Data from the same patients are connected with lines. *$p < 0.05$ by Wilcoxon signed-rank test. (c) Representative histograms of HLA-DR (left column) and FSC (right column) expression in each mB subpopulation ($CD38^{low}$ (upper row), $CD38^{int}$ (middle row) and $CD38^{high}$ (lower row)) of peripheral blood from a patient with MS, before fingolimod initiation. Values represent MFIs of HLA-DR and FSC. (d) Data shown are MFI of HLA-DR (left panel) and FSC (right panel) in mB subpopulations ($CD38^{low}$, $CD38^{int}$ and $CD38^{high}$) of peripheral blood from nine patients with MS, before fingolimod treatment. Data are represented as mean ± SD.
*$p < 0.05$ by one-way ANOVA and *post hoc* Tukey's test.
ANOVA: analysis of variance; FSC: forward scatter; HLA: human leukocyte antigen; mB: memory B cells; MFI: mean fluorescence intensity; MS: multiple sclerosis; pre: before treatment; 2wk: 2 weeks after treatment initiation.

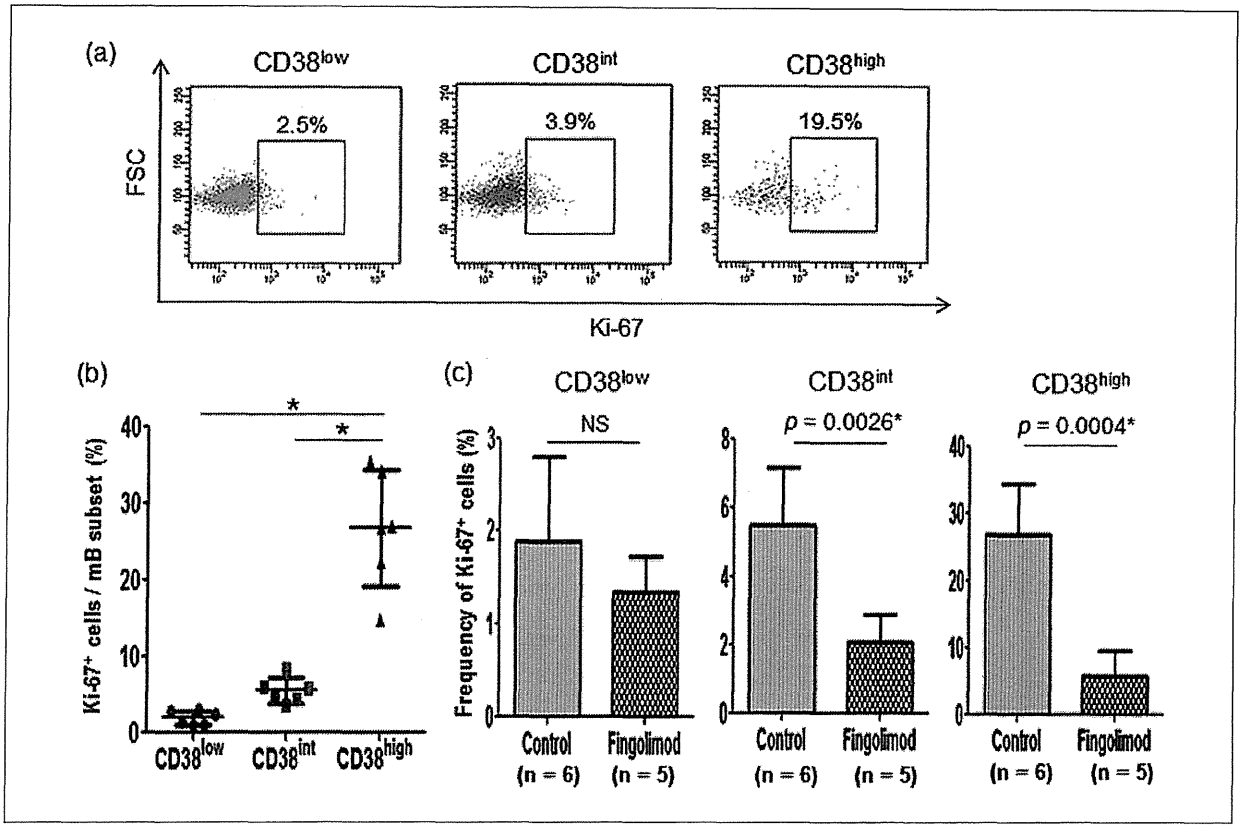

Figure 3. Ki-67 expression in mB subpopulations of peripheral blood from MS patients.
(a)Representative flow cytometry analyses of intracellular Ki-67 expression in mB subpopulations ($CD38^{low}$ (left panel), $CD38^{int}$ (middle panel), and $CD38^{high}$ (right panel)) of peripheral blood from an untreated patient with MS. Each mB subpopulation was analysed for FSC and Ki-67 expression. Values in each plot represent frequency of $Ki\text{-}67^+$ cells in each mB subpopulation. (b) Frequency of $Ki\text{-}67^+$ cells in each mB subpopulation of peripheral blood from six untreated patients with MS. Data are represented as mean ± SD. *$p < 0.05$ by one-way ANOVA and *post hoc* Tukey's test. (c) Frequency of the $Ki\text{-}67^+$ population in each mB subpopulation ($CD38^{low}$ (left panel), $CD38^{int}$ (middle panel), and $CD38^{high}$ (right panel)) is compared between untreated patients with MS (control; $n = 6$) and fingolimod-treated patients with MS (Fingolimod; $n = 5$). Mean duration with fingolimod treatment ± SD is 15.8 ± 8.8 (6 to 30) weeks. Data are represented as mean ± SD.
