REGULATORS OF IMMUNE SYSTEM FUNCTION IN AUTOIMMUNITY AND AGING - MOLECULAR AND CELLULAR RESEARCH

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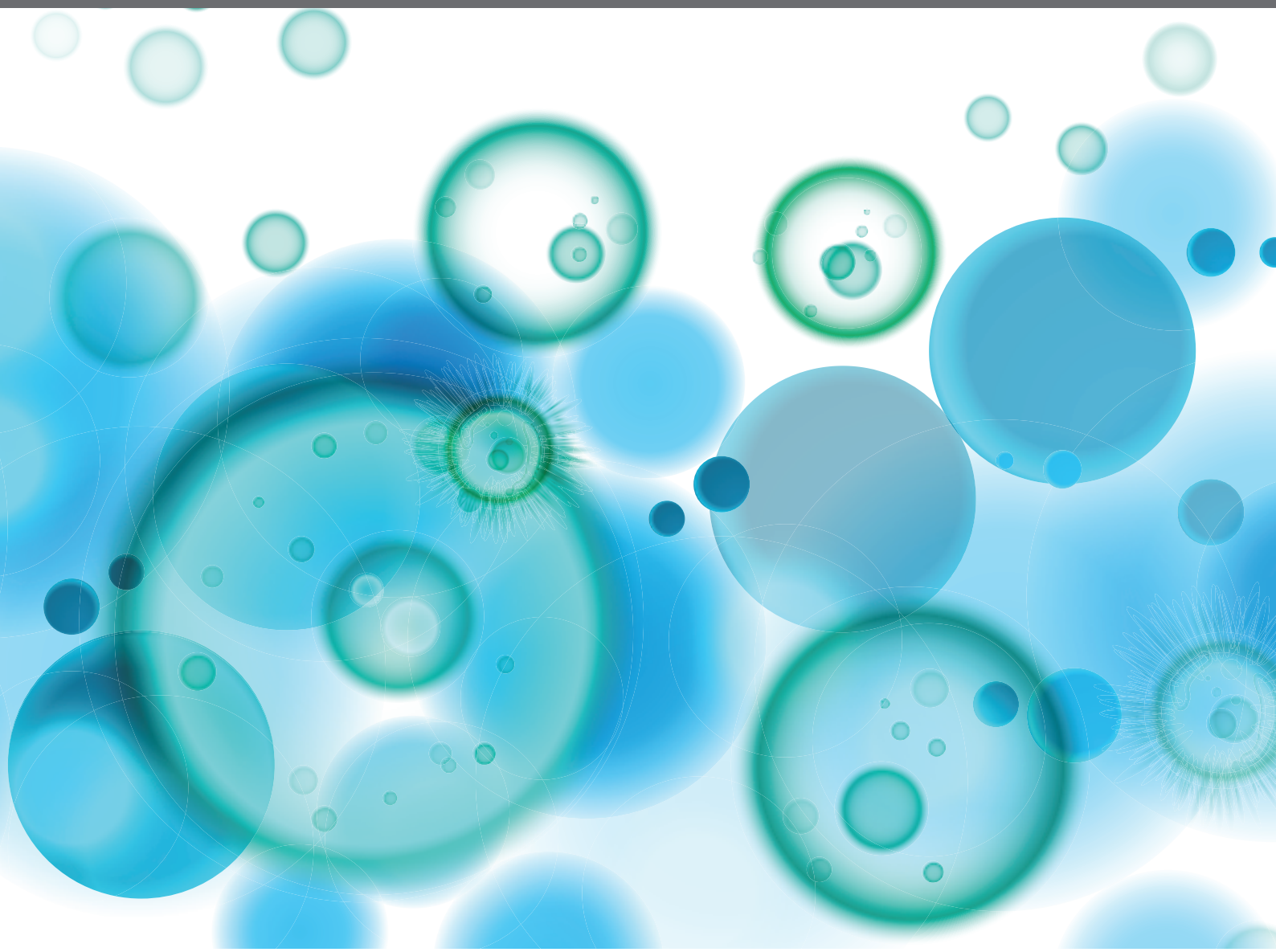

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REGULATORS OF IMMUNE SYSTEM FUNCTION IN AUTOIMMUNITY AND AGING - MOLECULAR AND CELLULAR RESEARCH

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Human PD-1^{hi}CD8^{+} T Cells Are a Cellular Source of IL-21 in Rheumatoid Arthritis

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Background: Rheumatoid arthritis (RA) is a prototypical autoantibody-driven autoimmune disease in which T-B interactions play a critical role. Recent comprehensive analysis suggests that PD-1^{+}CD8^{+} T cells as well as two distinct IL-21-producing PD-1^{+}CD4^{+} T cell subsets, follicular helper T (Tfh) and peripheral helper T (Tph) cells, are involved in the pathogenesis of RA. Herein, we aimed to clarify a generation mechanism of IL-21-producing CD8^{+} T cells in humans, and to characterize this novel subset in patients with RA.

Methods: CD8^{+} T cells in the peripheral blood (PB) and synovial fluid (SF) of healthy control (HC) and patients with RA were subject to the analysis of IL-21 mRNA and protein. We evaluated the surface marker, cytokine and transcription profiles of IL-21-producing CD8^{+} T cells in HCPB, RAPB and RASF.

Results: IL-21-producing CD8^{+} T cells were enriched in the CD45RA^{-}(memory) PD-1^{+}, especially PD-1^{hi} subpopulation, and IL-12 and IL-21 synergistically induced IL-21 production by naïve CD8^{+} T cells. Memory PD-1^{hi}CD8^{+} T cells in HCPB facilitated plasmablast differentiation and IgG production in an IL-21-dependent manner. In addition, PD-1^{hi}CD8^{+} T cells in RASF and RAPB produced large amounts of IL-21 and were characterized by high levels of CD28, ICOS, CD69, HLA-DR, and CCR2 but not CXCR5. Furthermore, PD-1^{hi}CD8^{+} T cells expressed high levels of transcripts of *MAF* and *PRDM1*, a feature observed in Tph cells.

Conclusions: Identification of IL-21-producing PD-1^{hi}CD8^{+} T cells expands our knowledge of T cell subsets with B helper functions in RA. Selective targeting of these subsets could pave an avenue for the development of novel treatment strategies for this disease.

Keywords: rheumatoid arthritis, IL-21, CD8, T cells, PD-1

INTRODUCTION

Rheumatoid arthritis (RA) is a prototypical autoimmune disease characterized by joint inflammation and bone destruction (1). The emergence of autoantibodies (auto-Abs) such as anti-citrullinated protein antibodies (ACPA) and rheumatoid factors (RF) in the preclinical stage of RA underscores the autoimmune-driven process of this disease (2). In the clinical stage, these auto-Abs can form immune complexes in the joints in which attraction and activation of various immune cells take place, culminating in synovial inflammation and bone destruction (3).

Mounting evidence including genome-wide association studies suggests that RA is an MHC class II-associated disease in which $CD4^+$ T cells play a critical role (4). Follicular helper T (Tfh) cells are a $CXCR5^+PD\text{-}1^+CD4^+$ T cell subset with abundant production of IL-21 that in turn helps the generation of memory B cells and plasma cells in the germinal center (GC) (5). Of note, humans have Tfh cells in the peripheral blood (PB) termed circulating Tfh (cTfh) (6). cTfh cells harbor the potential to support antibody secretion (7) and altered numbers of cTfh cells have been reported in patients with autoimmune diseases including RA (8–12).

Previous studies, however, showed that $CXCR5^-PD\text{-}1^+CD4^+$ T cells can also produce IL-21 that is instrumental in the generation of ectopic lymphoid structures in RA synovium (13, 14). Thus, these findings pose the question of which $CD4^+$ T cell subset ($CXCR5^+$ versus $CXCR5^-$) is critical in the pathogenesis of RA. To address this issue, Rao et al. recently characterized the features of Tfh and $CXCR5^-PD\text{-}1^+CD4^+$ T cells, and coined the latter 'peripheral helper T' (Tph) cells (15, 16). Despite possession of their B cell helper functions *via* IL-21 production, Tfh and Tph cells exhibit distinct features. Tph cells lack CXCR5, but instead express high levels of CCR2 that allows the recruitment of this subset to inflammatory sites. In addition, two subsets exhibit both similar and different expression patterns of transcription factors (TFs). Both cells highly express MAF (15, 17), while Tph cells highly express BLIMP1, a transcription factor typically downregulated in Tfh cells (15, 17).

Since RA is an MHC II-associated disease, the role of $CD8^+$ T cells in this disease has attracted relatively little attention. However, $CD8^+$ T cells comprise ~40% of all T cells in RA synovium and the abundance of these cells in SF and PB is closely associated with disease activity in RA (18, 19). $CD8^+$ T cells are generally considered a prototypical cytotoxic cell type. A recent high-quality study using single-cell transcriptomics and mass-cytometry identified several distinct $CD8^+$ T cell subsets in the synovium of patients with RA (20). Of note, $CD8^+$ T cells constitute $PD\text{-}1^+$ and $PD\text{-}1^-$ subpopulations; the latter only enriches granzyme-producing cytotoxic cells. What then does the former ($PD\text{-}1^+CD8^+$ T cell) do in this case? Apart from their cytotoxicity, several lines of evidence suggest that $CD8^+$ T cells can be another source of IL-21. In mice, IL-6 induces IL-21-producing $CD8^+$ T cells that help in the production of virus-specific IgG Abs (21). In humans, IL-21-producing $CD8^+$ T cells are detected in the tissues of patients with nasal polyps and Hodgkin lymphoma (22, 23). Interestingly, IL-21-producing $CD8^+$ T cells in polyp tissues express PD-1 and ICOS at high levels and promote IgG production *in vitro* (22). Given that $CD8^+$ T cells and B cells are abundant in lymph nodes of early RA patients (24) and $CD8^+$ T cells play a role in modulating ectopic GC formation in RA (25, 26), $PD\text{-}1^+CD8^+$ T cells may play a pathogenic role in RA *via* IL-21 production. At present, however, it remains unknown how $PD\text{-}1^+CD8^+$ T cells are generated in humans and whether the features of these cells could be similar to or distinct from Tfh and Tph cells in human autoimmune diseases such as RA.

In this study, we demonstrate that IL-21-producing $CD8^+$ T cells were enriched in the $CD45RA^-$(memory) T cells, especially in the $PD\text{-}1^{hi}$ subpopulation, whereas granzyme B-producing $CD8^+$ T cells were abundant in the terminal effector subpopulation. IL-12 and IL-21 synergistically induced IL-21 production by naïve $CD8^+$ T cells. Memory $PD\text{-}1^{hi}$ $CD8^+$ T cells in HCPB facilitated plasmablast differentiation and IgG production in an IL-21-dependent manner. In addition, $PD\text{-}1^{hi}CD8^+$ T cells in RASF and RAPB produced large amounts of IL-21 and were characterized by high levels of CD28, ICOS, CD69, HLA-DR, and CCR2 but not CXCR5. Furthermore, $PD\text{-}1^{hi}CD8^+$ T cells expressed high levels of transcripts of *MAF* and *PRDM1*, a feature observed in Tph cells. Together, these findings suggest that $PD\text{-}1^{hi}CD8^+$ T cells in RASF play, in concert with Tfh/Tph cells, a pivotal role in the pathogenesis of RA.

MATERIALS AND METHODS

Patients and Controls

We studied 18 patients with RA who fulfilled the 1987 American College of Rheumatology classification criteria and ACR & EULAR 2010 classification criteria. Eighteen synovial fluid (SF) samples were obtained from the patients. Patient details are shown in **Supplementary Table 1**. Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. The Institutional Review Board of Kyushu University Hospital approved all research on human subjects (No 29-544). We obtained information from the medical records of the patients, including clinical manifestations, laboratory findings and medication history.

Reagents

Dynabeads Human T-Activator CD3/28 was purchased from Invitrogen (Carlsbad, CA, USA). An affiniPure F $(ab')^2$ Fragment Goat Anti-Human IgA/IgG/IgM (H+L) (anti-BCR, 10 μg/ml) was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Recombinant human cytokines (IL-12 (50 ng/ml), IFN-γ (20 ng/ml), IL-4 (20 ng/ml), IL-6 (50 ng/ml), IL-10 (10 ng/ml), IL-21 (50 ng/ml), TGF-β (50 ng/ml), IL-2 (100 U/ml), IL-15 (1 ng/ml) and recombinant human IL-6 Receptor (100 ng/ml) were obtained from R&D Systems (Minneapolis, MN, USA). CpG oligonucleotide type B (0.1 μM) was purchased from Gene Design Inc. (Osaka, Japan). Phorbol myristate acetate (PMA) and Ionomycin were purchased from Calbiochem (Nottingham, UK). A human monoclonal

antibody (mAb) against IL-21 (anti-IL-21, used at the dose of 10 μg/ml) was purchased from Mabtech AB (Nacka Strand, Sweden).

Isolation and Cell Sorting of T and B Cell Subsets

Peripheral blood mononuclear cells (PBMCs) were obtained using a density centrifugation with LSM (MP Biomedicals, LLC, Santa Ana, CA, USA). $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells were isolated by positive selection with anti-CD4, CD8 and CD19 microbeads and a MACS magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells exhibited greater than 99.5% viability and more than 95% purity, confirmed by flow cytometry. Cells were stained with mouse or rabbit mAbs against human CD3, CD4, CD8, CD19, CD20, CD27, CD28, CD45RA, CD69, CD95, CD192 (CCR2), CD197 (CCR7), ICOS (CD278), PD-1 (CD279) and HLA-DR (all from BioLegend, San Diego, CA, USA). Zombie Dyes purchased from BioLegend were used to exclude dead/dying cells in intracellular cytokine staining. Naïve $CD8^+$ T cells were defined as $CD45RA^+CCR7^+$, memory $CD4^+$ T cells as $CD45RA^-$, memory $CD8^+$ T cells as $CD45RA^-$, and memory $CD19^+$ B cells as $CD27^+$. For some experiments, $CD45RA^+$ $CD8^+$ T cells, $CD45RA^-PD\text{-}1^-CD8^+$ T cells, $CD45RA^-PD\text{-}1^{int}CD8^+$ T cells, $CD45RA^-PD\text{-}1^{hi}CD8^+$ T cells and $CD45RA^-PD\text{-}1^{hi}CD4^+$ T cells were purified by flow cytometry.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from primary $CD8^+$ T cells using Isogen II reagent (Nippon Gene, Tokyo, Japan). Quantitative real-time PCR was performed in the MX3000P Sequence Detector (Agilent technologies, Santa Clara, CA, USA). TaqMan target mixes for *IL-21* (Hs00222327_m1), *MAF* (Hs04185012_s1), *BCL6* (Hs00153368_m1), *PRDM1* (Hs00153357_m1), *TCF7* (Hs01556515_m1) were all purchased from Applied Biosystems. 18S ribosomal RNA was separately amplified in the same plate as an internal control for variation in the amount of cDNA in PCR. The collected data were analyzed using Sequence Detector software (MX3000P). Data were expressed as the fold change in gene expression relative to the expression in control cells.

Intracellular Staining of IL-21, IFN-γ and IL-17

Phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Calbiochem, Nottingham, UK), ionomycin (1 μM, Calbiochem) and Golgi Stop (Brefeldin-A, eBioscience, Carlsbad, CA, USA) were added 6 h before staining. Cell surface staining was performed before intracellular cytokine staining for 20 min. After washing two times, fixation/permeabilization buffer (BD Biosciences) was added to fix the cells for 20 min. Antibodies to detect IL-21, IFN-γ and IL-17 (Biolegend) were added to cell suspension and intracellular staining was performed for 15 min. After washing two times, cells were analyzed by FACS Aria II (BD Biosciences). We evaluated IL-21 production in $CD8^+$ T cells in IFN-γ^+, IFN-γ^-, IL-17^+ and IL-17^- fractions to investigate the relationship between IL-21-producing cells and IFN-γ/IL-17-producing cells.

Co-Culture Experiments

Upon stimulation of PBMCs with CD3/28 beads for 72 h, $CD45RA^+CD8^+$ T cells, $CD45RA^-PD\text{-}1^{hi}$ $CD8^+$ T cells and $CD45RA^-PD\text{-}1^{hi}$ $CD4^+$ T cells were purified by flow cytometry. Memory B cells were co-cultured with purified $CD45RA^+CD8^+$ T cells, $CD45RA^-PD\text{-}1^{hi}CD8^+$ T cells and $CD45RA^-PD\text{-}1^{hi}CD4^+$ T cells for 6 days with anti-BCR (10 μg/ml), CD3/28 beads and CpG (0.1 μM). $CD19^+$ B cells and T cells at a ratio of 2:1 (3.2×10^5 cells/mL: 1.6×10^5 cells/mL) were cultured in a 96-well plate and the expression of surface markers including CD27 and CD38 in $CD19^+$ cells was analyzed by flow cytometry.

Enzyme-Linked Immunosorbent Assay (ELISA)

Pre-stimulated T cell and memory B cells were co-cultured under the conditions mentioned above and IgG production in the culture supernatants was measured using an IgG (Total) Human ELISA kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

Statistical Analysis

Numerical data in the *in vitro* experiments were presented as mean of the different experiments and standard error of the mean (SEM). Multiple group comparisons were analyzed using the Kruskal-Wallis test. The significance of the differences was determined by Student's t-test for comparing differences between two groups. Numerical data in patient-sample analyses were presented as mean, and the significance of differences (SD) was determined by Student's t-test or nonparametric Mann-Whitney U-test according to distributions. The correlations between two groups were analyzed using Spearman's correlation coefficient. For all tests, *P* values less than 0.05 were considered significant. All analyses were performed using GraphPad Prism 8 (Prism, La Jolla, CA USA).

RESULTS

$CD8^+$ T Cells Committed to Produce IL-21 Exist in PB

We first compared IL-21 production in $CD4^+$ and $CD8^+$ T cells from the peripheral blood of HCs (HCPB). The gating strategy for intracellular cytokine staining of T cells is shown in **Supplementary Figure 1A**. In the absence of stimuli, there were small numbers of $CD4^+$ but few $CD8^+$ T cells capable of producing IL-21 (**Figure 1A**). Upon CD3/28 stimulation, however, they produced significant amounts of IL-21, although the levels in $CD8^+$ T cells were still lower than those in $CD4^+$ T cells (**Figure 1A**). The FMO control of IL-21 in $CD4^+$ and $CD8^+$ T cells with or without CD3/28 stimulation is depicted in **Supplementary Figure 1B**. We found that 72 h was the optimal time point for IL-21 production and all of the experiments were carried out in this condition thereafter (data

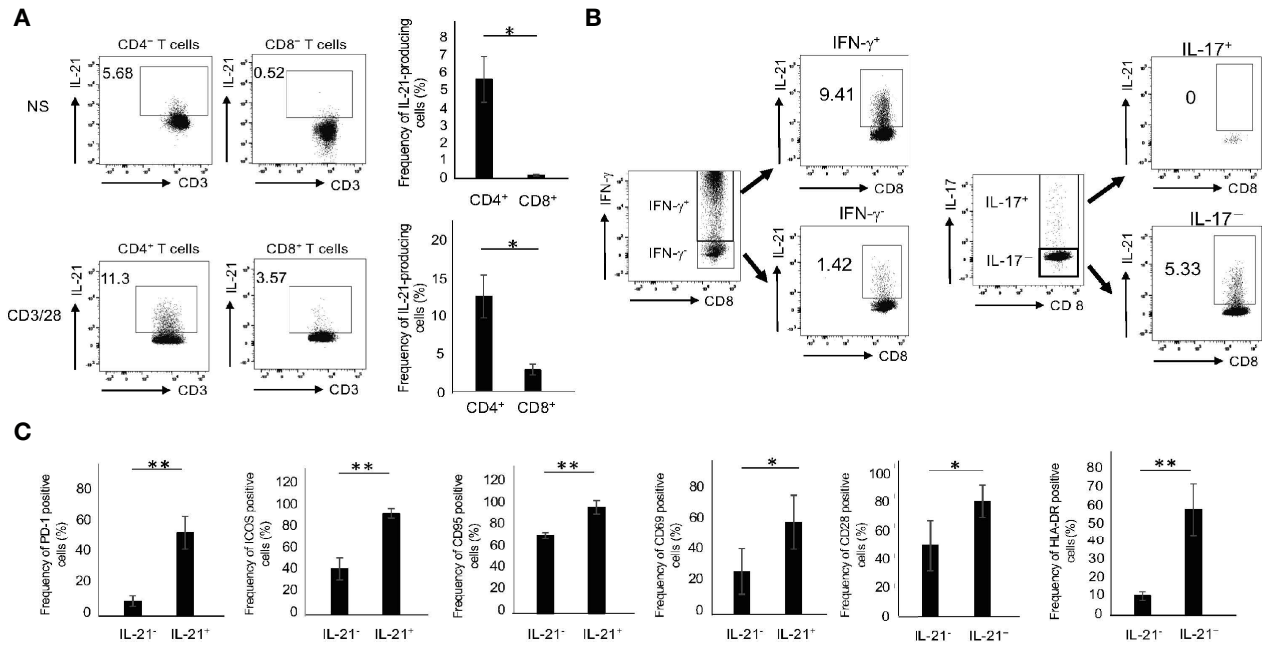

FIGURE 1 | The phenotype of IL-21-producing CD8$^+$ T cells. **(A)** PBMCs from HCs were stimulated in the absence or presence of CD3/28 beads for 72 h and then IL-21 production from CD4$^+$ and CD8$^+$ T cells was analyzed by intracellular staining (added PMA and Ionomycin for the last 6 h). Left panels are representative data on IL-21 production from CD4$^+$ and CD8$^+$ T cells stimulated with CD3/28 and the right graph summarizes the results (N=4). **(B)** PBMCs from HCs were stimulated under the same condition as **(A)**, and IFN-γ and IL-17 production from IL-21$^+$ and IL-21$^-$ CD8$^+$ T cells was analyzed by intracellular staining. **(C)** PBMCs from HCs were stimulated under the same condition as **(A)**, and expression levels of PD-1, ICOS, CD95, CD69, CD28 and HLA-DR in IL-21$^+$ and IL-21$^-$ CD8$^+$ T cells were analyzed by flow cytometry. The graph summarizes the results (N=4). *P < 0.05, **P < 0.01, NS, no stimulation.

not shown). In this situation, CD4$^+$ and CD8$^+$ T cells revealed an opposite trend in the production of IL-17 and IFN-γ: the former cells produced high levels of IL-17, while the latter cells produced high levels of IFN-γ (**Supplementary Figure 2**). Notably, IL-21 was predominantly produced by IFN-γ$^+$ and IL-17$^-$ CD8$^+$ populations (**Figure 1B**). We also determined several surface markers that are associated with activated Tfh/Tph cells (5, 6, 15, 16) in IL-21-producing CD8$^+$ T cells in HCPB and found that the frequency of PD-1$^+$, ICOS$^+$. CD95$^+$, CD69$^+$, CD28$^+$ and HLA-DR$^+$ in IL-21$^+$CD8$^+$ T cells was significantly higher than that in IL-21$^-$CD8$^+$ T cells (**Figure 1C**). These results suggest that CD8$^+$ T cells, like CD4$^+$ T subsets, which are committed to produce IL-21, exist in human PB.

IL-21-Producing CD8$^+$ T cells Are Enriched in Memory PD-1^{hi} Fraction

Given that CD8$^+$ T cells comprise the subpopulations with distinct functions such as naïve, memory and effector cells (27), we determined the production of IL-21, IFN-γ and granzyme B in these subpopulations. As shown in **Figure 2A**, IL-21 was predominantly produced by memory (CD45RA$^-$) CD8$^+$ T cells. On the other hand, IFN-γ was produced by memory and terminal effector (CD45RA$^+$CCR7$^-$) CD8$^+$ T cells, while granzyme B was produced by terminal effector CD8$^+$ T cells (**Figure 2A**). Expression levels of PD-1 in Tfh/Tph cells correlate well with their potential for IL-21 production (28). We thus investigated IL-21 production in the four fractions: CD45RA$^+$, CD45RA$^-$PD-1$^-$, CD45RA$^-$PD-1^{int} and CD45RA$^-$PD-1^{hi} (**Figure 2B**). Short-term treatment of PMA and ionomycin that are commonly used in intracellular staining did not affect PD-1 expression (data not shown). We found that 72 h was, again, the optimal time point for the induction of CD45RA$^-$PD-1^{hi}CD8$^+$ T cells (data not shown). Interestingly, IL-21 production was largely confined to CD45RA$^-$PD-1^{hi}CD8$^+$ T cells (**Figure 2B**). These results suggest that IL-21-producing CD8$^+$ T cells are enriched in the memory, especially PD-1^{hi} fraction.

IL-12 and IL-21 Are Critical in the Generation of IL-21-Producing CD8$^+$ T Cells

Given that naïve CD8$^+$ T cells produced less IL-21 (**Figure 2A**), we hypothesized that a specific cytokine plays a pivotal role in their commitment towards IL-21 production. To this end, purified naïve CD8$^+$ T cells were cultured with IL-12, IFN-γ, IL-4, IL-6, IL-21, IL-10 and TGF-β combined with CD3/28 stimulation and followed by the analysis of IL-21 production. As shown in **Figure 3A**, IL-12 and, to a lesser extent, IL-21 alone significantly up-regulated the levels of CD3/28-induced *IL21* mRNA in naïve CD8$^+$ T cells. In addition, combination of IL-12 and IL-21 synergistically induced *IL21* mRNA expression in these cells. This trend observed in *IL21* mRNA was also true for

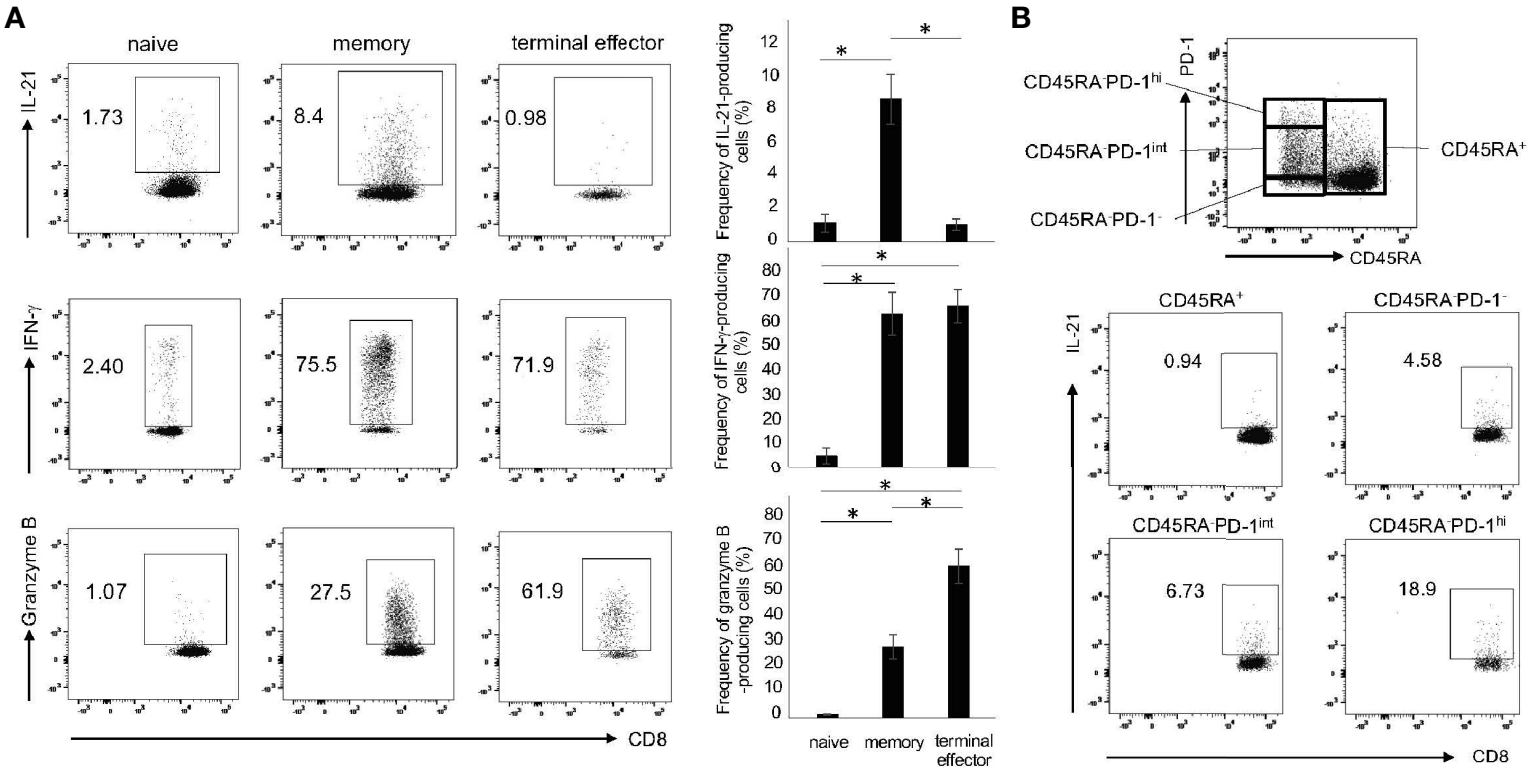

FIGURE 2 | The subsets of IL-21-producing CD8$^+$ T cells. **(A)** PBMCs from HCs were stimulated with CD3/28 beads for 72 h. Production of IL-21, IFN-γ and granzyme B in naïve (CD45RA$^+$CCR7$^+$), memory (CD45RA$^-$) and terminal effector (CD45RA$^+$CCR7$^-$) cells was analyzed by intracellular staining (added PMA and Ionomycin for the last 6 h). Left panels are representative data and right graphs summarize the results (N=3). **(B)** PBMCs from HCs were stimulated under the same condition as **(A)**, and then analyzed by flow cytometry. The panel shows four fractions of CD8$^+$ T cells (CD45RA$^+$, CD45RA$^-$PD-1$^{-/low}$, CD45RA$^-$PD-1^{int} and CD45RA$^-$PD-1^{hi}). The panels show IL-21 production from four populations including CD45RA$^+$, CD45RA$^-$PD-1$^{-/low}$, CD45RA$^-$PD-1^{int} and CD45RA$^-$PD-1^{hi} CD8$^+$ T cells by intracellular staining. *$P < 0.05$.

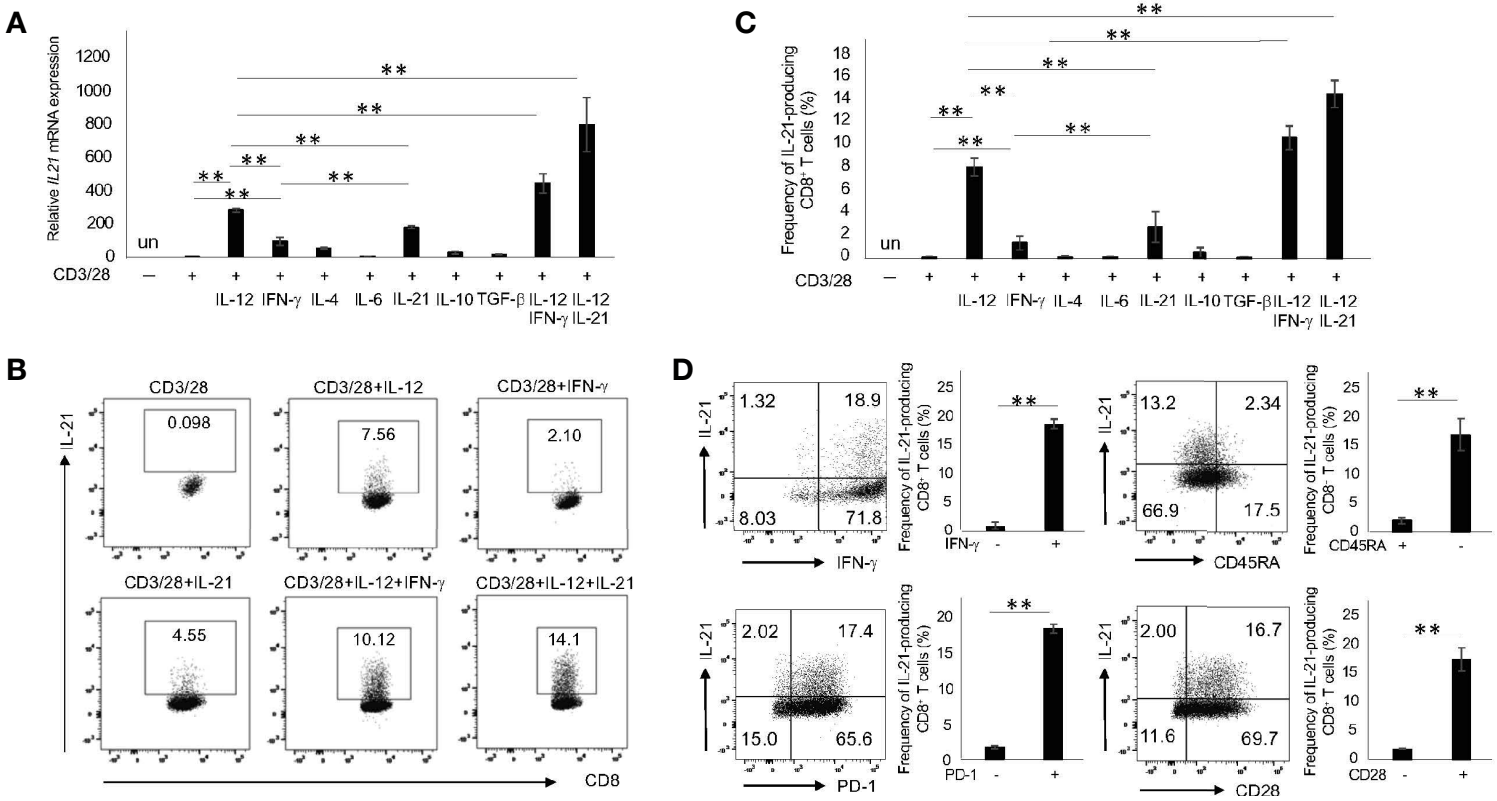

FIGURE 3 | IL-21-producing CD8$^+$ T cells are most efficiently induced by IL-12 and IL-21 in combination with CD3/28. **(A)** Naïve CD8$^+$ T cells (CD45RA$^+$CCR7$^+$ CD8$^+$ T cells) in HCs were purified by flow cytometry and then stimulated with the indicated cytokines in combination with CD3/28 beads for 72 h. Cells were harvested, and transcriptions of *IL21* were evaluated by qPCR. **(B)** Purified naïve CD8$^+$ T cells in HCs were stimulated with the indicated cytokines for 5 days, and IL-21 production was analyzed by intracellular staining. Representative data are shown and summarized results are shown in **(C)** (N=4). **(D)** Naïve CD8$^+$ T cells in HCs were stimulated with IL-12 and IL-21 in conjunction with CD3/28 beads. The panels show expression of CD45RA, PD-1, CD28 and IFN-γ on IL-21$^+$ and IL-21$^-$ CD8$^+$ T cells. Representative data are depicted, and summarized graphs of the results are shown (N=4). $^{**}P < 0.01$, un, undetected.

IL-21 protein production (**Figures 3B, C**). On the other hand, naïve $CD8^+$ T cells produced significant IFN-γ upon CD3/28 stimulation only (**Supplementary Figure 3A, B**). Interestingly, IL-12 enhanced, but IL-21 inhibited IFN-γ production. Combination of IL-12 and IL-21, however, induced IFN-γ production to a similar extent to IL-12 alone (**Supplementary Figure 3A, B**). We thus reasoned that this combination induces the generation of IFN-γ^+IL-21-producing $CD8^+$ T cells and, indeed, this was the case (**Figure 3D**). Moreover, in this condition, IL-21-producing $CD8^+$ T cells were mainly observed in the $CD45RA^-$, $CD28^+$ and PD-1^+ fractions (**Figure 3D**). These results suggest that IL-12 and IL-21 are critical in the generation of IL-21-producing $CD8^+$ T cells.

Memory PD-$1^{hi}CD8^+$ T Cells Facilitate Plasmablast Differentiation and IgG Production in an IL-21-Dependent Manner

IL-21 is a critical cytokine for the differentiation of B cells to Ab-secreting cells (29, 30). Since 72 h was determined to be the optimal point for the induction of $CD45RA^-$PD-$1^{hi}CD8^+$ T cells, we pre-stimulated PBMC with CD3/28 beads for 72 h. The $CD45RA^+CD8^+$ T cells, $CD45RA^-$PD-$1^{hi}CD8^+$ T cells and $CD45RA^-$PD-$1^{hi}CD4^+$ T cells were then purified by flow cytometry. Memory B cells were co-cultured with $CD45RA^+CD8^+$ T cells, $CD45RA^-$PD-$1^{hi}CD8^+$ T cells and $CD45RA^-$PD-$1^{hi}CD4^+$ T cells. Compared with $CD45RA^+CD8^+$ T cells, $CD45RA^-$PD-1^{hi} $CD8^+$ T cells as well as $CD45RA^-$PD-1^{hi} $CD4^+$ T cells induced large numbers of plasmablasts ($CD19^+CD27^+CD38^{hi}$) (**Figures 4A, B**). In addition, $CD45RA^-$ PD-$1^{hi}CD8^+$ T cells promoted IgG production, albeit to a lesser extent as compared with $CD45RA^-$PD-$1^{hi}CD4^+$ T cells (**Figure 4C**). Furthermore, the anti-IL-21 antibody suppressed plasmablast differentiation and IgG production (**Figure 4D**). These results suggest that memory PD-$1^{hi}CD8^+$ T cells have the potential to facilitate the differentiation of memory B cells to plasmablasts and IgG production in an IL-21-dependent manner.

Memory PD-$1^{hi}CD8^+$ T Cells Produce IL-21 in RAPB and RASF

Based on the findings above, we next compared IL-21 production in $CD4^+$ and $CD8^+$ T cells from RAPB and RASF. As compared with HCPB (**Figure 1A**), there were significant numbers of IL-21-producing $CD4^+$ and $CD8^+$ T cells in RAPB in the absence of CD3/28 stimulation (**Figure 5A**). Notably, under these conditions, there were much more IL-21-producing $CD4^+$ and $CD8^+$ T cells in RASF than in RAPB (**Figure 5A**). In addition, the frequency of IL-21-producing $CD8^+$ T cells significantly correlated with that of $CD4^+$ T cells in RASF (**Figure 5B**). Previous studies showed that IL-21-producing $CD4^+$ T (Tfh/Tph) cells are more frequently observed in seropositive RA patients (15, 31). Indeed, this was also the case

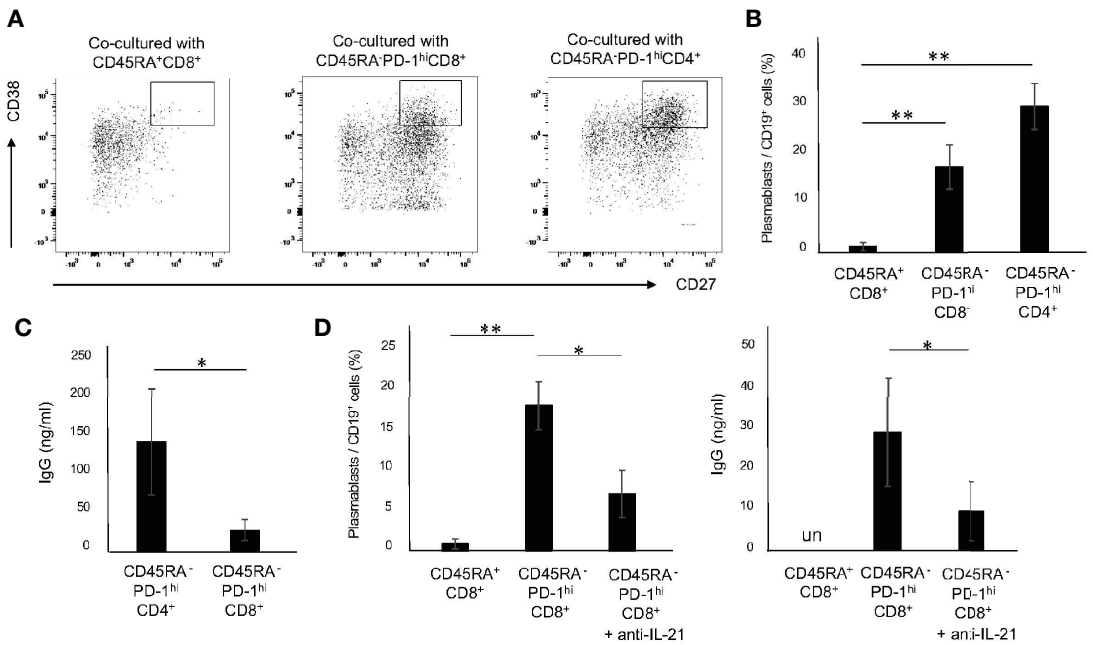

FIGURE 4 | Memory PD-$1^{hi}CD8^+$ T cells promote the differentiation of B cells to plasmablasts and IgG production in an IL-21-dependent manner. **(A)** Upon stimulation of PBMCs from HCs with CD3/28 beads for 72 h, $CD45RA^+CD8^+$ T cells, $CD45RA^-$ PD-1^{hi} $CD8^+$ T cells and $CD45RA^-$ PD-1^{hi} $CD4^+$ T cells were purified by flow cytometry. Memory B ($CD19^+CD20^+CD27^+$) cells were co-cultured with $CD45RA^+$ $CD8^+$ T cells, $CD45RA^-$PD-1^{hi} $CD8^+$ T cells and $CD45RA^-$ PD-$1^{hi}CD4^+$ T cells in the presence of anti-BCR, CD3/28 beads and CpG for 6 days. Representative data on the expression of CD27 and CD38 are depicted in **(A)** and the results are summarized in **(B)** (N=4). **(C)** Comparison of IgG production from plasmablasts induced by co-cultured $CD45RA^-$PD-$1^{hi}CD4^+$ T cells and $CD45RA^-$PD-$1^{hi}CD8^+$ T cells. **(D)** Effect of IL-21 blockade on the frequency of plasmablasts and IgG production induced by co-cultured $CD45RA^-$PD-$1^{hi}CD8^+$ T cells. $^*P < 0.05$, $^{**}P < 0.01$. un, undetected.

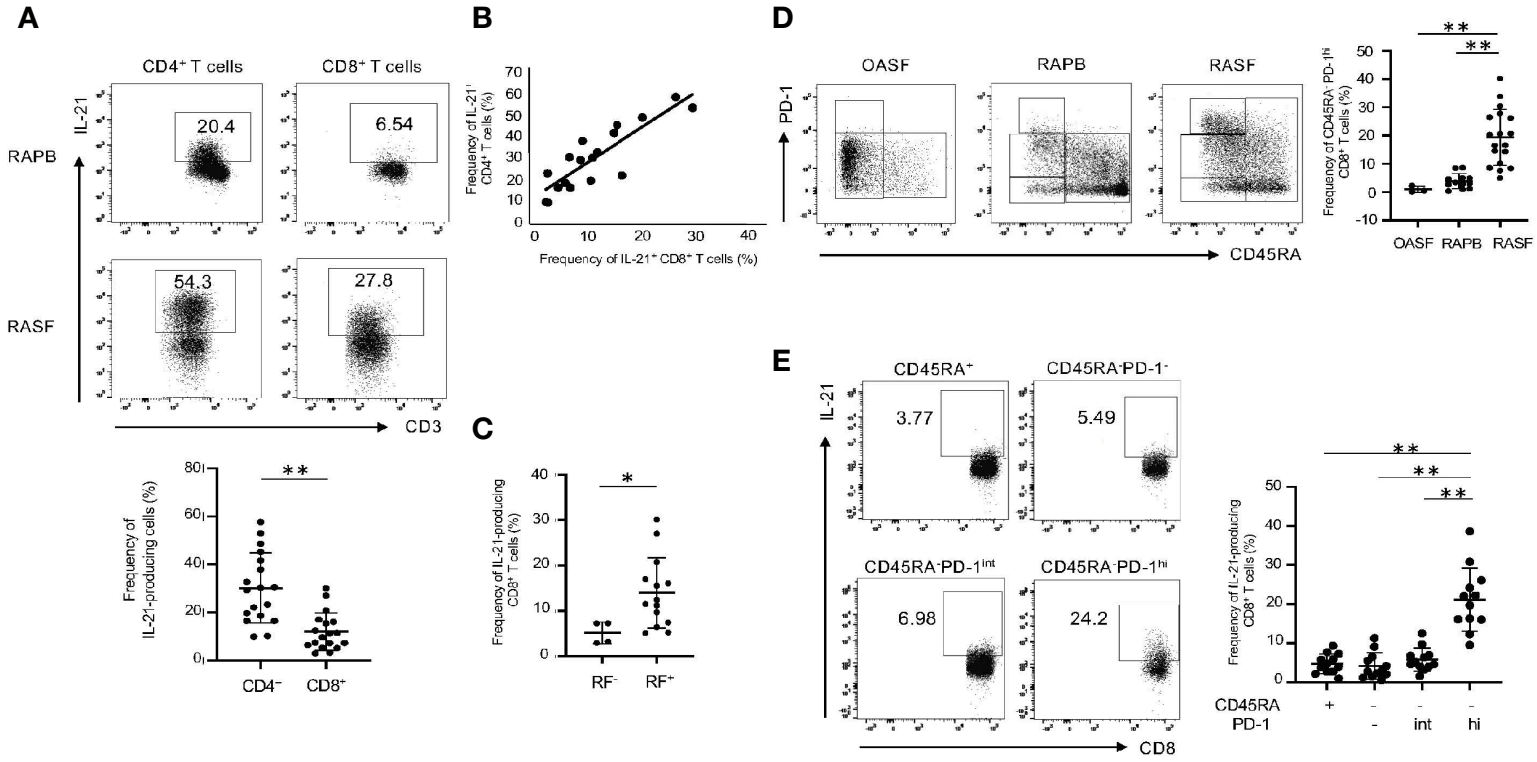

FIGURE 5 | IL-21-producing CD8$^+$ T cells are enriched in memory PD-1^{hi} fraction in RAPB and RASF. **(A)** The upper panels show the representative data regarding the percentage of IL-21-producing CD4$^+$ and CD8$^+$ T cells in PB and SF of patients with RA and the lower panel summarizes the results (N=18). **(B)** Correlation of the percentage of IL-21$^+$ CD4$^+$ T cells with that of IL-21$^+$ CD8$^+$ T cells in RASF (N=18). **(C)** The panel summarizes the frequency of synovial IL-21-producing CD8$^+$ T cells in RF-positive and -negative patients with RA. **(D)** The left panels are representative data of the percentage of CD45RA$^-$PD-1^{hi} fraction among whole CD8$^+$ T cells in OASF (N=3), RAPB (N=12) and RASF (N=18) and right panel summarizes the results. **(E)** The left panels are representative data of the percentage of IL-21-producing CD8$^+$ T cells among CD45RA$^+$, CD45RA$^-$PD-1$^{-/low}$, CD45RA$^-$PD-1^{int} and CD45RA$^-$PD-1^{hi} fractions in RASF and the right panel summarizes the results (N=12). *$P < 0.05$. **$P < 0.01$.

in IL-21-producing $CD8^+$ T cells: the ratio of IL-21-producing $CD8^+$ T cells was higher in seropositive (RF^+) RA patients than in seronegative (RF^-) RA patients (**Figure 5C**). However, no correlation was found between the frequency of IL-21-producing $CD8^+$ T cells and the number of swollen/tender joints, the titer of CRP, or anti-CCP (**Supplementary Figure 4A, B**). Notably, $CD45RA^-PD\text{-}1^{hi}CD8^+$ T cells were more abundant in RASF than in RAPB and SF from patients with osteoarthritis (OA) (**Figure 5D**). As similar to $CD8^+$ T cells in HCPB (**Figure 2B**), IL-21 production was highest in $CD45RA^-PD\text{-}1^{hi}CD8^+$ T cells in RASF (**Figure 5E**). These results suggest that in addition to $CD4^+$ T cells, memory $PD\text{-}1^{hi}CD8^+$ T cells are a potent producer of IL-21 in RAPB and RASF.

Memory $PD\text{-}1^{hi}CD8^+$ T cells Exhibit Similar Features to Tph Cells

We further characterized IL-21-producing memory $PD\text{-}1^{hi}CD8^+$ T cells in RASF in terms of their expression of surface markers and transcriptional factors. $PD\text{-}1^{hi}CD8^+$ T cells in RASF expressed the highest levels of $CD28^+$, $CD69^+$, $ICOS^+$ and $HLA\text{-}DR^+$ (**Figure 6A**), a feature observed in IL-21-producing $CD8^+$ T cells induced *in vitro* (**Figure 1C**). In addition, memory $PD\text{-}1^{hi}CD8^+$ T cells in RASF did not express CXCR5 (**Figure 6B**) but significantly expressed CCR2 (**Figure 6A**), whereas $CD4^+$ T cells in RAPB expressed CXCR5 (**Figure 6B**), suggesting that $PD\text{-}1^{hi}CD8^+$ T cells in RASF share similar feature with Tph cells, but not Tfh cells, in terms of expression of chemokine receptors (5, 6, 15). Moreover, memory $PD\text{-}1^{hi}CD8^+$ T cells expressed the transcripts of *MAF* and *PRDM1* at high levels, while they expressed *TCF7* (encoding TCF-1) mRNA at low levels and *BCL6* mRNA at comparable levels among all $CD8^+$ subsets (**Figure 6C**), the feature of which is similar to that of Tph cells (15, 16). These results suggest that memory $PD\text{-}1^{hi}CD8^+$ T cells in RASF exhibit similar features to Tph cells.

DISCUSSION

RA is a prototypical autoantibody-driven autoimmune disease in which T cell help to B cells is a fundamental event. In this respect, two distinct IL-21-producing $CD4^+$ T cell subsets, Tfh and Tph cells, have recently gained much attention due to their potential for generating Ab-producing cells (5, 6, 15, 16). In this study, we showed that $PD\text{-}1^{hi}CD8^+$ T cells are also equipped to produce significant amounts of IL-21 that allows B cell differentiation, and these T cells were more abundant in RAPB and RASF as compared with HCPB.

A recent study showed that $CD8^+$ T cells in RA synovium constitute $PD\text{-}1^+$ and $PD\text{-}1^-$ subsets wherein the latter only enriches granzyme-producing cytotoxic cells (20, 32). We found that $PD\text{-}1^{hi}CD8^+$ T cells were significantly enriched in the memory fraction that exhibit low levels of cytotoxic molecules in HC and RA patients (**Figures 2**, **5**). PD-1 is a coinhibitory molecule in activated T cells and a well-known marker of $CD8^+$ T cell exhaustion, a condition that is characterized by impaired effector function and is frequently observed in the setting of both chronic viral infection and malignant tumors (33, 34). Recent evidence, however, suggests that $PD\text{-}1^+CD8^+$ T cells are not totally defective in their effector functions but rather exert unique functions. $PD\text{-}1^+CD8^+$ T cells are metabolically active and represent clonally expanding effectors in patients with juvenile idiopathic arthritis (35). Consistent with these findings, our current findings suggest that $PD\text{-}1^+CD8^+$ T cells actively contribute to RA pathogenesis *via* their production of IL-21.

One of our interests in this study was to elucidate the generation mechanism of IL-21-producing $CD8^+$ T cells. Among the tested cytokines, combination of IL-12 and IL-21 most effectively generated IL-21-producing $CD8^+$ T cells from naïve subsets *in vitro* (**Figure 3**). Notably, IL-21-producing $CD8^+$ T cells co-produced IFN-γ but not IL-17 (**Figure 1B**). The IL-12-STAT4 pathway is the most potent in inducing both IFN-γ and IL-21 in human $CD4^+$ T cells (36, 37). $CD4^+$ T cells from patients deficient in IL-12 receptor β1 chain produced less IFN-γ and IL-21 upon stimulation (38). Given that Tfh1 and Tph cells produce large amounts of IFN-γ and IL-21 and they help B cells give rise to plasmablasts [6, 15], IL-21-producing $CD8^+$ T cells may be equipped with similar features to Tfh1 and Tph cells. In addition to STAT4, STAT3-inducing cytokines such as IL-21 play a critical role in the generation of Tfh in humans (39). Together, these findings suggest that human $CD8^+$ T cells share a similar mechanism of IL-21 production with $CD4^+$ T cells. We found high levels of IL-21 production in $CD8^+$ T cells in RASF (**Figure 5**). This may be explained by previous studies showing that *STAT4* SNP and STAT3 hyperactivation are associated with RA (40, 41), thus leading to accentuated response to IL-12 and IL-21.

Another compelling interest in this study was whether IL-21-producing memory $PD\text{-}1^{hi}CD8^+$ T cells in RA resemble the features of Tfh or Tph cells. Despite possession of B cell helper functions *via* IL-21 production, these cells exhibit distinct features. Tfh and Tph cells are characterized by the positivity of CXCR5 and CCR2, respectively. Compared with PB, RASF and RAST are significantly enriched with Tph cells (15, 16). We found that $PD\text{-}1^{hi}CD8^+$ T cells in RASF expressed CCR2, but not CXCR5 (**Figures 6A, B**). Notably, this trend was also true for $PD\text{-}1^{hi}CD8^+$ T cells in RAPB (data not shown). BCL6 was originally reported as a pivotal TF in the generation of Tfh cells (42). Human cTfh cells, however, express BCL6 protein at very low levels, although a role of this TF in cTfh cells remains to be clarified. Instead, MAF is a critical TF in the function of Tfh and Tph cells, particularly for IL-21 production (15, 16, 43, 44). We found a clear correlation between MAF levels and IL-21 expression in $PD\text{-}1^{hi}CD8^+$ T cells of RASF (**Figure 6C**), again suggesting a conserved molecular mechanism of IL-21 induction between $CD4^+$ and $CD8^+$ T cells in humans. The expression of TCF-1 and BCL6 correlates inversely with that of BLIMP1 (45). Notably, Tph cells highly express BLIMP1, a transcription factor typically downregulated in Tfh cells (15, 17). We found that $PD\text{-}1^{hi}CD8^+$ T cells in RASF express BLIMP1 at high levels, while they express TCF1 at low levels, a trend observed in Tph cells (**Figure 6C**). Along with these characteristics, this suggests that memory $PD\text{-}1^{hi}CD8^+$ T cells in RASF are equipped with the features of Tph cells.

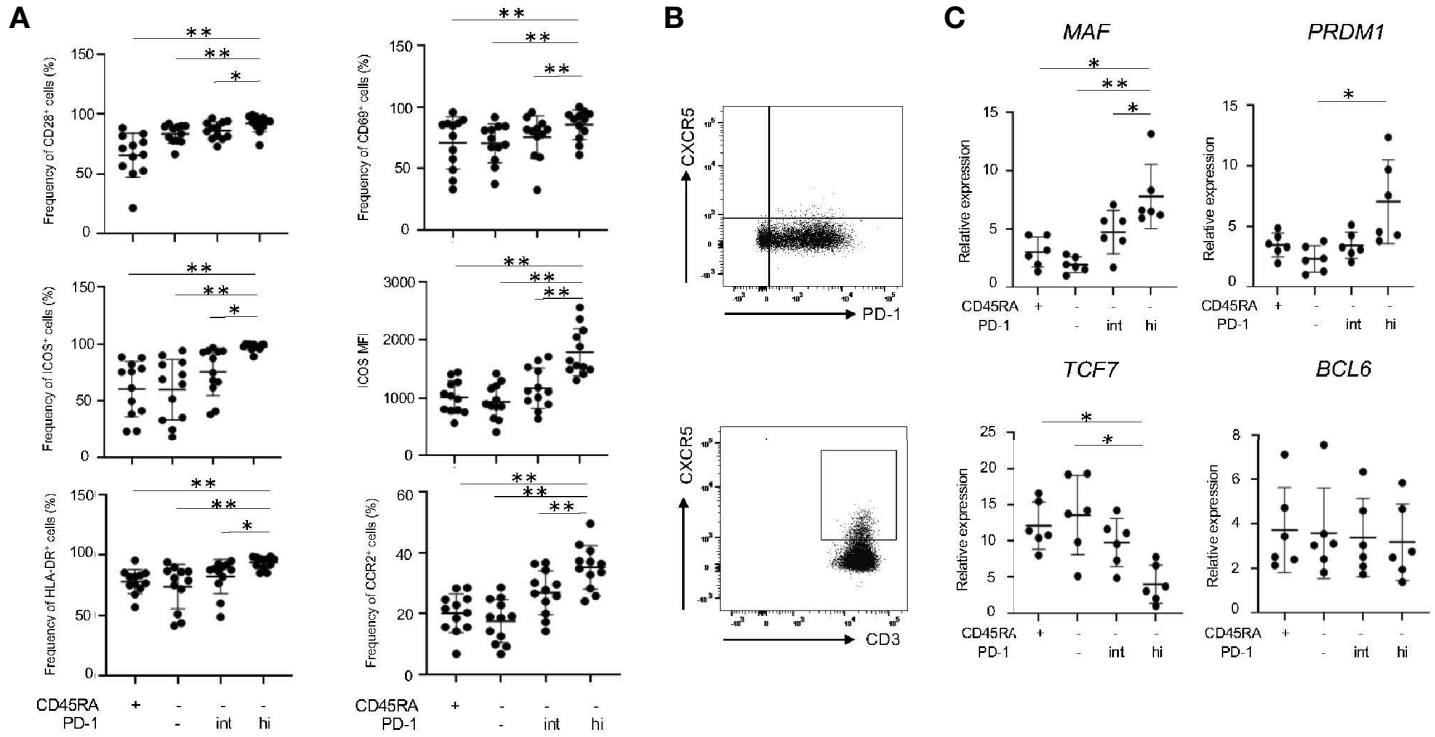

FIGURE 6 | Memory PD-1^{hi} CD8^{+} T cells share several features with Tph cells in RASF. **(A)** The panels summarize the percentage of CD28^{+}, CD69^{+}, ICOS^{+}, HLA-DR^{+} and CCR2^{+} among CD45RA^{+}, CD45RA^{-}PD-1$^{-/low}$, CD45RA^{-}PD-1^{int} and CD45RA^{-}PD-1^{hi} fractions in RASF (N=12). **(B)** The upper panel shows the representative data regarding the percentage of CXCR5^{-}CD8^{+} T cells in RASF and the lower panel shows representative data on CXCR5^{+}CD4^{+} T cells in RAPB. **(C)** Comparison of levels of *MAF*, *PRDM1*, *TCF7*, *and BCL6* mRNA in CD45RA^{+}, CD45RA^{-}PD-1$^{-/low}$, CD45RA^{-}PD-1^{int} and CD45RA^{-}PD-1^{hi} CD8^{+} T cells in RASF (N=6). $^{*}P < 0.05$; $^{**}P < 0.01$.

What's a role of PD-1^{hi}CD8^{+} T cells in RA? IL-21 plays an essential role in the differentiation of B cells into Ab-secreting plasma cells (46). We showed that memory PD-1^{hi}CD8^{+} T cells in HCPB upon CD3/28 stimulation promoted B cell differentiation into plasmablasts and IgG production in an IL-21-dependent manner, albeit to a bit lesser degree than memory PD-1^{hi}CD4^{+} T cells (**Figure 4**). Based on these findings, we propose our hypothetical model depicted in **Figure 7**. Both IL-12 and IL-21 are critical cytokines that play a pivotal role in the generation of IL-21-producing PD-1^{+}CD8^{+} T cells. Given the dominance of Tph over Tfh cells in RA synovium (15, 16), the provision of IL-21 to CD8^{+} T cells in this case could be from Tph cells. Indeed, the ratio of IL-21-producing CD8^{+} T cells correlated well with that of IL-21 producing CD4^{+} T cells in RASF (**Figure 5B**), implying the possibility that memory PD-1^{hi}CD8^{+} T cells, in concert with Tph cells, promoted plasmablast differentiation in RASF, which is in accord with our finding that the ratio of IL-21-producing CD8^{+} T cells was significantly higher in seropositive (RF^{+}) than seronegative (RF^{-}) patients with RA (**Figure 5C**). Although correlation between the frequency of IL-21-producing CD8^{+} T cells and the number of swollen/tender joints, the titer of CRP and anti-CCP was not found (**Supplementary Figure 4A, B**), an adequately powered clinical study is needed to confirm these findings.

The current study has several limitations. First, we still need to carefully elucidate whether memory PD-1^{hi}CD8^{+} T cells in RASF are generated by the same mechanism as *in vitro* generated PD-1^{hi}CD8^{+} T cells. IL-21 is shown to induce the expression of BCL6 and TCF-1 (47, 48), however mRNA levels of these genes in PD-1^{hi}CD8^{+} T cells were not high in our results (**Figure 6C**), indicating the possibility that other cytokines asides from IL-12 and IL-21 are also involved in the generation of PD-1^{hi}CD8^{+} T cells in RASF. Second, IL-21-producing CD8^{+} T cells are detected in the tissues of patients with nasal polyps and malignant tumors (22, 23). Intriguingly, however, these cells express high levels of CXCR5 which resemble the feature of Tfh. It thus remains to be determined whether Tfh-like CD8^{+} T cells also exist in patients with RA. Based on a recent study (20), this could be the case. Third, an intriguing issue of how much PD-1^{hi}CD8^{+} T cells are involved in the pathogenesis of RA as compared with Tfh/Tph cells remains somewhat elusive at the moment. We showed the B-cell helper functions of PD-1^{hi}CD8^{+} T cells in HCPB upon CD3/28 stimulation (**Figure 4**) as well as the existence and characteristics of PD-1^{hi}CD8^{+} T cells in RASF (**Figures 5**, **6**). However, we still need more extensive analysis using more RASF samples to address this issue.

In conclusion, we demonstrate the characterization and generation mechanism of human IL-21-producing memory PD-1^{hi}CD8^{+} T cells, which have similar features to Tph cells and accumulate abundantly in RASF. PD-1^{+}CD8^{+} T cells characterized by impaired effector function are frequently observed in the setting of chronic infection and malignant tumors (33, 34), but our results suggest that memory PD-1^{hi}CD8^{+} T cells in RASF, in concert with CD4^{+} T cells, play an active role in the pathogenesis of RA. Taken together, identification of this CD8^{+} T subset expands our knowledge of

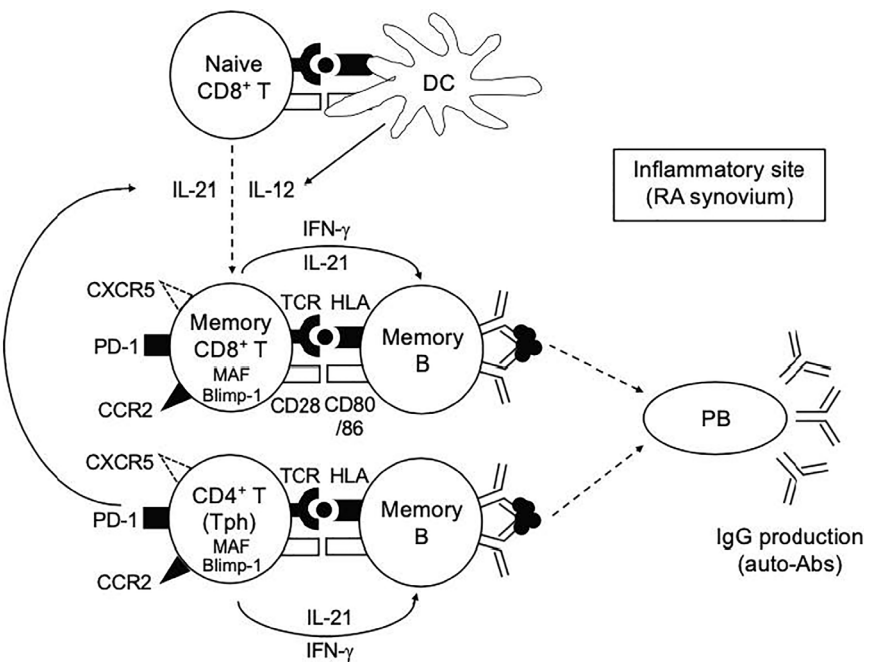

FIGURE 7 | Our hypothetical model in this study. Both IL-12 and IL-21 play a pivotal role in the generation of IL-21-producing PD-1^{+}CD8^{+} T cells. The provision of IL-12 and IL-21 to CD8^{+} T cells in this case could be from DCs and Tph cells, respectively. Memory PD-1^{hi}CD8^{+} T cells, in concert with Tph cells, play a role in promoting plasmablast differentiation and antibody production at the inflammatory sites such as RA synovium.

T cell subsets with B cell helper functions in RA, a prototypic systemic autoimmune disease. Selective targeting of these subsets could pave an avenue for the development of novel treatment strategies for this devastating disorder.

DATA AVAILABILITY STATEMENT

All data sets presented in this study are included in the article. Requests to access the datasets should be directed to niiro.hiroaki.811@m.kyushu-u.ac.jp.

ETHICS STATEMENT

The Institutional Review Board of Kyushu University Hospital approved all research on human subjects (no 29-544). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KH performed the experiments, statistical analysis, and drafted the manuscript. MY, MA, YK, HM, NO, YA, MK, TH, KA, and HN designed the study and helped to draft the manuscript. TS and HY provided synovial fluid cells of patients with RA and helped to draft the manuscript. HN contributed to data analysis and interpretation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.654623/full#supplementary-material

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Serum Levels of Inflammatory Proteins Are Associated With Peripheral Neuropathy in a Cross-Sectional Type-1 Diabetes Cohort

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Chronic low-grade inflammation is involved in the pathogenesis of type-1 diabetes (T1D) and its complications. In this cross-section study design, we investigated association between serum levels of soluble cytokine receptors with presence of peripheral neuropathy in 694 type-1 diabetes patients. Sex, age, blood pressure, smoking, alcohol intake, HbA1c and lipid profile, presence of DPN (peripheral and autonomic), retinopathy and nephropathy was obtained from patient's chart. Measurement of soluble cytokine receptors, markers of systemic and vascular inflammation was done using multiplex immunoassays. Serum levels were elevated in in DPN patients, independent of gender, age and duration of diabetes. Crude odds ratios were significantly associated with presence of DPN for 15/22 proteins. The Odds ratio (OR) remained unchanged for sTNFRI (1.72, p=0.00001), sTNFRII (1.45, p=0.0027), sIL2Rα (1.40, p=0.0023), IGFBP6 (1.51, p=0.0032) and CRP (1.47, p=0.0046) after adjusting for confounding variables, HbA1C, hypertension and dyslipidemia. Further we showed risk of DPN is associated with increase in serum levels of sTNFRI (OR=11.2, p<10), sIL2Rα (8.69, $p<10^{-15}$), sNTFRII (4.8, $p<10^{-8}$) and MMP2 (4.5, $p<10^{-5}$). We combined the serum concentration using ridge regression, into a composite score, which can stratify the DPN patients into low, medium and high-risk groups. Our results here show activation of inflammatory pathway in DPN patients, and could be a potential clinical tool to identify T1D patients for therapeutic intervention of anti-inflammatory therapies.

Keywords: autoimmunity, chronic inflammation, type-1 diabetes, cytokines, receptors, peripheral neuropathy

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INTRODUCTION

The morbidity and mortality associated with type-1 diabetes (T1D) is mainly related to the development of long-term micro- and macro-vascular complications (1). The microvascular damage due to hyperglycemia can lead to neuropathy, retinopathy, and nephropathy (2–4). The macro-vascular damage can lead to thrombosis (5). Neuropathies (DPN) are one of the first and more common diabetic complications and present in up to 50% of patients (6), is frequently underdiagnosed by physicians (7) and can be unrecognized by the patients (8). The major characteristic of diabetic peripheral neuropathy (DPN) is the progressive loss of nerve fibers from both the autonomic and peripheral nervous system. This loss of sensory function has major detrimental effects, including risk of foot ulcerations, amputations, and increased mortality rates (6).

The Diabetic Control and Complications Trial/Epidemiology of Diabetes and Complications (DCCT/EDIC) study suggests that despite intensive glycemic control, incidence and prevalence of DPN increased in both the subjects under intensive (7% incidence) and permissive (17% incidence) glycemic control (9, 10). The reported prevalence of diabetic peripheral neuropathy (DPN) in type 1 diabetes (T1D) patients is highly variable (11). It was 13% in a Scottish registry population study (12), 11% in the 25,000-person T1D Exchange Clinic Network in the U.S (13) and 28% in EURODIAB IDDM Complications Study (14). Recent reports from DCCT/EDIC suggests that prevalence of DPN is 28% in T1D patients after 20 years of diabetes (9, 15, 16). In a Danish study, presence of subclinical DPN was 55.1% whereas only 2.6% individuals were confirmed to have DPN (17). The Pittsburgh Epidemiology of Diabetes Complications Study, suggested that DPN was prevalent in 18% of the 18–29-year-old individuals (18).

The high rates of DPN among youth with diabetes are a cause of concern and requires early detection and better risk factor management. Interventions to address poor glycemic control and dyslipidemia may prevent or delay debilitating neuropathic complications (17–19). The long-term complications of T1D require lifestyle changes and medications that will modify risk factors such as hyperglycemia, hyperlipidemia, and hypertension (10, 17, 18). Yet, the current age and duration of type 1 diabetes remain important predictors of developing DPN. These studies suggest some T1D patients would still develop complications despite intensive treatment. Therefore, there is a need for additional markers to identify these at-risk population.

The precise mechanisms by which DPN occurs is multi-factorial (20, 21). However, chronic low-grade inflammation is considered the root cause of development of complications in both type-1 and type-2 diabetes patients (21, 22). Previous reports have shown that serum levels of inflammatory proteins, such as TNF-α, IL-16, soluble TNF receptors (sTNFR1 and 2) were increased in the serum of T1D patients with micro-vascular (23–25) and macro-vascular complications (26). In T1D subjects with DPN, serum levels of sTNFR1 and sTNFR2 were associated with DPN after adjusting for confounders (27). The majority of the studies on inflammatory proteins in DPN is on the type-2 diabetes (T2D) patients. We have shown that serum levels of insulin like growth factor binding proteins (IGFBP), TNF-α and IL-6 pathways were able to stratify T1D patients into risk categories for a number of complications (28), including nephropathy (24) and retinopathy (25). Results presented here show that serum levels of five inflammatory proteins are associated with increased risk of DPN in T1D individuals after adjusting for age, duration, gender, hypertension and dyslipidemia. We utilized a machine learning approach to combine the serum level of several proteins into a linear predictor to stratify T1D subjects.

MATERIALS AND METHODS

Study Population

Serum samples for this cross-sectional study were obtained from Caucasian subjects recruited into the Phenome and Genome of Diabetes Autoimmunity (PAGODA) study between 2002 and 2010. These subjects attended the Augusta University (AU) Medical Center and/or endocrinology clinics in the Augusta and metro-Atlanta areas of Georgia. The presence and year of diagnosis of diabetic peripheral neuropathy was identified by retrospective chart review. The vast majority of subjects were diagnosed with DPN based on a neurological history and exam by the treating endocrinologist. Many, but not all, had further evaluations and confirmation of DPN by a neurologist. Patients with diabetic foot ulcers and amputations were excluded from analysis. Patients with non-DPN microvascular complications were excluded from the control nDPN group. Medical history, clinical, and demographic profiles for T1D subjects were obtained from the medical charts (**Table 1**). All study participants gave written informed consent before enrolling in the study. The study was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki, 1997) and was approved by the institutional review board at AU.

Venous blood was collected in clot activator tubes, allowed to clot at room temperature for 30 minutes prior to centrifugation at 3000g. Separated serum was then aliquoted into wells of 96-well plate to create a master plate. Individual daughter plates were then created by aliquoting 5-10μl of serum from this master plate. All master and daughter plates were stored at -80°C until use.

Laboratory Measurements

We measured twenty-two proteins (IL1Ra, IL8, MCP-1, MIP-1β, CRP, SAA, MMP1, MMP2, MMP9, sgp130, sICAM1, sVCAM1, sIL2Rα, sIL6R, sTNFRI, sTNFRII, sEGFR, IGFBP1, IGFBP2, IGFBP3, IGFBP6 and tPAI1) in the serum of subjects recruited in the Phenome and Genome of Diabetes Autoimmunity (PAGODA) Study. These proteins have all been reported in the inflammation pathways associated with progression of T1D and its complications (24–26, 28, 29). Luminex assays for these proteins were obtained from Millipore (Millipore Inc., Billerica, MA, USA). Prior to the full-scale study, we optimized the dilution of sera for a distribution of intensity in the linear portion of the standard curve. Multiplex assays were performed according to the instructions provided with the kit. Briefly, serum samples were

TABLE 1 | Demographic and Clinical variable for 694 Caucasian T1D patients without (nDPN) and with Peripheral Neuropathy (DPN).

Characteristics	nDPN (n = 507)	DPN (n = 187)	p
Gender (n)			
Males	240	77	
Females	267	110	0.174‡
Age (years)	39.5 ± 12.5	52.1 ± 11.5	$6.5x10^{-30}$
Median Age (range, years)	38.8 (20.0 -73.7)	53.1 (20.5-74.5)	$1.4x10^{-27}$†
Median Age of diagnosis (range, years)	19.0 (0-62.8)	20.1 (0-67.4)	0.372†
Duration of T1D (years)	17.9 ± 10.9	29.3 ± 13.0	$1.2x10^{-22}$
UACR (ug/mg)	6.7 (1.2-2425.2)	13.1 (1.7-38205.6)	$1.2x10^{-8}$†
BUN (mg/dL)	13.0 ± 3.8	17.1 ± 7.3	$4.1x10^{-10}$
HbA1c (%, NGSP)	7.7 ± 0.6	8.1 ± 0.6	$2x10^{-3}$
HbA1c (%, median (range))	7.6 (3.2 - 15.5)	7.9 (5.4 - 16.7)	0.0013†
HbA1c (mmol/mol, IFCC)	61.3 ± 5.7	65.5 ± 6.5	$3x10^{-3}$
HbA1c (mmol/mol, median(range))	59.6 (31.2 - 144.5)	62.5 (38.8 - 159.0)	0.002†
Total Cholesterol (mg/dL)	174.8 ± 34.6	176.5 ± 39.7	0.645
LDL (mg/dL)	98.0 ± 27.7	95.7 ± 33.2	0.457
Triglycerides	91.3 ± 73.7	104.1 ± 74.1	0.07
HDL	58.8 ± 17.2	59.4 ± 18.3	0.72
Blood Pressure (mmHg)			
Systolic BP	119.2 ± 10.5	125.1 ± 11.7	$4.5x10^{-8}$
Diastolic BP	73.5 ± 6.6	73.1 ± 7.7	0.6
MAP	88.2 ± 8.31	90.3 ± 7.8	0.005
DPN duration (years)		8.9 ± 8.5	
Other complications			
Nephropathy (n)	No	37	
Retinopathy (n)	No	100	
Hypertension (n)	74	90	
Dyslipidemia (n)	137	98	
CAD (n)	No	50	
Diabetic Foot Ulcer (n)	No	27	

CAD, Coronary artery disease.
†Kruskall-Wallis test, ‡Chi-sq Test, all other p values are from T-tests.

incubated with antibody coated microspheres, followed by biotinylated detection antibody. Detection of the proteins was accomplished by incubation with phycoerythrin-labeled streptavidin. The resultant bead immuno-complexes were then read on a FLEXMAP3D (Luminex, TX, USA) with the instrument settings recommended by manufacturer.

The captured median fluorescence intensity (MFI) data was passed through quality control steps as described in our earlier study (30). Briefly, wells with individual bead counts below 30, or bead count coefficient of variation (CV) above 200 were flagged for exclusion. Replicate wells with CV≥25% were excluded from further analyses. The standard concentration and MFI were log2 transformed prior to regression. Protein concentrations were estimated using a regression fit to the standard curve with serial dilution of known concentration for each protein (30).

Construction of Inflammation Score

Ridge regression (R package "glmnet") was used to create a multi-protein score that accounted for serum levels of multiple proteins (31). The algorithm combines the serum levels of multiple proteins in a linear combination by using the sum of the squares of the coefficients of individual proteins generated in the model based on a penalty term. The effect of the penalty term is adjusted by providing a matrix of values for lambda from 0 to infinity. The optimum lambda was determined using the lambda.min function in R, which chooses the minimum lambda value on cross validation. We optimized the model by using recursive feature elimination. We fit all 22 proteins to the peripheral neuropathy outcome using a least absolute shrinkage and selection operator (LASSO) by setting alpha=0 in the cv.glmnet function. We manually removed the proteins contributing the lowest weight to the linear predictor (**Supplementary Figure 2**). After the selection of proteins for the final model, we ran ridge regression using cv.glmnet function to obtain a linear predictor. We used the composite score obtained from the ridge regression model to calculate the odds ratios (OR) of having DPN for the upper four quintiles using the first quintile as reference.

Statistical Analysis

Data is presented as count, percentages, and means ± standard deviation for normally distributed variables. Median and range is presented for non-normal variables. Prior to any statistical analysis, protein concentration data was log2 transformed to follow the normal distribution. For generation of the figures, the data was back transformed to natural units. For all statistical analysis we removed T1D subjects with diabetic foot ulcer (n=27). Potential univariate differences between T1D patients without any complication (nDPN) and T1D patients with diabetic neuropathy (DPN) was examined using boxplots and t-test. Pairwise correlations between individual proteins were determined using Pearson correlation coefficient, and presented

as a heatmap with hierarchical clustering. The association between the serum levels of each candidate molecule and age and T1D duration was determined using a linear regression including sex and disease status as covariates.

For all association analysis, log2 transformed concentration data was converted into z-scores by centering and scaling to unit standard deviation. The potential relationship between DPN and inflammatory proteins was evaluated using logistic regression models where presence/absence of DPN was incorporated as dependent variable. Covariates, Age, sex, duration of T1D, HbA1c, hypertension (HTN), and dyslipidemia were adjusted in separate models in the logistic regression.

We divided serum level for each protein into 5 quintiles containing 20% DPN patients in each quintile (20^{th} percentile). The cutoff protein levels from DPN patients were then used to count nDPN and DPN subjects in each quintile. The 1st quintile was used as reference and OR for DPN was calculated for the upper four quintiles. Pearson's Chi-squared test with Yates' continuity correction was used to calculate the ORs. The chi-squared test for trend in proportions was used to calculate the p-value of overall trend.

All P-values were two-tailed and a $P< 0.05$ was considered statistically significant. All statistical analyses were performed using the R language and environment for statistical computing (v 4.0.3; R Foundation for Statistical Computing; www.r-project.org).

RESULTS

Description of the Study Participants

Serum samples from T1D subjects (n=694) were analyzed in this study. These subjects were divided into, T1D with no complications (nDPN, n=507) and T1D patients who were diagnosed with peripheral neuropathy (DPN, n=187). The patients in DPN group were older (median age: 53.1 vs 38.8 years) and had type-1 diabetes for a longer duration (29.3 ± 13.0 vs 17.9 ± 10.9 years) than the nDPN group (**Table 1**). Subjects in DPN group have higher values for HbA1c and systolic blood pressure. There were no differences in the serum level of HDL, LDL, or cholesterol between the DPN and nDPN groups (**Table 1**). Of the 187 T1D subjects with DPN, 37 had diabetic nephropathy, 100 had retinopathy, 50 had coronary artery disease, and 27 had diabetic foot ulcer(s). Hypertension and dyslipidemia were present in both the groups (**Table 1**).

Relationships of Inflammatory Proteins in DPN Patients

We evaluated the serum level of 22 inflammatory proteins which have been associated with T1D progression and complications (24–26, 29). Six out of twenty-two proteins (sTNFRI, sTNFRII, MMP2, IGFBP2, sIL2Rα, IGFBP6, CRP) showed elevated (1.2-1.8 fold) mean serum levels in the DPN group compared to the nDPN group (**Figure 1** and **Supplementary Figure 1**). There was a slight but significant elevation in serum levels of sgp130 (1.16-fold), sICAM1 (1.13-fold), MCP1 (1.14-fold) and sVCAM1 (1.16-fold). Six proteins, MMP1, IGFBP1, SAA, IL1Ra, sEGFR, and IL8 showed non-significant elevation in DPN group (**Figure 1** and **Supplementary Figure 1**).

We next evaluated the correlation structure of these inflammatory proteins by pairwise Pearson's correlation analysis in the nDPN and DPN groups separately. Hierarchical clustering of the correlation matrix suggests a single cluster with 11 proteins in the T1D group. Within this cluster, the first sub-cluster, including proteins sIL6R, tPAI1, MMP2, IGFBP6 and sTNFRII, had correlation coefficient (r) values ranging from 0.41 to 0.65 ($p<10^{-32}$). The second sub-cluster of highly correlated group of proteins contained sICAM1, sgp130, sVCAM1, and IGFBP3 (r=0.42-0.90, $p<10^{-32}$, **Figure 1B**, left). In the DPN group, we observed a stronger correlation among the 11 proteins compared to the nDPN group (**Figure 1B**, right). The first sub-cluster had r-values in range of 0.41-0.7 ($p=0\text{-}4x10^{-8}$), the second cluster had r-values in range of 0.38-0.83 ($p=0\text{-}8x10^{-7}$). The most notable difference was the strength of correlation among sEGFR, sIL2Rα, sTNFRI, sIL6R, tPAI1, MMP2, IGFBP6 and sTNFRII in the DPN group. These results suggest that inflammation plays a more coordinated role in patients with DPN compared to nDPN subjects.

Influence of Age, Duration of Diabetes and Gender on Serum Levels

In nDPN group, the subject's age at the time of sample collection was significantly correlated with serum levels of IGFBP2 (r=0.27, $p=1x10^{-9}$), MMP1 (r=0.161, p=0.0003), and SAA (r=0.12, p=0.009) (**Supplementary Table 1**). The subject's duration of T1D disease burden was significantly correlated with IGFBP1 (r=0.16, p=0.0003), IGFBP2 (r=0.26, $p=2.2x10^{-9}$), MMP2 (r=0.22, $p=1x10^{-6}$), and sTNFR2 (r=0.18, $p=7.2x10^{-5}$). Females have a higher serum level on average than males for IL-1Ra (F/M ratio=1.48, $p=2.8x10^{-5}$), IGFBP1 (F/M ratio=17.6, $p=1.2x10^{-8}$), IGFBP2 (F/M ratio=156, $p=1.2x10^{-3}$), IGFBP6 (F/M ratio=0.76, $p=2.4x10^{-7}$).

In DPN group, the subject's age at the time of sample collection was significantly correlated with serum levels of IGFBP3 (r=-0.2, p=0.008) and MMP2 (r=0.2, p=0.007). Only MMP2 (r=0.19, p=0.008) showed significant correlation with disease duration in DPN group. Only IGFBP6 (F/M ratio=0.74, p=0.007) showed significant differences between females and males in the DPN group.

We also evaluated the relationship between hemoglobin A1c (HbA1c) and serum levels of inflammatory proteins. A positive correlation was observed for CRP in the nDPN group (r=0.18, p=0.0002, **Supplementary Table 4**). A negative correlation was observed for MIP1β with HbA1c in DPN group (r=-0.3, p=0.001, **Supplementary Table 4**). Only serum concentration of MCP1 was correlated with systolic blood pressure (r=0.2, $p=3.7x10^{-5}$).

