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Impact of coding risk variant IFNGR2 on the B cell-intrinsic IFN-γ signaling pathway in multiple sclerosis

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\textbf{A R T I C L E   I N F O}

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\textbf{A B S T R A C T}

B cells of people with multiple sclerosis (MS) are more responsive to IFN-γ, corresponding to their brain-homing potential. We studied how a coding single nucleotide polymorphism (SNP) in IFNGR2 (rs9808753) co-operates with Epstein-Barr virus (EBV) infection as MS risk factors to affect the IFN-γ signaling pathway in human B cells. In both cell lines and primary cells, EBV infection positively associated with IFN-γ receptor expression and STAT1 phosphorylation. The IFNGR2 risk SNP selectively promoted downstream signaling via STAT1, particularly in transitional B cells. Altogether, EBV and the IFNGR2 risk SNP independently amplify IFN-γ signaling, potentially driving B cells to enter the MS brain.

1. Introduction

In many autoimmune diseases, both central and peripheral B-cell tolerance checkpoints are impaired [1], resulting in the escape of autoreactive, naive B cells that enter the circulation. Strikingly, in people with multiple sclerosis (MS), an inflammatory disease of the central nervous system (CNS), only defects in peripheral B-cell selection have been found [1,2]. In addition, B cells of people with MS are highly sensitive to IFN-γ, a trigger that mediates escape from peripheral tolerance [3,4] and promotes differentiation into memory subsets expressing T-box transcription factor T-bet [5]. Probably as a result, these types of B cells infiltrate the CNS to mature into antibody-secreting cells (mostly IgG) and contribute to MS pathology [5–8]. IFN-γ induces T-bet transcription through dimerization of its receptor subunits IFNGR1 (α-chain) and IFNGR2 (β-chain), which is followed by phosphorylation of downstream signaling molecule STAT1 [9–12]. Importantly, while IFNGR1 is required for ligand binding, IFNGR2 is crucial for initiating downstream activity [9,10]. It is currently unclear how the IFN-γ signaling pathway in B cells is affected and contributes to enhanced IFN-γ responsiveness in MS.

For the development of MS, B cells are likely affected by a complex interaction between genetic and environmental risk factors [13–16]. Results from large genome-wide association studies (GWAS) indicate that one of the few coding risk single nucleotide polymorphisms (SNPs) for MS is located in IFNGR2 (rs9808753; A > G) [17,18]. It causes a change in amino acid, although its functional implications are not known. Furthermore, there is a 32-fold increased risk to develop MS after infection with the Epstein-Barr virus (EBV) [14], a B-lymphotropic human herpesvirus that can interact with risk variants either in a direct or indirect manner [16,19]. Notably, the presence of rs9808753 is associated with a lower abundance of EBV in human B-cell lines [20]. Here, we elaborated on the enhanced IFN-γ responsiveness of B cells in MS by exploring the impact of the IFNGR2 risk SNP and EBV on the IFN-γ signaling pathway, both in human B-cell lines and blood B cells from healthy and MS donors.

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\textsuperscript{3} Shared contribution.

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Abbreviations

B-LCL B lymphoblastoid cell line
CNS central nervous system
EBV Epstein-Barr virus
eQTL expression quantitative trait loci
FACS fluorescence activated cell sorting
IFNGR interferon-gamma receptor
MACS magnetic activated cell sorting
MS multiple sclerosis
PBMC peripheral blood mononuclear cell
cPCR quantitative polymerase chain reaction
RQ-PCR real-time quantitative reverse transcriptase polymerase chain reaction
SNP single nucleotide polymorphism
STAT1 signal transducer and activator of transcription 1
T-bet T-box transcription factor

2. Materials and methods

2.1. Human B-cell lines and patients

To uncover the effect of EBV infection and IFNGR2 risk SNP rs9808753 on IFN-γ signaling in B cells, we analyzed IFN-γ receptor, (p-)STAT1 and T-bet expression profiles in human B-cell lines and primary B cells. 19 different human B lymphoblastoid cell lines (B-LCLs), authenticated by cytotgenetics and tested for mycoplasma contamination, were analyzed for EBV load and the presence of rs9808753 (Table S1). We selected 4 B-LCLs (Karpas-422, MC116, EHEB and JVM13) based on the presence (homozygote risk: GG) or absence (non-risk: AA) of rs9808753 genotyping could not be performed. An additional cohort of untreated early MS patients, samples were genotyped on the Immuno-Chip (Illumina) as described previously [18].