*$p < 0.05$ by unpaired *t*-test.
FSC: forward scatter; Ki-67: a marker present only during cell growth or proliferation; mB: memory B cells; MS: multiple sclerosis; NS: not statistically significant.

affecting the total numbers of monocytes and natural killer (NK) cells.[16,28,29] Furthermore, in MS, fingolimod selectively reduces naïve T cells, as well as $CD4^+$ central memory T cells that are enriched for Th17 cells.[6,30] In addition, fingolimod treatment may induce a relative increase in $CD27^-CD28^-$ $CD8^+$ T cells[31] and a decrease in $CD56^{bright}CD62L^+CCR7^+$ NK cells.[32]

The role of autoreactive $CD4^+$ T cells in MS pathogenesis has been emphasised over decades.[33] In contrast, B-cell involvement in MS was highlighted lately, after the clinical effectiveness of rituximab was demonstrated in RRMS patients. Rituximab's effectiveness in MS may result from the depletion of autoantibody-producing B cells, but it can also be explained by depletion of B cells that are able to induce or support activation of autoreactive T cells.[15] In fact, B cells exhibit the ability to present antigen to T cells, and mBs are more capable than nBs of supporting the proliferation of neuroantigen-specific $CD4^+$ T cells, in vitro.[23] The presence of oligoclonal bands in the CSF suggests local production of antibodies within the CNS.[34] Consistent with this, brain lesions[13] and CSF[14] of patients with MS contain clonally-expanded B cells. These results collectively support the postulate that mBs can potentially trigger the inflammation of MS, either via autoantibody production or via autoantigen presentation to autoreactive T cells.

The focus of this study is to investigate the alterations of peripheral blood B-cell types in fingolimod-treated patients with RRMS. We showed that activated $CD38^{int}$ and $CD38^{high}$ mB subpopulations were highly susceptible to