DPN Is Associated With Increase in Serum Levels of Inflammatory Proteins

We assessed the difference in protein level between the DPN and nDPN groups adjusting for clinical confounders in a multivariate logistic regression model (**Table 2**). The crude OR (95% CI) for

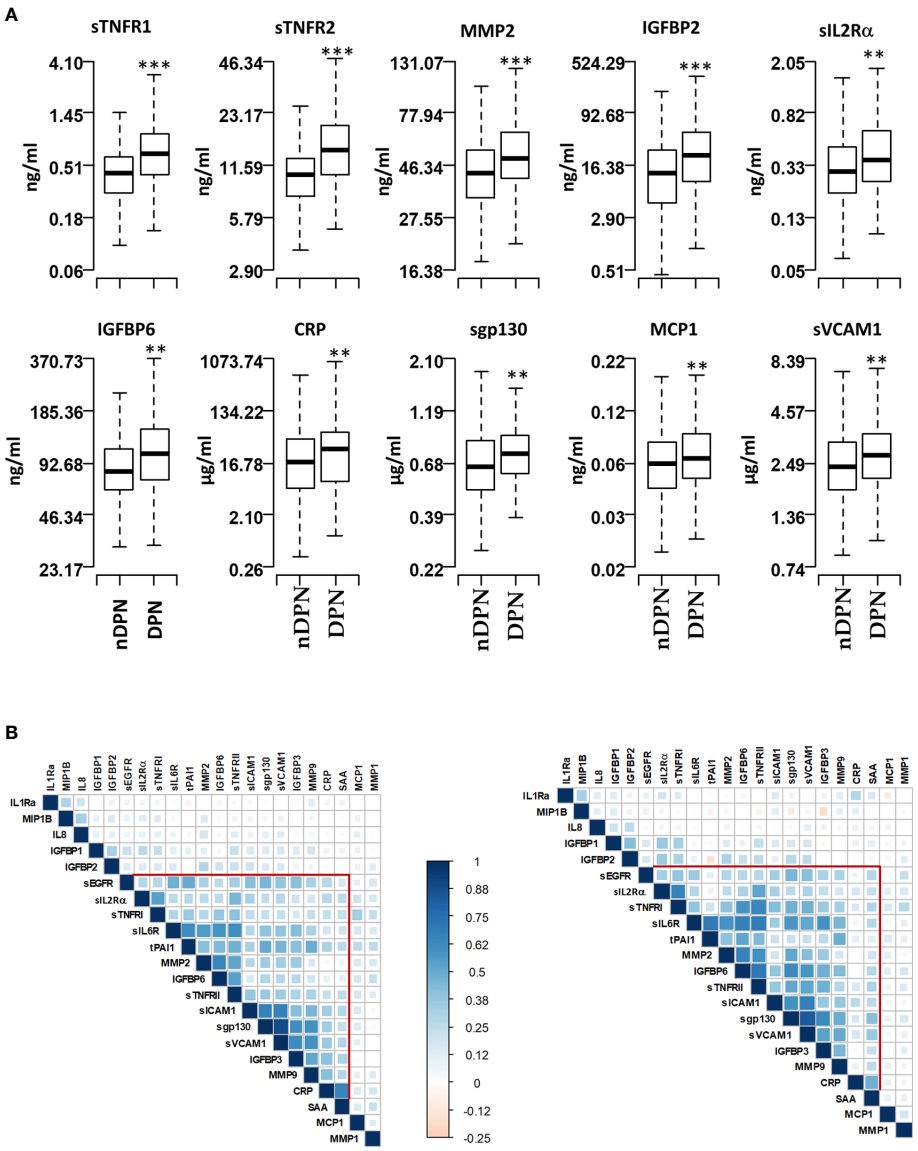

FIGURE 1 | Serum levels of proteins and pairwise correlations. **(A)** Boxplots showing the measured levels of selected proteins in T1D patients without diabetic neuropathy (nDPN, n=507) and with diabetic neuropathy (DPN, n=187). **(B)** Heatmaps showing the pairwise correlation coefficients in nDPN (left) and DPN (right) patients. The correlation coefficients are clustered based on the supervised hierarchical clustering. The color density represents the strength of correlation (r) values and the size of the squares represent the significance. ***P < 0.000001, **P < 0.01.

the presence of DPN was significant for per SD increase in protein concentration for 15 proteins (Model 1, **Table 2**). The positive associations remained significant after multivariate adjusting of age, duration of diabetes and gender (Model 2, **Table 2**), glycemic control (Model 3, **Table 2**), hypertension (HTN) and dyslipidemia (Model 4, **Table 2**). The associations remained unchanged for sTNFR1, sIL2Rα, sTNFRII, IGFBP6 and CRP (**Table 2**). We did not observe any weakening of the associations for these five proteins in any of the models presented in **Table 2**.

Non-linear associations are difficult to detect on the logistic regression models we described. Thus, we determined odds ratio (OR) after dividing the protein concentrations into quintiles (**Figure 2** and **Table 3**). The serum levels of each protein in the DPN group were divided into quantiles containing 20 percent of subjects. The cutoff values obtained from the DPN patients was used to assign the T1D patients into five quantiles. For all comparisons, we used the first quintile as a reference to calculate the OR for quintiles 2-5. **Figure 2** and **Table 3** shows the odds ratio with 95% CI. We observed that DPN is associated

TABLE 2 | DPN is independently associated with per SD increase in serum levels, after adjusting for age at sample, duration of T1D, Gender, glycemic control, hypertension and dyslipidemia.

Proteins	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model 3 OR (95% CI)	Model 4 OR (95% CI)
sTNFRI	2.12(1.71-2.67)¶	1.82(1.45-2.32)¶	1.76(1.37-2.3)‡	1.72(1.36-2.19)‡
sIL2Rα	1.48(1.23-1.78)†	1.36(1.11-1.68)†	1.39(1.11-1.75)†	1.4(1.13-1.73)†
sTNFRII	2(1.53-2.65)¶	1.47(1.17-1.91)†	1.39(1.1-1.81)†	1.45(1.16-1.9)†
IGFBP6	1.75(1.38-2.24)¶	1.56(1.21-2.06)†	1.53(1.17-2.07)†	1.51(1.17-2.01)†
CRP	1.53(1.23-1.92)†	1.47(1.15-1.91)†	1.45(1.1-1.92)†	1.47(1.13-1.92)†
IGFBP1	1.32(1.09-1.6)†	1.13(0.91-1.43)	1.14(0.9-1.47)	1.2(0.94-1.52)
sgp130	1.32(1.06-1.68)*	1.25(0.98-1.61)	1.2(0.93-1.58)	1.21(0.95-1.59)
IL1Ra	1.53(1.16-2.03)†	1.32(0.95-1.83)	1.25(0.89-1.77)	1.24(0.89-1.73)
SAA	1.37(1.1-1.73)†	1.23(0.95-1.6)	1.25(0.94-1.67)	1.18(0.91-1.55)
IGFBP2	1.82(1.41-2.36)¶	1.14(0.87-1.51)	1.16(0.85-1.58)	1.2(0.9-1.61)
sICAM1	1.24(1.03-1.5)*	1.16(0.94-1.44)	1.08(0.86-1.37)	1.14(0.92-1.42)
sVCAM1	1.25(1.03-1.55)*	1.16(0.93-1.47)	1.12(0.88-1.45)	1.15(0.91-1.47)
MMP2	1.56(1.24-1.99)†	1.1(0.88-1.39)	1.1(0.86-1.42)	1.12(0.89-1.42)
MCP1	1.36(1.12-1.66)†	1.14(0.91-1.42)	1.09(0.86-1.4)	1.1(0.88-1.38)
MMP1	1.26(1.03-1.54)*	1.16(0.92-1.47)	1.11(0.86-1.44)	1.08(0.85-1.38)
IGFBP3	0.89(0.74-1.06)	0.97(0.79-1.23)	0.96(0.76-1.25)	0.94(0.77-1.2)
MIP1B	1.22(0.92-1.62)	1.2(0.87-1.65)	1.43(0.99-2.07)	1.21(0.87-1.68)
MMP9	1.05(0.87-1.28)	1.18(0.94-1.5)	1.09(0.86-1.41)	1.14(0.91-1.46)
sIL6R	1.09(0.91-1.34)	1.13(0.91-1.43)	1.06(0.84-1.34)	1.15(0.92-1.46)
sEGFR	0.86(0.71-1.02)	0.85(0.7-1.05)	0.8(0.64-1.01)	0.84(0.69-1.03)
tPAI1	0.96(0.81-1.16)	1.07(0.86-1.36)	1.02(0.8-1.3)	1.04(0.83-1.32)
IL8	1.15(0.95-1.39)	0.99(0.79-1.24)	1.01(0.79-1.28)	1.01(0.8-1.27)

*Values presented are Odds ratio (OR, 95% CI). Model 1: protein concentration only, Model 2 = protein concentration + age+ duration of T1D + gender, Model 3= Model 2+ HbA1c, Model 4 = Model 2 + hypertension and dyslipidemia. *P < 0.05, †P < 0.01, ‡P < 0.001, and ¶P < 1x10^{-4}.*

with an increase in serum levels of 15 of the 22 proteins measured. The strongest association was noted for sTNFRI ($p<1.5x10^{-15}$), followed by sTNFRII ($p<1.5x10^{-15}$), IGFBP6 ($p<7.1x10^{-8}$), IGFBP2 ($p<5.7x10^{-6}$) and MMP2 ($p<9.4x10^{-5}$). The OR for these proteins increased with the serum levels in the fifth quintile to 11.2 (sTNFRI), 8.69 (sTNFRII), 4.8 (IGFBP6) and 3.13 (IGFBP2). Results on MMP2 levels suggested that any increase over 20th percentile has an OR of 2-4.5 of having DPN in these individuals. Markers of endothelial dysfunction (sgp130, sICAM1, sVCAM1) showed an increase in OR of from 1.5 to 2.5 as the levels of these proteins increased in the serum (**Supplementary Figure 3**).

We implemented a machine-learning approach using ridge regression to find the a combination of proteins optimally associated with DPN. The combination of 22 proteins gave the highest OR of 36.3 in 5th quintile, which was higher than that of any single protein. We then proceeded to determine the minimum number of proteins that can be combined together to improve the OR of having DPN. We found an optimal combination of 16 molecules (IL1Ra, MCP1, MIP1β, CRP, IGFBP2, IGFBP3, IGFBP6, MMP1, MMP2, MMP9, sEGFR, sgp130, sIL2Rα, sTNFRI, sTNFRII and tPAI1) which still have comparable ORs in the 3rd-5th quintile (**Figure 2** and **Table 3**). Our composite score results suggest three different risk groups in DPN patients, group 1 includes low risk DPN patients who have serum levels in 2nd quintile (OR<1.5). The individuals in 3rd and 4th quintile represents medium risk group of DPN patients with OR between 3.5-9.0. The individuals in the highest quintile belongs to the highest risk and depending on the combination of protein may have ORs between 20-36.

DISCUSSION

We report increased serum levels of five out of twenty-two proteins assessed in T1D patients with DPN. The differences were significant after accounting for age, duration of diabetes, and gender. The strongest associations with DPN were with the two TNF-α receptors (TNFR1 and TNFR2). Moderate associations were observed for solubleIL2 receptor (sIL2Rα), a marker of activated helper T-cell marker, as well as IGF binding protein (IGFBP6) and, CRP, a marker of inflammation. Using a quintile-based risk assessment approach, we identified several proteins associated with increased risk of DPN in T1D patients. Increase in serum levels of SAA, MMP2, sIL2Rα, sIL1Ra, sgp130, sICAM-1 and sIL6R in the 2nd-5th quintiles were significantly associated with DPN, whereas serum levels in 3rd to 5th quintile showed associations with DPN for IGFBP1, IGFBP2, IGFBP6 and sVCAM1. The associations observed in this study are potential therapeutic targets for DPN prevention. The TNF-α/IL-6 pathway is reportedly involved in DPN pathogenesis (20, 26).

The higher levels of inflammation markers in the DPN group supports association between inflammation and pathogenesis of DPN. Our results on the serum level of sTNFRI and sTNFRII are in agreement with previous report showing activation of TNF-α system in T1D patients with nephropathy, retinopathy and neuropathy (23–27). The soluble form of TNFRs are known to act as stabilizers of TNF-α activity, which prolongs systemic and vascular inflammation associated with complications (32). We did not noticed any weakening of the associations with DPN for sTNFRII for any of the models (27).

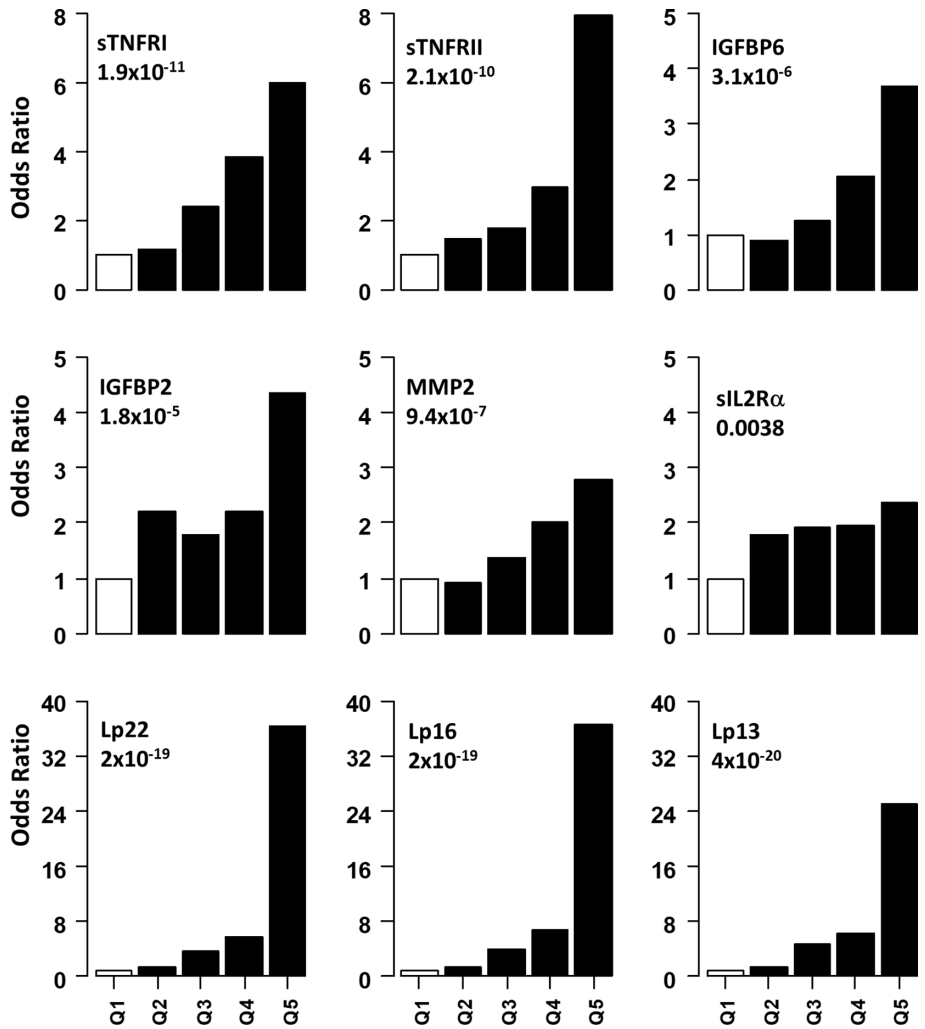

FIGURE 2 | Association of serum level of proteins with presence of DPN. Odds ratios associated with each of the top four quintiles compared to the bottom 1st quintile for each of the twelve individual proteins. The open bar represents the 1st quintile as reference (OR = 1). From left to right, each of the other four solid bars represents the 2nd to 5th quintile (20% of the DPN patients). Odds ratios associated with the risk scores calculated based on different combinations of 22 (Lp22), 16, (Lp), 13(Lp) and 11 (Lp) proteins using ridge regression. Vertical axes are odds ratios.

Activation of the TNF-α system is associated with activation of T-cells (33), which is evident with increase in serum levels of sIL2Rα, a marker for activated T-cells in several autoimmune diseases including T1D. We have not identified any other study that has evaluated the association between sIL2Rα and presence of DPN in T1D patients. Contrary to a previously published report (27) CRP was strongly associated with presence of DPN in our study. This indicates role of systemic inflammation in DPN. In our study, markers of endothelial dysfunctions (IL6, sgp130, sVCAM1, sICAM1) were not significant after multivariate adjustment. We measured serum levels of two proteins that are required for IL6 signaling, sgp130 and sIL6R. Classical IL6 signaling is only limited to immune cells. The majority of the cell types' action of IL6 is dependent on its binding to soluble IL6 receptor (sIL6R) and a signal transducing glycoprotein, sgp130, on the cell *via* trans-signaling (34). In our logistic regression analysis, we did not see any significant association of these two proteins with the presence of DPN. However, our quintile analysis suggests that any increase above the 20th percentile in the serum levels for sIL6R and sgp130 is strongly associated with presence of DPN (OR=1.5-2.25, **Table 3**). The results of our study, suggests there is increase in the inflammation promoting IL6 trans-signaling in DPN patients (35). Taken together, out results suggests an activation of TNF-α/IL6 inflammatory pathways in DPN, similar to those reported in retinopathy (25) and nephropathy (24).

Chronic hyperglycemia is shown to be one of the pathogenic factors in activation of inflammatory pathways. In our analysis, the association with DPN remained unchanged for sTNFRI, sTNFRII, sIL2Rα, IGFBP6 and CRP, after adjusting for HbA1c levels in study subjects, suggesting that factors other than hyperglycemia are also involved in pathogenesis of DPN (36).

TABLE 3 | Odds ratio (95% CI) of having DPN based on serum levels in quintile (20th percentile) categories.

Protein	Quintile-2 OR	Quintile -3 OR	Quintile-4 OR	Quintile-5 OR	Adj P Trend
sTNFRI	1.35 (0.82 - 2.23)	2.21 (1.32 - 3.71)	4.12 (2.37 - 7.15)	11.19 (5.75 - 21.77)	$1.5x10^{-16}$
sTNFRII	1.32 (0.8 - 2.17)	3.03 (1.78 - 5.16)	4.3 (2.47 - 7.49)	8.69 (4.64 - 16.28)	$1.5x10^{-16}$
IGFBP6	1.03 (0.62 - 1.7)	1.51 (0.9 - 2.53)	2.49 (1.45 - 4.27)	4.79 (2.65 - 8.67)	$7.1x10^{-9}$
IGFBP2	0.91 (0.55 - 1.5)	1.5 (0.89 - 2.52)	2.38 (1.38 - 4.09)	3.13 (1.79 - 5.48)	$5.7x10^{-7}$
MMP2	1.99 (1.19 - 3.33)	2.16 (1.29 - 3.63)	2.11 (1.26 - 3.54)	4.52 (2.57 - 7.95)	$9.4x10^{-7}$
sIL2Rα	1.69 (1.01 - 2.83)	1.72 (1.03 - 2.88)	1.78 (1.06 - 2.98)	3.07 (1.78 - 5.3)	0.00030
sgp130	1.53 (0.89 - 2.63)	2.29 (1.31 - 4)	2.32 (1.33 - 4.06)	2.25 (1.29 - 3.93)	0.00088
sVCAM1	1.02 (0.6 - 1.74)	1.42 (0.82 - 2.46)	2.57 (1.43 - 4.61)	1.6 (0.92 - 2.79)	0.00620
SAA	1.61 (0.93 - 2.78)	1.7 (0.98 - 2.95)	1.89 (1.09 - 3.29)	2.19 (1.25 - 3.84)	0.00640
MCP1	1.42 (0.83 - 2.42)	1.64 (0.96 - 2.81)	1.16 (0.69 - 1.96)	2.76 (1.56 - 4.87)	0.0130
CRP	1.39 (0.8 - 2.41)	1.08 (0.63 - 1.86)	2 (1.13 - 3.53)	1.94 (1.1 - 3.41)	0.0130
sICAM1	1.58 (0.91 - 2.73)	2.04 (1.17 - 3.57)	2.04 (1.17 - 3.57)	1.83 (1.05 - 3.18)	0.0130
MMP1	1.46 (0.87 - 2.46)	1.29 (0.77 - 2.16)	1.56 (0.92 - 2.63)	1.95 (1.14 - 3.33)	0.0200
IGFBP1	1.01 (0.61 - 1.68)	1.53 (0.9 - 2.59)	1.39 (0.82 - 2.35)	1.63 (0.96 - 2.77)	0.0240
IL1Ra	1.31 (0.77 - 2.24)	1.51 (0.88 - 2.6)	1.33 (0.78 - 2.28)	1.94 (1.12 - 3.37)	0.0340
sIL6R	1.51 (0.9 - 2.54)	1.71 (1.01 - 2.9)	1.52 (0.9 - 2.56)	1.67 (0.99 - 2.83)	0.057
IGFBP3	0.93 (0.52 - 1.67)	0.51 (0.29 - 0.9)	0.71 (0.4 - 1.26)	0.61 (0.34 - 1.08)	0.066
sEGFR	1.6 (0.91 - 2.8)	1 (0.59 - 1.71)	0.86 (0.51 - 1.46)	0.78 (0.46 - 1.32)	0.066
MIP1β	1.08 (0.63 - 1.84)	1.3 (0.76 - 2.23)	1.19 (0.7 - 2.03)	1.54 (0.89 - 2.66)	0.110
IL8	1.05 (0.6 - 1.83)	1.15 (0.66 - 2.02)	1.44 (0.81 - 2.55)	1.28 (0.73 - 2.25)	0.180
MMP9	0.98 (0.56 - 1.7)	1.05 (0.6 - 1.84)	0.96 (0.55 - 1.67)	1.15 (0.66 - 2.02)	0.660
tPAI1	1.24 (0.71 - 2.18)	0.94 (0.54 - 1.63)	1.06 (0.61 - 1.85)	1 (0.58 - 1.74)	0.720
Lp22	1.51 (0.82 - 2.79)	3.76 (1.95 - 7.25)	5.74 (2.88 - 11.46)	36.34 (12.17 - 108.5)	$1.9x10^{-19}$
Lp16	1.45 (0.79 - 2.68)	3.96 (2.05 - 7.66)	6.71 (3.31 - 13.6)	36.56 (12.25 - 109.15)	$1.9x10^{-20}$
Lp13	1.38 (0.75 - 2.55)	4.74 (2.42 - 9.3)	6.41 (3.18 - 12.94)	25.16 (9.57 - 66.13)	$4x10^{-20}$
Lp11	1.42 (0.77 - 2.62)	4.19 (2.16 - 8.13)	8.94 (4.26 - 18.77)	19.47 (7.98 - 47.47)	$1.9x10^{-20}$

Quintile 1 was used as a reference. Lp: linear predictor derived from combination of 22 (Lp22), 16 (Lp16) and 13 (Lp13) proteins.

Similar to earlier studies we did not see any effect of HTN and dyslipidemia (26, 27, 37). The results presented here suggests that our combined model may be a useful clinical tool to identify patients at risk for developing DPN.

We applied a ridge regression model to calculate the risk of DPN in T1D individuals. Ridge regression allows for individual coefficients to model the association between each individual protein and DPN. This model allows more model flexibility compared to the composite z-score approach to predict diabetes-related microvascular complications as reported by Schram et al. (26).

Our study limitations include its cross-sectional nature and the classification of DPN only based on chart review without further clinical validation. Although age was included as a confounder, DPN patients were still 14 years older than nDPN patients on average and aging is associated with inflammatory markers (26). The study has several strengths including measurement of multiple markers in a multiplex format, which enhances the replicability of the results. We have included proteins that are involved in the activation of inflammation (sTNFRI, sTNFRII and sIL2Rα), systemic inflammation (SAA and CRP), insulin like growth factor signaling (IGFBPs) and vascular function (sIL6R, sgp130, sVCAM1 and sICAM1). Our study has a larger DPN population (n=187) and more inflammatory markers than previously reported studies (26, 27). The most important contribution is the machine learning approach to combine the information to an individualized linear predictor score.

We report here that serum levels of sTNFRI, sTNFRII, sIL2Rα, IGFBP6 and CRP are increased in DPN patients with T1D after adjusting for confounding co-variates. We have successfully shown that the serum levels can also be used for stratifying DPN patients into risk groups. Our study indicates that DPN patients have an activation of inflammatory pathways, unaffected by glycemic control and hypertension. This suggests a new therapeutic strategy to prevent DPN. Our inflammatory signature may be of clinically useful tool to screen for activated inflammation in diabetes patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Augusta University IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SP and J-XS were involved with conception of the project. SP and MH acquired the Luminex data. SP, LT, and PT were responsible for data analysis. WZ, KB, DH, MG, JB, RB, BB, JM, and JR

contributed to clinical samples. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.654233/full#supplementary-material

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REVIEW
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Cholinergic System and Its Therapeutic Importance in Inflammation and Autoimmunity

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Neurological and immunological signals constitute an extensive regulatory network in our body that maintains physiology and homeostasis. The cholinergic system plays a significant role in neuroimmune communication, transmitting information regarding the peripheral immune status to the central nervous system (CNS) and vice versa. The cholinergic system includes the neurotransmitter\ molecule, acetylcholine (ACh), cholinergic receptors (AChRs), choline acetyltransferase (ChAT) enzyme, and acetylcholinesterase (AChE) enzyme. These molecules are involved in regulating immune response and playing a crucial role in maintaining homeostasis. Most innate and adaptive immune cells respond to neuronal inputs by releasing or expressing these molecules on their surfaces. Dysregulation of this neuroimmune communication may lead to several inflammatory and autoimmune diseases. Several agonists, antagonists, and inhibitors have been developed to target the cholinergic system to control inflammation in different tissues. This review discusses how various molecules of the neuronal and non-neuronal cholinergic system (NNCS) interact with the immune cells. What are the agonists and antagonists that alter the cholinergic system, and how are these molecules modulate inflammation and immunity. Understanding the various functions of pharmacological molecules could help in designing better strategies to control inflammation and autoimmunity.

Keywords: choline acetyltransferase (ChAT), cholinergic system (CS), muscarinic acetylcholine receptors (mAChR), neuroimmunology, neurotransmitters, nicotinic acetylcholine receptors (nAChR)

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INTRODUCTION

The complex bi-directional neuroimmune communication maintains each organ's physiological balance and functions in the body. The central and peripheral neuronal circuits, immune cells and cytokines, neuro-endocrine hormonal systems, gut microbiota and their metabolites, and the blood-brain and intestinal mucosal barriers are important players throughout this regulatory network. Any disturbance in these systems alters the delicate balance between health and disease (1).

The physiological mechanism of cross-talk within the neural network and reticuloendothelial system that regulates immune response, metabolism, and a vast array of pivotal functions constitute the inflammatory reflex (IR). The parasympathetic afferent and efferent arms of the Vagus nerve (VN) serve as a control center that connects impulses between the brain and internal organs (2). The afferent fibers of the VN have innervation in the reticuloendothelial system and major organs of the body. It is activated by low cytokines or endotoxins present in the tissues and communicates *via* neuronal signals sent to the poor cytokine milieu of the central nervous system (CNS) (3).

ACh has also been detected in cells of non-neural origins and microbes. It is vastly found in cardiomyocytes, the entire gastrointestinal (GI) tract, bladder urothelial cells, and various human leukemic cells, demonstrating its diverse function within an organism. The non-neuronal cholinergic system (NNCS) is made up of neurotransmitter acetylcholine, its synthesizing and degrading enzymes, transporters, and receptors within epithelial cells in airways, intestine, skin, urothelium, vagina, placenta, cornea, granulosa cells, endothelial cells, immune cells and mesenchymal cells (4). Signal transduction in keratinocytes, lymphocyte proliferation and differentiation, regulation of cytoskeleton of epithelial cells, differentiation and migration of cells in the epidermis for wound healing, ciliary activities, and regulation of the permeability in the epithelial lining of airways in an autocrine/paracrine manner (5). The details of neuronal origin cholinergic systems, their components, and signaling in the tissues have been discussed earlier (6, 7).

In this review, several immune cells that express components of NNCS and respond to neurotransmitters, specific agonists, and antagonists and their contribution to inflammation and autoimmunity are discussed. We further explored the different cholinergic agonists, antagonists, and AChE inhibitors (AChEI) that modulate the immune system and their effect on the differentiation and function of various immune cells.

Abbreviations: α-BTX, α-bungarotoxin; ACh, Acetylcholine; AChE, AcetylcholinesteraseAChEI, Acetylcholinesterase inhibitor; AChRs, Acetylcholine receptor; AD, Alzheimer Disease; BBB, Blood-Brain Barrier; BChE, Butyrylcholinesterase; BChEI, Butyrylcholinesterase inhibitor; cAMP, Cyclase adenosine monophosphate; CD, Crohn's disease; ChAT, Choline acetyltransferase; ChE, cholinesterase; ChEI, cholinesterase inhibitor; ChT, Choline transporter; CIA, Collagen-induced arthritis; CNS, Central nervous system; COPD, Chronic obstructive pulmonary disease; CS, Cholinergic status; CTL1, Choline transporter-like protein 1; CTLA-4, Cytotoxic T lymphocyte-associated protein 4; EAE, Experimental autoimmune encephalomyelitis; ENS, Enteric nervous system; FOXP3: Forkhead box P3; GI, Gastrointestinal; GPCRs, G protein-coupled receptors; HPA, Hypothalamic–pituitary–adrenal axis; IBD, Inflammatory bowel disease; IBS, Irritable bowel syndrome; ILs, Interleukins; JAK-STAT, Janus kinases signal transducer and activator of transcription; LTB-4, Leukotriene B4; MAPK, Mitogen-activated protein kinase; mAChRs, Muscarinic acetylcholine receptor; MG, Myasthenia Gravis; MS, Multiple sclerosis; nAChRs, Nicotinic acetylcholine receptor; NICUs, neuro-immune cell units; NK cells, Natural killer cells; NMJ, Neuromuscular junction; NNCS, Non-neuronal cholinergic system; NO, nitric oxide; OXO-M, Oxotremorine; PBMCs, Peripheral blood mononuclear cells; PHA, phytohemagglutinin; PKC, protein kinase C; PLC, Phospholipase C; RA, Rheumatoid arthritis; SLE, Systemic lupus erythematosus; VAChT; Vesicular acetylcholine transporter; VN, Vagus nerve; VNS, Vagus nerve stimulation; WT, wild-type.

COMPONENTS OF THE NON-NEURONAL CHOLINERGIC SYSTEM (NNCS) IN IMMUNITY

Acetylcholine (ACh)

The cholinergic system, which is found in both neuronal and non-neuronal cells, forms a network that performs various complex functions in the body. The ChAT enzyme synthesizes ACh from the precursor molecules, choline (8). The majority of choline is formed by the degradation of lipid, especially lecithin, and hydrolysis of acetylcholine (9). Acetyl-coenzyme A (Acetyl-CoA), produced by mitochondria, is used for the esterification of choline by the cytoplasmic enzyme ChAT in the parasympathetic nervous system and motor neurons (**Figure 1**). In addition to the VN, T cells, B cells, dendritic cells (DCs), and macrophages in the follicular and marginal zones of the spleen are other major sources of ACh (10). Immune cells have the machinery to synthesize ACh and directly release it into the bloodstream. In contrast, neuronal cells store ACh after synthesis in a specialized neurosecretory vesicle and release it *via* exocytosis at specialized synaptic clefts (11).

The relative concentration of ACh in humans is found to be 8.66 ± 1.02 pmol/ml in the blood and 3.12 ± 0.36 pmol/ml in plasma (12). Ach is also produced by gut microbes like *Lactobacillus plantarum* (13). Physiological levels of ACh present in the bloodstream affect immune cells in the lymphoid tissues and those that are migrating to the site of inflammation in an autocrine and paracrine manner. Recent studies have correlated lower levels of ACh in chronic inflammatory neurodegenerative diseases like Alzheimer's disease (AD), vascular dementia (VD), and multiple sclerosis (MS) (14–16). The elevated ACh level is also linked to inflammatory diseases like atopic dermatitis, chronic obstructive pulmonary disease (COPD), and periodontal disease (17–19). Patients with acute ischemic stroke had higher levels of lymphocyte-derived-ACh, which was linked to an increase in post-stroke infection and mortality (20). The diverse ways in which ACh binds to and activates different types of receptors on the surface of various cells and tissues explain its differential outcome and functions within an organism.

Choline Acetyltransferase (ChAT) Enzyme

ChAT is responsible for the biosynthesis of ACh. The ACh content in cells is proportional to the expression of ChAT within the cells (21). This enzyme is synthesized in the perikaryon of cholinergic neurons and is under the control of multiple regulatory elements (22). The enzyme occurs in both soluble and membrane-bound forms and is transcribed from various ChAT mRNA species that share identical coding regions but differ in the 5'-noncoding regions (23). R, N0, N1, N2, and M-types are some of the ChAT mRNA species that have been identified (24). N1 and N2 type mRNA transcript of ChAT is expressed by T cells, thus differing from the R type in CNS (25). In a murine model, ChAT mRNA is constitutively expressed in T

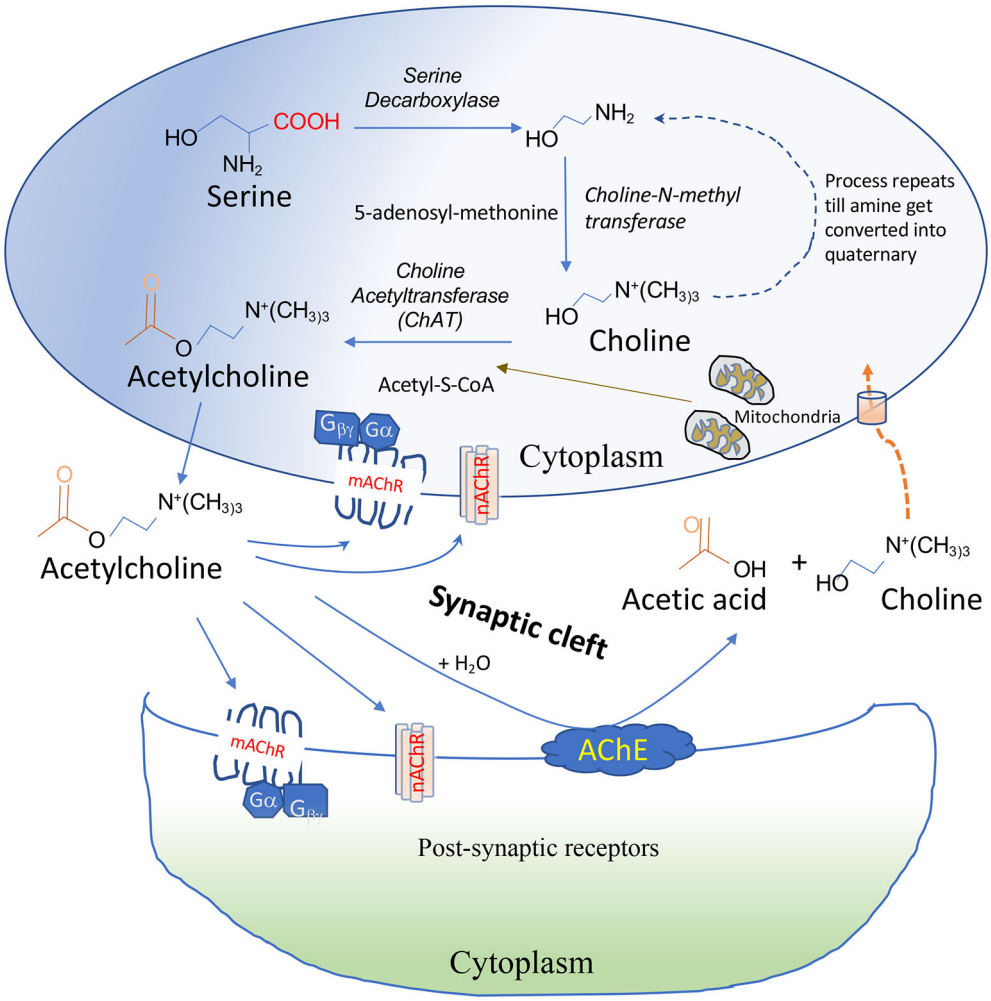

FIGURE 1 | Synthesis and degradation of ACh. ACh is synthesized from Acetyl-S-CoA and choline by the choline acetyltransferase (ChAT) enzyme in the cytoplasm. ACh is secreted out immediately after synthesis in non-immune cells but stored in a specialized vesicle in neuronal cells secreted at presynaptic neurons after activation. Release of ACh requires an influx of Ca2+ ion in the cells followed by docking of ACh-containing vesicle docking at membrane and fusion and release of neurotransmitter into the synaptic cleft *via* a process known as exocytosis. ACh binds *via* the autocrine or paracrine mechanism to nicotinic acetylcholine receptors (nAChRs) or muscarinic acetylcholine receptors (mAChRs) on post-synaptic neurons or immune cells. Acetylcholinesterase (AChE) present on the membrane can degrade ACh into choline and acetic acid. Extracellular choline formed is transported into the cells by choline transporters.

and B cells and mononuclear lymphocytes isolated from the renal vasculature (21). Upon immunological activation, peritoneal macrophages and bone marrow-derived DCs increase ChAT transcription compared to cells in the resting stages (26). ChAT mRNA expression is also detected in human leukemic T cell lines, human peripheral blood T cell and B cells, human lung and alveolar macrophages, and monocytes (27–29). COPD patients' neutrophils were observed to have over-expression of ChAT. In contrast, epithelial cells of ulcerative colitis patients displayed downregulation of ChAT, indicating, ChAT has differential involvement in different diseases affecting epithelial linings and smooth muscles (30, 31). Several natural and synthetic compounds have been identified as having ChAT stimulatory or inhibitory functions, consequently affecting the immune cells. The summary of the effect of ChAT activators and inhibitors is listed in **Table 1**.

Cholinesterase (ChE) and Cholinesterase Inhibitors (ChEI)

The degradation of ACh into choline and acetate ions is regulated by acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.7) enzymes, as shown in **Figure 1**. AChE is a 537-amino-acid protein that functions as a primary serine hydrolase. It has a recovery time of around 100 microseconds and can hydrolyze 6 X 10^5 ACh molecules per minute (63). BChE is a nonspecific serine hydrolase capable of hydrolyzing broad choline-based esters, thus serving as a co-regulator of cholinergic transmission (64). With a half-life of 20

TABLE 1 | Effect of ChAT activators and inhibitors on the immune system.

Molecules	Cholinergic effects	Effect of immune status	Experimental model
Estradiol	Increases ChAT activity in the forebrain (32, 33).	$CD4^+$ T cells, B cells, and macrophages express estrogen receptors (34). Regulates innate immunity, antigen presentation, and adaptive immune response and has a protective anti-inflammatory effect (35).	1. Ovariectomized RA mice (36). 2. Cancer model (37) 3. Autoimmune disease (38)
Trimethyltin (TMT)	TMT increases ChAT activity in the dentate gyrus (39).	TMT treatment causes atrophy of the thymus, spleen, and lymph nodes. Show reduced antibody levels, lymphocyte proliferation, NK cell function, and peritoneal macrophages' phagocytic activity (40, 41). Induce microglial/astroglial activation (42).	1.TMT-induced neurotoxic and seizure model (43). 2. Autophagy-induced Alzheimer and epilepsy (44)
A23187	Affect calcium ionophore and increases ChAT expression in leukemic T cells.	A23187 induces the expression of IL-2 receptors in purified T cells (45). It stimulates the proliferation of allogeneic T cells and increases DC-stimulated cytotoxic T lymphocytes (46). A23187 treatment in macrophages causes leukotriene C4 release and enhanced macrophage anti-tumor activity (47).	
Anti-thymocyte globulin (ATG)-Fresenius	Upregulate ChAT expression mediated by CD11a and ACh release through transient increases in intracellular Ca^{2+} (48).	ATG induced a semi-mature phenotype DC with a tolerogenic phenotype that actively suppressed the T cell proliferation (49). Negatively influence B-cell immune reconstitution and deplete cytotoxic T cells (50).	Solid-organ transplantation and allogeneic stem cell transplantation in human (51).
Dibutyryl cAMP	PKA activator upregulates ChAT mRNA expression and ChAT activity and ACh production in the human leukemic cell (52). Dibutyryl cAMP treatment on adipocytes induced Chrna2 expression that controls whole-body metabolism (53).	Dibutyryl-cAMP induces the endogenous production of cAMP and mimics the inhibitory effect of epinephrin on cytotoxic T lymphocytes (54). Cyclic AMP suppress the production of IL-2 in T cells but stimulate antigen-specific and polyclonal antibody production in B cells (55).	–
Phorbol 12-myristate 13-acetate (PMA)	Nonspecific PKC activator. It promotes the expression of both M_3/M_5 mAChR and ChAT mRNA in endothelial cells and spinal cord neurons. Thus, activating cholinergic signaling (56, 57).		
Phytohemagglutinin (PHA)	Antigen-induced T cell activation via TCR/CD3ε complexes enhances upregulation of ChAT and M_5 mAChR expression (58).	PHA-activated lymphocytes respond to cholinergic stimulation with an increase in their free cytoplasmic Ca^{2+} levels.	
Naphthyl-vinyl-pyridine derivatives (NVP)	NVP's method of ACh antagonism involves inhibiting the enzyme.	LPS challenged Splenic Lymphocyte-derived ACh was prevented by cotreatment with NVP (59).	
α-NETA	α-NETA exhibits a potent inhibitory activity of ChAT	α-NETA treatment significantly delays the onset of EAE. It antagonizes Chemokine-like receptor-1 (CMKLR1) and inhibits β-arrestin-2 cell migration (60).	
Bromoacetylcholine and Bromoacetylcarnitine	Inhibits ChAT and carnitine acetyltransferase (CarAT) activity to synthesize ACh	Synthesis of ACh was reduced by 50 percent in various leukemic T cell lines upon inhibition of ACh synthesizing enzymes (61).	
FK-506 (tacrolimus)	Reduces PHA-induced expression of ChAT mRNA and ACh synthesis through the calcineurin-mediated pathway		Treatment of MG (62).

to 60 days, AChE is predominantly found in the neuromuscular junction (NMJ), plasma, liver, and erythrocytes, while BChE is primarily found in the liver and blood plasma, with a reduced half-life of 10 to 14 days in these tissues (65, 66). The cholinergic system-specific catalytic activity of AChE/BChE degrades signal transmission by ACh and determines one's cholinergic status (CS) (67). Ubiquitous expression of AChE is found within mouse lymphocytes, DCs, and macrophages (68).

In two independent studies, serum AChE levels and CS were substantially higher in patients with irritable bowel syndrome (IBS), whereas CS was significantly lower in IBD patients (69, 70). AChE immunoreactivity was also higher in cirrhotic livers, suggesting a connection between CS dysregulation and GI diseases (71). The possible link of reduced AChE and BChE enzyme activity to proinflammatory processes through hydrolysis of ACh was evident in diseases like MS and AD (72, 73). ChE activity, in turn, can be modulated by ChE inhibitors (AChEIs and BChEIs), thereby increasing ACh levels in the body. The pharmacokinetic properties of ChEIs are thus exploited for the treatment of neurodegenerative and inflammatory diseases like myasthenia gravis (MG) and AD (74). Some ChEIs, such as donepezil, galantamine, and rivastigmine, are currently being used to treat AD (75, 76). Some of the synthetic molecules that enhance or inhibit ChE and affect cholinergic transmission are listed in **Table 2**. While many of these molecules have been studied in the context of neurological diseases, how they modulate inflammation and autoimmunity is still under investigation.

TABLE 2 | Modulators of the AChE enzyme.

Molecules	Cholinergic effect	Effect on immune status	Applications
GAL (Galantamine)	Weak competitive and reversible ChEI also allosterically modulates nicotinic acetylcholine receptors.	Treg suppressive activity is enhanced post GAL incubation (77). GAL sensitizes microglial α7-nAChRs and induces Ca^{2+} influx signaling cascades that stimulate Aβ phagocytosis in the AD model (78) GAL resulted in reduced mucosal inflammation associated with decrease MHC II levels and proinflammatory cytokine secretion by splenic $CD11c^{+}$ cells (79).	Improves cognitive function in AD and dementia (80).
Rivastigmine	Rivastigmine inhibits both AChE and BChE in CNS. It preferentially inhibits the G1 enzymatic form of AChE, predominantly found in AD patients.	Rivastigmine significantly decreases nitric oxide release, IL-1β, IL-6, and TNF-α from stimulated macrophages (81). In the EAE model, it reduces microglial activation, encephalitogenic T cells proliferation, and TNF-α and IFN-γ production (82).	Used in improving functional and clinical symptoms of AD and Parkinson's (83, 84)
Hup A	A highly selective, centrally-acting AChE inhibitor also antagonizes NMDA receptors.	HupA administration showed a reduction of proinflammatory cytokines TNF-α and IL-1β in sepsis-associated encephalopathy. Increased expressions of ChAT and CHRM1 attributed to reduced neuronal apoptosis and septic symptoms relief (85, 86). HupA reduces proinflammatory cytokines (IFN-γ and IL-17) and chemokines in the EAE while increases anti-inflammatory cytokines (IL-4 and IL-10 (4).	Hup A is administered for the treatment of AD and schizophrenia (86, 87)
Neostigmine	Blocks the active site of AChE and has limited ability to pass the blood-brain barrier.	Neostigmine increases HLA-DR expression and stimulates TNF-α production in resting DCs. It significantly reduced TNF-α and IL-12p70 production and prevented up-regulation of HLA-DR expression triggered by LPS (26).	It is administered for neurophysiological modifications in MG. It is also used to treat acute colonic pseudo-obstruction, Ogilvie syndrome, and GI disorders (88, 89).
Pyridostigmine (PY)	A potent carbamate peripheral inhibitor of AChE increases the transmission of impulses from cholinergic neurons across the synaptic cleft.	PY enhances anti-inflammatory response in HIV-1-infected patients by reducing T cell proliferation and IFN-γ production and increases IL-4 and IL-10 expression (90). It has a pro-eosinophilic effect through downregulation of IL-5, IL-13, and eotaxin in DSS-induced colitis. It also attenuates DSS-induced microbiota dysbiosis and improves epithelial integrity (91). Cholinergic modulation with PY induces greater recruitment of M2 macrophages and circulatory Treg cells soon after myocardial infarction in rats (92).	PY is used for the management of MG (93). Oral PY to be helpful in different GI dysmotility (94).
Physostigmine	Interfere with acetylcholine signaling such as atropine, scopolamine.	Physostigmine significantly decreases the expression of IL-1β, TNF-α, and IL-10 in the spleen and plasma in mice models, along with reduced neurodegeneration in the hippocampus (95).	ChEI was first investigated for the treatment of AD however discontinued for multiple adverse effects (96).
Ambenonium chloride	AChE inhibitor and down-regulates α6β2 -nAChR mediated dopamine release.		Reduce the aggregation of the β-amyloid peptide (Aβ) and a prion-peptide in AD (97)
Acotiamide hydrochloride	A selective, reversible AChE inhibitor improved clonidine-induced hypomotility.		Used for treatment of functional dyspepsia (98).
Corydaline	Inhibits AChE in a dose-dependent manner.	Inhibits pro-inflammatory cytokines expression (TNF-α, IL-6) in LPS-challenged macrophages.	
Donepezil	Centrally acting reversible AChEI and also upregulates nAChR in neurons (99, 100).	It shows anti-inflammatory effects and prevents BBB degradation by modulating MMP-2/9, NGF/proNGF, IFN-γ/IL-4, and p-Akt in EAE (101). Pre-treatment with donepezil suppressed TNF-α–induced sustained intracellular Ca^{2+} elevation via the PI3K pathway in rodent microglial cells. It also suppresses NO production and increases the phagocytic activity of mouse primary microglial cells (102). In macrophages, donepezil reduces inflammatory cytokines (IL-1β, IL-2, IL-6, IL-18, and TNF-α) and attenuates LPS-induced nuclear translocation of NF-κB (103). It inhibits RANK-induced bone degradation by inhibiting osteoclast differentiation (104).	It is mainly used to treat AD, PD, Schizophrenia, and depression (105–106).
Choline alfoscerate (α-GPC)	Parasympathomimetic acetylcholine precursor, acting as acetylcholine release promoter (107).	–	–
Cisapride	Stimulate serotonin receptors mediated increases of acetylcholine release in the enteric nervous system (108).	–	–
Curcumin	Stimulates vagus nerve and enhance ACh biosynthesis by upregulating	–	–

(Continued)

TABLE 2 | Continued

Molecules	Cholinergic effect	Effect on immune status	Applications
	enhanced ChAT activity and expression of VAChT in murine RA model. (109) Upregulates gene expression of M_1 and M_3 mAChR, choline acetyltransferase, and GLUT3 in the cerebral cortex of diabetic rats (110).		

Choline Transporter (ChT) and Vesicular Acetylcholine Transporter (VAChT)

ChTs are expressed on the cell membranes of cholinergic neurons in presynaptic terminals and regulate the ACh reservoirs during autonomic, cognitive, and motor functions (111). This membrane protein helps transport the precursor molecule choline into the neurons for the synthesis of ACh (111). ChTs are predominantly found on the plasma membrane of microvascular cells. They are also highly expressed on the mitochondrial membrane, where they are involved in choline oxidation upon absorption. Bone marrow-derived macrophages treated with lipopolysaccharide (LPS) show increased transcript and protein expression of the choline transporter-like protein-1 (CTL1) (112). The human leukemic T-cell line expresses *CHT1* mRNA and mediates choline uptake in T cells (113). Activation of protein kinase C causes inhibition of CTL1 in macrophages, thereby causing altered cytokine secretion (114). The distribution of ChT on different immune cells and its importance in the specific tissue microenvironment in controlling inflammation and immunity need to be further investigated.

ACh is packaged into the secretory vesicles by a specific transporter protein VAChT using an exchange of protons (H^+) (115). Most cholinergic neurons in the brain, spinal cord, and NMJ with skeleton muscle express VAChT (116). Alteration of VAChT expression has consequences on the concentration of acetylcholine loaded in the secretory vehicle, thus indirectly maintaining the neurotransmitter release. Dysregulation of VAChT has been reported in several diseases like AD, Epilepsy, and Sepsis (117–119). VAChT knockdown (VAChT-KD) mice, upon LPS challenge, show increased susceptibility to inflammation and greater mortality. LPS challenge increases the levels of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in the spleen, brain (120). Human melanocytes, keratinocytes, and alpha-cells also express non-neuronal VAChT, thereby regulating the acetylcholine release and cholinergic activity (121, 122). The expression and function of VAChT on immune cells are still unclear and thus have potential physiological consequences in the peripheral immune response.

Acetylcholine Receptors (AChRs)

The diversified role of ACh is governed by different types of receptors, known as cholinergic receptors (AChRs), which are classified according to their affinity for various chemical ligands (123).

Nicotinic Acetylcholine Receptors (nAChRs)

These receptors respond to the ligand, nicotine. These ligand-gated ion channels are composed of four distinct subunits (α1–10, β1–4, γ, and δ) bound in different stoichiometric ratios around a central pore with the help of ϵ subunits (**Figure 2**). These receptors exist as homomers (with all subunits of one type), such as $(\alpha7)_5$, or as heteromers with at least one α and one β subunit among the five subunits that are combined in various combinations, such as $(\alpha4)_3(\beta2)_2$, $(\alpha4)_2(\beta2)_3$, $(\alpha3)_2(\beta4)_3$, $\alpha4\alpha6\beta3(\beta2)_2$ (124). The structure of the human α3β4-nAChR complex, solved using cryoelectron microscopy (125), is shown in **Figure 2**. This structure shows a central channel formed by different subunits of nAChRs. The channels help move ions from the extracellular environment to the cytoplasmic side or vice versa in the cells after stimulation with specific ligands (**Figure 2**). The α3β4-nAChR subtype is located in the autonomic ganglia and adrenal glands, which forms the main relay between the central and peripheral nervous systems in the hypothalamic-pituitary-adrenal axis (HPA) axis upon activation (126). Diverse nAChR subtypes confer differential selectivity for nicotinic drugs in the central and peripheral nervous system, muscles, and many other tissues (127). The ligand binds to the specific site of the receptor leading to the triggering of all subunits of nAChR to change conformation, resulting in the opening of a non-selective cation channel, which then regulates the movement of ions (128).

In the immune system, nAChRs are known to regulate inflammatory processes (129). Pathological causes in acquired neurodegenerative diseases, such as autoimmune ganglionopathy, autoimmune encephalitis, and MG, are caused by an autoimmune reaction against nAChRs in the NMJ (130–132). Non-neuronal nAChRs are also involved in the pathogenesis of palmoplantar pustulosis, psoriasis, and rheumatic diseases (133–135). Overexpression of nAChR in gastric, colorectal, pancreatic, liver, lungs, and breast tumors appears to regulate cancer cell processes such as proliferation, apoptosis, angiogenesis epithelial-mesenchymal transformation (136, 137). Several of the nAChR agonists and antagonists are known to work in a receptor-specific and selective manner. Some of the agonists and antagonists are listed in **Tables 3** and **4**. Treatment with these ligands and their

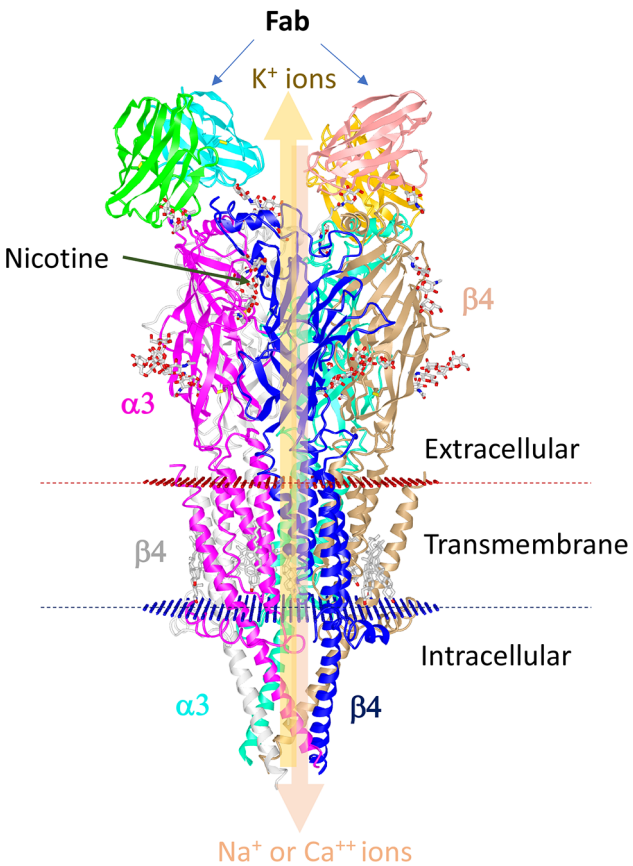

FIGURE 2 | Structure of human α3β4-nAChR complexes with nicotine. The human nAChR complex with α3β4 nicotine acetylcholine receptor (Protein data bank Id: 6PV7) is displayed using the online iCn3D software. The structure is composed of two α3 chains and three β4 chains. The Fab fragments of the antibody used for stabilization of the sample are shown at the top. Nicotine is shown in balls and sticks. Red and blue discs represent the plasma membrane. The thick arrow depicts the regulation of the movement of ions by the central pore.

effect on immune cells is not very well studied and needs detailed investigation.

Muscarinic Acetylcholine Receptors (mAChRs)

These are metabotropic receptors consisting of seven transmembrane subunit G protein-coupled receptors (GPCRs) that respond to ACh and muscarine (248). M_1 to M_5 mAChR subtypes share 64 to 68 percent sequence identity and 82 to 92 percent sequence similarity, indicating that they have a high degree of sequence homology. Their G-protein coupling preferences and physiological functions, however, are different. mAChRs have been separated into two groups based on their functional coupling. The M_1 mAChR, M_3 mAChR, and M5 mAChR are coupled to $G_{q/11}$ proteins, which mediate the activation of phospholipase C (PLC) activity (249). The M_2 mAChR and M_4 mAChR are coupled to the $G_{i/o}$ protein, which mediates inhibition of adenylate cyclase (AC) and thus causes a decrease in cyclic adenosine monophosphate (cAMP) (248). Based on the physiology and distribution of the individual receptor, mAChRs can trigger different signal transduction pathways in the cells in a tissue-specific manner. Recently, the structures of the human M_1 mAChR and M_2 mAChR2 with G-protein complexes were published (250). The structure visualization of human M_1 mAChR with the G-protein complex is shown in **Figure 3**. mAChRs have an extracellular ligand-binding domain and a transmembrane and intracellular signaling domain. The intracellular domain interacts with G proteins and other signaling molecules and helps intracellular signaling (**Figure 3**). mAChRs are abundant in the hippocampus, cortex, thalamus, gastric and salivary glands, smooth muscle, and cardiac tissue, each having a specific downstream signaling cascade. Thus, the structural differences, ligand specificity, and functioning mechanism help understand each receptor's roles within specific tissues. In the murine endotoxemia model, muscarinic receptor-mediated cholinergic signaling in the forebrain regulates peripheral immune function and inflammation to suppress serum TNF-α levels (251). Conversely, the major cause for the pathogenesis of autoimmune Sjögren's syndrome is the production of auto-antibodies against the M_3 mAChR (252). Autoantibodies

TABLE 3 | Agonists of acetylcholine receptors (AChRs).

Agonists	Cholinergic effects	Effect of immune status	Experimental models
Pan-cholinergic agonists			
Acetylcholine chloride	Non-selective cholinergic agonist mimics the effect of the endogenous compound acetylcholine.		Its multi-faceted action, toxicity, and rapid inactivation by cholinesterase do not offer a therapeutic value.
Carbachol	Non-selective cholinomimetic agonist stimulates both muscarinic and nicotinic receptors.	Carbachol treatment reduces the expression of IL-1β; MHC-II, CD86, and IL-12p70 in splenic DCs at the early phases of sepsis (138). It reduces the release of inflammatory cytokines (TNF-α, IL-1β, and IL-6) and expression of caspase-3 in myocardial cells and improves the cardiac function and survival rate from sepsis in rats (139). Carbachol increases the expression of inflammatory genes (IL-6, IL-8, and cyclooxygenase-2) in smooth muscles (140).	Used for treatment of glaucoma in humans (141).
Selective to nicotinic receptors			
Nicotine	Nicotine induces non-selective activation of nAChRs.	Nicotine-treated cells produce lower Th1 cytokines (IL-2 and IFN-γ), but significantly higher Th2 cytokines (IL-4 and IL-10) (142). Nicotine suppresses IL-18-mediated systemic inflammatory responses and downregulates expression of TNF-α, IL-12, and IFN-γ in PBMCs (143).	Nicotine has anti-inflammatory and depressive activity in neurodegenerative and depressed patients (144, 145).
Cotinine	Activates, desensitizes nAChR at a much lower potency than nicotine.	A high cotinine concentration stimulates extracellular ROS generation and oxidative stress-mediated tissue damage by activated neutrophils (146). Pre-treatment of monocytes with cotinine mounts IL-10-dominated anti-inflammatory response via α7-nAChR through PI3K/GSK-3β-dependent pathway (147).	
ABT-418		ABT-418 has a neuroprotective effect on rat cortical cells to glutamate (Glu)-induced cytotoxicity mediated via interaction with α7-nAChRs (148).	A clinical trial of ABT-418 was conducted to treat adults with attention deficit hyperactivity disorder and AD (149, 150).
Epibatidine	Binds to the α4/β2-nAChR and also binds to the α3/β4-nAChR subtype.	Stimulation of α4β2 nAChRs with epibatidine increases the IgM-mediated proliferation of B cells (151). *In vivo* administration of epibatidine (5 μg/kg, s.c.) increases plasma corticosterone levels and reduces the lymphocyte proliferation in the presence of concanavalin A (152).	
Succinylcholine chloride	Irreversible and competitive agonist on muscle type (α1)2β1δε-nAChR (153). It is resistant to acetylcholinesterase and is quickly degraded by plasma butyrylcholinesterase.	Patients who received succinylcholine as an anesthetic had lower CD4/CD8 frequency and IgE levels in their peripheral blood. It also changes the oxidative state of lymphocytes by impairing glutathione levels and prompting T cells to produce more reactive oxygen species (ROS) (154, 155).	
PNU-282987	Selective α7-nAChR agonist	PNU-282987 has a protective function in the lung injury model. PNU–282987 inhibits TNF–α and IL–6 release and decreases the phosphorylation levels of p38, JNK, and ERK in peritoneal macrophages (156). In the bronchoalveolar microenvironment, PNU-282987 reduces the neutrophil recruitment and inflammatory cytokines secretion (157). It also has an anti-inflammatory role in NK cells by reducing the NF-κB levels and its translocation to the nucleus, down-regulating the expression of NKG2D receptors, and inhibiting IFN-γ secretion and NKG2D-dependent NK cell cytotoxicity (158)	It has been used as an anti-inflammatory therapy in animal models of diseases such as airway inflammation, cardiomyopathies, and AD (159–160).
Cris-104	Neuronal α4β2-nAChR agonist	Cris-104 increases nor-epinephrine concentration and increases neuronal activity in the brain, thus having an anti-nociceptive efficacy in rodent models of acute and chronic pain (161).	
PHA-543613	Selective α7-nAChR agonist	PHA-543613 suppresses CDC42 and MMP2 mRNA expression in macrophages (162). Administration of PHA-543613 induces activation of PI3K/AKT/GSK-3β to reduce neuroinflammation and oxidative stress (163, 164).	It's being studied as a potential cure for cognitive deficits in schizophrenia, PD, and intracerebral hemorrhage (165, 166).
NS6740	Silent non-ionotropic agonist of α7-nAChRs but an effective modulator of the cholinergic anti-inflammatory.	NS6740 shows an anti-inflammatory property in LPS challenge microglial cells by reducing TNF-α release (167).	
GAT107	Positive modulator of α7-nAChR	GAT107 shows a dose-dependently attenuation of CFA-induced inflammatory pain by reducing phosphorylation of intracellular p38MAPK (168). In macrophages,	

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TABLE 4 | Antagonists of selective acetylcholine receptors (AChRs).

Antagonists	Cholinergic effects	Effect on immune status	Experimental models
Selective to nicotinic receptors			
Hexamethonium	Nicotinic receptor blocker	Hexamethonium blockade of peripheral nAChR increases neutrophil migration and mechanical hyperalgesia in the RA model (207). It suppresses stress-induced mast cell activation and inhibits elevated PGE2 levels after exposure to stress (208).	–
α-Bungarotoxin (α –BTX)	Bind to nAChRs in an irreversible antagonistic manner, blocking ACh's activity at the post-synaptic membrane, inhibiting channel opening and ion flow, and cause paralysis (209).	α –BTX treatment to T cells activated by sub-optimal PHA concentrations causes blockade of α7-nAChR that enhance T cell proliferation (210).	–
Mecamylamine	To neutralize the effects of nicotine, it is used as a competitive non-selective (α3β4, α4β2, α3β2, and α7) nAChR antagonist	Mecamylamine reverses the ant-inflammatory role of nicotine in the nAChR-mediated cholinergic pathway.	Mecamylamine is licensed for the treatment of hypertension. It attenuates all of the nicotine and cigarettes symptoms, including seizures, rendering it an important pharmacotherapy for tobacco addiction (211).
Dihydro-beta-erythroidine	Selective α4β2-nAChR antagonist (161).	–	–
Dextromethorphan (DXM)	α3β4-nAChR, α4β2-nAChR, and α7-nAChR antagonist in the cholinergic pathway (212). Also, It is a selective antagonism of N-methyl-D-aspartate receptors and/or show interaction with opiate receptors (213).	DXM decreases the expression of CD40, CD80, CD86, MHC class I, and MHC class II in both murine BMDCs and human monocyte-driven DCs upon LPS challenge. DXM pre-treatment results in dose-dependent substantial reductions in TNF-α, IL-6, IL-12, and ROS production. It inhibits the ability of LPS-stimulated BMDCs to promote ovalbumin-specific T cell proliferation by downregulating MAPK and NF-κB pathways (214). DXM is neuroprotective in cerebral ischemia models, spinal cord injury, PD, and epilepsy by downregulating NADPH oxidase, thus, reducing superoxide free radicals and intracellular (ROS) (215, 216). It prevents immune cell filtration, inhibits NOX2 activity, and has an anti-inflammatory effect in EAE (217). Proinflammatory cytokines (TNF-α, IL-6, and IL-17A) expression levels decrease in CIA mice and RA patients. In collagen-reactive $CD4^+$ T cells, DXM reduced the production of anti-CII IgG, IFN-γ, and IL-17A (218).	DXM is under development for the treatment of depression, AD, ALS, and neuropathic pain (219–220).
Methyllycaconatine (MLA)	α7-nAChR antagonist	MLA (2.4 mg/kg per day) treatment in acute viral myocarditis increases the frequency of Th1 and Th17 cells, lowers the frequency of Th2 and Treg cells in the spleen. It also increases proinflammatory cytokines, cellular infiltration, and severity of myocardium lesions in viral myocarditis (221).	–
N,N-decane-1,10-diyl-bis-3-picolinium diiodide (bPiDI)	Selective α6β2-nAChR antagonist (222). Nicotine-evoked dopamine activation and nicotine reinforcement are mediated by α6β2-nAChRs expressed by dopaminergic neurons. bPiDI blocks nicotine's effects on these receptors, making them therapeutic targets for nicotine addiction (223).		
α-conotoxins	Specific to α3β2-nAChR, α9α10-nAChR, and α3β4-nAChR (224).	α-conotoxins (5.5 μM) increases IL-10 production in Tregs and decreased IL-17 production in T cells (225). In PMA-activated macrophages, α-conotoxins upregulate the TNF-α and IL-6 in a concentration-dependent manner (226).	–
SR16584	High affinity for α3β4-nAChR and 10 nM for α4β2-nAChR (227, 228).	–	–

(Continued)

TABLE 4 | Continued

Antagonists	Cholinergic effects	Effect on immune status	Experimental models
18-methoxycoronaridine (18-MC)	Highly selective α3β4-nAChR antagonist (229).	–	–
AT-1001	High-affinity and selective to α3β4-nAChR (230).	–	In humans with Th1-mediated celiac disease, it plays a therapeutic role by inhibiting cell permeability (231).
MG 624	α7-nAChR antagonist (232).	–	–
Selective to muscarinic receptors			
Atropine	A nonspecific antagonist that competitively inhibits acetylcholine (ACh) at postganglionic muscarinic sites and CNS (232). Abolish the effect of vagus nerve stimulation.	Prior to the LPS-induced activation of the inflammatory response, atropine decreases TNF-α and raises IL-10 plasma levels without affecting IL-6 production. This reduction in TNF-α improved the rate of survival from endotoxic shock in mice (233). Suppresses T cell proliferation and proinflammatory cytokine production in turpentine-induced inflammation. In reaction to the potent neutrophil/macrophage chemoattractant fMLP, atropine therapy decreases both chemokinesis and chemotaxis of PBMCs (234, 235).	–
Hyoscyamine	Non-competitively inhibits acetylcholine (ACh).	In the acute lung injury model in rats, hyoscyamine derivatives cause substantial reductions in TNF-α, IL-6, IL-1, and p38MAPK, NFB, and AP1 activation, as well as TLR4 expression (236).	–
Scopolamine hydrobromide	A non-selective muscarinic acetylcholine receptor (mAChR).	Scopolamine hydrobromide treatment shows upregulation of TLR3, TLR7, TLR8, and cytokines such as IL-4 and IL-10 (237). Mice treated with scopolamine show an increased density of $CD4^+$, $CD11c^{+,}$ and $CD11b^+$ cells. And also show elevated levels of IL-1β, IL-2, IL-6, IL-12Rβ1, IL-17A, IL-17R, IFN-γ, and TNF-α transcripts (238).	Used for treatment of motion sickness, and GI obstruction (239, 240)
Gallamine Triethiodide	Non-competitive inhibition by altering the affinity of the agonist for its binding site.	–	–
VU0255035	Selective M_1 mAChR antagonist	–	–
Pirenzepine	Selective M_1 mAChR selective antagonist.	–	Used in peptic ulcers and also reduces muscle spasms (241, 242).
Methoctramine	Selective M_2 mAChR antagonist	Methoctramine increases the high-frequency component of heart rate variability and inhibits systemic TNF–α release by activating muscarinic receptors (182). Methoctramine abolishes the ACh-elicited anti-apoptotic property and reduces the TNF-α-activated apoptotic pathway via EGFR-PI3K signaling in cardiomyocytes (243).	–
AF-DX 384	Selective M_2 mAChR and M_4 mAChR antagonist.	–	–
Darifenacin	Selective M_3 mAChR antagonist	–	Effectively used for the treatment of overactive bladder disorder (244).
4-diphenylacetoxy-N-(2-chloroethyl)-piperidine (4-DAMP mustard)	Selective M_1/M_3 mAChR antagonist.	4-DAMP abolishes mAChR-mediated immunoglobulin class switching to IgG in B cells. It inhibits the production of IL-6 and the maturation of B cells into IgG-producing plasma cells (245). M_3 mAChR-mediated IL-8 expression in regulating inflammatory response via PKC/NF-κB signaling axis is completely antagonized by 4-DAMP (246). It also inhibits human T cell growth by inhibiting M1 mAChR-mediated expression of both IL-2 and IL-2R (179).	–
Tropicamide	M_4 mAChR antagonist responsible for increased phosphorylation of AMPA receptor.	–	Inhibiting cholinergic stimulation responses, producing dilation of the pupil and relaxation of the ciliary muscle in ophthalmic surgery (247).

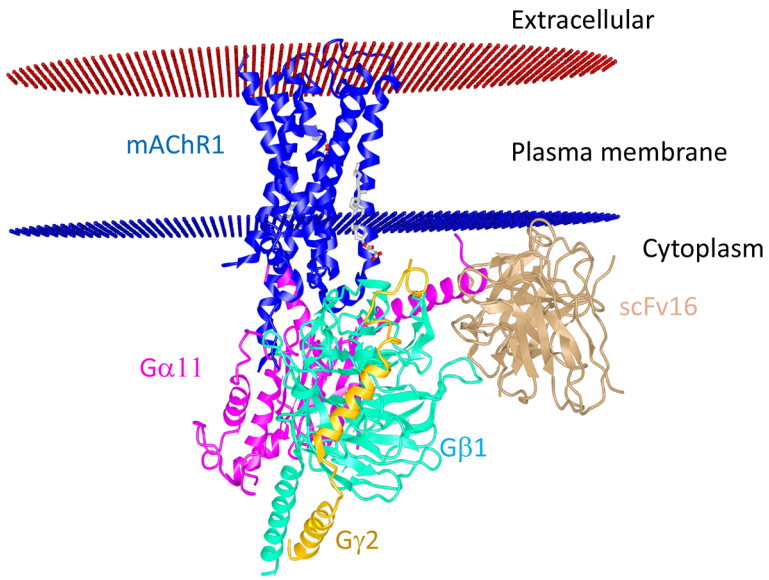

FIGURE 3 | Structural interaction of M_1 mAChR with the G protein-coupled receptor. The human muscarinic acetylcholine receptor 1G11 protein complex (Protein data bank Id: 6OIJ) structure (3.3 Å resolution) is displayed using the online iCn3D software. M_1 mAChR interacts with G-proteins α11 and γ2-β1. The scFV16 nanobody used for stabilizing the structure is also shown. The allosteric ligand is shown in the ball and stick. Red and blue discs represent the plasma membrane.

against muscarinic receptors also triggered chronic immune activation in patients with chronic fatigue syndrome and periodontitis (253, 254). Patients with airway inflammatory infections/allergic rhinitis had increased expression of M_3 mAChR mRNA and protein (255). However, the specific patterns of mAChR subunit distribution in tissues and expression in particular immune cell types are not well defined. Some of the selective mAChR agonists and antagonists are listed in **Tables 3** and **4**, respectively. Given the diverse distribution of mAChRs in different immune cells, the mechanism by which selective ligands alter specific immune cells in the tissue microenvironment during inflammation and immunity needs detailed investigation.

The Role Played by the Cholinergic System in Various Immune Cells

The neuronal and lymphoid cholinergic system evokes various downstream functional and biochemical effects through AChRs present on immune cells. The importance of different components of the complex cholinergic system in different immune cells is discussed below-

Role in T Cells

T cells and their effector and regulatory function play an important role in inflammation and autoimmunity (256, 257). Some splenic and intestinal T cell subsets have been found to express functional ChAT and produce ACh ($ChAT^+$ T cells) (21, 258). Most of these T cell subsets are found in the vicinity of catecholamine splenic nerve fibers forming a cholinergic non-neuronal reservoir (259). The ACh produced in the microenvironment activates α7-nAChR on T cells and facilitates the activation and proliferation of T cells (260). *In vitro* administrations of nicotine or ACh in a micromolar range inhibits DC mediated-T cell proliferation and differentiation, as well as reduce CD28 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) expression and diminished maturation in T cells (261, 262). Endogenously released ACh in human leukemic T cell upregulates several Ca^{2+}-permeable ACh-gated ion channels. Nicotine also impairs antigen receptor-mediated signal transduction in lymphocytes and causes T cell anergy by arresting cells in the G0/G1 phase (263). It is also known to alter the expression of co-stimulatory and adhesion molecules (such as ICAM and CD44) on immune cells and suppress the production of inflammatory cytokines (TNF-α, IFN-γ, and IL-6) (264). Nicotine binds to various forms of nAChR on T cells with variable affinity and mediates the apparently paradoxical effects of fostering cell survival while also causing apoptosis by inducing expression of FasL and survivin gene (265). The receptors, α7-nAChRs and α4-nAChR are predominantly known to be involved in $CD4^+$ T cell proliferation and function (266, 267). The α7-nAChR antagonist, α-bungarotoxin (α-BTX), and methyllycaconitine (MLA) show an increased proliferative response to the T cell mitogen, phytohemagglutinin (PHA) (268). Activation of the α7-nAChR by nicotine in experimental autoimmune encephalomyelitis (EAE) mice model ameliorate clinal symptoms by directing naive $CD4^+$ T cells towards the IL-4-producing Th2 phenotype and subsequently leads to decreased production of Th1 cytokines (such as TNF-α, IFN-γ, IL-2) and Th17 cytokines (such as IL-17, IL-17F, IL-21, and IL-22). However, activation of α7-nAChR with agonist does not affect the differentiation of Th17 cells (269). α7-nAChR activation with nicotine in human PBMC and $CD4^+$ T cells results in a similar reduction of IL-17 production, suggesting that it has an anti-inflammatory property (270).

Nicotine-induced activation of α7-nAChRs also increases the suppressive function of $CD4^+CD25^+$Tregs/$CD4^+CD25^-$ T-cell by up-regulation of CTLA-4 as well as Foxp3 expression and decreased IL-2 secretion (271). These studies suggest that nAChRs activation can modulate the function of various subsets of $CD4^+$ T cells.

In humans, all five subtypes of mAChRs are known to be expressed on lymphocytes. However, each receptor's expression pattern differs among different cell types in an individual (29). PHA is known to increase M_5 mAChR mRNA expression *in vitro*, while lymphocytes stimulated with phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator, plus ionomycin, a calcium ionophore, increase M_3 and M_5 mAChR mRNA expression, demonstrating that differential expression of mAChR is caused by different immunological stimulations (272). Activation of the M_3 mAChR using methacholine on $CD4^+$ T cells isolated from airway inflammatory infections/ allergic rhinitis patients leads to increased production of IL-4 and TNF-α (255). Treatment of Jurkat cell lines with the mAChR agonist, oxotremorine (OXO-M), increases the expression of IL-2 receptors on lymphocytes and enhances the PMA-induced IL-2 secretion (273). Interestingly, it was found that arecoline, a non-selective muscarinic agonist improves cognitive function and memory in AD (274). Chronic treatment with arecoline leads to a reduction in the size of the spleen, thymus, and mesenteric lymph nodes, as compared to untreated control mice. *In-vitro* arecoline treatment was shown to reduce lymphocyte proliferation and IL-2 production (275). M_3 mAChR knockout ($M3^{-/-}$) mice were reported to have trouble in clearing bacterial and helminth infections. The absence of M_3 mAChR also led to delayed expulsion of *Nippostrongylus brasiliensis* due to inhibition of smooth muscle contraction, a reduction in the activation of $CD4^+$ T cells, and lower levels of expression of IL-4 and IL-13. The activation of the M_3 mAChR, specifically with ACh on activated T cells, increases IL-13 and IFN-γ cytokine production (276). The distribution of the M_1-M_5 mAChR on each subset of $CD4^+$ T cells in humans and mice has not been systemically studied and is therefore open for further investigation.

Role in B Cells

Mouse B cells are known to express α4, α5, α7, β2, and β4 subunits of nAChR, with the expression of different subunits being regulated at the various stages of B cell maturation (277). At the primary level, nAChRs are required for the development and survival of B lymphocytes within the primary lymphoid tissues and spleen. α4 and β2 knockout mice have reduced B cell populations, underscoring their critical role in regulating lymphocyte survival (278). The $CD19^+B220^+IgM^+$ B lymphocytes mainly expressed α7, α4, and β2 subunits of nAChRs. Mice deficient in these subunits of nAChRs showed reduced amounts of serum IgG, while $\beta2^{-/-}$ mice had a reduced number of IgG-producing cells in the spleen. However, the IgG response to horse cytochrome *c* in α4 and β2 knockouts was stronger than in wild-type (WT) mice, with the $\beta2^{-/-}$ mice having high cytochrome *c*-specific antibodies after immunization (279). Also, α7-nAChR influences IgM antibody production but not IgM to IgG class switching (280). α7-nAChR is constitutively expressed on $CD5^+CD1d^+$ regulatory B lymphocytes, which increases with the activation of B lymphocytes. Inhibiting α7-nAChR with methyllicaconitine inhibits CD40-mediated B lymphocyte proliferation (281). α4β2-nAChR, α7-nAChR, and α9α10-nAChR on B lymphocytes are differentially involved in B cell-mediated immune cell interactions. α7 and α9(α10) subunits of nAChRs are linked to CD40-mediated B cell proliferation, while the α4β2-nAChR is linked to IgM antibody production (151). However, the initial levels of IgM in WT and $M_1/M_5^{-/-}$ double knockout mice were similar. The OVA-antigen-specific IgG1 levels in $M_1/M_5^{-/-}$KO mice were significantly lower, suggesting that mAChRs are not required for antibody production but are involved in immunoglobulin class switching (282). M_1 mAChR knockout ($M_1^{-/-}$) mice show increased splenic noradrenaline production and a decrease in the number of IgG-producing B cells (283). M_3 mAChR in B cells show an increase in calcium signaling and c-Fos gene expression, thereby affecting several downstream signaling pathways (284). However, an autoantibody against the M_3 mAChR has been reported in the immunopathogenesis of Sjögren's syndrome and correlates with a significant risk of developing B cell lymphoma (285). Together, these studies suggest that AChRs contribute to mounting an effective humoral response. A detailed investigation of cholinergic systems' expression in the different developmental and differentiation stages of B cells needs to be investigated, and its clinical importance in the inflammatory disease yet to be established.

Role in Dendritic Cells (DCs)

Nicotine impairs the capability of DCs to capture antigens and reduces the responsiveness of DCs to maturation stimuli. The nicotine-treated DCs fail to produce IL-12, IL-1β, IL-10, and TNF-α and thus unable to induce APC-dependent T cell responses and Th1-cell polarization. DCs exposed to nicotine tend to polarize $CD4^+$ T cells towards the Th2 phenotype. These show increased expression of OX40L and significantly high amounts of IL-4, IL-5, and IL-13 (261, 286). $CD205^+$ DCs express mRNA encoding secreted lymphocyte antigen-6/ Urokinase-type plasminogen activator receptor-related peptide (SLURP)-1, an endogenous α7-nAChR allosteric ligand that stimulates DCs to produce ACh in an autocrine manner (260). Treatment with GTS-21, an α7-AChR agonist, showed a robust anti-inflammatory action during collagen-induced arthritis (CIA) in DBA/1 mice by modulating DCs. GTS-21 treatment down-regulated CD80 and MHC II expression on the surface of DCs, leading to suppression of its infiltrating capacity and differentiation. These inhibitory effects were successfully reversed by the α7-nAChR antagonist, methyllycaconitine (287, 288). Treatment of DCs with ACh also resulted in the upregulation of the macrophage-derived chemokine (CCL22) in the thymus and the activation-regulated chemokine (CCL17). CCL22 and CCL17 help in the recruitment of Th2 cells at the site of inflammation (289). In both mice and humans, DCs treated with nicotine utilize upregulation of notch ligands and nuclear receptor peroxisome proliferator-activated receptors γ (PPAR γ)

to modulate the Th1/Th2 balance in favor of Th2 lineage (290). Activation of nAChRs on immature DCs (imDCs) has also been shown to have anti-tumorigenic effects. Activation of nAChR promotes the expression of co-stimulatory molecules CD80/CD86 and 4-1BBL on imDCs, thereby increasing their ability to stimulate T-cell proliferation. Transfer of nicotine-treated imDCs has been shown to reduce tumor growth by generating an effective cytotoxic T cell response in the tumor microenvironment (291).

DCs have been found to express the M_3 mAChR, M_4 mAChR, M_5 mAChR, ChAT, and AChE (289). Methacholine, a synthetic choline ester, and a non-selective mAChR agonist were shown to increase expression of the OX40L on DCs, which helps in the interaction co-stimulation with T cells (255). Cholinergic activation using the M_1 mAChR-specific agonist, McN-A-343, in colitis was found to decrease IFN-γ, IL-17, IL-12p70, and IL-23 in the splenic $CD11c^+$ DCs (181). These studies suggest that AChRs affect the immune response by altering innate immune cells like DCs. Further detailed molecular mechanism of cholinergic receptor signaling in the differentiation and function of DCs under different inflammatory conditions and tissues needs to be investigated.

Role in Macrophages

The intracellular signaling of alveolar macrophages is mediated by α9, α10, and β2-nAChR. Macrophage populations previously exposed to nicotine show a lower rise in ATP-induced intracellular Ca^{2+} release, which is independent of STAT3 phosphorylation (292). The α7-nAChR on macrophages is vastly sensitive to ACh released by the $ChAT^+$ T cells, and its interaction with ACh leads to reduced production and release of proinflammatory cytokines. Antigen-stimulated spleen cells from α7 knockout mice produce significantly higher amounts of TNF-α, IL-6, and IFN-γ than splenocytes from WT mice (264). Vagus nerve stimulation (VNS) for a duration of 0.1–60s in WT mice modulates cytokine release from macrophages *via* the α7-nAChR receptor. However, the α7 subunit deficiency rendered the VN ineffective in inhibiting TNF-α release (293). Priming macrophages with a cognate ligand for α7-nAChRs results in a pharmacological inhibition of AC which in turn increases the cAMP levels in the cells. Thus, activation of α7-nAChR in macrophages promotes expression and phosphorylation of c-FOS and CREB, required for a sustained decrease in the endotoxin-induced release of TNF-α (294). In LPS-stimulated human macrophages, ACh-induced activation diminishes pro-inflammatory cytokines like TNF-α, IL-1β, IL-6, and IL-18, but not the anti-inflammatory cytokine, IL-10 (295). VNS causes activation of nAChRs on macrophages, thus hampering their activation *via* the JAK2-STAT3 signaling pathway. nAChR antagonist treatment of macrophages causes enhanced expression of JAK2 and STAT3, which negatively regulate metalloproteinase 9 (MMP-9) production and inhibit macrophage migration (296, 297). It was found that nicotine treatment led to overexpression of IL-1 receptor-associated kinase M (IRAK-M), a negative regulator of TLR4 signaling, *via* α7-nAChRs. Upregulation of IRAK-M expression is required for the anti-inflammatory effect of nicotine on LPS-induced TNF-α production by peritoneal macrophages (298). In these macrophages, nicotine treatment significantly lowered ATP-induced intracellular Ca^{2+} signaling *via* β2-nAChR only (299, 300).

The muscarinic agonist, carbachol, causes a moderate enhancement in phagocytosis of zymosan particles by primary peritoneal macrophages (301). Depending on the local microenvironment, several mAChR subtypes were reported on differentiated resident macrophages. Activation of M_1–M_3 mAChR causes tumor macrophages to proliferate mainly by activating the arginase pathway, producing high prostaglandin E2, and promoting potent angiogenesis. Likewise, in normal macrophages, activation of M1–M_2 mAChR- triggers protein kinase C activity and induces moderate prostaglandin E_2 liberation for proliferation (302).

Role in Mast Cells

Treatment of patients suffering from allergic diseases with nicotine leads to suppressing the production of Th2 cytokines and cysteinyl leukotriene LTC_4. Crosslinking of the high-affinity receptor of IgE on mast cells causes its activation. Upon activation, mast cells at the early phase release preformed inflammatory mediators, and in the late phase, they synthesize and secrete cytokines/chemokines and leukotrienes. Treatment with low concentrations of nicotine leads to suppressing the late-phase, but not of the degranulation response. α7/α9-nAChR antagonists, methyllycaconitine, and alpha-bungarotoxin, successfully reverses nicotine's suppressive effect on the late-phase response (303). mAChRs have also been characterized in human mast cells as tissue-based mediators that regulate histamine release and control hypersensitivity (304, 305). Upon treatment with nicotine, the human basophil cell line, KU-812, and the human mast cell line, HMC-1, are known to express nAChRs, thereby corroborating ACh expression reports by several cell types outside the neuromuscular system (306).

Atropine, a non-selective mAChR antagonist, has been shown to reduce the permeability of colon tissue in patients with ulcerative colitis and also further diminish histamine release and disrupt the interactions between mast cells corticotropin-releasing factor (CRF), and eosinophils in the mucosal barrier (307). OXO-M, a stable agonist of mAChR, and physostigmine, an AChE inhibitor, suppress histamine release (308). Methoctramine, an M_2 mAChR antagonist, has been reported to activate phosphoinositide breakdown at high concentrations *via* pertussis toxin-sensitive G proteins, with subsequent histamine (309). M_1 mAChR signaling modulates phosphoinositide (PI) 3-kinases, which are critical regulators of mast cell degranulation (310). Mast cell degranulation requires IgE signaling and receptor-mediated calcium mobilization (311). The lethal toxin of *Clostridium sordellii* is known to inhibit Rac, thereby disrupting calcium turnover and blocking M_1-mediated exocytosis in RBL 2H3-hm1 mast cells (312). Together, these studies suggest that AChRs can alter mast cell function and contribute to the pathogenesis of mast cell-mediated diseases.

Role in Neutrophils

The mRNA and protein expression of several nAChR subunits, such as α1, α3, α4, α7, β2, and β4, are also reported in human polymorphonuclear neutrophils (PMN). The α7, α4β2, and α3β4 subunits of nAChR on PMNs have been shown to have regulatory roles in their maturation at the site of inflammation (313). During inflammation, cell surface adhesion molecules play an integral role in the migration of neutrophils from lymphoid organs to the peripheral inflammatory site. nAChRs are known to regulate the expression of the cell surface protein, CD11b, on the surface of neutrophils. Nicotine administration and VNS significantly reduce surface expression of CD11b on neutrophils *via* suppression of F-actin polymerization, thereby reducing neutrophil attachment to the endothelium surface and transmigration to inflamed sites caused by microbial infection (314).