2.4. EBV load determination

As reported previously [15], DNA was isolated from B-LCLs and used for a multiplex real-time quantitative reverse transcriptase polymerase chain reaction (RQ-PCR). EBV load was measured with a QuantStudio™ 5 Real-Time PCR Systems (Thermo Fisher Scientific). Data were analyzed with QuantStudio (Thermo Fisher Scientific) using the B-LCL Namalwa as the standard curve.

2.5. Fluorescence activated cell sorting (FACS) and Epstein-Barr virus (EBV) infection

Naïve mature B cells (CD3-CD19+CD38low/−/CD27−IgD−) were sorted from PBMCs of healthy controls with a FACS Aria™ Fusion cell sorter (BD Biosciences) using fluorochrome-labeled monoclonal anti-human antibodies (Table S2). In order to infect with EBV, naïve mature B cells were incubated with EBV (strain B95.8) and 2.5 μg/mL CpG-ODN 2006-G5 (InvivoGen via Bio-Connect) for 3 h at 37 °C and 5 % CO2 [22]. Afterwards, the cells were cultured for 6 weeks in the presence of 2.5 μg/mL CpG-ODN 2006-G5 in RPMI supplemented with 10 % FCS.

2.6. Phosphoflow

B-LCLs and primary B cells, risk and non-risk evenly distributed across experiments, were stimulated and stained in 96-wells round-bottom plates. We seeded 1-5x10^5 cells in 50 μL RPMI with 2 % FCS in each well on ice. For stimulation, the plate was placed in a water bath at 37 °C. 100 ng/ml human recombinant IFN-γ (PeproTech via Tebu-Bio) was added for 10 min and the cells were directly placed on ice afterwards. Subsequently, cells ± IFN-γ stimulation were washed and stained extracellularly for 30 min at 4 °C in the dark using fluorochrome-labeled monoclonal anti-human antibodies (Table S3). Then, the cells were washed and stained with fixable viability dye (Zombie-NIR, Biolegend) for 10 min at 4 °C in the dark. Afterwards, fixation and permeabilization were done using the Transcription Factor Phospho Buffer Set (BD Biosciences) according to the manufacturer’s protocol. The cells were stained intracellularly for p-STAT1 (Tyr701, AF647, Cell Signaling Technologies) for 30 min at 4 °C in the dark (Table S3). Stained cells were measured with a 5-laser Cytek Aurora flow cytometer (Cytek Biosciences). Data analysis was done using the OMIQ software from Dotmatics (www.omiq.ai, www.dotmatics.com).

2.7. Multicolor flow cytometry

B-LCLs and primary B cells, risk and non-risk evenly distributed
across experiments, were stained in 96-wells round-bottom plates. We seeded 0.5–2*10^5 cells in each well and stained them with fixable viability dye (eFluor520, Invitrogen) for 15 min at 4 °C in the dark. Then, the cells were washed and stained extracellularly using fluorochrome-labeled monoclonal anti-human antibodies (Table S4). Afterwards, fixation and permeabilization were done using the eBioscience™ Foxp3 Transcription Factor Staining Buffer Set (Invitrogen) according to the manufacturer’s protocol. The cells were stained intracellularly for IFNγR1 (PE, Biolegend) and IFNγR2 (PE, Biolegend) for 45 min at 4 °C in the dark (Table S4). Stained cells were measured with a LSRII-Fortessa flow cytometer (BD Biosciences). Data analysis was done using the OMIQ software from Dotmatics (www.omiq.ai, www.dotmatics.com).