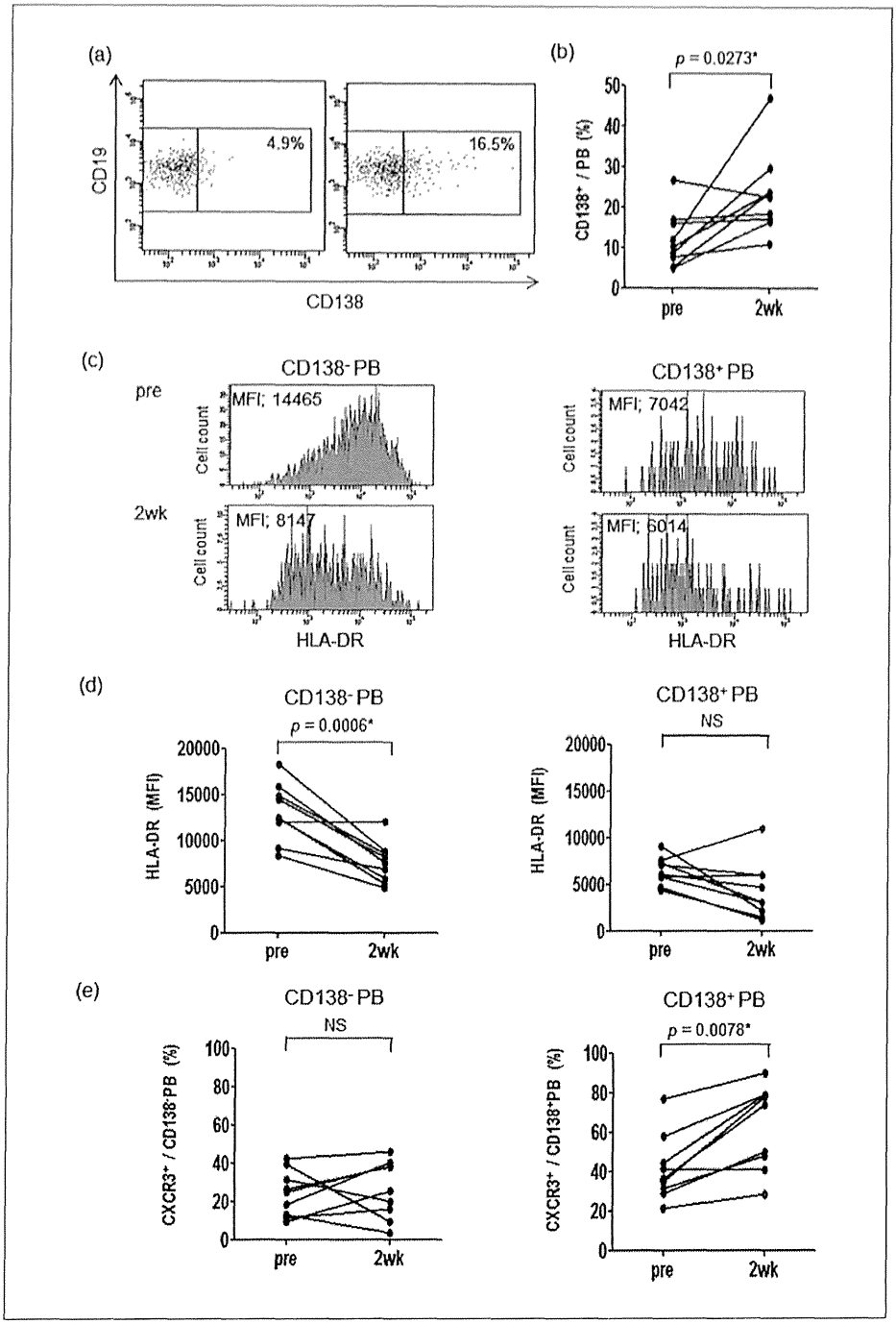

Figure 4. Phenotypic alteration of the remaining PBs in peripheral blood following fingolimod treatment.
(a)Representative dot plots of $CD19^+CD27^+CD180^-CD38^{high}$ PB, analysed for CD19 and CD138 expression before (pre) and 2 weeks after (2wk) fingolimod initiation. Values represent frequencies of the $CD138^+$ subpopulation in total PB. (b) Data are frequencies of the $CD138^+$ subpopulation in total PB of peripheral blood from nine patients with MS before (pre) and 2 weeks after (2wk) fingolimod initiation. Data from the same patients are connected with lines. *$p < 0.05$ by Wilcoxon signed-rank test. (c) Data are representative histograms of HLA-DR expression in $CD138^-$ and $CD138^+$ PB of peripheral blood, from a patient with MS before (pre) and 2 weeks after (2wk) fingolimod initiation. Values represent MFI of HLA-DR. (d) Data are MFI of HLA-DR in $CD138^-$ and $CD138^+$ PB of peripheral blood from nine patients with MS, before (pre) and 2 weeks after (2wk) fingolimod initiation. Data from the same patients are connected with lines. *$p < 0.05$ by paired *t*-test. (e) Data are frequencies of $CXCR3^+$ cells in $CD138^-$ PB and $CD138^+$ PB of peripheral blood from nine patients with MS before (pre) and 2 weeks after (2wk) fingolimod initiation. Data from the same patients are connected with lines. *$p < 0.05$ by Wilcoxon signed-rank test.
MFI: mean fluorescence intensity; MS: multiple sclerosis; NS: not statistically significant; PB: plasmablast; pre: before treatment; 2wk: after 2 weeks of treatment.

fingolimod, as indicated by their reduction in the peripheral blood following fingolimod treatment. It is demonstrated in mice that surface expression levels of S1P1 on B cells in the SLT are controlled by transcription levels and CD69-mediated internalisation of S1P1. Stimulation of B-cell receptors induces not only a cessation of S1P1 transcription, but also an upregulation of CD69. Both of these changes reduce the expression levels of surface S1P1 in the SLT to some extent.[2]

Although we were not able to directly analyse B cells in the SLT of the patients, we speculated that surface S1P1 expression on mBs within the SLT in human may also decrease greatly, following antigen activation and exposure to fingolimod, which would result in these B lymphocytes having a reduced responsiveness to S1P. In fact, the activated mB subpopulations that we isolated from the patients' peripheral blood, in particular $CD38^{high}$ mB, were found to contain a substantial proportion of $Ki\text{-}67^+$ cells (Figure 3(a) and (b)). We confirmed that the proportions of $Ki\text{-}67^+$ cells in the activated $CD38^{int}$ and $CD38^{high}$ mB subpopulations were significantly decreased following fingolimod treatment, suggesting that recently-activated cells were selectively trapped in the SLT following fingolimod treatment. Because activation of autoreactive mBs in the SLT followed by their migration to the CNS could trigger a relapse of RRMS,[35] we assumed that inhibition of activated mB cell egress from the SLT was at least partly involved in the reduced relapses of RRMS after fingolimod treatment.

We also identified a PB subpopulation that is relatively resistant to fingolimod as being $CD138^+$ PBs. The frequency of the $CD138^+$ subpopulation in the total PBs, and that of $CXCR3^+$ cells in $CD138^+$ PBs, was significantly increased by fingolimod treatment. Of note, the $CD138^+CXCR3^+$ PBs are enriched in the CSF of NMO during relapse,[27] and fingolimod could induce exacerbation of NMO, accompanied by the appearance of large brain lesions.[11,12] Although knowledge on the biology of PBs is limited, the percentages of $CCR7^+$ cells are much lower as compared with nBs or mBs, indicating that fingolimod may differentially alter the in vivo migration of PBs and other B cells.

It is of relevance to note that despite reductions of circulating lymphocytes, RRMS patients receiving fingolimod may develop clinical relapses. These relapses are not always mild, but could be serious and accompany huge brain lesions.[7–10] Although the trapping of regulatory lymphocytes in the SLT[8,9] or the enrichment for $CD45RO^-CCR7^-CD8^+$ T cells in the CSF[7] is proposed as a possible mechanism for formation of tumefactive brain lesions, we were very curious to know if the increased proportion of $CD138^+$ PBs over other lymphocytes in the peripheral blood might influence the character of the CNS pathology and induce large demyelinating lesions. In fact, it was recently reported that $CD45^+CD19^+CD138^+$ PBs are relatively enriched in the CSF of fingolimod-treated MS patients,[16] raising the possibility that the dominance of $CD138^+$ PBs in the peripheral blood is preserved or even promoted in the CNS of patients with MS who develop tumefactive brain lesions[7–10] and NMO patients who deteriorate[11,12] after being treated with fingolimod. Therefore, resistance of activated PBs in fingolimod-treated patients with MS or NMO may give us a clue to understanding the individual patients' differences regarding the effectiveness of fingolimod therapy.