In mice, treatment with the non-selective cholinergic antagonist, atropine, led to an increase in the neutrophil population and increased serum corticosterone (CORT) concentrations in treated mice compared to WT mice (315). Neutrophil chemotactic activity is regulated by acetylcholine-mediated IL-8 release from epithelial cells *via* mAChR. ACh significantly stimulates the ERK1/2 and NFkB pathways, leading to an increase in chemotaxis by neutrophils, which can be reversed by tiotropium, an antagonist of the M_3 mAChR (316). The M_3 mAChR takes active participation in triggering cell death and contributes to the pathophysiology observed in several autoimmune diseases like vasculitic inflammation and thrombosis. The stimulation of M_3 receptors on neutrophils induces neutrophil extracellular trap formation *via* the Akt, RAF/MEK/ERK pathway, ROS induction, and peptidyl arginine deiminase activation (317). Blocking the M3 mAChR reduces the proinflammatory effect of ACh on smooth muscles, as well as epithelial and endothelial cells. M_3 mAChR knockout mice show altered neutrophil recruitment due to the downregulation of cell adhesion molecules like fibrinogen-α and CD177 (318). Sputum samples in healthy smokers and chronic obstructive pulmonary disease (COPD) patients have been shown to have increased TGF-β1 and ACh concentrations, consequently increasing neutrophil adhesion to epithelial cells. TGF-β1 depletion significantly reduces M_3 mAChR and ChAT expression on epithelial cells, thereby establishing autocrine/paracrine feedback during neutrophilic inflammation (319). It is also noticed that during airway hyper-responsiveness due to infections, TNF-α production by neutrophils negatively regulates the M_2 mAChR, causing vagally-mediated bronchoconstriction (320).

Role in Natural Killer (NK) Cells

NK cells play a very important role in several inflammatory and chronic diseases (321). Human NK cells show the complete cholinergic machinery expression, including ChAT, VAChT, AChE, and ChT1 (322). Upon acute inflammation, $ChAT^+$ NK cells upregulate the synthesis of ACh to stimulate monocytes, modulate cytokine expression in the tissue microenvironment, and reduce inflammatory damages (322). Highly purified NK cells have been reported to express α4, α5, α6, β2, and β3 subunits of nAChR receptor (323). Under inflammatory disease conditions, NK cells increase the production of ACh by upregulation of ChAT enzymes. ChAT expression also increases along with the maturation of NK cells. Adoptive transfer of ChAT-expressing NK cells ($ChAT^+$ NK cells) into the cerebral ventricles of $CX3CR1^{-/-}$ mice reduces inflammation and autoimmune responses in the experimental autoimmune encephalomyelitis (EAE) model (322). $ChAT^+$ NK cells have been shown to successfully reduce the infiltration of $CCR2^+Ly6C^{hi}$ monocytes and lower the secretion of proinflammatory cytokines. The anti-inflammatory effect of NK cells is mediated *via* α7-nAChRs. NK stimulation with cytokines (IL-12, IL-18, and IL-15) increases the transcription and translation of α7-nAChR (322). Activation of α7-nAChR in NK cells decreases their NK group 2D member (NKG2D)-dependent cell-mediated cytotoxicity and IFN-γ production, thereby showing anti-inflammatory properties during inflammation (158). β2-nAChR modulates NK cell functions *via* NF-κB-induced transcriptional activity in NK cells (323). Aberrant functioning of NK cells is the major cause of tumorigenesis and multiple cancers. It has been reported that single nucleotide polymorphisms (SNPs) of mAChR in natural killer cells result in dysregulation of Ca^{2+} signaling and reduced NK cell cytotoxic activity, leading to the pathophysiology observed in myalgic encephalomyelitis/chronic fatigue syndrome (324). The cytotoxicity of NK cells towards YAC-1 target cells was inhibited by the addition of ACh, suggesting that AChRs on NK cells control the cytotoxic function of NK cells. Furthermore, pilocarpine, an agonist of the mAChR, showed a similar effect on the cytotoxicity of NK cells when atropine was used to block the inhibitory effect of ACh (325). Together, these studies suggest that AChRs can affect the NK cell function in different inflammatory diseases.

Role in Eosinophils

Human peripheral blood eosinophils express the M_3-M_5 mAChRs, and activation of these mAChRs has an inhibitory effect on the activation of these cells (307, 326). Eosinophils play an important role in allergic disorders such as rhinitis, atopic dermatitis, and asthma. In ulcerative colitis, the cholinergic system in eosinophils at the mucosal barrier may contribute to mucosal inflammation (307). However, the role of mAChRs in eosinophils need detailed investigation.

Pre-Clinical and Clinical Importance of the Cholinergic System

The vagal efferent nerves originate at the medulla and innervate the GI tract, connecting it to the ENS. This gut-brain axis is known to regulate GI motility and secretion *via* vagal efferent fibers, which form cholinergic synapses in the ENS and respond to inflammatory stimuli (327). Cholinergic transmission between VN and reticuloendothelial organs is extensively required in maintaining arterial blood pressure, heart rate variability and modulate the innate and adaptive immune response (328). An increased proinflammatory cytokine storm is known to correspond to reduced VN activity in several inflammatory diseases, including rheumatoid arthritis (RA), systemic lupus

erythematosus (SLE), sepsis, IBD (329–332). Activating the vagal efferent releases ACh at the distal end of the VN and effector ENS, inhibiting the release of proinflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-18), forming the CAP (295, 333, 334). The CAP is a highly conserved pathway and plays an important role in controlling morbidity and mortality associated with various human diseases, such as endotoxemia, sepsis, IBD, and RA (293). VNS implants in patients with resistant epilepsy showed LPS-induced release of TNF-α, IL-1β, and IL-6 in post-VNS blood. Also, in the RA cohort, patients showed reduced disease severity with reduced TNF-α levels in peripheral blood samples (335, 336).

This pathway can aggressively target innate immune cells and proinflammatory cytokine production. Therefore, it is proposed as a potential therapeutic target for mitigating several infections, including sepsis and the cytokine storm recently reported in SARS-CoV-2 infection (337, 338). The neuronal circuits that control TNF-β production in macrophages and other innate cells in the spleen lack the enzymatic machinery for ACh production. Rosas-Ballina et al. identified an important connection of the inflammatory reflex by discovering the cholinergic machinery in the memory $CD4^+$ T cells in the spleen (259, 339). Further, this lymphocyte-produced ACh regulates the innate immune response in the local tissue microenvironment (340). Given the importance of cholinergic signaling in inflammatory reflexes, several drugs and molecules originally designed for neurological diseases draw attention as potential drugs for inflammatory diseases. Some of the drugs that interfere with neuroimmune communication and affect inflammation and immunity are listed in **Tables 3** and **4**. Further, we discussed the notable cholinergic agents used in humans.

Cholinergic Agonists

Nicotine acts as a pan-agonist of various homomeric nAChRs including α7-nAChR, β2-nAChR, α3-nAChR, α4-nAChR, and α5-nAChR. However, in epidemiological and clinical trials, nicotine-induced addiction and toxicity leading to several autonomic dysfunctions, cardiovascular malfunctions, tumorigenesis, and neuropathic pain (341–344). Various selective cholinergic agonists are exploited in clinical research to reduce the adverse effects of non-selective receptor activation and cytokine dysregulation in various inflammatory conditions. Some of these selective agonists are discussed below-

GTS-21 [3-(2,4-dimethoxy-benzylidene) anabaseine]

GTS-21 (also known as DMBX-A) is an orally active small molecule and a selective α7-nAChR agonist used in clinical trials for AD and schizophrenia, shown to enhance memory and cognitive activity (345). GTS-21 is also known to attenuate the production of proinflammatory cytokines TNF-α and IL-1β from monocytes stimulated with Toll-like receptor (TLR) agonists (346). GTS-21 inhibits Akt and NF-κB signaling pathway, thereby reducing the LPS-induced cytokine production in macrophages (347). It has recently been shown that GTS-21 ameliorates polymicrobial sepsis-induced hepatic injury by modulating autophagy (348). GTS-21 is known to inhibit the differentiation of DCs and controls collagen-induced arthritis in mice (287). In human endotoxemia, GTS-21 induces an anti-inflammatory function (349), and higher GTS-21 concentration in the plasma significantly correlated with the lower amount of TNF-α, IL-6, and IL-1RA but not IL-10 (349, 350). It has been reported that chronic obstructive pulmonary disease (COPD) patients have high levels of IL-6 and nitric oxide (NO), and GTS-21 treatment suppresses the IL-6 and NO levels in plasma by modulating the function of PBMCs (351). In RA patients, GTS-21 suppresses the differentiation of Th1 cells and IFN-γ production in PBMCs (352). This drug has also displayed promising results in clinical trials for AD, schizophrenia, ameliorating disease severity in sepsis, pancreatitis, and inflammation induced by traumatic brain injury (353–355).

ABT-126

The α7-nAChR is an extensively studied cholinergic receptor for developing new drugs that will ameliorate cognitive deficiencies, neuropsychiatric disorders, inflammation, and autoimmune diseases. ABT-126 (trade name Neonicline) is a small molecule allosteric modulator of α7-nAChR. ABT-126 is a safe and well-tolerated α7-nAChR agonist. A phase II randomized controlled multi-center clinical trial showed a pro-cognitive effect in mild to moderate dementia AD patients (356). Phase II trials with ABT-126 also improved schizophrenia-associated cognitive impairment in non-smokers compared to smokers (357). A detailed study on the effect of ABT-21 on different immune parameters is yet to be studied. Given its importance, ABT-126 will be of great value in exploring an effective target for treating critical inflammatory and autoimmune diseases.

CNI-1493

CNI-1493 (also known as Semapimod) is an anti-amyloidogenic and vagal output stimulant that inhibits systemic inflammation *via* CAP (358). CNI-1493 was synthesized as an endogenous inhibitor of the synthesis of nitric oxide (NO) and inflammatory cytokines in the CNS (359, 360). In the pre-clinical AD model, CNI-1493 has a neuroprotective effect by inhibiting amyloid oligomers' formation and subsequently suppressing the production of IL-6 and TNF-α (361). CNI-1493 has been shown to inhibit LPS-induced TNF-α, IL-1α, IL-1, IL-6, and IL-8 in macrophages and monocytes but not in T cells (362, 363). In acute bacterial infection, CNI-1493 has been reported to reduce the inflammatory response by inhibiting NO synthesis in macrophages and promoting ROS production in granulocytes (364). In the EAE model, CNI-1493 treatment has been shown to reduce DC maturation and T cell priming (365). CNI-1493 was also found to have a protective effect in clinical trials in gut inflammatory diseases like Crohn's disease and pancreatitis (366, 367).

Pilocarpine

Pilocarpine is a natural alkaloid extracted from the plant *Pilocarpus*. It acts as a muscarinic agonist and is used to treat the autoimmune Sjogren's syndrome. It stimulates saliva secretion, aqueous tears from lacrimal glands, and mucin from goblet cells (368, 369). Pilocarpine hydrochloride has been shown to inhibit *Candida albicans* biofilm formation and its pathogenicity (370). In the rat model of epilepticus seizure,

intraperitoneal pilocarpine injection promotes activation of cholinergic neurons and dysregulation of brain homeostasis (371–373). It is known to have no severe side effects in humans as a parasympathomimetic drug (374). Its role in modulating immunological components in infection, cancer, and autoimmunity needs further investigation.

Acetylcholinesterase (AChE) inhibitors

Inhibitors of acetylcholinesterase (EC 3.1.1.7), such as galantamine, donepezil, huperzine, and rivastigmine, are some of the drugs approved for human use to treat AD, MS, and dementia (375). Most AChEIs are competitive inhibitors of AChE and allosteric modulators of nAChRs. During inflammation, the increased levels of ACh in the plasma cause these molecules to form a complex with exovesicular AChE, leading to increased nitric oxide efflux from endothelial cells (376). The AChE molecules can terminate activation of the cholinergic anti-inflammatory pathway on red blood cells (RBCs) surface (377). The AChE bound on RBCs' surface can inactivate the plasma ACh and may enhance inflammation (376, 378). AChEI linked to the RBC membrane through a glycosylphosphatidylinositol (GPI) anchor also serves as an age marker for RBCs (377). AChEI modulates the anti-inflammatory pathway and helps in the release of ACh to compensate for the reduced number of AChRs in inflammatory and neurodegenerative diseases. Several AChEIs cross the blood-brain barrier and inhibit AChE and BChE in both the central and peripheral nervous systems. These are listed in **Table 3**. AChEIs are known to lower proinflammatory cytokines such as IFN-γ, IL-17, MCP-1, RANTES, TWEAK, and increase anti-inflammatory cytokines IL-4 and IL-10 (379).

Cholinergic Antagonists

Overexpression and altered parasympathetic inputs are often associated with the progression of ovarian, lung, skin cancers, and solid tumors (380, 381). Increased ACh signaling *via* M_1 mAChR, M_2 mAChR, and M_3 mAChR also contributes to asthma and COPD (382). Abnormal cholinergic activity leads to immune-related pathological conditions in several skin diseases like atopic dermatitis, psoriasis, pemphigus, and palmoplantar pustulosis (19, 383, 384). Aberrant expression of ACh components, namely CHT1, ChAT, VAChT, nAChR, mAChR, and OCT1, and its release in GI tracts contribute to pathological conditions like IBD, colon cancer, and pancreatitis (70, 385, 386). Below, we discussed some of the promising antagonistic agents currently used in humans.

Mecamylamine

Mecamylamine (also known as inversine) is an orally available, non-selective, and non-competitive antagonist of heteromeric α4β2 and α3β4 subtype of nAChRs, and it can cross the blood-brain barrier even at a low dose (387). It is extensively used as an anti-hypertensive, anti-addictive, and anti-depressant drug (387). Mecamylamine is known to abolish the impairment of macrophages and decrease the *Mycobacterium tuberculosis* burden induced by nicotine (388). In the presence of IL-18, mecamylamine abolishes the nicotine-induced inhibition of adhesion molecules on monocytes and cytokine production by PBMC (143). The potential for its effectiveness in treating neuroimmune diseases requires further investigation.

Atropine

It is widely used in treating bradycardia and inhibiting respiratory and oral secretion (389). *In vitro* treatment with atropine has been shown to reduce the production of IL-2 by concanavalin-A stimulated T cells and reduced the cytotoxicity of NK cells (390). Along with the suppression of T cell response, it significantly reduces tissue injury, leucocyte accumulation, and inflammatory reactions at the site of turpentine-induced inflammation (180). Atropine administration before LPS challenge in mice has been reported to reduce TNF-alpha and elevated IL-10 levels in the plasma, thus, having a protective role in endotoxic shock (233). Atropine is also shown to lower the IgA production in the small intestine of BALB/c mice (391). Currently, atropine is successfully used to treat myopia, IBD, and MG patients (392–394). However, its role in several neuroimmune and autoimmune diseases needs to be investigated.

Dicyclomine

Dicyclomine (also known as dicycloverine) is a selective M_1 mAChR antagonist having an antispasmodic effect and is effectively used for treating several GI conditions such as irritable bowel syndrome (IBS) and intestinal cramping (395, 396). However, its exact mode of action in controlling mucosal homeostasis remains elusive. Apart from its antimuscarinic activities, it also has anti-fungal properties against the human pathogen *Candida albicans.* It prevents the growth, adhesion, biofilm formation, and yeast to hyphal transformation in *C. albicans* by targeting gene transduction in both the cAMP pathway and MAPK cascade (397). Dicyclomine hydrochloride is also found to have antibacterial potential in animals challenged with *Salmonella typhimurium via* competitive inhibition of lipase activity (398, 399). The anti-cholinergic implication of dicyclomine on immunological components requires further study.

Tiotropium

Tiotropium (trade names, Spiriva, Braltus) is a long-acting M_3 mAChR antagonist that has an immunosuppressive function in allergic asthma and chronic obstructive pulmonary disease (COPD) and which improves airway remodeling (400). Tiotropium also reduces the Th2 cytokine production in mice (401), and antagonizes the LTB4 production in human alveolar macrophages (402). *In vitro* studies have demonstrated that the M_3 mAChR expressed on macrophages is responsible for producing pro-inflammatory cytokines like IL-8 and leukotriene B4 (LTB-4). LTB-4 is an inflammatory mediator that causes leukocyte adhesion, activation, and inflammatory cell recruitments. M_3 mAChR drives neutrophil recruitment *via* macrophage-derived chemotactic mediators (402). Tiotropium, an M_3 mAChR antagonist, shows anti-chemotactic properties and reduces the ROS-mediated cytotoxicity in alveolar macrophages, thus reducing cellular inflammation (403, 404).

SUMMARY AND FUTURE PERSPECTIVE

There is growing evidence suggesting bidirectional interactions between the nervous system and the immune system at the cellular and molecular levels. Understanding the multicellular and multidimensional signals involved and the regulatory mechanisms of immunological reflex in chronic and acute inflammatory diseases offer ample opportunity for basic and clinical research. Many neurodegenerative diseases have a close relationship with the activation of inflammation in the central nervous system and the peripheral immune system (405, 406). Given the importance of functional circuitry in the secondary lymphoid tissues (407), the cholinergic system's influence on the immune system cannot be ignored while designing therapeutic strategies to treat even neurological disorders. In clinical trials (clinical trial registry numbers NCT00783068, NCT04470479, NCT00000172, NCT00892450), some cholinergic stimulators and pharmaceutical antagonists were used in various inflammatory diseases. These molecules can also alter the innate and adaptive response and need to be investigated further.

Further, the various activation mechanisms of the cholinergic system in different subsets of innate and adaptive immune cells need to be elucidated. A multidimensional and multifactorial systems biology approach could help connect various individual components, such as genetic disposition, cholinergic deficits, inflammatory mechanism, oxidative stress, mitochondrial dysfunction, and other neurotransmitter defects. Such an approach would serve well to understand neuro-immune diseases and may also help in customizing therapeutic regimens. The high degree of homology (64 to 68% sequence identity and 82 to 92% sequence similarity) between the transmembrane domains of mAChRs (250), makes designing small-molecule ligands that could selectively target specific mAChRs incredibly challenging. Recently, Biased M_1 mAChR-mutant mice were used to develop next-generation drugs for AD, which hold promise for the future (408).

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Expression Analysis of Protein Inhibitor of Activated STAT in Inflammatory Demyelinating Polyradiculoneuropathy

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Protein inhibitors of activated STAT (PIAS) are involved in the regulation of the JAK/STAT signaling pathway and have interactions with NF-κB, p73 and p53. These proteins regulate immune responses; therefore dysregulation in their expression leads to several immune-mediated disorders. In the present study, we examined expression of *PIAS1-4* in peripheral blood of patients with acute/chronic inflammatory demyelinating polyradiculoneuropathy (AIDP/CIDP) compared with healthy subjects. We demonstrated down-regulation of all *PIAS* genes in both AIDP and CIDP cases compared with controls. Similarly, comparisons in gender-based groups revealed down-regulation of these gene0s in patients of each gender compared with gender-matched controls. There was no significant difference in expression of *PIAS* genes between AIDP and CIDP cases. Based on the area under the receiver operating characteristic curves, *PIAS1-4* genes could distinguish between inflammatory demyelinating polyradiculoneuropathy and healthy status with accuracy values of 0.87, 0.87, 0.79 and 0.80, respectively. In differentiation between AIDP cases and healthy controls, these values were 0.92, 0.92, 0.83 and 0.86, respectively. Finally, *PIAS1-4* genes could discriminate CIDP from healthy status with accuracy values of 0.82, 0.83, 0.75 and 0.75, respectively. The current study underscores the role of *PIAS* genes in the pathogenesis of inflammatory demyelinating polyradiculoneuropathy and their potential usage as biomarkers.

Keywords: protein inhibitor of activated STAT, PIAS, inflammatory demyelinating polyradiculoneuropathy, AIDP, CIDP

INTRODUCTION

Being firstly recognized as the inhibitors of signal transducer and activator of transcription (STAT) proteins, the protein inhibitors of activated STAT (PIAS) are involved in the regulation of the JAK/STAT signaling pathway (1, 2). PIAS1, PIAS2 (PIASxα, PIASxβ), PIAS3 and PIAS4 (PIASy) are the main members of this protein family (3). In addition to the STAT family of transcription factors (TFs), PIAS proteins have functional interactions with NF-κB, p73 and p53 (4). PIAS proteins have crucial roles in

the regulation of the immune responses particularly through modulation of a number of cytokine-associated genes (4). Among PIAS proteins, PIAS1 has a prominent role in the regulation of innate immune responses as it specifically suppresses expression of IFN-inducible genes. These speculations are based on the observed enhancement of the antiviral function of IFN-β and IFN-γ in Pias1−/− cells (5). PIAS2 has been shown to suppress the IL-12-associated STAT4-dependent gene induction (6). Similarly, PIAS3 has a specific inhibitory effect on STAT3 signaling (7). STAT3 has been primarily identified as a TF induced by the IL-6 family of cytokines (8). Subsequent studies revealed its essential roles in the development of Th17 cells (9), regulation of humoral immune responses (10) and modulation of innate immunity (11). PIAS4 has prominent roles in suppression of STAT1, and inhibition of LEF1 and SMAD3 signaling pathways (12). Consistent with the critical functions of PIAS proteins in the regulation of immune responses, they have been shown to be involved in the pathogenesis of multiple sclerosis (MS) (13). However, their expression pattern and functional relevance with inflammatory demyelinating polyradiculoneuropathies are largely unknown. In the current study, we examined expression of PIAS1-4 in patients with acute/chronic inflammatory demyelinating polyradiculoneuropathy (AIDP/CIDP) compared with healthy subjects. Autoimmune inflammatory responses are considered as the principal mechanisms for development of these neurologic conditions (14, 15). In addition to the pathophysiological events, these two conditions share several features in terms of clinical manifestations and treatment modalities (14). Therefore, identification of the role of *PIAS* genes in the pathogenesis of AIDP/CIDP might facilitate the process of design of therapeutic options and prediction of disease course in these conditions.

MATERIALS AND METHODS

Enrollment of AIDP/CIDP Patients and Healthy Subjects

In total, 22 AIDP cases, 31 CIDP cases and 50 healthy individuals with no sign of inflammatory conditions were enrolled in the current study. Diagnostic evaluations were performed using the criteria offered by the American Academy of Neurology (16) and National Institute of Neurological Disorders and Stroke (17). AIDP/CIDP cases were in remission at the time of sampling. Diagnosis was based on clinical manifestations as well as electrophysiological and biochemical tests. Those with recent or chronic infection, cancer, or any systemic diseases were exempted from participation in the study. Persons enlisted in the control group were healthy individuals with no history of recent or chronic infection, malignancy, or systemic diseases. The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1398.853). All AIDP/CIDP cases and controls signed the informed consent forms.

Expression Assay

Peripheral blood samples were obtained from controls and AIDP/CIDP patients at the time of remission. Samples were collected in EDTA-containing tubes. RNA was isolated from blood samples using the RNA extraction kit delivered by the GeneAll Company (Seoul, Korea). Then, RNA was converted to cDNA by using the Thermo Fisher Scientific kit (Brussels, Belgium). Expression levels of *PIAS* genes were measured in AIDP/CIDP cases and control subjects using the master mix supplied by the Ampliqon Company (Odense, Denmark). Expression of *PIAS* genes were measured using the Step One Plus™ Real-Time PCR system (Applied Biosystems, Foster city, CA, USA). Details of expression analysis of *PIAS* genes were explained formerly (13, 18). **Table 1** shows the primers and probes.

Statistical Methods

R programming language was used for statistical analysis. Transcript quantities of *PIAS1*, *PIAS2*, *PIAS3*, and *PIAS4* in relation to the *HPRT* reference gene was calculated from CT values using the equation: $\frac{amp_{gene}^{-CT_{gene}}}{amp_{HPRT}^{-CT_{HPRT}}}$. Then, the values were log2 transformed and used for subsequent analysis. Four comparisons between CIDP/AIDP/All patients and healthy individuals and between CIDP and AIDP patients were done and the significant difference between means was computed using the t-test. Correlations between expressions were evaluated through the

TABLE 1 | The information about primers and probes.

Gene name	Primer and probe sequence	Primer and probe length	Product length
HPRT1	F: AGCCTAAGATGAGAGTTC	18	88
	R: CACAGAACTAGAACATTGATA	21	
	FAM -CATCTGGAGTCCTATTGACATCGC- TAMRA	24	
PIAS1	F: AGCCTAAGGGAAAGCCATAGC	21	83
	R: ATGTCTGGTATGATGCCAAAGATG	24	
	FAM-TGCCGTGTCCGTGCTGCTCCTGT- TAMRA	24	
PIAS2	F: CACGAACTCTTGAAGGACTTTCTG	24	126
	R: AAGTGGAAGGCAACGAGTGG	20	
	FAM - CCAGCCACGGCCAAGTCAGGTTCT -TAMRA	24	
PIAS3	F:GCCCTACCTGGAAGCAAAGG	20	120
	R: GTACTCATGTAGTGGGAGACTGG	23	
	FAM- CCCACCCAACGTGCCCATAGCAGG - TAMRA	24	
PIAS4	F: GAGAAGAAGCCCACCTGGATG	21	77
	R: AGGAGCCCGTCGATGATGAG	20	
	FAM- CCCGTGTGCGACAAGCCAGCCC - TAMRA	22	

calculation of Spearman correlation coefficients. Three predictive machine learning methods namely Bayesian Generalized Linear Model, Generalized Linear Model, and Linear Discriminant Analysis with 10-fold cross validation were used to compute the sensitivity and specificity of each model. The receiver operating characteristic curve was plotted. The Linear Discriminant Analysis Model (LDA) provided the most efficient estimates and in the best setting, the AUC was 0.94. Youden's J statistic was employed to find the optimum threshold. LDA was then selected based on previous results to investigate efficiency of each gene for separating groups.

RESULTS

General Demographic and Clinical Information of Cases and Controls

The current project enrolled 17 female and 36 male patients along with 13 female and 37 male subjects who enlisted as controls. Cases and controls were matched in the term of age parameter. Demographic information of patients and controls are demonstrated in **Table 2**.

TABLE 2 | General demographic and clinical information of cases and controls.

Variables	AIDP Cases	CIDP cases	Controls	P value
Female/Male [no. (%)]	6 (27%)/16 (73%)	11 (35%)/20 (65%)	13 (26%)/37 (74%)	0.39
Age (mean ± SD, Y)	49.72 ± 14.6	50.5± 15.8	44.48 ± 2.3	0.13

Expression Assays

Figure 1 shows the relative expression amounts of *PIAS* genes in AIDP/CIDP patients and healthy subjects. Expression levels of all *PIAS* genes were significantly decreased in both CIDP cases compared with controls. For *PIAS1*, ratio of mean expressions (RME) in cases versus controls was 1.12E-03 (P value=8.2E-14). RME (P values) for *PIAS2*, *PIAS3* and *PIAS4* genes in CIDP cases versus controls were 2.22E-03 (4.5E-13), 9.02E-03 (1.7E-08) and 1.05E-02 (3.8E-09), respectively. Expression of all genes was also decreased in AIDP cases compared with controls. The corresponding RME and P values for *PIAS1*-*PIAS4* genes in the AIDP cases versus controls were 1.52E-03 (3.3E-07), 2.60E-03 (5.9E-07), 1.47E-02 (5.9E-05) and 1.21E-02 (2.6E-05), respectively.

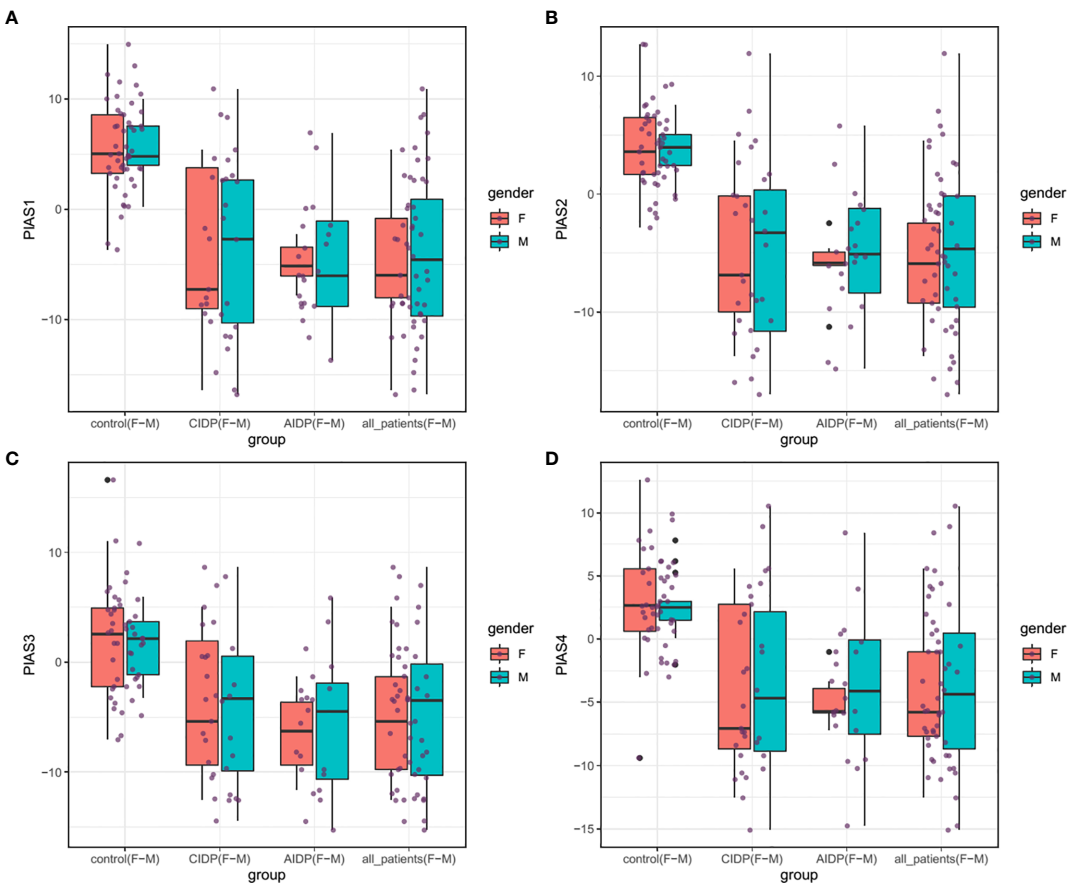

FIGURE 1 | Relative expressions of *PIAS* genes in AIDP/CIDP patients and healthy subjects. Depicted box plots and whiskers show all data points from maximum to minimum. Boxes are depicted from Q1 to Q3. The horizontal lines in the middle of boxes show the median values. Each black dot shows expression in a certain sample. Mean values and interquartile range are displayed.

Similarly, comparisons in gender-based groups revealed down-regulation of these genes in patients of each gender compared with gender-matched controls. There was no significant difference in expression of *PIAS* genes between AIDP and CIDP cases. When assessing expression of *PIAS* genes in total CIDP and AIDP cases versus total controls, all genes were found to be down-regulated in affected individuals which is consistent with their expression pattern in related patients' subgroups. **Table 3** shows the detailed statistics of expression analysis of *PIAS* genes among study groups.

Afterwards, we appraised whether expression of *PIAS* transcripts can separate AIDP/CIDP patients from controls (**Figure 2**). Based on the AUC values, *PIAS1-4* genes could distinguish between inflammatory demyelinating polyradiculoneuropathy and healthy status with accuracy values of 0.87, 0.87, 0.79 and 0.80, respectively. In differentiation between AIDP cases and healthy controls, these values were 0.92, 0.92, 0.83 and 0.86, respectively. Finally, *PIAS1-4* genes could discriminate CIDP from healthy status with accuracy values of 0.82, 0.83, 0.75 and 0.75, respectively. **Figure 3** and **Table 4** show details of ROC curve analysis.

DISCUSSION

PIAS proteins have been shown to modulate the function of many TFs such as STATs, NF-κB and SMADs. The Jak-STAT cascade as a main target of PIAS proteins is the main intracellular cascade induced by class I cytokine receptor proteins. Through this signaling pathway, the activated kinase phosphorylates tyrosine amino acids in the intracellular domain of cytokine receptors (19). STATs have important roles in STATs in the development of neutrophils and regulation of their function, polarization of macrophages, and activity of dendritic cells (19). NF-κB+ signaling has also been shown to regulate expression of cytokines and antimicrobial molecules. Moreover, this signaling pathway controls differentiation, subsistence and proliferation of cells that are involved in innate and adaptive immune reactions (20). The SMAD pathway controls IgA secretion by B cells and enhances differentiation of CD4+ T cells into T17 cells and regulatory T cells (21). Therefore, PIAS proteins can affect immune responses *via* various routes. These proteins exert their regulatory roles *via* different routes such as inhibition of the DNA-binding activity of TFs and functional interaction with transcriptional corepressors or co-activators. They also have prominent roles in the regulation of innate immune reactions (4). In the current project, we demonstrated down-regulation of *PIAS1-4* transcripts in the peripheral blood of AIDP and CIDP patients, with no significant difference between these two groups of patients. We have recently examined expression levels of *STAT* genes in the same cohort of CIDP and AIDP patients and reported over-expression of *STAT1* in female patients compared with sex-matched controls (22). It is worth mentioning that since *PIAS1* was similarly down-regulated in males and females in the current study, this system may not be the only one responsible for elevated *STAT1* levels. STAT1 has a crucial role in induction of gene expression in response to IFN

TABLE 3 | The results of Bayesian Regression model for comparison of expression of *PIAS* genes in AIDP/CIDP patients and healthy persons (Expressions of *PIAS* genes have been compared between CIDP cases and controls, AIDP cases and controls, CIDP cases and AIDP cases as well as total patients and total control.

Number of Samples		*PIAS1*				*PIAS2*				*PIAS3*				*PIAS4*			
		SE*	Ratio of Mean Expressions	P Value	95% CI**		SE	Ratio of Mean Expressions	P Value	95% CI		SE	Ratio of Mean Expressions	P Value	95% CI		SE	Ratio of Mean Expressions	P Value	95% CI	
CIDP/Control																					
Total	31/50	1.10	1.12E-03	**8.2E-14**	-11.99	-7.62	1.02	2.22E-03	**4.5E-13**	-10.84	-6.78	1.10	9.02E-03	**1.7E-08**	-8.98	-4.61	1.01	1.05E-02	**3.8E-09**	-8.58	-4.58
F	11/13	1.65	9.83E-04	**3.2E-06**	-13.41	-6.57	1.43	1.55E-03	**1.0E-06**	-12.28	-6.38	1.64	7.54E-03	**1.8E-04**	-10.40	-3.70	1.47	8.07E-03	**7.2E-05**	-9.98	-3.92
M	20/37	1.43	1.21E-03	**1.9E-08**	-12.58	-6.81	1.35	3.10E-03	**1.6E-07**	-11.06	-5.61	1.39	1.10E-02	**2.7E-05**	-9.30	-3.70	1.31	1.28E-02	**1.6E-05**	-8.91	-3.66
AIDP/Control																					
Total	22/50	1.53	1.52E-03	**3.3E-07**	-12.46	-6.27	1.43	2.60E-03	**5.9E-07**	-11.49	-5.68	1.38	1.47E-02	**5.9E-05**	-8.87	-3.31	1.35	1.21E-02	**2.6E-05**	-9.08	-3.65
F	6/13	2.41	1.22E-03	**1.7E-03**	-14.92	-4.44	2.02	1.98E-03	**7.7E-04**	-13.37	-4.59	2.15	1.29E-02	**1.1E-02**	-10.89	-1.66	2.09	1.06E-02	**8.1E-03**	-11.10	-2.03
M	16/37	2.00	1.73E-03	**1.1E-04**	-13.30	-5.05	1.95	3.54E-03	**3.5E-04**	-12.18	-4.11	1.77	1.76E-02	**2.8E-03**	-9.46	-2.19	1.76	1.42E-02	**1.8E-03**	-9.76	-2.52
CIDP/AIDP																					
Total	31/22	1.80	2.07E+00	5.6E-01	-2.56	4.66	1.71	1.46E+00	7.5E-01	-2.89	3.99	1.74	3.23E+00	3.4E-01	-1.80	5.18	1.64	1.43E+00	7.6E-01	-2.79	3.81
F	11/6	2.45	1.85E+00	7.2E-01	-4.42	6.20	2.26	2.00E+00	6.6E-01	-3.81	5.81	2.57	4.57E+00	4.1E-01	-3.30	7.68	2.17	2.15E+00	6.2E-01	-3.57	5.78
M	20/16	2.38	2.24E+00	6.3E-01	-3.66	5.99	2.31	1.35E+00	8.5E-01	-4.27	5.13	2.26	2.87E+00	5.0E-01	-3.07	6.11	2.18	1.25E+00	8.8E-01	-4.11	4.75
All Patients/Controls																					
Total	53/50	1.23	7.33E-04	**1.0E-09**	-12.92	-7.91	1.17	1.78E-03	**8.9E-09**	-11.51	-6.75	1.41	4.54E-03	**3.8E-06**	-10.65	-4.91	1.22	8.49E-03	**3.0E-06**	-9.36	-4.40
F	17/13	1.11	6.60E-04	**1.9E-07**	-12.95	-8.18	1.34	9.90E-04	**7.0E-05**	-13.06	-6.90	1.86	2.82E-03	**1.8E-03**	-12.75	-4.19	1.15	4.92E-03	**2.4E-05**	-10.18	-5.15
M	36/37	1.68	7.72E-04	**3.3E-06**	-13.82	-6.86	1.55	2.63E-03	**1.7E-05**	-11.79	-5.35	1.82	6.13E-03	**5.4E-04**	-11.12	-3.58	1.64	1.13E-02	**7.1E-04**	-9.86	-3.06

*SE, standard error; **95% CI, 95% confidence interval.

*Expression of each of PIAS genes was significantly correlated with other PIAS genes in all study groups. Notably, based on the measured correlation coefficients, such correlations were more robust among patients (**Figure 2**).*

Significant P values are shown in bold letters).

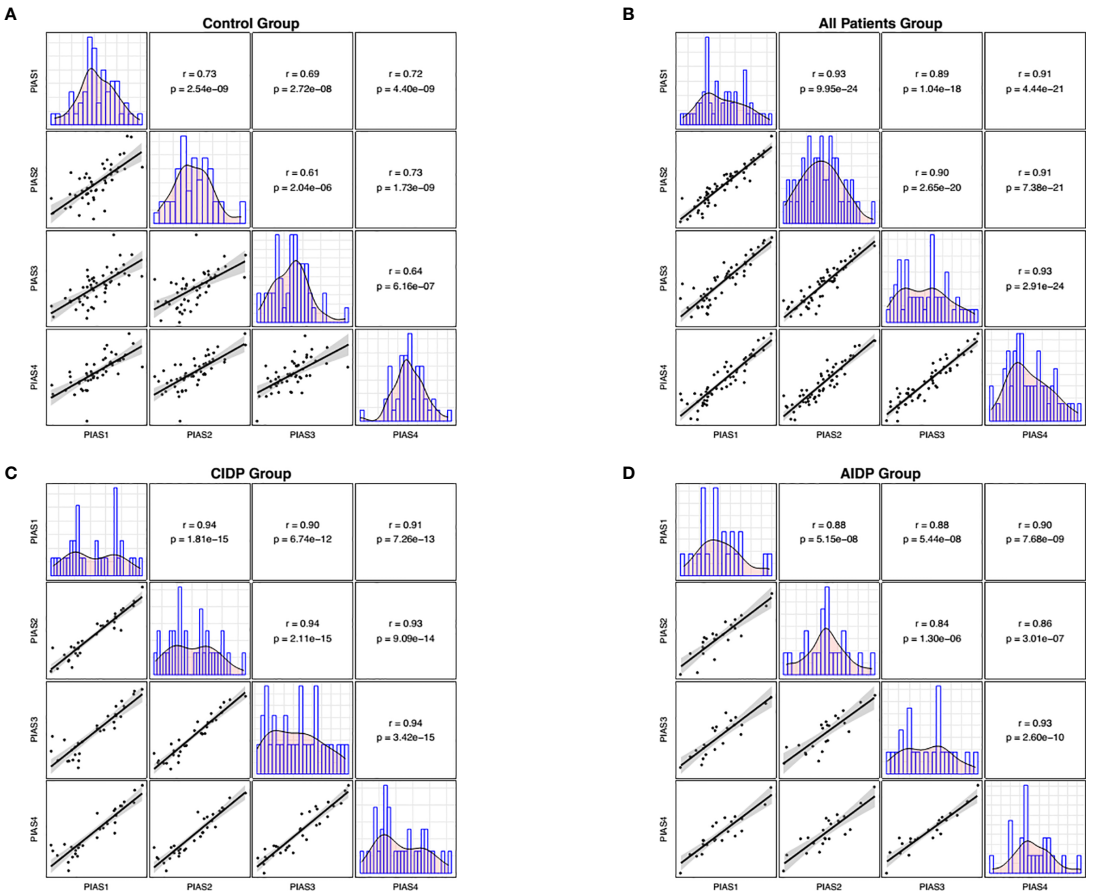

FIGURE 2 | Correlations between transcript quantities of *PIAS* genes. The distributions of parameters are represented on the diagonals. The bivariate scatter plots with a fitted line are shown on the lower parts of the diagonals. Correlation coefficients and p values of the correlations are displayed on the upper sections of the diagonal.

stimulation. PIAS1 is the only member of the PIAS family of proteins that can block the DNA binding function of STAT1 and suppress STAT1-associated stimulation of gene expression in response to IFN (2). Consistent with the anti-inflammatory role of PIAS1, down-regulation of this transcript has been associated with allograft rejection (23). PIAS proteins have also prominent roles in decreasing the effects of pro-inflammatory cytokines (24). Moreover, PIAS agonists and inducers have immunosuppressive effects (24). Therefore, the observed down-regulation of *PIAS* transcripts in AIDP and CIDP cases might exacerbate the autoimmune responses in these patients, thus contributing in the pathogenesis of these conditions. In line with the inhibitory role of PIAS proteins on the activity of NF-κB and the observed down-regulation of *PIAS* transcripts in AIDP/CIDP patients, Andorfer et al. have shown a remarkable increase in NF-κB levels in Guillain-Barré syndrome and CIDP cases compared to controls. They also suggested a critical role for NF-κB in the pathogenesis of these inflammatory conditions (25). Although we did not perform functional studies to assess whether down-regulation of *PIAS* transcripts is the cause or effect of CIDP/AIDP, based on the role of these transcripts in suppression of STAT signaling we hypothesize that this dysregulation is the cause of this autoimmune condition. Abnormal levels of PIAS-targeting miRNAs might be a possible mechanism of down-regulation of *PIAS* transcripts. Several miRNAs including miR-146a have been shown to affect expression of PIAS, STAT and other components of this pathway (26). Meanwhile, expression of a number of these miRNAs including miR-146a has been found to be dysregulated in GBS patients (27). Besides, miR-18a has been found to negatively regulate PIAS3 expression and therefore influencing expression of STAT3 target genes (28). Notably, miR-18a has a distinctive inhibitory effects on differentiation of T17 cells (29). Therefore, abnormal functional network between PIAS, STAT and miRNAs might affect expression and function of these pathways.

We also demonstrated significant correlations between *PIAS1-4* transcript levels particularly among patients. Such finding implies the presence of a solitary regulatory mechanism for these genes and the robustness of this mechanism in the context of AIDP/CIDP. Finally, we appraised the diagnostic power of *PIAS1-4* genes in distinguishing between disease and healthy conditions. The

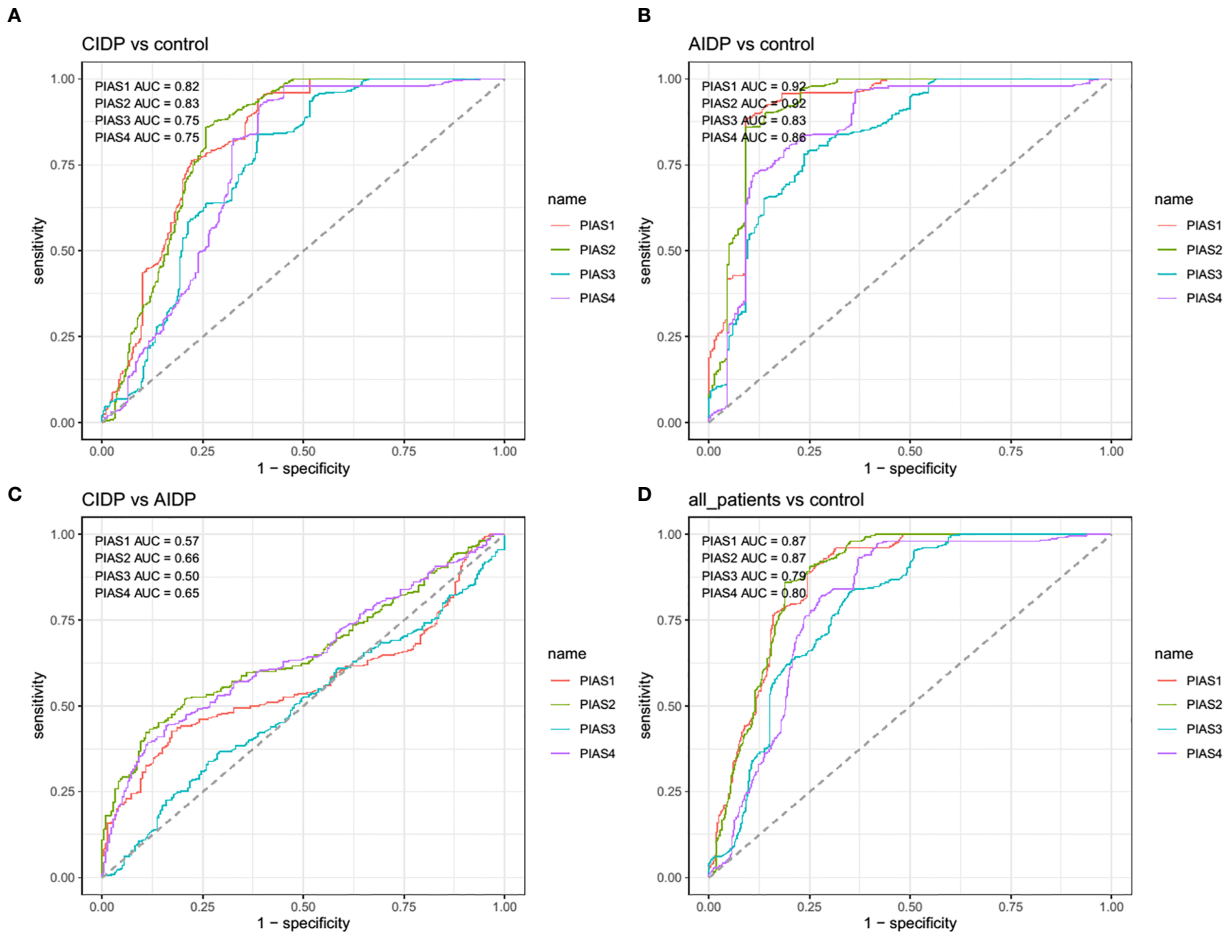

FIGURE 3 | The receiver operating characteristic (ROC) curve of *PIAS1-4* in diagnosis of inflammatory demyelinating polyradiculoneuropathy.

best values were detected for *PIAS1* and *PIAS2* for differentiation of AIDP cases from healthy condition.

Taken together, the observed down-regulation of *PIAS* genes in AIDP/CIDP cases implies their possible contribution in the pathogenesis of these conditions and their potential usage as biomarkers. Therapeutic options that alter expression of these genes might improve the response of patients to available therapeutic options and attenuate the course of these disorders. These speculations should be appraised in animal models of inflammatory demyelinating polyradiculoneuropathy.

TABLE 4 | Detailed statistics of ROC curve analysis.

Number of Samples	*PIAS1*			*PIAS2*			*PIAS3*			*PIAS4*			All Four genes		
	AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity
CIDP/ Control Total 31/50	0.82	0.94	0.61	0.83	0.86	0.74	0.75	0.84	0.61	0.75	0.93	0.61	0.85	0.80	0.75
AIDP/ Control Total 22/50	0.92	0.88	0.91	0.92	0.86	0.91	0.83	0.78	0.76	0.86	0.73	0.89	0.94	0.96	0.82
CIDP/AIDP Total 31/22	0.57	0.43	0.83	0.66	0.52	0.80	0.50	0.37	0.71	0.65	0.44	0.84	0.49	0.68	0.47
All Patients/ Controls Total 53/50	0.87	0.91	0.73	0.87	0.86	0.81	0.79	0.84	0.65	0.80	0.93	0.63	0.89	0.98	0.65

A limitation of this study is lack of assessment of expression levels of *PIAS* transcripts in different time points during the course of disorder to find whether their expression is changed during the time.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1398.853). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MT and FN performed the experiment. AS and NN collected the data and analyzed it. SG-F wrote the draft and revised it. All authors contributed to the article and approved the submitted version.

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REVIEW
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The OX40/OX40L Axis Regulates T Follicular Helper Cell Differentiation: Implications for Autoimmune Diseases

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T Follicular helper (Tfh) cells, a unique subset of $CD4^+$ T cells, play an essential role in B cell development and the formation of germinal centers (GCs). Tfh differentiation depends on various factors including cytokines, transcription factors and multiple costimulatory molecules. Given that OX40 signaling is critical for costimulating T cell activation and function, its roles in regulating Tfh cells have attracted widespread attention. Recent data have shown that OX40/OX40L signaling can not only promote Tfh cell differentiation and maintain cell survival, but also enhance the helper function of Tfh for B cells. Moreover, upregulated OX40 signaling is related to abnormal Tfh activity that causes autoimmune diseases. This review describes the roles of OX40/OX40L in Tfh biology, including the mechanisms by which OX40 signaling regulates Tfh cell differentiation and functions, and their close relationship with autoimmune diseases.

Keywords: OX40, OX40L, Tfh cells, differentiation, autoimmune diseases

INTRODUCTION

Many autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are characterized by autoantibody production. A specialized cell subset named T follicular helper (Tfh) cells has attract much attention because of their requirement for B cell help and the production of high affinity class-switched antibodies. Tfh cells, located in lymphoid follicles, belong to a distinct $CD4^+$ T subset. They are essential for generation of effective and long-lived humoral immune responses. Several pairs of costimulatory molecules have been demonstrated to control Tfh development and function. OX40/OX40L is one of them. OX40 and OX40L play a critical role in enhancement of immune responses and participate in the development of autoimmune diseases. Recently, it was reported that the OX40/OX40L interaction is required for the functions of Tfh cells. This article focuses on the effects of OX40/OX40L signaling on Tfh cells and their roles in the pathogenesis of autoimmune diseases.

DIFFERENTIATION AND FUNCTIONS OF Tfh CELLS

In 2000, Schaerli and Breifeld found that there is a subset of $CD4^+$ T cells in lymphoid follicles, called Tfh cells, which express CXCR5, ICOS and CD40L (1). They are obviously distinct from other Th cells in two aspects. First, while CXCR5 is only expressed temporarily on other Th cell subpopulations when they are activated, while its expression on Tfh cells persists for a long time. Upregulated CXCR5 and downregulated CCR7 facilitate the migration of Tfh cells from the T cell area to CXCL13-rich B lymphoid follicles where they interact with B cells. Second, unlike Th1, Th2 and Th17 cells, the differentiation of Tfh cells proceeds through multiple stages, including initiation, maintenance and full polarization. A variety of cytokines, transcription factors and surface molecules are involved in these process (2–4).

ICOS, PD-1, Bcl-6, BTLA, CD40L, IL-21, IL-6R, SAP and IL-21R are shown to be highly expressed in mouse and human Tfh cells, indicating that these molecules may play critical roles in promoting the development and maintenance of Tfh cells and regulating their functions (4–7). Bcl-6 is recognized as an essential transcription factor for regulation of Tfh cell differentiation. Bcl6 controls Tfh differentiation by antagonizing Blimp-1 and other transcription factors which are also important for Th1, Th2 and Th17 cells. GCs are absent in Bcl-6-deficient mice since Bcl-6 deficient $CD4^+$ T cells do not support the GC reaction. The expression of Bcl-6 in Tfh cells is mainly driven by IL-6, IL-21 and certain downstream transcription factors including STAT1 and STAT3 (8, 9). Recently, a feed-forward loop mediated by the transcription factors Bcl-6 and Tox2 is reported to promote the Tfh program (10). Bcl-6 upregulates Tox2 expression, which further drives Bcl-6 expression and Tfh development. ICOS/ICOSL molecules are involved in every stage of Tfh cell differentiation. Mathieu et al. transferred ICOS-Y181F into mice to block ICOS-mediated PI3K activation and found that the number of Tfh cells in the spleen was significantly reduced. At the same time, ICOS-PI3K was also found to be essential for upregulating IL-21 and IL-4, which are key factors for Tfh function (4, 11). These multiple factors drive Tfh differentiation. The first stage of Tfh differentiation is initiated by interaction with a professional antigen-presenting cell (APC), such as a dendritic cell (DC). After T cell priming, Bcl-6 and CXCR5 expression is upregulated on $CD4^+$ T cells to facilitate Tfh cell migration to the T-B border. Then, the second stage begins. This is a B cell-dependent phase of Tfh differentiation that is regulated by ICOS/ICOSL signaling. In the third stage, Tfh cells and B cells migrate to GCs, where B cells continuously help Tfh cells to promote their full polarization. GC Tfh cells are in a further polarized Tfh cell state, with elevated expression of Bcl-6, CXCR5, PD-1 and ICOS.

The most important function of Tfh cells is to provide help to B cells (12). They are necessary for GC formation, high-affinity B cell selection, and generation of memory B cells and plasma cells (13, 14). At the T-B border, B cells present antigens to Tfh cells and only those cells presenting antigens with high affinity obtain Tfh help and then enter GCs (15). Most B cell responses cannot progress without the help of Tfh cells. GCs are recognized as the essential sites of B cell mutation and antibody affinity maturation. After GC Tfh cells recognize the antigens presented by GC B cells in the follicle light zone, they provide signals for GC B cell proliferation and migration to the dark zone, where B cells will undergo somatic hypermutation (16). Moreover, GC Tfh cells promote the development of long-term humoral immunity by generating memory B cells and high-affinity plasma cells. The effect of Tfh cells on B cell differentiation within GCs and extrafollicular areas depends on numerous signals, such as IL-21 and CD40 signals. IL-21 is the most potent cytokine driving plasma cell differentiation in both mice and humans (17–21). IL-21 induces both Bcl-6 and Blimp-1 expression in B cells, in which Bcl-6 promotes GC B cell proliferation and Blimp-1 is critical for plasma cell differentiation (17, 22, 23). IL-21 signaling is dependent on the activation of STAT3 and STAT5 (24). CD40L/CD40 engagement is central to the maintenance of GC B cells. Provision of CD40L protein *in vitro* was found to inhibit apoptosis of GC B cells (25–27). Schirock et al. reported that a critical ECM:α_v integrin axis specifically regulated prolonged Tfh positioning within the GCs and supported the generation of long-lived plasma cells but not memory B cells (28).

OX40 AND OX40L MOLECULES

Structure and Expression of OX40 and OX40L

OX40 (also called ACT35, CD134 or TNFRSF4), belonging to the TNFR superfamily, is a type 1 transmembrane protein containing 249 amino acids with a 49 amino acids in cytoplasmic tail and a 186 amino acids in extracellular region (29, 30). OX40 protein was first recognized on activated rat $CD4^+$ T cells in 1987. Subsequently, OX40 expression was also found expressed in mice and humans (31–33). OX40 is mainly expressed on activated $CD4^+$ T cells and $CD8^+$ T cells, whereas its expression level is relatively low on NK cells and NKT cells (29, 34–37). OX40L (also named as CD252, TNFSF4, CD134L or gp34), the ligand of OX40, is a type II glycoprotein with a 23 amino acids cytoplasmic tail and a 133 amino acids extracellular domain (38). As a member of the TNF superfamily, it is expressed as a trimer. OX40L was initially identified as gp34 protein on human T-cell leukemia virus transformed cells in 1985 (39). Later, it was found that OX40L is mainly expressed on antigen presenting cells, such as B cells and dendritic cells (40–42). Similar to other members of the TNF family, the OX40 signal transduces through TNF receptor related factors (TRAFs). OX40 signal is transduced to T cells *via* TRAF2 and TRAF5 *in vivo* and TRAF1, TRAF3 and TRAF5 *in vitro* (43–47).

The expression of OX40 and OX40L is regulated by many factors. OX40 expression is induced on T cells by TCR, CD28/CD80, CD40/CD40L and other signals and peaks at 48-72 hours following T cell activation (34, 48–50). TCR signaling can initiate

the expression of OX40 on a variety of cells, while CD28 and other cytokines can further promote its expression on activated T cells (32). It has also been reported that IL-2, IL-4 and TNF can enhance or prolong OX40 expression. Sun et al. found that IL-2, TNF-α and IFN-γ were highly expressed in liver tissues of animal models of nonalcoholic steatohepatitis, but only exogenous IL-2 stimulation could upregulate OX40 expression on $CD4^+$ T cells (51). CD40 signaling and inflammatory signals transmitted by Toll-like receptors induce OX40L expression on antigen-presenting cells (41). Factors such as IL-18, IFN-γ, thymic stromal lymphopoietin (TSLP) and prostaglandin E2 can also promote the expression of OX40L (49, 52–55). In an inflammatory environment, upregulated OX40L expression on APCs ensures that activated $OX40^+$ T cells receive OX40 signals from nearby cells (33).

Functions of OX40 and OX40L

As a pair of costimulatory molecules, OX40/OX40L is required for T cell activation especially in the later phase of the immune response. OX40/OX40L plays critical roles in enhancing the function of effector T cells, maintaining their survival and inhibiting their apoptosis. Rogers et al. detected a significantly decreased percentage of antigen-specific T cells in OX40-deficient mice. Moreover, antiapoptotic molecules, such as Bcl-xL and Bcl-2, were obviously downregulated in $OX40^{-/-}$ T cells and $CD28^{-/-}$ T cells after antigen stimulation. When $CD28^{-/-}$ T cells were stimulated with an anti-OX40 agonist antibody, the expression of Bcl-xL and Bcl-2 was increased, and T cell apoptosis was inhibited (56). OX40-deficient T cells normally proliferated and differentiated into effector T cells 2-3 days after activation of TCR signaling. However, the survival rate was significantly reduced after 12-13 days of activation, which indicated that OX40 signaling might not be essential for the early stage of T cell activation but might promote the proliferation of T cells and maintain their survival in the later stage (57). OX40/OX40L signaling is critical for differentiation of various Th cells. This signaling preferentially induces differentiation of naive $CD4^+$ T cells into Th2 cells but promotes Th1 differentiation under the influence of antigens or IL-12 (58). OX40 was also reported to play important roles in differentiation of Th9 cells through the nonclassical NF–κB pathway by activating tumor necrosis factor receptor-associated factor 6 (TRAF6) (59). Which type of Th cell differentiation is promoted by this signal may be dependent on the environment it is involved. OX40 expression is usually downregulated after the effect phase of the primary T cell response and can be rapidly upregulated on memory T cells after secondary challenge with the same antigen again to subsequently activate and recruit memory effector T cells, suggesting that the OX40/OX40L interaction is required in the recall response (59). OX40 is also constitutively expressed on mouse Treg cells (60). Evidence has shown that OX40 signaling is essential for inhibiting Treg cell function. Jaquemin et al. reported that engagement of the OX40/OX40L axis resulted in Foxp3 downregulation in Tregs and decreased Treg-mediated suppression of effector T cell proliferation(OX40 upregulates BATF3 and BATF, which produce a closed chromatin configuration to repress Foxp3 expression in a Sirt1/7-dependent manner (61). However, there are also reports showing that OX40 agonists can enhance Treg cell proliferation and inhibit function. Gavin MA et al. found that the number of Treg cells in the spleen of OX40-deficient mice decreased, while the number of Treg cells in the thymus of OX40L-overexpressing mice increased, indicating that abnormal OX40/OX40L signaling interfered with the development of Treg cells (62).

In addition to its critical role in T cells, OX40/OX40L signaling can also promote the differentiation and maturation of DCs. Human immature DCs have no OX40L expression, whereas the expression of OX40L is rapidly induced after sCD40L stimulation. Ligation of OX40L upregulated the expression of CD80, CD86, CD54 and CD40 on mononuclear-derived DCs in the reversible phase, and could enhance the secretion of IL-4, IL-6, IL-12, TNF-α and IL-1β by 4- to 35- fold (41). This result indicates that the OX40L reverse signal enhanced the maturation of DCs. B cells are also an OX40L-expressing antigen-presenting cells that play an important role in formation of the GC (63). Cross-linking of OX40L on stimulated B cells significantly enhanced proliferative response and promoted immunoglobulin secretion (64). Morimoto et al. found CD134L engagement on human B cells increased IgG production rate per cell rather than increasing the number of plasma cells (65). Therefore, the OX40/OX40L bidirectional signal not only acts on T cells but also plays critical roles in differentiation and maturation of APCs, especially DCs and B cells (**Table 1**).

OX40/OX40L SIGNALING IN Tfh CELLS

Expression of OX40 On Tfh Cells

OX40 is transiently expressed on $CD4^+$ T cells after 12-24 hour of activation. As a distinct $CD4^+$ T cell subset, Tfh cells in both mice and humans have been confirmed to express OX40.

TABLE 1 | OX40/OX40L functions in different cell types.

Cell types	OX40 or OX40L expression	Functions
T cells	OX40 and OX40L	Promotion of T cell activation and proliferation (57) Inhibition of T cell apoptosis (56) Enhancement of recall response (59) Promotion of Th differentiation (58, 59)
Tfh cells	OX40	Promotion of Tfh differentiation and maintenance Enhancement of Tfh function of helping B cells
Tregs	OX40	Inhibition of Treg function (60, 61) Promotion of Treg proliferation (62)
DCs	OX40L	Promotion of the differentiation and maturation of DCs (41)
B cells	OX40L	Promotion of B cell proliferation and Ig secretion (64, 65) Promotion of GC formation (63)

Adam L et al. found that OX40 and ICOS were coexpressed on peripheral blood Tfh cells of patients with primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC). Compared with PBC patients, PSC patients had significantly upregulated OX40 and ICOS expression (66). Analysis of patients with rheumatoid arthritis (RA) showed an abundance of OX40-overexpressing Tfh cells, especially Tfh17 cells (67). Jiang et al. also reported OX40 expression on Tfh cells in a mouse model of myelodysplastic syndrome (MDS) (68). Tahiliani et al. found that in mice infected with vaccinia virus, OX40 was already expressed on pre-Tfh cells, and the expression level gradually increased with maturation of the Tfh cells (69). Therefore, OX40 is expressed during differentiation of Tfh cells and may play a role in Tfh development and functions.

Differentiation and Maintenance of Tfh Cells

Many cytokines and costimulatory signals, such as IL-12, IL-6 and ICOS/GL50, have been reported as the key factors in differentiation of Tfh cells. Recently, OX40/OX40L is characterized as another important costimulatory signal to promote Tfh differentiation. It has been reported that Rouqin regulates Tfh cell differentiation by inhibiting the expression of ICOS and OX40 mRNA, suggesting a close correlation between OX40 and Tfh cells (70). Defects in Rc3h1 and Rc3h2 in T cells elevate the expression of OX40 and Irf4, leading to activation of the NF-κB pathway. Tfh cells and GC-B cells spontaneously differentiate in the absence of immunization (70). Therefore, OX40 may promote the differentiation of Tfh cells.

CXCR5 is one of the most widely used markers to identify Tfh cells. Initially, OX40 *in vitro* was reported to induce CXCR5 mRNA transcription in activated mouse T cells, indicating that OX40 signaling may promote differentiation of Tfh cells by upregulating CXCR5 expression (71, 72). Then, the OX40/OX40L signal was found to upregulate multiple Tfh genes, including *CXCR5, Bcl-6, IL-21, CXCL13* and *PDCD1*, in both naïve and memory Th cells and to downregulate the expression of the transcription factor *PRDM1*, which inhibited the generation of Tfh cells (72). The upregulation of Bcl-6 and downregulation of PRDM1 fully demonstrated the important role of OX40 signaling in Tfh cell differentiation. Jacquemin et al. further compared the expression of Tfh genes after stimulation with OX40, IFN-γ and IL-12 which is an inducer of Tfh cells, and found that OX40 and IL-12 promoted naïve Th cells to express Tfh cell-related genes at similar levels (72). OX40 signaling is more efficient than IL-12 signaling at inducing memory Th cells to upregulate Tfh genes. The cooperation of the two signals can further increase the expression of *CXCR5* and *IL-21* on memory Th cells. In addition, it was shown that after 8-15 days of infection with vaccinia virus (VacV) in OX40-deficient mice, the numbers of Tfh and GC Tfh cells were significantly reduced compared with those in wild-type mice, indicating a critical role of OX40 in Tfh maturation (69). Prior studies reported that the interaction of OX40 and OX40L can also promote accumulation of $CD4^+$ T cells in the T/B boundary and B cell follicles in mouse models with protein Ag immunization (73). Recently, Tahiliani V et al. visualized OX40L-expressing DCs and B cells at the T/B borders and in the follicle and GC, in direct association with $OX40^+$ Tfh cells in these areas (69). The interaction between Tfh cells and DCs or B cells is very important for further Tfh differentiation and Tfh maintenance. Therefore, OX40/OX40L signaling promotes not only Tfh generation but also Tfh maturation and maintenance (**Figure 1**).

Enhancement of Tfh Functions

OX40L was found to be expressed in the GCs and surrounding areas, suggesting that OX40/OX40L signaling may play a role in formation of the GC. Li Y et al. constructed a recombinant rabies virus (RABV) mouse model (LBNSE-OX40L) which overexpressed OX40L and found that Tfh cells and GC-B cells significantly increased after RABV infection (74). Deletion of OX40L in B cells in the SLE mouse model resulted in an improved disease index and a decreased number of plasma cells and GC-B cells (75). In OX40-deficient mice, GCs could not be built up even if activated B cells from wild-type mice were injected. In contrast, GCs could be formed and expanded when B cells from OX40-deficient mice were injected into wild-type mice (69), suggesting that the OX40/OX40L signal is essential for the formation of GCs. In addition, reduced Ab production has been found in animals that lack the OX40 molecule. The interaction of $OX40L^+$ B cells and $OX40^+$ Tfh cells has been observed in T-B border and GCs in mice infected with VacV, further indicating the roles of OX40/OX40L signaling in Tfh helping B cells. During their interaction, a bidirectional OX40/OX40L signal occurs. On the one hand, OX40 on Tfh cells can receive signals from OX40L expressed on B cells to promote secretion of cytokines such as IL-21, which further assists B cell activation and antibody production. On the other hand, B cells can receive the OX40L reverse signal from Tfh to directly expand B cell clones and promote GC formation (**Figure 1**).

OX40-Initiated Signaling Pathways in Tfh Cells

There are two main OX40/OX40L signal transduction pathways in T cells. One is the antigen-independent NF-κB pathway, and the other is the antigen-dependent PI3K-Akt pathway. Binding of OX40L results in trimerization of OX40 monomers and recruitment of TRAF2, 3 and 5 (46, 47). TRAF2 has been characterized as an adaptor molecule that can lead to activation of NF-κB signal and recruitment of PI3K. For the NF-κB pathway, engagement of OX40 on activated/effector T cells by OX40L recruits not only the TRAF-RIP-IKKα/β/γ complex, but also the PCARMA1–BCL10–MALT1–PKCθ complex (76). This signalosome directly controls NF-κB activation without antigen/TCR engagement. The TRAF-RIP-IKKα/β/γ signaling complex mediates phosphorylation and degradation of IκBα, leading to activation of NF-κB1 and entry of p50 and RelA into the nucleus, which is sufficient to provide survival signals to T cells in the absence of antigens. The CARMA1–MALT1–BCL10–PKCθ complex forms the signalosome with OX40 in the immune synapse, which plays a major role in promoting prolonged NF-κB activity and survival of effector T cells during late-phase T cell

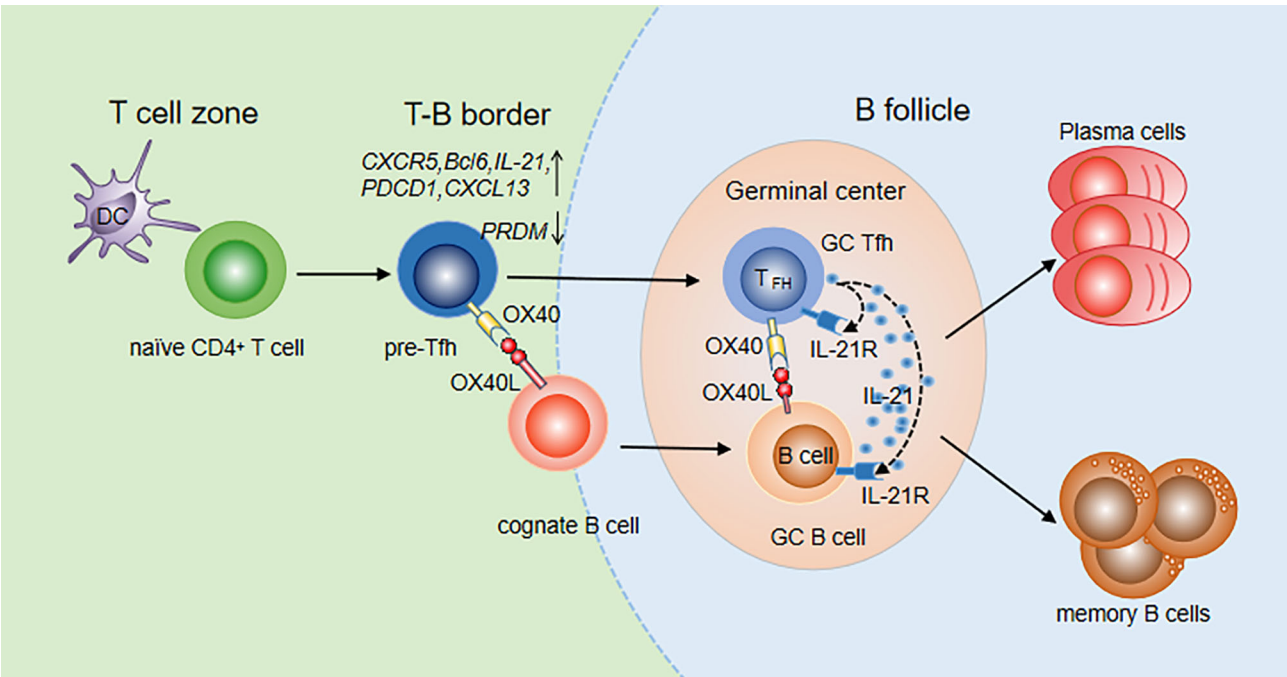

FIGURE 1 | OX40/OX40L signaling in Tfh differentiation and function. Tfh differentiation occurs at the time of DC priming. Upregulation of OX40 on pre-Tfh cells promotes their accumulation at the T-B border. With the interaction of cognate B cells, OX40 signaling contributes to Tfh maintenance, maturation and migration to B follicles. Meanwhile, B cells also move to B follicles to further interact with Tfh cells. Bidirectional OX40/OX40L signaling promotes both GC Tfh and GC B cell differentiation. With the help of GC Tfh cells, B cells differentiate into plasma cells and memory cells.

responses when antigen is cleared. OX40 can also induce phosphorylation of IKKα and activation of NIK, which activates the noncanonical NF-κB2 pathway (46, 77, 78). For the PI3K-Akt pathway, after ligation of OX40L, OX40 was found to assemble a signaling complex that contains TRAF2, PKB and its upstream activator PI3K (79, 80). It only induced strong phosphorylation and functional activation of the PI3K-Akt pathway when Ag was presented. Thus, OX40 can augment TCR signaling *via* the PI3K-Akt pathway. In addition, OX40 synergizes with TCR to allow Ca^{2+} influx and nuclear accumulation of NFATc1 and NFATc2 (78) (**Figure 2**).

The NF-κB pathway is also involved in Tfh cell proliferation and survival (81). Blocking molecules in the NF-κB1 and NF-κB2 pathways inhibits the Bcl-6 expression on $CD4^+$ T cells. However, the effect is independent of OX40 signaling (72). Strong and durable TCR signals can also contribute to promoting Th cell differentiation to the Tfh lineage and their proliferation (82–84). A recent study showed that stimulation with anti-CD3 and anti-CD28 beads promoted the expression of multiple Tfh molecules including CXCR5, IL-21, CD40L and Bcl-6, in a dose-dependent manner. The combination of OX40 and TCR signals further upregulated the expression of Tfh molecules, indicating that OX40 signaling promotes Tfh differentiation by enhancing TCR signaling. PI3K activity is an essential component of pathways driving Tfh cell and GC formation (85). Both TCR and OX40 are characterized as strong activators of both the PI3K and Akt signaling pathways. Thus, OX40 may mediate Tfh cells by augmenting TCR signaling *via* the PI3K-Akt pathway. Another way for OX40 to regulate Tfh cells may be through NFAT, which has been shown to be essential for effective Tfh development (**Figure 2**) (86).

IMPLICATION OF Tfh IN AUTOIMMUNE DISEASES AND IMMUNE THERAPY THROUGH OX40/OX40L SIGNALING

Tfh cells and OX40/OX40L have both been reported to be associated with autoimmune diseases both in humans and mice (**Table 2**). Since many autoimmune diseases such as SLE, RA and Graves' diseases are autoantibody-mediated, the critical roles of Tfh cells in these diseases are obvious (87–101). Tfh cells enhance the intensity and duration of the GC response and promote autoantibody production. In lupus nephritis lesions, Tfh-like cells expressing PD-1, ICOS, IL-21 and Bcl-6 were observed to form ectopic GCs. In addition, an increased population of circulating Tfh cells was identified in SLE patients (102). In salivary gland tissues and peripheral blood of patients with Sjogren's syndrome (SS), the numbers of $CD4^+CXCR5^+$ Tfh cells were significantly increased along with abnormal B cells and plasma cells, suggesting that Tfh cells participate in the pathogenesis of SS by promoting B cell maturation (103). Moreover, multiple studies have demonstrated that OX40 is expressed on pathogenic T cells in autoimmune

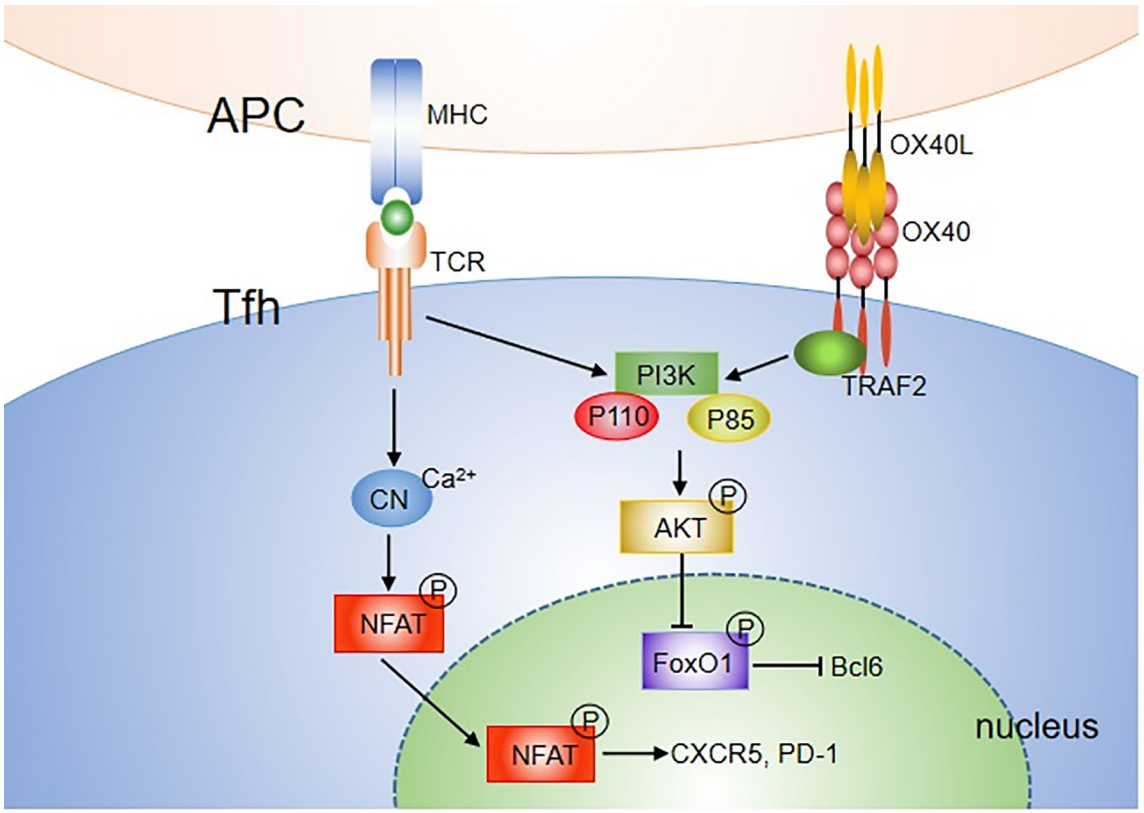

FIGURE 2 | OX40 signaling pathways mediating Tfh differentiation. Both TCR and OX40 can activate PI3K, including P110 and P85 subunit, further leading to phosphorylation of AKT. pAKT then phosphorylates the FOXO1 transcription factor, which can subsequently be exported out of the nucleus and degraded. FOXO1, which represses Bcl6, is recognized as an inhibitor of Tfh differentiation. In addition, OX40 synergizes with TCR to allow Ca^{2+} influx and nuclear accumulation of NFATc1 and NFATc2. Overall, OX40 may mediate Tfh cell activity by augmenting TCR signaling *via* the NFAT or PI3K-Akt pathway.

disease (42, 104–112). Farres et al. found that compared with healthy people, $CD4^+$ T cells in SLE patients express high levels of OX40, and the disease activity index is positively correlated with the number of $CD4^+$ T cells expressing OX40. The disease is improved after treatment with an anti-OX40L monoclonal antibody (113, 114). Yoshioka et al. found that T lymphocytes in synovial fluid and synovial tissue of RA patients express OX40, and secondary lymphocytes in synovial tissue express OX40L, suggesting that the OX40/OX40L interaction may play a key role in RA occurrence and development (34). Graves' disease (GD) is an autoimmune thyroid disease, with clinical manifestations that primarily include ophthalmopathy, goiter and hypermetabolic syndrome (115, 116). We have found that OX40/OX40L was abnormally and persistently coexpressed on $CD4^+$ T cells from GD patients, and the coexpression level was closely related to TRAb (117).

Recent data for SLE and RA showed that high OX40 and OX40L expression may be involved in the pathogenesis of autoimmune diseases by enhancing Tfh functions. Jacquemin et al. also reported that $OX40L^+$ myeloid cells are visualized in skin and kidney tissues from SLE patients. OX40 engagement upregulated the expression of several Tfh-associated molecules in T cells from lupus patients, including Bcl6, CXCR5 and IL-21, showing that Th cells in an OX40L-rich environment may receive OX40 signaling to promote Tfh development. The percentage of $OX40L^+$ myeloid cells in blood was significantly higher in active patients than in inactive patients and positively correlated with peripheral Tfh cell frequencies, indicating that the Tfh response was enhanced by the OX40 signal. Moreover, myeloid cells expressing OX40L can also impair Treg and Tfr functions by suppressing Tfh-dependent B cell activation and immunoglobulin secretion in SLE. OX40-overexpressing Tfh cells, especially Tfh 17 cells, were found to be increased in RA and a murine model. In vitro coculture experiments showed enhanced hyposialylation by the Tfh cells *via* OX40. Blockade of OX40 signaling prevented arthritis development by reducing Tfh17 cells and recovering autoantibody salivation (67). Therefore, upregulated OX40 signaling plays a crucial role in the development of autoimmune diseases by enhancing Tfh functions directly or indirectly. Thus, targeting OX40/OX40L signaling may be an effective strategy for these diseases.

OX40/OX40L blockade *in vivo* is generally effective in many models with autoimmune diseases, mainly by inhibiting activation and migration of $CD4^+$ T cells and altering cytokine

TABLE 2 | OX40, OX40L expression and Tfh cells in autoimmune diseases.

Disease	OX40	OX40L	Tfh
Systemic lupus erythematosus (SLE)	Upregulated OX40 expression on peripheral T cells (108)	Upregulated OX40L expression on myeloid APCs (72)	Increased Tfh in patients with active SLE (108)
Rheumatoid arthritis (RA)	Upregulated OX40 expression on T cells in synovial fluid and blood (34, 67, 119) Upregulated OX40 expression on circulating $CD4^+CD28^-$ T cells (116) Upregulated OX40 expression on circulating Tfh17 cells (67)	Upregulated OX40L expression on sublining cells in synovial tissue (34) and on monocytes and B cells in blood (90)	Increased circulating Tfh cells and Tfh17 cells (118)
Type 1 Diabetes (T1D)	Increased circulating $CD4^+CD25^{high}$ $OX40^+$ T cells in children with newly diagnosed T1D (94)		Increased circulating Tfh cells in newly diagnosed T1D children (102) Increased circulating Tfh cells in T1D patients (88, 89)
Graves' diseases	Upregulation of OX40 on circulating $CD4^+$ T cells (117)	Upregulation of OX40L on circulating $CD4^+$ T cells (117)	Increased circulating Tfh and Tfh2 cells (90) Elevated Tfh cells in thyroid tissues (77, 78)
Multiple sclerosis	Downregulation of OX40 expression on circulating $CD4^+$ T cells after treatment with natalizumab (95) The presence of $OX40^+$ T cells in brain tissue (106)		Increased circulating IL-21-producing Tfh-like cells (96) Increased Tfh/Tfr ratio associates with abnormal IgG production in blood and CSF (92)
Myasthenia gravis	Upregulation of OX40 expression on circulating $CD4^+$ T cells (107) and thymic $CD4^+$ T cells adjacent to GC (108).	The presence of $OX40L^+$ mononuclear cells in thymic GC (108).	Increased circulating Tfh cells (102) Increased circulating Tfh17 cells in MuSK-antibody positive patients (93).
Sjogren syndrome	Upregulation of OX40 expression on circulating CD4+ T cells (108)	Upregulation of OX40L expression on circulating B cells and monocytes (108)	Increased circulating Tfh and Tfh17 cells (94) Localization of Tfh cells in salivary glands (95)
lupus mouse	Upregulation of OX40 expression on $CD4^+$ T cells in the spleen and kidney of NZB/WF1 mouse (109)		Expanded Tfh cells in spleen of MRL/lpr mouse (96) Tfh cells infiltrating the brain of murine neuropsychiatric lupus in MRL/lpr mouse (97)
Collagen-induced arthritis mouse	Upregulation of OX40 expression on $CD4^+$ T cells in joints (110) and spleen (111) Upregulation of OX40 expression on $CD4^+CD28^-$ T cells in spleen (118)	Upregulation of OX40L expression on APCs in spleen (98)	Increased Tfh cells in the spleen (111)
T1D mouse	Upregulation of OX40 expression on $CD4^+$ and $CD8^+$ T cells in pancreatic lymph nodes and spleen of NOD mouse prior to diabetes onset (120)	Upregulation of OX40L expression on dendritic cells in pancreatic lymph nodes late during NOD development (120)	Increased Tfh cells in the pancreatic lymph node and the pancreas of DO11×RIP-mOVA mouse (99)
Autoimmune encephalomyelitis (EAE) model	Upregulation of OX40 expression on CD4+ T cells in spleen and brain tissue (42, 106) OX40 expression selectively on autoantigenic $CD4^+$ T cells from the inflammatory site in spinal cord or brain (111, 112)	Upregulation of OX40L expression on $CD11b^+$ cells and vascular endothelial cells in central nerous system (106, 121)	Increased Tfh cells in ectopic lymphoid structures in spinal cords (100)

production (34, 118, 119) (**Table 3**). When a blocking anti-OX40L antibody was given to NOD mice at 12 weeks of age, the incidence of diabetes was reduced (120). In EAE mice, anti-OX40L antibody blockade leaded to decline of clinical score and reduction of spinal cord T cell infiltration (121, 122). There are also some clinical trials targeting OX40/OX40L in development. OX40L-blocking antibodies were reported to ameliorate antigen-driven Th2 responses in mouse and nonhuman primate models of asthma (123). An anti-OX40 antibody, GBR 830 in phase II study showed significant clinical improvement in patients with moderate to severe atopic dermatitis (125) (**Table 3**). Combined OX40L and mTOR blockade in nonhuman primate graft-versus-host disease (GVHD) model prolonged survival by controlling effector T cell activation while preserving Treg reconstitution (126). However, the treatment with a humanized anti-OX40L mAb has no effect on allergen-induced airway responses in mild asthmatic patients (124). Timing and dosing of clinical intervention may be critical for the efficacy.

CONCLUSION

Providing help for B cell development and GC reactions is the most crucial function of Tfh cells, which lead to high-affinity antibody production. Thus, increased activity of Tfh cells plays a pathogenic role in a wide range of autoimmune diseases, in both mice and humans. The differentiation of Tfh cells requires not only TCR signaling, cytokines and antigen stimulation but also costimulatory

TABLE 3 | Therapeutic effects of OX40/OX40L blockade *in vivo*.