2.8. Immunoblotting

B-LCLs were stimulated in 5 mL tubes. We added 1*10^6 cells in 1 mL RPMI with 20 % FCS in each tube on ice. For stimulation, 100 ng/mL human recombinant IFN-γ (PeproTech via Tebu-Bio) was added for 10 min at 37 °C. Afterwards, cells ± IFN-γ stimulation were lysed and used for immunoblotting as reported previously [21]. For IFN-γ stimulated immunoblotting, cell lysates were loaded on a 4–15 % precast polyacrylamide gel and blotting was done on a trans-blot turbo mini PVDF membrane (Bio-Rad), which was blocked in 4 % BSA. Primary antibodies used were purified mouse anti-total Stat1 N-terminus (1/STAT1, BD Biosciences), purified mouse anti-Stat1 pY701 (4a, BD Biosciences), Armenian hamster anti-human IFN-γR (2HUB159, Santa Cruz), and mouse anti-human β-actin (AC-15, Abcam). Horseradish peroxidase (HRP)-conjugated goat-anti-mouse Ig (Dako) and goat anti-hamster Ig (Jackson ImmunoResearch) were used as secondary antibodies. Protein bands were visualized using Western Lightning Plus-ECL (PerkinElmer). To further assess this in conjunction with EBV infection, we next analyzed the expression level and activity of downstream molecule STAT1 in our 4 selected B-LCLs. First, STAT1 gene expression was increased in the EBV$^{\text{high}}$ B-LCL carrying the risk SNP (Fig. S1C). Second, basal p-STAT1 levels were higher in EBV$^{\text{high}}$ compared to EBV$^{\text{low}}$ B-LCLs, as determined by phosphoflow (Fig. 1A). Third, STAT1 phosphorylation was triggered by IFN-γ, especially in EBV$^{\text{low}}$ B-LCLs, in which levels were approximately two times more induced in the presence of rs9808753 (p = 0.0003). This effect was less, but still significant in EBV$^{\text{high}}$ B-LCLs (Fig. 1A). Thus, not only STAT1, but also p-STAT1 seems to be more constitutively expressed in the presence of EBV, thereby dampening the risk SNP-related IFN-γ responsiveness. To validate these results and analyze the dependency of the risk SNP effect on total STAT1 protein, we measured both STAT1 and p-STAT1 using immunoblotting (Fig. 1B). In line with the flow cytometry data, EBV$^{\text{low}}$ B-LCLs carrying the risk SNP showed the most pronounced induction of p-STAT1/STAT1 ratios upon IFN-γ stimulation (Fig. 1B). Interestingly, p-STAT1/STAT1 ratios showed a relative increase already at baseline in B-LCLs carrying rs9808753 (Fig. 1B).

In primary B cells (Fig. 2A–B), in vitro EBV infection induced STAT1 phosphorylation (Fig. 2C), which was not influenced by the presence of rs9808753 or different between patients and controls (data not shown). In line with our B-LCL data, p-STAT1 was less induced by IFN-γ in EBV-infected B cells (Fig. 2C). At baseline, B cells showed a trend increase in p-STAT1 levels in risk versus non-risk SNP carriers (Fig. 2D). To further specify the latter difference, we subsequently analyzed the phosphorylation of STAT1 in blood B-cell subpopulations, which showed no differences in frequencies between non-risk and risk SNP carriers (Fig. S5). Interestingly, of all subpopulations analyzed, we found the highest p-STAT1 levels in transitional (early emigrant) B cells (CD38^highCD127^-), which, to our knowledge, has not been described before (Fig. 2E). These levels were even further increased in transitional B cells from MS patients carrying rs9808753, both at baseline and after IFN-γ stimulation (Fig. 2F). When discriminating between two successive stages of transitional B cell development, we found that transitional type 1 (T1; IgM$^{\text{high}}$/IgD$^{\text{dim}}$) cells expressed higher levels of p-STAT1 than transitional type 2 (T2; IgM$^{\text{dim}}$IgD$^+$) cells, irrespective of stimulation with IFN-γ (Fig. 2G) [25,26]. Accordingly, T1 versus T2 transitional B-cell ratios were associated with the increased p-STAT1 levels in risk versus non-risk SNP carriers (Fig. 2H). Counterintuitively, independent of the risk SNP, T1 transitional B cells showed the lowest IFNγR1 and IFNγR2 protein levels (Figs. S6A–D), which could possibly be explained by a more efficient internalization and recycling of the receptor complex [27].
These data imply that the risk SNP is associated with impaired differentiation of T1 (p-STAT1<sup>hi</sup>) into T2 (p-STAT1<sup>lo</sup>) transitional B cells. T1 transitional B cells have been proposed as targets of negative selection and seem to depend on STAT1 for further development in the spleen [28]. Hence, it is tempting to speculate that due to MS risk SNPs such as rs9808753, p-STAT1<sup>hi</sup> transitional B cells have an enhanced capacity to escape from selection and interact with IFN-α-producing T follicular helper (Tfh) cells to promote subsequent maturation in the periphery [2,29,30]. Transitional B cells highly express the type I IFN receptor, which may further promote the survival of autoreactive populations via another coding MS risk SNP in TYK2 [31–33]. In type I IFN-treated MS patients, changes in transitional B cells are also associated with risk of active MRI lesions [34]. Not only IFNGR, but also CD40 plays a critical role in regulating T cell-mediated B-cell tolerance in humans [35] and is downregulated on B cells of people carrying an MS risk variant in CD40 [36].