Acknowledgements

We thank Toshiyuki Takahashi at the Department of Neurology, Tohoku University, for examining serum anti-AQP4-Abs in our patients. We also thank Hiromi Yamaguchi, Yasuko Hirakawa, and Tomoko Ozawa for their technical support.

Conflict of interest

The authors declare that there are no conflicts of interest.

Funding

This work was supported by the Ministry of Health, Labour and Welfare of Japan (grant on intractable neuroimmunological diseases number H23-nanchi-ippan-017); and the Japanese Society for the Promotion of Science (grant number: S24229006).

References

1. Kivisakk P, Mahad DJ, Callahan MK, et al. Expression of CCR7 in multiple sclerosis: Implications for CNS immunity. *Ann Neurol* 2004; 55: 627–638.
2. Cyster JG and Schwab SR. Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. *Ann Rev Immunol* 2012; 30: 69–94.
3. Cohen JA and Chun J. Mechanisms of fingolimod's efficacy and adverse effects in multiple sclerosis. *Ann Neurol* 2011; 69: 759–777.
4. Kappos L, Radue EW, O'Connor P, et al. A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. *N Engl J Med* 2010; 362: 387–401.
5. Cohen JA, Barkhof F, Comi G, et al. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. *N Engl J Med* 2010; 362: 402–415.
6. Mehling M, Lindberg R, Raulf F, et al. Th17 central memory T cells are reduced by FTY720 in patients with multiple sclerosis. *Neurology* 2010; 75: 403–410.
7. Pilz G, Harrer A, Wipfler P, et al. Tumefactive MS lesions under fingolimod: A case report and literature review. *Neurology* 2013; 81: 1654–1658.
8. Jander S, Turowski B, Kieseier BC, et al. Emerging tumefactive multiple sclerosis after switching therapy from natalizumab to fingolimod. *Mult Scler* 2012; 18: 1650–1652.
9. Visser F, Wattjes MP, Pouwels PJ, et al. Tumefactive multiple sclerosis lesions under fingolimod treatment. *Neurology* 2012; 79: 2000–2003.
10. Leypoldt F, Munchau A, Moeller F, et al. Hemorrhaging focal encephalitis under fingolimod (FTY720) treatment: A case report. *Neurology* 2009; 72: 1022–1024.