Disease	Model	Intervention and effect
Rheumatoid arthritis (RA)	Collagen-induced arthritis (CIA) mouse	Anti-OX40L mAb ameliorated clinical score and suppress IFN-γ and anti-CII Ig2a production (34)
	CIA mouse	Anti-OX40L mAb reduced the proinflammatory responses and ameliorated arthritis development (118)
	CIA mouse	Treatment with the anti-OX40 Fab′PEG blocking antibody and the OX40L:Ig fusion protein delayed the time of onset of arthritis and reduced the overall clinical score (119)
Type 1 Diabetes (T1D)	NOD mouse	Anti-OX40L mAb given to NOD mice at 12 weeks of age prevented diabetes development (120)
Multiple Sclerosis	EAE mouse	Anti-OX40L antibody leaded to decline of clinical score and reduction of spinal cord T cell infiltration (121, 122)
Asthma	mouse and nonhuman primate models	Anti-OX40L mAb inhibited Th2 lung inflammation (123)
	Mild atopic asthmatic patients	A humanized anti-OX40L mAb has no effect on allergen-induced airway responses despite partial and transient reduction in total IgE and airway eosinophils (124)
Atopic dermatitis	Patients with moderate to severe syndrome	Blocking anti-OX40 antibody showed significant clinical improvement (125)
Graft-versus-host disease (GVHD)	nonhuman primate model	Combined OX40L and mTOR blockade prolonged survival by controlling effector T cell activation (126)

signals, such as ICOS/ICOSL and OX40/OX40L. As an important marker of Tfh cells, OX40 can promote Tfh generation and contribute to maintenance of Tfh and GC B cells at later times. OX40 synergizes with ICOS to maximize and prolong the Tfh response. Therefore, upregulation of OX40 and OX40L may induce abnormal activation of Tfh cells and excessive production of autoantibodies, leading to the development of autoimmune diseases.

Given that blocking OX40/OX40L signaling has shown great therapeutic effects in some mouse models of autoimmune diseases, targeting OX40/OX40L is promising as a new therapeutic approach for these diseases. However, the efficacy data of clinical trials are currently limited. Further studies are needed for clinical intervention since many factors, such as dose and time point, influence the effect. Moreover, controversial results have been obtained regarding the roles of the OX40/OX40L axis in regulation of Tfh responses. Whether other factors may impact the roles of OX40L in Tfh cells needs also to be further investigated.

AUTHOR CONTRIBUTIONS

QW and ZS organized and supervised the entire manuscript. NF contributed to the sections on Tfh cell differentiation and functions and the OX40 and OX40L molecules. XF contributed to the sections on OX40 signaling in Tfh cells and the implication of Tfh cells in autoimmune diseases. All authors contributed to the article and approved the submitted version.

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Haplotype-Specific Expression Analysis of MHC Class II Genes in Healthy Individuals and Rheumatoid Arthritis Patients

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HLA-DRB1 alleles have been associated with several autoimmune diseases. For anti-citrullinated protein antibody positive rheumatoid arthritis (RA), *HLA-DRB1* shared epitope (SE) alleles are the major genetic risk factors. In order to study the genetic regulation of major histocompatibility complex (MHC) Class II gene expression in immune cells, we investigated transcriptomic profiles of a variety of immune cells from healthy individuals carrying different *HLA-DRB1* alleles. Sequencing libraries from peripheral blood mononuclear cells, CD4+ T cells, CD8+ T cells, and CD14+ monocytes of 32 genetically pre-selected healthy female individuals were generated, sequenced and reads were aligned to the standard reference. For the MHC region, reads were mapped to available MHC reference haplotypes and AltHapAlignR was used to estimate gene expression. Using this method, *HLA-DRB* and *HLA-DQ* were found to be differentially expressed in different immune cells of healthy individuals as well as in whole blood samples of RA patients carrying *HLA-DRB1* SE-positive *versus* SE-negative alleles. In contrast, no genes outside the MHC region were differentially expressed between individuals carrying *HLA-DRB1* SE-positive and SE-negative alleles, thus *HLA-DRB1* SE alleles have a strong cis effect on gene expression. Altogether, our findings suggest that immune effects associated with different allelic forms of HLA-DR and HLA-DQ may be associated not only with differences in the structure of these proteins, but also with differences in their expression levels.

Keywords: major histocompatibility complex, RNA-sequencing, allelic expression, T cell, monocyte, rheumatoid arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder affecting approximately 0.5-1% of the population worldwide (1). Although the exact cause of RA remains unknown, a set of so-named shared epitope (SE) alleles, *HLA-DRB1**01 (*01:01 and *01:02), *04 (*04:01, *04:04, *04:05, and *04:08), and *10 (*10:01), have been associated with RA (2) and more specifically with anti-

citrullinated protein antibody (ACPA)-positive RA (3). These alleles share a sequence encoding five amino acids in position 70-74 of the antigen-binding groove of the HLA-DR beta chain. More recent studies have shown the importance also of amino acids in positions 11 and 13 in the HLA-DR beta chain (4). It has been suggested that citrullinated antigens may bind preferentially to HLA-DRB1 SE sequences leading to the activation of autoreactive T-cells (5). *HLA-DRB1* SE alleles are not the only alleles associated with ACPA-positive RA, a meta-analysis has described that *HLA-DRB1**13:01 alleles provide protection against ACPA-positive RA (6). Indeed, many autoimmune diseases are associated with certain *HLA-DRB1* alleles. For example, *HLA-DRB1**15:01 confers the strongest risk for developing multiple sclerosis (7), but is not associated with RA. Moreover, the genetic architecture of the HLA locus is complex as allelic variants of *HLA-DRB1* involve linkage with either none or one of the paralogs (*HLA-DRB3*, *HLA-DRB4* or *HLA-DRB5*). Therefore, *HLA-DRB1* is expressed in cells with all *HLA-DRB1* haplotypes, whereas expression of the paralogs is haplotype-specific (**Figure 1**).

The genetic association of *HLA-DRB1* to RA is proposed to at least partly reflect a favored binding of citrullinated peptides to the HLA binding groove. However, the precise molecular mechanisms by which *HLA-DRB1* SE alleles predispose to ACPA-positive RA are currently unclear. There is evidence suggesting that expression of genes in the major histocompatibility complex (MHC) region vary significantly between different *HLA-DRB1* alleles (8–11). In addition, a recent study showed allele-specific expression of *HLA-DRB1* in Korean ACPA-positive RA patients (12). However, these differences have not been studied in healthy individuals with susceptibility alleles in the MHC locus and for specific cell types. In this study, we aimed to identify differentially expressed genes in peripheral blood mononuclear cells (PBMCs) and isolated CD4+ T cells, CD8+ T cells, and CD14+ monocytes from PBMCs of healthy individuals with *HLA-DRB1* SE alleles compared to healthy individuals not carrying these alleles, which will be referred to as SE negative alleles. In addition, the total expression of the MHC Class II molecules in whole blood samples was investigated in both healthy individuals and in RA patients. We used RNA-seq data with a pipeline [AltHapAlignR (8)] that aligns reads to available MHC reference haplotypes for more accurate analysis of gene expression in the MHC region. This analysis will help us to understand the mechanisms of immune response in individuals with susceptibility alleles in the MHC locus.

MATERIALS AND METHODS

Ethics Statement

This project was undertaken with ethical approval of the Regional Ethical Review Board in Uppsala (2009/013) and all healthy donors gave written informed consent according to the declaration of Helsinki.

Blood Donors

Blood samples from 32 healthy previously genotyped donors (females between 55 and 73 years of age) were provided by the Uppsala Bioresource (Uppsala University Hospital, Uppsala, Sweden). The samples were selected by positivity for certain *HLA-DRB1* alleles (*HLA-DRB1**04, *HLA-DRB1**13:01, and *HLA-DRB1**15:01). The main characteristics of the participants are presented in **Table 1**.

Blood Sampling and Cell Separation

Buffy coats were processed by density gradient centrifugation using Ficoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and PBMCs were subsequently recovered. CD4+, CD8+, and CD14+ cells were isolated from the PBMCs *via* positive selection using CD4, CD8 or CD14 Microbeads (Miltenyi Biotec Norden AB, Lund, Sweden) on the autoMACS® Pro Separator (Miltenyi Biotec Norden AB).

RNA Sequencing

Total RNA was extracted with the RNeasy Mini kit (Qiagen AB, Sollentuna, Sweden) according to manufacturer's instructions. Samples were treated with DNase (Qiagen) for 20 min at room temperature to avoid contamination with genomic DNA. The quality of each RNA sample was assessed using the Agilent Bioanalyzer 2100

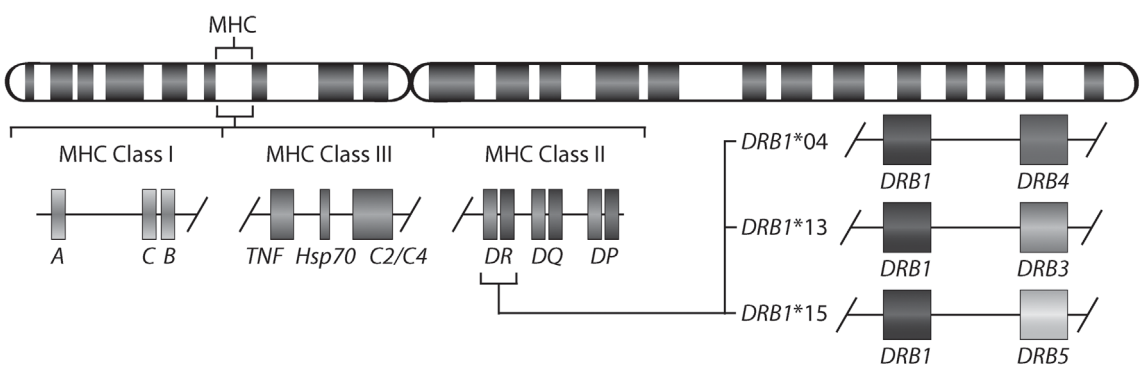

FIGURE 1 | Gene map of the major histocompatibility complex (MHC) region. The MHC region on the short arm of human chromosome 6 contains the HLA-DR (HLA-DRA and HLA-DRB) molecules. The allelic variants of *HLA-DRB1* (*DRB1**04, *13, and *15) are linked with only one of the genes *HLA-DRB4*, *HLA-DRB3*, or *HLA-DRB5*.

TABLE 1 | Characteristics of study participants.

Sample ID	Sex	Age	*HLA-DRB1* alleles	*HLA-DRB1* SE	PBMCs	CD4+ T cells	CD8+ T cells	CD14+ monocytes
1	F	60	*03:01/*04:01	Positive	x	x	x	NA
2	F	58	*03:01/*04:01	Positive	x	NA	NA	NA
3	F	59	*03:01/*15:01	Negative	x	NA	x	NA
4	F	65	*03:01/*15:01	Negative	NA	NA	x	NA
5	F	56	*01:01/*04:01	Double positive	x	x	x	NA
6	F	65	*03:01/*13:01	Negative	x	x	NA	NA
7	F	65	*04:01/*07:01	Positive	x	x	x	NA
8	F	60	*04:01/*13:03	Positive	NA	x	x	NA
9	F	65	*04:01/*07:01	Positive	x	x	NA	x
10	F	59	*03:01/*04:01	Positive	x	x	NA	x
11	F	63	*03:01/*15:01	Negative	x	x	NA	NA
12	F	61	*04:01/*14:01	Positive	x	x	x	NA
13	F	67	*03:01/*13:01	Negative	NA	x	NA	NA
14	F	61	*03:01/*15:01	Negative	x	x	NA	NA
15	F	65	*01:01/*04:01	Double positive	x	x	x	x
16	F	56	*03:01/*04:01	Positive	x	x	x	NA
17	F	62	*03:01/*15:01	Negative	x	x	NA	x
18	F	62	*03:01/*13:01	Negative	x	x	x	NA
19	F	70	*03:01/*04:01	Positive	x	x	NA	NA
20	F	55	*03:01/*13:01	Negative	x	x	x	NA
21	F	73	*03:01/*13:01	Negative	x	x	NA	NA
22	F	59	*04:01/*04:04	Double positive	x	NA	x	NA
23	F	58	*03:01/*13:01	Negative	x	x	x	NA
24	F	66	*04:01/*14:01	Positive	x	NA	NA	x
25	F	61	*04:01/*09:01	Positive	x	x	x	NA
26	F	61	*03:01/*04:01	Positive	x	NA	x	NA
27	F	72	*01:01/*04:01	Double positive	x	x	x	x
28	F	67	*03:01/*13:01	Negative	x	x	x	x
29	F	56	*04:08/*07:01	Positive	x	x	x	NA
30	F	67	*03:01/*15:01	Negative	x	x	x	x
31	F	62	*03:01/*15:01	Negative	x	x	x	x
32	F	57	*01:01/*04:01	Double positive	x	x	x	x

F, female; NA, not available; PBMCs, peripheral blood mononuclear cells; SE, shared epitope; x, included in analyses.

and RNA 6000 Nano Chips (Agilent Technologies Sweden AB, Kista, Sweden). The RNA integrity number (RIN) ranged between 3.4 and 9.5 (median of 7.9). The RNA was fragmented and prepared into sequencing libraries using the Illumina TruSeq stranded total RNA sample preparation kit with ribosomal depletion using RiboZero (2 x 125 bp) and sequenced on an Illumina HiSeq 2500 sequencer (SNP&SEQ Technology Platform, Uppsala, Sweden). Between 24.5 and 60.5 (median of 41) million reads were produced per sample. Raw read quality was evaluated using FastQC. Pre-filtering on quality of reads using cutadapt (version 1.9.1) was applied (-q 30 -a AGATC GGAAGAGCACACGTCTGAACTCCAGTCAC -A AGATC GGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATC TCGGTGGTCGCCGTATCATT -m 40). Filtered reads were aligned to the hg38 assembly, containing the MHC reference haplotype PGF [GENCODE release 28 (GRCh38.p12)], using STAR in two-pass mode (version 2.5.4b) (13) with default settings. STAR was also used to obtain the gene counts. All sequencing data generated in this study are available at NCBI Gene Expression Omnibus accession number GSE163605.

Whole Blood RNA Sequencing Data of Healthy Individuals and RA Patients

FASTQ files for 439 whole blood samples were downloaded from the GTEx project (14). For RA patient samples, 158 FASTQ files from the EIRA/RECOMBINE project were used. Seq2HLA (15) was used to impute classical HLA alleles from RNA-seq data. Individuals with *HLA-DRB1**03, *04, *07 and *15 alleles were selected (based on the available MHC reference haplotypes APD, COX, DBB, MANN, MCF, PGF, QBL and SSTO). Mapping to the human genome, MHC region and differential gene expression analysis were performed as described above.

Mapping to the MHC Region

Counts of genes in the MHC region (chr6: 28,500,000-33,500,000) were replaced with counts obtained from AltHapAlignR (8). In short, unmapped reads and reads mapped to the MHC region (using MHC reference haplotype PGF) were extracted and realigned to all available MHC reference haplotypes (APD, COX, DBB, MANN, MCF, QBL, SSTO) independently using STAR in two-pass mode. Reads mapped to multiple regions, with mapping quality less than 20, and duplicate reads were removed before further analyses.

Differential Gene Expression Analysis

Raw expression counts obtained from STAR (non-MHC genes) and AltHapAlignR (MHC genes) were adjusted for library size using the R package DESeq2 (version 1.26.0) (16). Pre-filtering of low count genes was performed to keep only genes that had at least 50 counts in total over all samples. The counts were regularized-logarithm transformed for principal component

analysis (PCA). For each specific cell subset, RIN score was highly correlated with principal component 1 and used as covariate as binary category (< 7 and ≥ 7) in the analyses. The default DESeq2 options were used, including log fold change shrinkage using apeglm (17) and independent hypothesis weighting (18). *P*-values were obtained with the Wald test. Differences in gene expression with Benjamini-Hochberg adjusted *P*-value < 0.05 and fold change (log2) > 1 were considered significant.

HLA-Typing and Imputation

For the 32 healthy donors, HLA-DRB1 genotypes were initially imputed from Immunochip data by SNP2HLA (19) and later HLA low resolution typing was performed for validation. Additionally, DR4 subtyping was performed for *HLA-DRB1**04 positive individuals. *HLA*-typing was performed by sequence-specific primer polymerase chain reaction assay (DR low-resolution kit and DR4 kit; Olerup SSP, Stockholm, Sweden) and analyzed by agarose gel electrophoresis (20). An interpretation table was used to determine the specific genotype according to the manufacturer's instructions. In addition, seq2HLA (15) was used to impute classical HLA alleles from RNA-seq data for all cohorts.

Quantitative Real-Time PCR

RNA of the PBMC, CD4+, CD8+, and CD14+ cell subset samples was converted into cDNA using iScript™ Reverse Transcription Supermix (Bio-Rad, Solna, Sweden). Quantitative real-time PCR (qPCR) was carried out using SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad) and primers detecting *HLA-DQA* (forward primer 5'-CAACATCACATGG CTGAGCA-3' and reverse primer 5'-TGCTCCACCTTGCAGT CATAA-3'), *HLA-DQB* (forward primer 5'-TCTCCCCATCCA GGACAGAG-3' and reverse primer 5'-TTCCGAAACCACCGG ACTTT-3'), and *HLA-DRA* (forward primer 5'-CCTGTCACC ACAGGAGTGTC-3' and reverse primer 5'-TCCACCCTG CAGTCGTAAAC-3') on a CFX384 Touch™ system (Bio-Rad) with the following protocol: 95°C for 30 sec, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. The endogenous controls *ACTIN* (forward primer 5'-GGACTTCGAGCAAGAGATGG-3' and reverse primer 5'-AGCACTGTGTTGGCGTACAG-3'), *UBE2D2* (forward primer 5'-TGCCTGAGATTGCTCG GATCT-3' and reverse primer 5'-TCGCATACTTCTGAGTC CATTCC-3'), and *ZNF592* (forward primer 5'-GTAAAGGA GAATTGCCTGCA-3' and reverse primer 5'-GAATGCACAT TTGTGGAAAA-3'). Data was analyzed with the SDS 2.4 software of Applied Biosystems before applying the ΔΔCt method (21).

Cell Type Enrichment Analysis

The xCell tool (22) was used to estimate cellular heterogeneity in the PBMC, CD4+, CD8+, and CD14+ cell subsets from RNA sequencing data. xCell uses the expression levels ranking [Transcripts Per Million (TPM)] and these were obtained using Salmon (version 0.8.2) (23).

RESULTS

Differentially Expressed Genes in PBMCs of *HLA-DRB1* SE-Positive *Versus* SE-Negative Healthy Individuals

To identify differentially expressed genes in PBMCs of healthy individuals with and without *HLA-DRB1* SE alleles, we conducted RNA-seq on 29 PBMC samples and aligned reads to available MHC reference haplotypes using AltHapAlignR (8). We identified five MHC Class II genes (*HLA-DRB4*, *HLA-DQA2*, *HLA-DRB1*, *HLA-DQA1*, and *HLA-DQB1*) that were differentially expressed to a great degree in PBMCs of *HLA-DRB1* SE-positive *versus* SE-negative individuals (**Figure 2A** and **Table S1**). Of these differentially expressed genes, *HLA-DRB4*, *HLA-DQA2*, *HLA-DRB1*, and *HLA-DQA1* were higher expressed in *HLA-DRB1* SE-positive individuals, whereas *HLA-DQB1* was lower expressed in *HLA-DRB1* SE-positive individuals, in comparison to *HLA-DRB1* SE-negative individuals. We further confirmed the expression differences of *HLA-DQA* and *HLA-DQB* depending on *HLA-DRB1* alleles by qPCR (**Figures S1–S3**). We identified no significantly differentially expressed genes outside the MHC region in PBMCs of *HLA-DRB1* SE-positive *versus* SE-negative healthy individuals with an adjusted *P*-value < 0.05 and a fold change (log2) > 1 (**Table S1**).

To reduce heterogeneity from the effect of the second allele in the *HLA-DRB1* SE-positive group, we performed differential gene expression analysis on PBMC samples with only *HLA-DRB1**03:01 as second allele. We found that the five MHC Class II genes (*HLA-DRB4*, *HLA-DQA2*, *HLA-DRB1*, *HLA-DQA1*, and *HLA-DQB1*) were differentially expressed in PBMCs of *HLA-DRB1* SE-positive *versus* SE-negative individuals carrying one *HLA-DRB1**03:01 allele [adjusted *P*-value < 0.05 and a fold change (log2) > 1 (**Table S2** and **Figure 2B**)].

As the *HLA-DRB1* sequence appeared to be absent in the MHC reference haplotype APD (*HLA-DRB1**13:01), we also performed differential gene expression analysis on PBMC samples of individuals with the alleles *HLA-DRB1**03:01/*04:01 *versus* *03:01/*15:01. Four MHC Class II genes (*HLA-DRB5*, *HLA-DRB4*, *HLA-DQA2*, and *HLA-DQB1*) were differentially expressed in PBMCs of healthy individuals carrying *HLA-DRB1**03:01/*04:01 compared to *03:01/*15:01 [adjusted *P*-value < 0.05 and a fold change (log2) > 1 (**Table S3**)]. In addition, there is a trend towards *HLA-DRB1* being higher expressed in PBMCs of individuals carrying *HLA-DRB1**03:01/*04:01 compared to *HLA-DRB1**03:01/*15:01, although no statistically significant difference was observed [adjusted *P*-value of 0.068 and fold change (log2) of 0.904 (**Figure 2C**)].

Differentially Expressed Genes in CD4+ and CD8+ T Cells of *HLA-DRB1* SE-Positive *Versus* SE-Negative Healthy Individuals

As PBMCs are a heterogeneous mixture of immune cell types, we isolated CD4+ T cells, CD8+ T cells, and CD14+ monocytes from the same healthy individuals *via* positive selection using microbeads, sequenced RNA samples that passed quality control and performed differential gene expression analyses. We found five MHC Class II genes (*HLA-DRB4*, *HLA-DQA2*, *HLA-DRB1*, *HLA-DQA1*, and *HLA-DQB1*) that were differentially expressed

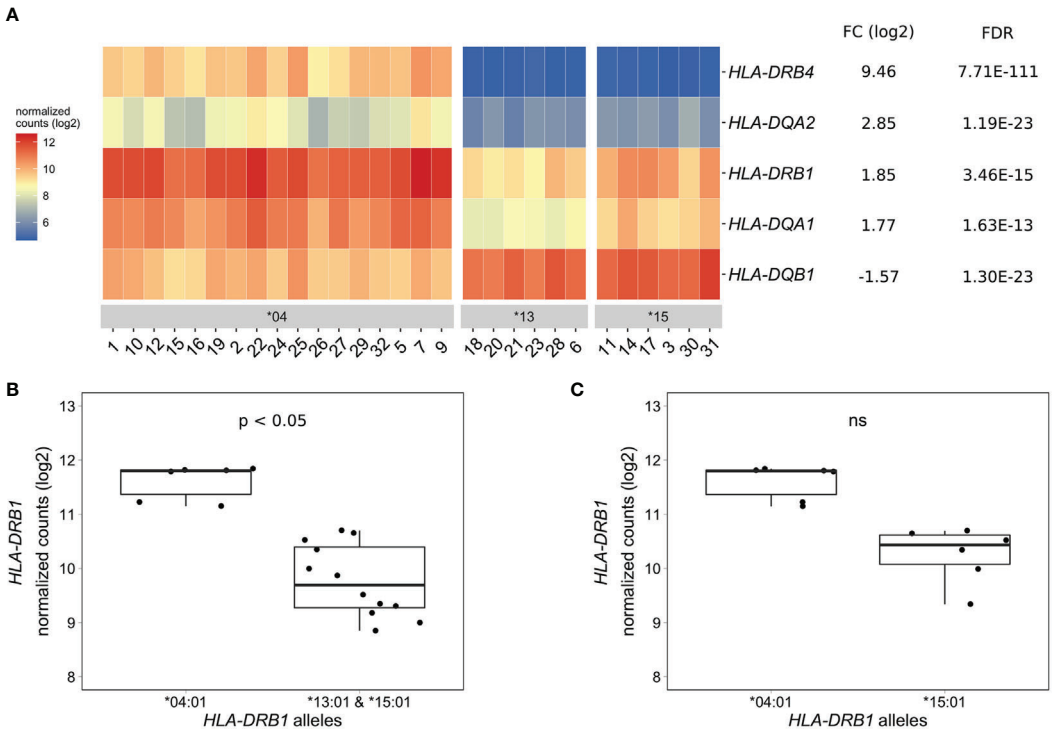

FIGURE 2 | Differentially expressed MHC Class II genes in PBMCs of *HLA-DRB1* SE-positive *versus* SE-negative healthy individuals. **(A)** Heat map of differentially expressed genes (log transformed normalized gene counts) in PBMCs of *HLA-DRB1* SE-positive [*04 (*n* = 17)] *versus* SE-negative [*03:01/*13:01 and *03:01/*15:01 (*n* = 12)] individuals. Genes shown in red have higher gene counts and those shown in blue have lower gene counts. **(B)** *HLA-DRB1* gene expression in PBMCs of *HLA-DRB1* SE-positive [*03:01/*04:01 (*n* = 6)] and SE-negative [*03:01/*13:01 and *03:01/*15:01 (*n* = 12)] individuals. **(C)** *HLA-DRB1* gene expression in PBMCs of *HLA-DRB1* SE-positive [*03:01/*04:01 (*n* = 6)] and SE-negative [*03:01/*15:01 (*n* = 6)] individuals. FC, fold change; ns, non-significant adjusted *P*-value (FDR).

to a great degree in CD4+ T cells of *HLA-DRB1* SE-positive *versus* SE-negative individuals (**Figures 3A, C**, and **Tables S4–S6**). Again, we identified no differentially expressed genes outside the MHC region in CD4+ T cells of *HLA-DRB1* SE-positive *versus* SE-negative individuals (adjusted *P*-value < 0.05 and a fold change (log2) > 1 (**Tables S4–S6**)]. In CD8+ T cells, we found five MHC Class II genes (*HLA-DRB4*, *HLA-DRB1*, *HLA-DQB1*, *HLA-DQA2*, and *HLA-DQA1*) and two genes outside the MHC region (*RPL10P6* at chromosome 2q35 and *ADRB1* at chromosome 10q25) to be differentially expressed between *HLA-DRB1* SE-positive and SE-negative individuals (**Figure 3B** and **Tables S7–S9**). The expression differences of *HLA-DQA* and *HLA-DQB* depending on *HLA-DRB1* alleles were confirmed by qPCR (**Figures S1–S3**). In addition, there is a trend towards *HLA-DRB1* being higher expressed in CD8+ T cells of individuals carrying *HLA-DRB1**03:01/*04:01 compared to *HLA-DRB1**03:01/*15:01, although no statistically significant difference was observed [adjusted *P*-value of 0.538 and fold change (log2) of 0.811 (**Figure 3D**)].

Differentially Expressed Genes in CD14+ Monocytes of *HLA-DRB1* SE-Positive *Versus* SE-Negative Healthy Individuals

In CD14+ monocytes, we identified five genes that were differentially expressed between *HLA-DRB1* SE-positive and SE-negative healthy individuals [adjusted *P*-value < 0.05 and a fold change (log2) > 1 (**Figure 4A** and **Table S10**)]. Of these five differentially expressed genes, three were MHC Class II genes (*HLA-DRB4*, *HLA-DQA2*, and *HLA-DQA1*), one was an MHC Class I gene (*HLA-A*), and one was a non-MHC gene (*TENT4B* at chromosome 16q12). *HLA-DRB4*, *HLA-DQA2*, *HLA-DQA1*, and *HLA-A* were higher expressed in *HLA-DRB1* SE-positive individuals, whereas *TENT4B* was lower expressed in *HLA-DRB1* SE-positive individuals, in comparison to *HLA-DRB1* SE-negative individuals. In addition, there is a trend towards *HLA-DRB1* being higher expressed in CD14+ monocytes of individuals carrying *HLA-DRB1**03:01/*04:01 compared to *HLA-DRB1**03:01/*15:01 [adjusted *P*-value of 0.122 and fold change (log2) of 1.658 (**Figure 4B**)]. The expression differences of *HLA-DQA* and *HLA-DQB* depending on *HLA-DRB1* alleles were confirmed by qPCR (**Figures S1–S3**).

HLA-DRB1 Is Differentially Expressed in Whole Blood Samples of Healthy Individuals Carrying Different *HLA-DRB1* Alleles

To explore if this difference in *HLA-DRB1* gene expression could also be seen in whole blood samples of healthy individuals, we used RNA-seq data from 439 whole blood samples from the

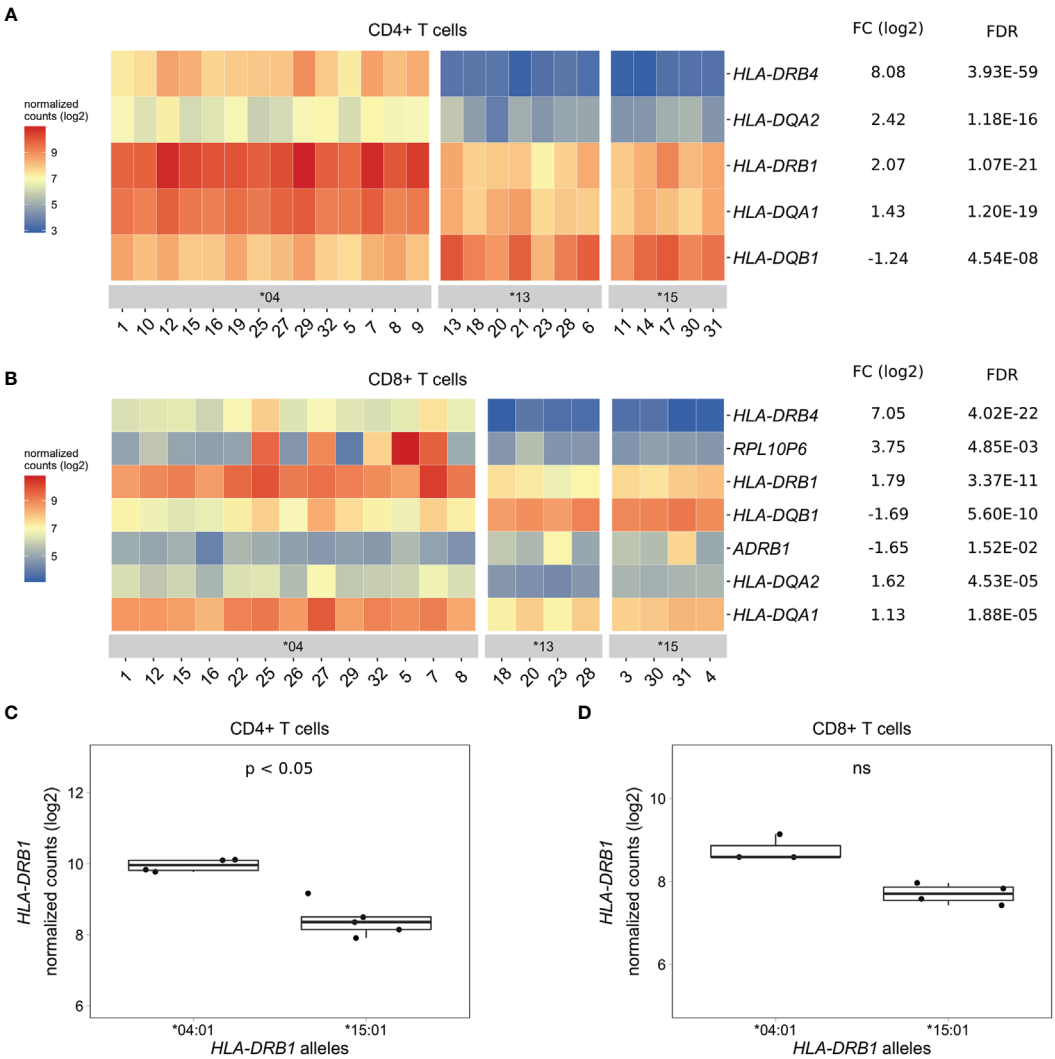

FIGURE 3 | Differentially expressed genes in CD4+ and CD8+ T cells of *HLA-DRB1* SE-positive *versus* SE-negative healthy individuals. **(A)** Heat map of differentially expressed genes (log transformed normalized gene counts) in CD4+ T cells of *HLA-DRB1* SE-positive [*04 (n = 14)] *versus* SE-negative [*03:01/*13:01 and *03:01/*15:01 (n = 12)] individuals. **(B)** Heatmap of differentially expressed genes (log transformed normalized gene counts) in CD8+ T cells of *HLA-DRB1* SE-positive [*04 (n = 13)] *versus* SE-negative [*03:01/*13:01 and *03:01/*15:01 (n = 8)] individuals. In both heatmaps, genes shown in red have higher gene counts and those shown in blue have lower gene counts. **(C)** *HLA-DRB1* gene expression in CD4+ T cells of *HLA-DRB1* SE-positive [*03:01/*04:01 (n = 4)] and SE-negative [*03:01/*15:01 (n = 5)] individuals. **(D)** *HLA-DRB1* gene expression in CD8+ T cells of *HLA-DRB1* SE-positive [*03:01/*04:01 (n = 3)] and SE-negative [*03:01/*15:01 (n = 4)] individuals. FC, fold change; ns, non-significant adjusted *P*-value (FDR).

GTEx project (14). Classical HLA alleles were imputed from RNA-seq data and samples with *HLA-DRB1**03, *04, *07 and *15 alleles were selected for mapping reads to the MHC region to avoid alignment biases. Our data show that *HLA-DRB1* is significantly higher expressed in *HLA-DRB1* SE-positive individuals (carrying at least one *HLA-DRB1**04 allele) compared to *HLA-DRB1* SE-negative individuals [carrying no *HLA-DRB1**04 alleles (**Figure 5A**)]. In addition, we examined the expression of *HLA-DRB1* in individuals carrying different *HLA-DRB1* alleles and determined that the expression of *HLA-DRB1* is strongly dependent on the presence of different *HLA-DRB1* alleles (**Figure 5B**). Within whole blood samples from the GTEx project, there is a trend towards higher expression levels of *HLA-DRB1* in *HLA-DRB1**04 and *07 alleles compared to *HLA-DRB1**03 and *15 alleles.

HLA-DRB1 Is Differentially Expressed in Whole Blood Samples of RA Patients Carrying Different *HLA-DRB1* Alleles

To analyze *HLA-DRB1* expression levels in RA patients carrying different *HLA-DRB1* alleles, we used RNA-seq data from 158 whole blood samples from the EIRA/RECOMBINE project.

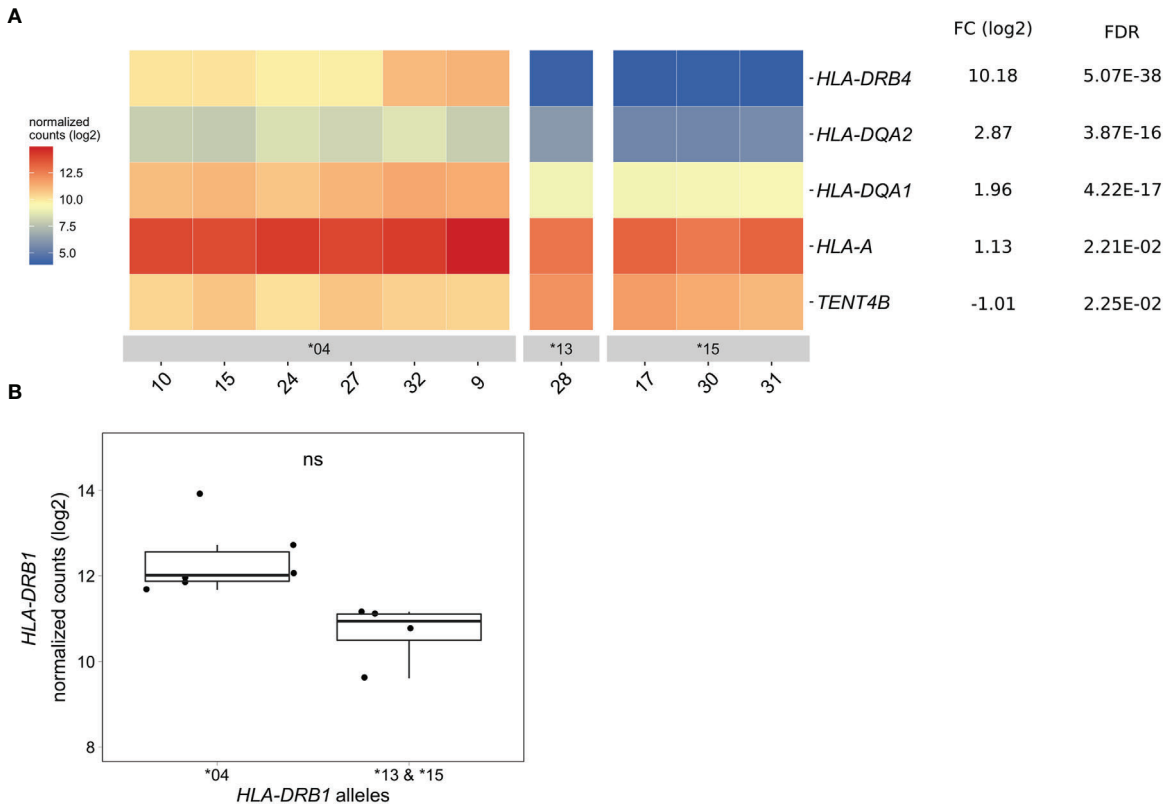

FIGURE 4 | Differentially expressed genes in CD14+ monocytes of *HLA-DRB1* SE-positive *versus* SE-negative healthy individuals. **(A)** Heat map of differentially expressed genes (log transformed normalized gene counts) in CD14+ monocytes of *HLA-DRB1* SE-positive [*04 (*n* = 6)] and SE-negative [*03:01/*13:01 and *03:01/*15:01 (*n* = 4)] individuals. Genes shown in red have higher gene counts and those shown in blue have lower gene counts for the contrast SE+ *vs.* SE-. **(B)** *HLA-DRB1* gene expression in CD14+ monocytes of *HLA-DRB1* SE-positive [*04 (*n* = 6)] and SE-negative [*03:01/*13:01 and *03:01/*15:01 (*n* = 4)] individuals. FC, fold change; ns, non-significant adjusted *P*-value (FDR).

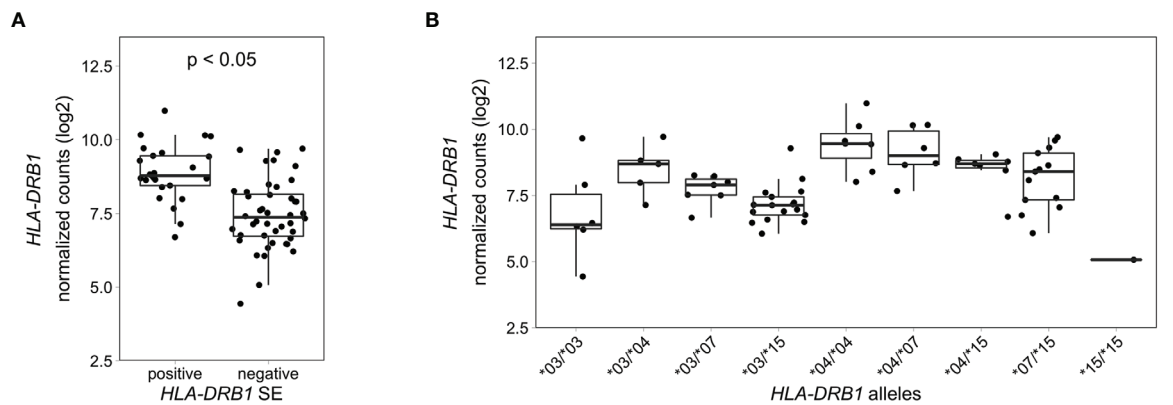

FIGURE 5 | *HLA-DRB1* expression levels in whole blood samples of healthy individuals of the GTEx project. **(A)** *HLA-DRB1* gene expression in whole blood samples of *HLA-DRB1* SE-positive [*04 (*n* = 25)] and SE-negative [*03, *07, and *15 (*n* = 44)] individuals. **(B)** *HLA-DRB1* gene expression in whole blood samples of individuals carrying different combinations of *HLA-DRB1* alleles [*03/*03 (*n* = 6), *03/*04 (*n* = 5), *03/*07 (*n* = 7), *03/*15 (*n* = 17), *04/*04 (*n* = 7), *04/*07 (*n* = 6), *04/*15 (*n* = 7), *07/*15 (*n* = 13), and *15/*15 (*n* = 1)]. P, adjusted *P*-value (FDR).

Classical HLA alleles were imputed from RNA-seq data and samples with *HLA-DRB1**03, *04, *07 and *15 alleles were selected for mapping reads to the MHC region. Also in these samples, *HLA-DRB1* is significantly higher expressed in *HLA-DRB1* SE-positive RA patients (carrying at least one *HLA-DRB1**04 allele) compared to *HLA-DRB1* SE-negative RA patients (carrying no *HLA-DRB1**04 alleles) (**Figure 6A**). In addition, we examined the expression of *HLA-DRB1* in RA patients carrying different *HLA-DRB1* alleles and showed that the expression of *HLA-DRB1* is strongly associated with *HLA-DRB1* alleles (**Figure 6B**). In whole blood samples of RA patients, there is a trend towards higher expression levels of *HLA-DRB1* in *HLA-DRB1**04 and *07 alleles compared to *HLA-DRB1**03 and *15 alleles.

DISCUSSION

The major finding of our study is the identification of relatively high differences in gene expression levels for several MHC Class II genes in immune cells of healthy individuals depending on MHC haplotypes, which are known as genetic risk factors for autoimmune diseases. More specifically, we found that the gene for the HLA-DR beta chain is expressed higher in several types of immune cells with the RA-associated haplotype in comparison to RA-irrelevant haplotypes. By pre-selection of individuals with specific haplotypes in our study, we were able to decrease the level of heterogeneity and by accurate and haplotype-specific alignment to different MHC reference sequences, we were able to reliably identify expression levels of these genes. Our results provide for the first time evidence for differential expression of several MHC Class II genes in whole blood, PBMCs, CD4+ T cells, CD8+ T cells, and CD14+ monocytes of individuals with genetic predisposition to an autoimmune disease, i.e. RA.

The MHC is an extremely polymorphic region and quantification of expression of various allelic forms causes major issues for standard mapping methods and for studying expression of these genes. Using the standard human transcriptome reference, most of the sequencing reads will misalign to the MHC Class II locus. This could be recognized by for example *HLA-DRB5* expression within individuals carrying *HLA-DRB1**04 alleles, where *HLA-DRB5* is not present (**Figures 1** and **S4**). The sequencing reads should align to *HLA-DRB4* but instead align to the most similar gene on the standard human transcriptome reference which is *HLA-DRB5*. In addition, the expression of other MHC class II genes, including *HLA-DRB1*, are also influenced by this (**Figure S4**). Here, we used the AltHapAlignR pipeline (8) for a more accurate and reliable expression analysis within the MHC region, which is especially important for the MHC Class II locus. It employs the eight available MHC reference haplotypes (APD, COX, DBB, MANN, MCF, PGF, QBL, and SSTO (24)) to generate less biased estimates of gene expression from this locus. However, the number of MHC reference haplotypes is still limited and obviously does not cover all patterns of MHC variations in the human genome. The standard MHC reference haplotype is PGF (*DRB1**15:01:01-*DQA1**01:02:01-*DQB1**06:02). The MHC reference haplotypes COX and QBL contain the *HLA-DRB1**03:01:01 allele (*DRB1**03:01:01-*DQA1**05:01:01-*DQB1**02:01:01), DBB and MANN contain the *HLA-DRB1**07:01:01 allele (*DRB1**07:01:01-*DQA1**02:01-*DQB1**03:03:02 or *DQB1**02:02, respectively), and SSTO contains the *HLA-DRB1**04:03:01 allele (*DRB1**04:03:01-*DQA1**03:01:01-*DQB1**03:05:01). In addition, *HLA-DRB1* is technically not present on the current versions of the MHC reference haplotypes APD (*HLA-DRB1**13:01) and MCF [*HLA-DRB1**04:01 (GRCh38.p12)]. Therefore, our findings showing that *HLA-DRB1* is lower expressed in *HLA-DRB1**13:01 individuals compared to *HLA-DRB1**04:01 and *15:01 are inconclusive. Furthermore, in our study the *HLA-DRB1**04:01 samples are mapping to the closest matched MHC reference haplotype SSTO (*HLA-DRB1**04:03:01), which could potentially decrease efficiency of alignment in the MHC Class II locus towards a lower number of mapped sequencing reads. In this

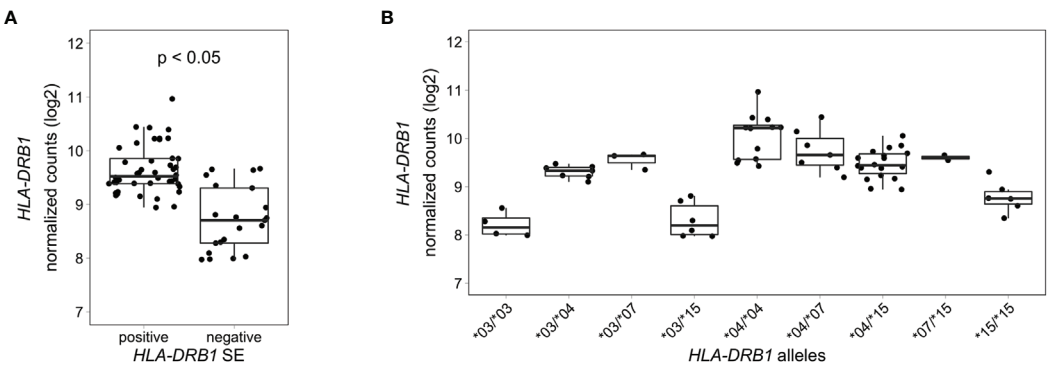

FIGURE 6 | *HLA-DRB1* expression levels in whole blood samples of RA patients. **(A)** *HLA-DRB1* gene expression in whole blood samples of *HLA-DRB1* SE-positive [*04 (*n* = 44)] and SE-negative [*03, *07, and *15 (*n* = 21)] RA patients. **(B)** *HLA-DRB1* gene expression in whole blood samples of RA patients carrying different combinations of *HLA-DRB1* alleles [*03/*03 (*n* = 4), *03/*04 (*n* = 7), *03/*07 (*n* = 3), *03/*15 (*n* = 6), *04/*04 (*n* = 12), *04/*07 (*n* = 7), *04/*15 (*n* = 18), *07/*15 (*n* = 2), and *15/*15 (*n* = 6)]. P, adjusted *P*-value (FDR).

case, the detected level of *HLA-DRB1* expression for individuals with *HLA-DRB1**04:01 will be underestimated. The expression of *HLA-DRB4* gene, which is present at the *HLA-DRB1**04 haplotype and absent at *HLA-DRB1**03, *13, *15 haplotypes, was expected to be highly different between individuals carrying *HLA-DRB1* SE-positive and SE-negative alleles. Even though there are several possible issues with the MHC reference haplotypes, our data shows that the expression of *HLA-DRB* and *HLA-DQ* genes is different between individuals carrying *HLA-DRB1**04:01 and *15:01 alleles in different cell types. Moreover, we found that *HLA-DRB1* tended to be higher expressed in *HLA-DRB1**04 and *07 alleles compared to *HLA-DRB1**03 and *15 alleles in both healthy individuals from the GTEx project and RA patients from EIRA/RECOMBINE study. Importantly, we studied the overall expression of *HLA-DRB1* and did not consider possible transcript heterogeneity due to alternative splicing that may be the source of the difference in expression.

To confirm the results of RNA-seq data, we performed qPCR analysis on the same PBMC and cell subset RNA samples. No primers could be designed to measure expression of the different forms of *HLA-DQA* (*HLA-DQA1* and *HLA-DQA2*) and *HLA-DQB* (*HLA-DQB1* and *HLA-DQB2*) due to high degree of gene homology. Therefore, we measured overall expression of *HLA-DQA* and *HLA-DQB*, and could confirm differences in expression by qPCR (**Figures S1, S2**). In addition, no differences in expression of *HLA-DRA* could be detected by qPCR (**Figure S3**), which is also concordant with our RNA-seq data. No primers can be designed for simultaneous robust measure of expression of *HLA-DRB* in samples with different *HLA-DRB1* alleles because of the high number of variations between the different forms of *HLA-DRB1*, *HLA-DRB3*, *HLA-DRB4* and *HLA-DRB5*. Therefore the expression of *HLA-DRB* was not determined by qPCR in our study.

Using RNA-seq, we found *HLA-DRB1* and other MHC Class II genes to be differentially expressed in CD4+ T cells of healthy individuals carrying *HLA-DRB1* SE-positive *versus* SE-negative alleles. Although there are CD4+ T-cell subsets expressing HLA-DR (25–27), by using xCell (22), a tool that performs cell type enrichment analysis from RNA-seq data, we noticed that the suspensions of CD4+ T cells in our experiment are slightly contaminated with monocytes (**Figure S5**). Monocytes express *HLA-DRB1* at a significantly higher level than CD4+ T cells and therefore we cannot totally exclude that the observed differences in the CD4+ T-cell subset are caused by the monocyte contamination.

HLA-DR is present on the surface of cells as heterodimers consisting of an alpha chain (HLA-DRA) and a beta chain (HLA-DRB1 together with either HLA-DRB3, HLA-DRB4 or HLA-DRB5, depending on the haplotype). Increased expression of HLA-DR is considered to be an activation marker on different cell types. Therefore, we correlated *HLA-DRB1* expression levels with the expression of other activation markers of different cell types (*CD25*, *CD38*, *CD69*, *CD86*, *CD40*, and *CD63*). We found no correlation with any of these activation markers (**Figures S6–S9**), suggesting that the higher expression of *HLA-DRB1* in samples with *HLA-DRB1**04 alleles in our study cannot be due to uncontrolled cell activation.

Our study shows that HLA-DR is not only expressed in professional antigen presenting cells, such as CD14+ monocytes, but also in CD4+ and CD8+ T cells. Molecular mechanisms of differences in gene expression levels for *HLA-DRB1* in these immune cells of healthy individuals and RA patients depending on MHC haplotypes are not addressed in this study. Several studies have suggested that the levels of *HLA-DRB1* might be regulated by DNA methylation (28–30), which might be distinctive for different haplotypes. They show that hypomethylation of the *HLA-DRB1* promoter, which leads to higher expression of *HLA-DRB1*, is involved in the pathogenesis of for example multiple sclerosis. In addition, there is evidence that the expression of *HLA-DRB1* is regulated by super enhancers located in the MHC region between the genes *HLA-DRB1* and *HLA-DQA1* (31–33). Histone modifications in these enhancers are associated to differential 3D chromatin conformation and gene expression of HLA genes, and more importantly, there seem to be differences in individuals carrying different MHC haplotypes (34). The super enhancers were found to regulate HLA-DR and HLA-DQ protein levels as well (32, 33). This suggests that not only differences at *HLA-DRB* and *HLA-DQ* gene expression level can be observed in individuals with different MHC haplotypes, but also at protein level. However, studies identifying functional consequences of higher gene expression levels of *HLA-DRB1* in *HLA-DRB1**04 individuals are still pending.

Using the available MHC reference haplotypes, we were able to identify by RNA-seq that *HLA-DRB* and *HLA-DQ* genes are differentially expressed in whole blood samples, PBMCs, CD4+ T cells, CD8+ T cells, and CD14+ monocytes of *HLA-DRB1* SE-positive *versus* SE-negative healthy individuals. In addition, we identified that *HLA-DRB1* is also differentially expressed in whole blood samples of *HLA-DRB1* SE-positive *versus* SE-negative RA patients. While MHC Class II genes consistently demonstrate differential expression between *HLA-DRB1* SE-positive and SE-negative healthy individuals, we found little difference for expression of non-MHC genes. In conclusion, our study shows that not only structural differences but also differences in expression of MHC Class II molecules in different immune cells may explain the relationships between immunity and autoimmune disease and presence of certain MHC Class II alleles in an individual.

DATA AVAILABILITY STATEMENT

All sequencing data generated in this study are available at NCBI Gene Expression Omnibus accession number GSE163605.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional Ethical Review Board in Uppsala. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MH: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing – Original Draft, Visualization. EH: Investigation, Writing – Review & Editing. LR: Resources, Writing – Review & Editing. LK: Conceptualization, Writing – Review & Editing. VM: Writing – Review & Editing. LP: Conceptualization, Methodology, Data Curation, Writing – Review & Editing, Supervision, Project administration, Funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIALS

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.707217/full#supplementary-material

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TRIM21/Ro52 - Roles in Innate Immunity and Autoimmune Disease

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TRIM21 (Ro52/SSA1) is an E3 ubiquitin ligase with key roles in immune host defence, signal transduction, and possibly cell cycle regulation. It is also an autoantibody target in Sjögren's syndrome, systemic lupus erythematosus, and other rheumatic autoimmune diseases. Here, we summarise the structure and function of this enzyme, its roles in innate immunity, adaptive immunity and cellular homeostasis, the pathogenesis of autoimmunity against TRIM21, and the potential impacts of autoantibodies to this intracellular protein.

Keywords: innate immunity, E3 ubiquitin ligase, autoimmune disease, Sjogren's syndrome, Fc receptor, systemic lupus erythematosus, intracellular antibodies

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AUTOIMMUNE DISEASES ASSOCIATED WITH TRIM21

The immune system is a balanced network of interacting cells which distinguish between self and non-self to effectively respond to invading pathogens. Failures in these peripheral and central tolerance mechanisms can lead to immune cells reacting to self-antigens, causing the extensive inflammation and tissue damage observed in autoimmune diseases (1).

TRIM21 (also called Ro52) autoantibodies have been detected in at least thirteen autoimmune diseases, with frequencies of detection in patients ranging from 5% to 95% (2, 3). The most common TRIM21-associated autoimmune diseases are systemic lupus erythematosus (SLE) affecting the central nervous system, skin, kidneys and joints, and Sjögren's syndrome (SS), which primarily affects the tear and salivary glands (4, 5). Beyond SS and SLE, anti-TRIM21 antibodies have also been identified in patients with primary biliary cirrhosis, idiopathic inflammatory myopathies (mainly polymyositis and dermatomyositis), and infants with congenital heart block (CHB) associated with maternal autoantibody transfer (6–9). In fact, for primary Sjögren's syndrome (pSS), serological detection of anti-TRIM21 antibodies is a diagnostic criterion, with detection rates in patients ranging from 50-70% according to assay method (10, 11).

For TRIM21-associated SS, single nucleotide polymorphism (SNP) and genome-wide association (GWAS) studies have identified polymorphisms in HLA, interferon regulatory factor-5 (IRF5) (suggesting TRIM21 is an interferon-stimulated gene; see below), and B cell activating factor (BAFF) loci (12, 13). For example, it is postulated that specific gene polymorphisms, including a CGGGG indel repeat in the IRF5 gene promoter, can alter IRF5 mRNA expression. This specific repeat may act as a binding site for transcription factor SP1, driving chronic type I interferon (IFN-I) proinflammatory cytokine production (14, 15).

Despite the identification of genetic risk factors (e.g. the influence of SNPs in autoantibody induction) and some of the cellular interactions involved, antigen-specific mechanisms which initiate and drive autoimmune pathologies remain poorly understood. Analysis of peripheral blood mononuclear cells (PBMCs) isolated from SLE and SS patients provides evidence of elevated

TRIM21 transcript expression; however, questions remain as to whether TRIM21 is a key autoantigen driving B-cell activation, autoantibody production and autoimmune pathogenesis in these diseases (16).

TRIM PROTEIN FAMILY

Comprising more than 80 members, the TRIM protein family is a group of E3 ubiquitin ligases with roles in multiple cellular processes including cell cycle regulation, autophagy and innate immunity (17, 18). They have a conserved multidomain architecture, exclusively found in metazoans (both vertebrate and invertebrate species), consisting of the tripartite motif (N-terminal Really Interesting New Gene (RING) domain, B-box domain and coiled-coil domain) that may be associated with a variable C-terminal domain.

Specific C-terminal domains mediate substrate recognition, localisation within the cell and are used to classify TRIM proteins into sub-families according to function (19). This is illustrated by the C-terminal subgroup One Signature domain which enables localisation of TRIM18 (MID1) to microtubules for polyubiquitination and proteasomal degradation of the protein phosphatase 2A catalytic subunit (PP2Ac) (20, 21). Involved in multiple signalling pathways, PP2Ac regulates the mammalian Target of Rapamycin Complex 1 (mTORC1) pathway which controls tumour cell growth and metabolism, intracellular transport, cell migration, autophagy and cell cycle dynamics. When active, TRIM18 polyubiquitinates PP2Ac leading to its degradation, thus inhibiting mTORC1 complex formation to prevent downstream signalling (22). Therefore, it is unsurprising that mutations that alter or inhibit TRIM protein functions have complex downstream effects.

Other family members contain single or combinations of the following C-terminal domains; fibronectin type 3, plant homeodomain, transmembrane, ADP ribosylation factor-like, MATH (meprin and tumour-necrosis factor receptor (TNFR)-associated factor (TRAF) homology), filamin-type immunoglobulin, NHL repeats, or most commonly, a PRY/SPRY domain (23). Given the number of C-terminal domains and proteins in this family, it is conceivable that there is little or no functional redundancy between family members, yet this remains to be confirmed.

TRIM21 STRUCTURE

TRIM21 consists of an N-terminal RING domain, B-box domain, central coiled-coil domain (anticipated based on TRIM family homology) and terminal PRY/SPRY domain (24). The full crystal structure of TRIM21 is yet to be solved. However, TRIM21's structure can be predicted by combining confirmed structures, binding kinetics and homology. The coiled-coil domain is believed to mediate TRIM21 homodimerization. This enables the dimer's two PRY/SPRY domains to form a high-affinity binding pocket for immunoglobulin Fc domains, **Figure 1** (25, 27).

RING Domain

The RING domain is characterised by a series of conserved cysteine and histidine residues (28). These residues coordinate zinc atom binding, enabling the folding of an E3 ubiquitin ligase domain. The E3 zinc finger motifs mediate interactions with ubiquitin-bound E2 enzymes, catalysing the transfer of ubiquitin to target proteins (29, 30). Ubiquitin transfer involves the formation of lysine-specific, covalent polyubiquitin chains, which determine specific cellular activities. For example, proteins modified with Lysine 48 (K48)-specific ubiquitin chains are targeted for proteasomal degradation, whilst K63-specific chains regulate signalling pathways including DNA repair and inflammatory signalling (31).

A specific mechanism for self-anchored TRIM21 ubiquitin transfer has been recently uncovered, showing the RING domain acts as both substrate and catalyst for ubiquitination. Following mono-ubiquitination at the N-terminus (RING domain) by the E2 enzyme Ube2W, TRIM21 is activated. This ubiquitin-priming promotes recruitment of an E2 Ube2N/Ube2V2 heterodimer during K63-specific ubiquitination. Two dimerised RING domains hold the E2-Ub heterodimer in place, catalysing the transfer of K63-linked ubiquitin chains to a third acceptor mono-ubiquitinated RING, during self-anchored *trans*-ubiquitination. Such K63-linked ubiquitination is required for targeting the TRIM21-protein (e.g. antibody) complex for degradation (32).

The RING-domain of TRIM21 also mediates K48-linked ubiquitination. This was demonstrated by the ubiquitination and degradation of DEAD-box protein DDX41, an intracellular dsDNA sensor in myeloid dendritic cells (DCs) and monocytes (33). Binding to DNA *via* the DEAD domain, DDX41 activates the STING pathway for proinflammatory IFN-I stimulation (34). By targeting DDX41 for degradation, TRIM21-mediated DDX41 ubiquitination inhibits the IFN-I response to dsDNA. IFN-I responses to self-DNA are central in SLE pathogenesis (35). It is seemingly contradictory that such IFN-I responses are elevated in SLE patients when TRIM21 mRNA expression is also increased, as one would expect greater DDX41 ubiquitin-mediated degradation (16). This discrepancy may suggest inherent TRIM21 functions in downregulating DNA sensors such as DDX41 are impaired, by some yet unknown mechanism.

B-Box Domain

The B-box domain is less well-characterised but may have distinct functions in different TRIM family members. It may help coordinate TRIM self-association, may contain a zinc finger motif, in some TRIM proteins might confer E3 ubiquitin ligase activity, or in others has a regulatory role (26, 36, 37).

The B-box domain can mediate higher-order complex formation (38). This was demonstrated for TRIM5α which recognizes and binds to capsids of multiple retroviruses including HIV-1 (39). TRIM5α spontaneously assembles into a hexagonal lattice *via* hydrophobic interactions utilizing a key Arg residue located at the B-box2 domain (40). This hexagonal array mimics that of the target viral capsids and enables efficient binding of the SPRY domains to multiple capsid sites for

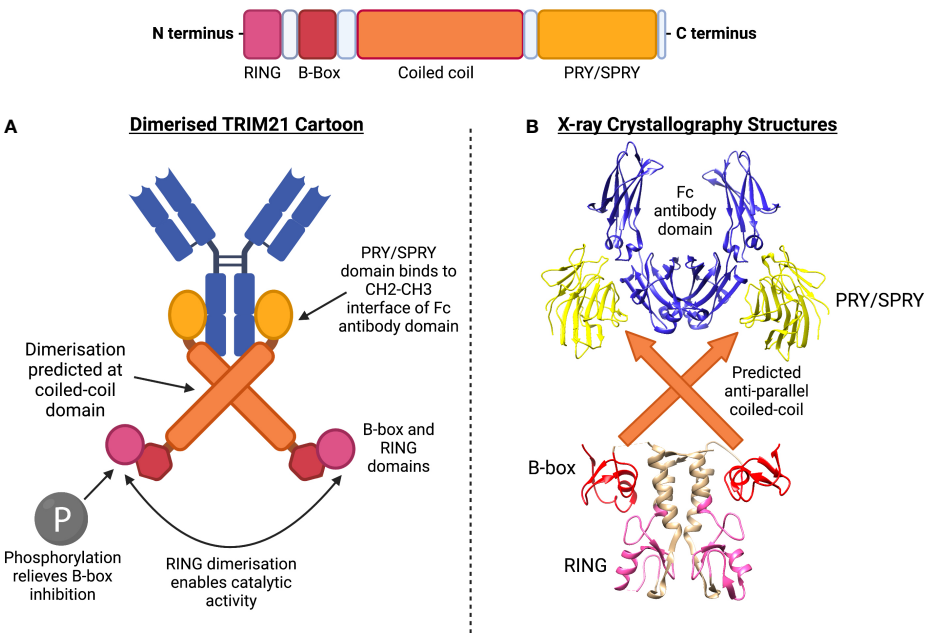

FIGURE 1 | TRIM21 structure includes RING, B-box, coiled-coil and PRY/SPRY domains. **(A)** Dimerised TRIM21 cartoon shows binding of PRY/SPRY domain at constant region (Fc) of antibody. Dimerisation at the coiled-coil is predicted according to homology to other TRIM family members. Phosphorylation of the LxxIS motif of the RING domain relieves B-box inhibition, allowing RING dimerisation for catalytic activity. **(B)** X-ray crystallography structures have been obtained for the (1) PRY/SPRY domain, identified in complex with the Fc antibody domain (PDB:2IWG) (25). (2) The B-box and RING domains have been crystallised in a dimer confirmation (PDB:5OLM) (26). The central predicted coiled-coil structure has not been formally identified.

subsequent ubiquitination and degradation during antiretroviral defense (39).

Despite also having a B-box2 domain, TRIM21 does not form higher-order assemblies. Instead, TRIM21's B-box domain has a regulatory role *via* interactions with the RING domain (41). Whilst most TRIM proteins are constitutively active, X-ray crystallography has shown that the TRIM21 B-box domain represses its ubiquitin ligase activity by occupying the E2 binding site. Overexpression of kinases IKKβ or TBK1 led to phosphorylation of a LxxIS motif in the RING domain and was sufficient to relieve B-box inhibition (26). These findings may be of interest in an autoimmunity context, as disruption of this autoinhibition mechanism could contribute to excessive proinflammatory signalling. Polymorphisms located in regions encoding the LxxIS motif of TRIM21 have not yet been identified and in general, GWAS studies relating to TRIM21 in autoimmunity are limited. However, one SS patient study identified polymorphisms in multiple coding regions, with certain SNPs correlating with the presence of anti-TRIM21 antibodies (42). Therefore, GWAS studies should be expanded to identify additional polymorphisms associated with TRIM21, including those potentially encoding the LxxIS motif.

Coiled-Coil

The central coiled-coil domain of TRIM21 is predicted to be an α-helical, supercoiled structure involved in dimerisation and TRIM self-association, **Figure 1** (43). Except for TRIM19 which forms a torus-shaped homo-tetramer, TRIM proteins which associate *via* coiled-coil domains exist either as monomers, homodimers or in monomer: homodimer equilibria, with hetero-dimerisation being uncommon (44, 45). TRIM homodimers (and possibly higher-order oligomers) formed by anti-parallel coiled-coils also allow dimerisation at the RING domain (46, 47).

Studies have shown dimerisation of the catalytic RING domains to be necessary for ubiquitinating activity in most TRIM proteins studied so far (48). This holds true for TRIM21, with a recent study demonstrating that dimerisation at TRIM21's coiled-coil directly enabled catalytic activity (49). Mutation of residues at the antiparallel coiled-coil dimer interface was sufficient to inhibit auto-ubiquitination of the RING domain, following infection of HEK293T cells with antibody-coated adenovirus. Furthermore, forced RING domain dimerisation (*via* RING-linker-RING constructs) increased ubiquitin discharge activity. A proposed model of "clustering-induced activation" suggests dimerisation of RING domains makes E2-ubiquitin engagement with TRIM21 and subsequent polyubiquitin discharge more energetically favourable (49).

C-Terminal PRY/SPRY Domain

The variable C-terminal region of TRIM family proteins is involved in protein-protein interaction and subcellular localisation, with TRIM21 containing a PRY/SPRY C-terminal domain, **Figure 1** (25, 45).

The specificity and roles of PRY/SPRY domains have been demonstrated in a number of studies (50). Domain-swapping experiments show that TRIM5α's PRY/SPRY domain is essential for viral restriction (51). Mutations in TRIM18, most often in the PRY/SPRY domain, alter TRIM18's subcellular distribution and cause X-linked Opitz Syndrome (52, 53). Furthermore, PRY/SPRY mutations in TRIM20 cause Familial Mediterranean Fever (54), and in TRIM36 cause anencephaly (55).

For TRIM21, the PRY/SPRY domain contains a high-affinity immunoglobulin Fc binding site, the structure of which has been solved, **Figure 1** (25). TRIM21's PRY/SPRY domain binds to the CH2-CH3 interface of the Fc, and does not overlap with the Fc domain's binding sites for FcγR and C1q. The CH2-CH3 interface is highly conserved, allowing TRIM21 binding to 98% of circulating immunoglobulins regardless of their antigen specificity (25).

Human TRIM21 displays promiscuous antibody interactions, binding to all four human IgG subclasses and even binding IgGs from different mammalian species in a 2:1 binding ratio (TRIM21: antibody Fc) (56). TRIM21 also binds both IgA (Kd: 54 μM) and IgM (Kd: 17 μM) (57, 58). These affinities are substantially lower than that of IgG binding, which has been measured at Kd: 37 nM for TRIM21-Fc fragment interactions (25). Importantly, except for IgG binding to its cognate high-affinity FcγRI (Kd: 4.2 nm), most FcRs bind IgG Fc with lower-affinity binding in the micromolar range (59, 60). Therefore, the nanomolar TRIM21-Fc binding highlights the strength and high affinity of IgG for this intracellular receptor. Whether TRIM21 can bind to IgE or IgD remains to be investigated (24, 25).

The TRIM21 PRY/SPRY domain is also subject to regulation by acetylation. Histone deacetylase 6 (HDAC6) interacts with TRIM21 *via* the PRY/SPRY domain, where it deacetylases TRIM21 at K385 and K387, promoting TRIM21 homodimerization. When HDAC6 is inhibited, TRIM21 remains hyperacetylated, preventing TRIM21 dimerisation, thus impairing its enzymatic activity (61).

TRIM21 ANTI-PATHOGEN ACTIVITY

Antibodies extracellularly neutralise antigens, preventing targeted pathogens from penetrating cell membranes. This occurs either directly or indirectly through opsonisation and/or complement system activation, whilst antibody-mediated neutralisation within intracellular compartments was thought unlikely due to membrane exclusion (62). Extracellular neutralisation is not always possible, as many neutralising epitopes are shielded, for example by glycans (63).

In vivo studies have demonstrated that even in the presence of saturating concentrations of neutralising antibody, TRIM21-deficient mice are highly susceptible to mouse adenovirus 1 infection. In contrast, wild-type mice upregulate TRIM21, thus controlling viraemia. Interestingly, *Trim21*$^{+/-}$ heterozygous mice display an intermediate phenotype, leading to an increased viral load but lower than that of *Trim21*$^{-/-}$ mice, suggesting that TRIM21 levels directly influence the efficiency of antiviral defence (64).

Intracellular Antibody Receptor

When bound to non-neutralising antibodies, intracellular pathogens including viruses, bacteria and parasites, may be rapidly sensed by cytosolic TRIM21 (64–67). The mechanisms by which antibody-bound pathogens access the cytoplasm vary and are pathogen-specific (68). Some enveloped viruses, such as the α-herpesvirus HSV, deliver their capsids directly to the cytosol by fusing their envelope with the plasma membrane (69). Others use endocytosis pathways such as clathrin-mediated endocytosis, micropinocytosis or lipid raft-mediated endocytosis (70). Multiple bacterial species use caveolin-mediated endocytosis to enter cells, including *E. coli*, *C. jejuni*, *S. typhimurium*, and *P. aeruginosa*, as have a number of viruses, such as SV40 (71). This mechanism might be preferred for pathogens, as unlike clathrin-coated pit entry, caveolae-internalized bacteria may avoid lysosomal degradation (72).

TRIM21 binds internalised antibody-coated pathogens within the cell *via* PRY/SPRY-Fc interactions. However, why and how antibody-coated pathogens are available for binding in the cytosol, remains largely unsolved. TRIM21 recruitment may be limited to non-enveloped DNA and RNA-viruses that enter the cytosol with attached immunoglobulins. McEwan et al. showed that the enveloped Respiratory Syncytial virus shed attached antibodies upon entry and did not activate TRIM21, whereas non-enveloped feline calicivirus did (73). Additionally, TRIM21 can detect picornavirus HRV14, which promotes the lysis of endosomal membranes for cytosolic virion release, whilst it cannot not detect HRV2, which delivers its genome into the cytoplasm through a pore (74).

TRIM21 facilitates antibody-dependent intracellular neutralisation of human adenovirus type 5 (Ad5). Ad5 bound to as few as 1.6 antibody molecules recruits TRIM21 in the cytosol and is degraded in a TRIM21-dependent manner. This involves TRIM21 autoubiquitination and possibly ubiquitin transfer also to the TRIM21-associated viral particle prior to proteasomal degradation, **Figure 2** (58, 67). Adenoviral capsids can be released from early endosomes into the cytosol during "endosomal escape" (75). This stepwise endosomal rupture process is promoted after initial engagement of Ad5 with coxsackievirus adenovirus receptor (CAR) and coreceptor αv integrin at the host cell membrane (76). Interaction with these receptors may promote early capsid disassembly, occurring just as Ad5 enters the cell membrane (77, 78). Disassembly leads to the exposure of protein VI, a lytic factor which disrupts the endosomal membrane, releasing the capsid into the cytosol for subsequent TRIM21 engagement, **Figure 2** (58, 79).

TRIM21 also localises with antibody-bound *S. typhimurium* in the cytosol, **Figure 2** (73). Normally within host cells, *S. typhimurium* grows within Salmonella-containing vacuoles (SCVs) which protect the bacteria from antibacterial responses (80). However, mutant *S. typhimurium* defective in SifA, an effector translocated by type III secretion systems, have been identified. ΔSifA *S. typhimurium* cannot maintain SCV membrane integrity due to an absence of lysosomal membrane glycoproteins (lgps) and vacuolar ATPase (vATPase), necessary for membrane fusion during bacterial replication. Therefore,

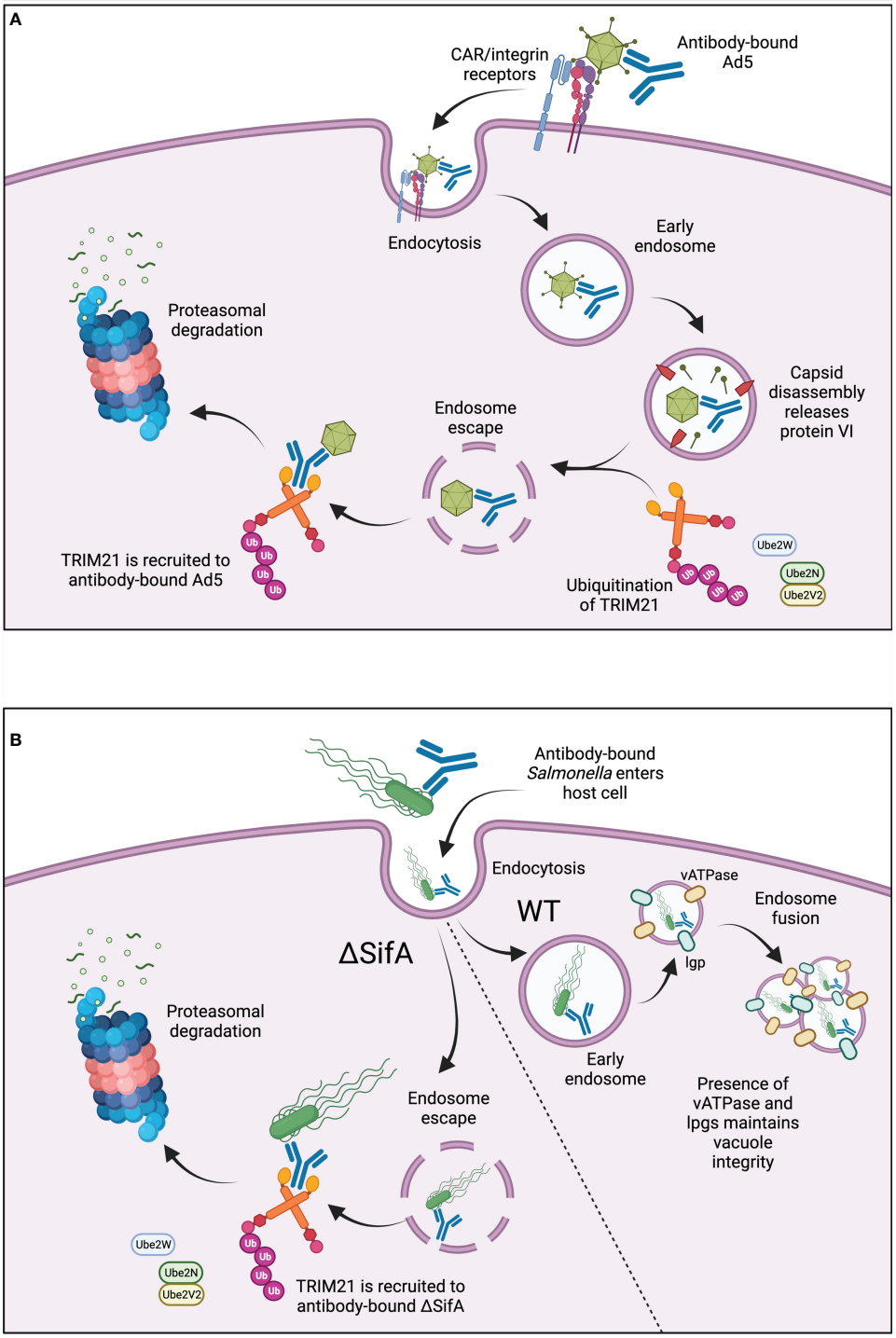

FIGURE 2 | **(A)** TRIM21 in antiviral responses. TRIM21 is recruited to cytosolic antibody-bound-Ad5 after virus entry into the cell. Ad5 binds to CAR/αv integrin receptors at the cell surface, triggering capsid disassembly upon cell entry. Lytic protein VI is released leading to endosomal disassembly and "endosomal escape" of the antibody-bound-Ad5. Active, ubiquitinated TRIM21 binds to the Fc region of the antibody and promotes subsequent proteasomal degradation. **(B)** TRIM21 in responses to intracellular bacteria. TRIM21 is recruited to antibody-coated SifA-mutant *S. typhimurium*. Wild-type *S. typhimurium* are protected from TRIM21-mediated degradation due to the presence of vATPase and lgps, which maintain SCV integrity and enable endosome fusion for bacterial replication. ΔSifA bacteria cannot maintain SCV integrity, leading to endosomal escape into the cytosol for TRIM21-mediated degradation.

ΔSifA mutants are released into the cytosol, **Figure 2** (81). Surprisingly, a follow-up study showed that ΔSifA *S. typhimurium* actually replicated more rapidly within the cytosol of epithelial cells compared to those residing within vacuoles, suggesting there may be some bacterial advantage for vacuole-escape (82). TRIM21 has been shown to recognise antibody-bound-ΔSifA mutants more readily than wild-type *S. typhimurium*, leading to greater activation of NF-κB proinflammatory signalling (73). Although the bactericidal effects of this recognition and enhanced signalling were not explored, it does suggest TRIM21 is important for rapid intracellular detection and binding of pathogens which escape into the cytosol, **Figure 2**.

By binding IgM, TRIM21 provides protection from primary infections, and by binding IgG it can protect against secondary infection. *In vivo* challenge protection has been demonstrated using antibodies targeting the viral nucleoprotein of various viruses including influenza, coronaviruses, and lymphocytic choriomeningitis virus (LCMV) (65, 83–89). Nucleoprotein-specific antibodies are non-neutralising, as viral nucleoprotein is an internal virus antigen, and protection does not require the FcγR (90, 91). TRIM21 and anti-nucleoprotein antibodies play a critical role in stimulating nucleoprotein-specific cytotoxic T cells during LCMV infection (65).

Once bound to cytosolic antibody-coated pathogens, TRIM21 induces coordinated effector and signalling responses, both dependent on TRIM21's E3 ubiquitin ligase activity. Upon cellular infection with Ad5-antibody complexes, two independent, concurrent activities occur. Firstly, the effector response results in proteasomal degradation of the virus. Secondly, an intracellular antiviral response is induced *via* innate-immune signalling molecules such as IFN-I (67, 68). Additionally, TRIM21 initiates signalling cascades, activating the transcription factor NF-κB, upregulating more inflammatory cytokines and inducing an antiviral state (73).

Viral Subversion of TRIM21

Some viruses have hijacked TRIM21 to enhance their replication. In the case of severe fever with thrombocytopenia syndrome virus, its non-structural protein (NSs) interacts with and inhibits TRIM21. This prevents the activation of nuclear factor erythroid 2-related factor 2, which is responsible for the expression of a number of antioxidant effectors, thus promoting viral replication and pathogenesis (92). Enterovirus 71 (EV71) is restricted by the host restriction factor SAMHD1. Upon infection, EV71 upregulates TRIM21 in an IFN-dependent manner; TRIM21 interacts with and degrades SAMHD1 through K48-ubiquitination and proteasomal degradation, thus promoting EV71 replication (93). Human papillomavirus presents an alternative method for immune escape by utilising the oncoprotein E7. This recruits TRIM21 to promote the K33-linked ubiquitination and degradation of the host IFI16 inflammasome (94).

Other Interactions

TRIM21 also has indirect effects on innate immunity by promoting pattern recognition receptor (PRR) detection of exposed immunostimulatory ligands that are otherwise shielded by viral capsids. This induces two waves of TRIM21-dependent transcription, the first caused by TRIM21 antigen recognition *via* Fc binding and a later wave following recognition of viral PAMPs by cGAS and STING (74). TRIM21 also interacts through its PRY/SPRY domain with another PRR, MAVS. TRIM21 is upregulated following early detection of host cell viral invasion from Hepatitis C virus, Newcastle disease virus or Sendai virus (SEV). Then its RING domain conjugates K27-linked polyubiquitin onto MAVS, promoting TBK1 binding for subsequent MAVS downstream signalling (95, 96).

Effects of TRIM21 on virus-encoded proteins are less well-understood than those of the IFN pathway. TRIM21 reportedly interacts with hepatitis B virus (HBV) to prevent HBV DNA replication, by promoting the K48-linked ubiquitination and degradation of HBV DNA Pol (97). Additionally, Porcine Epidemic Diarrhoea Virus (PEDV) is inhibited by TRIM21, which targets the nucleoprotein for proteasomal degradation. Conversely, following *in vitro* PEDV infection, the endogenous expression of TRIM21 was downregulated, increasing both PEDV viral titres and nucleoprotein levels (98).

Co-immunoprecipitation has shown TRIM21 to interact with intracellular proteins that regulate antiviral responses. This includes direct interactions with IFN-inducible protein 35 (IFI35) and indirectly, the N-Myc and STAT interactor (NMI). Subsequent K63-linked ubiquitination of NMI stabilises the TRIM21/IFI35/NMI complex and downregulates innate antiviral signalling pathways by inhibiting IFN-I production (99).

TRIM21 – AN INTERFERON STIMULATED GENE

Expression

IFNs bind to cell surface receptors, triggering signalling cascades for downstream transcription of genes involved in innate and adaptive immunity. IFN-α and IFN-β are type I IFNs (IFN-I) and IFN-γ is type II (IFN-II) (100). Early indications that TRIM21 is regulated by IFN signalling were shown by Rhodes et al. In unstimulated HeLa cells, TRIM21 was detected at low concentrations, whilst TRIM21 mRNA was rapidly upregulated in response to IFN-II (101). Furthermore, TRIM21 was upregulated in cultured macrophages and DCs, in response to either influenza virus infection or CpG oligonucleotide TLR9 agonists. TRIM21 induction was dependent on IFN-I signalling, as it was diminished in $IFNAR^{-/-}$ cells (102). IFN-I and to a lesser extent IFN-II, induced TRIM21 mRNA in the mouse T cell line EL4. Promoter-mapping and luciferase-reporter experiments identified an IFN-stimulated response element (ISRE) in the TRIM21 promoter, **Figure 3** (103).

IFN-I stimulation induces phosphorylation of the JAK1 and TYK2 kinases, which in turn phosphorylate STAT1 and STAT2. pSTAT1 and pSTAT2 complex with IRF9, forming the IFN-stimulated gene factor 3 (ISGF3) complex which translocates to the nucleus. ISGF3 activates transcription by binding ISREs in promoters of IFN-stimulated genes, **Figure 3** (104). IFN-II signalling induces JAK1 and JAK2 phosphorylation, leading to STAT1 phosphorylation and the formation of pSTAT1

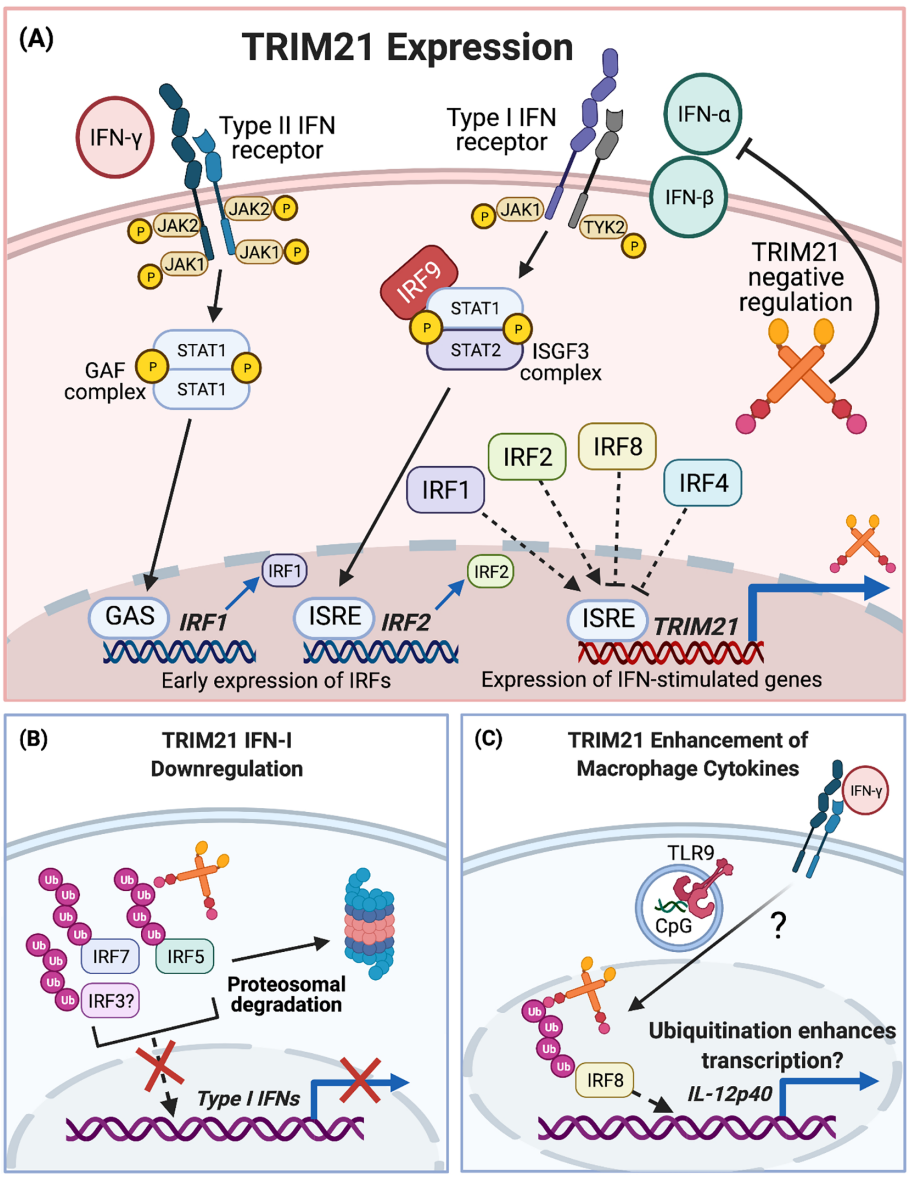

FIGURE 3 | TRIM21 and IFN signalling. **(A)** TRIM21 expression is upregulated by IFN-I and IFN-II signalling. IFN-I signalling involves JAK/STAT phosphorylation and ISGF3 complex formation for early IRF expression. Nuclear translocation of IRF1/2 and binding to the ISRE leads to expression of IFN-stimulated genes including TRIM21. IFN-II signalling also occurs *via* JAK/STAT signalling, leading to formation of the GAF complex. This binds to GAS elements for IRF1 expression and subsequent TRIM21 expression. TRIM21 upregulation is inhibited by IRF4 and IRF8, although much of the upstream signalling pathway remains to be fully elucidated. **(B)** After upregulation, TRIM21 downregulates the IFN-I response *via* ubiquitination of IRF7, IRF5 and possibly IRF3. **(C)** TRIM21 enhances proinflammatory macrophage cytokine expression after IRF8 ubiquitination.

homodimers, referred to as the gamma-activated factor (GAF) complex. GAF translocates to the nucleus, binding to gamma IFN activation sites (GAS) to activate transcription, **Figure 3** (105).