This work has some limitations. First, the used human B-cell lines and B cells of MS patients were specifically genotyped and/or selected for the risk SNP in IFNGR2, without considering any other risk variants that could impact the IFN-γ signaling pathway (see also above). Second, we do not have information on the genotype of healthy individuals who were included as a control group. However, also given the low odds ratio for individual (non-HLA) risk SNPs, we did not aim and expect to find disease-specific effects of this SNP. Third, we show a clear association of this risk SNP with changes in IFN-γ signaling, but do not prove causality. Fourth, the very low number of circulating EBV-infected B cells did not allow for a proper analysis of EBV as an independent determinant of T-bet<sup>+</sup> B cells <em>ex vivo</em>. Previously, we and others did find that T-bet<sup>+</sup> B cells have an early response to EBV infection to affect the B-cell differentiation program [37,38,40]. This could explain their propensity to become CXCR3<sup>+</sup> class-switched B cells that enter and terminally differentiate into antibody producers in the MS brain [5,7,15]. Obviously, more research is needed to further unveil this underlying B cell-restricted mechanism and how this differs in MS compared to other autoimmune diseases.

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**CRediT authorship contribution statement**

Fig. 2. EBV infection and the presence of rs9808753 induce STAT1 phosphorylation in primary B cells. (A) Peripheral blood mononuclear cells (PBMCs) were isolated from healthy controls (HC, n = 8) and natalizumab-treated MS patients genotyped and selected for the presence of rs9808753: non-risk (AA, n = 8) or risk (AG/GG, n = 8). FACS-sorted naive mature B cells were infected with EBV in vitro. Phosphorylation was performed on total and EBV-infected B cells to analyze phosphorylated STAT1 (p-STAT1) before and after IFN-γ stimulation for 10 min. (B) Within total B cells (CD19⁺CD3⁻), we analyzed transitional (CD38highCD27⁻), naive mature (CD38dim/CD27⁺IgD⁺), double negative (CD38dim/CD27⁺IgD⁻), non-switched memory (CD38dim/CD27⁺IgD/IgM⁺), and class-switched memory (CD38dim/CD27⁺IgD⁻IgM⁻) populations. (C) p-STAT1 levels in B cells from healthy control blood before and after in vitro infection with EBV. (D) p-STAT1 expression in total B cells from the blood of healthy controls (dotted line) and both non-risk and risk patients groups. (E) p-STAT1 expression in B-cell subsets from healthy control blood. (F) Dot plot illustrating p-STAT1 levels in B-cell subsets among non-risk and risk patient groups relative to healthy controls. Dot size corresponds to the fold change in p-STAT1 expression between patients and healthy controls, while dot color signifies the p-value associated with this fold change. In addition, p-STAT1 expression in total B cells from the blood of healthy controls (dotted line) and both non-risk and risk patients groups. (G) p-STAT1 expression in T1 (IgMhighIgDlow) and T2 (IgMdimIgD+) transitional B cells from all groups. (H) Correlations between T1 versus T2 ratios and p-STAT1 expression in transitional B cells. Data were analyzed with (C, D, F) Mann-Whitney U tests, (E) Kruskal Wallis and Dunn’s post-hoc tests, (G) Wilcoxon signed-rank tests, and (H) Spearman correlation coefficients. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.


Declaration of competing interest

Marvin M. van Luijn received research support from EMD Serono, Merck, Novartis, GSK and Idorsia Pharmaceutical Ltd. Joost Smolders received lecture and/or consultancy fees from Biogen, Merck, Novartis, Sanofi-Genzyme and Roche. The remaining authors declare no competing interests.
Data availability
Data will be made available on request.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2024.103279.

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