11. Izaki S, Narukawa S, Kubota A, et al. [A case of neuromyelitis optica spectrum disorder developing a fulminant course with multiple white-matter lesions, following fingolimod treatment]. *Rinsho Shinkeigaku* 2013; 53: 513–517.
12. Min JH, Kim BJ and Lee KH. Development of extensive brain lesions following fingolimod (FTY720) treatment in a patient with neuromyelitis optica spectrum disorder. *Mult Scler* 2012; 18: 113–115.
13. Baranzini SE, Jeong MC, Butunoi C, et al. B-cell repertoire diversity and clonal expansion in multiple sclerosis brain lesions. *J Immunol* 1999; 163: 5133–5144.
14. Qin Y, Duquette P, Zhang Y, et al. Clonal expansion and somatic hypermutation of V(H) genes of B cells from cerebrospinal fluid in multiple sclerosis. *J Clin Invest* 1998; 102: 1045–1050.
15. Hauser SL, Waubant E, Arnold DL, et al. B-cell depletion with rituximab in relapsing–remitting multiple sclerosis. *N Engl J Med* 2008; 358: 676–688.
16. Kowarik MC, Pellkofer HL, Cepok S, et al. Differential effects of fingolimod (FTY720) on immune cells in the CSF and blood of patients with MS. *Neurology* 2011; 76: 1214–1221.
17. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol* 2011; 69: 292–302.
18. Takahashi T, Fujihara K, Nakashima I, et al. Establishment of a new sensitive assay for anti-human aquaporin-4 antibody in neuromyelitis optica. *Tohoku J Exp Med* 2006; 210: 307–313.
19. Chihara N, Aranami T, Sato W, et al. Interleukin 6 signaling promotes anti-aquaporin 4 autoantibody production from plasmablasts in neuromyelitis optica. *Proc Natl Acad Sci USA* 2011; 108: 3701–3706.
20. Gohda M, Kunisawa J, Miura F, et al. Sphingosine 1-phosphate regulates the egress of IgA plasmablasts from Peyer's patches for intestinal IgA responses. *J Immunol* 2008; 180: 5335–5343.
21. Kabashima K, Haynes NM, Xu Y, et al. Plasma cell S1P1 expression determines secondary lymphoid organ retention versus bone marrow tropism. *J Exp Med* 2006; 203: 2683–2690.
22. Corcione A, Casazza S, Ferretti E, et al. Recapitulation of B-cell differentiation in the central nervous system of patients with multiple sclerosis. *Proc Natl Acad Sci USA* 2004; 101: 11064–11069.
23. Harp CT, Ireland S, Davis LS, et al. Memory B cells from a subset of treatment-naive relapsing–remitting multiple sclerosis patients elicit CD4(+) T-cell proliferation and IFN-gamma production in response to myelin basic protein and myelin oligodendrocyte glycoprotein. *Eur J Immunol* 2010; 40: 2942–2956.
24. Ruffin N, Lantto R, Pensieroso S, et al. Immune activation and increased IL-21R expression are associated with the loss of memory B cells during HIV-1 infection. *J Intern Med* 2012; 272: 492–503.
25. Gerdes J, Lemke H, Baisch H, et al. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 1984; 133: 1710–1715.
26. Odendahl M, Mei H, Hoyer BF, et al. Generation of migratory antigen-specific plasma blasts and mobilization of resident plasma cells in a secondary immune response. *Blood* 2005; 105: 1614–1621.
27. Chihara N, Aranami T, Oki S, et al. Plasmablasts as migratory IgG-producing cells in the pathogenesis of neuromyelitis optica. *PLoS One* 2013; 8: e83036.
28. Budde K, L Schmouder R Nashan B, et al. Pharmacodynamics of single doses of the novel immunosuppressant FTY720 in stable renal transplant patients. *Am J Transpl* 2003; 3: 846–854.
29. Vaessen LM, Van Besouw NM, Mol WM, et al. FTY720 treatment of kidney transplant patients: A differential effect on B cells, naive T cells, memory T cells and NK cells. *Transpl Immunol* 2006; 15: 281–288.
30. Mehling M, Brinkmann V, Antel J, et al. FTY720 therapy exerts differential effects on T-cell subsets in multiple sclerosis. *Neurology* 2008; 71: 1261–1267.
31. Johnson TA, Lapierre Y, Bar-Or A, et al. Distinct properties of circulating CD8+ T cells in FTY720-treated patients with multiple sclerosis. *Arch Neurol* 2010; 67: 1449–1455.
32. Johnson TA, Evans BL, Durafourt BA, et al. Reduction of the peripheral blood CD56(bright) NK lymphocyte subset in FTY720-treated multiple sclerosis patients. *J Immunol* 2011; 187: 570–579.
33. Nylander A and Hafler DA. Multiple sclerosis. *J Clin Invest* 2012; 122: 1180–1188.
34. Meinl E, Krumbholz M and Hohlfeld R. B-lineage cells in the inflammatory central nervous system environment: Migration, maintenance, local antibody production and therapeutic modulation. *Ann Neurol* 2006; 59: 880–892.
35. Von Budingen HC, Bar-Or A and Zamvil SS. B cells in multiple sclerosis: Connecting the dots. *Curr Opin Immunol* 2011; 23: 713–720.