Both IRF1 and IRF2 bind to a *TRIM21* gene ISRE to upregulate TRIM21 expression, **Figure 3** (103, 106). Despite the abrogation of IFN-II-stimulated TRIM21 induction in IRF1$^{-/-}$ macrophages, the TRIM21 locus does not contain a GAS site. This suggests IFN-II stimulates TRIM21 expression by an indirect mechanism involving IRF1. TRIM21 expression was inhibited by IRF4 and IRF8, which bound to the ISRE and blocked IRF1 and IRF2-mediated TRIM21 upregulation, **Figure 3** (103). The roles of non-canonical IFN signal transduction pathways in *TRIM21* gene regulation are not yet known (107).

Negative Regulation

TRIM21 participates in a negative feedback loop to downregulate IFN-I production, modulating the IFN response and inhibiting

further TRIM21 expression, **Figure 3** (108, 109). Downregulation of IFN-I results from TRIM21-mediated IRF7 ubiquitination and subsequent proteasomal degradation, **Figure 3**. In TRIM21-deficient cells, these inhibitory effects were abrogated and the IFN-α4 promoter was overstimulated by IRF7 (109).

Similarly, TRIM21 ubiquitinates IRF5 to mediate its degradation and prevent IRF5-driven IFN-α expression, **Figure 3** (110, 111). Interestingly, one study showed that although TRIM21 interacted with and ubiquitinated all IRF5 isoforms studied, the subsequent effects on protein stability were isoform specific. For example, IRF5 isoforms lacking 48 nucleotides in a central Proline, Glutamic acid, Serine, Threonine-rich (PEST) domain were ubiquitinated by TRIM21 but resistant to subsequent degradation (110). Increased stability of certain isoforms may have relevance in SLE pathogenesis, whereby patients show enhanced expression and alternative splicing of IRF5, due to increased spliceosome activity (112). IRF5 polymorphisms affecting PEST domain expression are associated with elevated risk for SLE and may modulate IFN-I promoter activity. However, it remains unclear whether these isoforms contribute to SLE pathology through increased IFN-I expression (113).

Evidence for IFN-β downregulation following TRIM21-mediated IRF3 degradation, is more conflicting. In a human glial cell line (CHE3), Japanese encephalitis virus infection stimulated IFN-β upregulation *via* IRF3 phosphorylation. TRIM21 overexpression abrogated IFN-β induction, whilst TRIM21 silencing enhanced it (108). These findings agreed with a previous study in HEK293 cells, whereby IRF3-induced IFN-β expression was negatively regulated by TRIM21 following SEV infection, or stimulation by LPS or the TLR3 agonist polyI:C, **Figure 3** (114). In contrast, after SEV infection of HEK293 cells, TRIM21 bound IRF3, blocking Pin1-mediated pIRF3 degradation and enhancing IRF3-stimulated IFN-β expression (115, 116). Reasons for these conflicting results remain unknown and the impact of TRIM21-mediated ubiquitination on the stability of IRF3 is uncertain.

Nuclear Translocation

Studies have also investigated IFN-mediated upregulation of TRIM21 and the downstream effects leading to apoptosis and macrophage cytokine expression (117, 118). Imaging using fluorescent anti-TRIM21 monoclonal antibodies showed that IFN-I stimulation of HeLa cells led to increased cytoplasmic TRIM21 protein levels. After 48 hours of stimulation TRIM21 had undergone nuclear translocation which preceded apoptosis, detected by TUNEL-assays in parallel-treated cultures (117). However, whether TRIM21 nuclear translocation actively induced apoptosis remains to be confirmed. The mechanisms of nuclear translocation, and the functions, if any, of intranuclear TRIM21 are yet to be explored.

Intranuclear roles of TRIM21 have been demonstrated in macrophages following IFN-II and TLR stimulation. IRF8-TRIM21 complexes were identified in nuclear extracts of macrophages stimulated with IFN-II plus TLR9 agonist CpG oligonucleotides (118, 119). This was verified in live macrophages whereby YFP fluorescent signals dependent on IRF8-TRIM21 interactions were detected in the nucleus, also corresponding to increased IRF8 ubiquitination. However, unlike previous reports, this led to enhanced proinflammatory IL-12p40 expression, suggesting that ubiquitination actually enhances IRF8-mediated transcription, **Figure 3** (118). Much of the upstream signalling pathway and how IRF8-ubiquitination enhances IL-12p40 transcription remains unclear. It may be worth confirming whether similar examples of enhanced TRIM21-mediated transcription exist for other proinflammatory gene targets in activated macrophages. These could include promotors requiring coordinated regulation by both IRF8 and IRF1, including SOCS7 for NF-κB signalling, H28 for antigen presentation, c-Myc for cell growth and survival, and CXCL16, which is elevated in SLE patient sera and expressed by macrophages for adhesion, chemotaxis and inducing inflammatory cell infiltration (120, 121).

Importance of Regulation

Inducing IFN-I signalling helps eliminate virus and prevents host morbidity and mortality (122–124). However, IFN levels must be balanced; too much may over-stimulate innate immunity and inflammation, leading to pathological outcomes including autoimmunity. Therefore, signal termination is necessary to prevent an excessive IFN response. TRIM21 controls innate antiviral responses by degrading the transcription factors IRF7 and IRF3 (108, 125, 126). For Influenza A virus, NMI is upregulated, promoting TRIM21 ubiquitination of IRF7 (125), whilst for SEV infection, FoxO1 destabilises IRF3 by promoting TRIM22 or TRIM21-mediated proteasomal degradation, following K-48 ubiquitination (126).

Autoimmunity as a result of aberrations in these fine-tuned signalling pathways has been demonstrated for SS using gene expression array analysis. Compared to healthy controls, PBMCs isolated from SS patients displayed elevated TRIM21 transcript expression, correlating with increased IRF1 and IRF2 mRNA (103). One might expect enhanced ubiquitination of TRIM21-regulated IRFs, leading to greater downregulation of the IFN response, yet this was not the case. Instead, IRF3 and IRF5 protein levels remained high even in the presence of increased TRIM21. This may suggest other signalling pathways contribute to impaired proteasomal degradation and altered IFN signalling homeostasis in autoimmune individuals (127). A clearer understanding of these signalling pathways is needed.

TRIM21 AND DYSREGULATION OF CELLULAR HOMEOSTASIS

In Cancer

TRIM21 downregulation has been implicated in unfavourable outcomes for multiple cancer types including breast cancer, hepatocellular carcinoma, diffuse large B-cell lymphomas (DLBCL) and most recently, colitis-associated cancers (128–131). Lower TRIM21 mRNA expression was correlated with reduced patient survival in DLBCLs and in hepatocellular carcinomas; *in vitro* studies implicated enhanced cell proliferation as a possible cause (129, 130). In liver cancer cell lines, siRNA-mediated TRIM21 silencing abrogated apoptosis,

whilst promoting cellular proliferation and migration in a transwell system (129). Conversely, TRIM21 overexpression in a B cell line reduced proliferation and increased apoptosis compared to control cells, and promoted apoptosis following anti-CD40 treatment (16). Together, these studies suggest TRIM21 has tumour suppressor capabilities, influencing cellular and tissue homeostasis.

Autoantibodies against nuclear and cytoplasmic autoantigens have been detected in cancer patients, with possible causal mechanisms including autoantigen overexpression from tumour cells and autoantigen release following chemotherapy-induced cell death (132). Recently, anti-TRIM21 antibodies have been detected in patients with ovarian cancer, the presence of which correlated with better patient survival. Analysis showed these antibodies differed in epitope profile to anti-TRIM21 antibodies detected in SLE and SS, although why this is the case remains to be discovered (133).

TRIM21 Deficiency and Autoimmune B Cells

Dysregulation of B cell function has been identified in both SS and SLE patients. This is characterised by higher levels of circulating activated B cells which express co-stimulatory markers and secrete autoantibodies, driving inflammation and autoimmune pathology (2). Similarly to the cancer studies, TRIM21 deficiency has been explored as a potential driver of SLE, by altering B cell regulation. TRIM21$^{-/-}$ mice have been developed with differing phenotypes, most likely due to specific gene disruption strategies (134). One knockout showed no phenotypic abnormalities, likely due to compensatory upregulation of related TRIMs (135). However, other TRIM21$^{-/-}$ mice showed inflammatory phenotypes when subjected to minor injury by ear tagging. They developed site-specific severe dermatitis and displayed SLE-specific autoimmune characteristics, including elevated proinflammatory cytokine production (136). This discrepancy may occur due to production of a truncated TRIM21 protein (RING, B-box and partial coiled-coil domain) in the second strategy, which enabled and promoted autoantibody production.

Another study showed how *TRIM21* gene disruption in a lupus-prone mouse model (TRIM21$^{-/-}$MRL/*lpr*) altered SLE pathology. Compared to lupus-prone (MRL/*lpr)* mice retaining functional TRIM21 activity, the spleens of TRIM21$^{-/-}$MRL/*lpr* mice had significantly increased mature B cell numbers. Once isolated, these cells differentiated into antibody-secreting plasma cells at higher rates *in vitro*, compared to cells from the MRL/*lpr* spleens (137). One model proposed TRIM21 may regulate plasma cell differentiation by ubiquitinating IRF5, thus blocking IRF5-induced upregulation of IRF4 and subsequent Blimp-1 mRNA expression, **Figure 4** (110, 137–139). However, much of this pathway, especially how IRF5 alters IRF4 expression and whether TRIM21 has a definitive role in this IRF4/5 axis, requires clarification.

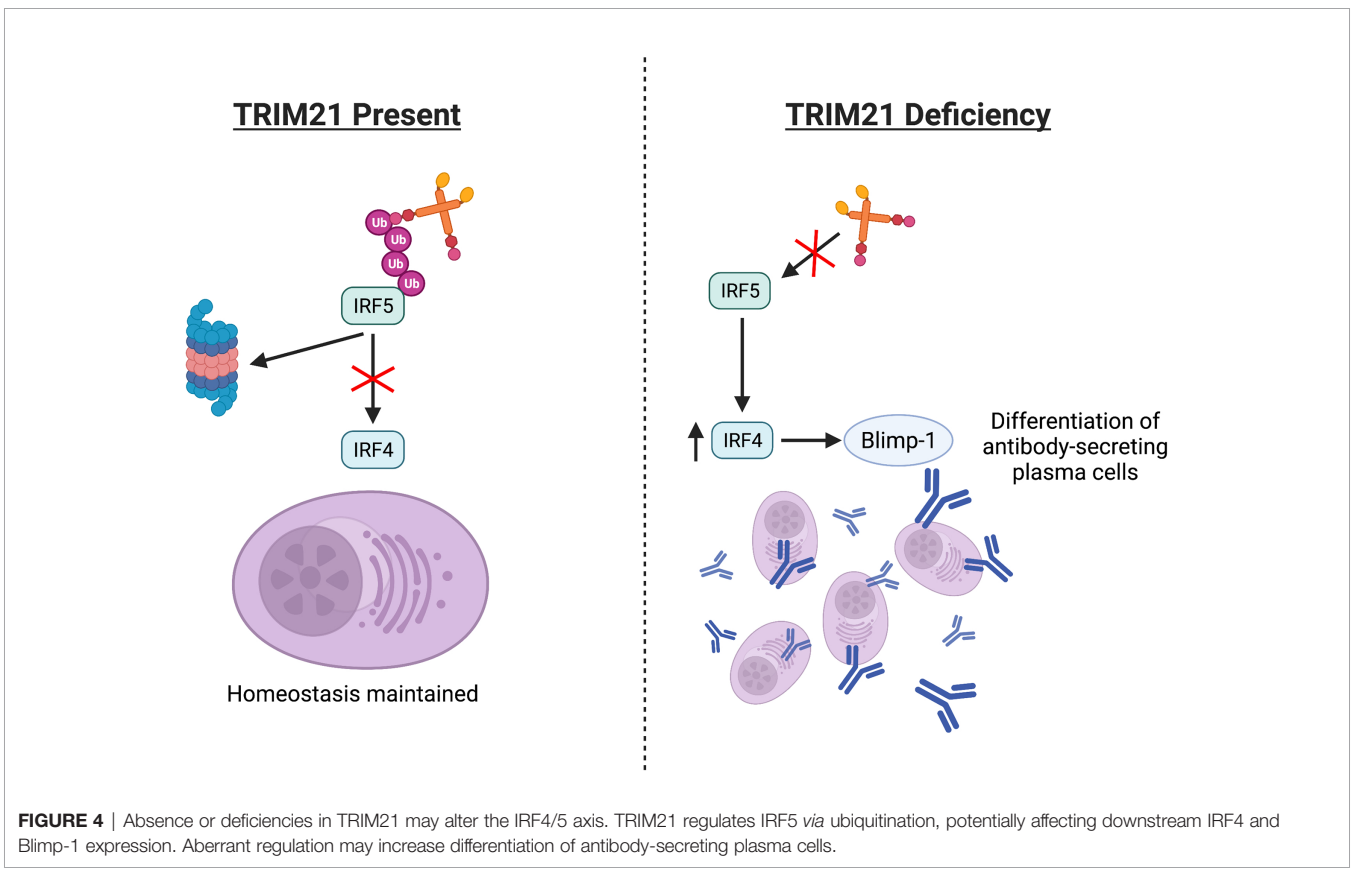

FIGURE 4 | Absence or deficiencies in TRIM21 may alter the IRF4/5 axis. TRIM21 regulates IRF5 *via* ubiquitination, potentially affecting downstream IRF4 and Blimp-1 expression. Aberrant regulation may increase differentiation of antibody-secreting plasma cells.

Impaired Function

Alternatively to expression deficiencies, impaired TRIM21 function may involve antibody-dependent mechanisms. For example, anti-TRIM21 seropositivity in SLE patients correlates with impaired TRIM21 ubiquitinylating activity. PBMCs isolated from anti-TRIM21 seropositive patients display enhanced IFN-I gene expression, suggesting an association between the presence of TRIM21 autoantibodies and attenuated negative regulation of IFN-pathway proteins by TRIM21 (127).

How autoantibodies may downregulate native TRIM21 function has been explored *in vitro*. In a cell-free system, anti-TRIM21 antibodies from SS patients sterically hindered TRIM21's interaction with the E2 ubiquitin-conjugating enzyme, preventing E3 ligase autoubiquitination *via* the RING domain (140). This may impair the subsequent ubiquitination of target proteins such as IRF5, although whether anti-TRIM21 antibodies can indeed access and inhibit TRIM21 *in vivo* has not been directly tested.

HOW CAN ANTIBODIES ACCESS INTRACELLULAR TRIM21?

Under normal conditions, TRIM21 is uniformly distributed throughout the cytoplasm, with small amounts detected within the nucleus (141). Thus, whether and how autoantibodies access this cytoplasmic protein remains a key question for TRIM21-associated autoimmune diseases.

Cell Surface Expression

Recently, TRIM21 was detected at the surface of antigen-presenting cells in pSS patient blood (142). Anti-TRIM21 antibodies are associated with pSS pathogenesis, inducing damage and driving inflammation. This could involve recognition of anti-TRIM21 Fc domains by lymphocytes, driving inflammatory pathways such as antibody-dependent cellular cytotoxicity (143, 144). Therefore, cell surface TRIM21 might exacerbate proinflammatory pathways in autoimmune diseases.

IFN-β, the most potent inducer of TRIM21 expression, induces changes in TRIM21 expression and cellular location in PBMCs (142, 145). TRIM21 was strongly upregulated both intracellularly and at monocyte cell surfaces, with a modest increase observed in plasmacytoid DCs (142). Although this evidence suggests IFN upregulates TRIM21 expression, it remains unclear how TRIM21 may be transported and expressed at the cell surface.

Exposure After Apoptosis

These recent novel findings contradict previous publications suggesting TRIM21 is only exposed at the cell surface during cell death, with anti-TRIM21 antibodies possibly driving apoptosis (141–143). TRIM21 exposure following apoptosis has been demonstrated in the context of foetal CHB which is strongly associated with maternal SLE and SS (146). High titres of cross-reactive maternal anti-TRIM21 antibodies induced apoptosis of foetal cardiomyocytes *in vitro*, **Figure 5** (9). Although CHB pathogenesis remains unclear, one proposed mechanism involves cross-reactive, inhibitory binding of maternal anti-Ro52/60 and anti-La antibodies to α1-subunits of L-Type foetal cardiomyocyte calcium channels (147). Channel inhibition may then dysregulate calcium signalling, leading to abnormal atrioventricular conduction and eventual apoptosis, **Figure 5** (148, 149). Apoptosis of cultured, non-permeabilised human foetal cardiomyocytes led to the redistribution of Ro52/60 and La antigens into apoptotic blebs and their emergence at the cell surface (150). Furthermore, in a co-culture system, apoptotic foetal cardiomyocytes treated with anti-Ro/La antibodies stimulated macrophages to secrete TNF-α (151). Apoptotic blebs rich in autoantigens such as TRIM21 may be insufficiently cleared, facilitating antigen-autoantibody interactions, or enabling DC ingestion and presentation to activate T cells and autoreactive B cells, **Figure 5** (152–154).

Exposure After Stress-Induced Translocation

TRIM21 may also be exposed in non-apoptotic cells following UV-B/C exposure or oxidative stress (155, 156). For example, UV-irradiated keratinocytes displayed TRIM21 at the cell surface, a phenomenon which was inhibited in a concentration-dependent manner by the reactive oxygen species (ROS) scavenger *N*-acetyl-L-cysteine (157, 158). Surface TRIM21 exposure was also induced in keratinocytes treated with diamide, a thiol-oxidising agent (157). Diamide disrupts intracellular glutathione redox reactions, inducing oxidative stress and promoting mitochondrial-induced apoptosis pathways (159). Together, these results indicate that oxidative stress and altered redox homeostasis may induce cell surface TRIM21 exposure. Increased expression of molecular chaperones may also be involved, aiding TRIM21 protein synthesis and translocation, although this has not been confirmed experimentally (157).

Oxidative damage has been identified in the salivary glands of SS patients, with increased saliva detection of the oxidative stress biomarker 8-Hydroxy-2-deoxyguanosine, generated following ROS-induced oxidation of DNA guanine residues (160, 161). The findings by Saegusa et al., suggest that ROS may drive increased exposure of TRIM21 during SS pathogenesis (157). Other studies have confirmed the translocation of SLE autoantigens to keratinocyte cell surfaces following stimulation by UV-B, heat-shock or certain inflammatory cytokines (144). However, the optimal irradiation dose (200mJ/cm^2) used in one study far exceeds levels required to induce significant keratinocyte cell death (162, 163). Thus, the autoantigen redistribution observed in these studies might have occurred during early apoptotic events.

Whilst the mechanism by which TRIM21 is translocated to the surface remains uncertain, one study reported that TRIM21 cytoplasmic bodies localise along microtubule networks. Visualised using live cell fluorescence microscopy, these bodies were highly mobile, undergoing multidirectional movements across a range of distances within the cytosol. However, motility was only observed beneath the plasma membrane and

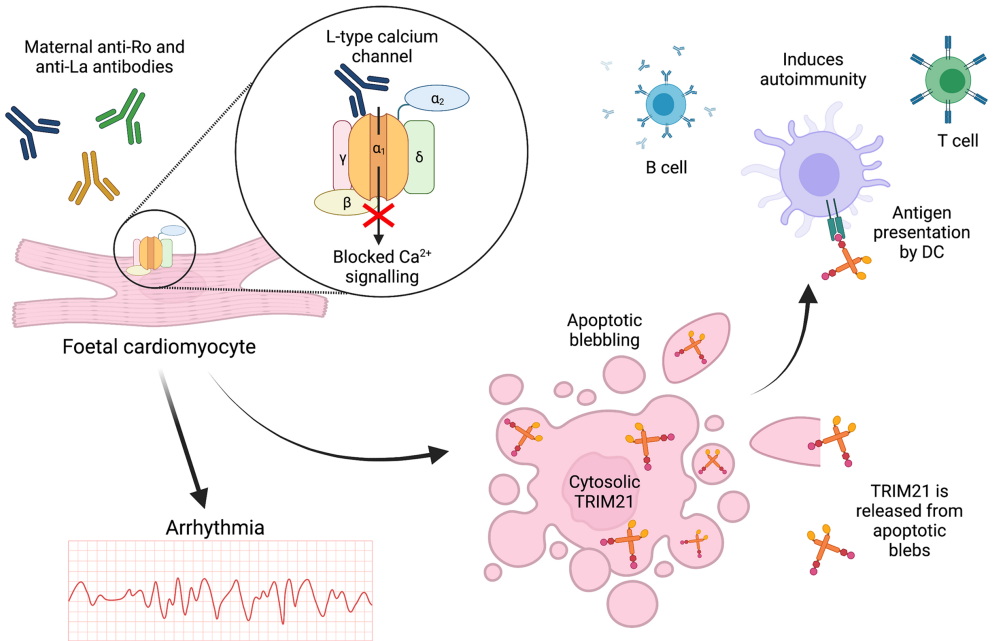

FIGURE 5 | TRIM21 exposure at the cell surface may occur due to apoptosis. In foetal CHB, cross-reactive maternal autoantibodies may bind to and block L-type calcium channels leading to arrhythmia and cardiomyocyte apoptosis. This releases intracellular antigens such as TRIM21 for antigen presentation, driving subsequent autoimmunity.

not at the cell surface (164). Therefore, whether this motility contributes to TRIM21 membrane exposure in response to stimulation or oxidative stress remains unclear.

Together these reports suggest that TRIM21 translocation to the cell surface may occur following apoptosis or cellular stress. Coming back to the recent findings by Hillen et al., it is possible the observed TRIM21 surface expression actually occurred during early apoptotic events (142). Repeating this study using Annexin V as a discriminator between apoptotic and non-apoptotic cells may elucidate whether TRIM21 is indeed expressed at the cell surface under non-apoptotic conditions (165).

ANTI-TRIM21 ANTIBODY DEVELOPMENT IN PATIENTS AND ANIMAL MODELS

Role of BAFF in Anti-TRIM21 Responses

Defective tolerance mechanisms, elevated antigen presentation and inflammatory cytokine expression are thought to drive autoreactive B cell activation, leading to pathogenic autoantibody production (166). Therefore, it is noteworthy that TRIM21 protein levels are significantly elevated in the salivary gland ductal epithelium of SS patients, correlating with inflammation and lymphocyte infiltration (167). Studies have directly correlated serum anti-TRIM21 antibody levels with disease severity. Higher anti-TRIM21 autoantibody titres in pSS are associated with greater localised and systemic disease manifestations including parotid enlargement, haematological abnormalities and central nervous system involvement (168). Studies of patients undergoing hematopoietic stem cell transplantation suggest a link between elevated BAFF expression and anti-TRIM21 autoantibodies (169). Increased BAFF may support the survival of TRIM21-targeting autoreactive memory B cells. However, questions remain as to where this tolerance breakdown occurs, whether TRIM21 specifically drives autoreactivity and whether BAFF expression is necessary for anti-TRIM21 responses.

Epitope Spreading

Another potential mechanism driving anti-TRIM21 autoantibody production is epitope spreading, whereby during chronic inflammation, the exposure of normally sequestered self-antigens induces a secondary immune response against endogenous epitopes (170, 171). Such epitope spreading was demonstrated in mice immunised with the Ro60 autoantigen plus Freund's complete adjuvant. After repeated Ro60 booster immunizations, mice developed an antibody response against the unrelated TRIM21/Ro52 autoantigen (172). This corresponds with SS patient data whereby individuals may display anti-TRIM21 antibodies alone, or in combination with anti-Ro60 antibodies (173). Interestingly, the autoantibodies from anti-TRIM21/anti-Ro60 doubly-seropositive patients exhibit a distinct epitope profile with frequent reactivity to the TRIM21 RING domain, in contrast to patients reactive to TRIM21 alone (174). Thus, Ro60 autoimmune reactivity might influence subsequent reactivity against TRIM21.

Importantly, TRIM21 and Ro60 are structurally and functionally distinct proteins occupying different cellular

compartments. Ro60 (TROVE2) a ring-shaped protein which forms ribonucleoprotein complexes with non-coding (Y) RNA. By binding misfolded Y-RNAs, Ro60 may assist in their degradation (175). Direct interactions and complex formation between Ro60 and TRIM21/Ro52 have not been established, although transient interactions may be possible (175, 176). One hypothesis for how epitope spreading could induce double-positive TRIM21/Ro60 autoreactivity, is that if an autoimmune patient displays reactivity against one of these antigens (Ro60 or TRIM21), transient antigen interactions may expose epitopes from the other antigen, driving a secondary autoimmune response (177). However, questions remain regarding the autoimmune contributions of reactivity against certain TRIM21 epitopes and why only some autoimmune patients are double-positive for anti-Ro60 and anti-TRIM21 antibodies.

Modelling Anti-TRIM21 Autoimmunity

Autoimmune induction of anti-TRIM21 antibodies has been explored in mouse models (178). In particular, the New Zealand Mixed 2758 (NZM2758) mouse strain, obtained by backcrossing and selectively breeding New Zealand Black and New Zealand White mice, has been used to model SS development (179, 180). These mice display classical SS manifestations including glandular histopathology and dysfunction, lymphocyte infiltration, inflammation and autoantibody production (180). TRIM21-immunized NZM2758 mice developed salivary gland dysfunction which correlated with levels of anti-TRIM21 produced (181). Passive serum transfer from TRIM21-immunized NZM2758 mice induced salivary gland dysfunction in adjuvant-primed (intraperitoneally), non-immunized recipient NZM2758 mice (181).

Of note, TRIM21 immunization induced IgG deposition in, and dysfunction of, the lacrimal glands in female but not male NZM2758 mice (182). This observation is interesting in light of the known sex differences in pSS presentation, with women accounting for approximately 90% of cases but men typically presenting with more severe disease (183). Male mice in the Trzeciak et al., study showed no significant clinical disease and therefore do not model the severe disease observed in men with pSS (182). Sex differences in the pathogenesis of rheumatic diseases such as SS and SLE may be related to hormonal effects (184) and to gene dosage effects of immune-related genes such as TLR7 on the X chromosome (185).

Anti-TRIM21 Epitope Targets

Immunisation of NZM2758 mice with MBP-fusion proteins corresponding to different murine TRIM21 domains, was used to determine anti-TRIM21 antibody epitope targets and how they relate to autoimmune pathologies. No differences were detected in the levels of autoantibodies generated against the different TRIM21 domains, however certain epitopes correlated with salivary gland dysfunction in these mice. Specifically, antibodies raised against the coiled-coil domain of TRIM21 reduced saliva production, suggesting a link between specific antibody targets and SS pathology (186). This corresponds with patient findings, confirming that the coiled-coil domain is the most antigenic epitope. For example, 97% of patients with autoimmune rheumatic diseases, and who are also anti-TRIM21 antibody positive, generate antibodies targeting the coiled-coil domain, **Figure 1** (187).

Extensive SS-patient epitope analysis has demonstrated the presence of autoantibodies directed against other TRIM21 regions, including the immunoglobulin binding C-terminal (PRY/SPRY) domain, **Figure 1**. Using pull-down assays, it was demonstrated that antibodies from patient serum bound with high affinity to the C-terminal region of both wild-type TRIM21 and a mutant which cannot bind to immunoglobulin Fc. One model proposes that TRIM21's role in targeting antibody-coated pathogens for proteasomal degradation may promote autoantibody production against these antigenic regions (24, 188). However, whether or not autoantibodies directed against the C-terminal domain are pathogenic and actively drive SS clinical manifestations (e.g. glandular dysfunction), remains to be elucidated.

CONCLUSION AND FUTURE PERSPECTIVES

TRIM21 is an intracellular receptor that binds with high affinity to immunoglobulin Fc regions *via* its PRY/SPRY domains. This binding is vital for intracellular detection of pathogens that escape extracellular antibody neutralisation, enter host cells and access the cytosol. Subsequently, ubiquitination targets the pathogens for proteasomal degradation and immune signalling is activated. In particular, TRIM21 is both enhanced by and regulates IFN signalling for effective immune responses to infection. This also includes the upregulation of other proinflammatory transcription factors such as NF-κB, inducing an antiviral state for pathogen destruction.

TRIM21 dysregulation has been implicated in cancers and autoimmune diseases which include SS and SLE. In particular, excessive IFN responses are correlated with elevated TRIM21 transcript expression. However, these findings are seemingly counter-intuitive as more TRIM21 should promote greater IRF ubiquitination, thus decreasing the IFN response. Therefore, clearer understanding of TRIM21's roles in these signalling pathways is required to explain these contradictory findings.

The associations between anti-TRIM21 seropositivity and impaired intracellular TRIM21 ubiquitination activities are intriguing. However, the main question these findings raise is how autoantibodies are generated against an intracellular antigen. Whether TRIM21 is expressed at the cell surface remains unclear, whilst exposure after apoptosis, as shown by apoptosis of foetal cardiomyocytes in the presence of maternal anti-Ro52 antibodies, provides the most suitable explanation for the generation of anti-TRIM21 antibodies.

Why only some autoimmune patients generate antibodies against both Ro60 and TRIM21/Ro52, whilst others generate antibodies against just one, is another unanswered question. So far, NZM2758 mice have provided a model of female-specific SS. Further studies using these animal models may answer key questions related to anti-TRIM21 antibody generation,

autoantibody epitope specificity, and how this relates to SS clinical manifestations. Certainly, answering these questions will go some way to enhancing our understanding of TRIM21 biology, its roles in innate and adaptive immunity, and its contributions to autoimmunity.

AUTHOR CONTRIBUTIONS

EJ, SL, and LD: Researched the topic, wrote, and edited the manuscript. EJ: Prepared the illustrations. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Multifaceted Roles of B Cells in the Thymus: From Immune Tolerance to Autoimmunity

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The thymus is home to a significant number of resident B cells which possess several unique characteristics regarding their origin, phenotype and function. Evidence shows that they originate both from precursors that mature intrathymically and as the entry of recirculating mature B cells. Under steady-state conditions they exhibit hallmark signatures of activated B cells, undergo immunoglobulin class-switch, and express the Aire transcription factor. These features are imprinted within the thymus and enable B cells to act as specialized antigen-presenting cells in the thymic medulla that contribute negative selection of self-reactive T cells. Though, most studies have focused on B cells located in the medulla, a second contingent of B cells is also present in non-epithelial perivascular spaces of the thymus. This latter group of B cells, which includes memory B cells and plasma cells, is not readily detected in the thymus of infants or young mice but gradually accumulates during normal aging. Remarkably, in many autoimmune diseases the thymus suffers severe structural atrophy and infiltration of B cells in the perivascular spaces, which organize into follicles similar to those typically found in secondary lymphoid organs. This review provides an overview of the pathways involved in thymic B cell origin and presents an integrated view of both thymic medullary and perivascular B cells and their respective physiological and pathological roles in central tolerance and autoimmune diseases.

Keywords: B cells, thymus, central tolerance, aging, autoimmune disease

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1 INTRODUCTION

Several decades ago, B cells were identified as a normal component of the human and mouse thymus (1, 2). Given their specific detection in the medulla it was acknowledged that they are a resident subset rather than B cells casually circulating through the thymus. Perhaps the most surprising aspect of thymic B cells is their unconventional origin through an intrathymic developmental pathway. Although B cells represent a small proportion of total thymocytes, they are a significant component in the medulla, occupying on average 30% of the area in that compartment (3), and are more abundant than thymic dendritic cells (3, 4). Their localization in the thymus and their intrinsic

capacity to present antigens has driven much of the thymic B cell research to establish their involvement in central tolerance.

A generally overlooked aspect in studies of thymic B cells is the effect of aging on the thymic structure and cellular composition, which have mainly been conducted using infant thymic tissue and young mice. During the physiological process of thymic involution, there is an expansion of non-epithelial non-thymopoietic areas known as perivascular spaces (PVS). These PVS are not inert, they contain lymphocytes which are thought to be mature cells that recirculate back to the thymus (5). Surprisingly, many B cells accumulate in the PVS during normal aging, including IgG^+ class-switched memory B cells and plasma cells with reactivity to common pathogens (3, 6). In several autoimmune diseases the recruitment of B cells to the PVS is exacerbated and often leads to the formation of germinal centers that sustain the differentiation of autoreactive plasma cells (1, 7–18). Thus, thymic B cells that accumulate in the PVS play a physiological role by maintaining protective humoral memory and a pathological role in the context of autoimmunity as a site where self-reactive antibodies are produced.

We review the current understanding of the unique properties of thymic B cells in mice and humans including their origin and development, phenotype, and various functions. We aim to propose a cohesive model in which the thymus is populated by two types of B cells: resident B cells located in the thymic medulla that appear in early life during fetal development that contribute to central tolerance as specialized APCs, and perivascular B cells that migrate from the periphery which play a more conventional role in the adaptive humoral response both in health and disease.

2 ORIGIN OF THYMIC B CELLS

Though the presence of B cells in the thymus has been well established, their origin remains a matter of analysis. Some evidence suggests that, unlike the vast majority of B cells that differentiate in the bone marrow, thymic B cells differentiate within the thymus from early thymic precursors (ETPs) (8–10, 12, 13). However, it has also been shown that a small number of B cells can enter the thymus from the circulation and acquire a similar phenotype of thymic resident B cells (4, 19) (**Figure 1**).

2.1 Intrathymic B Cell Development

In the bone marrow, B cells arise from common lymphoid progenitors that undergo several stages of development. One of the earliest is the pro-B (progenitors of B cells) stage in which cells express some B-cell-specific markers such as B220 and CD19, but lack surface immunoglobulin (Ig) and other mature B cell markers. Pro-B cells initiate Ig heavy chain rearrangement and transition to the pre-B (precursors of B cells) stage characterized by the expression of a pre-BCR. Pre-B cells subsequently undergo Ig light chain rearrangement and become immature B cells that express a functional BCR. Immature B cells exit the bone marrow and reach the spleen, where they complete their development into mature B cells (20, 21). B cells colonize the thymus very early in life during fetal development. In mice, $B220^+$ B cells are detected as early as gestational day 18, suggesting that the bone marrow is not essential for their development (22, 23). Moreover, in adult mice the thymus harbors populations of pro-B ($B220^{int}CD43^+IgM^-$) and pre-B ($B220^{int}CD43^-IgM^-$) cells similar to B cell progenitors that are normally found in the bone marrow (19, 24). Although B cell development classically occurs in the bone marrow and fetal liver, these findings suggest that thymic B cells could originate from precursors that differentiate locally. When thymic B cell progenitors ($B220^{int}CD43^+$) are isolated and reinjected into a thymus lobe of neonatal congenic mice, they can differentiate intrathymically into mature $B220^+IgM^+$ B cells (24). More recently, Perera et al, provided further evidence of intrathymic B cell maturation by using recombination-activating gene 2-GFP reporter mice to label B cells that have recently undergone BCR rearrangement. They identified $B220^{lo}CD43^{hi}$ cells positive for GFP which express lower levels of CD19 and MHC-II, indicating that they are equivalent to pre-B and pro-B rearranging their BCR. As expected, mature $B220^{hi}$ cells were negative for GFP. A third population of $B220^{hi}$ thymic B cells with intermediate GFP expression was also distinguishable, likely representing newly mature B cells that have recently completed BCR rearrangement. Consistently, BrdU labeling showed that immature $B220^{lo}GFP^+$ cells could divide rapidly, while mature B cells had a much slower turnover rate (25). Arguing against B cell development in the thymus, Yamano et al. compared the phenotype of putative thymic progenitors with those found in the bone marrow based on c-Kit expression. c-Kit, a receptor for stem cell factor is expressed at the pro-B stage and has an essential role in adult mice in the maintenance of B lymphopoiesis (26, 27). The authors observed that in the bone marrow $CD19^+IgD^-IgM^-$ B cell progenitors can be subdivided into pre-B cells ($CD2^+cKit^-$) and pro-B cells ($CD2^-cKit^+$), that lack any Ig on their surface. However, among the $CD19^+IgD^-IgM^-$ population in the thymus, none express a pro-B cell phenotype and most $CD2^+cKit^-$ express IgG on their surface, thus corresponding to mature class-switched B cells rather than progenitors (4). It is possible that B cell progenitors are scarce in the adult thymus and that their phenotype does not fully overlap with those found in the bone marrow.

In the human thymus, it is also possible to identify B cells from different developmental stages, including subsets with an immature phenotype equivalent to pre-B ($CD10^+IgM^-CD34^-$) and pro-B ($CD10^+IgM^-CD34^+$) cells usually found in the bone marrow (2); however, further studies are needed to address whether the human thymus can support B cell development.

It appears that some progenitors that colonize the thymus maintain B cell lineage potential; however, they are outcompeted by T cell progenitors in the thymic environment. Luc et al. reported that ETPs that seed the thymus for T cell development, also preserve their potential for B cell lineage differentiation in young and adult mice. The ETPs that maintain B cell lineage potential are contained in the $Lin^-CD4^-CD8\alpha^-CD25^-c\text{-}Kit^{hi}Flt3^{hi}$ subset (28). Notably, the frequency of $Flt3^{hi}$ ETPs is tenfold lower in the thymus of adult mice than in newborn mice (28).

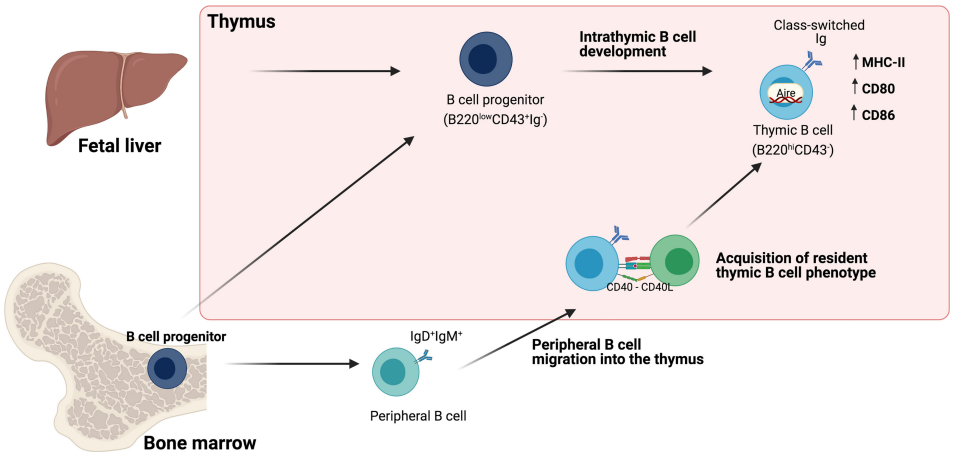

FIGURE 1 | Origin of Thymic B cells. Schematic representation of different pathways involved in the origin of thymic B cells. One pathway proposes intrathymic differentiation from fetal liver and bone marrow B cell progenitors that give rise to resident thymic B cells. A second pathway is the migration of mature B cells, which acquire the phenotype of resident thymic B cells through the interaction with developing thymocytes (discussed in Section 2).

Moreover, when single Flt3^{hi} ETP cells are sorted and seeded onto a bone marrow stromal cell line, a lower frequency of Flt3^{hi} ETPs from adults is able to differentiate into B cells (28). Thus, the B cell potential of ETPs decreases with age. Other studies have also shown that thymic stromal cell lines support the development of mature IgM$^+$ B cells from hematopoietic precursors *in vitro* (29, 30).When fetal thymic lobes are cultured in the presence of Flt3 and IL-7, progenitors expand and differentiate into mature and functional B cells, supporting that the fetal thymus provides an environment that can support the development of T and B cells (31). IL-7, which is essential for the development of T cells and γδ cells in the thymus, also influences the number of thymic B cells. Transgenic mice that overexpress IL-7 have significantly increased numbers of thymic B cells (32). A similar effect on the number of thymic B cells is observed in mice with conditional deletion of IL-7R in CD4$^+$CD8$^+$, CD4$^+$ and CD8$^+$ thymocytes (33). In the bone marrow IL-7 promotes B cell specification and commitment, survival of pro-B cells, and proliferation of pro-B and pre-B cells (34, 35). Therefore the balance between T and B lymphopoiesis is most likely controlled by the availability of IL-7 in the thymus. The frequency of thymic B cells is also increased in the thymus of mice that present a defect in T cell development such as TCR$^{-/-}$ mice and Tgε26 transgenic mice, which have a blockade in the early development of thymocytes due to their expression of the human CD3E gene (19, 36, 37). This phenomenon is also observed in mice deficient in Notch-1 –an essential receptor that promotes T cells lineage commitment – where a decrease in T cells precursor is associated with an expanded number of thymic immature B cells (38, 39). While these studies suggest that the changes in thymic B cell frequency in these models are due to an increase in the lymphopoiesis of B cells as a consequence of a disruption of the T cell niche, a caveat that should be considered is that these models also present a significant decrease in thymic cellularity, which may result in a relative increase in the frequency of thymic B cells due to overall loss of thymocytes.

2.2 Migration of Circulating B Cells to the Thymus

Another alternative that would explain the origin of B cells is the migration of mature peripheral B cells to the thymus. When splenic B cells are transferred intravenously to a congenic mouse to evaluate what percentage of B cells from the donor is capable of migrating into to thymus and other organs, at 18 days post transfer it was observed that only 0.6% of the donor-derived cells migrated to the thymus, while about 5% of the total lymph node and spleen cells came from the donor (19). Similarly, Yamano et al. reported that the frequency of transferred splenic B cells migrating to the thymus was 5-10-fold lower than the spleen (4). In parabiosis assays, which allow a more physiological study of the migration of circulating B cells to the thymus, it was observed that about 50% of mature B cells in the spleen were donor-derived cells, rapidly equilibrating, while the thymus showed a lower exchange where only 20% of mature B220$^+$ cells come from the donor (25, 40). These studies conclude that there is a contribution to the thymic B cell pool through the migration of peripheral B cells, although it appears to be minimal in mice. Inefficient recirculation of mature lymphocytes to the thymus is thought to be partly due to limited available niches to accommodate immigrating cells. The recirculation of B cells to the thymus has been studied in young mice in which thymic involution is not significantly noticeable. It is possible that if similar studies were performed in aged mice the proportion of B cells derived from circulation would be greater. In agreement with this, Cepeda et al. find a significant increase in the frequency of thymic B cells when mice are at least 12-24 months old and show and evident thymic decline (41). When thymic cellularity is reduced by inducing the death of thymocytes through irradiation or acute involution triggered by systemic LPS treatment, enhanced migration of peripheral B and T cells occurs (4, 42). In LPS-

treated mice, the MCP-1/CCR2 axis is one of the mechanisms that directs the migration of peripheral B cells (42). This suggests that in the under inflammatory or infectious conditions, thymic involution combined with the induction of chemokines can drive the accumulation of peripheral B cells in the thymus. Further evidence of this is the abnormally expanded frequency of thymic B cells in autoimmunity (discussed in Section 5). Altogether, we still lack a clear understanding of how the thymic B cell population is maintained over time and the relative contribution of intrathymic differentiation from precursors, self-renewal and import of peripheral B cells to the thymic B cell pool.

3 PHENOTYPE OF THYMIC B CELLS

3.1 Mouse Thymic B Cells

Thymic B cells have an unusual phenotype compared to circulating and secondary lymphoid organ B cells. Initial analysis of thymic B cells in mice revealed a high expression of CD5 and CD43, which are hallmark surface markers of the B1-a lineage located predominantly in the peritoneum, suggesting that thymic B cells may arise from fetal liver progenitors (43, 44). However, a more careful comparison of bona fide peritoneal B1 cells and thymic B cells shows a non-overlapping pattern in the expression of CD5 and CD43, which is lower and less well-defined in thymic B cells. Also, thymic B cells unlike peritoneal B1 cells lack CD11b (25). Moreover, when $RAG^{-/-}$ mice are lethally irradiated and reconstituted with cells from either fetal liver or bone marrow, both can partially restore thymic B cells, albeit not to their normal numbers. In contrast, peritoneal B cells are fully restored by the fetal liver but not bone marrow cells (25). Thus, it appears that thymic B cells and B1 B cells are from different lineages and that bone marrow and fetal liver cells contribute to the development of thymic B cells. Analysis of CD21/CD23, which distinguishes classic splenic subsets, shows that thymic B cells are composed of $CD21^{+}CD23^{+}$cells that resemble follicular B cells and $CD21^{-}CD23^{-}$ B cells but do not contain $CD21^{++}CD23^{-}$ marginal zone B cells (25).

Thymic B cells also differ from resting peripheral B cells in that under steady-state conditions, they display an activated phenotype, expressing high levels of MHC-II as well as significantly higher levels of costimulatory molecules CD80, CD86 compared to B cells from the spleen (4, 25, 43, 45). Most thymic B cells express CD69 and low levels of CD62L, which have been associated with an early activation state (43, 45). However, recent studies show that resident memory B cells located in lymphoid and non-lymphoid tissues are also characterized by a $CD69^{+}CD62L^{lo}$ phenotype (46–48). Given that thymic B cells are not readily exchanged with circulatory B cells, it is likely these markers indicate that thymic B cells are resident, as opposed to recently activated, cells.

3.2 Intrathymic B Cell Licensing and the Central Role of CD40/CD40L Interaction

An unexpected property of thymic B cells is that in mice without prior infection or immunization about one-third are antigen-experienced $IgD^{-}IgM^{-}$ class-switched B cells and express other subtypes of IgG and IgA on their surface, IgG2b being the most frequent, while most peripheral B cells are naïve $IgD^{+}IgM^{+}$ (4, 40).

A pivotal study by Yamano et al. demonstrated that the phenotype of B cells is modified within the thymus through a process recently termed as thymic licensing. When naïve B cells are transferred intravenously, they retain their original phenotype in the spleen; however, those that immigrate into the thymus acquire the features of resident thymic B cells, including increased levels of MHC-II and co-stimulatory molecules CD80 and CD86, thus enabling them to act as more efficient APCs (a function that will be discussed later). Furthermore, they also identified a fraction of thymic B cells that undergo immunoglobulin class-switch and acquire the expression of the autoimmune regulator (Aire) gene (4). Aire is classically induced in medullary thymic epithelial cells (mTEC), allowing these cells to express autoantigens in the thymus that are only found in peripheral tissues promoting self-tolerance to a broad range of autoantigens (49). However, Aire protein levels are significantly lower in B cells than mTECs and Aire regulates fewer genes in thymic B cells (4). Most importantly, the same programming of the B cell phenotype occurs when naïve B cells are injected directly into the thymus, reinforcing that licensing is elicited by local cues (4).

Thymic B cells differ in their responsiveness to conventional B cell stimuli compared to splenic B cells. They do not proliferate or differentiate into antibody-secreting cells to the same extent as splenic B cells in response to LPS or anti-IgM+IL-4. However, they are significantly more responsive when they interact with activated T cell blasts (50), or are activated by a combination of anti-CD40 and IL-10 (24, 51). This superior response to CD40 stimulation is also demonstrated in the strong requirement of CD40/CD40L signaling to maintain a normal frequency of thymic B cells provided through interaction with developing T cells. Mice lacking either CD40 or CD40L have significantly reduced numbers of thymic B cells while maintaining a standard B cell frequency in peripheral lymphoid organs (52, 53).

Notably, cognate interaction with developing thymocytes provides necessary signaling for intrathymic B cell licensing, i.e., higher levels of MHC-II, induction of Aire, and isotype class-switching (4, 40). Thymic B cells fail to acquire these features under conditions where antigen presentation to CD4 SP thymocytes is blunted, such as a lack of MHC-II on B cells, CD40 deficiency, as well as in $TCR\alpha^{-/-}$ mice. Licensing is also altered in OT–II mice indicating that a normal T cell repertoire and cognate B-T interaction is required (4, 40).

3.3 Human Thymic B Cells

Few studies have analyzed the phenotype of human thymic B cells, mainly through immunohistological methods rather than more comprehensive flow cytometry. These reports show that B cells in the human thymus, similar to mice, are found in a small proportion presenting a unique phenotypic characteristic compared to peripheral B cells. About 50% of thymic B cells express CD5 on their surface, while peripheral blood B cells are mostly $CD5^{-}$ (54). In the human thymus, B cells uniformly express maturation markers such as CD19, CD22, CD20,

CD40, and CD2 (22, 55–57). About 10% express Ki-67, indicating that some thymic B cells are actively proliferating (22, 55, 57). Work from our group found that human thymic B cells also display elevated levels of MHC-II, CD80, CD83, and CD86. Notably, in young infants, thymic B cells consistently express higher amounts of MHC-II molecules than thymic $CD11c^+$ dendritic cells, suggesting that they are poised to present antigens efficiently, similar to what has been as reported in mice (3).

In a longitudinal analysis of the distribution and phenotype of human thymic B cells spanning seven decades of life, our group also demonstrated the existence of two distinct subsets of B cells in the human thymus located in the medullary and the perivascular compartments respectively. Medullary B cells co-localize with mTECs and immature thymocytes. Medullary B cells represent the vast majority of thymic B cells in neonates and infants, yet their relative proportion decreases as the thymic epithelial areas decline in adulthood (3). The second subset of B cells begins to appear in the thymic PVS after the first year of life, which expands with age, allowing an increasing number of B cells to accumulate in this area (discussed in Section 4). PVS B cells are primarily composed of class-switched IgG^+CD27^+ memory cells, the main subset of thymic B cells in adults. Terminally differentiated plasma cells that secrete primarily IgG also begin to populate the PVS during childhood and are maintained in adults (3).

It is noteworthy that in infants where most thymic B cells are located in the medulla, even in the first weeks of life, between 15-30% have already undergone Ig class-switch to IgG or IgA (3). Such observation warrants further research to determine whether human B cells also go through an equivalent thymic licensing process as described in mice.

4 THYMIC B CELL FUNCTIONS

4.1 The Role of Thymic B Cells in Central Tolerance

Several experimental models have demonstrated that thymic B cells play a non-redundant role in the negative selection of self-reactive clones (**Figure 2**). This function was first investigated using mouse strains bearing different Minor lymphocyte stimulating antigens (Mls). Neonatal intrathymic injection of thymic B cells that express Mls-1 antigen results in a reduced frequency of Mls-1 reactive $v\beta6^+$ thymocytes, however Mls-1+ splenic B cells are unable to effectively induce clonal deletion either *in vivo* or in fetal thymus organ culture (58, 59). This suggests that thymic B cells induce clonal deletion of self-reactive thymocytes, although it should be considered that this effect may be partly attributed to the presentation of Mls-1 antigen by endogenous dendritic cells. In turn, T cells from mice that have been tolerized with Mls-1 thymic B cells have a restrained ability to mount a graft-versus-host response when transferred into Mls-1-bearing mice (58). Likewise, thymic B cells expressing viral superantigen Mtv-7 effectively mediate clonal deletion of MtV-7-reactive transgenic thymocytes *in vitro*, whereas splenic B cells showed weaker capacity unless previously activated through CD40 engagement (43). These early studies suggested that

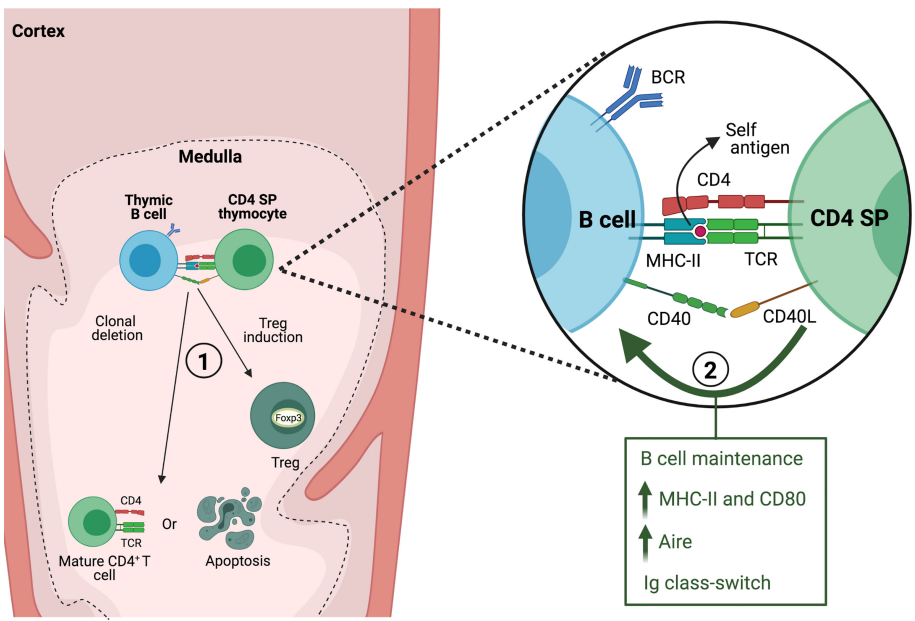

FIGURE 2 | Interaction between thymic B cells and CD4 SP thymocytes. In the thymic medulla, B cells present self-antigen to CD4 SP thymocytes through MHC-II and engagement of CD40/CD40L. The interaction between both cells leads to 1) clonal deletion or generation of thymic regulatory T cells (Treg) and 2) is required for maintaining normal thymic B cell numbers and acquisition of a thymic resident B cell phenotype.

thymic B cells might be conditioned by their environment to enhance their ability to interact with cognate T cells and participate in negative selection.

More recent studies have established the contribution of endogenous thymic B cells to central tolerance. In transgenic mice in which I-E molecules are expressed exclusively by B cells, there is significant deletion of I-E-reactive vβ5 and vβ11 T cell clonotypes (60). B^{MOG} mice which have forced presentation of a Myelin oligodendrocyte glycoprotein (MOG) peptide on MHC-II by B cells are tolerized against induction of EAE (61), which suggested the ability of B cells to purge MOG-reactive T cells. This was corroborated by generating 2D2/B^{MOG} mice, bearing a MOG-specific TCR, in which a drastic reduction of thymic and peripheral transgenic $CD4^+$ T cells was observed along with a high frequency of Tregs among the surviving self-reactive T cells (62). Perera et al. used a novel approach to understand how reactivity towards self-antigens in the thymic B cell repertoire may favor their ability in negative selection. Mice in which B cells express a transgenic BCR with specificity for a self-antigen display enhanced negative selection of cognate CD4 SP thymocytes. They also found that when mice express a self-reactive transgenic TCR, a lack of B cells results in an incomplete negative selection, indicating that the endogenous B cell repertoire contributes to this process (25). There is evidence that autoreactive B cells are enriched among thymic B cells in mice and humans (40, 63). By cloning Ig genes from single cells and testing their reactivity against a panel of self-antigens it was determined that significantly more antibodies derived from human thymic B cells show reactivity to nuclear antigens from a Hep-2 cell lysate compared to B cells from bone marrow. A high frequency of thymic antibodies also binds to dsDNA, while only few recognize insulin peptide or malondialdehyde-modified BSA (an oxidized lipid antigen found in apoptotic cells) (63). Though it is not clear what types of self-antigens could recognized by thymic B cells, it has been proposed that a broad recognition of self-antigens would enhance their capacity to purge cognate thymocytes. In mice, a bias towards autoreactivity (i.e., binding to nuclear antigens in Hep-2 cells) occurs among Ig class-switched thymic B cells, consistent with an enhanced capacity to present self- antigens, interact strongly with thymocytes and undergo thymic licensing (40). Additionally, Yamano et al. have demonstrated that self-antigens induced by Aire specifically in B cells, therefore dependent on thymic licensing, can also drive clonal deletion of cognate CD4 SP thymocytes (4).

A distinct property of thymic B cells is that they are the first antibody-secreting cells (ASCs) that spontaneously secrete IgG, IgA and IgE which appear early in the postnatal thymus, before to the appearance of ASCs in the spleen (64, 65). In a pig model, thymic B cells are also the first to undergo Ig class-switch and secrete IgG and IgA spontaneously during the gestational period and in newborn germ-free piglets, whereas splenic B cells only secrete IgM (66–68). Moreover, colonization of germ-free piglets with *E. coli* leads to IgG and IgA diversification in the mesenteric lymph node and blood and selective expansion of clones. In contrast, the thymic B cell repertoire remains polyclonal and unaffected by colonization (68). Therefore, thymic Ig class-switch and secretion do not appear to be driven by external antigenic stimuli o microbiota colonization. One explanation for why Ig class-switch and secretion may be functionally relevant in the thymus is to establish T tolerance to immunoglobulins. In the case of IgE, Haba et al. show that mice become tolerant to IgE, i.e., fail to generate anti-IgE antibodies when immunized, at postnatal day 10, which coincides with the appearance of IgE ASCs in the thymus (65). Also, during the generation of a humoral response, B cells internalize, process and present antigen-derived peptides. In this process, peptides from the BCR are also processed and presented, therefore, establishing T cells tolerance to such antigens is critical to avoid inadequate and potentially harmful activation of immunoglobulin-specific T cells. Thymic B cells have been shown to negatively select TCR-transgenic T cells that recognize Ig determinants, therefore ensuring tolerance for subsequent cognate B-T interaction (69, 70).

Thymic B cells have also been shown to contribute to the differentiation and expansion of thymic Tregs. Transgenic BAFF mice, which have a 7-fold increase in the frequency of thymic B cells, have twice as many Tregs, whereas, in B cell-deficient uMT mice, Tregs are reduced by nearly half (45, 71). Histological staining shows that B cells are co-localized with Tregs in the thymic medulla, and they can induce the differentiation of CD4 SP thymocytes intro Tregs *in vitro* (45). The expansion of thymic Tregs requires cognate B cell help through MHC-II molecule as well as a polyclonal BCR repertoire, suggesting that this interaction may be initiated after self-antigen uptake, processing, and presentation by thymic B cells (45, 71). In the human thymus, IL-15 promotes the differentiation of Treg precursors into mature $Foxp3^+$ Tregs and the proliferation and survival of Tregs. Thymic B cells express high levels of IL-15 mRNA compared to other thymic subsets and co-localize in IL-15-rich areas in the thymic medulla, therefore B cells may promote Treg differentiation through the production of this cytokine (72).

Thymic B cells also appear to regulate normal mTEC development and expression of tissue-restricted antigens (TRAs) partly through the production of lymphotoxin α (LTα) and β (LTβ). Akirav et al. have shown that LTα and LTβ mRNA is expressed by thymic B cells in addition to CD4 and CD8 SP thymocytes. Moreover, they observe that the number of mature mTEC is reduced nearly by half in mice with diminished numbers or lacking thymic B cells. μMT mice lacking B cells also exhibit a lower expression of Aire and TRAs such as insulin and MOG in the thymus. Notably, in mice with conditional deletion of LTβ expression specifically in B cells, there is also a reduced expression of TRAs, suggesting that LTβ deficiency in B cells impairs mTEC development (52, 73).

4.2 Perivascular B Cells: A Niche of Protective Humoral Memory

As mentioned previously, numerous B cells are also found in the human thymus PVS (3, 5, 6). However, to date few studies have investigated the nature of these B cells. The area of the thymus

occupied by PVS progressively increases with age, starting to expand from early childhood (3, 5). These structural changes are accompanied by an increased frequency of thymic B cells in older children and adults (3, 6). Immunohistochemical staining of several B cell markers combined with cytokeratin staining shows that most PVS B cells express CD21, CD72 and CD37, while these markers are absent in medullary B cells (6). These phenotypical differences would support that medullary and PVS B cells are separate subsets with little or no exchange. After the first year of life, the accumulation of B cells in PVS correlates with an age-associated increase in the frequency of $CD27^+IgG^+$ memory B cells, suggesting that most PVS B cells consist of memory B cells (3). Furthermore, a significant proportion of B cells that accumulate in the thymus during aging express the chemokine receptor CXCR3, suggesting that PVS B cells migrate to the thymus after being activated in the periphery (3). $CD138^+$ plasma cells are also found in the thymic PVS and secrete IgG without the need for additional stimulation. Thymic plasma cells secrete primarily IgG1 and IgG3, the two main complement-fixing effector IgG subclasses. Antigen-based ELISPOT assays show that human thymic plasma cells contain a high frequency of cells reactive to common viral antigens encountered through infection and vaccination, including influenza, rubella and measles (3). Notably, the PVS also harbors eosinophils that are located in close proximity to plasma cells (3, 5). Eosinophils are essential for the maintenance of long-lived plasma cells in the bone marrow and gut-associated tissue (74, 75), therefore the PVS may acts as a functional niche for viral-specific plasma cells (**Figure 3**).

4.3 Aging and Thymic B Cell Function

During aging, immune responses against new antigens are diminished while the risk for developing autoimmune disease generally increases (76). Thymic involution, a hallmark of aging, is characterized by a steady loss of cellularity and a decline in the export of mature T cells. Age-associated functional decline in the thymus is also believed to be related to the increased susceptibility to autoimmune disorders (77). Since B cells play a critical role in the induction of central tolerance, a relevant question is whether a decline in the function of thymic B cells could contribute to defects in T cell selection. We have found that aging significantly impacts the phenotype of human thymic B cells, particularly in regard to their antigen-presenting function. We determined a nearly 10-fold reduction in the expression of MHC-II between infants and adults, and a significant reduction in the levels of co-stimulatory molecules CD80, CD83, and CD86 (3). In addition, Cepeda et al. recently reported that while the relative proportion of B cells increases in aged mice, the expression of Aire declines in thymic B cells, including in IgD^-IgM^- class switched cells that have presumably undergone licensing (41). Transcriptional analysis of human thymic B cells from young and aged individuals also shows diminished Aire expression. The observed loss of Aire expression is accompanied with changes in the expression of Aire-dependent genes both in mice and humans (41). These findings suggest that the ability of thymic B cells to enforce central tolerance is dampened by aging.

5 AUTOIMMUNITY-DRIVEN ACCUMULATION OF B CELLS IN THE THYMUS

In several autoimmune diseases and relevant animal models, there is a drastic deterioration of the thymic epithelial network associated with elevated numbers of B cells that accumulate in the PVS and organize into follicles and active germinal centers (**Figure 3**). Depending on the disease and its severity, thymic alterations can range from a modest increase in the frequency of thymic B cells to a complete remodeling of the thymus into what resembles a secondary lymphoid organ (**Table 1**). In this section we will review the diseases in which concomitant thymic pathology has been described.

5.1 Myasthenia Gravis

The most studied disease that is characterized by its associated thymic pathology is myasthenia gravis (MG). Approximately 60-80% of MG patients develop a condition defined as thymic follicular hyperplasia in which the normal thymic lymphoepithelial architecture is disrupted and replaced by B-cell follicles containing active germinal centers that give rise to plasma cells (1, 7, 82). The incidence of thymic pathology reported in MG patients varies in the literature due to an evident gender and age bias (8). Thymic pathology occurs mainly in females and peaks between the ages of 20-40 years. The number of thymic germinal centers wanes in patients over the age of 50 years when the lymphoepithelial areas have been broadly replaced by adipose and connective tissue, which likely fails to provide a favorable environment for GC maintenance (8). The thymus of MG patients contains activated B cells undergoing clonal proliferation and hypermutation and plasma cells that produce autoantibodies against the acetylcholine receptor (AChR) (83–85). Consequently, the incidence of thymic B cell germinal centers correlates with anti AChR titers (8). Converging evidence that the thymus is actively contributing to the pathogenesis in MG has been validated in retrospective analysis and, more recently, a randomized clinical trial of thymectomy, which shows significant improvements in the clinical outcome after surgery compared to steroid therapy alone (82, 105).

Several mechanisms at play in the development of thymic pathology in MG have been identified. The abnormal recruitment of B cells results from an elevated production of the B-cell attracting chemokine CXCL13 and B-cell activating factor (BAFF) by thymic epithelial cells (78, 79). IFN-β, a signature cytokine in many autoimmune diseases, can induce the expression of CXCL13 and BAFF in TECs (78). A possible scenario is that chronically elevated levels of inflammatory cytokines condition the thymic environment to promote the recruitment of B cells. Transgenic mice in which mTECs overexpress CXCL13 have normal numbers of thymic B cells, however, inflammation induced by the administration of TLR3 agonist Poly(I:C) promotes infiltration of B cells in the thymus and increases the susceptibility of severe MG when they are immunized against AChR (86). Therefore, thymic pathology requires a combination of increased B cell chemotactic signals

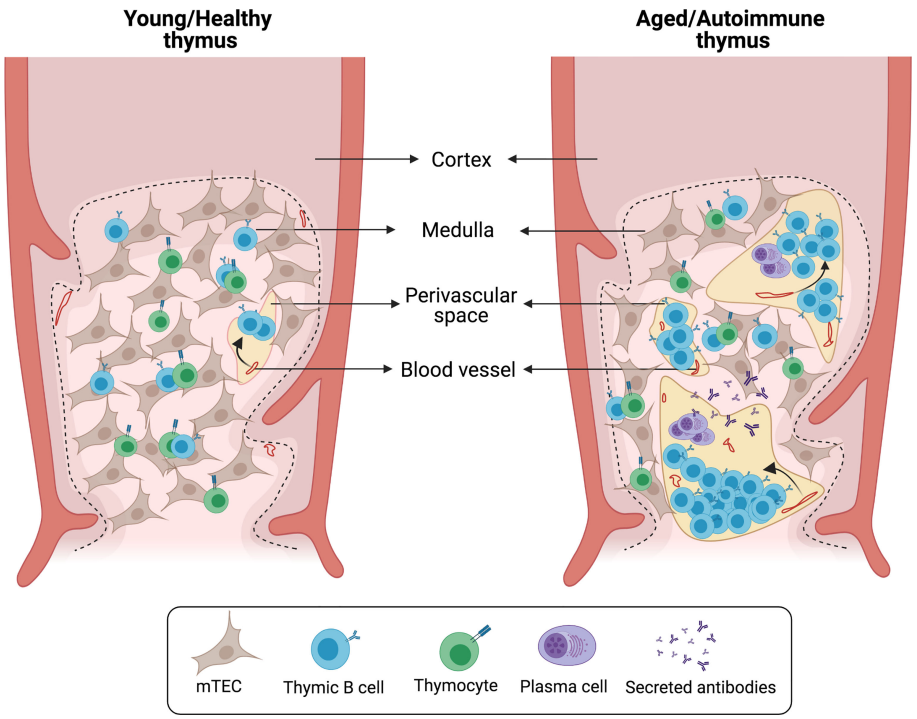

FIGURE 3 | Histological changes in aged/autoimmune thymus. Schematic representation of characteristic alterations in the aged/autoimmune thymus. The medullary epithelial network is disrupted by the enlargement of perivascular spaces (PVS). B cells and plasma cells infiltrate the thymus and localize within the PVS forming germinal center-like structures.

in the thymic environment as well as B cell activation in response to systemic inflammation.

Another key feature of thymic follicular hyperplasia in MG patients is the increased development of lymphatic vessels and high endothelial venules (HEVs) (80, 81). Blood vessels associated with follicular cell aggregates originate in the PVS and become equipped with the tall endothelium of the post-capillary-venule type. In physiological conditions HEVs are found in secondary lymphoid organs, yet they can also appear in chronically inflamed tissues where they are associated to the infiltration of peripheral cell aggregates (106). Therefore, the presence of HEVs in the MG thymus suggests active immigration of lymphocytes from the bloodstream.

Conventional corticosteroid treatment normalizes several features of thymic pathology in MG patients, reducing the number of germinal centers, the expression of chemokines that orchestrate GC formation, including CXCL13, CCL21 and CCL19 (79, 107), and also the number of HEVs (81). These findings are not trivial as they strongly argue that thymic disturbances are a consequence rather than a cause of autoimmune disease.

5.2 Systemic Lupus Erythematosus

Thymic abnormalities have been also been reported in patients with systemic lupus erythematosus (SLE). The most representative manifestation is a high frequency (6-fold increase) of plasma cells compared to the thymus of both healthy subjects and MG patients. While some SLE patients also develop germinal centers in the thymus, it is not a defining feature as in MG (9, 10). The thymus also presents signs of extreme atrophy in SLE patients with complete loss of the cortex and disorganization and aggregation of epithelial cells in the medulla (10).

Although there are limited human studies to understand the underlying causes and implications of thymic pathology associated with SLE, it has been further examined in mouse strains that spontaneously develop a systemic autoimmune disorder that resembles human SLE. Early histological studies in the thymus of NZB, BWF1, MRL/MP-lpr/lpr, BXSB/MpJ Yaa and C3H HeJ-gld/gld mice revealed that these models recapitulate a progressive loss of the normal stromal architecture. Typical stromal alterations include a disorganized network of epithelial cells and enlarged perivascular spaces that are densely packed with lymphocytic follicles and plasma cells (87–91). Later works have examined more closely the abnormal recruitment of B cells to the thymus. In aged BWF1 mice, the group of Ishikawa reported a 2000-fold increase in the expression of CXCL13 in the thymus. The primary source of CXCL13 is myeloid $CD11c^+$ dendritic cells which are also more abundant in the thymus of aged mice. They found that B cells that accumulate in the thymus are composed of B1 ($B220^{int}CD5^+$) and B2 ($B220^+CD5^-$) B cell subsets and suggested that B1 cells may be

TABLE 1 | Various autoimmune disease and relevant animal models that are associated with thymic pathology.

Disease	Thymic pathology		Animal model	Thymic pathology	
	Thymic stromal changes	Cellular changes		Thymic stromal changes	Cellular changes
Myasthenia gravis	Epithelial areas are replaced by perivascular spaces (8) Higher expression of CXCL13 and BAFF in TECs (78, 79) Increase of lymphatic vessels and high endothelial venules (HEV) (80, 81)	Formation of germinal centers containing activated B cells (1, 7, 82) Plasma cells that produce autoantibodies against AchR (83–85)	K5-CXCL13 mice immunized with AchR (86)	Not specified	Recruitment of B cells in the thymus (86)
SLE	Severe atrophy, loss of the cortex and disorganization of epithelial cells in the medulla (10)	Germinal centers in a reduced subset of patients (9, 10) Increased number of plasma cells (9, 10)	BWF1, MRL/MP-lpr/lpr, BXSB/MpJ Yaa and C3H HeJ-gld/gld mice (87–91)	Loss of normal stromal structure with a disorganized network of epithelial cells (87–91) Enlarged PVS (11, 92, 93) High expression of CXCL13 in myeloid DCs (93, 94) Expression of PNAd in blood vessels in PVS (93)	B cells organized into germinal center-like structures (11) Increased frequency of TFH cells (11) Plasma cells producing anti-dsDNA IgG (11)
T1D	Not studied	Not studied	NOD mice (12, 95–97)	Enlarged PVS (95–97)	Increased number of B cells, plasma cells and TFH cells (12) Formation of ectopic germinal centers (12)
Sjogren's syndrome	Severe atrophy with formation of thymic epithelial cysts (13–15, 98)	Germinal centers associated to thymic epithelial cysts (13–15)	IQI/*Jic*, and Aly/*aly* mice (99)	Disorganized stromal network (99)	Increased number of B cells (99)
Ulcerative colitis	Not specified	Formation of lymphoid follicles (16)	DSS induced colitis, G$\alpha i2^{-/-}$ mice (100–102)	Acute thymic involution (100–102) Loss of $CD4^+CD8^+$ thymocytes (100–102)	Not studied
Rheumatoid arthritis	Thymic hyperplasia; low-grade B cell lymphoma (17, 18, 103)	High density of B cells, germinal centers, plasma cells (17, 18, 103, 104)	–	–	–

preferentially recruited due to a higher expression of CXCR5 (94). The same group also demonstrated that in healthy mice B cells do not actively immigrate into the thymus, however; in diseased BWF1 adoptively transferred B cells are readily detectable in the thymus and mainly localize in the perivascular spaces (92, 93). Trafficking of peripheral lymphocytes to the perivascular spaces is supported by the expression of peripheral node addressin in localized blood vessels (93).

The degree of thymic structural alterations in BWF1 mice correlates with the levels of anti-dsDNA autoantibodies (91), however, a pivotal question to be addressed is the contribution of the thymus to the progression of the disease. We have recently shown that in diseased BWF1 mice, the thymus contains large numbers of B cells organized into germinal center-like structures as well as a distinct subset of follicular helper T cells, reminiscent of active germinal centers found in the thymus of MG patients. Moreover, we detected antibody-secreting cells in the thymus of diseased-BWF1 mice that produce anti-dsDNA IgG autoantibodies (11). Therefore, the thymus may constitute an important niche that supports the maintenance of the pathogenic humoral response in SLE.

5.3 Type 1 Diabetes

Type 1 diabetes (T1D) is a condition that results from the attack of pancreatic beta cells by innate and adaptive immune cells. How the disease progresses, and its underlying immunological mechanisms have been possible to study using the non-obese diabetic (NOD) mouse strain. Several studies aiming to understand whether thymus dysfunction contributes to immune dysregulation observed an accelerated deterioration of epithelial organization and formation of giant perivascular spaces (95–97). Furthermore, a recent study found that in NOD mice B cells begin to accumulate in the thymus at the prediabetic stage. B cells accumulate in follicle-like structures in non-epithelial areas which likely correspond to enlarged PVS described in earlier studies (12). In addition, they found that the NOD thymus contained significantly more follicular helper T cells and a slight increase in the number of plasma cells, altogether suggestive of the formation of ectopic germinal centers in the thymus (12). It remains to be studied if B cell accumulation in NOD mice is driven by similar changes in the thymic environment as those observed in MG and SLE, such as the production of CXCL13. The reactivity of B cells and plasma cells that infiltrate the thymus in NOD mice also need to be addressed, since it is not clear whether thymic abnormalities contribute to T1D progression or it is rather an epiphenomenon that occurs as a byproduct of peripheral B cell activation. A possibility is that in T1D, the thymus also harbors pathogenic B cells or plasma cells. In this sense, autoreactive islet antibodies are present in individuals that develop T1D. However, the pathogenicity of these antibodies is unclear, since they are dispensable for disease development in NOD mice (108). Nonetheless, B cells do play a critical role in T1D pathogenesis as antigen-presenting cells (108). In this regard, the capacity of B

cells to interact with and potentially stimulate autoreactive T cells in the PVS is yet to be explored.

5.4 Other Diseases

Several case reports have followed patients with Sjogren's syndrome requiring extended thymectomy due to the presence of multilocular thymic cysts in which thymic medullary epithelia undergo a cystic transformation as a result of inflammation (98). Thymic cysts are associated with lymphoid tissue containing active germinal centers (13–15). Furthermore, an increased number of thymic B cells and disorganized thymus structure have been observed in IQI/*Jic* and Aly/*aly* mouse strains that model Sjogren's syndrome (99).

Thymic abnormalities have also been noted in patients with rheumatoid arthritis (RA). A clinical report of an RA patient not receiving corticosteroid therapy found that the thymic cortex was completely absent and identified active germinal centers (104). In other case reports, patients with RA and juvenile idiopathic arthritis develop thymic hyperplasia diagnosed as low-grade B cell lymphoma. In these patients, thymus biopsy reveals increased density of $CD20^+$ B cells, lymphoid follicles with germinal centers and plasma cells scattered in the tissue (17, 18, 103).

Thymic pathology has also been observed in patients with ulcerative colitis. One study reported the presence of lymphoid follicles in 40% of UC patients (16). In a subset of patients with poor response to conventional treatment, close to 90% went into remission after thymectomy. indicating a contribution of the thymus to disease severity (109). Ulcerative colitis can be induced in mice by the administration of dextran sulphate sodium or occurs spontaneously in $G\alpha i2^{-/-}$ mice. In both models the thymus undergoes atrophy associated with a dramatic reduction of double positive thymocytes (100–102), however, other alteration in the thymic organization and infiltration of B cells have not been addressed.

6 CHALLENGES AND FUTURE PERSPECTIVES

Thymic B cells have been studied for several decades yet their unconventional nature still leaves many open questions. The process of thymic B cell licensing is particularly intriguing because it is not fully understood if thymic B cell isotype switching is driven by antigen encounter, or occurs in a random manner as a result of strong T cell help signaling. On average one third of thymic B cells have switched to IgG and IgA, yet it is not clear what determines that a thymic B cells will either retain IgD^+IgM^+ expression or undergo class-switch. Moreover, whether switched Ig regulate other properties of thymic B cells such as their survival or the ability to present antigens found in low concentration needs to be elucidated.

Although there is substantial evidence that B cells reside in two different compartments of the thymus, the medulla and PVS, few studies have addressed their coexistence or whether there is any exchange between them. The thymic B cell pool is the result of local differentiation and import from circulation, therefore a reasonable hypothesis is that medullary B cell arise primarily from B cell progenitors while PVS B cells are exclusively derived from circulating mature B cells. Analysis of the Ig repertoire between both subsets could provide insight into their different clonal origin and identify any overlapping clonotypes if exchange does in fact occur.

The balance between medullary B and PVS B cells evolves during the normal aging process and in autoimmune diseases. Starting from early childhood there is a decline in the area occupied by the medulla which is progressively replaced by the PVS, which occurs in an accelerated manner in individuals with autoimmune conditions. This has important implications for studies that aim to examine how aging and autoimmunity affects the phenotype and functionality of thymic B cells, as it requires that a distinction be made between medullary and PVS B cells. A recent study concluded that aging was associated with a decline in Aire expression and other genes such as CD80 (41). In our own studies we have found that thymic B cells in adults have lower levels of MHC-II (3). It is conceivable that this is actually a reflection of an increased ratio of PVS B cells, exhibiting have a different phenotype than medullary B cells. Future studies should consider the relevance of distinguishing both types of thymic B cells, for instance, through intravenous labeling in mice which stains cells in the PVS while sparing other thymic compartments (110).

Accumulation of B cells with reactivity to commonly encountered viruses during healthy aging and self-reactive B cells in MG and SLE strongly suggests that the PVS is a target niche for activated B cells, memory B cells and plasma cells generated during a physiological and pathogenic immune response. The development of chronic inflammation in autoimmune disease may amplify the mechanisms that normally regulate B cell recruitment to the thymus, thus it may be relevant to define common pathways, i.e. chemokines, cytokines, that are involved in both situations.

7 CONCLUSION

As our understanding of thymic B cells advances, it is evident that they cannot be regarded as a single homogeneous population. Different subsets are positioned in separate regions of the thymus, and most likely have a distinct origin, phenotype, and function. In this Review we propose that thymic B cells should be classified as medullary and PVS B cells. Medullary B cells populate the thymus early in ontogeny, originate mainly from intrathymic B cell differentiation of progenitors, and exert the role of specialized antigen-presenting cells in central tolerance. Remarkably, the thymic environment, in particular cognate interactions with immature thymocytes, appears to enhances the capacity of thymic B cells to mediate negative selection. On the other hand, PVS B cells populate the thymus later in life and accumulate gradually throughout aging. They are most likely peripheral B cells that migrate to the thymus, and include memory B cells and plasma cells. The presence of antiviral thymic plasma cells suggests that the PVS compartment may resemble the bone marrow as a functional niche of humoral memory. Future studies are urgently needed to fully understand the role of PVS B cells. Finally, the mechanisms by which the thymic PVS fosters antigen-experienced B cells and plasma cells appear to be detrimentally amplified in some

autoimmune diseases. Often, the thymus of patients and animal models with active autoimmune pathology is burdened by numerous B cell follicles with active germinal centers and plasma cells that are a source of pathogenic autoantibodies.

AUTHOR CONTRIBUTIONS

JC, YH, DS, MB, MR, and SN conceptualized, wrote and edited the manuscript. JC and SN designed the figures. All authors contributed to the article and approved the submitted version.

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Therapeutic Potential of a Novel *Bifidobacterium* Identified Through Microbiome Profiling of RA Patients With Different RF Levels

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The potential therapeutic effects of probiotic bacteria in rheumatoid arthritis (RA) remain controversial. Thus, this study aimed to discover potential therapeutic bacteria based on the relationship between the gut microbiome and rheumatoid factor (RF) in RA. Bacterial genomic DNA was extracted from the fecal samples of 93 RA patients and 16 healthy subjects. Microbiota profiling was conducted through 16S rRNA sequencing and bioinformatics analyses. The effects of *Bifidobacterium* strains on human peripheral blood mononuclear cells and collagen-induced arthritis (CIA) mice were assessed. Significant differences in gut microbiota composition were observed in patients with different RF levels. The relative abundance of *Bifidobacterium* and *Collinsella* was lower in RF-high than in RF-low and RF-negative RA patients, while the relative abundance of *Clostridium* of Ruminococcaceae family was higher in RF-high than in RF-low and RF-negative patients. Among 10 differentially abundant *Bifidobacterium*, *B. longum* RAPO exhibited the strongest ability to inhibit IL-17 secretion. Oral administration of *B. longum* RAPO in CIA mice, obese CIA, and humanized avatar model significantly reduced RA incidence, arthritis score, inflammation, bone damage, cartilage damage, Th17 cells, and inflammatory cytokine secretion. Additionally, *B. longum* RAPO significantly inhibited Th17 cells and Th17-related genes—*IL-17A*, *IRF4*, *RORC*, *IL-21*, and *IL-23R*—in the PBMCs of rheumatoid arthritis patients. Our findings suggest that *B. longum* RAPO may alleviate RA

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by inhibiting the production of IL-17 and other proinflammatory mediators. The safety and efficacy of *B. longum* RAPO in patients with RA and other autoimmune disorders merit further investigation.

Keywords: rheumatoid arthritis, rheumatoid factor, microbiome, *Bifidobacterium longum*, T helper 17 cell

INTRODUCTION

Rheumatoid factor (RF) is an autoantibody detected in approximately 80% of patients with the autoimmune disease rheumatoid arthritis (RA) (1, 2). In addition to RA, RF has been implicated in other autoimmune diseases, including Sjögren's syndrome and systemic lupus erythematosus (3, 4). The presence of RF has also been reported in older individuals; however, its role remains unclear (5). Additionally, the mechanisms contributing to variation in RF levels in patients with RA are poorly understood (6–8).

Mounting evidence suggests the critical role of human RF as an IgG antigen in RA development and pathogenesis (9). Additionally, antigens of infectious microorganisms, such as Epstein–Barr virus, cytomegalovirus, parvovirus B19, and *Porphyromonas gingivalis*, can trigger RA development (10–14). The deposition of immune complexes containing RF in the joints activates the complement, resulting in type III hypersensitivity reactions and causing joint inflammation (15). During this inflammatory reaction, the membrane attack complex consisting of C5a and C5b-C9 is formed due to complement activation (15). C5a induces the migration of neutrophils and polymorphonuclear neutrophils to synovial cavities, and C5b-C9 activates peptidyl arginine deaminating enzymes (PADs) in neutrophils by attacking their cell membranes (9). Hypercitrullinated proteins activated by PADs and non-resolving inflammation increase the antigen pool in RA (9). A recent clinical study has shown that the presence of RF was linked to RA severity (16). Furthermore, RF has been identified as a predictor of drug response in patients with RA treated with anti-TNFα drugs (7).

The gut microbiome has emerged as a key regulator of RA development and progression. Notably, commensal segmented filamentous bacteria have been reported to activate T helper (Th)-17 lymphocytes in the lamina propria, inducing autoantibody production and aggravating RA (17). In a mouse RA model, Maeda et al. identified gut dysbiosis as an RA risk factor, as it activates autoreactive T cells and Th17 cells in the large intestine (18). Human studies have confirmed the strong association between gut microbiota and RA. Staggering differences in the diversity and composition of the gut microbiome have been reported in patients with new-onset RA (NORA). In particular, Scher et al. reported that the relative abundance of *Prevotella copri* was higher in patients with NORA than in healthy subjects, suggesting *P. copri* as a potential pathogen driving RA (19). Furthermore, a 27-kDa protein of *P. copri* has been shown to induce potent immune responses by stimulating Th1 cells in SKG mice (20). Meanwhile, the higher abundance of Oxalobacteraceae family showed a protective effect on RA in a large-scale association analysis with 24 population-based cohorts (21).

Notwithstanding the increasing evidence of the role of microbiome in RA, the relationship between the gut microbiome and RF is understudied. In this study, we conducted microbiome analyses using bioinformatics to investigate the association between intestinal microflora and RF levels. We also performed *in vivo* studies to determine whether a single strain of *Bifidobacterium*, which was found based on the microbiome analysis, could modulate RA symptoms in an RA mouse model.

MATERIAL AND METHODS

Inclusion and Exclusion Criteria

In this study, we enrolled 93 RA patients and 16 healthy subjects (**Table 1**). Patients with RA fulfilled the 2010 American College of Rheumatology and European League Against Rheumatism classification criteria (22). RA patients were classified based on RF levels into RF-negative ($n = 16$), RF-low ($n = 24$), and RF-high ($n = 53$) groups. Patients with RF ≤20 IU/ml were considered RF-negative, those with RF levels between 20 and 60 IU/ml were considered RF-low, and those with RF >60 IU/ml were considered RF-high. Subjects who had recently used antibiotics, probiotics, or prebiotics were excluded from the study. The demographic and clinical characteristics of the study subjects are summarized in **Table 1**. The age of healthy subjects (37–62) and RA patients including RF-negative (41–57), RF-low (40–59), and RF-high (40–60) was not significantly different (**Table 1**). The ratio of female among the subjects is 94% from healthy control subjects, 100% from RF-negative, 92% from RF-low, and 85% from RF-high. Additionally, disease activity score in 28 joints (DAS28), erythrocyte sedimentation rate (ESR), C-reactive protein levels, and current use of methotrexate or a biological disease-modifying anti-rheumatic drug were not statistically different between RF-negative, RF-low, and RF-high RA patients. The study design was approved by the Institutional Review Board of Seoul St. Mary's Hospital, The Catholic University of Korea (approval ID: KC17TNSI0570). Written informed consent was obtained from all study participants.

Microbiota Analysis

Individual human fecal samples were sent immediately with ice to the research site after collection in a plastic container and were stored at −70°C within 12 h of arrival. Isolation of the bacterial genomic DNA from the fecal samples was performed using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer, followed by

TABLE 1 | Clinical features of research subjects.

	RA patients				Control
	Positive			Negative (NG) (RF ≤ 20)	Normal (NM)
	Low positive (LP) (20 < RF ≤ 60)	High positive (HP) (RF > 60)	Total		
N	24	53	77	16	16
Age	51.1 ± 5.9	51.2 ± 5.7	51.2 ± 5.7	50.9 ± 5.5	49.2 ± 9.6
Female	22 (92)	45 (85)	67 (87)	16 (100)	15 (94)
DMARD-naive	5 (21)	16 (30)	21 (27)	3 (19)	–
Current use of MTX	19 (79)	33 (62)	52 (68)	12 (75)	–
Current use of bDMARD	9 (38)	15 (28)	24 (31)	6 (38)	
RF** (IU/ml)**	34.8 ± 10.4	279.5 ± 230.9	203.2 ± 222.5	8.4 ± 5.9	–
Anti-CCP (%, NG/LP/HP)	29.2/0/70.8	18.9/7.6/73.6	22.08/5.19/72.73	25.0/12.5/62.5	
DAS28 (score)	2.4 ± 0.8	2.8 ± 1.3	2.63 ± 1.16	2.8 ± 1.5	–
ESR (mm/h)	12.7 ± 8.6	16.1 ± 15.6	15.0 ± 13.8	12.9 ± 8.3	–
CRP (mg/dl)	0.3 ± 0.5	0.6 ± 1.6	0.5 ± 1.3	0.4 ± 0.7	–

All values are presented as mean ± standard deviation, n (%), or n. All statistical analyses were done with Kruskal–Wallis test and chi-square test. The quadruple asterisks indicate p-value under 0.0001.

DMARD, disease-modifying anti-rheumatic drug; MTX, methotrexate; bDMARD, biological DMARD.

bead beating on a TissueLyser system (Qiagen). Quantification of the bacterial genomic DNA was carried out using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). For sequencing, 16S rRNA gene amplification and index PCR were conducted following the Illumina 16S Metagenomic Sequencing Library preparation guide (Illumina, San Diego, CA, USA). For amplification of V3 and V4 regions, the 16S sequence was amplified using forward primer (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and reverse primer (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′). For indexing, Nextera XT Index 1 and 2 Primers from a Nextera XT Index kit (Illumina) were used. Each PCR product was purified using AMPure XP beads (Beckman Coulter, Pasadena, CA, USA). DNA sequencing was conducted in using the paired-end method (300 bpx2) with an Illumina MiSeq instrument according to the Illumina protocol. Raw 16S rRNA sequences were bioinformatically analyzed using QIIME 2 version 2019.4 (23), as previously described (24).

Bacteria Isolation From Human Fecal Samples

Transgalactooligosaccharide (TOS) antibiotic mupirocin agar medium, a selective medium for *Bifidobacterium*, was used to separate *Bifidobacterium* from 100 mg of human fecal samples. After plating fecal samples diluted with phosphate-buffered saline (PBS) and culturing for 30 h at 37°C in an anaerobic jar under anaerobic condition, colonies were randomly taken from a plate from each sample and inoculated with cysteine De Man, Rogosa, and Sharpe (cMRS) broth and cultured for 24 h. The above process was performed in a glove box for blocking oxygen. Identification of bacteria strain was done with 16S rRNA sequencing service of Macrogen Inc. (Seoul, Korea).

Bacterial Preparation

Bacterial samples (50μl) were inoculated into 5 L of cMRS liquid medium and incubated for 20 h at 37°C. After incubation, the bacteria were collected *via* centrifugation (2236HR high-speed centrifuge; Gyrozen, Gimpo, Korea) at 20°C and 7,000 rpm. After two washes with PBS, the bacterial samples were dried at 36°C and centrifuged at 2,000 rpm for 24 h using a Scanvac Speed Vacuum Concentrator (LaboGene Aps, Lillerød, Denmark). All bacteria used in this study were isolated from healthy subjects and RA patients. Donor characteristics are summarized in **Table 1**.

Mice

Seven-week-old male DBA/1J mice (Orient Bio, Gyeonggi-do, Korea) and 7-week-old male NOD-scid IL2rγ^{null} (NSG) mice (Jackson Laboratory, Bar Harbor, ME, USA) were maintained under specific pathogen-free conditions in an animal facility with controlled humidity (55 ± 5%), light (12 h/12 h light/dark), and temperature (22 ± 1°C). The air in the facility passed through a HEPA filter system designed to exclude bacteria and viruses. Animals were fed mice chow (normal chow and 60 kcal chow) and water *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the School of Medicine and the Animal Research Ethics Committee of the Catholic University of Korea and were conducted in accordance with the Laboratory Animals Welfare Act according to the Guide for the Care and Use of Laboratory Animals. All experimental procedures were evaluated and conducted in accordance with the protocols approved by the Animal Research Ethics Committee at the Catholic University of Korea (Permit Number: CUMC 2019-0242-01). All procedures performed in this study followed the ethical guidelines for animal use.

Generation of Collagen-Induced Arthritis and Obese Collagen-Induced Arthritis Mice

Collagen-induced arthritis (CIA) was established in male DBA/1J mice. Briefly, mice were inoculated with 100 μg of chicken type II collagen (CII; Chondrex Inc., Redmond, WA, USA) liquified

overnight in 0.1 N acetic acid (4 mg/ml) in complete Freund's adjuvant. Immunizations were performed intradermally *via* injection into the bottom of the tail. At 2 weeks following immunization, the mice were boosted with 100 μg of CII in incomplete Freund's adjuvant (Chondrex Inc.). In addition, the obese CIA mouse group was fed a high-fat diet (60 kcal of fat) at primary immunization. A HFD was maintained until the sacrifice.

Generation of Humanized Avatar Arthritis Model

To induce arthritis in humanized mice, 5×10^5 peripheral blood mononuclear cells (PBMCs) of RA patients were injected into the tail vein. At 2 weeks after, orbital blood was collected to confirm engraftment by flow cytometry. Orbital blood was immunostained with CD4 T cells and CD8 T cells, and the cells were stained with Alexa Flour 700-conjugated anti-human CD4 antibody (BD Biosciences, San Diego, CA, USA) and allophycocyanin (APC)-Cy7-conjugated anti-human CD8 (BD Biosciences). Thereafter, 1 week after confirmation of engraftment, the mice were sensitized with 50 μg of lipopolysaccharide (Sigma Aldrich, St. Louis, MO, USA). Each *in vivo* experiment was repeated a total of three times.

Clinical Assessment of Arthritis

Arthritis severity was determined using the mean arthritis index, which ranges from 0 to 4, as follows: 0, no evidence of erythema or swelling; 1, erythema and mild swelling confined to the midfoot (tarsals) or ankle joint; 2, erythema and mild swelling extending from the ankle to the midfoot; 3, erythema and moderate swelling extending from the ankle to the metatarsal joint; and 4, erythema and severe swelling encompassing the ankle, foot, and digits. The severity of arthritis was determined as the sum of scores from all legs assessed by two independent observers blinded to the experimental groups.

Histological Analysis

Mouse joint tissues were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), decalcified in a histological decalcifying solution (Calci-Clear Rapid; National Diagnostics, Atlanta, GA, USA), and embedded in paraffin wax for histological analyses. Sections (7 μm) were prepared and stained with hematoxylin (YD Diagnostics, Yongin, Korea), eosin (Muto Pure Chemicals Co., Ltd., Tokyo, Japan), and safranin O (Sigma-Aldrich). Cartilage damage was marked as described previously (25).

Immunohistopathological Analysis of Arthritis

Joint tissues were incubated overnight at 4°C with primary antibodies against TNFα (Abcam, Cambridge, UK), IL-17 (Abcam), IL-6 (Abcam), and IL-1β (Novus Biologicals, Littleton, CO, USA). Subsequently, samples were incubated with a biotinylated streptavidin–peroxidase complex for 1 h, and the signals were developed using chromogen 3,3′-diaminobenzidine (Thermo Scientific, Rockford, IL, USA). The sections were examined under a photomicroscope (Olympus, Tokyo, Japan). The number of positive cells in high-power digital images (magnification, ×400) was counted using Adobe Photoshop software (Adobe, San Jose, CA, USA). Stained cells were counted independently by three observers, and the mean values were evaluated.

Measurement of Anti-CII Antibodies and Immunoglobulin

Serum was gathered 7 weeks after the first immunization for concentration of the IgA, IgM, IgG2a, and collagen-specific IgG2a levels, and the serum was stored at −70°C until further use. The levels of the IgA, IgM, IgG2a, and collagen-specific IgG2a antibodies in the serum were measured using ELISA (Bethyl Laboratories, Montgomery, TX, USA).

Flow Cytometry Analysis

Cytokine expression in mice was analyzed *via* intracellular staining with the following antibodies: fluorescein isothiocyanate-conjugated anti-IL-17, APC-conjugated anti- interferon-gamma (IFN-γ), and PerCP-conjugated anti-CD4. Before staining, cells were stimulated for 4 h with phorbol myristate and ionomycin in the presence of GolgiStop (BD Biosciences). To analyze regulatory T (Treg) populations, splenocytes were stained with PerCP-conjugated anti-CD4, APC-conjugated anti-CD25, and PE-conjugated anti-Foxp3 antibodies. T helper and Treg populations were determined using specific antibodies (eBioscience, San Diego, CA, USA). After surface staining for 30 min, the cells were permeabilized with Cytofix/Cytoperm solution (BD Biosciences). Thereafter, the cells were intracellularly stained with fluorescent antibodies. Flow cytometry was performed with the aid of a cytoFLEX Flow Cytometer (Beckman Coulter, Brea, CA, USA), and flow cytometry data were analyzed using FlowJo (Tree Star, Ashland, OR, USA).

Isolation and Stimulation of Peripheral Blood Mononuclear Cells

PBMCs were isolated from healthy donors. Briefly, blood from donors was mixed with PBS, and the mixture was cautiously layered onto 10 ml of Ficoll Plaque Plus solution (GE Healthcare Life Sciences, Marlborough, MA, USA). Blood samples were centrifuged for 30 min at 2,500 rpm and 20°C to separate blood contents. After centrifugation, the layer containing PBMCs was collected, and the cells were washed with PBS and cultured in RPMI medium containing 10% fetal bovine serum. PBMCs were incubated with plate-bound anti-CD3 (0.5 μg/ml) and then subjected to IL-17 analysis by ELISA for 3 days or subjected to RNA sequencing and real-time polymerase chain reaction analysis for 2 days.

Enzyme-Linked Immunosorbent Assay

The supernatant was collected 3 days after *B. longum* RAPO or vehicle treatment with anti-CD3 (0.5 μg/ml). IL-17 was analyzed by sandwich enzyme-linked immunosorbent assay (ELISA; IL-17 DuoSet ELISA; R&D Systems, Lille, France). The absorbance at 450 nm was determined using an ELISA microplate reader (Molecular Devices).

mRNA Sequencing Data

We preprocessed the raw reads from the sequencer to remove low-quality and adapter sequence before analysis and aligned the processed reads to *Homo sapiens* (*hg38*) using HISAT v2.1.0 (26). HISAT utilizes two types of indexes for alignment (a global, whole-genome index and tens of thousands of small local indexes). These two types of indexes are constructed using the same BWT (Burrows–Wheeler transform) and a graph FM index (GFM) as Bowtie2. Because of its use of these efficient data structures and algorithms, HISAT generates spliced alignments several times faster than Bowtie and BWT that are widely used. The reference genome sequence of *Homo sapiens* (*hg38*) and the annotation data were downloaded from the UCSC table browser (http://genome.uscs.edu). Transcript assembly and abundance estimation used StringTie (27, 28). After alignment, StringTie v1.3.4d was used to assemble aligned reads into transcripts and to estimate their abundance. It provides the relative abundance estimates as read count values of transcript and gene expressed in each sample. Also, transcript assembly of known transcripts, novel transcripts, and alternative splicing transcripts was processed by StringTie v2.1.3b.

Statistical Analysis of Gene Expression Level

The relative abundances of gene were measured in read count using StringTie. We performed statistical analysis to find differentially expressed genes using the estimates of abundances for each gene in the samples. Genes with one more than zeroed read count values in the samples were excluded. To facilitate log2 transformation, 1 was added to each read count value of filtered genes. Filtered data were log2-transformed and subjected to TMM normalization. Statistical significance of the differential expression data was determined using exactTest using edgeR and fold change in which the null hypothesis was that no difference exists among groups. False discovery rate (FDR) was controlled by adjusting *p*-value using the Benjamini–Hochberg algorithm. For the DEG set, hierarchical clustering analysis was performed using complete linkage and Euclidean distance as a measure of similarity. Gene enrichment and functional annotation analysis and pathway analysis for significant gene list were performed based on gProfiler (https://biit.cs.ut.ee/gprofiler/gost) and KEGG pathway (http://www.genome.jp/kegg/pathway.html).

Real-Time Polymerase Chain Reaction

Total RNA was extracted using TRI reagent (Molecular Research Center, USA) and cDNA was synthesized with the Dyne First Strand cDNA Synthesis Kit (Dyne Bio, South Korea) according to the protocol of the manufacturer. mRNA was quantified using the StepOnePlus™ Real-Time PCR systems (Applied Biosystems, USA) with SensiFAST SYBR Hi-ROX (Bioline, USA). mRNA levels were normalized to that of β-actin. The following primers were used: β-actin 5′-GGACTT CGAGCAAGAGATGG-3′ and 5′-TGTGTTGGGGTACAGG TCTTTG-3′; IL-17A 5′-CAACCGATCCACCTCACC TT-3′ and 5′-GGCACTTTGCCTCCCAGAT-3′; RORC 5′-AGTCGG AAGGCAAGATCAGA-3′ and 5′-CAAGAGAGGTTCTGG GCAAG-3′; IL-21 5′-TGTGAATGACTTGGACCCTGAA-3′ and 5′-AAACAG GAAATAGCTGACCACTCA-3′; IRF-4 5′-CCTGCAAGCTCTTTGACACA-3′ and 5′-GAGTCACCTGG AATCTTGGC-3′; and IL-23R 5′-AGGTACTGGCAG CCTTGGAGTT-3′ and 5′-CCCTGTAGAGATGG AAGCAACTG-3′.

Statistical Analysis

Non-parametric statistical tests (Mann–Whitney test and Kruskal–Wallis test) were used to compare the alpha diversity of each group and the relative abundance of each taxon. Pairwise permutational analysis of variance (PERMANOVA) was used to compare beta diversity between groups. Spearman correlation analysis was used to analyze the relationship between the relative abundance of each taxon and RF levels. Data were analyzed using GraphPad Prism (V.5 for Windows; GraphPad Software Inc., La Jolla, CA, USA) and are presented as means ± standard deviations. Comparisons of numerical data between two groups were performed using Student's *t*-test or the Mann–Whitney *U*-test. Differences in the mean values of more than two groups were assessed using analysis of variance with a *post-hoc* test. *p*-values <0.05 (two-tailed) were considered to indicate statistical significance.

RESULTS

Comparison of Bacterial Alpha and Beta Diversities

To examine the relationship between RF levels and changes in intestinal microbial diversity, we performed gut microbiome profiling through 16S rRNA sequencing of fecal samples from 77 RF-positive patients, 16 RF-negative patients, and 16 healthy volunteers (**Table 1**). Raw sequences were filtered and merged, and 1,284,582 reads were grouped into operational taxonomic units (OTUs) based on 97% similarity, with a mean of 11,059 across 110 samples. Alpha diversity (Faith's PD), OTU number, Pielou's evenness, and Shannon's diversity index did not differ significantly between the RF-negative, RF-low, and RF-high groups (**Supplementary Figures 1A–D**). However, there were significant differences in beta diversity between RF-negative and RF-low RA patients and healthy subjects (**Supplementary Figures 1F, G**). Notably, RF-negative RA patients had a significantly lower Shannon index compared with healthy subjects (**Supplementary Figure 1A**). RF-low individuals exhibited a significantly lower Shannon index and OTU number compared with healthy volunteers (**Supplementary Figures 1A, B**). Jaccard distance-based beta diversity analysis revealed significant differences in the microbial community between healthy subjects and RF-low RA patients ($p = 0.027$, PERMANOVA; **Supplementary Figure 1F**), as well as between healthy subjects and RF-high RA patients ($p = 0.010$, PERMANOVA; **Supplementary Figure 1F**). Unweighted UniFrac distance-based microbial structure analysis showed significant differences between healthy subjects and

RF-negative RA patients (p = 0.045, PERMANOVA; **Supplementary Figure 1G**), as well as between healthy subjects and RF-high RA patients (p = 0.003, PERMANOVA; **Supplementary Figure 1G**). Other beta diversity matrices did not differ significantly among the groups (**Supplementary Figures 1E, H**).

Association Between RF Levels and Microbial Composition

Although there were no differences in microbial diversity in RA patients according to RF level, the Kruskal–Wallis test revealed significant differences in the composition of individual taxa. We identified 11 differentially abundant taxa at the false discovery rate of 10%; 10 taxa were enriched in healthy volunteers, and one taxon was enriched in RA patients (**Table 2**). At the phylum level, Actinobacteria were less abundant in RF-high RA patients than in healthy individuals and RF-negative RA patients (**Figures 1A, B**). The relative abundance of Actinobacteria in all RA patients was negatively correlated with RF level (R = −0.29, p = 00051; **Figure 1C**). By contrast, the abundance of the phylum Firmicutes in all RA patients was positively correlated with RF levels (R = 0.22, p = 0.0322; **Figure 1D**). Within the Actinobacteria phylum, *Bifidobacterium* were significantly more abundant in RF-negative than in RF-high RA patients (**Figure 2A**). Another genus of the Actinobacteria phylum, *Collinsella*, was more abundant in healthy volunteers than in RF-negative or RF-high RA patients; however, *Collinsella* abundance did not differ significantly among RA patients (**Figure 2B**). Similarly, *Clostridium* species were more abundant in healthy individuals than in the different RA patient groups, although their abundance was similar across RA patient groups (**Figure 2C**). Compared with RF-negative RA patients and healthy individuals, RF-high RA patients exhibited a lower prevalence of the genera *Bifidobacterium*, *Collinsella*, and *Clostridium* (**Supplementary Figures 3A–C**). The prevalence of *Veillonellaceae* family was the highest among the control and RA patients (**Supplementary Figure 3D**).

Among the genera of the Actinobacteria phylum, the genus *Bifidobacterium* exhibited a negative correlation with RF levels (R = −0.22, p = 0.0326; **Figure 2D**). Furthermore, the abundance of the genus *Ruminococcus* was positively correlated with RF levels (R = 0.25, p = 0.0161; **Figure 2D**).

Bifidobacterium longum RAPO Therapy Reduced the Severity of CIA

Bifidobacterium genera can be cultivated despite being anaerobes and are widely known for immune regulation ability. Hence, we decided to focus on *Bifidobacterium* genus among the differentially abundant genera for further study. To identify the most potent *Bifidobacterium* strain among the *Bifidobacterium* isolated from healthy individuals and RA patients, we treated PBMCs (stimulated with anti-CD3) with *B. pseudocatenulatum* 20T2, *B. pseudocatenulatum* 20T1, *B. pseudocatenulatum* 18T6, *B. pseudocatenulatum* 5T4, *B. longum* 4L7, *B. longum* 4L6, *B. adolescentis* 8T3, *B. adolescentis* 8T1, *B. bifidum* 7T2, and *B. longum* RAPO. Among these strains, *B. longum* RAPO exhibited the most potent inhibitory effect on IL-17 secretion (**Figure 3**).

To evaluate the effects of *B. longum* RAPO in CIA, we orally administrated 1×10^8 CFU/mouse *B. longum* RAPO alone, methotrexate 3 mg/kg alone, or vehicle to CIA mice at 3 weeks after immunization. Mice developed RA at 21 days after immunization; however, oral administration of *B. longum* RAPO to CIA mice decreased CIA, as demonstrated by a significantly reduced arthritis score compared with the vehicle-treated mice (**Figure 4A**). In mice that were given oral administration of *B. longum* RAPO, the severity of arthritis damage was attenuated, and cartilage protection was improved, compared with the control mice. Inflammation score, bone damage, and cartilage damage were also significantly decreased in mice treated with *B. longum* RAPO (**Figure 4B**). Importantly, the frequencies of $CD4^+$ T cells producing IFN-γ and IL-17 were significantly lower in mice treated with *B. longum* RAPO than in control mice treated with the vehicle. Furthermore, administration with *B. longum* RAPO resulted in an increased

TABLE 2 | Differentially enriched taxa in rheumatoid patients and healthy subjects.

Taxa		*p*-value	FDR	NM mean (%)	NG mean (%)	LP mean (%)	HP mean (%)	NM prevalence (%)	NG prevalence (%)	LP prevalence (%)	HP prevalence (%)
Enriched taxa in NM											
Phylum	Actinobacteria	0.0001	0.0006	3.69^{a}	3.43^{a}	1.7^{ab}	1.34^{b}	87.50	100.00	83.33	81.13
Class	Actinobacteria	0.0051	0.0281	2.04^{ac}	2.27^{ab}	1.26^{ac}	0.99^{c}	87.50	93.75	79.17	71.70
	Coriobacteriia	0.0000	0.0004	1.65^{a}	1.16^{ab}	0.44^{bc}	0.35^{cd}	87.50	75.00	50.00	47.17
Order	Bifidobacteriales	0.0084	0.0546	1.97^{ab}	2.24^{b}	1.21^{ab}	0.99^{ac}	87.50	93.75	79.17	71.70
	Coriobacteriales	0.0000	0.0005	1.65^{a}	1.16^{ab}	0.44^{bc}	0.35^{cd}	87.50	75.00	50.00	47.17
Family	Bifidobacteriaceae	0.0084	0.0756	1.97^{ab}	2.24^{b}	1.21^{ab}	0.99^{ac}	87.50	93.75	79.17	71.70
	Coriobacteriaceae	0.0000	0.0007	1.65^{a}	1.16^{ab}	0.44^{bc}	0.35^{cd}	87.50	75.00	50.00	47.17
Genus	*Bifidobacterium*	0.0084	0.0840	1.97^{ab}	2.24^{b}	1.21^{ab}	0.99^{ac}	87.50	93.75	79.17	71.70
	Collinsella	0.0005	0.0094	1.14^{a}	0.58^{ab}	0.26^{bc}	0.24^{bc}	81.25	50.00	41.60	33.96
	Clostridium	0.0088	0.0587	3.42^{a}	1.39^{b}	1.52^{b}	2.03^{b}	93.75	93.75	83.33	83.02
Enriched taxa in RA											
Family	Veillonellaceae	0.0156	0.0936	1.34^{ab}	1.73^{bc}	3.14^{bc}	3.03^{c}	62.50	81.25	79.17	90.57

Comparisons were analyzed using Kruskal–Wallis tests. The different alphabet characters indicate statistically significant differences of each value among NM, NG, LP, and HP with p-value less than 0.05. The FDR was calculated with Benjamini–Hochberg procedure.

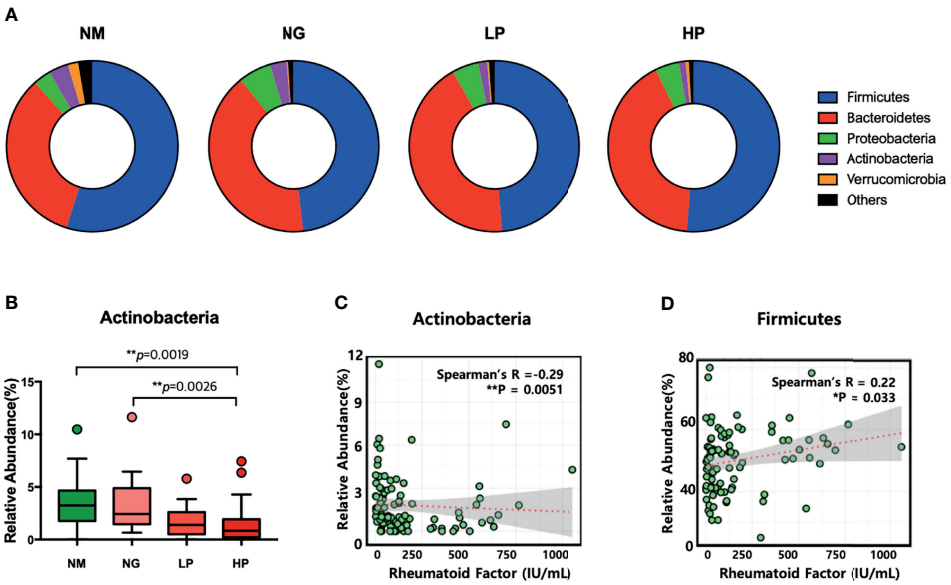

FIGURE 1 | Microbial composition at the phylum level in patients with different rheumatoid factor (RF) levels. **(A)** Comparison of intestinal microflora composition at the phylum level. **(B)** Relative abundance of Actinobacteria. Comparisons in relative abundance were performed using the Kruskal–Wallis test. **(C)** Correlation between the relative abundance of Actinobacteria and RF levels. **(D)** Correlation between the relative abundance of Firmicutes and RF levels. *$p < 0.05$; **$p < 0.01$. NM, normal control; NG, rheumatoid arthritis (RA) patients showing 20 or less of RF level; LP, RA patients showing over 20 and not exceeding 60 of RF level; HP, RA patients showing over 60 of RF level.

Treg population, compared with the control (**Figure 4C**). Serum IgA, rheumatoid factor IgM, IgG2a, and CII-specific IgG2a levels were reduced by the administration of *B. longum* RAPO (**Figure 4D**). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (*vs.* vehicle-treated group).

Effect of *Bifidobacterium longum* RAPO on Obese CIA

An obese arthritis model was reported according to a previous report (29). Obese CIA mice had a more serious state of disease and a higher arthritis score compared with CIA. To investigate the effect of *B. longum* RAPO in obese CIA, obese CIA-induced RA mice were fed the HFD for 7 weeks. There was a significant decrease in the arthritis score and incidence in the *B. longum* RAPO-administered mice compared with the vehicle-treated obese CIA mice (**Figure 5A**). We then performed effector T-cell staining on splenocytes from *B. longum* RAPO- and vehicle-treated mice. Flow cytometry analysis of splenocytes showed that the Th17 (IL-17$^+$ in CD4$^+$ T cells) population was reduced by the administration of *B. longum* RAPO; on the other hand, the populations of Th1 (IFN-γ^+ in CD4 T cells) and Th2 (IL-4$^+$ in CD4$^+$ T cells) did not differ significantly between *B. longum* RAPO- and vehicle-treated mice with obese CIA (**Figure 5B**). In mice that received *B. longum* RAPO, the severity of arthritis was attenuated, and cartilage conservation was improved, compared with the vehicle-treated mice (**Figure 5C**). We also analyzed *B. longum* RAPO effects on inflammatory cytokine staining of joints which showed that *B. longum* RAPO treatment diminished the levels of IL-1β, IL-6, IL-17, and TNFα (**Figure 5D**). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (*vs.* vehicle-treated group).

Inhibition of Th17 Cells by *Bifidobacterium longum* RAPO Treatment on Human PBMCs *In Vitro*

The PBMCs of RA patients were cultured under anti-CD3 0.5 μg/ml conditions with *B. longum* RAPO for 72 h. Flow cytometry showed that *B. longum* RAPO treatment significantly suppressed Th1 and Th17 cell proliferation (**Figure 6A**). We next analyzed by RNA sequencing the gene expression profiles of RA PBMC treatment with *B. longum* RAPO in the presence of anti-CD3 0.5 μg/ml for 48 h. When demonstrated by absent/present classification and using at least two-fold difference in expression as the cutoff, 5,089 genes were differentially expressed in vehicle-treated RA PBMCs versus *B. longum* RAPO-treated RA PBMCs. Among those in the Th17 pathway, *Il17*, *IRF4*, *RORC*, *Sll21*, and *Il23* were downregulated by *B. longum* RAPO (**Figure 6B**). Also, Th17-related genes confirmed that *Il17*, *IRF4*, *RORC*, *Sll21*, and *Il23* were downregulated by qPCR (**Figure 6C**). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (*vs.* vehicle-treated group).

Bifidobacterium longum RAPO Ameliorates Human Avatar Arthritis Mice

Arthritis score and incidence were examined to investigate whether oral administration of *B. longum* RAPO can have an effect on human avatar arthritis mice. The engraftment of the patient PBMC was confirmed by analyzing the expression of

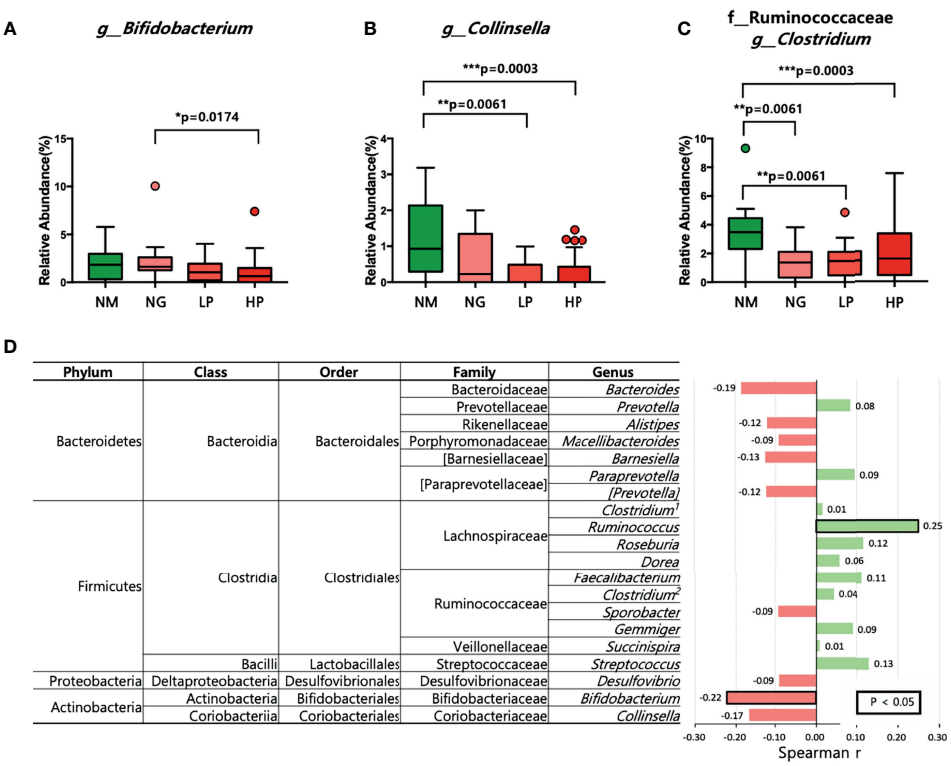

FIGURE 2 | Microbial composition at the genus level in patients with different RF levels. **(A–C)** Comparison of the relative abundance of **(A)** *Bifidobacterium*, **(B)** *Collinsella*, and **(C)** *Clostridium* using the Kruskal–Wallis test. **(D)** Correlations between the relative abundance of each of the 20 most abundant genera and RF levels. Positive correlations are shown in green, and negative correlations are shown in pink. The numbers in bold indicate statistically significant correlations ($p < 0.05$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. NM, normal control; NG, RA patients showing 20 or less of RF level; LP, RA patients showing over 20 and not exceeding 60 of RF level; HP, RA patients showing over 60 of RF level.

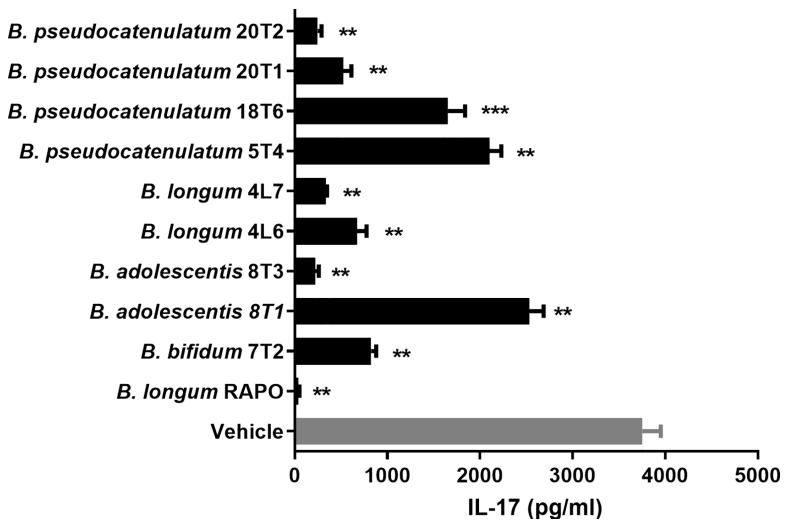

FIGURE 3 | *Bifidobacterium longum* RAPO suppresses IL-17 expression in human PBMCs. PBMCs were stimulated with anti-CD3 for 72 h. IL-17 levels in the culture supernatant were analyzed using ELISA. Data are presented as the means ± standard deviations (SDs) from three independent experiments. **$p < 0.03$; ***$p < 0.01$.

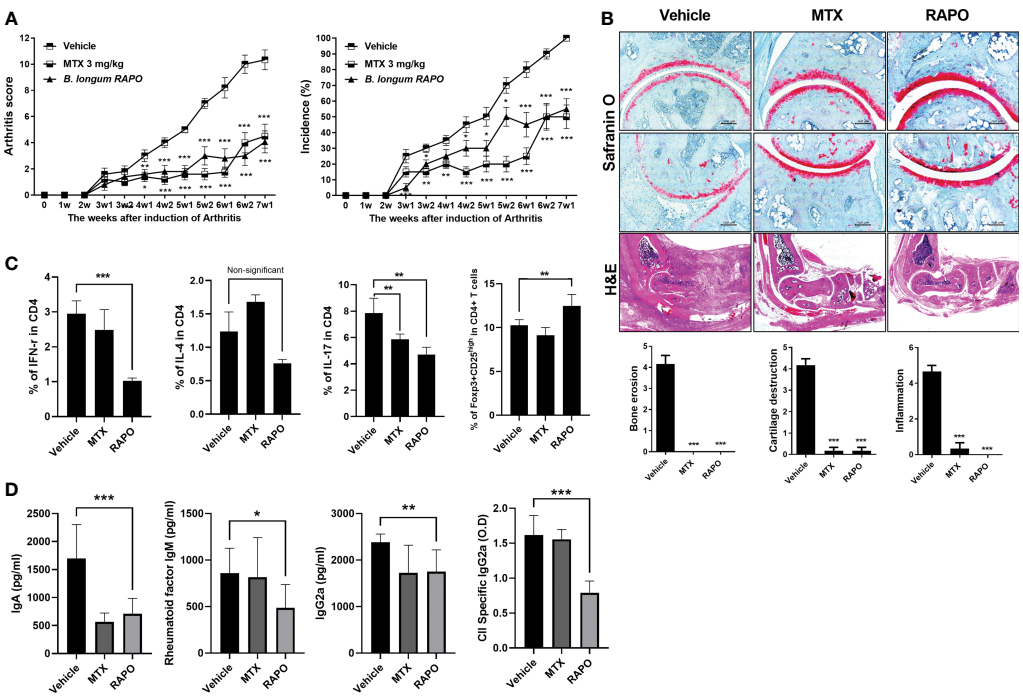

FIGURE 4 | *Bifidobacterium longum* RAPO alleviates RA in collagen-induced arthritis (CIA) mice. CIA mice (n = 5 per group) were orally administered *B. longum* RAPO (1×10^8 CFU/mouse) or methotrexate (MTX; 3 mg/kg) once daily for 7 weeks after the immunization boost. **(A)** Reductions in arthritis score and arthritis incidence in CIA mice treated with *B. longum* RAPO. Effects of *B. longum* RAPO on RA development in CIA mice. **(B)** Tissue specimens were acquired from the hind paw joints of mice and stained with hematoxylin and eosin and safranin O. Representative histological quality and histological grades are shown. **(C)** Splenocytes isolated at 7 weeks after immunization were stimulated with phorbol myristate, ionomycin, and GolgiStop for 4 h. The percentages of Th1 ($CD4^+IFN\text{-}\gamma^+$), Th2 ($CD4^+IL\text{-}4^+$), and Th17 ($CD4^+IL\text{-}17^+$) cells were analyzed using flow cytometry, including also Treg ($CD4^+CD25^{high}$ $Foxp3^+$). **(D)** Levels of IgA, IgM, IgG2a, and anti-CII-specific IgG2a antibodies in the serum of CIA mice at 7 weeks after the first immunization. Data are presented as the means ± SDs. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (*vs*. vehicle-treated group).

CD4 and CD8 T cells using human antibodies (**Supplementary Figure 4D**). The *B. longum* RAPO-treated group had a lower arthritis score and incidence than the vehicle group (**Figure 6D**).

Also, it was confirmed that Th17 cells were effectively inhibited like CIA and obese CIA (**Figure 6E**). Histological analysis was performed using safranin O staining to confirm the degree of cartilage destruction in avatar mice of RA patient. The cartilage damage score was low in the *B. longum* RAPO-administered group (**Figure 6F**). **, $p < 0.01$; ***, $p < 0.001$ (*vs*. vehicle-treated group).

DISCUSSION

In this study, we investigated the gut microbiota composition in RA patients with varying RF levels and the effects of *B. longum* RAPO on RA. Previous studies have shown that targeted therapies can modulate the gut microbiota composition and increase the abundance of beneficial microorganisms (30). Here, we found significant differences in the gut microbiota composition between healthy individuals and RA patients with varying RF levels. In accordance with our previous findings (24), we found that RA patients had altered levels of *Collinsella* among other Actinobacteria. Species of the genus *Collinsella* have been demonstrated to modify host levels of bile acids and plasma cholesterol by producing various beneficial short-chain fatty acids, such as butyric acid, acetic acid, formic acid, and lactic acid (31–34). Chen et al. reported that the relative abundance of *Collinsella aerofaciens* is higher in RA patients (35). However, as the genus *Collinsella* consists of nine diverse species, species-specific or strain-specific effects are possible (34). Members of Actinobacteria, and the genus *Collinsella* in particular, seem to be enriched in the gut microbiota of RA patients.

Several studies have profiled the gut microbiome in RA patients, mainly in early-stage RA patients. Scher et al. found that *Prevotella copri* was more abundant in NORA patients than in healthy subjects, and Alpizar-Rodriquez et al. confirmed that *P. copri* was enriched in individuals at risk of RA (19, 36). However, microbiome profiling studies of RA patients with different levels of clinical indices remain limited. The presence of RF is associated with RA severity independent of anti-citrullinated protein antibodies (16). To elucidate the relationship between RF and gut microbiome composition, we controlled for anti-citrullinated protein antibodies and other clinical indices other than RF. The gut microbiome of RA patients with different RF levels exhibited significant

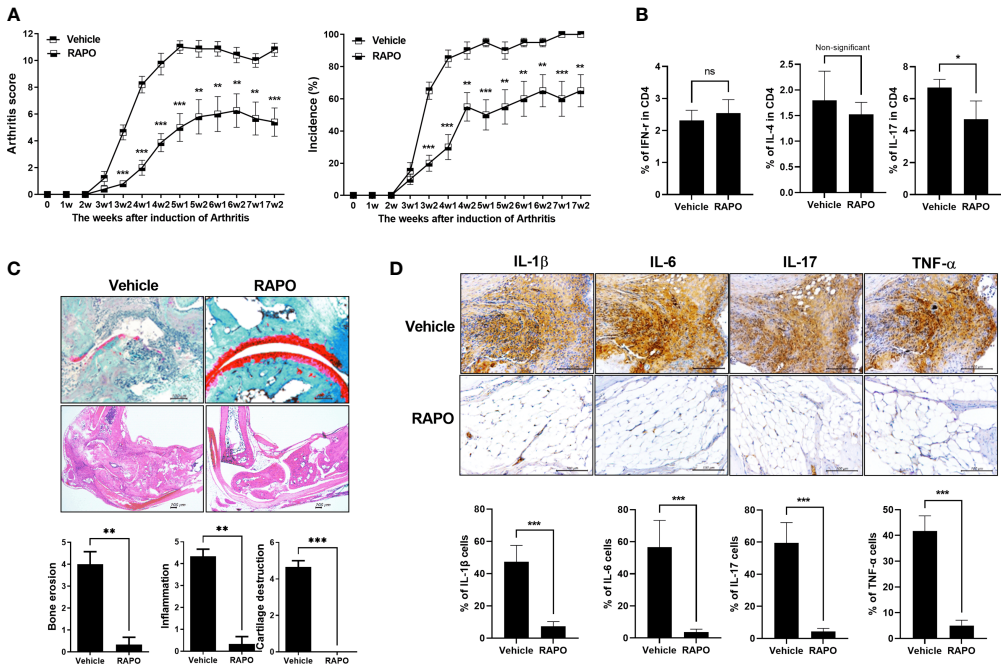

FIGURE 5 | Effects of *Bifidobacterium longum* RAPO in obese CIA mice. Mice were administered orally with *B. longum* RAPO (1×10^8 CFU/mouse) once daily for 7 weeks after the immunization boost. **(A)** Arthritis score and incidence of *B. longum* RAPO-treated mice compared with those of obese CIA mice ($n = 5$ for each group). **(B)** *B. longum* RAPO reduces IL-17 expression in CD4 T cells from the spleen of mice with obese CIA. Flow cytometry of Th1 cells (IFN-r$^+$CD4$^+$), Th2 cells (IL-4$^+$CD4$^+$), and Th17 cells (CD4$^+$IL17$^+$) from the spleen of mice with obese CIA. **(C)** Effect of *B. longum* RAPO on RA in mice with obese CIA. Tissue from the hind paw joints was stained with hematoxylin and eosin, as well as safranin O. **(D)** *B. longum* RAPO inhibits the proinflammatory cytokines IL-1β, IL-6, IL-17, and TNFα in CIA mice. Representative immunohistochemistry images showing that *B. longum* RAPO alleviates RA in obese CIA mice. Synovium sections treated with a vehicle, *B. longum* RAPO, or vehicle were stained for IL-1β, IL-6, IL-17, and TNFα. Scale bar, 100 μm. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (*vs.* vehicle-treated group).

differences in terms of alpha and beta diversities. Microbial composition differed profoundly among the groups. Notably, Actinobacteria and *Bifidobacterium* were less abundant in RF-positive RA patients than in their RF-negative counterparts, with RA patients with RF >60 IU/ml exhibiting the lowest relative abundance of those taxa. Moreover, the Actinobacteria phylum and *Bifidobacterium* genus exhibited a strong negative correlation with RF levels, suggesting that these taxa may be involved in RA in an RF-dependent manner. Hence, in RA patients with high RF titers, a low abundance of *Bifidobacterium* could be considered a risk factor of RA.

A reduced relative abundance of *Bifidobacterium* has been reported in patients with RA and other immune-related diseases. Vogt et al. reported that bifidobacteria were less abundant in patients with Alzheimer's disease than in healthy subjects and that the relative abundance of bifidobacteria was negatively correlated with the extent of amyloid deposition (37). Interestingly, Matson et al. showed that melanoma patients had a lower abundance of *Bifidobacterium* than did control subjects (38). Additionally, alterations in the abundance of bifidobacteria are prominent in elderly people (39–41). A large-scale association study with 18,340 individuals from 24 population-based cohorts of European, Hispanic, Middle Eastern, Asian, and African ancestries also identified that the association between the lactase (LCT) gene locus and the abundance of *Bifidobacterium* was age-dependent (21). These findings suggest that a reduction in the abundance of *Bifidobacterium* in patients with high RF levels may reflect an unhealthy status.

To assess whether the reduction in the abundance of *Bifidobacterium* is the cause or a consequence of RA, we administered bifidobacteria to mice with RA. *Bifidobacterium longum* RAPO was identified as the *Bifidobacterium* strain with the strongest ability to inhibit IL-17 production. In mice, *B. longum* RAPO treatment significantly reduced RA incidence, RA score, and the production of autoantibodies. Importantly, administration of *B. longum* RAPO in RA mice significantly inhibited joint inflammation and prevented bone damage, cartilage damage, and loss of large intestine tissue. These findings strongly suggest that oral administration of *B. longum* RAPO may alleviate RA and other autoimmune diseases.

Additionally, oral administration of *B. longum* RAPO significantly reduced the frequencies of IFN-γ-positive and IL-17-positive CD4$^+$ T cells, suggesting that *B. longum* RAPO components may modulate the development and function of Th17 cells and regulatory T cells.

Through RNA sequencing analysis, we also indicated that *B. longum* RAPO treatment inhibited the expression of *IL-17A*, *IRF4*, *RORC*, and *IL-23R*. These results significantly reduced IL-17 in collagen-induced arthritis, obese arthritis, and avatar

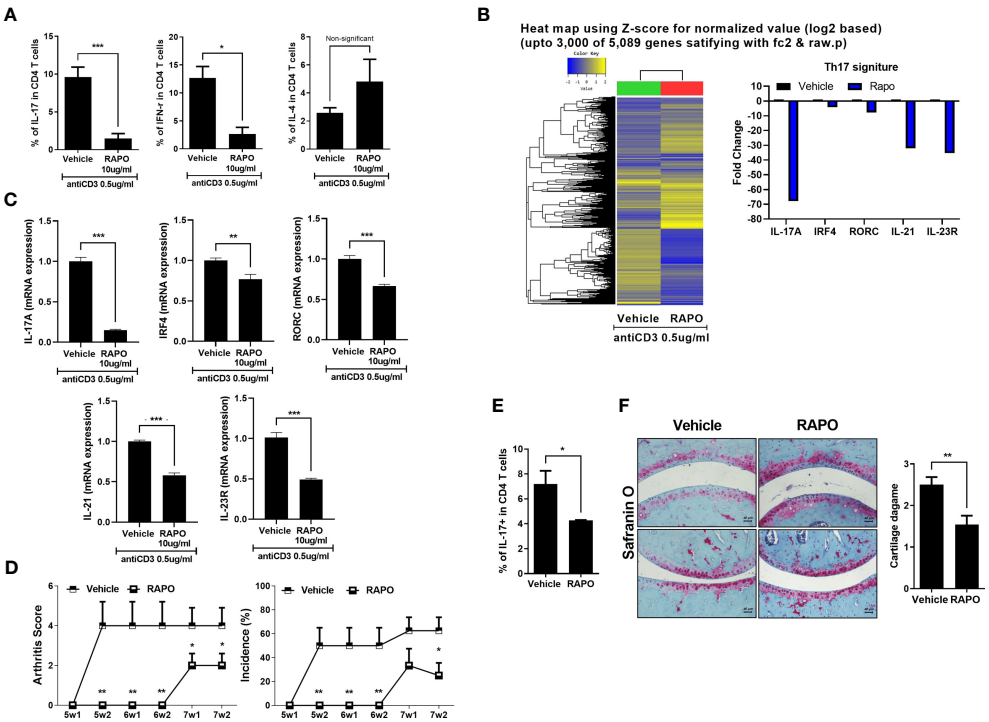

FIGURE 6 | Effects of *Bifidobacterium longum* RAPO in human PBMCs and avatar mice of RA patient. **(A)** The PBMCs of the RA patient were cultured with anti-CD3 antibody for 72 h and the resulting Th1 cells (IFN-r$^+$CD4$^+$), Th2 cells (IL-4$^+$CD4$^+$), and Th17 cells (CD4$^+$IL17$^+$) were analyzed. **(B)** The PBMCs of the RA patient were cultured with anti-CD3 antibody for 48 h. Hierarchical cluster heatmap of the PBMC-stimulated anti-CD3 antibody of RA patient treated with *B. longum* RAPO or vehicle. The expression of Th17 pathway was analyzed by RNA sequencing. Significantly differentially expressed gene and significant differences in Th17 pathway activities. The fold change of Th17-related genes decreased in the treatment of the *B. longum* RAPO. **(C)** Relative mRNA expression of Th17 pathway genes was analyzed by real-time PCR. **(D)** NSG mice were administered with *B. longum* RAPO (1×10^8 CFU/mouse) once daily for 7 weeks after the sensitization injection. **(E)** Splenocytes from the avatar mice of RA patient treated with *B. longum* RAPO. The cells were stained with Abs against CD4, IL-17. A graph from a representative experiment showing the frequency of IL-17$^+$ cells in CD4 T cells. **(F)** Joint sections from the avatar mice of RA patient with *B. longum* RAPO-treated mice were stained with safranin O. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (*vs*. vehicle-treated group).

arthritis mice. In addition, RNA sequencing results confirmed that it regulates the increase and decrease of genes related to necroptosis, autophagy, and AMPK/mTOR. Indeed, inhibition of necroptosis factor is also implicated in the treatment mechanism of rheumatoid arthritis (42, 43). Further molecular mechanism studies are needed to understand these mechanisms.

The potential therapeutic effects of probiotic bacteria in RA have been previously reported. Nevertheless, this is the first study to report the anti-RA effects of a bacterial strain identified through high-throughput screening. Additionally, there are many discrepancies in the findings of previous studies of the therapeutic effects of probiotic bacteria in RA. For example, Pan et al. reported that *Lactobacillus casei* (ATCC334) restored gut microbial dysbiosis and prevented bone destruction in a rodent model of RA (44). Additionally, *Lactobacillus helveticus* SBT2171 has been reported to alleviate RA in a CIA mouse model (45). In patients with RA, supplementation of a probiotic formulation containing *Lactobacillus acidophilus*, *L. casei*, and *B. bifidum* significantly improved the DAS28 (46). By contrast, Pineda et al. reported that oral administration of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 in RA patients did not improve the clinical features of RA (47). A meta-analysis showed that although probiotics could lower the levels of the proinflammatory cytokine IL-6 in RA patients, they failed to alleviate the clinical manifestations of RA (48). Even though these discrepancies among studies may have resulted from differences in the strain or dose used, the lack of mechanistic evidence from well-designed studies also hinders the establishment of effective probiotic regimens. Additionally, most previous studies have focused on strains known to inhibit inflammation. By contrast, we conducted unbiased screening based on microbiome profiling data and identified a novel *Bifidobacterium* strain that alleviates RA.

An important limitation of this study is the lack of evidence in humans. Although *B. longum* RAPO exerted strong anti-RA effects in mice, these effects should be confirmed in human studies. Therefore, well-designed, controlled clinical trials are required to investigate the safety and efficacy of *B. longum* RAPO in patients with RA or other immune-related diseases involving RF, such as Sjögren's syndrome and systemic lupus erythematosus. In conclusion, we identified *B. longum* RAPO as a potential anti-RA strain through gut microbiome profiling of

RA patients and investigated its RA-modulating effects in a mouse RA model. Our findings strongly suggest that *B. longum* RAPO supplementation may alleviate RA by inhibiting the production of proinflammatory mediators. Future studies are required to confirm these findings in different mouse models, as well as investigate the safety and efficacy of *B. longum* RAPO in patients with RA and other autoimmune disorders.

DATA AVAILABILITY STATEMENT

The 16sRNA sequence data presented in the study are deposited in the Sequence Read Archive (SRA) repository, accession number PRJNA759926. The RNA-sequencing data presented in the study is deposited in the GEO repository, accession number GSE183154.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Seoul St. Mary's Hospital, The Catholic University of Korea (approval ID: KC17TNSI0570). The patients/participants provided their written informed consent to participate in this study. All experimental procedures were evaluated and conducted in accordance with the protocols approved by the Animal Research Ethics Committee at the Catholic University of Korea (Permit Number: CUMC 2019-0242-01). All procedures performed in this study followed the ethical guidelines for animal use.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important content. M-LC, GJ, and S-HP had full access to all the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis. Conception and design of the study: YJ and JJ. Acquisition of data: YJ, JJ, S-YL, HN, JC, K-HC, SL, AL, and S-JP. Analysis and interpretation of data: J-WK, MP, and BK. Drafting the article: YJ and JJ. Revising the article critically: M-LC, GJ, and S-HP. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Supplementary Figure 1 | Comparison of alpha and beta diversity among the healthy control people and RA patients grouped according to the RF level. Comparison of **(A)** Shannon index diversity, **(B)** number of observed OTUs, **(C)** Faith's PD and **(D)** pielou's evenness were performed for NM, NG, LP, and HP groups using Kruskal-Wallis tests. Lines inside the box represent the median, while whiskers represent the lowest and highest values within the 1.5 interquartile range (IQR). Outliers and individual sample values are shown as dots. Principal coordinate plot based on the **(E)** Bray Curtis, **(F)** Jaccard, **(G)** Unweighted-UniFrac distance matrix, and (H) Weighted UniFrac.

Supplementary Figure 2 | Microbial composition at the top 20 expressed genera in patients with different RF levels. **(A–G)** Comparison of the relative abundance of genera under Bacteroidetes phylum, **(H–P)** Comparison of the relative abundance of genera under Firmicutes phylum, **(Q)** Comparison of the relative abundance of genera under Proteobacteria phylum. The numbers in bold indicate statistically significant correlations ($p < 0.05$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. NM: Normal control, NG: RA patients showing 20 or less of RF level, LP: RA patients showing over 20 and not exceeding 60 of RF level, HP: RA patients showing over 60 of RF level.

Supplementary Figure 3 | Microbial composition at the genus level in patients with different RF levels. Prevalence of **(A)** *Bifidobacterium*, **(B)** *Collinsella*, **(C)** *Clostridia*, and **(D)** *Veillonellaceae*. Prevalence was expressed as the percentage of samples containing each bacterial genus. The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/JlxAE8.

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PDGF Promotes Dermal Fibroblast Activation *via* a Novel Mechanism Mediated by Signaling Through MCHR1

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Systemic sclerosis (SSc) is an autoimmune disease characterized by vasculopathy and excessive fibrosis of the skin and internal organs. To this day, no effective treatments to prevent the progression of fibrosis exist, and SSc patients have disabilities and reduced life expectancy. The need to better understand pathways that drive SSc and to find therapeutic targets is urgent. RNA sequencing data from SSc dermal fibroblasts suggested that melanin-concentrating hormone receptor 1 (MCHR1), one of the G protein-coupled receptors regulating emotion and energy metabolism, is abnormally deregulated in SSc. Platelet-derived growth factor (PDGF)-BB stimulation upregulated MCHR1 mRNA and protein levels in normal human dermal fibroblasts (NHDF), and MCHR1 silencing prevented the PDGF-BB-induced expression of the profibrotic factors transforming growth factor beta 1 (TGFβ1) and connective tissue growth factor (CTGF). PDGF-BB bound MCHR1 in membrane fractions of NHDF, and the binding was confirmed using surface plasmon resonance (SPR). MCHR1 inhibition blocked PDGF-BB modulation of intracellular cyclic adenosine monophosphate (cAMP). MCHR1 silencing in NHDF reduced PDGF-BB signaling. In summary, MCHR1 promoted the fibrotic response in NHDF through modulation of TGFβ1 and CTGF production, intracellular cAMP levels, and PDGF-BB-induced signaling pathways, suggesting that MCHR1 plays an important role in mediating the response to PDGF-BB and in the pathogenesis of SSc. Inhibition of MCHR1 should be considered as a novel therapeutic strategy in SSc-associated fibrosis.

Keywords: scleroderma, systemic sclerosis, skin fibrosis, MCHR1, PDGF, fibroblast

1 INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disease characterized by vasculopathy and excessive fibrosis of the skin and internal organs (1). Skin fibrosis is the most common finding in SSc patients and can be associated with fibrosis of internal organs, which results in high mortality (2, 3). Fibroblasts are considered the effector cells in fibrosis (4). Several growth factors, such as transforming growth factor beta (TGFβ) (5), connective tissue growth factor (CTGF) (6) and platelet-derived growth factor (PDGF), can activate the profibrotic response of fibroblasts and thus contribute to the pathogenesis of SSc (7). Currently, no effective therapies exist that can halt fibrosis or reverse it (3).

Melanin-Concentrating Hormone Receptor 1 (MCHR1) is a G protein-coupled receptor (GPCR), identified first as a receptor for melanin-concentrating hormone (MCH) in 1999 (8). MCH is a cyclic neuropeptide originally isolated from the salmon pituitary that mediates skin color changes due to environmental conditions (9). MCH and MCHR1 are mainly expressed in the central nervous system (10, 11), but are also expressed in peripheral tissues, including human immune cells (12), human skin melanocytes (13), and human intestinal myofibroblasts (14). Some reports indicate that this pathway could modulate the immune system (12), inflammatory responses (15), and melanocyte function (13). The contribution of MCHR1 to fibrotic responses is demonstrated in patients with inflammatory bowel disease (14), and severe hepatic steatosis in mice (16). Aberrant MCHR1 expression is reported in lung tissues of patients with idiopathic pulmonary fibrosis, which has clinical and pathogenic features that overlap with SSc-associated interstitial lung disease (17). On the basis of these findings, we examined the levels of MCHR1 in SSc dermal fibroblasts. Based on the RNA sequencing data of these fibroblasts (Malaab et al., in press), we identified MCHR1 as a hub gene in our network analysis. Our goal was to elucidate the role of MCHR1 signaling in dermal fibroblast activation.

2 MATERIALS AND METHODS

2.1 Primary Human Dermal Fibroblast Culture

Primary human dermal fibroblasts were cultured from skin tissues of patients with SSc or healthy donors as previously described (18), under a protocol approved by the Institutional Review Board (IRB) of the University of Pittsburgh. Informed consent was obtained from all participants. Clinical features of the patients included in this study are shown in **Supplementary Table 1**. For healthy donors, skin samples were obtained without identifiers and deemed as non-human subject research by the IRB of the Medical University of South Carolina. All research included in this manuscript conforms with the Declaration of Helsinki. Fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), penicillin, streptomycin, and antimycotic agent (Invitrogen, Carlsbad, CA, USA) and used in passages 3 to 8.

Primary normal human dermal fibroblasts (NHDF) from healthy donors were treated with the following reagents; PDGF-BB (40 ng/mL) (R&D Systems, Minneapolis, MN, USA), MCH (100nM) (TOCRIS, Minneapolis, MN, USA), PI3K inhibitor (LY294002, 10μM), MEK inhibitor (U0126, 10μM), STAT3 inhibitor (StatticV, 5μM), TGFβ receptor inhibitor (ALK4/5/7 inhibitor, SB431542, 10μM), PDGF receptor inhibitor (CP-673451, 100nM), MCHR1 inhibitor (ATC0065, 50nM), dimethyl sulfoxide (DMSO) or Ethanol as a vehicle control, and used for immunoblotting, PCR and ELISA analyses as appropriate. Inhibitors were used at the indicated concentrations based on previous reports (19–25). Cell viability was determined with Cell Counting Kit-8 (Dojindo, Rockville, MD, USA) assay as previously described (26). Detailed information about the reagents is shown in **Supplementary Table 2**.

2.2 RNA Sequencing

Total RNA was extracted from each dermal fibroblast cell strain in passage 3 for gene expression analysis using the CsCl-gradient purification method (18). RNA integrity (RINs ≥ 8) was verified using Agilent 2200 TapeStation (Agilent Technologies, Palo Alto, CA). RNAseq libraries were prepared for all dermal fibroblast samples using the TruSeq RNA Sample Prep Kit following the manufacturer's protocol (Illumina, San Diego, CA) at the Hollings Cancer Center Genomics Core at MUSC (**Supplementary Figure 1A**). Libraries were clustered at a concentration to ensure at least 100 million reads per sample on the cBot as described by the manufacturer (Illumina, San Diego, CA). Clustered RNAseq libraries were paired-end sequenced using version 4 with 2×125 cycles on an Illumina HiSeq2500. Demultiplexing was performed utilizing bcl2fastq-1.8.4 to generate Fastq files.

A second paired-end RNAseq analysis for MCHR1 silenced NHDF and controls treated with/without PDGF-BB was performed at Novogene (Sacramento, CA, USA) with the NEBNext Ultra TM RNA library prep kit (New England Biolabs, MA, USA) on the Illumina NovaSeq 6000 instrument (Illumina) (**Supplementary Figure 1B**).

2.3 Differential Expression Analysis

2.3.1 Gene Level Analysis

Gene level analyses were completed using the OnRamp BioInformatics Genomic Research Platform (OnRamp Bioinformatics, San Diego, CA) (27) and the Novogene pipeline. Briefly, Fastq files quality control was performed by FastQC, adapters were trimmed and filtered by CutAdapt, and alignment to the hg19 human genome was done by STAR RNAseq aligner. Gene-level count data were generated by HTSeq and FeatureCounts, and Batch-corrected by ComBat-seq (28). Differential expression analysis was carried out by DESeq2 (29), using R studio version 1.2.1335 2009-2019. For each gene, DESeq2 reported estimated log2 fold change (log2FC) and provided a false discovery rate (FDR) adjusted p-value (q-value). Transcript count data were sorted according to their q-value. FDR is the expected fraction of false positive tests among significant tests and was calculated using the Benjamini-Hochberg

multiple testing adjustment procedure. Differentially expressed (DE) genes were defined by q-value < 0.1.

2.3.2 Systems Level Analysis

Systems level analysis was performed using iPathwayGuide (Advaita Bioinformatics, Ann Arbor, MI), a tool that uses a systems biology approach to identify pathways that are significantly impacted in any condition from high-throughput gene expression data (30). The impact analysis incorporates the classical probabilistic component of the magnitude of the expression changes of each gene, the position of the DE genes on the given pathways, the topology of the pathway that describes how these genes interact, and the type of signaling interactions between them. Gene Ontology (GO) terms with a *p*-value < 0.05 were considered to be significantly perturbed. Network analysis was used to identify the hub gene; genes with the largest number of incoming edges are found in the center, and those with the fewest are at the periphery.

2.4 Quantitative Polymerase Chain Reaction

Total RNA was extracted using TRIzol (Life Tchnologies), and qPCR was performed in duplicate using TaqMan® gene expression assays using StepOne Plus Real-time PCR machine (Applied Biosystems, Carlsbad, CA), using the following protocol; A. Holding stage: 1) 15 mins at 48°C 2) 10 mins at 95°C. B. Cycling Stage: 1) 1 min at 95°C 2) 1 min at 60°C for a total of 40 cycles. Gene expression levels were normalized to *Beta 2 Microglobulin (B2M)* and compared using the 2−ΔΔCt method. TaqMan® probes for human *Actin Alpha 2 (ACTA2), Collagen Type I Alpha 1 (Col1α1), CTGF, Fibronectin 1 (FN1), TGFβ1, MCHR1, and B2M* were obtained from Applied Biosystems. The assay catalog numbers are shown in **Supplementary Table 3**.

2.5 Immunoblotting

Fibroblast lysates were harvested directly in 2× sodium dodecyl sulfate gel-loading buffer (100 mmol/L Tris-Cl, pH 6.8, 200 mmol/L mercaptoethanol, 4% sodium dodecyl sulfate, 0.2% bromophenol blue, 20% glycerol). Samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose blotting membranes (GE Healthcare Life science). Membranes were then blocked with 5% milk and incubated with one of the following antibodies; MCHR1, Alpha Smooth Muscle Actin (αSMA), Fibronectin (FN), CTGF, Collagen Type I Alpha 1 (Col1A1), TGFβ1, Caspase 3, and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). Product details are shown in **Supplementary Table 2**. Signals were detected using horseradish peroxidase-conjugated secondary antibody and chemiluminescence (Perkin-Elmer, Waltham, MA, USA) on an iBright750 (Thermo Fisher Scientific). Signals were quantified using ImageJ software (designed at the National Institutes of Health) for densitometry (31, 32).

2.6 Western Ligand Blotting

Western ligand blotting was performed as previously described (33) with some modifications. Briefly, 1×10^6 NHDF were cultured and collected using the Subcellular Protein Fractionation Kit (Thermo Fisher Scientific), then membrane fraction samples were electrophoresed under non-reducing conditions and transferred to a nitrocellulose membrane. Similarly, recombinant human MCHR1 (Abnova, Taipei City, Taiwan) was electrophoresed under non-reducing conditions and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in Tris-buffered saline/5% Tween-20 and incubated for 1 hour with biotinylated PDGF-BB (R&D). The membrane was washed and incubated for 1 hour with horseradish peroxidase-conjugated streptavidin (Invitrogen), and the signal was detected using chemiluminescence (Perkin-Elmer) on an iBright750 (Thermo Fisher Scientific).

2.7 MCHR1 Silencing

NHDF were seeded in a 6-well plate at a density of 1×10^5 cells/well in DMEM supplemented with 10% fetal bovine serum. MCHR1-specific small-interfering RNA (ON-TARGET plus) and control RNAi were purchased from Dharmacon (Lafayette, CO, USA). For transfection, Lipofectamine®2000 (Invitrogen) was used in accordance with the manufacturer's instructions. A mixture of 10 or 100 nM of each RNAi and Lipofectamine®2000 was added to cells, and cells were cultured for 72 h. Fibroblasts were serum-starved at least 2 h before further stimulation. MCHR1-silenced or control siRNA fibroblasts were treated with 40 ng/mL PDGF-BB (R&D) and harvested 6hrs or 24hrs after stimulation. The culture supernatants were collected by centrifugation and aliquoted. All samples were stored at - 80°C until further analysis.

2.8 Surface Plasmon Resonance (SPR) Assay

All experiments were done at the Biacore Molecular Interaction Shared Resource at Georgetown University using a Biacore T200 instrument (Cytiva, Marlborough, MA, USA) with a sensor chip CM5 (Cytiva) at 25°C. Recombinant human MCHR1 Protein (Abnova) was used as a ligand to capture onto the CM5 chip, using standard amine coupling chemistry. Recombinant human PDGF-BB (Sigma-Aldrich) was used as an analyte to flow over the ligand captured surface. Flow Cell (FC) 1 was used as the reference for FC2. Recombinant human MCHR1 was diluted (1:25 dilution, ~1.2 μg/ml diluted concentration) in 10 mM sodium acetate buffer at pH 4.0 and immobilized onto FC2 to a level of ~5500 RU. PBS-P (20 mM Phosphate buffer pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.05% v/v surfactant P20) was used as the immobilization running buffer. Based on the Immobilized response value, theoretical R_{max} values were calculated. The R_{max} values assume 1:1 interaction mechanism. Overnight kinetics for PDGF-BB binding to MCHR1 were performed in the presence of PBS-P. The contact and dissociation times were 60 s and 300 s, respectively. The flow rates of all analytes solutions were maintained at 50 μL/min. Two 20 s pulses of 1:1000 H_3PO_4 (H_3PO_4:ddH_2O, v/v) were injected for surface regeneration. Injected analyte concentrations were from 100 nM to 3.125 nM (two-fold dilutions). Analytes were injected in duplicate. For analysis, sensorgrams from the overnight kinetics were evaluated using 1:1 kinetics model fitting.

2.9 Measurement of cAMP Production

NHDF were plated in 6 well plate at a density of 1×10^5 cells/well and cultured overnight. Cells were washed twice with HBSS and pre-treated with 50nM ATC0065 for 1 hour, followed by addition of PDGF-BB, MCH, or vehicle control for 30min. Fibroblasts were harvested with 0.1M HCL and centrifuged at 1000g for 10 min, and supernatants were used to measure cyclic adenosine monophosphate (cAMP) levels. The levels of cAMP in fibroblasts were measured in duplicate samples using Cyclic AMP ELISA kits (Cayman chemical, Ann Arbor, Michigan, USA) according to the manufacturer's instructions. The absorbance at 410 nm was measured with a SYNERGY H1 microplate reader (Biotec, Winooski, VT, USA).

2.10 Cell Proliferation Assay

Cell proliferation was measured by using the Cell Counting Kit-8 (Dojindo, Rockville, MD, USA). NHDF were seeded in a 96-well plate at a density of 5×10^3 cells/well in 100 µl culture medium and were allowed to adhere overnight. Cells were treated with 40ng/ml PDGF-BB or 10^{-7}M MCH or vehicle control with or without 50nM ATC0065, then incubated for 24, 48, or 72 hrs. 10µl of Cell Counting Kit-8 reagent was added to each well 2 hrs prior to measurement of absorbance. The absorbance at 450 nm was measured with a SYNERGY H1 microplate reader (Biotec).

2.11 Statistical Analysis

In addition to the described differential expression analysis, statistical comparisons were performed using Mann-Whitney U test, unpaired Student's t-test, multiple t-test, one-way analysis of variance (ANOVA) (posthoc Tukey or Dunnett), or two-way analysis of variance (post-hoc Sidak) as indicated. All tests were carried out using GraphPad Prism version 8.0 software (GraphPad Software, San Diego, CA). A *P* value <0.05 was considered significant.

3 RESULTS

3.1 Network Analysis Revealed MCHR1 as a Hub Gene

To identify potential genes involved in the pathogenesis of skin fibrosis in SSc, we performed RNA sequencing (RNAseq) using dermal fibroblasts from twins discordant for SSc and healthy subjects. We identified 742 DE genes in dermal SSc fibroblasts ($q < 0.1$, $\log2FC < |0.6|$). We determined that *MCHR1* was a hub gene significantly upregulated in our network analysis (**Supplementary Table 4** and **Supplementary Figure 2**), suggesting MCHR1 might play a pivotal role in the pathogenesis of SSc based on the "centrality principle" stating that highly connected vertices are often functionally important in biological systems (34). Thus, we focused our study on MCHR1.

3.2 MCHR1 Expression Is Upregulated in SSc Dermal Fibroblasts and Induced by PDGF-BB

To confirm the upregulation of MCHR1 in SSc patients, we examined *MCHR1* mRNA levels in dermal fibroblasts from SSc patients with early disease compared to fibroblasts from healthy subjects using quantitative PCR (qPCR). *MCHR1* expression was significantly higher in the dermal fibroblasts of SSc patients (**Figure 1A**). To determine which growth factors may increase MCHR1, we examined the effects of several fibrosis-promoting factors on *MCHR1* expression levels in NHDF, including TGFβ1, interleukin 6 (IL-6), bleomycin (BLM), and PDGF-BB. PDGF-BB and BLM significantly increased *MCHR1* levels, while TGFβ1 decreased its expression, albeit not significantly ($p = 0.0586$) (**Figure 1B**). A time-course experiment showed that PDGF-BB induced an increase in *MCHR1* expression levels as early as 2 hrs post-treatment, and the difference reached significance after 24 hrs (**Figure 1C**). After 96 hrs of stimulation, *MCHR1* levels returned to basal levels. In parallel, MCHR1 protein abundance was increased in NHDF stimulated with PDGF-BB for 48 and 72 hrs (**Figure 1D**).

3.3 PI3K and MEK Activation Mediates the PDGF-BB Induction of MCHR1

To determine which PDGF signaling cascades mediate the induction of MCHR1, NHDF were cultured with PDGF-BB in combination with specific inhibitors of PI3K (LY294002), MEK (U0126), and STAT3 (Stat3 inhibitor V, static) signaling. Inhibition of the PI3K and MEK signaling pathways significantly reduced the PDGF-BB-induced increase in *MCHR1* mRNA and protein levels (**Figures 1E, F**), suggesting that these two pathways mediate PDGF-BB induction of MCHR1. We also confirmed that the inhibitors had no off-target effect on MCHR1 levels (**Supplementary Figure 3**).

3.4 PDGF-BB Induces the Expression of Fibrotic Genes

To further delineate the role of PDGF-BB in fibrosis, we investigated whether PDGF-BB induces fibrotic gene expression in NHDF. PDGF-BB significantly increased the expression levels of *Col1α1* at 6 hrs and 24 hrs, *CTGF* at 4 and 6 hrs, and *TGFβ1* at 6 and 24 hrs (**Figure 2A**). Immunoblotting showed that 48 hrs of PDGF-BB stimulation significantly increased the protein abundance of Col1A1, FN1, CTGF, and TGFβ1 in NHDF (**Figure 2B**). Additionally, PDGF-BB increased the expression levels of the myofibroblast marker *ACTA2* (**Figure 2A**) and its corresponding protein αSMA (**Figure 2B**), although the increase was not statistically significant. Together, our data showed that PDGF-BB can induce fibrotic mediators and ECM deposition in NHDF.

3.5 PDGF-BB Increases MCHR1 and Fibrotic Genes Through PDGF Receptor

To determine if the fibrotic responses elicited by PDGF-BB were induced through PDGF receptor signaling, NHDF were pre-incubated with PDGF receptor inhibitor CP-673451, and treated with PDGF-BB. PDGF-BB induced MCHR1 and TGFβ1 protein abundance, and this effect was significantly attenuated by PDGF receptor inhibition (**Figure 2C**). PDGF-BB-induced FN1 and CTGF, but not Col1A1, were also reduced by PDGF receptor inhibition, albeit not significantly (**Figure 2C**).

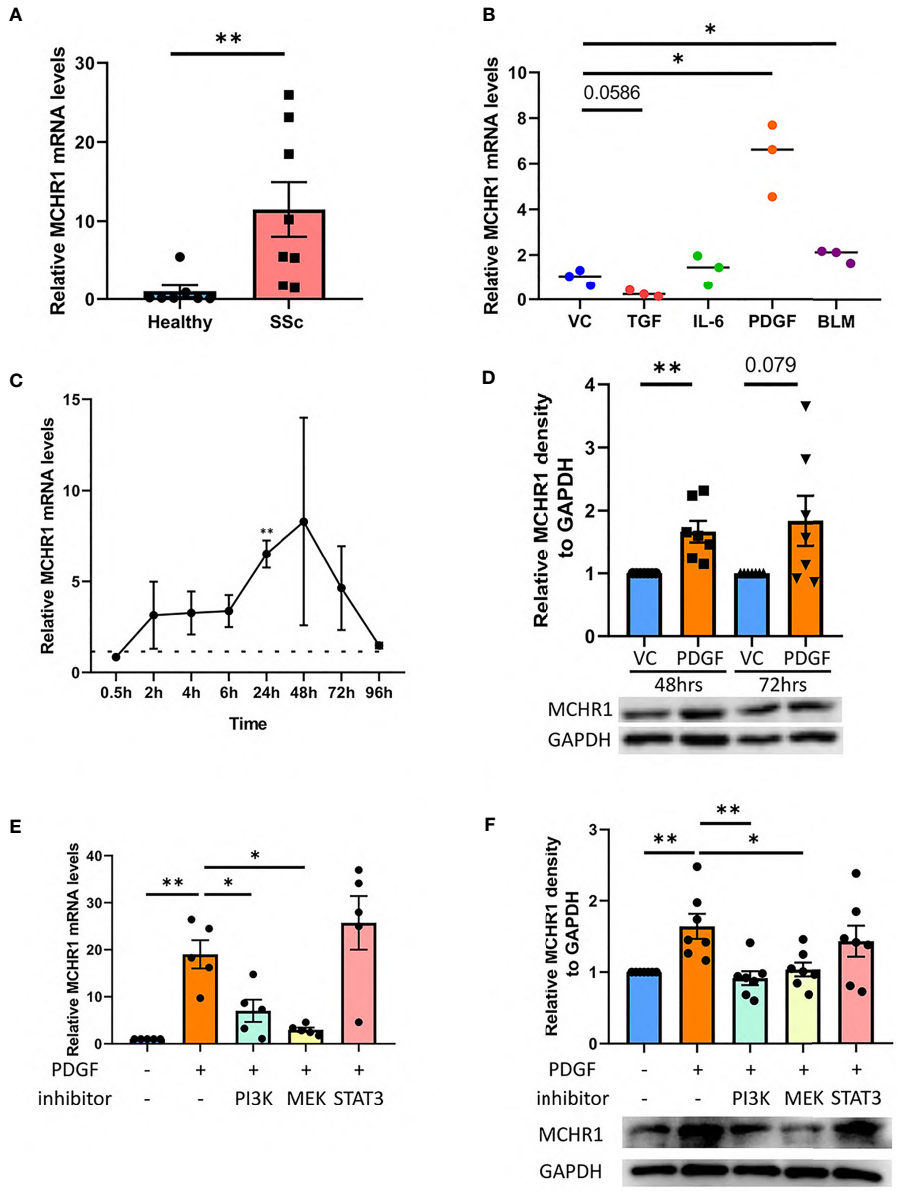

FIGURE 1 | MCHR1 expression in dermal fibroblasts. **(A)** MCHR1 expression in dermal fibroblasts of SSc patients (SSc) and healthy subjects (Healthy) was measured using qPCR (n = 8). **(B)** MCHR1 expression in NHDF treated with TGFβ1 (5ng/ml), interleukin 6 (IL-6, 20 ng/mL), PDGF-BB (PDGF, 40 ng/mL), bleomycin (BLM, 10 mU/mL), or vehicle control (VC) for 24 hrs (n=3). **(C)** MCHR1 expression levels in NHDF treated with PDGF-BB (40ng/ml) compared to vehicle control at the indicated time points (n=3). **(D)** Quantification of MCHR1 protein abundance relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in PDGF-BB-treated NHDF (40 ng/mL) for 48 and 72 hrs (n=7). Representative immunoblots are shown. **(E)** MCHR1 expression levels in NHDF incubated with 10 μM of the following inhibitors: LY294002 (PI3K), U0126 (MEK), 5 μM of StatticV (STAT3), DMSO as a vehicle control (n=5). PDGF-BB (40 ng/mL) was added 1 hour after inhibitors. NHDF were treated with PDGF-BB for 24 hrs. **(F)** Quantification of MCHR1 protein abundance relative to GAPDH in PDGF-BB-treated NHDF (40 ng/mL) for 48hrs in combination with inhibitors PI3K, MEK, STAT3 and DMSO as vehicle control (n=7). Representative immunoblots are shown below. *P < 0.05, **P < 0.01, error bars = SEM.

3.6 PDGF-BB Increases CTGF and TGFβ1 Independently of TGFβ Receptor Signaling

TGFβ1 is the prototype fibrotic factor that increases the expression of several profibrotic genes in fibroblasts (35). Since PDGF-BB increased TGFβ1 abundance (**Figure 2B**), we sought to determine if the induction of fibrotic genes by PDGF-BB is mediated by TGFβ1. NHDF were pre-incubated with the ALK5 inhibitor, an inhibitor of TGFβ receptor signaling. PDGF-BB-induced Col1A1 and FN1 protein levels were significantly decreased by ALK5 inhibitor (**Supplementary Figure 4**), suggesting that PDGF-BB-induced upregulation of Col1A1 and FN1 is dependent on PDGF-BB activation of TGFβ receptor. PDGF-BB-induced CTGF and

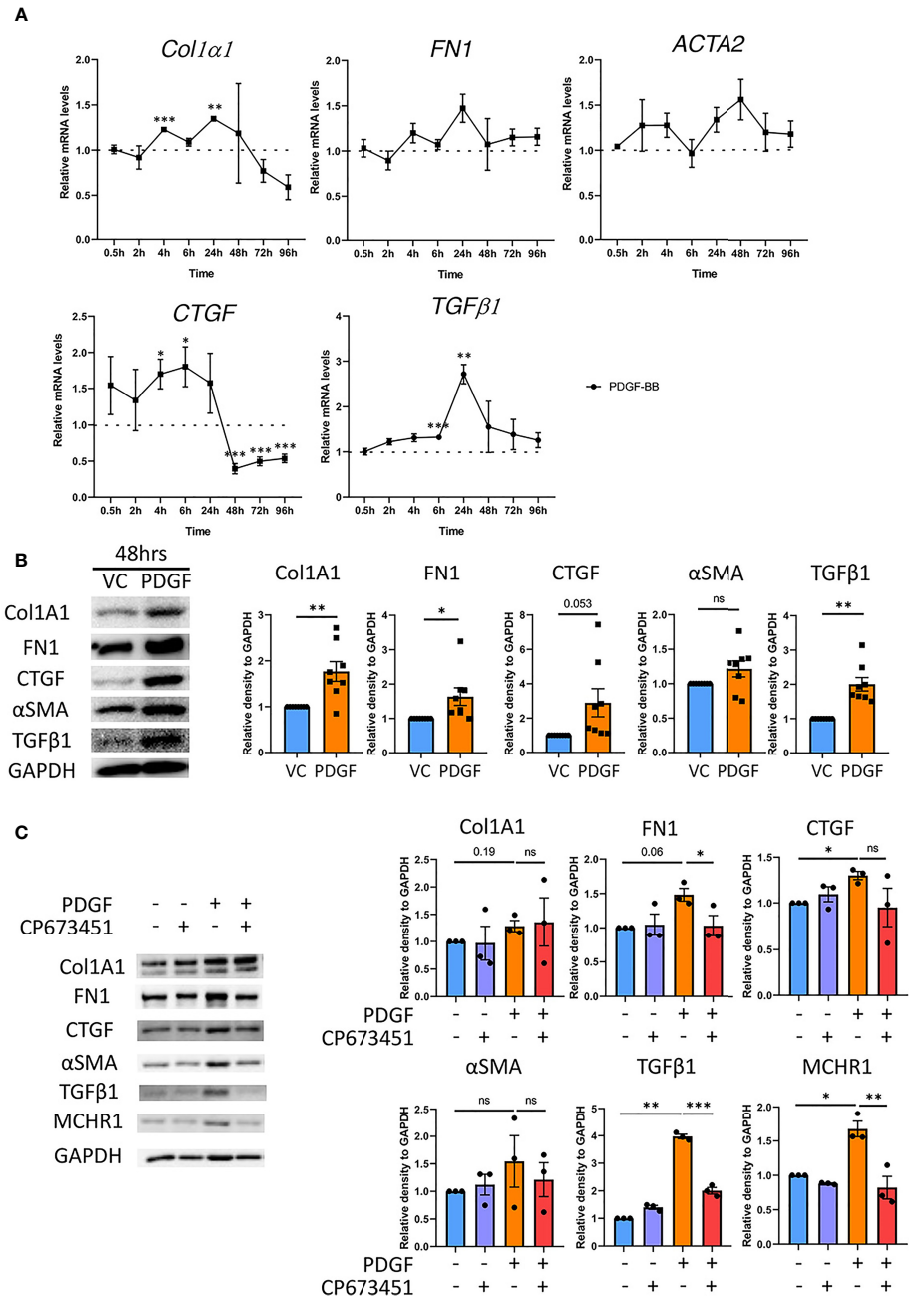

FIGURE 2 | Effect of PDGF-BB on fibrotic genes in NHDF. **(A)** The expression levels of fibrotic genes in NHDF treated with PDGF-BB compared to vehicle control at the indicated time points (n=3). **(B)** Quantification of Col1A1, FN1, CTGF, αSMA and TGFβ1 protein abundance relative to GAPDH in PDGF-BB-treated NHDF (PDGF, 40 ng/mL) for 48 hrs (n=8). Representative immunoblots are shown. **(C)** Quantification of Col1A1, FN1, CTGF, αSMA, TGFβ1 and MCHR1 protein abundance relative to GAPDH in NHDF treated with PDGF-BB and PDGF receptor inhibitor (CP-673451) (n=3). Representative immunoblots are shown. NHDF were treated with 10 nM of CP-673451 1 hr prior to PDGF-BB (40ng/ml for 48 hrs). Ethanol was used as vehicle control. *P < 0.05, **P < 0.01, ***P < 0.001, ns, not significant, error bars = SEM.

TGFβ1 levels were modestly reduced by ALK5 inhibition, albeit not significantly. ALK5 inhibition did not affect PDGF-BB regulation of αSMA. Together these results show that PDGF-BB induces the profibrotic factors CTGF and TGFβ1 independently of TGFβ1 receptor signaling, while the induction of Col1A1 and FN1 by PDGF-BB is due to activation of TGFβ1 signaling.

3.7 PDGF-BB Induces CTGF and TGFβ1 Through MCHR1

Since PDGF-BB induction of CTGF and TGFβ1 was independent of TGFβ receptor signaling, we sought to determine if this response is mediated by MCHR1. *MCHR1* expression in NHDF was silenced using small-interfering RNA

prior to stimulation with PDGF-BB (see transfection efficacy in **Supplementary Figure 5**). *MCHR1* silencing alone did not affect the expression levels of profibrotic genes, but PDGF-BB-induced *CTGF* and *TGFβ1* gene expression levels were significantly decreased by *MCHR1* silencing (**Figure 3A**). In conditioned media of cells treated with PDGF-BB, the protein abundance of TGFβ1 and CTGF were increased compared to vehicle-treated cells, and *MCHR1* silencing significantly prevented this increase (**Figure 3B**). We also examined the role of MCHR1 in SSc dermal fibroblasts. Our data show that *MCHR1* silencing only reduced PDGF-BB induction of TGFβ1 (**Figures 3C, D**). Interestingly, CTGF was not induced by PDGF-BB in SSc dermal fibroblasts at the time point examined.

3.8 PDGF-BB Binds MCHR1

The findings in MCHR1 silenced fibroblasts suggest that PDGF-BB is working through MCHR1 to induce the expression of the profibrotic factors CTGF and TGFβ1. We, therefore, examined whether MCH, the ligand of MCHR1, also regulates fibrotic genes in NHDF. Our data show that MCH does not increase profibrotic factor expression in NHDF (**Supplementary Figure 6**), suggesting that this effect is specific to MCHR1 activation by PDGF-BB. To examine whether PDGF-BB binds to MCHR1, we performed western ligand blotting using NHDF membrane fractions. Our data show that biotinylated PDGF-BB binds to a protein of the same molecular weight as MCHR1 (**Figure 4A**). Higher molecular weight bands correspond to the sizes of PDGFR. We further confirmed the interaction by western ligand blot using recombinant MCHR1. Again, PDGF-BB bound a band corresponding to recombinant human MCHR1 in a dose-dependent manner (**Figure 4B**). To confirm the binding of PDGF-BB to MCHR1, we performed SPR assay. SPR assay revealed that PDGF-BB binds to MCHR1 with an average K_D of 46.6 nM (SEM ±8.1, n=3) (**Figure 4C**). Taken together, our data demonstrate that PDGF-BB directly binds to MCHR1, an association that regulates the expression of CTGF and TGFβ1.

3.9 MCHR1 Modulates cAMP Levels

The MCH-MCHR1 pathway is known to reduce cAMP levels in some cell types (8). To determine whether PDGF-BB activates cAMP signaling downstream of MCHR1, we measured cAMP levels in NHDF by ELISA. PDGF-BB reduced cAMP levels in NHDF, as did MCH (**Figure 5A**). Pre-incubation of NHDF with a selective MCHR1 inhibitor (ATC0065) prevented the reduction of cAMP in response to both PDGF-BB and MCH, indicating that PDGF-BB can also reduce cAMP levels through MCHR1.

3.10 MCHR1 Does Not Mediate Cell Proliferation Induced by PDGF-BB

Fibroblast cell proliferation in SSc is associated with PDGF-BB signaling (7), and the MCH/MCHR1 pathway is reported to affect cell proliferation in some cell types (14, 36). Thus, we investigated the effects of PDGF-BB and MCHR1 on cell proliferation in NHDF. As shown in **Figure 5B**, PDGF-BB induced cell proliferation, and the increase was significant 72 hrs after stimulation (**Figure 5B**). MCHR1 silencing in NHDF and inhibition of MCHR1 with ATC0065 did not affect PDGF-BB-induced cell proliferation (**Figures 5C, D**). Furthermore, treatment of NHDF with MCH had no effect on cell proliferation (**Supplementary Figure 7**). We further validated our data by examining caspase-3 protein abundance in NHDF. Consistent with our cell proliferation assay results, caspase-3 protein abundance significantly increased in PDGF-BB treated NHDF (**Supplementary Figure 8**). These findings suggest that, although MCHR1 mediates PDGF-BB induction of CTGF and TGFβ1 expression, PDGF-BB regulation of cell proliferation is independent of MCHR1.

3.11 MCHR1 Regulates the Expression of Several PDGF-BB Downstream Targets

3.11.1 Genes Regulated by PDGF-BB *via* MCHR1

To identify which genes are regulated by PDGF-BB through its association with MCHR1, we performed total RNAseq of NHDF in which *MCHR1* was silenced with siMCHR1 and control (siCtr). NHDF were then treated with PDGF–BB or vehicle (VC) for 24 hrs. We identified 9,065 DE genes regulated by PDGF-BB in siCtr NHDF ["PDGF-BB vs VC" in siCtr NHDF] (q<0.1) and 8,927 DE genes in PDGF-BB-treated siMCHR1 ["PDGF-BB vs VC" in siMCHR1 NHDF] (**Supplementary Table 5**).

To identify genes regulated by PDGF-BB *via* MCHR1, we performed a meta-analysis using iPathway Guide for the DE genes in ["PDGF-BB vs VC" in siCtr NHDF] and ["PDGF-BB vs VC" in siMCHR1 NHDF]. We identified 1,473 DE genes that are unique to ["PDGF-BB vs VC" in siCtr NHDF] and thus considered to be driven by PDGF-BB through MCHR1 (**Supplementary Table 6**). Gastrin releasing peptide receptor (*GRPR*) (log2FC = 4.843; q = 0.00689), integrin subunit beta (*ITGB*) 4 (log2FC = -5.015; q = 0.00014), and vitronectin (*VTN*) (log2FC = -4.901; q = 0.00029) were in the list of DE genes (**Supplementary Table 6**), all genes reported to be associated with fibrosis or inflammation (37–39).

To investigate the functional roles of the DE genes, a Gene Ontology (GO) analysis was performed and revealed the enrichment of biological processes related to "cell communication", "development", "biosynthesis and metabolism", "DNA and RNA regulation", "immune responses" and "cell proliferation" among the top 40 most perturbed GO terms unique to "PDGF-BB vs VC" in siCtr NHDF (**Table 1** and **Supplementary Table 7**). Other terms related to ubiquitination, NF-kappaB signaling and hippo signaling were also enriched, a signature also observed in blood samples from SSc patients (40, 41).

The predicted upstream regulators analysis performed using iPathwayGuide is based on the gene expression data from our DE genes. iPathwayGuide predicts the activation or inhibition of each regulator based on the number of DE target genes whose fold change is consistent with the regulator predicted activity (activated or inhibited), and the sign of the interaction between the regulator and the targets (positive or negative). This analysis identified 336 upstream regulators, out of which 34 were unique to ["PDGF-BB vs. VC" in siCtr NHDF] (**Supplementary Table 8**).

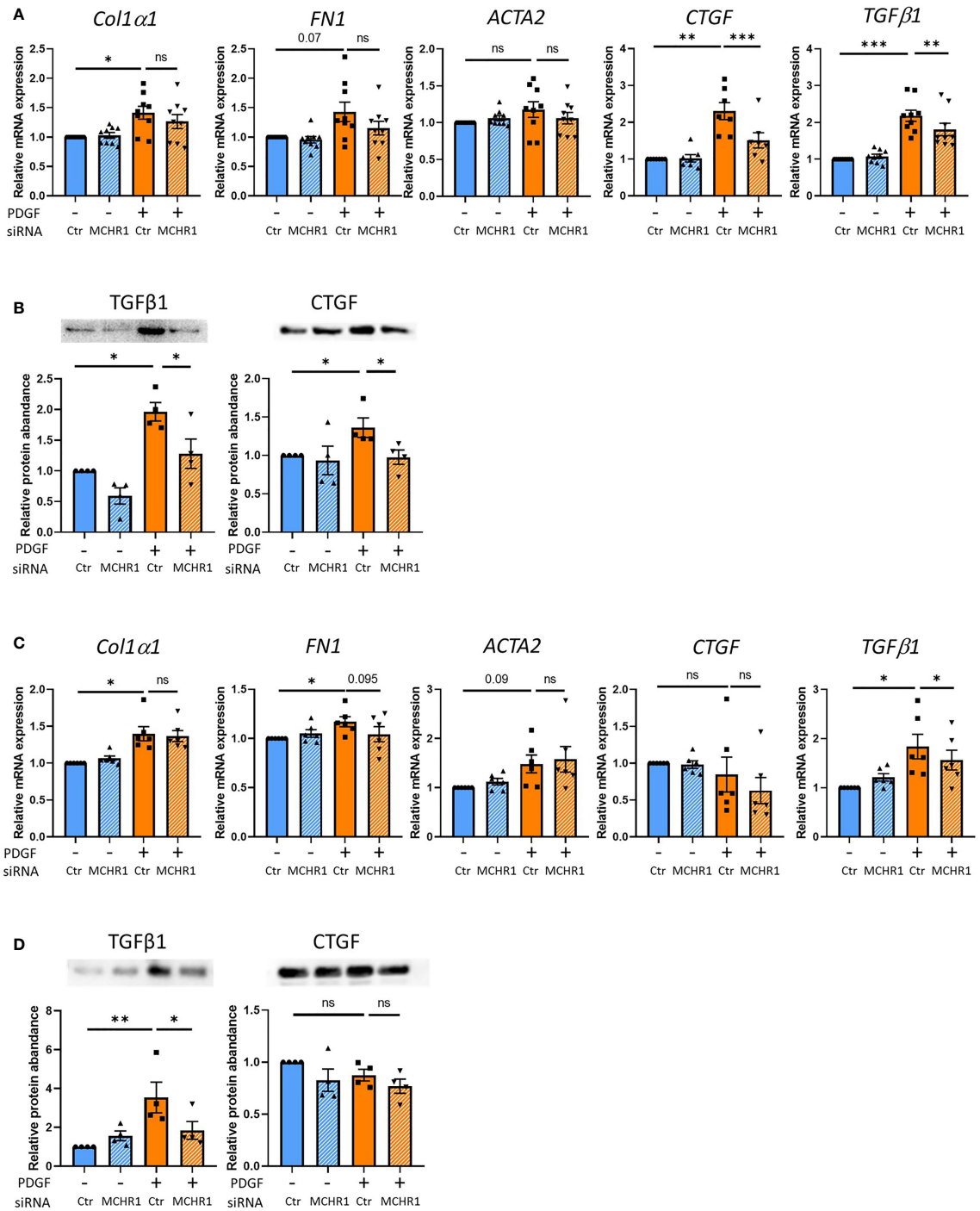

FIGURE 3 | Effect of MCHR1 silencing (siMCHR1) on fibrotic expression levels in NHDF and SSc dermal fibroblasts. NHDF were transfected with siMCHR1 for 72 hrs then stimulated with PDGF-BB (40ng/ml) for 6 hrs (n=6) and 24 hrs (n=9). SSc dermal fibroblasts were transfected with siMCHR1 for 48 hrs then stimulated with PDGFBB (40ng/ml) for 6 hrs (n=6) and 24 hrs (n=6). Transfection efficacy is shown in **Supplementary Figure 5**. **(A)** Effects of MCHR1 silencing on the expression levels of fibrotic genes in NHDF treated with PDGF-BB or vehicle. **(B)** Quantification of TGFβ1 and CTGF protein abundance in the conditioned media of NHDF transfected with siMCHR1 and treated with PDGF-BB (n=4). Representative immunoblots are shown. **(C)** Effects of MCHR1 silencing on the expression levels of fibrotic genes in SSc dermal fibroblasts treated with PDGF-BB or vehicle. **(D)** Quantification of CTGF and TGFβ1 protein abundance in the conditioned media of SSc dermal fibroblasts transfected with siMCHR1 and treated with PDGF-BB (n=4). Representative immunoblots are shown. *P < 0.05, **P < 0.01, ***P < 0.001, ns, not significant, error bars = SEM.

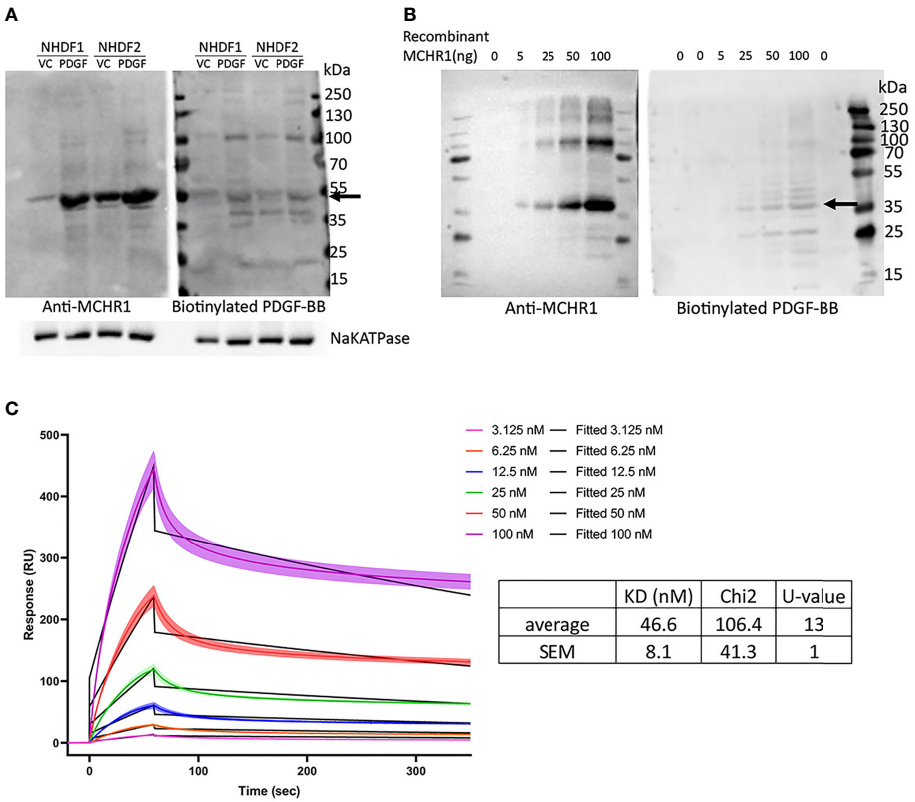

FIGURE 4 | PDGF-BB binding to MCHR1. **(A)** PDGF-BB binding to MCHR1 was assessed using biotinylated PDGF-BB in a western ligand blot. PDGF-BB (40ng/ml) or vehicle control-treated NHDF membrane fractions were separated by electrophoresis on the same gel. The gel was transferred to a membrane and the membrane was cut in half. MCHR1 was detected on one membrane using anti-MCHR1 antibody, and proteins interacting with PDGF-BB were detected on the second membrane using biotinylated PDGF-BB. The molecular weight of MCHR1 corresponds to the 50- to 55-kd bands indicated by arrows. **(B)** PDGF-BB binding to recombinant human MCHR1 was assessed using biotinylated PDGF-BB in a western ligand blot. The indicated amount of recombinant MCHR1 was separated by electrophoresis on the same gel. The gel was transferred to a membrane and the membrane was cut in half. MCHR1 was detected on one membrane using anti-MCHR1 antibody, and proteins interacting with PDGF-BB were detected on the second membrane using biotinylated PDGF-BB. The molecular weight of recombinant MCHR1 corresponds to the 35- to 45-kd bands indicated by arrows. **(C)** Surface plasmon resonance (SPR) measurements of PDGF-BB and recombinant MCHR1. Black lines represent the model data and colored lines show the response of PDGF-BB binding to MCHR1 over time. Recombinant MCHR1 was immobilized on the CM5 chip and the indicated concentrations of PDGF-BB were added. Kinetic values are the mean ± SEM from 3 independent experiments, each ran in duplicate.

3.11.2 Genes Impacted by MCHR1 Silencing

We also performed another analysis to identify genes downstream of PDGF-BB that are impacted by MCHR1 silencing. This DE analysis returned 1,095 DE genes (q<0.1) (**Supplementary Table 9**). Consistent with our qPCR data, *TGFβ1* was downregulated in siMCHR1 NHDF (log2FC = -0.207; q = 8.073×10^{-4}). *Col1α1*, *Col2α1*, *Col3α1*, and *FN1* expression was also downregulated in PDGF-treated siMCHR1 NHDF compared to PDGF-BB treated siCtr NHDF. GO analysis revealed the enrichment of biological processes related to "ECM remodeling", "development", "cell communication", "immune responses" and "secretion" among the top 40 most perturbed biological processes in PDGF-BB treated siMCHR1 NHDF vs. PDGF-BB treated siCtr NHDF (**Table 2** and **Supplementary Table 10**). General terms pertaining to cell signaling were also enriched, as was the MAPK cascade, albeit not in the top 40 most enriched terms (82/599; DE count/All count, p = 0.00019). Taken together, our findings identified pathways and biological processes regulated by PDGF-BB in an MCHR1 dependent and independent manner.

4 DISCUSSION

4.1 PDGF-BB Promotes Fibrosis Independently of TGFβ Receptor

This study is the first to demonstrate a role for MCHR1 in mediating the profibrotic effects of PDGF-BB and its reduction of cAMP. The PDGF-BB mediated increase in CTGF and TGFβ1 levels was MCHR1-dependent and TGFβ receptor-independent. We observed that PDGF-BB and BLM stimulation significantly increased *MCHR1* expression in NHDF while TGFβ1 decreased its expression, suggesting that TGFβ1 might provide negative feedback for MCHR1 expression since TGFβ1 is induced by PDGF-BB in NHDF (**Figure 6A**, arrow #1). BLM is the reagent commonly used to

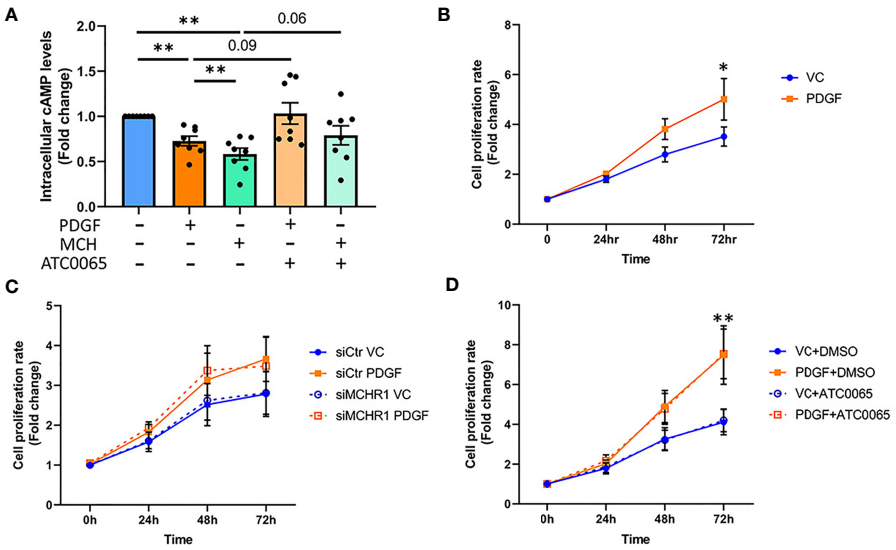

FIGURE 5 | Effect of PDGF-BB on fibroblast cAMP levels and cell proliferation. **(A)** Cyclic adenosine monophosphate (cAMP) levels in NHDF treated with PDGF-BB (40ng/ml) or melanin concentrating hormone (MCH, 10^{-7}M) for 30min. Cells were incubated with 50nM MCHR1 inhibitor (ATC0065) or DMSO as a vehicle control prior to treatment (n=8). Normalized cAMP levels in control cells were set at 1. **(B)** The cell proliferation rates of NHDF treated with PDGF-BB (40ng/ml) were measured at the indicated time points (n=5). **(C)** The cell proliferation rate in MCHR1 silenced NHDF (siMCHR1) and control NHDF (siCtr) stimulated with PDGF-BB (40ng/ml) or vehicle control (VC) were assessed at the indicated time points (n=5). **(D)** The cell proliferation rate was assessed in NHDF treated with 50nM of MCHR1 inhibitor (ATC0065) or DMSO as vehicle control 1 hr prior to PDGF-BB (n=3). Normalized absorbance at 0 h was set at 1. *P < 0.05, **P < 0.01, error bars = SEM.

induce SSc-like fibrotic responses in murine and human skin (42). BLM is reported to induce pro-inflammatory cytokines such as TGFβ1 and CTGF in human dermal fibroblasts *in vitro* (43), suggesting that the combination of several pro-inflammatory mediators, even in the presence of TGFβ1, can increase the expression of MCHR1, consistent with our data in SSc dermal fibroblasts showing high levels of MCHR1. Similar to our findings, Ziogas et al. showed that dextran sodium sulfate (DSS) induces MCHR1 expression and inflammatory colitis *via* several inflammatory mediators, including TGFβ1, in myofibroblasts (14, 44).

TGFβ is thought to be a master regulator of the fibrotic response and is a prototype fibrotic factor (5). However, PDGF is also important in the pathogenesis of fibrosis. PDGF was initially thought to only promote fibroblast proliferation, but activation of PDGF signaling alone can induce skin and internal organ fibrosis in mice (45). We observed that PDGF-BB induced the fibrotic genes *TGFβ1* and *CTGF* in NHDF (**Figure 6A**, arrow #2). The increase in *CTGF* occurred earlier than *TGFβ1*, both of which were induced earlier than other fibrotic genes such as *Col1α1* and *FN1*. This led us to speculate that TGFβ1 may mediate, at least in part, the response to PDGF-BB in NHDF. In fact, our data showed that TGFβ1 mediated ECM production in response to PDGF-BB (**Figure 6A**, arrows #3,4), but the increase in CTGF and TGFβ1 was independent of TGFβ signaling. Interestingly, we did not see CTGF induction by PDGF-BB in SSc dermal fibroblasts. We speculate this is because SSc fibroblasts produce more CTGF at baseline (46), and thus the amplitude of the response to PDGF stimulation may not be as robust as in NHDF, or because a negative feedback loop is in place in SSc fibroblasts to block further induction of CTGF.

4.2 MCHR1 Mediates the Profibrotic Effects of PDGF-BB

We explored whether the profibrotic effects of PDGF-BB were mediated by MCHR1 for two reasons: 1) PDGF-BB increased MCHR1 levels in NHDF and 2) MCH stimulation alone did not induce the expression of fibrotic genes nor MCHR1. However, previous studies suggested that MCH had profibrotic properties. MCH infusion increased profibrotic genes including *TGFβ1* in mouse liver (16), and co-stimulation of MCH with IGF1 or TGFβ1 enhanced cell proliferation rate or collagen production, respectively, in CCD-18Co human myofibroblasts (14). Human microvascular endothelial cells produced MCH following stimulation with Th2 cytokines such as IL4 and IL13 (47). IL4 and IL13 contribute to fibrosis by promoting cell differentiation and collagen production and are found in both serum and lesional skin tissues of SSc patients (48). These studies suggest that IL4 and IL13 can induce MCH, contributing to the pathogenesis of fibrosis in SSc patients. However further investigation will be needed since MCH levels of peripheral tissue or plasma in SSc patients have not been reported, and the contribution of MCH in SSc is unexplored.

The interaction between MCHR1 and PDGF-BB or PDGF receptors has not been reported, however, it is quite possible that PDGF signaling activates MCHR1 since transactivation of GPCR by growth factor receptor-tyrosine kinase has been shown (49, 50). We determined that *MCHR1* silencing in NHDF

TABLE 1 | Top 40 most enriched biological processes which are unique to [PDGF-BB treated vs VC siCtr NHDF].

GO name	Count DE	Count All	p-value
cell communication	2948	4913	0.00011
regulation of macromolecule biosynthetic process	2058	3401	0.0002
regulation of cell division	101	139	0.00021
aromatic compound biosynthetic process	2163	3581	0.00023
regulation of biosynthetic process	2171	3596	0.00026
nucleobase-containing compound biosynthetic process	2128	3524	0.00029
organic cyclic compound biosynthetic process	2232	3702	0.00031
signaling	2926	4890	0.00035
anatomical structure maturation	117	165	0.00036
heterocycle biosynthetic process	2158	3578	0.00037
RNA metabolic process	2430	4044	0.00043
positive regulation of endothelial cell proliferation	65	86	0.00046
regulation of cellular macromolecule biosynthetic process	1998	3309	0.00049
regulation of cellular biosynthetic process	2124	3525	0.00055
negative regulation of cell adhesion	152	222	0.00072
regulation of substrate adhesion-dependent cell spreading	40	50	0.00083
base-excision repair	33	40	0.00087
regulation of cytokinesis	56	74	0.00107
regulation of transcription by RNA polymerase I	25	29	0.00108
positive regulation of chemotaxis	81	112	0.00108
cellular response to vascular endothelial growth factor stimulus	43	55	0.0013
negative regulation of intracellular signal transduction	300	464	0.00149
protein polyubiquitination	214	325	0.00185
positive regulation of transcription by RNA polymerase I	18	20	0.00207
neurogenesis	857	1393	0.00209
regulation of immune system process	696	1123	0.0021
axonogenesis	267	412	0.00218
I-kappaB kinase/NF-kappaB signaling	167	250	0.00226
catechol-containing compound biosynthetic process	11	11	0.00245
catecholamine biosynthetic process	11	11	0.00245
inositol metabolic process	11	11	0.00245
positive regulation of immune system process	457	726	0.00254
immune effector process	576	924	0.00254
developmental maturation	143	212	0.00256
immune response-regulating signaling pathway	192	291	0.00268
hippo signaling	34	43	0.00297
attachment of mitotic spindle microtubules to kinetochore	14	15	0.00327
immune response-regulating cell surface receptor signaling pathway	190	289	0.00357
regulation of nucleobase-containing compound metabolic process	2045	3415	0.00366
positive regulation of epithelial cell proliferation	114	167	0.00366

Table is sorted based on p-value. Group color: Biological processes related to cell communication (orange), biosynthetic and metabolic process (red), cell proliferation (light blue), development (green), DNA and RNA regulation (yellow) and immune response (dark blue). The Count DE column shows the number of DE genes in each biological process, and the Count All column shows how many genes are in this GO term. The full output table is shown in ***Supplementary Table 7****. DE, differentially expressed; GO, gene ontology.*

significantly reduced PDGF-BB-induced *TGFβ1* and *CTGF* levels, indicating that PDGF-BB signaling could be modulated by MCHR1. GPCRs are involved in the transmission of PDGF signaling (51), leading us to speculate that MCHR1 may be required to activate downstream signaling of PDGF. We assume that MCHR1 upregulation by PDGF-BB is the result of a positive feedback of PDGF signaling, consistent with other reports showing that a ligand can induce the expression of its receptor (52, 53). Moreover, our western ligand blot and SPR assay results suggest that PDGF-BB directly binds to MCHR1 to activate downstream pathways. MCHR1 then interacts with the Gi/o/q protein and regulates intracellular signaling (8).

TABLE 2 | Top 40 most enriched biological processes in PDGF-BB-treated [siMCHR1 NHDF vs siCtr NHDF].

GO name	Count DE	Count All	p-value
extracellular matrix organization	75	259	5.50E-20
extracellular structure organization	75	259	5.50E-20
regulation of multicellular organismal process	297	2010	4.60E-19
response to chemical	368	2736	4.50E-17
blood vessel development	103	478	1.00E-16
Signaling	466	3763	1.40E-15
vasculature development	104	504	1.60E-15
cardiovascular system development	104	504	1.60E-15
response to external stimulus	239	1608	2.80E-15
blood vessel morphogenesis	90	412	4.30E-15
response to type I interferon	32	72	4.50E-15
immune response	179	1102	6.20E-15
biological adhesion	148	850	6.60E-15
cell adhesion	147	847	1.10E-14
response to organic substance	294	2129	1.30E-14
cellular response to chemical stimulus	290	2101	2.40E-14
response to stimulus	613	5373	3.20E-14
wound healing	78	343	3.30E-14
type I interferon signaling pathway	30	68	4.10E-14
cellular response to type I interferon	30	68	4.10E-14
immune system process	254	1783	5.10E-14
anatomical structure morphogenesis	254	1783	5.10E-14
cell surface receptor signaling pathway	262	1862	7.80E-14
system development	382	3005	1.00E-13
Secretion	160	976	1.00E-13
response to wounding	87	413	1.20E-13
cell communication	460	3789	1.20E-13
tube morphogenesis	109	584	4.00E-13
Angiogenesis	77	352	4.30E-13
response to stress	345	2672	4.50E-13
cytokine-mediated signaling pathway	93	467	5.30E-13
regulation of developmental process	247	1755	5.40E-13
tube development	126	719	5.70E-13
export from cell	152	929	5.80E-13
response to cytokine	131	761	7.20E-13
multicellular organismal process	501	4258	1.10E-12
cellular response to organic substance	245	1749	1.20E-12
regulation of localization	246	1759	1.30E-12
secretion by cell	148	906	1.40E-12
tissue development	186	1231	1.90E-12

Table is sorted based on p-value. Group color: Biological processes related to ECM remodeling (purple), cell communication (orange), development (green), immune response (dark blue), and secretion (gray). The Count DE column shows the number of DE genes in each biological process, and the Count All column shows how many genes are in this biological process. The full output table is shown in ***Supplementary Table 10****. DE, differentially expressed; GO, gene ontology.*

4.3 cAMP Levels Are Reduced in PDGF-BB-Stimulated NHDF

In *MCHR1* transfected cells, MCH binding to MCHR1 can inhibit the accumulation of cAMP, activate MAP kinase signaling, induce IP3 production, and increase intracellular Ca^{2+} (8). We observed a reduction in cAMP levels following PDGF-BB and MCH treatment in NHDF, and MCHR1 chemical inhibition neutralized cAMP reduction due to both PDGF-BB and MCH, suggesting that PDGF-BB can directly activate the MCHR1 signaling pathway. This data also suggests that MCHR1 inhibition has anti-fibrotic effects by increasing cAMP levels. Elevated intracellular cAMP levels have been shown to exert anti-fibrotic effects, decrease fibroblast

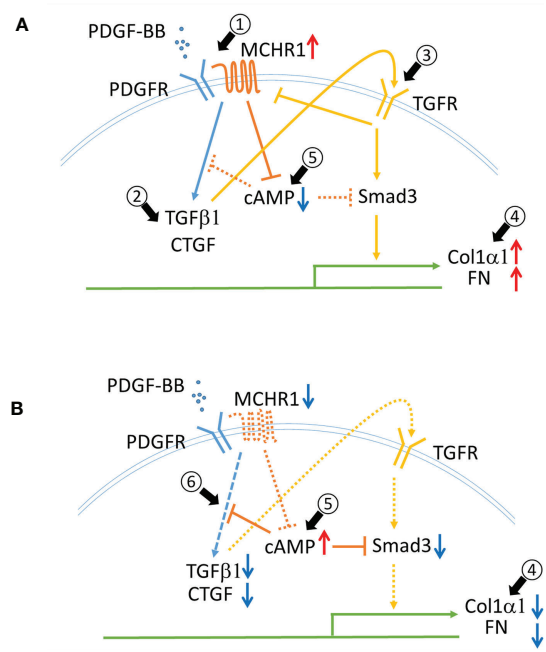

FIGURE 6 | Schematic representing the role of MCHR1 in the fibrotic response elicited by PDGF-BB in NHDF. **(A)** PDGF-BB induces MCHR1, TGFβ1 and CTGF (arrow #1,2). PDGF-BB induced TGFβ1 increases ECM deposition (arrow #3,4). MCHR1 reduces intracellular cAMP levels and enhances the cell response to TGFβ1 (arrow #5). **(B)** Without MCHR1 signaling, intracellular cAMP levels increase (arrow #5), and PDGF signaling is attenuated (arrow #6). Increased cAMP levels reduce PDGF-BB induced TGFβ1 and CTGF and prevent the cell response to TGFβ1 (arrow #4).

proliferation, promote fibroblast cell death, and inhibit ECM production (54). Increased cAMP levels in MDCK cells prevented TGFβ–mediated increases in αSMA levels, suggesting that increased cAMP can inhibit the fibrotic response induced by TGFβ, likely as a result of inhibition of Smad3 activation (54). Increased cAMP levels in lung fibroblasts can inhibit PDGF-BB-induced CTGF and TGFβ1 (55). Based on our findings and those of others, we speculate that increased MCHR1 expression levels might modulate cAMP levels in NHDF and enhance the fibrotic response to PDGF-BB (**Figures 6A, B** arrow #5, 6).

Interestingly, Janus kinase-1 (JAK1) and PDGF-receptor-α (PDGFRA) were among the list of 34 genes predicted to be upstream regulators of DE genes regulated by PDGF-BB *via* MCHR1 ["PDGF-BB vs. VC" in siCtr NHDF] (**Supplementary Table 8**). JAK1 and PDGFRA are involved in PDGF signaling (56, 57), indicating that MCHR1 might modulate PDGF-BB signaling. In support of this observation, Zigoas et al. showed that the inhibition of MCH/MCHR1 signaling attenuated Smad3 expression levels in mouse fibroblasts of a colitis murine model (14). In contrast, the cAMP signaling pathway was activated by PDGF in several cell types as a result of a negative feedback loop (58, 59). Our observation of decreased cAMP levels could be due to the use of different cell types, PDGF-BB concentrations, and experimental time points.

4.4 Targeting MCHR1 as a Therapeutic Strategy Against Fibrosis

In experimental colitis, MCHR1 antagonist reduced colonic inflammation, probably by blocking IL10 upregulation, suggesting that inhibition of MCH/MCHR1 signaling could be a novel anti-inflammatory therapeutic approach (60, 61). Anti-fibrotic effects of MCH/MCHR1 inhibition have been reported. Anti-MCH antibody suppressed the production of fibrotic genes in experimental colitis (14), and oral administration of MCHR1 antagonist decreased *Col1α1* and *TGFβ1* expression levels in a dose-dependent manner in the liver of C57BL/6 J mice with severe hepatic steatosis (16). Previous reports suggest that the anti-fibrotic effects of alpha-melanin stimulating hormone (α-MSH) are actually due to inhibition of MCH/MCHR1 signaling (14, 61), since α-MSH can neutralize the functional effects of MCH (62, 63). The anti-fibrotic effects of α-MSH were reported in NHDF and in a murine model of skin fibrosis induced by TGFβ1 or BLM (64, 65), suggesting that inhibition of MCH/MCHR1 signaling has anti-fibrotic effects in skin.

The functional enrichment analysis of our RNAseq data revealed the involvement of MCHR1 in the fibrotic and inflammatory responses induced by PDGF-BB. Genes and biological processes involved in fibrosis were exclusively enriched by PDGF-BB in NHDF. For example, PDGF-BB decreased ITGB4 expression, a signature that is associated with enhanced lipopolysaccharide-induced inflammation (38). GRPR is the G protein-coupled receptor that binds to gastrin-releasing peptide (GRP). GRP was shown to induce the fibrotic response in a murine model of lung fibrosis and in human cell lines (37, 66), and GRPR antagonism can reverse the effect of GRP on cell proliferation (67), indicating increased GRPR may be involved in the fibrotic response.

GO term analysis revealed some biological processes are deregulated by PDGF-BB *via* MCHR1. Consistent with previous reports, our data indicated MCHR1 could modulate the immune system (12) and inflammatory responses (15). The biological processes related to biosynthetic, metabolic, and development are also perturbed. This is not surprising as MCHR1 modulates energy metabolism (11), likely in association with leptin (68). The biological process related to ubiquitination may affect SSc pathogenesis by modulating TGFβ signaling and TLR-dependent signaling (69, 70). We also observed that the biological processes related to ECM remodeling and PDGF-BB signaling were perturbed when *MCHR1* expression is silenced in NHDF, indicating that silencing of MCHR1 mediated the effects of PDGF-BB in the regulation of these genes, namely PDGFRA, PDGFRB, PDGFD, and MAPK9. Interestingly, we found several biological processes related to vascular development. Vasculopathy is one of the most common features of SSc (1), and MCHR1 has not been previously reported to contribute to angiogenesis or vasculopathy.

4.5 PDGF-BB Induces Cell Proliferation Independently of MCHR1 in NHDF

In the present study, we observed that PDGF-BB induced cell proliferation of NHDF, in agreement with previous studies (45),

whereas MCH did not. In addition, *MCHR1* silencing or chemical inhibition did not affect cell proliferation. It is reported that PDGF-BB-induced cell proliferation is suppressed by inhibition of PDGF receptor (71). Taken together, these findings lead us to conclude that PDGF-BB induces cell proliferation in an MCHR1-independent manner. MCH/MCHR1 signaling in cell proliferation is still controversial. In some cell types, MCH/MCHR1 signaling inhibited cell proliferation, e.g., MCH had inhibitory effects on CD3+ lymphocyte proliferation (12) and MCHR1 antagonism induced proliferation of progenitor cells in mouse brain (72). In contrast, MCH induced cell proliferation in DSS-treated human myofibroblasts (14). There are some possible explanations for these conflicting results: MCH/MCHR1 signaling can promote different responses depending on cell type, or cell proliferation rate might depend on the basal expression levels of MCHR1 since DSS treated human myofibroblasts showed higher levels of MCHR1. However, our study has some limitations. Although we found that transcription levels of MCHR1 were upregulated in SSc dermal fibroblasts, we did not examine the role of MCHR1 *in vivo*. We showed that PDGF-BB induces MCHR1 and confirmed that blocking PDGF-BB signaling by inhibiting PDGF receptor prevented the upregulation of MCHR1, but there is a possibility that MCHR1 is further activated by PDGF-BB-induced molecules or other factors. We also showed that PDGF-BB directly binds to MCHR1 by western ligand blot and SPR, however, we must also consider the possibility that PDGFR and MCHR1 are co-localized and PDGF-BB signals *via* both receptors simultaneously or sequentially.

5 CONCLUSION

In conclusion, we are the first to show that MCHR1 contributes to the PDGF-BB-induced fibrotic response and the resulting increase in the potent profibrotic factors CTGF and TGFβ1. Increased levels of MCHR1 in SSc fibroblasts can promote PDGF-BB signaling, increase TGFβ1 and CTGF levels, modulate intracellular cAMP production, and increase the fibrotic response. Increased levels of MCHR1 can also potentially mediate the vasculopathy characteristic of SSc. Together, our data show that inhibition of MCHR1 should be considered as a potential therapeutic strategy for skin fibrosis.

DATA AVAILABILITY STATEMENT

RNAseq data used in this study have been deposited on the NCBI GEO under access number GSE180488.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Institutional Review Board of the University of Pittsburgh, the Institutional Review Board of the Medical University of South Carolina. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Study design: NT and CF-B. Performed experiments: NT. Gene level analysis and systems level analysis: LR and WS. Writing of the manuscript, generation of figures: NT and CF-B. Reviewing the draft, comments: LR and WS. Manuscript editing: NT, LR, and CF-B. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.745308/full#supplementary-material

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Vitamin D Metabolic Pathway Genes Polymorphisms and Their Methylation Levels in Association With Rheumatoid Arthritis

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Abnormal vitamin D metabolism is involved in the pathogenesis of rheumatoid arthritis (RA). In this study, we evaluated the association of single nucleotide polymorphisms (SNPs) and methylation levels in vitamin D metabolic pathway genes with RA susceptibility. Ten SNPs in vitamin D metabolic pathway genes (*CYP2R1*, *CYP24A1*, *VDR*, *CYP27B1*) were genotyped in 477 RA patients and 496 controls by improved multiple ligase detection reaction (iMLDR). The methylation levels of the promoter regions of these genes were detected in 122 RA patients and 123 controls using Illumina Hiseq platform. We found that the *CYP2R1* rs1993116 GA genotype, *CYP27B1* rs4646536 GA genotype, rs4646536 A allele frequencies were significantly increased in RA patients when compared to controls. The decreased risk of rs1993116, rs4646536 was found under the dominant mode in RA patients. However, no significant association was found between *CYP2R1* rs7936142, rs12794714, *CYP24A1* rs2762934, rs6068816, rs2296239, rs2296241, *VDR* rs11574129, rs3847987 polymorphism, and RA susceptibility. The *VDR*, *CYP27B1* methylation levels in RA patients were significantly lower than those in controls, while *CYP2R1*, *CYP24A1* methylation levels were not associated with RA. There were no statistical associations between *CYP2R1*, *CYP24A1*, *VDR*, *CYP27B1* methylation levels and their respective genotype in RA patients. In addition, plasma 25OHD level in RA patients was significantly lower than that in healthy controls. In summary, our results showed that *CYP2R1*, *CYP27B1* genetic variations were associated with the genetic background of RA, while altered *VDR*, *CYP27B1* methylation levels were related to the risk of RA.

Keywords: rheumatoid arthritis, autoimmune disease, vitamin D metabolic pathway, methylation, single nucleotide polymorphisms

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INTRODUCTION

Rheumatoid arthritis (RA) is a common autoimmune, chronic inflammatory disease, with a prevalence of 1% around the world (1). The disease is characterized primarily by affecting peripheral joints of the hands, wrists, and feet and can eventually lead to the accumulation of joint damage and irreversible disability in patients (2). At present, the pathogenesis of RA has not been fully elucidated, and individual progress is highly variable during the development of RA; thence, early diagnosis and treatment to maximize the chance of inducing remission is important to prevent permanent disabling joint damage. Many studies have explored the influence of genetic susceptibilities, epigenomic features, abnormal immune response, and environmental factors on the onset, disease activity, and prognosis of RA (3–5). A large number of genes/loci and even some specific variants underlying RA in different races have been identified, with different study protocols like genome-wide association study (GWAS), and candidate gene approaches, while these genes/loci only account for a fraction of phenotypic variance in RA (6, 7). Therefore, it is necessary to study whether immune-modulating gene variation is related to RA susceptibility.

With the development of molecular biology, the regulation of epigenetic changes in gene expression and disease progression has received more and more attention (8). DNA methylation, which is an important key epigenetic trait, involves the addition of a methyl group to the cytosine of CpG dinucleotides and is associated with many biological processes, including X-chromosome inactivation, genomic imprinting, aging, and canceration (9). According to recent studies, epigenetic changes by DNA methylation were dynamic, individual, and highly important in inflammatory processes, and influencing mechanisms of DNA methylation such as DNA methyltransferases activity could directly affect the RA development and might be a very promising therapeutic target for RA (10, 11).

In another context, the association between DNA methylation and inflammation-regulating immune pathways might play a significant role in the pathogenesis of RA (11). Recent studies had suggested that the vitamin D metabolic pathway might be a potential contributor to RA (12). *In vitro*, vitamin D metabolites modulate inflammation by altering the function of T helper and regulatory T cell (12, 13). *In vivo*, vitamin D metabolites including the 25-hydroxyvitamin D (25OHD, the main circulating metabolite) and 1,25-dihydroxyvitamin D (1,25(OH)2D, the active form of vitamin D) had been reported to be associated with RA disease progression, and vitamin D supplementation might have beneficial effects for RA patients (14–16). Moreover, some studies also analyzed the correlation between vitamin D metabolism gene polymorphisms and RA, although the results were inconsistent (17, 18).

The methylation levels of several key genes in the vitamin D metabolic pathway were found to associate with the risk and prognosis of tuberculosis (19). However, the role of vitamin D metabolic pathway gene methylation levels in RA had not been systematically studied. Thus, we performed this molecular epidemiological study to explore the effect of genetic variation, aberrant DNA methylation in four key vitamin D metabolic pathway genes (*CYP2R1*, *CYP24A1*, *VDR*, *CYP27B1*) on the risk of RA in a Chinese population.

MATERIALS AND METHODS

RA Patients and Normal Controls

In this case-control study, a total of 973 subjects including 477 RA patients and 496 normal controls were consecutively included to explore the relationship between *CYP2R1*, *CYP24A1*, *VDR*, *CYP27B1* gene polymorphisms and RA susceptibility. Then, 122 RA patients and 123 normal controls were enrolled to detect their promoter methylation levels. The RA patients' diagnosis was based on the 1987 American College of Rheumatology revised criteria (20), and peripheral blood samples and clinical information of all subjects were obtained from Anhui Provincial Laboratory of Inflammatory and Immune Diseases. The clinical data [including anticyclic citrullinate peptide (anti-CCP), rheumatoid factor (RF), *etc.*] and drug treatment (including glucocorticoid, methotrexate) of RA patients were collected. The normal controls did not have a history of inflammatory/autoimmune diseases or cancer.

SNP Selection

We first sought the genotype data of Han Chinese people in Beijing from CHBS_1000g and Ensembl Genome Browser 85. Then, we selected the tag SNPs, capturing all the common SNPs located in the chromosome locus transcribed into vitamin D metabolic pathway genes (*CYP2R1*, *CYP24A1*, *VDR*, *CYP27B1*) and their flanking 2000 bp region. The selection was performed with Haploview 4.0 software (Cambridge, MA, USA). Moreover, the existing studies on the association between *CYP2R1*, *CYP24A1*, *VDR*, *CYP27B1* gene polymorphism and RA susceptibility were also reviewed to search for significant SNP. Finally, we selected one tagSNP (rs4646536) in *CYP27B1*, three tagSNPs (rs12794714, rs7936142, rs1993116) in *CYP2R1*, four tagSNPs (rs2762934, rs6068816, rs2296239, rs2296241) in *CYP24A1*, and two tagSNPs (rs11574129, rs3847987) in *VDR* for genotyping in the present study. Above SNPs accorded with MAF ≥ 0.05 in CHB and r^2 threshold > 0.8.

Genotyping and Methylation Analysis

The genomic DNA was extracted from the peripheral blood leukocytes by the Flexi Gene-DNA Kit (Qiagen, Valencia, CA, USA). Improved multiple ligase detection reaction (iMLDR) genotyping assay was used for genotyping with technical support from the Center for Genetic & Genomic Analysis, Genesky Biotechnologies (Inc., Shanghai). Those individuals with 100% genotyping success rate for the above SNPs were included for final analysis.

The methylation level of vitamin D metabolic pathway genes was detected using MethylTarget® with technical support from the Center for Genetic & Genomic Analysis, Genesky Biotechnologies (Inc., Shanghai). We sequenced the CpG islands in the promoter region of *CYP2R1*, *CYP24A1*, *VDR*,

CYP27B1 by the Illumina Hiseq platform. Primers were designed to amplify the specific sites of interest from the bisulfite-converted DNA (**Table 1**), and the mean methylation level of all CpG sites on the fragment was calculated as the methylation level of the specific sites of each gene.

Enzyme-Linked Immunosorbent Assay

In this study, an additional 2 ml peripheral blood was collected from 84 RA patients and 84 normal patients by EDTA anticoagulant tube, and then plasma was obtained by Ficoll-Hypaque density gradient centrifugation. Plasma 25OHD level was determined by ELISA kits (MyBioSource Inc., USA), and the result was expressed as nanograms per milliliter.

Statistical Analysis

Statistical analysis was conducted with the SPSS 23.0 (Armonk, NY: IBM Corp, USA). Hardy-Weinberg equilibrium test was performed in normal controls with Chi-square (χ^2). Logistic regression analysis was used to calculate the association between genotype, allele distribution frequencies of each SNP and RA risk. Two genetic models (dominant model, recessive model) were also analyzed, and SHEsis software was used to calculate haplotype analysis. The methylation levels of candidate genes were shown as median value and interquartile range, and the differences in methylation levels between two groups and three groups were analyzed by Mann-Whitney *U* test and Kruskal-Wallis *H* test, respectively. The diagnostic value of candidate gene methylation levels in RA patients was calculated by receiver operating characteristic (ROC) analysis. P value < 0.05 was considered as statistically significant.

RESULTS

Association of Vitamin D Metabolic Pathway Gene Polymorphisms With RA Susceptibility

In this step, we included 477 RA patients and 496 controls. The RA patients consisted of 389 females and 88 males, with an average age of 52.70 ± 12.25 years, and 384 females and 112 males were enrolled in controls with a mean age of 50.61 ± 14.76 years. **Table 2** shows the allele and genotype frequency distributions of all SNPs in RA patients and normal controls, and all SNPs were conformed to Hardy Weinberg equilibrium in controls.

We found that *CYP2R1* rs1993116 GA genotype frequency was significantly higher in RA patients than that in normal controls (GA *versus* GG: P = 0.047), while a decreased risk of rs1993116 was observed under the dominant mode (GG *versus* AA+GA: P = 0.047). In the *CYP27B1* gene, the rs4646536 GA genotype and A allele frequencies were significantly increased in RA patients in comparison to normal controls (GA *versus* GG: P = 0.027; A *versus* G: P = 0.024, respectively). Moreover, a decreased risk of rs4646536 variant was found under dominant mode (GG *versus* AA+GA: P = 0.016). However, there was no significant difference in allele and genotype distribution of the *CYP2R1* rs7936142 and rs12794714 between RA patients and normal controls (all P>0.05). Similarly, *CYP24A1* gene rs2762934, rs6068816, rs2296239, rs2296241, and *VDR* gene rs11574129, rs3847987 polymorphisms were not significantly associated with RA.

To explore the potential relationship between *CYP2R1*, *CYP24A1*, *VDR*, and *CYP27B1* genetic variation and anti-CCP, RF status in RA patients, we also conducted a case-only analysis (**Table 3**). We found that allele and genotype frequencies of all SNPs did not have statistically significant differences between anti-CCP-positive RA patients and anti-CCP-negative RA patients, as well as RA patients with RF-positive and with RF-negative.

Haplotype Analysis

We constructed the haplotypes of *CYP2R1*, *CYP24A1*, *VDR* through SHEsis software and analyzed the relationship between these haplotypes and RA susceptibility. Seven main haplotypes (CAAC, CAGC, CGGC, CGGT, TAGC, TGGC, TGGT) for *CYP24A1*, three main haplotypes (AAG, AGA, AGG) for *CYP2R1*, and three main haplotypes (AA, AG, CA) for *VDR* were detected by SHEsis in this study (**Table 4**). The results demonstrated that there was no statistically significant difference in the frequency distribution of the above haplotypes between RA patients and normal controls (all P>0.05).

The Methylation Levels of Vitamin D Metabolic Pathway Genes in RA Patients and Normal Controls

In this step, the RA group included 100 females and 22 males, with a mean age of 52.61±13.05 years, and the control group included 82 females and 41 males, with an average age of 46.93±14.29 years. The methylation levels of specific sites between RA patients and

TABLE 1 | The primers of specific sites in each gene.

Gene	Fragment	Forward primer	Reverse primer
CYP24A1	CYP24A1_1	AGGTTGGGGGTATTTGGTTTTT	CCCRAAAATAACCCCCAAAA
	CYP24A1_2	TTTTTGTTGATGGGGGAGTTT	CAACCCCTACRACCAATACAAAA
	CYP24A1_3	GAGGYGGGAGGAGGGAAAG	AAAATCAACAACCCRTAACCTTCTTT
	CYP24A1_4	GGGAGAGGGGTTTTGGTATT	ACACCTAAACTCRCCATACCTACTAAAAAC
CYP27B1	CYP27B1_1	GGGTTTTTGGGGGTAGAGA	ATCCRCTCCCCCAAATACAA
CYP2R1	CYP2R1_1	TTTGTAGGGGGAGTTTYGTTTTT	ACCTACTATTAACCATCTAAAACTCAAAAC
	CYP2R1_2	AAAATAAAATAGGTGAGTTTTGTTTTAGG	AATAACTCATTTAAAACTCATAACCAACC
VDR	VDR_1	GATTAGGGAAGTTGAGATTTAGTTTTT	AAAAACTCAACCTAATCCCACAAA
	VDR_2	AGGTGTTGGGTTGTTTTTGTTTG	ACTTCAACTTTCTCAAACCTCAATACC

TABLE 2 | Genotypes and allele frequencies of vitamin D metabolic pathway genes in RA patients and controls.

SNP	Analyze model		RA (n = 477)	Control (n = 496)	*P* value	*OR* (95% *CI*)
CYP2R1						
rs7936142	Genotypes	TT	6 (1.26)	7 (1.41)	0.849	1.112 (0.370,3.341)
		AT	102 (21.38)	102 (20.56)	0.763	0.953 (0.700,1.299)
		AA	369 (77.36)	387 (78.02)		Reference
	Alleles	T	114 (11.95)	116 (11.69)	0.861	0.976 (0.741,1.285)
		A	840 (88.05)	876 (88.31)		Reference
	Dominant model	AA	369 (77.36)	387 (78.02)	0.803	1.039 (0.768,1.405)
		AT+TT	108 (22.64)	109 (21.98)		Reference
	Recessive model	TT	6 (1.26)	7 (1.41)	0.835	1.124 (0.375,3.368)
		AA+AT	471 (98.74)	489 (98.59)		Reference
rs12794714	Genotypes	AA	54 (11.32)	66 (13.31)	0.280	1.254 (0.832,1.891)
		GA	225 (47.17)	237 (47.78)	0.573	1.081 (0.825,1.415)
		GG	198 (41.51)	193 (38.91)		Reference
	Alleles	A	333 (34.91)	369 (37.20)	0.293	1.105 (0.918,1.329)
		G	621 (65.09)	623 (62.80)		Reference
	Dominant model	GG	198 (41.51)	193 (38.91)	0.409	0.898 (0.695,1.160)
		AA+GA	279 (58.49)	303 (61.09)		Reference
	Recessive model	AA	54 (11.32)	66 (13.31)	0.347	1.202 (0.819,1.765)
		GG+GA	423 (88.68)	430 (86.69)		Reference
rs1993116	Genotypes	AA	69 (14.47)	68 (13.71)	0.289	0.810 (0.548,1.196)
		GA	233 (48.85)	215 (43.35)	**0.047**	0.758 (0.577,0.996)
		GG	175 (36.69)	213 (42.94)		Reference
	Alleles	A	371 (38.89)	351 (35.38)	0.110	0.860 (0.716,1.034)
		G	583 (61.11)	641 (64.62)		Reference
	Dominant model	GG	175 (36.69)	213 (42.94)	**0.047**	1.299 (1.004,1.680)
		AA+GA	302 (63.31)	283 (57.06)		Reference
	Recessive model	AA	69 (14.47)	68 (13.71)	0.735	0.939 (0.655,1.348)
		GG+GA	408 (85.53)	428 (86.29)		Reference
CYP24A1						
rs2296239	Genotypes	CC	62 (13.00)	63 (12.70)	0.897	0.974 (0.649,1.460)
		CT	232 (48.64)	242 (48.79)	0.997	0.999 (0.762,1.311)
		TT	183 (38.36)	191 (38.51)		Reference
	Alleles	C	356 (37.32)	368 (37.10)	0.920	0.991 (0.824,1.191)
		T	598 (62.68)	624 (62.90)		Reference
	Dominant model	TT	183 (38.36)	191 (38.51)	0.963	1.006 (0.777,1.303)
		CC+CT	294 (61.64)	305 (61.49)		Reference
	Recessive model	CC	62 (13.00)	63 (12.70)	0.890	0.974 (0.669,1.418)
		TT+CT	415 (87.00)	433 (87.30)		Reference
rs2296241	Genotypes	AA	94 (19.71)	87 (17.54)	0.394	0.853 (0.593,1.229)
		GA	229 (48.01)	242 (48.79)	0.858	0.975 (0.734,1.294)
		GG	154 (32.29)	167 (36.67)		Reference
	Alleles	A	417 (43.71)	416 (41.94)	0.429	0.930 (0.777,1.113)
		G	537 (56.29)	576 (58.06)		Reference
	Dominant model	GG	154 (32.29)	167 (33.67)	0.646	1.065 (0.815,1.391)
		AA+GA	323 (67.71)	329 (66.33)		Reference
	Recessive model	AA	94 (19.71)	87 (17.54)	0.386	0.867 (0.627,1.197)
		GG+GA	383 (80.29)	409 (82.46)		Reference
rs2762934	Genotypes	AA	5 (1.05)	7 (1.41)	0.644	1.314 (0.413,4.176)
		GA	106 (22.22)	99 (19.96)	0.403	0.876 (0.644,1.194)
		GG	366 (76.73)	390 (78.63)		Reference
	Alleles	A	116 (12.16)	113 (11.39)	0.599	0.929 (0.705,1.224)
		G	838 (87.84)	879 (88.61)		Reference
	Dominant model	GG	366 (76.73)	390 (78.63)	0.477	1.116 (0.825,1.509)
		AA+GA	111 (23.27)	106 (21.37)		Reference
	Recessive model	AA	5 (1.05)	7 (1.41)	0.609	1.351 (0.426,4.287)
		GG+GA	472 (98.05)	489 (98.59)		Reference
rs6068816	Genotypes	TT	66 (13.84)	73 (14.72)	0.304	1.153 (0.879,1.513)
		CT	204 (42.77)	225 (45.36)	0.460	1.156 (0.786,1.701)
		CC	207 (43.40)	198 (39.92)		Reference
	Alleles	T	336 (35.22)	371 (37.40)	0.318	1.099 (0.913,1.322)
		C	618 (64.78)	621 (62.60)		Reference
	Dominant model	CC	207 (43.40)	198 (39.92)	0.271	0.867 (0.672,1.118)
		TT+CT	270 (56.60)	298 (60.08)		Reference

(Continued)

TABLE 2 | Continued

SNP	Analyze model		RA (n = 477)	Control (n = 496)	*P* value	*OR* (95% *CI*)
	Recessive model	TT	66 (13.84)	73 (14.72)	0.695	1.075 (0.750,1.540)
		CC+CT	411 (86.16)	423 (85.28)		Reference
VDR						
rs3847987	Genotypes	AA	26 (4.45)	25 (5.04)	0.818	0.935 (0.527,1.658)
		CA	168 (35.22)	180 (36.29)	0.762	1.042 (0.798,1.360)
		CC	283 (59.33)	291 (58.67)		Reference
	Alleles	A	220 (23.06)	230 (23.19)	0.948	1.007 (0.816,1.243)
		C	734 (76.94)	762 (76.81)		Reference
	Dominant model	CC	283 (59.33)	291 (58.67)	0.834	0.973 (0.754,1.256)
		AA+CA	194 (40.67)	205 (41.33)		Reference
	Recessive model	AA	26 (5.45)	25 (5.04)	0.774	0.921 (0.524,1.618)
		CC+CA	451 (94.55)	471 (94.96)		Reference
rs11574129	Genotypes	GG	19 (3.98)	18 (3.63)	0.866	0.944 (0.487,1.832)
		GA	132 (27.67)	151 (30.44)	0.357	1.140 (0.862,1.508)
		AA	326 (68.34)	327 (65.93)		Reference
	Alleles	G	170 (17.82)	187 (18.85)	0.557	1.071 (0.851,1.348)
		A	784 (82.18)	805 (81.15)		Reference
	Dominant model	AA	326 (68.34)	327 (65.93)	0.423	0.896 (0.686,1.171)
		GG+GA	151 (31.66)	169 (34.07)		Reference
	Recessive model	GG	19 (3.98)	18 (3.63)	0.773	0.908 (0.470,1.752)
		AA+GA	458 (96.02)	478 (96.37)		Reference
CYP27B1						
rs4646536	Genotypes	AA	72 (15.09)	64 (12.90)	0.085	0.710 (0.480,1.048)
		GA	227 (47.59)	209 (42.14)	**0.027**	0.735 (0.560,0.965)
		GG	178 (37.32)	223 (44.96)		Reference
	Alleles	A	371 (38.89)	337 (33.97)	**0.024**	0.809 (0.672,0.973)
		G	583 (61.11)	655 (66.03)		Reference
	Dominant model	GG	178 (37.32)	223 (44.96)	**0.016**	1.372 (1.062,1.773)
		AA+GA	299 (62.68)	273 (55.04)		Reference
	Recessive model	AA	72 (15.09)	64 (12.90)	0.325	0.833 (0.580.1.198)
		GG+GA	405 (84.91)	432 (87.10)		Reference

Bold value means P < 0.05.

controls are shown in **Table 5**. The results demonstrated that CYP24A1_1, CYP27B1_1, and VDR_1 methylation levels were significantly lower in RA patients than that in normal controls (P=0.032, P<0.001, P<0.001, respectively).

We further calculated the cumulative methylation levels of each gene by calculating the mean methylation levels of all CpG sites on the included fragments. The methylation levels of VDR and CYP27B1 were significantly reduced when compared to normal controls, while the differences in CYP2R1 and CYP24A1 methylation levels between RA patients and controls were not statistically significant (**Figure 1**). The diagnostic value of VDR and CYP27B1 methylation levels for RA diagnosis was also assessed, and the AUCs of VDR and CYP27B1 were 0.628 (0.559–0.698) and 0.645 (0.575–0.714), respectively (**Figure 2**). Moreover, the optimal cutoff value of VDR for RA diagnosis was 0.018, and the corresponding sensitivity and specificity were 74.8 and 49.2%, respectively. The optimal cutoff value of CYP27B1 for RA diagnosis was 0.031, and the corresponding sensitivity and specificity were 75.6 and 51.2%, respectively.

We also analyzed the influences of the main drug treatment on the methylation levels of these genes, and there were no significant associations regarding CYP24A1, CYP27B1, CYP2R1, and VDR methylation levels between RA patients being treated with glucocorticoid and without, as well as the patients being treated with methotrexate and without (**Table 6**). In addition, the results revealed that CYP24A1, CYP27B1, CYP2R1, and VDR methylation levels were not associated with anti-CCP, RF in RA patients (**Table 6**). Nevertheless, the CYP2R1 methylation level was positively associated with erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) in RA patients (P = 0.003, P = 0.018, respectively) (**Table 7**). No significant correlations were observed about CYP27B1, CYP2R1, and VDR methylation levels and ESR and CRP in RA patients.

Associations Between Vitamin D Metabolic Pathway Gene Polymorphisms With Their Methylation Levels in RA Patients

To explore the associations between the genotype frequencies of *CYP2R1*, *CYP24A1*, *VDR*, and *CYP27B1* genes and their methylation levels among RA patients, we included 122 RA patients for analysis. The results showed no statistical associations between *CYP2R1*, *CYP24A1*, *VDR*, *CYP27B1* methylation levels and their respective genotype in RA patients (**Table 8**).

Plasma Level of 25OHD From RA Patients and Normal Controls

Finally, plasma 25OHD level was measured in 84 RA patients and 84 normal controls in this study. There were 76 females and 8

TABLE 3 | Association between vitamin D metabolic pathway gene polymorphisms and anti-CCP, RF in RA patients.

SNPs	Allele	Clinical features	Group	Genotypes			*P* value	Alleles		*P* value
	(M/m)			MM	Mm	mm		M	m	
CYP2R1										
rs7936142	A/T	anti-CCP	Positive	274	72	5	0.569	620	82	0.613
			Negative	62	16	0		140	16	
		RF	Positive	291	81	6	0.518	663	93	0.473
			Negative	62	16	0		140	16	
rs12794714	G/A	anti-CCP	Positive	143	172	36	0.082	458	244	0.185
			Negative	30	33	15		93	63	
		RF	Positive	156	178	44	0.888	490	266	0.636
			Negative	30	38	10		98	58	
rs1993116	G/A	anti-CCP	Positive	124	173	54	0.762	421	281	0.510
			Negative	31	36	11		98	58	
		RF	Positive	144	177	57	0.139	465	291	0.770
			Negative	24	46	8		94	62	
CYP24A1										
rs2296239	T/C	anti-CCP	Positive	133	176	42	0.779	442	260	0.973
			Negative	31	36	11		98	58	
		RF	Positive	146	186	46	0.945	478	278	0.924
			Negative	29	40	9		98	58	
rs2296241	G/A	anti-CCP	Positive	106	175	70	0.157	387	315	0.189
			Negative	32	31	15		95	61	
		RF	Positive	122	179	77	0.599	423	333	0.401
			Negative	27	39	12		93	63	
rs2762934	G/A	anti-CCP	Positive	267	80	4	0.789	614	88	0.572
			Negative	62	15	1		139	17	
		RF	Positive	287	87	4	0.614	661	95	0.422
			Negative	63	14	1		140	16	
rs6068816	C/T	anti-CCP	Positive	151	152	48	0.593	454	248	0.532
			Negative	38	29	11		105	51	
		RF	Positive	167	162	49	0.506	496	260	0.414
			Negative	33	31	14		97	59	
VDR										
rs3847987	C/A	anti-CCP	Positive	211	123	17	0.353	545	157	0.165
			Negative	40	33	5		113	43	
		RF	Positive	228	131	19	0.216	587	169	0.082
			Negative	39	33	6		111	45	
rs11574129	A/G	anti-CCP	Positive	239	100	12	0.897	578	124	0.645
			Negative	51	24	3		126	30	
		RF	Positive	260	104	14	0.675	624	132	0.366
			Negative	50	24	4		124	32	
CYP27B1										
rs4646536	G/A	anti-CCP	Positive	132	162	57	0.528	426	276	0.621
			Negative	29	40	9		98	58	
		RF	Positive	143	178	57	0.949	464	292	0.794
			Negative	28	38	12		94	62	

males in the RA group, with an average age of 53.15 ± 11.98 years. The control group included 77 females and 7 males, with a mean age of 52.57 ± 9.56 years. We found that the plasma level of 25OHD in RA patients (34.20 ± 5.15 ng/ml) was significantly lower than that in healthy controls (41.09 ± 7.52 ng/ml) (P<0.001).

DISCUSSION

Epidemiological studies had shown a high prevalence of vitamin D deficiency in autoimmune diseases, which could lead to worse disease clinical activity and progression of RA, systemic lupus erythematosus (SLE), and multiple sclerosis (MS) (21). Vitamin D deficiency in patients with autoimmune diseases and general population might be caused by several factors, including skin pigmentation, lack of exposure to sunlight, glucocorticoids use, genetic background, and age (22). Previous studies had identified the potential role of multiple genes, which could regulate vitamin D levels, and suggested that SNPs in these genes (CYP27B1, CYP2R1, VDR, etc.) were related to decreased vitamin D level (17, 23). Therefore, in-depth exploration of the association between vitamin D metabolic pathway gene SNPs and genetic risk of autoimmune diseases was helpful to further reveal the pathogenesis of these diseases. In this study, we analyzed the relationship between 10 SNPs in vitamin D metabolic pathway genes (*CYP2R1*, *CYP24A1*, *CYP27B1*, *VDR*) and RA

TABLE 4 | Haplotype analysis of *CYP2R1, CYP24A1, VDR* in RA patients and controls.

Haplotype	RA [n(%)]	Controls [n(%)]	*P* value	*OR* (95% CI)
CYP2R1 rs7936142-rs12794714-rs1993116				
AAG	332.99 (34.9)	368.99 (37.2)	0.293	0.905 (0.752,1.090)
AGA	370.99 (38.9)	350.99 (35.4)	0.109	1.162 (0.967,1.397)
AGG	250.01 (26.2)	272.01 (27.4)	0.545	0.940 (0.769,1.149)
CYP24A1 rs2296239-rs2296241-rs2762934-rs6068816				
CAAC	85.12 (8.9)	76.67 (7.7)	0.387	1.153 (0.835,1.593)
CAGC	74.28 (7.8)	85.27 (8.6)	0.457	0.884 (0.638,1.224)
CGGC	95.08 (10.0)	102.59 (10.3)	0.706	0.945 (0.703,1.270)
CGGT	60.57 (6.3)	60.03 (6.1)	0.846	1.037 (0.717,1.501)
TAGC	224.08 (23.5)	218.03 (2.5)	0.525	1.072 (0.865,1.328)
TGGC	128.85 (13.5)	124.54 (12.6)	0.610	1.071 (0.822,1.397)
TGGT	232.19 (24.3)	256.14 (25.8)	0.348	0.905 (0.736,1.114)
VDR rs3847987- rs11574129				
AA	50.00 (5.2)	40.00 (4.3)	0.348	1.221 (0.804,1.854)
AG	170.00 (17.8)	187.00 (18.9)	0.557	0.933 (0.742,1.175)
CA	734.00 (76.9)	762.00 (76.8)	0.948	1.007 (0.816,1.243)

Frequency < 0.03 in both controls and RA patients has been dropped.

susceptibility in a Chinese population, and detected the methylation levels of these genes in RA patients.

Among the vitamin D metabolic pathway genes, the presence of SNPs might influence autoimmune disease genetic susceptibility through causing vitamin D deficiency, and modulate the disease activity in type 1 diabetes (TID), SLE, MS. Chen et al. investigated the association of *VDR* polymorphism and genetic risk of SLE in a Chinese population and found that *VDR* rs2228570, rs1544410 polymorphism, and their interaction were all associated with increased SLE risk (24). The role of vitamin D metabolic pathway genes in RA had also been reported in previous studies, such as *CYP2R1* rs10741657 played an effect on vitamin D levels in RA patients (25). In the present study, we found that *CYP2R1* rs1993116, *CYP27B1* rs4646536 polymorphisms were significantly associated with RA susceptibility. In addition to this result, the decreased risk of rs1993116, rs4646536 variant was found to be related to RA risk in dominant mode. Previous studies suggested that rs1993116, rs4646536 variants were closely associated with vitamin D deficiency in human diseases (26, 27). Therefore, we assumed that rs1993116, rs4646536 might be involved in the development of RA by affecting vitamin D status, and the mechanism needed to be validated by more rigorous studies with a larger sample size and different ethnic population. *CYP2R1* rs12794714, *CYP24A1* rs2762934, rs6068816, and *VDR* rs11574129 had been reported to be involved in the genetic background of multiple diseases including diabetic ischemic stroke, gastric cancer, and other diseases (28–31). Unfortunately, this study did not demonstrate a significant association between these SNPs and RA risk. The inconsistencies might be explained by the genetic background of different diseases, sample size, different races, and experimental methods. RA patients could be divided into different genetic subsets according to the antibody status, including RF and anti-CCP (4), while we did not find any significant association with RF and anti-CCP status among RA patients.

In addition to the DNA sequence, genetic information also existed in epigenetic variation, and the role of epigenetic variation in the pathogenesis of diseases should not be overlooked (8, 32). For example, promoter methylation was involved in tumor development by silencing tumor suppressor genes (33). DNA hypomethylation was related to differentiation and proliferation of inflammatory processes and might lead to increased transcription and secretion of inflammatory proteins (34). A previous study was performed to detect the methylation status of lymphatic cells in SLE, RA patients, and found a significant hypomethylation in T cells (11, 35). The role of vitamin D metabolic pathway gene methylation in the development of human disease had been studied, and Wang et al. suggested that the methylation levels of the *CYP24A1*, *CYP27A1*, *CYP27B1*, *CYP2R1*, and *VDR* genes were associated

TABLE 5 | Methylation levels of specific sites between RA patients and controls.

Group	RA patients (n = 122)	Controls (n = 123)	*P* value
CYP2R1_1	0.0072 (0.0068,0.0076)	0.0072 (0.0065,0.0077)	0.708
CYP2R1_2	0.0071 (0.0066,0.0079)	0.0072 (0.0065,0.0079)	0.960
CYP24A1_1	0.0136 (0.0120,0.0155)	0.0143 (0.0127,0.0164)	**0.032**
CYP24A1_2	0.0615 (0.0560,0.0700)	0.0640 (0.0554,0.0737)	0.228
CYP24A1_3	0.0314 (0.0267,0.0376)	0.0318 (0.0271,0.0384)	0.607
CYP24A1_4	0.0662 (0.0577,0.0774)	0.0685 (0.0613,0.0791)	0.206
VDR_1	0.0316 (0.0260,0.0378)	0.0357 (0.0311,0.0421)	**<0.001**
VDR_2	0.0121 (0.0114.0.0129)	0.0121 (0.0108,0.0130)	0.516
CYP27B1_1	0.0307 (0.0263,0.0373)	0.0345 (0.0308,0.0412)	<0.001

Bold value means P < 0.05.

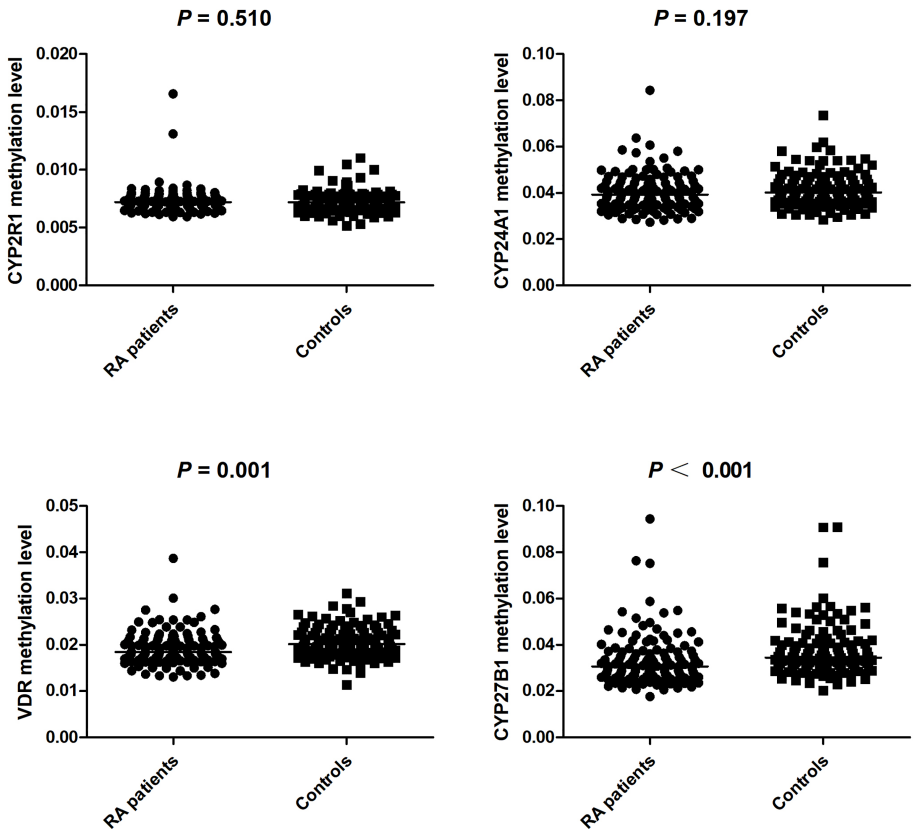

FIGURE 1 | The methylation levels of CYP2R1, CYP24A1, VDR, CYP27B1 between RA patients and controls.

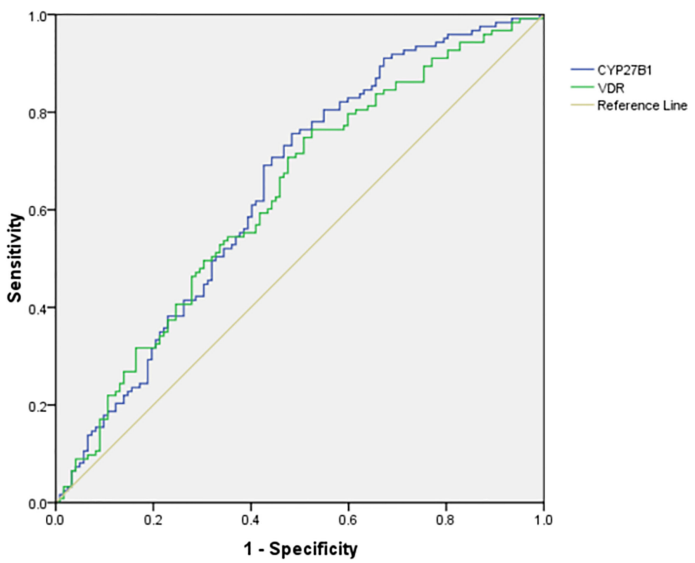

FIGURE 2 | The diagnostic accuracy of VDR and CYP27B1 methylation level in RA.

TABLE 6 | The association between CYP24A1, CYP27B1, CYP2R1, VDR methylation levels and antibody, drug treatment in RA patients.

Group	N	CYP2R1 methylation level	*P* value	CYP24A1 methylation level	*P* value	VDR methylation level	*P* value	CYP27B1 methylation level	*P* value
Antibody									
Anti-CCP			0.460		0.930		0.815		0.414
+	88	0.0073 (0.0069,0.0078)		0.0399 (0.0351,0.0451)		0.0189 (0.0171,0.0213)		0.0310 (0.0264,0.0387)	
–	14	0.0072 (0.0065,0.0076)		0.0393 (0.0350,0.0471)		0.0193 (0.0168,0.0250)		0.0345 (0.0271,0.0402)	
RF			0.266		0.556		0.504		0.770
+	99	0.0072 (0.0068,0.0076)		0.0396 (0.0348,0.0451)		0.0186 (0.0170,0.0212)		0.0312 (0.0263,0.0385)	
–	17	0.0075 (0.0070,0.0078)		0.0386 (0.0340,0.0454)		0.0174 (0.0167,0.0209)		0.0305 (0.0268,0.0354)	
Drug treatment									
glucocorticoid			0.794		0.859		0.067		0.924
+	68	0.0072 (0.0067,0.0077)		0.0305 (0.0261,0.0382)		0.0177 (0.0164,0.0207)		0.0305 (0.0261,0.0382)	
–	40	0.0072 (0.0068,0.0076)		0.0391 (0.0348,0.0450)		0.0192 (0.0177,0.0217)		0.0311 (0.0267,0.0362)	
Methotrexate			0.300		0.877		0.690		0.877
+	38	0.0072 (0.0065,0.0075)		0.0390 (0.0347,0.0444)		0.0187 (0.0167,0.0203)		0.0300 (0.0262,0.0370)	
–	70	0.0072 (0.0068,0.0078)		0.0394 (0.0345,0.0428)		0.0184 (0.0172,0.0212)		0.0309 (0.0266,0.0374)	

with the risk and prognosis of tuberculosis (19). Another study also showed that *cytochrome P450* gene silencing caused by hypermethylation in the promoter region might affect vitamin D activity (36). In the present study, we found that *VDR* and *CYP27B1* methylation levels of RA patients were significantly decreased in comparison to normal controls, and ROC curves showed that these genes could be potential biomarkers for the diagnosis of RA. Moreover, it was necessary to further explore the combined diagnostic effect of VDR, CYP27B1, and other indicators, to improve the sensitivity and specificity of these indicators in RA diagnosis. We also found that CYP24A1_1 (one fragment) level was decreased in RA patients, and CYP2R1 methylation level was significantly associated with ESR and CRP. These suggested that CYP24A1, CYP2R1 gene might be involved in the disease process of RA, but further verification was needed. The relationship between methylation and genetic variation among individuals had also been reported in previous studies (37). We further explored the associations between the genotype frequencies of *CYP2R1*, *CYP24A1*, *VDR*, *CYP27B1* genes and their methylation levels among RA patients; however, no statistical significance was found.

Methotrexate was the first-line therapy in early RA and was often prescribed in combination with glucocorticoid, hydroxychloroquine, *etc.* Previous studies had suggested that the anti-inflammatory mechanism of low-dose methotrexate treatment used in RA might relate to the inhibition of key enzymes in the purine *de novo* synthesis pathway and release of anti-inflammatory adenosine (38). In addition, methotrexate could inhibit methionine S-adenosyltransferase (MAT), followed by the inhibition of S-adenosyl methionine (SAM) *in vivo* and *in vitro*; moreover, SAM was responsible for the donation of methyl groups required for global DNA methylation (39, 40). Therefore, methotrexate was hypothesized to affect global DNA methylation, and one study found that higher baseline global DNA methylation was associated with methotrexate non-response (40). However, we observed that the use of methotrexate, as well as glucocorticoids did not have any significant influence on the methylation levels of these genes.

In conclusion, our results demonstrated that *CYP2R1* rs1993116, *CYP27B1* rs4646536 polymorphisms might contribute to the genetic predisposition to RA, while *CYP24A1* and *VDR* gene polymorphisms were not associated with RA susceptibility in a Chinese population. The methylation levels of *VDR* and *CYP27B1* genes were significantly related to the risk of RA and might be regarded as auxiliary biomarkers for RA diagnosis. Furthermore, we also found that the plasma vitamin D level in RA patients was significantly reduced, which was

TABLE 7 | The relationship between CYP24A1, CYP27B1, CYP2R1, VDR methylation levels and ESR and CRP in RA patients.

Parameters	*CYP2R1*		*CYP24A1*		*VDR*		*CYP27B1*	
	r_s	*P* value	r_s	*P* value	r_s	*P* value	r_s	*P* value
ESR	0.338	**0.003**	0.027	0.822	0.110	0.353	0.002	0.985
CRP	0.274	**0.018**	0.091	0.442	0.053	0.657	0.169	0.151

Bold value means P < 0.05.

TABLE 8 | Association between vitamin D metabolic pathway gene polymorphisms with their methylation levels in RA patients.

CYP2R1 SNP	Genotype	Number	CYP2R1 methylation level	P value
rs7936142	TT	1	0.0069	0.528
	AT	27	0.0072 (0.0069,0.0079)	
	AA	94	0.0072 (0.0068,0.0076)	
rs12794714	AA	12	0.0074 (0.0068,0.0079)	0.772
	GA	53	0.0072 (0.0068,0.0078)	
	GG	57	0.0072 (0.0069,0.0076)	
rs1993116	AA	19	0.0071 (0.0068,0.0075)	0.602
	GA	67	0.0072 (0.0068,0.0077)	
	GG	36	0.0073 (0.0069,0.0078)	
CYP24A1 SNP	Genotype	Number	CYP24A1 methylation level	*P* value
rs2296239	CC	18	0.0363 (0.0318,0.0475)	0.619
	CT	55	0.0382 (0.0347,0.0447)	
	TT	49	0.0396 (0.0355,0.0451)	
rs2296241	AA	20	0.0414 (0.0354,0.0457)	0.393
	GA	62	0.0381 (0.0334,0.0433)	
	GG	40	0.0412 (0.0350,0.0464)	
rs2762934	AA	2	0.0504 (0.0429,0.0579)	0.290
	GA	25	0.0377 (0.0325,0.0479)	
	GG	95	0.0392 (0.0348,0.0442)	
rs6068816	TT	18	0.0405 (0.0360,0.0474)	0.409
	CT	52	0.0387 (0.0337,0.0438)	
	CC	52	0.0396 (0.0355,0.0462)	
VDR SNP	Genotype	Number	*VDR* methylation level	*P* value
rs3847987	AA	6	0.0173 (0.0146,0.0197)	0.132
	CA	41	0.0198 (0.0173,0.0221)	
	CC	75	0.0181 (0.0172,0.0202)	
rs11574129	GG	6	0.0173 (0.0146,0.0197)	0.060
	GA	34	0.0198 (0.0175,0.0225)	
	AA	82	0.0181 (0.0170,0.0203)	
CYP27B1 SNP	Genotype	Number	CYP27B1 methylation level	*P* value
rs4646536	AA	14	0.0314 (0.0267,0.0384)	0.647
	GA	57	0.0311 (0.0268,0.0372)	
	GG	51	0.0294 (0.0256,0.0372)	

Median (interquartile range).

consistent with previous studies. The above results implied that it was of great significance to explore the role of vitamin D metabolism abnormality in the pathogenesis of RA. However, there were some limitations in this that study should be acknowledged. First, this was a case-control study, and we were unable to evaluate the relationship between the methylation levels of these genes and disease activity, medications, clinical efficacy of RA patients over a long period. Second, this study did not analyze the potential influence of ethnic background and environmental factors, as well as the interaction between environmental factors and genetic variation, in RA patients. Finally, the genotyping and ELISA tests were not performed in the same samples, and we were unable to further analyze the relationship between gene polymorphism, methylation, and vitamin D level. The precise role of vitamin D metabolic pathway genes in RA development needed to be further explored in repetitive, functional studies in the future.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the dbSNP repository, accession number 1063300. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of the First Affiliated Hospital of USTC. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

X-ML and T-PZ designed the study. T-PZ and H-ML conducted the experiment. H-ML performed the statistical analyses. QH and LW participated in the collection of samples. T-PZ drafted the manuscript. X-ML and LW contributed to manuscript revision. All authors contributed to the article and approved the submitted version.

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NFAT5 Controls the Integrity of Epidermis

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The skin protects the human body against dehydration and harmful challenges. Keratinocytes (KCs) are the most abundant epidermal cells, and it is anticipated that KC-mediated transport of Na^+ ions creates a physiological barrier of high osmolality against the external environment. Here, we studied the role of NFAT5, a transcription factor whose activity is controlled by osmotic stress in KCs. Cultured KCs from adult mice were found to secrete more than 300 proteins, and upon NFAT5 ablation, the secretion of several matrix proteinases, including metalloproteinase-3 (Mmp3) and kallikrein-related peptidase 7 (Klk7), was markedly enhanced. An increase in Mmp3 and Klk7 RNA levels was also detected in transcriptomes of *Nfat5*$^{-/-}$ KCs, along with increases of numerous members of the 'Epidermal Differentiation Complex' (EDC), such as small proline-rich (Sprr) and S100 proteins. NFAT5 and Mmp3 as well as NFAT5 and Klk7 are co-expressed in the basal KCs of fetal and adult epidermis but not in basal KCs of newborn (NB) mice. The poor NFAT5 expression in NB KCs is correlated with a strong increase in Mmp3 and Klk7 expression in KCs of NB mice. These data suggests that, along with the fragile epidermis of adult *Nfat5*$^{-/-}$ mice, NFAT5 keeps in check the expression of matrix proteases in epidermis. The NFAT5-mediated control of matrix proteases in epidermis contributes to the manifold changes in skin development in embryos before and during birth, and to the integrity of epidermis in adults.

Keywords: epidermis, keratinocytes, kallikrein 7, matrix proteases, Mmp3, NFAT5, skin

Abbreviations: Ab, antibody; KC, keratinocytes; Klk7, kallikrein 7; Mmp3, matrix metalloproteinase-3; NFAT, nuclear factor of activated T cells; NB mice, newborn mice; NGS, next generation sequencing; RPKM, reads per kilobase million; T, tail; TF, transcription factor; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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INTRODUCTION

The skin represents a physical barrier that shields the body against harmful environmental challenges and protects against dehydration (1). Large amounts of Na^+ ions are stored in the skin, and it has been anticipated that an active transport of Na^+ by KCs creates a physiological barrier within or below the epidermis with high osmolality (2–4). One factor that could orchestrate the storage of salt ions in the skin is the osmo-sensitive transcription factor NFAT5. However, so far there are no detailed studies on the function of NFAT5 in the skin.

To a large part, the skin barrier formation and function are conferred by the corneocytes in the *stratum corneum*, the outermost epidermal layer. In healthy adult epidermis, corneocytes are terminally differentiated KCs that assemble an insoluble complex of lipids and numerous adhesion proteins which are organized in corneodesmosomes (5, 6). Extracellular matrix proteases, such as metalloproteases and kallikreins, are secreted by KCs, cleave corneodesmosomes and, thereby, release corneocytes as skin scales (7, 8).

The differentiation of basal epidermal KCs to corneocytes is a fine-tuned process that lasts approximately 3-4 weeks in human and one week in murine skin. Only KCs of the *stratum basale* (the basal KCs) proliferate and, when detached, differentiate into cells of the *stratum spinosum*, followed by development to the *stratum granulosum*, and finally to the corneocytes of the *stratum corneum*. This well-coordinated Ca^{++}-dependent differentiation process is controlled by the activity of numerous genes that are specifically expressed in various epidermal layers.

Genes that are expressed predominantly or exclusively in the basal layer are several keratin, laminin and integrin genes. The K5/14 keratin heterodimers encoded by the keratin *Krt5* and *Krt14* genes constitute almost 50% of the protein content of basal KCs. Rising Ca^{++} levels in the upper epidermal layers promote differentiation. The synthesis of K5/14 keratins ceases and shifts to the expression of K1/10 keratin heterodimers (9–11). The laminin subunits gamma-2 (LAMC2) and beta-3 (LAMB3) as well as the $\alpha3\beta1$ and $\alpha6\beta4$ integrin heterodimers support basal KCs to assemble and to adhere to the basement membrane. Their synthesis is inhibited with the onset of differentiation, and in case of integrins, is replaced by other members of the integrin family (12).

We show here that the osmo-sensitive transcription factor NFAT5 (13) controls the transition of basal to suprabasal KCs, and finally to corneocytes in adult skin. While we did not detect an obvious role for NFAT5 in the intracellular transport of Na^+ ions in or through the epidermis, NFAT5 controls the expression of numerous genes that orchestrate the differentiation of KCs to corneocytes. In basal KCs, NFAT5 suppresses differentiation by inhibiting the formation of 'cornification proteins', such as Sprr and S100 proteins and the transglutaminases Tgm1 and Tgm2. The suppression of matrix proteases kallikrein 7 (Klk7) and matrix metallopeptidase 3 (Mmp3) in basal KCs of adult mice contributes to the integrity of skin that, upon NFAT5 ablation, shows a pre-mature shedding of skin scales. By contrast, both matrix proteases are 10-fold stronger generated in KCs from NB mice in which NFAT5 is poorly expressed. Similar to their coordinated expression in adult KCs, NFAT5 and Mmp3 are simultaneously expressed in KCs of murine and human embryos suggesting a role for NFAT5 in early skin formation and later on in the integrity of adult skin.

MATERIALS AND METHODS

Mice, Preparation and Short-Term Cultures of Primary Murine KCs and T Cells

If not stated otherwise, 8- to 12-week-old C57BL/6J mice were used in the experiments. All *Nfat5*$^{-/-}$ mice were on the 129/sv background, and in all assays in which they were used littermate WT mice were analysed. 129sv mice are smaller in size, show a kidney atrophy and are under permanent hypernatremia (14, 15). For the preparation of NFAT5-deficient KCs, the tails from *Nfat5*$^{-/-}$ and their wild type (WT) siblings were sent from Barcelona to Würzburg overnight in wet chambers on ice and used for the preparation of KCs. Primary KCs from tails of adult mice or from NB mice were prepared as described (16). However, instead of 0.25% porcine trypsin that in our hands killed primary murine KCs, TrypLE (ThermoFisher) was used for the preparation and splitting of cells. The skin of mice was incubated overnight in dispase solution (4 mg/ml, in KC medium) at 4°C. The epidermis was prepared, divided into small pieces and incubated in TrypLE for 15 min at 37°C. After suspension in S-MEM medium (Sigma-Aldrich), strong shaking and passage through a 70 µM cell strainer, the cells were plated on pre-coated plates for 5 h, followed by a PBS wash step and incubation in serum-free SFM KC medium (Gibco) containing 0.06 mM Ca^{++}.

Splenic murine $CD4^+$T cells were prepared as described previously, and cultivated in RPMI medium. They were activated either by antiCD3/CD28 (3/1 µg/ml), coated on plastic, or TPA (10 ng/ml) plus ionomycin (0.5 µM) for 24 h.

Transduction of KCs With NFAT5-Bio Retroviruses

Retroviruses expressing tagged NFAT5-bio and GFP were generated by transfections of plat E packaging cells (17) with viral NFAT5-bio/GFP or GFP constructs using GenJet Reagent (SigmaGen Laboratories). The next day, the medium was changed, and the following day the supernatant of plat E cells was passed through a 0.45 µm filter and, upon incubation with polybrene (4 µl per ml of a 1 mg/ml stock solution) for 20 min at room temperature, used in spin transfections. KCs grown in 2 ml SFM KC medium on pre-coated 6-well plates were transduced by adding 2 ml retroviral supernatant to 1 ml of culture medium, followed by centrifugation of parafilm-sealed culture plates at 32°C and 2200 rpm for 2.5 h. After centrifugation, the plates were maintained for 2 h at 37°C in a CO_2 incubator, followed by washing of cells with PBS and culture in KC medium. The efficiency of transduction was monitored by fluorescence

microscopy and by transducing NIH 3T3 control cells in parallel, which revealed GFP expression in more than 60% of the cells by flow cytometry (see **Figure 5E**).

For construction of the NFAT5-bio vector, full-length human NFAT5 (18) was amplified by PCR using the primers 5`Cla_hNFAT5: 5`ttatcgatggcggtgcttgcagctcc3` and 3`hNFAT5_Bio: 5`cagacctccaccgcccaattgaaaggagccagtcaagttg3`. The bio/avidin-tag (19) was generated by PCR using the primers 5`hNFAT5C_Bio: 5`caacttgactggctcctttcaattgggcgg tggaggtctg3` and 3`BamHI_Bio: 5`aaggatcccacgagcctccggcg tttgag3`. The overlapping products were amplified by a second PCR using the primers 5`Cla-hNFAT5 and 3`BamHI_Bio and cloned as a ClaI/BamHI fragment into the retroviral expression vector pEGZ (20). The retroviral pMSCV-F-BirA vector was purchased from BCCM/LMBP (Gent-Zwijnaarde, Belgium).

Secretion Assays

To determine the secretome of WT and *Nfat5*$^{-/-}$ KCs, KC proteins were labelled with azidohomoalanine (AHA) for 20 h, followed by click chemistry-based enrichment of secreted proteins, on-resin trypsinisation and liquid chromatography-mass spectrometry (LC-MS) analysis (21). To this end, 2-3 x10^6 KCs maintained in a 10-cm culture dish for one week *in vitro* were incubated with 7 ml RPMI-depletion medium (lacking methionine, arginine and lysine) for 30 min, followed by incubation for 20 h in methionine-free RPMI supplemented with lysine, arginine and 0.1 mM AHA, an azide-bearing analog of methionine. The cells were harvested, washed three times with PBS and quickly frozen in liquid nitrogen upon adding protease inhibitor.

Enrichment of Secreted Proteins and Quantitative Proteomic Analysis

Azide-tagged proteins were enriched from cell culture supernatants of primary KCs isolated from wild type and *Nfat5*$^{-/-}$ mice using a click chemistry approach according to manufacturer's instructions (Click Chemistry Capture Kit, Jena Bioscience, Jena, Germany) with minor modifications. Briefly, 7 ml of media were concentrated on a centrifugal filter unit (Amicon Ultra-15, 3 kDa MWCO, Merck, Darmstadt, Germany) at 4,000*g* and 4°C to a final volume of 250 µl. The click reaction was assembled in a 2 ml tube (Protein LoBind tubes, Eppendorf, Hamburg, Germany) by combining the concentrated sample, 250 µl of lysis buffer (200 mM Tris, 4% CHAPS, 1 M NaCl, 8 M urea, pH 8.0), 500 µl of 2× copper catalyst solution and 100 µl of washed alkyne agarose resin (Jena Bioscience). The reaction was incubated on a rotator at room temperature for 20 h. After reduction and alkylation of resin-bound proteins using dithiothreitol (DTT) and iodoacetamide (IAA), the resin was transferred to a spin column. The column was washed five times using 2 ml of agarose wash buffer (100 mM Tris, 1% SDS, 250 mM NaCl, 5 mM EDTA, pH 8.0), ten times 2 ml of urea buffer (100 mM Tris, 8 M urea, pH 8.0) and 10 times 2 ml of 20% (v/v) LC-MS grade acetonitrile/water. The resin was recovered in digestion buffer (50 mM ammonium bicarbonate and LC-MS grade water), transferred to a new tube and centrifuged at 1,000g for 5 min. The supernatant was discarded, 0.5 µg of trypsin (Trypsin Gold, Promega, Madison, WI, USA) was added to the remaining 200 µl of resin slurry and the samples were incubated overnight at 37°C. Afterwards, the resin was pelleted and the supernatant containing tryptic peptides was transferred to a clean tube. To improve peptide recovery, the resin was resuspended in 500 µl of LC-MS grade water. After centrifugation, the supernatant was transferred to the digest supernatant. Peptides were acidified with trifluoroacetic acid (TFA) to achieve a final concentration of 0.5% (v/v) and desalted on Sep-Pak tC18 96-well cartridges (Waters Corporation, Milford, MA, USA) using 0.1% (v/v) TFA in LC-MS grade water as wash solvent and 0.1% (v/v) TFA in 50% (v/v) acetonitrile/water as elution solvent. Purified peptides were lyophilized and reconstituted in 20 µl of 0.1% (v/v) formic acid in LC-MS grade water prior LC-MS analysis. To control for non-specifically enriched proteins, one-tenth of the cell culture supernatant was separated and processed in parallel in a similar manner, but without adding copper sulfate.

Peptide samples were analysed by consecutive LC-MS runs in triplicates using a nanoACQUITY UPLC system (Waters Corporation, Milford, MA, USA) coupled to a SYNAPT G2-S mass spectrometer (Waters Corporation) *via* a NanoLockSpray dual electrospray ionization source (Waters Corporation). Equal sample volumes of 0.3 µl were injected onto a HSS-T3 C18 250 mm × 750 µm reversed phase column (Waters Corporation) for each measurement and peptides separated by gradient elution over 90 min resulting in total analysis times of 110 min. Mass spectra were acquired in ion mobility-enhanced data-independent mode (UDMSE) (PMID: 24336358). [For details see (22)].

Raw data were processed by ProteinLynx Global Server (v3.0.2, Waters Corporation) and searched against the mouse Swiss-Prot protein sequence database (UniProtKB release 2018_07, 16,669 entries) and 171 common MS contaminants using following parameters: Trypsin was specified as digestion enzyme, two missed cleavages per peptide were allowed, carbamidomethylation of cysteines was set as fixed, and methionine oxidation as variable modification. The false discovery rate (FDR) was calculated in PLGS by searching a database of reversed protein sequences and a cutoff of 0.01 was applied. Label-free quantification (LFQ) including retention time alignment, feature clustering, cross-run normalization and protein inference was performed in ISOQuant v1.8 (22). At this time, control samples were not included in the analysis to avoid normalization artefacts. Only peptides without missed cleavages, a minimum sequence length of six amino acids, a minimum PLGS score of 6.0 and no variable modification were considered for quantification. Proteins identified by at least two different peptides were quantified by averaging the intensities of the three peptides with the highest intensities belonging to the respective protein (Top3 method, https://doi.org/10.1074/mcp.M500230-MCP200). An FDR cutoff of 0.01 was applied at the peptide and protein level in ISOQuant. The ISOQuant analysis was repeated without normalization including control samples in order to identify non-specifically enriched proteins. Proteins found in more than one biological replicate with at least one-

tenth of the Top3 intensity in the negative control compared with the sample were not considered potential candidates.

Differential protein abundance testing between wild type and *Nfat5*$^{-/-}$ animals was performed using Perseus v1.6.2.1 (PMID: 27348712) following $\log_2$-transformation of Top3 intensities and left-censored missing value imputation ("replace missing values from normal distribution" with default settings). *P*-values derived from two-sided Student's *t*-tests were adjusted for multiple hypothesis testing using the Benjamini-Hochberg method applying an FDR cutoff of 0.05.

The MS proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD028675 (Project name: Murine keratinocytes NFAT5 knockout secretome LC-MS/MS).

Western Blot and RT-PCR Assays

Western blots were performed either with whole protein extracts or with extracts of cytosolic and nuclear proteins on PAGE-SDS gels. For detecting NFAT5, the following Abs were used: Ab3446 (directed against the C-terminal aa1439-1455 of human NFAT5), Ab110995 (directed against an internal region of human NFAT5; both Abcam), sc-398171 (directed against aa67-300 of human NFAT5; Santa Cruz, Biotech) and PAI-023 (directed against a C-terminal peptide of human NFAT5; Affinity BioReagents). NFATc proteins were detected using the 7A6 mAb #556602 (for NFATc1) and the mAb #5062574 (for NFATc2; both BD Pharmingen). As loading control, filters were stained by Ponceau Red for 5 min and/or re-probed with the mAb #ab8227 specific for β-actin. Signals were developed using a chemiluminescence detection system (ThermoFisher Scientific).

For qRT-PCR assays, RNA was isolated from freshly harvested and PBS-washed or from deep-frozen cells using a standard TRIzol/isopropanol protocol. cDNAs were synthesized using the iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad). Real-time PCR assays were performed using the SYBR green master mix (Applied Biosystems) with the primers presented in **Supplementary Table 1**.

Transcriptome Assays by Next Generation Sequencing (NGS)

Total RNA was purified with RNeasy Plus Micro Kit according to the manufacturer's protocol (Qiagen) and quantified using a Qubit 2.0 fluorometer (Invitrogen). Quality was assessed on a Bioanalyzer 2100 (Agilent) using a RNA 6000 Nano chip (Agilent). Samples with an RNA integrity number (RIN) of > 8 were used for library preparation. Barcoded mRNA-seq cDNA libraries were prepared from 600 ng (WT vs Nfat5-KO experiment) or 140 ng (1 week vs 3 weeks experiment) of total RNA using the NEBNext® Poly(A) mRNA Magnetic Isolation Module and NEBNext® Ultra™ II RNA Library Prep Kit for Illumina® according to the manual with a final amplification of 11 (WT vs Nfat5) or 12 PCR cycles (1 week vs. 3 weeks). The quantity was assessed using Invitrogen's Qubit HS DNA assay kit. The library size was determined using Agilent's 2100 Bioanalyzer HS DNA assay. Barcoded RNA-Seq libraries were onboard clustered using HiSeq® Rapid SR Cluster Kit v2 using 8pM and 59bps were sequenced on the Illumina HiSeq2500 using HiSeq® Rapid SBS Kit v2 (59 cycles). The raw output data of the HiSeq was pre-processed according to the Illumina standard protocol. Sequence reads were trimmed for adapter sequences and further processed using Qiagen's software CLC Genomics Workbench (v12.0 with CLC's default settings for RNA-Seq analysis). Reads were aligned to the GRCm38 genome and expression values are RPKM. - All sequencing data were submitted to the GEO repository and are publicly available (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE184180).

Immunohistochemistry and Tape Stripping Assays

Primary KCs were fixed with 4% formaldehyde (in PBS) on culture dishes followed by incubation with 0.2% Triton X-100. After PBS washing, the cells were blocked with DAKO diluent solution followed by incubation for 90 min with primary Abs (diluted 1:100-1:400) in DAKO blocking solution. The primary Abs used are indicated in the figure legends.

Anonymized tissue specimens of human fetal skin were drawn from the pathology files of the Institute of Pathology (University Wuerzburg).

Tape stripping of skin was performed with ethanol-washed tails from WT and *Nfat5*$^{-/-}$ mice according to Ref (23). using corneofix tapes (Courage + Khazaka, Cologne).

Statistical Analysis

Statistical analysis was performed by using GraphPad Prism 6.0 (GraphPad Software, San Diego, USA). Data was presented as mean and error bars in figures represent ± SEM. Unpaired t-tests was used to evaluate the statistical differences. The values ≤0.05 were considered significant. ***p<0.0001, **p<0.001 and *p<0.05.

RESULTS

NFAT5 Is Expressed in Basal and Suprabasal KCs of Epidermis

Ca^{++} play an important role in epidermal differentiation (10). Therefore, one may speculate that the Ca^{++}/calcineurin/NFATc signaling cascade, which plays a central role in lymphocytes, might also control epidermal differentiation. However, when we investigated the expression of NFATc1 and NFATc2, the two most abundant NFAT factors in lymphocytes, in immunoblots, we observed a relatively poor NFATc expression in KCs, compared to lymphocytes. By contrast, the osmo-sensitive factor NFAT5, a distant relative of genuine NFATc factors (13), was expressed at a similar level in KCs and lymphocytes (**Supplementary Figures 1A, B**). This observation prompted us to study the role of NFAT5 in KCs.

Co-stainings of skin sections from adult humans with antibodies (Abs) raised against NFAT5 and markers of basal KC, as keratin 14 (K14) (6), or of suprabasal KC, as filaggrin (24), revealed NFAT5 expression throughout the epidermis. In numerous basal KCs, NFAT5 expression appeared to be restricted

to the cytoplasm, whereas in suprabasal KCs we detected NFAT5 expression predominantly – albeit not exclusively – in the nuclei of KCs (**Supplementary Figures 1C, D**). Under pathophysiological and experimental *in vitro* conditions, such as in epidermis from Ichthyosis patients and of sun-exposed persons, NFAT5 was predominantly expressed in suprabasal KCs (**Supplementary Figures 1D, E**).

We also stained NFAT5 in cytospins of cultured murine KCs. By using Abs raised against a C-terminal NFAT5 epitope (ab3446) or an Ab directed against an internal region of human NFAT5 (ab110995) we detected NFAT5 both in cytosol and nuclei of KCs. While the ab3446 Ab stained predominantly the nuclei of cultured KC, the ab110995 Ab stained their cytosol indicating the expression of NFAT5 in both cellular compartments of basal KCs in culture (**Supplementary Figures 2A, B**). This suggest a complex expression of NFAT5 in multiple isoforms in KCs, as described previously for other cells (25).

Ablation of NFAT5 in mice did not lead to gross changes of skin morphology (15, 26). However, when we prepared the skin and KCs from tails of *Nfat5*$^{-/-}$ mice, we detected the release of large skin sheets into the preparation medium that we never observed during the preparation of WT tail skin (**Figures 1A–C**). These sheets consisted of corneocytes and suprabasal KCs (see insert in **Figure 1C**). The fragile skin from *Nfat5*$^{-/-}$ mice was also observed in tape stripping assays (23) using tails from WT and *Nfat5*$^{-/-}$ mice (**Figures 1D, E**). The first tape strips from the skin of *Nfat5*$^{-/-}$ mouse tails revealed a tight layer of corneocytes packed by skin scales which differed in density and their large size from those of WT mice. While the skin of *Nfat5*$^{-/-}$ mice formed frequently a patchwork of large, released scales, the WT corneocytes showed a loose assembly of single scales (see inserts in **Figures 1D, E**). Sections through the tail skin of *Nfat5*$^{-/-}$ mice revealed a more fragile *stratum corneum* than in WT skin (compare **Figures 1F** with **1G**), and in numerous sections of tails from *Nfat5*$^{-/-}$ mice the *stratum corneum* was completely lost (**Supplementary Figure 3**).

NFAT5 Affects Protein Secretion by KCs

The cornification of KCs and desquamation of corneocytes is controlled by numerous proteases that are secreted by KCs (8). These proteases cleave the proteins of extracellular desmosomes to allow shedding of corneocytes. In order to elucidate whether NFAT5 affects the secretion of murine KCs we isolated KCs from tails of WT and *Nfat5*$^{-/-}$ mice and cultured them for 2-4 weeks in serum-free SFP KC medium (Gibco) containing 0.06 mM Ca^{++}. Under these low Ca^{++} conditions, the basal KCs generated a layer of cobblestone-like KCs within a few days. However, upon culture for 3 weeks and longer the confluent primary KCs started to enlarge their size and formed cornification-like envelopes in the lawn of KCs (**Supplementary Figures 4A, B**).

Using a novel metabolic labelling technique, we determined the secretome of WT and *Nfat5*$^{-/-}$ KCs. To this end, KC proteins were labelled with azidohomoalanine (AHA) for 20 h, followed by avidin-based affinity purification of secreted proteins, on-resin trypsinisation and mass spectroscopy (21). In those assays, we detected approximately 300 proteins that were secreted by WT and *Nfat5*$^{-/-}$ KCs within 24 h. Among the 24 proteins whose secretion was increased twofold and more by *Nfat5*$^{-/-}$ cells as compared to WT KCs we detected four extracellular matrix proteins and three enzymes (**Figure 2A**). The three enzymes matrix metallopeptidase 3 (Mmp3, encoded by the *Mmp3* gene; also known as stromelysin-1), kallikrein 7 (*Klk7*), and chitotriosidase 1 (*Chit1*) that showed a strong increase in secretion by *Nfat5*$^{-/-}$ compared to WT KCs are known to cleave and process extracellular matrix proteins and to control the sclerodermisation of epithelia (27–29). Three of the matrix proteins, suprabasin (*Sbsn*), dermokine (*Dmkn*) and desmocollin 2 (*Dsc2*) are known to be involved in the terminal differentiation of corneocytes, and two further cornification proteins, desmocollin 3 (*Dsc3*) and corneodesmin (*Cdsn*) (30–32), were also stronger secreted by *Nfat5*$^{-/-}$ than WT KCs (**Figure 2A**).

Within the pattern of 39 proteins whose secretion by *Nfat5*$^{-/-}$ KCs was diminished twofold and more, neither any cornification protein nor any protease, except Mmp9, was detected. Instead, *Nfat5*$^{-/-}$ KCs showed a decrease in several proteins that control RNA processing and translation, and enzymes that control cellular metabolism, as the glycolytic enzymes alpha enolase 1 (*Eno1*), fructose-biphosphate aldolase A (*Aldoa*), L-lactate dehydrogenase B (*Ldhb*), transketolase (*Tkt*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (**Figure 2B**). However, when we investigated the incorporation of glucose into cultured KCs using the fluorescent glucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2deoxy-D-glucose (2-NBDG) we observed a 2-3-fold increase in 2-NBDG uptake into *Nfat5*$^{-/-}$, compared to WT KCs (**Supplementary Figure 5**). This suggests a suppressive influence of NFAT5 on the metabolism of basal KCs.

NFAT5 Orchestrates KC Differentiation

To elucidate whether and how NFAT5 affects gene transcription in KCs, we used NGS assays to compare the transcriptomes of WT and *Nfat5*$^{-/-}$ KCs prepared from tails of adult mice. Due to the large variations that we observed in initial assays between various preparations of tail KCs, we first compared the transcriptomes of the same batches of KCs from WT C57BL/6J mice that were cultured either for one or for three weeks. To our surprise, we detected fundamental differences in the expression levels of numerous genes at these time points (**Supplementary Figure 6A**). The marked differences in gene expression profiles of 129/sv WT versus *Nfat5*$^{-/-}$ KCs (see **Supplementary Figure 6B**) might reflect the rapid differentiation events that change gene expression between basal and suprabasal KCs in murine skin during differentiation. Therefore, all transcriptomes that we obtained reflect a snapshot of a restricted life period of basal KCs. To keep this in mind, in the following we present and discuss the transcriptome results of KCs from two129/sv WT and *Nfat5*$^{-/-}$ sibling mice.

Among the 121 genes that were expressed in two and more RPKMs (Reads Per Kilobase Million, total reads in a sample divided by 1 mill.) in *Nfat5*$^{-/-}$ compared to WT KCs we detected

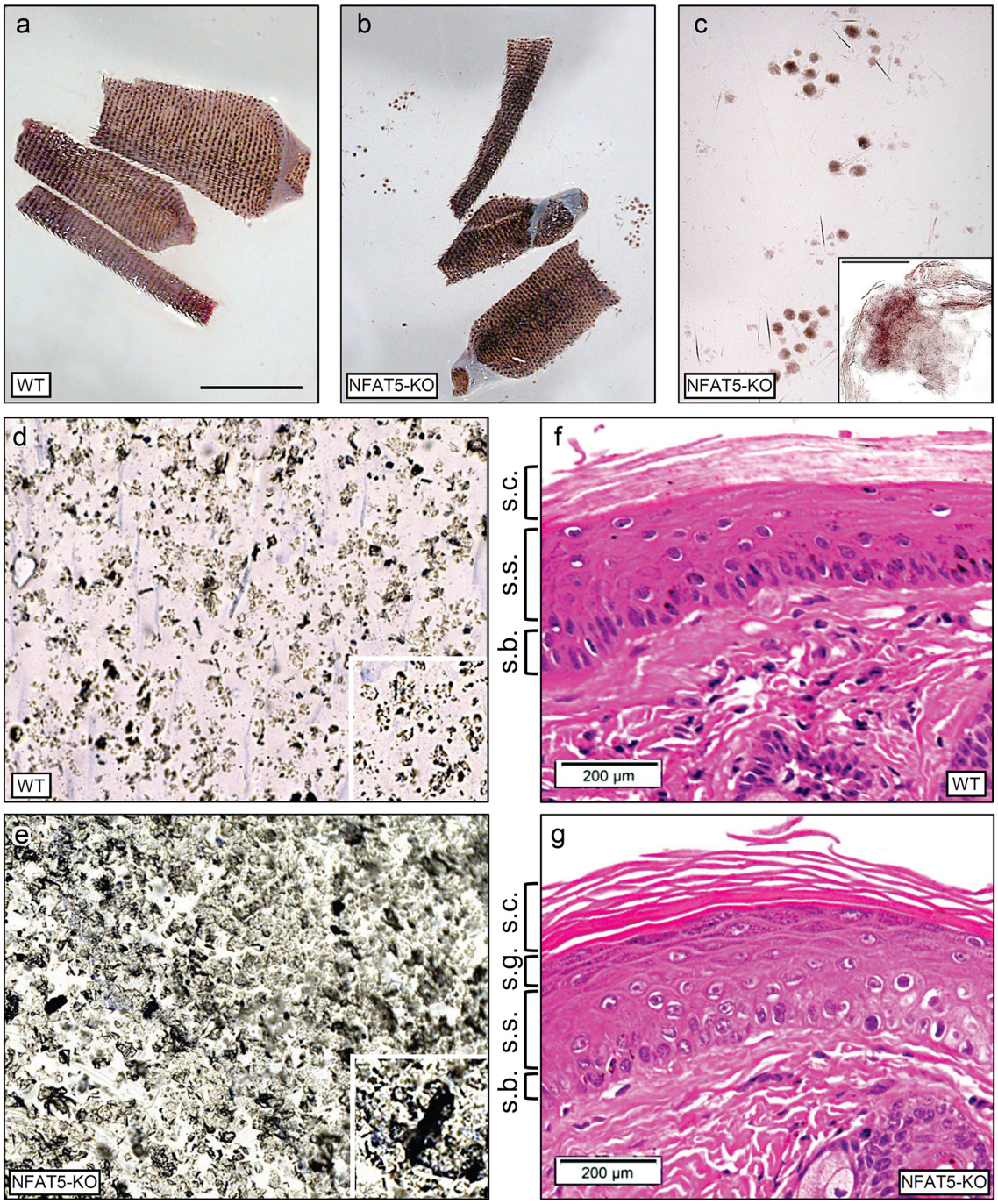

FIGURE 1 | NFAT5 ablation changes the integrity and morphology of murine epidermis. **(A, B)** Preparations of skin from 129sv WT **(A)** and 129sv *Nfat5*$^{-/-}$ mice **(B, C)**. In **(C)**, skin sheets released from tail skin of *Nfat5*$^{-/-}$ mice are presented at larger magnification. Bar= 100 µM. **(D, E)** Tape strips from the tails of WT **(D)** and *Nfat5*$^{-/-}$ mice **(E)**. One representative strip of more than ten is shown. **(F, G)** Representative H&E stains of skin sections from tails of WT **(F)** and *Nfat5*$^{-/-}$ mice **(G)**. s.c., *stratum corneum*; s.g., *stratum granulosum*; s.s., *stratum spinosum*; s.b., *stratum basale*.

nine genes encoding TFs (see blue arrows in **Figure 3**). Four of the TF genes, *Mxd1* (encoding the Myc partner Mad), *Hopx, Grhl3* and *Klf10* are known to control the proliferation of KCs (33–36), *Barx2* and *Hopx* regulate hair follicle differentiation (37, 38) while *Foxn1*, *Sox11* and *Egr1* are expressed during wound repair (39–41) and *Egr1* and *Zfp750* in psoriatic lesions (42, 43) (**Supplementary Table 2**). Only one TF gene, the *Irf9* gene, which is highly expressed in psoriatic lesions (44), appeared in

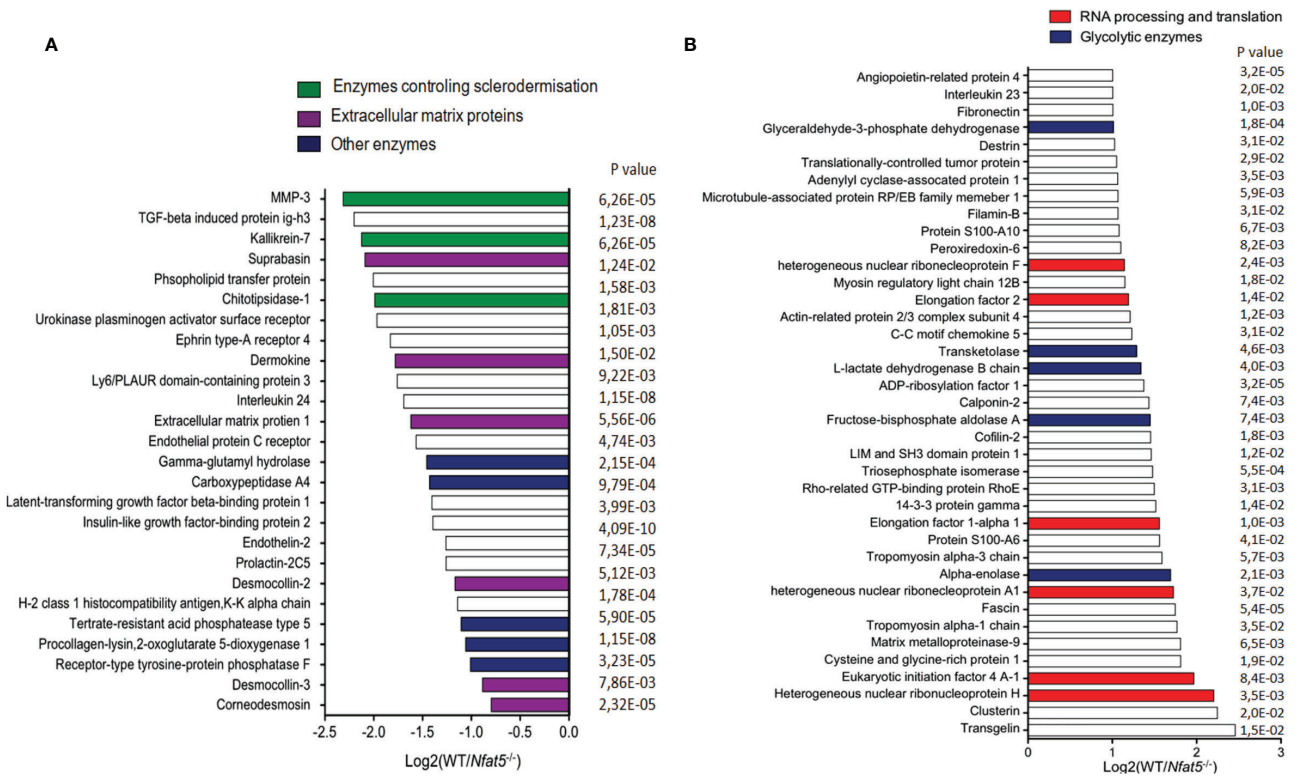

FIGURE 2 | Proteins that were secreted by isolated tail KCs within 24 h of culture. **(A)** Proteins that were 2-fold and more secreted by 129sv *Nfat5*$^{-/-}$ than 129sv WT KCs. **(B)** Proteins that were at least 2-fold less secreted by *Nfat5*$^{-/-}$ than WT KCs. Data of two independent experiments each analyzed in triplicates by LC-MS were compiled.

the list of the 47 genes that were expressed 2-fold stronger in *Nfat5*$^{-/-}$ than WT KCs (**Supplementary Figure 7** and **Supplementary Table 2**).

The differentiation of basal towards suprabasal KCs is closely associated with a reduction in cell proliferation. A diminished proliferation was observed *in vitro* upon prolonged culture of primary KCs at the cellular (**Figure 4A**) and molecular levels. KCs cultured for one week divided rapidly and expressed relative low levels of the cell cycle inhibitors *Cdkn1a*/p21^{WAF}, *Cdkn2a*/p16^{INK4a} and *Cdkn2b*/p15^{INK4b} whereas after 3 weeks and more they slowed down in proliferation while expressing up to 30-fold higher levels of cell cycle inhibitors. On the contrary, the expression of cyclins *Ccnd1*/cyclin D1, *Ccna1*/cyclin A2, *Ccnb1*/cyclin B1 and B2, and of cyclin-dependent kinase 1 (Cdk1) was much stronger in KCs cultured for 1 week than in cells cultured for 3 weeks (**Figure 4** and **Supplementary Figure 8**). The drop in KC proliferation was also reflected in a decrease of components of RNA polymerase I whose expression reflects cell cycle progression (45) (**Supplementary Figure 8D**). However, among the numerous genes that control KC proliferation the *Cdkn2a* gene seems to be the only gene whose expression is controlled directly by NFAT5. In *Nfat5*$^{-/-}$ KCs we detected a 4- to 6-fold increase in *Cdkn2a* transcripts whereas in KCs transduced with retroviruses expressing NFAT5 a marked reduction in proliferation and levels of *Cdkn2a* transcripts was observed (**Figures 4C, D**).

Numerous genes encoding cornification, tight junction and adhesion proteins belong to the group of genes that were stronger transcribed in *Nfat5*$^{-/-}$ than in WT KCs. Among those are ten genes that code for proteins of the Epidermal Differentiation Complex (EDC). These are seven small proline-rich proteins (Sprr1a, Sprr1b, Sprr2b, Sppr2d, Sppr2e, Sprr2h and Sprr2i), two S100 family proteins (S100a8 and S100a9), and the late cornified envelope protein Lce1h. Further genes that are suppressed by NFAT5 encode lipocalin 2 (*Lcn2*), the tight junction proteins claudins 3 and 6 (*Cldn3* and *6*), cornefilin (*Cnfn*), cadherin 2 (*Cdh2*), fibronectin 1 (*Fn1*), the KC differentiation-associated protein (*Krtdap*), and the *Tgm1* and *Tgm2* genes (**Figure 3** and **Supplementary Figure 9**).

Apart from the *S100a7a* gene encoding the S100 calcium binding protein A7A that is highly expressed in psoriatic lesions (46), none of these 'KC-differentiation' genes were found in the list of genes that were decreased in expression in *Nfat5*$^{-/-}$ KCs (**Supplementary Figure 7**). The majority of 'differentiation' genes did not increase but decrease upon prolonged culture of KCs for 3 weeks. Exceptions are the *Fn1, Tgm2* and *Lcn2* genes whose expression increased upon prolonged culture of tail KCs. On most of these genes, over-expressing NFAT5 upon

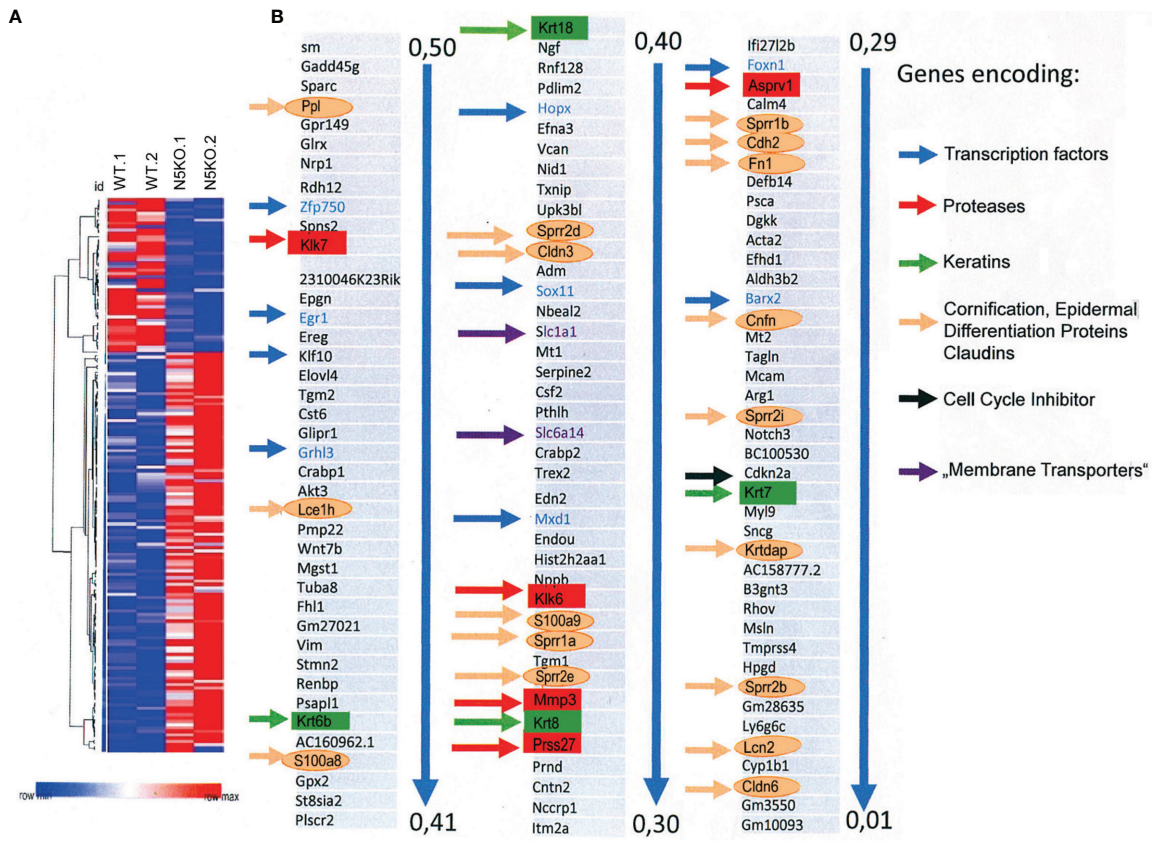

FIGURE 3 | Ablation of NFAT5 changed gene expression in cultivated KCs. **(A)** Heat map of 168 genes that were changed at least twofold in 129sv *Nfat5*$^{-/-}$ compared to 129sv WT KCs. **(B)** Compilation of 121 genes that were expressed in 2-fold more copies in *Nfat5*$^{-/-}$ KCs. Nine genes encoding TFs are shown in blue and indicated by blue arrows. Five protease genes are boxed and shown in red, four keratin genes are shown in green boxes and by green arrows. The gene coding for the cell cycle inhibitor *Cdkn2a* is shown in black, genes encoding membrane transporters are shown violet, and the numerous genes encoding Epidermal Differentiation Complex (EDC) proteins, claudins and other 'cornification` proteins are encircled and presented in orange.

transduction of NB and tail KCs exerted only a moderate effect on expression levels suggesting that they are no direct targets of NFAT5 (**Supplementary Figure 9**).

NFAT5 Modulates Keratin Expression in Epidermal KCs

Within the transcriptome of basal KCs, we detected a large number of transcripts for keratin K5/K14 heterodimers, the most abundant keratins of basal KCs (47). The K14 transcripts decreased slightly upon culture for 3 weeks, whereas a marked 2-3 fold decrease in K5 RNA levels was detected. In contrast, the transcripts of keratins 6a, K7, K8 and K18 increased 2 to 6-fold in KCs cultured for 3 weeks as compared to KCs cultured for only one week (**Supplementary Figure 10**). The transcripts of K5/14 heterodimers were at the same high level in WT and *Nfat5*$^{-/-}$ KCs, and they remained unaffected by NFAT5 expression in isolated tail KCs while they were slightly repressed in NB KCs. By contrast, the expression of K7, K8 and K18 keratins increased 2 to 6-fold in *Nfat5*$^{-/-}$ KCs, and upon NFAT5 over-expression a slight decrease in K8 and K18 RNA levels was observed (**Supplementary Figure 10**). It is noteworthy that no transcripts were detected for the keratins K1 and K10, the markers of suprabasal KCs.

Coordinated Expression of NFAT5 and Mmp3 in Fetal and Adult Skin

Among the 121 genes that were 2-fold stronger expressed in *Nfat5*$^{-/-}$ than WT KCs we detected five genes that encode matrix proteases (**Figure 3**). In line with the protein secretion data, these were the *Mmp3* and *Klk7*, and, in addition, the *Klk6*, *Asprv1* and *Prss27* genes. In qRT-PCR assays, we observed a strong increase in *Mmp3, Klk7* and *Asprv1* expression in KCs from NB mice as compared to adult KCs, and a decrease in tail KCs that were maintained for 3 weeks *in vitro* (**Figure 5**). The *Mmp3* gene was 30- to 50-fold stronger expressed in NB KCs as in adult KCs, and over-expressing NFAT5 in NB KCs led to a moderate increase in RNA levels. In contrast, the poor expression of *Mmp3* gene in adult KCs was suppressed 2 to 3-fold upon NFAT5 over-expression. A similar expression was observed for the *Klk7*

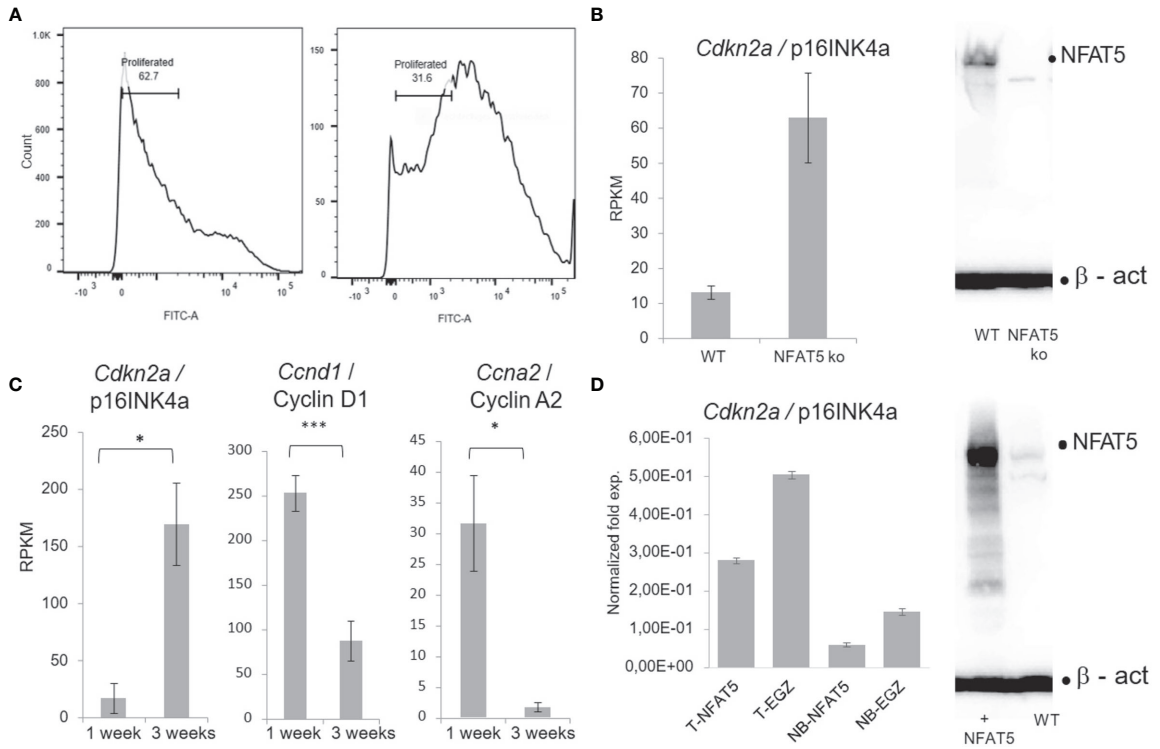

FIGURE 4 | NFAT5 supports the proliferation of KCs. **(A)** Measurement of KC proliferation cultivated for one week (left) or three weeks (right) in CFSE assays. **(B)** Increase of $p16^{INK4a}$ RNA levels in KCs upon NFAT5 ablation. The western blot (right) confirms the absence of NFAT5 expression in KCs isolated from *Nfat5*$^{-/-}$ mice. **(C)** Expression of cell cycle inhibitor $p16^{INK4a}$ and of cyclins D1 and A2 in KCs cultivated for one or three weeks. Next-generation-sequencing (NGS) data of four independent batches of KCs cultured for 1 or 3 weeks are shown. **(D)** Decrease in $p16^{INK4a}$ RNA levels in KCs isolated from the tails of adult (T) or from newborn mice (NB). The western blot illustrates increased NFAT5 levels in tail KCs that have been transduced with a retrovirus expressing NFAT5. The KCs in **(C, D)** were cultivated for 1 week. (***p<0.0001, **p<0.001 and *p<0.05).

gene: a stronger expression in NB than in adult KCs, a stimulatory effect of NFAT5 on *Klk7* expression in NB but a slight repression in adult KCs. Moreover, expression of the *Klk7* gene ceased in adult KCs upon prolonged incubation for 3 weeks. In 3 independent transcriptome NGS assays the RNA levels of *Mmp3* and *Klk7* genes were 2-3 fold higher in *Nfat5*$^{-/-}$ than WT tail KCs (**Figure 5**).

The striking variations in expression of matrix proteases prompted us to investigate whether and, if yes, where and when NFAT5, Mmp3 and Klk7 are co-expressed at various developmental stages. Therefore, we (co-) stained skin sections of adult, newborn and fetal mice with Abs specific for NFAT5, Mmp3 or Klk7. Since the anti-murine Mmp3 Abs did not allow co-stains of murine skin, we co-stained skin sections of human fetuses that revealed a distinct co-expression of NFAT5 and Mmp3 in KCs of *stratum basale*. In the cytosol of almost all KCs of the basal epidermal layer a strong NFAT5 and Mmp3 co-expression was detected, and a number of KCs showed also the nuclear occurrence of NFAT5 (and Mmp3) (**Figure 6**). A similar staining pattern was detected in sections of adult murine skin in which the NFAT5 and Mmp3 specific Abs stained predominantly basal KCs (**Figure 7** and **Supplementary Figure 11**). On the same hand, an Ab specific for Klk7 stained also the layer of basal KCs in sections of human fetuses in which NFAT5 is expressed in cytosol (**Figure 8**).

In striking contrast, we did not detect any NFAT5 expression in KCs of the *stratum basale* from NB mice while a strong and broad Mmp3 staining was apparent (**Supplementary Figure 11**). A similar staining pattern was observed in stainings of NB epidermis with an Ab specific for Klk7: while the Klk7 Ab stained broadly the epidermis of NB mice, no NFAT5 staining was detected. By contrast, the basal KC layer of epidermis from adult mice was co-stained by both Klk7 and NFAT5 (**Supplementary Figure 12**). These data suggest that in KCs from *stratum basale* of embryonic and adult mice – but not of NB mice - the co-expression of NFAT5 with Mmp3 and Klk 7 keeps in check the expression of matrix proteases in epidermis.

DISCUSSION

NFAT5 was originally described as an osmosensitive TF that affects the activity of T cells, macrophages, and other cells in the

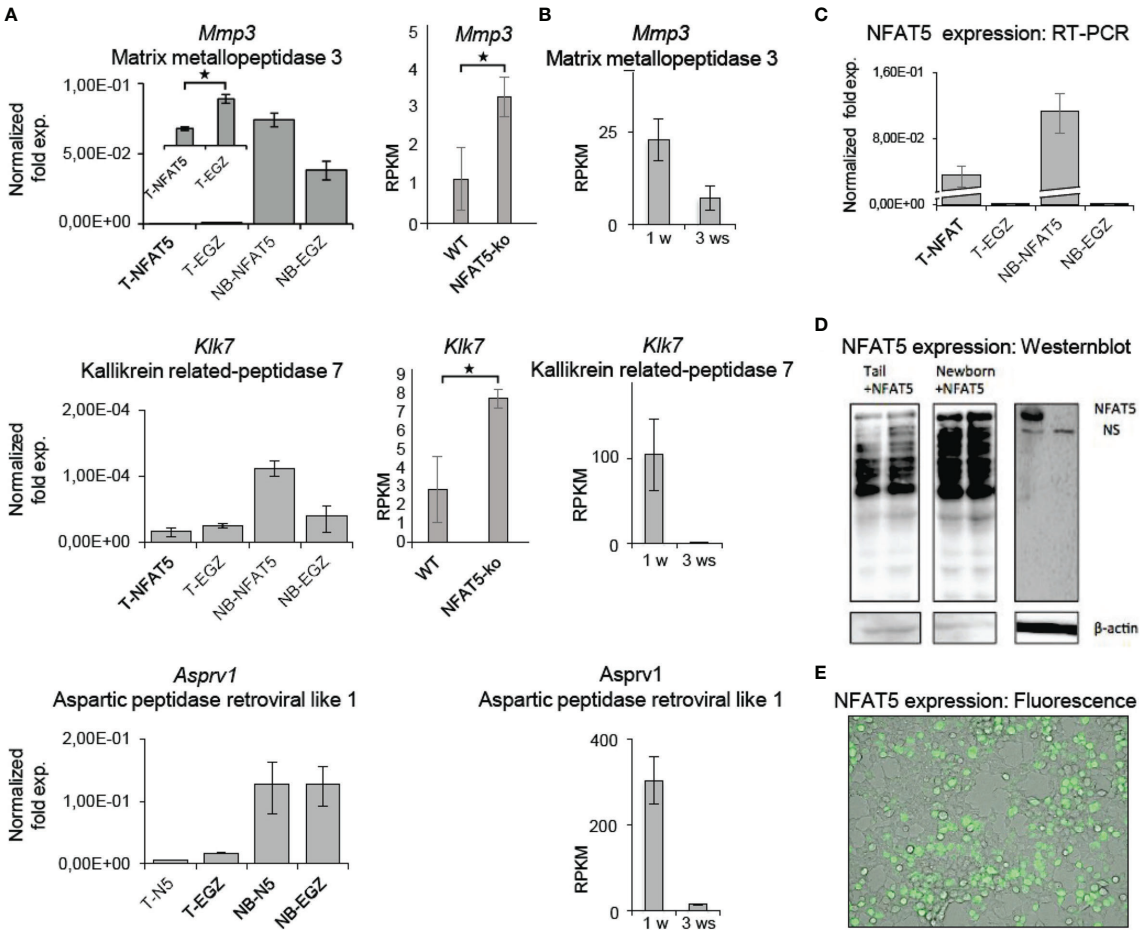

FIGURE 5 | NFAT5 affects the expression of *Mmp3, Klk5* and *Asprv1* protease genes in KCs. **(A)** Left panels, results of qRT-PCR assays showing the effect of NFAT5 on the expression of *Mmp3, Klk7* and *Asprv1* genes upon transduction of KCs obtained from the tails of adult ice (T) or from the skin of newborn (NB) mice. Right panels, effect of NFAT5 ablation on *Mmp3* and *Klk7* expression. Results of 3 independent NGS assays are shown. **(B)** Expression of protease genes in KCs cultured for 1 or 3 weeks (ws). NGS data of three independent batches of KCs cultured for 1 or 3 ws are shown. **(C–E)** Detection of NFAT5 expression upon transduction of KCs. Cultured KCs were transduced with retroviruses expressing NFAT5, or with an 'empty' EGZ virus. **(C)** Detection of human NFAT5 RNA by RT-PCR assays in tail (T) and NB KCs upon transduction. **(D)** Western blots showing the expression of NFAT5 upon transduction of KCs from newborn and tails of adult mice. **(E)** Fluorescence microscopy of NFAT5-GFP expression in transduced adult KCs. (*p<0.05).

skin. Thereby, increasing Na^+ concentrations support the antimicrobial skin barrier function (3, 48). However, to our surprise we did not detect any role for NFAT5 in controlling Na^+ transport by KCs, nor an effect on epidermal *Nos2* expression, a known NFAT5 target gene in macrophages (49). Therefore, similar to NFAT5's role in neutrophils where glucocorticoid hormones control NFAT5 expression and activity (4), in epidermal KCs NFAT5 exerts functions other than salt uptake and secretion.

The data of our study indicate that in KCs of inter-follicular epidermis NFAT5 controls the terminal differentiation of KCs to corneocytes. Although we investigated predominantly the role of NFAT5 in cultured basal KCs, the molecular mechanisms that control the differentiation of those cells to suprabasal KCs are initiated by a hierarchy of events that lead, finally, to the aberrant cornification of skin in *Nfat5*$^{-/-}$ mice. This is reflected by the skin phenotype of *Nfat5*$^{-/-}$ mice and by numerous changes in gene expression and protein secretion that we observed in cultured basal KCs (i.e., KCs that resemble those of the basal epidermal layer, in the *stratum basale*).

At the molecular level, NFAT5 (i) supports the proliferation and (ii) represses the differentiation of basal KCs. This is documented best by the massive increase of cell cycle inhibitor $p16^{INK4a}$ upon ablation of NFAT5, by the increase in expression and secretion of matrix proteases and of numerous members of the epidermal cornification complex in *Nfat5*$^{-/-}$ KCs. Moreover, NFAT5 controls the expression of a set of TFs, which are closely associated with proliferation and differentiation of KCs.

NFAT5 represses the expression of nine TF genes. One of them, MAD1 that is encoded by the *Mxd1* gene, is closely associated with the differentiation of KCs (33). MAD1 (MAX dimerization protein 1), a transcriptional repressor, is a

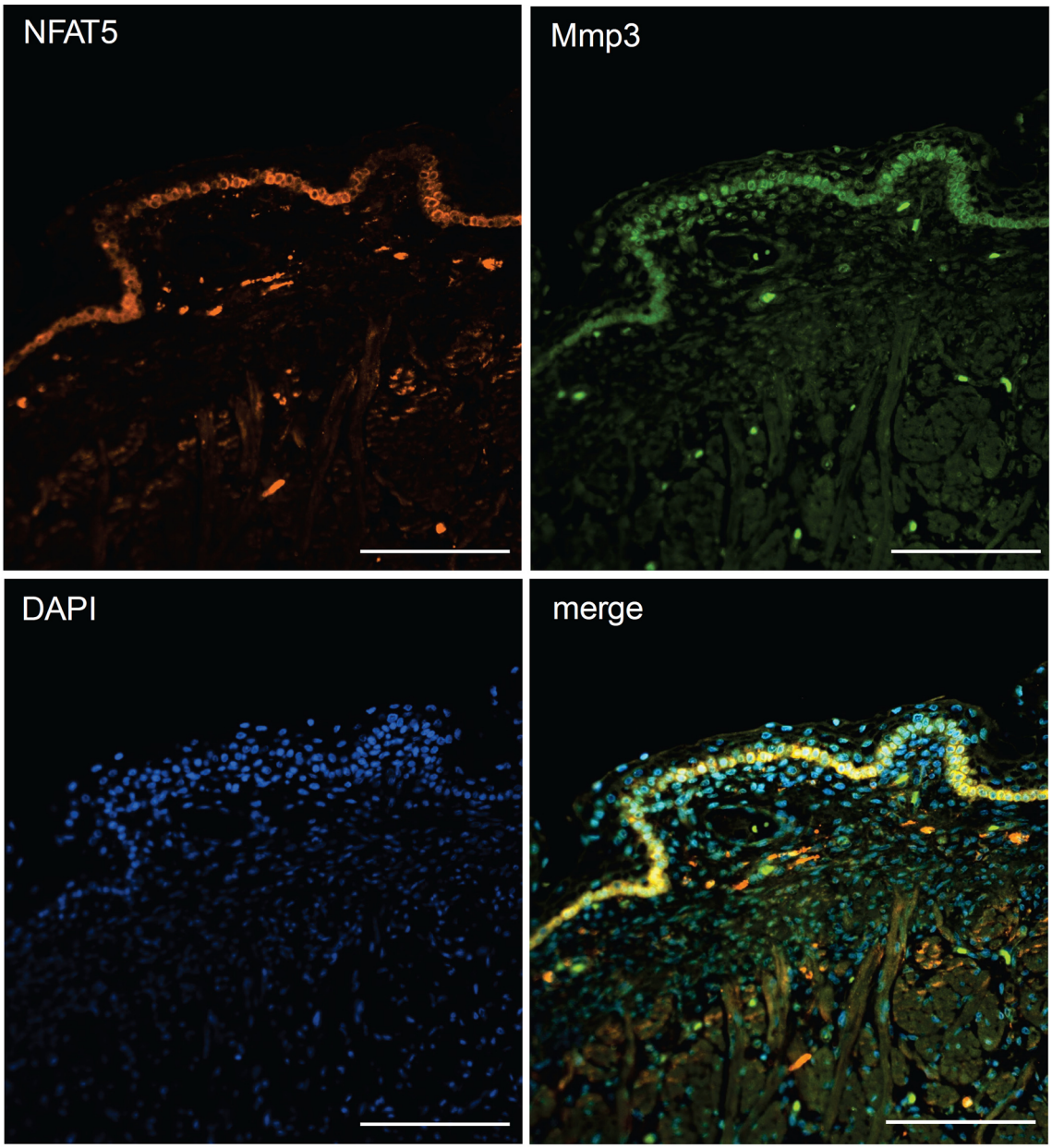

FIGURE 6 | Immunofluorescence of sections through the skin of a human fetus. Sections were stained with Abs against NFAT5 (ab110995, Abcam) and Mmp3 (AF513, Novus Biologicals) and counterstained with DAPI as indicated. Length of the bars: 100 μm.

dimerization partner of Myc and highly expressed in suprabasal KCs. Sox11 and its relative, Sox4, have been described to support an embryonic gene expression program that becomes activated during wound healing (40). A function in skin regeneration was also described for the expression of *Foxn1* (39), the 'nude locus' encoding Whn (50), that is particularly expressed in the *stratum spinosum*. While *Foxn1* appears to initiate KC differentiation by controlling more than 50 genes, it is unable to induce final differentiation steps (51, 52). An important function in wound healing and repair processes has been ascribed to *Grhl3*, which, in addition, controls the skin barrier function (35). *Hopx* expression, on the other hand, inhibits terminal differentiation of human KCs (34).

It remains to be shown by which signals the expression and function of NFAT5 is controlled in epidermal KCs. One upstream mediator is probably Blimp-1, a transcriptional repressor, whose epidermal ablation in mice resulted in defects in differentiation of KCs from the *stratum granulosum* to the *stratum corneum* and, thereby, to skin barrier defects. Among the 250 genes that were dysregulated in Blimp-1-deficient epidermis,

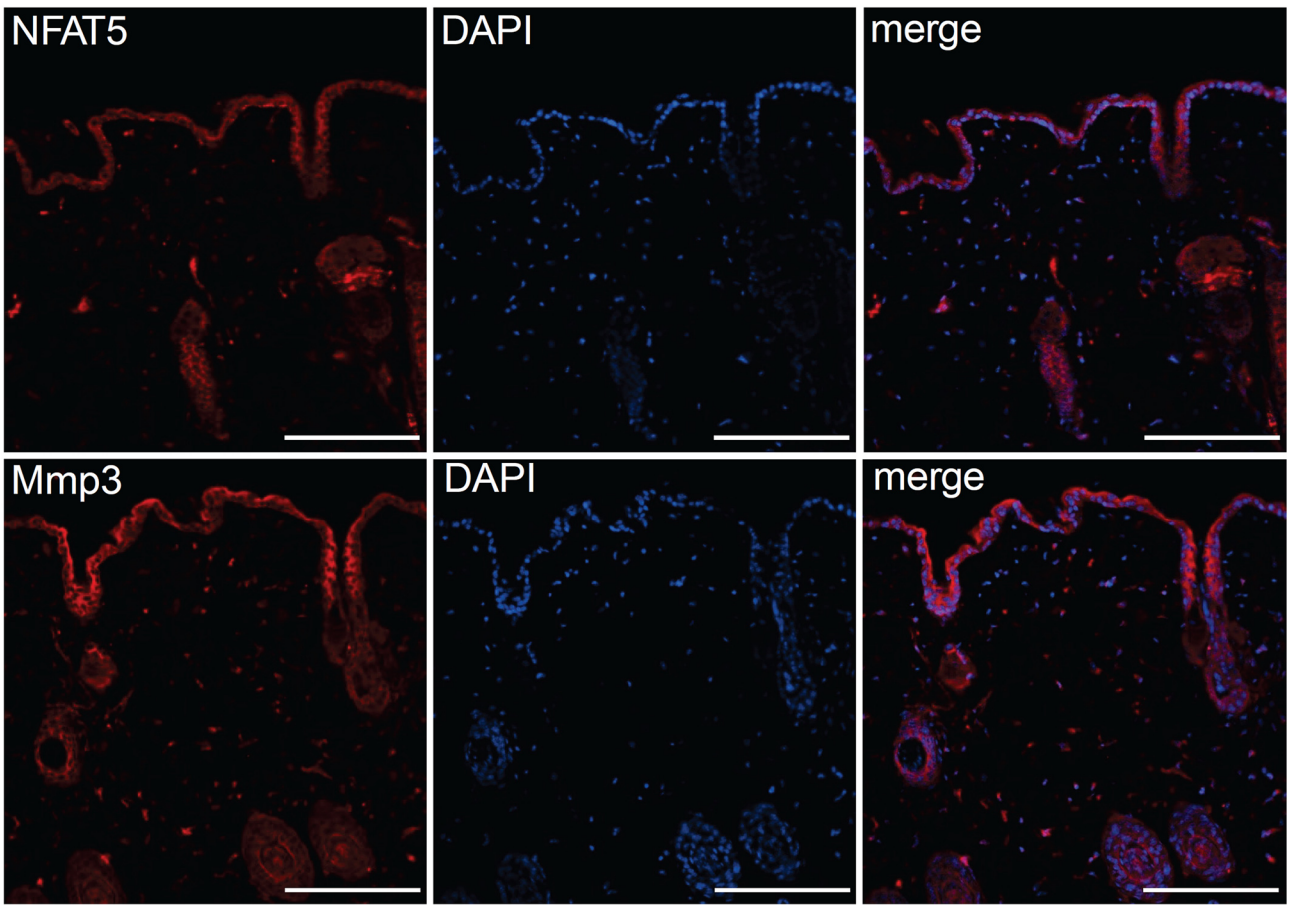

FIGURE 7 | Immunofluorescence of serial sections through the back skin of adult mice. Sections were stained with Abs against NFAT5 (ab110995, Abcam) and Mmp3 (ab53015 (Abcam) and counterstained with DAPI as indicated. Length of the bars: 100 μm.

Nfat5 was identified as a direct target of Blimp-1. However, the genes that we described here as NFAT5 targets in KCs were not detected as (direct or indirect) targets of Blimp-1 (53) thus questioning the involvement of NFAT5 in Blimp-1-mediated repression of KC genes.

Among the genes that are stronger transcribed in *Nfat5*$^{-/-}$ than WT KCs are numerous genes that control late cornification events. These are seven *Sprr* genes coding for small proline-rich region (SPRR) proteins, the *Lcn2*, the *Lce1h* gene encoding a member of late cornified envelope (LCE) proteins, *Cnfn*, encoding cornifelin, the *Fn1* gene encoding the extracellular matrix protein fibronectin 1, two genes encoding the tight junctions proteins claudin 3 and 7, and genes encoding S100 proteins. In addition, the *Tgm1* and *Tgm2* genes are further 'cornification genes' that are stronger expressed in *Nfat5*$^{-/-}$ than in WT KCs. They encode the transglutaminases 1 and 2, which cross-link cornification proteins by catalysing N6-(γ-glutamyl) lysine isopeptide bonds. Albeit both genes are repressed by NFAT5, their expression differs markedly between KCs that were kept in culture for either 1 or 3 weeks, respectively. While the *Tgm1* gene is predominantly expressed in freshly prepared KCs, *Tgm2* is much stronger expressed after 3 rather than 1 week of culture. Tgm1 defects have been associated with aberrant cornification and ichthyosis (54), whereas Tgm2 acts in many tissues and stabilizes extracellular matrices. Defects of the latter are closely linked with various diseases in humans (55).

Our KC secretion and transcriptome studies from adult *Nfat5*$^{-/-}$ mice revealed the peptidases Klk7 and Mmp3 as prominent extracellular matrix proteases whose synthesis is suppressed by NFAT5. Both proteases are known to cleave a multitude of extracellular matrix proteins, including corneodesmosomes (56, 57), and, thereby, control the shedding of corneocytes. The expression of both genes is controlled at the transcriptional level (58, 59), and it is likely that NFAT5 keeps in check their expression in adult (and embryonal) KCs. In contrast, we observed a strong, up to 50-fold increase in expression of both proteases in KCs from NB mice and a very low, if any NFAT5 expression in NB KCs. This suggests that NFAT5 does not repress Mmp3 expression in the epidermis of NB mice at the time point of birth when the skin barrier function is established. One may speculate that for the re-organization of

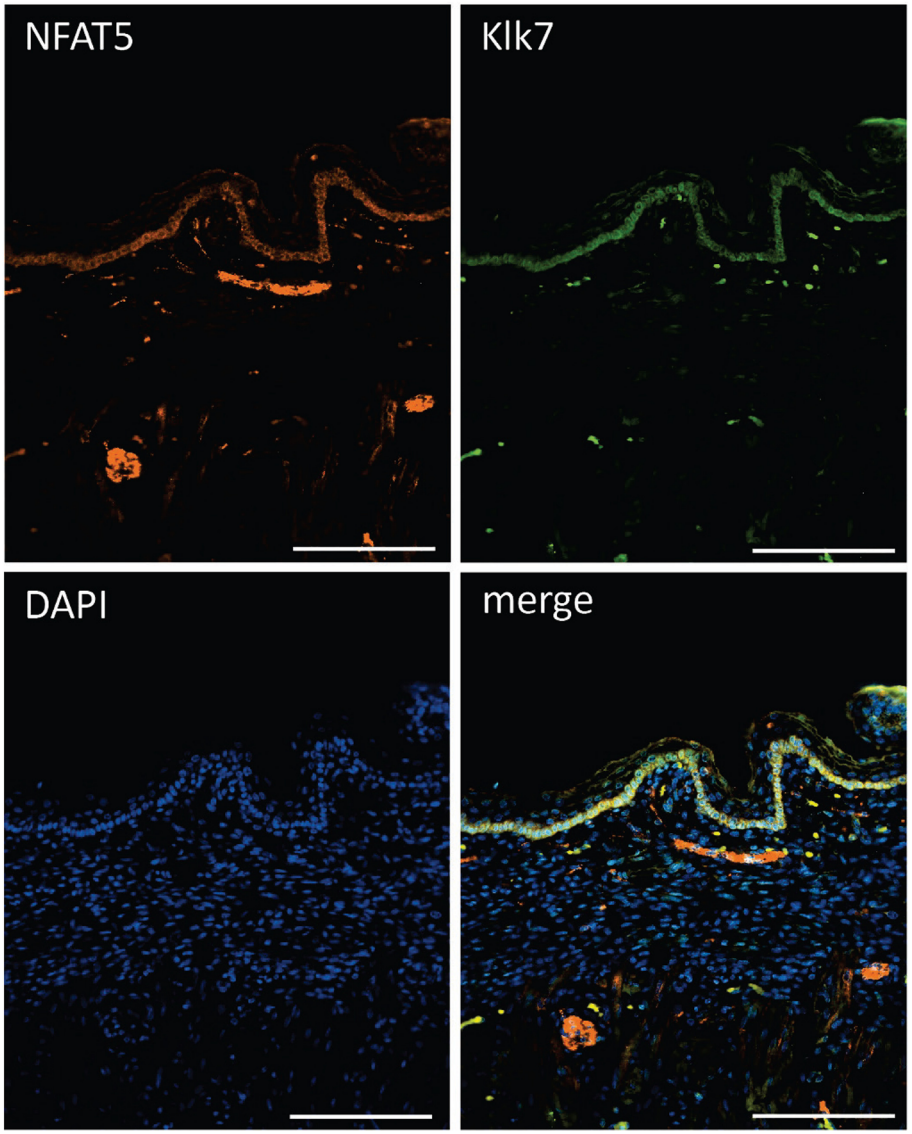

FIGURE 8 | Immunofluorescence of sections through the skin of a human fetus. Sections were stained with Abs against NFAT5 (ab110995, Abcam) and Klk7 (Biotechn/R+D Systems, No.: AF2624) and counterstained with DAPI as indicated. Length of the bars: 100 µm.

the skin barrier which at birth has to immediately adapt from an amniotic fluid environment to air contact high levels of matrix proteases are necessary which is made possible by low expression levels of epidermal NFAT5.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Sequencing data - NCBI GEO, accession no: GSE184180; Mass spectrometry data - PRIDE database, accession no: PXD028675.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Animal experiments were performed according to project licenses (Nr. 55.2-2531.01-80/10), which are approved and controlled by the Regierung von Unterfranken, Würzburg. Written informed consent was not obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

ES: led the investigation and wrote the manuscript. KMuh, DX, MA, TR, and AA: performed experiments. MS and ST: performed and evaluated mass spectrometric analyses of secretion assays. SK-H, MK, and TB: NGS assays. KMur and AK: Immunofluorescence and -chemistry stains. MG: supported the preparation of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.780727/full#supplementary-material

Supplementary Figure 1 | NFAT5 and NFATc1 expression in epidermis of interfollicular skin of mice and human. **(a)** Parallel Western blots with the same batches of whole cell protein extracts from primary murine KCs, compared to whole cell extracts from murine $CD4^+$T cells. Upper blot, detection of NFATc1 expression by incubation with the NFATc1-specific mAb 7A6. Lane 1, murine $CD4^+$ T cells activated by T+I for 24 h; lanes 2-5, KCs incubated for 3d in serum-free SFM KC medium (Gibco) containing 0.06 mM Ca^{++} without (lane 2) and with ionomycin (0.5 μM; lane 3), cyclosporine A (100 ng/ml; lane 4) or imiquimod (1 μg/ml; lane 5). In lanes 6-13, KCs were incubated in SFM medium containing 0.2 mM $CaCl_2$ without (lane 6) and with ionomycin (lane 7), with cyclosporine A (lane 8), imiquimod (lane 9), ionomyin+cyclosporine A (lane 10) or ionomycin+imiquimod (lane 11). In lane 12 and 13, KCs were incubated in S-MEM medium containing 0.2 mM $CaCl_2$ without (lane 12) or with ionomycin (lane 13). Lower blot, NFAT5 expression detected by pAb PAI-023 (Affinity Bio Reagents). For the lanes see **(a)**. **(b)** Immuno-histochemical staining of a section through human skin stained with the NFATc1-specific mAb 7A6. **(c)** Co-staining of sections through human back skin with Abs specific for NFAT5 (ab3446) and cytokeratin 14 (sc-53253). **(d)** Co-staining of human back skin with Abs for NFAT5 (ab3446) and filaggrin (sc-66192). **(e)** Staining of human sun-exposed face skin with an NFAT5-specific Ab (sc-398171). An enlargement of framed part of **(e)** is shown in **(é)**. Length of the bars: 50 μm.

Supplementary Figure 2 | Localization of NFAT5 in murine tail KCs upon culture for 1 week. Upon cytospin, the KCs were stained with the NFAT5-specific Abs ab3446 **(a)** or ab110995 **(b)**.

Supplementary Figure 3 | Representative H&E stains of sections through the skin from tails of WT129/sv **(a)** and *Nfat5*$^{-/-}$ 129/sv mice **(b)**. Note the missing corneocyte layer in skin from *Nfat5*$^{-/-}$ mice.

Supplementary Figure 4 | Primary KCs from tails of WT C57/Bl6 mice cultured for one week **(a)** or three weeks **(b)** *in vitro*.

Supplementary Figure 5 | Increased uptake of the fluorescent glucose analog 2-NBDG (2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2deoxy-D-glucose) into NFAT5-deficient 129/sv KCs, as compared to WT 129/sv KCs. **(a)** Flow cytometry of FITC-labeled 2-NBDG, and **(b)** quantification of 2-NBDG uptake.

Supplementary Figure 6 | Heat maps of more than 8400 genes that were expressed in 3 and more RPKMs in basal murine KCs. **(a)** Transcriptomes of KCs from WT C57BL/6 mice cultured for one or three weeks *in vitro*. Data from four independent experiments were compiled with cells being cultured for either 1 or 3 weeks. **(b)** Heat maps of transcriptomes from KCs of 129/sv mice. The two WT and *Nfat5*$^{-/-}$ KC cultures were maintained *in vitro* for 1 week.

Supplementary Figure 7 | Compilation of genes that were 2fold weaker expressed in *Nfat5*$^{-/-}$ than WT 129/sv KCs. **(a)** Heat map of 168 genes that were changed at least 2fold in expression between WT and *Nfat5*$^{-/-}$ KCs. **(b)** Compilation of 47 genes that were expressed in 2fold less copies in *Nfat5*$^{-/-}$ KCs. The gene encoding the TF Irf9 is marked by a blue arrow. Five genes encoding membrane transporters are highlighted by violet arrows. Genes coding for cytokine receptors and Tnf-signaling molecules are indicated by green arrows, and the laminin gene *Lamb3* and the gene encoding *S1007a*, a "cornification protein", are indicated by a grey or red arrows, respectively.

Supplementary Figure 8 | Extended culture of WT C57BL/6 KCs affects the expression of genes that control cell cycle. **(a)** Genes encoding cell cycle inhibitors. **(b)** Cyclin genes. **(c)** Cyclin-dependent kinase *Cdk1* gene. **(d)** Genes encoding subunits of RNA polymerase I.

Supplementary Figure 9 | Effect of NFAT5 ablation, of extended culture and transduction of KCs with control retroviruses (EGZ) or viruses expressing NFAT5-bio (N5) on the expression of "cornification proteins". The effect of transduction of NFAT5 into tail-KCs (T) or KCs from newborn mice (NB) is shown.

Supplementary Figure 10 | Effect of extended culture of KCs from tails of WT C57BL/6 mice, of NFAT5 ablation in 129/sv KCs and of transduction with a retrovirus expressing NFAT5 (N5) on keratin expression in tail KCs (T, left panels) or KCs from newborn mice (NB) (right panels). Data from at least two independent experiments are shown. Statistically different results are indicated by stars.

Supplementary Figure 11 | Immunofluorescence stainings of sections through the skin of newborn mice (upper part) or of tails of adult mice (lower part). Sections were counterstained with DAPI and overlays are shown as indicated. The following Abs were used: NFAT5, ab110995; Mmp3, ab53015 (both Abcam). Length of the bars: 100 μm.

Supplementary Figure 12 | Immunofluorescence co-stainings of sections through the skin of newborn (upper part) and adult mice (lower part). Sections were stained with Abs against NFAT5 (ab110995, Abcam) and Klk7 (Biotechn/R+D Systems, No.: AF2624) and counterstained with DAPI as indicated. The sections were counter- stained by DAPI. Length of the bars: 200 μm.

Supplementary Table 1 | List of primers used in qRT-PCR assays.

Supplementary Table 2 | List of TF genes whose expression was changed 2fold in KCs from tails of adult *Nfat5*$^{-/-}$ mice. Genes whose expression was enhanced 2fold and more: Gene whose expression was decreased 2fold and more.

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CASE REPORT
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Case Report: Semantic Variant of Primary Progressive Aphasia Associated With Anti-Glial Fibrillary Acid Protein Autoantibodies

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Background: Frontotemporal lobar degeneration is a heterogeneous disorder entailing a semantic variant of primary progressive aphasia (svPPA). A subtype of frontotemporal dementia associated with glutamate receptor subunit 3 (GluA3) antibody of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) was recently identified. Here, we describe the novelty of a svPPA associated with anti-glial fibrillary acid protein (GFAP) antibodies.

Methods: To diagnose this 68-year-old woman we conducted a clinical examination, neuropsychological testing, CSF analysis, MRI and 18F-fluorodeoxyglucose (18F-FDG) Positron Emission Tomography (PET)/computed tomography (CT) imaging.

Results: The clinical phenotype corresponds to a svPPA based on impaired confrontation naming and single-word comprehension. In addition, we observed spared speech production, impaired object knowledge, and surface dyslexia - further supporting the diagnosis of svPPA. Additional characteristic imaging features such as anterior temporal hypometabolism in 18F-FDG PET/CT confirmed patient's svPPA diagnosis. CSF analysis revealed signs of axonal degeneration, as both tau and phosphorylated tau proteins exceeded normal levels. Her serum showed anti-GFAP autoantibodies.

Conclusion: We diagnosed a svPPA in this patient and report an association between serum anti-GFAP antibodies and svPPA never reported in the literature so far, thereby expanding the clinical spectrum of svPPA and anti-GFAP-antibody related disease. Further research is needed to elucidate the underlying immunopathology of this disease entity to ultimately improve treatment.

Keywords: frontotemporal lobar degeneration (FTLD), semantic variant of primary progressive aphasia (svPPA), autoimmunity, anti-GFAP antibody, immunotherapy

INTRODUCTION

Frontotemporal lobar degeneration is a debilitating neurodegenerative disease of heterogeneous etiology. An autoimmune origin was recently postulated in a subgroup of frontotemporal lobar degeneration, most commonly in frontotemporal dementia's behavioral variant with antibodies directed against the glutamate receptor subunit 3 (GluA3) of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) (1, 2). Reports suggest that GFAP autoantibodies are detected in association with dementia, like senile dementia caused by Alzheimer´s and vascular dementia (3). Another study (4) depicted *via* immunoassay that patients with Alzheimer´s disease have higher levels of anti-GFAP autoantibodies. However, no GFAP autoantibodies have been documented so far in conjunction with frontotemporal lobar degeneration. Here we report the first case to our knowledge of a patient with frontotemporal lobar degeneration presenting a semantic variant subtype of primary progressive aphasia (svPPA) associated with repeatedly proven serum-GFAP autoantibodies. svPPA depicts one subtype of PPA along with the others, namely the non-fluent/agrammatic and logopenic PPA variant (5). These subtypes are differentiated clinically by their language and speech pattern and might be supported by neuroimaging evidence (5). These PPA subtypes reveal substantial clinical overlap that often makes them difficult to distinguish – a factor applying to PPA's logopenic variant especially (6). A useful neuropsychological instrument [demonstrated recently (7) to distinguish svPPA from the non-fluent PPA variant] was to test patients' naming capacity. Other novel diagnostic tools are having patients name animated objects: those with svPPA demonstrate a profound semantic deficit (8). The clinical spectrum of GFAP autoantibodies to date comprises patients with meningoencephalitis, headache, visual problems, often febrile temperature, abnormal movements (9), or hyponatremia (10). A recent study confirmed that the main clinical presentation associated with GFAP autoantibodies are subacute meningoencephalitis with dysfunction in cerebellar pathways (11). Myocloni and bulbar syndrome (12) as well as blindness (13) and vision loss (14) appear to be less frequent manifestations of GFAP-autoantibody disease. Thus, while GFAP autoantibody disease is heterogeneous, PPA has so far been reported to be associated with GFAP antibodies. Neuroinflammation's potentially pathogenic role in PPA was recently highlighted in a study of 63 patients with sporadic PPA (15). There is thus evidence of inflammation in svPPA, but none yet of any association with GFAP antibodies.

CASE REPORT

We describe a 68-year-old woman who came to our memory outpatient clinic with word-finding difficulties, worsening memory, and problems with activities of daily life, over a year before presentation. Spontaneous speech presented with semantic paraphasia. Her psychopathological examination revealed a thought disorder comprising slowed, circuitous and discursive thinking, easily irritated and talking at cross purpose. Her drive was reduced, she was intermittently agitated and easily irritated. She had sometimes revealed impulsive behavior. Her neurological examination revealed ideatoric apraxia. Her internal examination was unremarkable. Her prior medical diagnoses encompassed ulcerous colitis, hypercholesterinemia, vitamin D deficiency, arterial hypertension and disturbed glucose tolerance. She had earlier been diagnosed with gastritis and amotio retinae on the right side. She is married and has two children. Her father was diagnosed with dementia in his old age. At first presentation the drugs she was taking were: gingko biloba 120 mg/d, mesalazine 1000 mg/d, metoprolol 23,75 mg/d, cholecalciferol 1000 IE orally, candesartan 16 mg/d, and atorvastatin 10 mg/d. Upon cognitive screening, her mini mental status examination (MMSE) score was 23/30 (**Table 1**).

Neuropsychological testing initially revealed impaired semantic and syntactic spontaneous speech. She also exhibited reduced semantic and phonemic word fluency, impaired confrontation naming and single-word comprehension. Additional anomalies

TABLE 1 | Laboratory assessment and cognitive data.

PARAMETER	FIRST PRESENTATION	FOLLOW UP
CSF		
Cells/µl(<5µg/L)	2	0
Albumin mg/L	319	325
IgG mg/L	43.9	39.8
IgA mg/ L	6.2	5.7
IgM mg/L	0.93	1.4
QAlb %	8	7.6
QIgG %	4	2 3.7
QIgA %	1.8	1.5
QIgM %	1.2	1.6
Cell destruction marker CSF		
Tau protein pg/ml (<450pg/ml)	473	495
P-Tau 181 pg/ml (<61pg/ml)	72	70
Aß42 pg/ml (>450pg/ml)	400	573
Aß40 pg/ml	8633	8706
Ratio Aß42/40 x10 (>0.5)	0.46	0.66
Neural autoantibody		
Autoantibody CSF	–	–
Autoantibody Serum	anti-GFAP (1:320+)	anti-GFAP (1:320+)
Cognitive performance		
MMSE (sum score)	23/30	–
CERAD Boston naming test	-5.8	–
CERAD semantic fluency	-3.9	–
CERAD phonemic fluency	-1.7	–
CERAD list learning (trials 1-3)	-3.6	–
CERAD list recall (savings)	-3.4	–
CERAD list recognition/discriminability	-1.8	–
CERAD figure recall (savings)	-2.7	–
CERAD figure copy	1.1	–
TMT part A	0.0	–
TMT division part B/A	0.9	–

Aß42, ß-amylod 42; Aß40, ß-amyloid 40; CERAD, The Consortium to Establish a Registry for Alzheimer's Disease; CSF, cerebrospinal fluid; GFAP, glial fibrillary acid protein; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; P-Tau 181, phosphorylated tau protein 181; ratio Aß42/40, ratio ß-amyloid 42/40; QAlb, quotient albumin; QIgG, quotient immunoglobulin G; QIgA, quotient immunoglobulin A; QIgM, quotient immunoglobulin M; TMT, Trail Making Test. In the lab data, normal ranges are shown in brackets. In the neuropsychological data, z-values as normative data are depicted. Z-values <-1 mean performance below the normal range whereas z-values ≥-1 indicate performance within the normal range.

were superficial dysgraphia, a pathological result in the clock drawing test and impaired figural and verbal memory (especially encoding/learning deficits). Speech repetition and speech motor function were normal. MRI revealed pronounced left medial temporal atrophy and hippocampal atrophy with signal enhancement in temporal T2/fluid attenuated inversion recovery (FLAIR) sequences. An 18F-fluorodeoxyglucose (18F-FDG) Positron Emission Tomography (PET)/computed tomography (CT) (18F-FDG-PET/CT) was performed later and revealed asymmetric hypometabolism involving both temporal lobes, predominantly the anterior left temporal lobe (**Figure 1**). Her electroencephalography (EEG) was unremarkable, displaying an alpha-beta ground rhythm without epileptic potentials. She underwent a lumbar puncture for differential diagnosis. Cerebrospinal fluid (CSF) analysis revealed repeatedly (at her baseline presentation and her follow-up two months later) elevated tau protein and phosphorylated tau protein (**Table 1**). However, the decreased ß-amyloid 42/40 (Aß42/40) ratio we had noted in her first CSF analysis was normal at follow-up 2 months later. We searched for these autoantibodies *via* immunoblots, immunofluorescence tests and cell-based assays in the Euroimmun laboratory: anti- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor 1/2 (AMPAR1/2), amphiphysin, aquaporin 4, contactin associated protein 2 (CASPR2), CV2, dipeptidyl-peptidase–like protein-6 (DPPX), gamma aminobutyric acid B1/2 (GABAB1/2), glutamic acid decarboxylase (GAD65), HuD, leucin rich glioma inactivated protein 1 (LGI1), Ma1/2, NR1 subunit of the N-methyl-D-aspartate receptor (NMDAR), Ri, Ro, SOX1, TR, Yo and Zic4.

We detected anti-immunoglobulin G glial fibrillary acid protein (GFAP) antibodies in both serum analyses (done at the same time as her CSF analyses at baseline and follow-up) (1:320). Further blood investigations revealed slight hyperhomocysteinemia. We diagnosed a svPPA according the international consensus classification of Gorno-Tempini et al. (5), as these two essential features, namely impaired confrontation naming and single-word comprehension, were deficient. She fulfilled all four additional features such as spared speech production and repetition, impaired object knowledge, and surface dyslexia. In addition to these clinical features of the PPA's semantic variant, she exhibited imaging characteristics such as anterior temporal hypometabolism in 18F-FDG-PET, supporting our diagnosis of an svPPA variant in an early stage. Nevertheless, an impaired figural memory is unusual in svPPA. However, an Alzheimer´s dementia (AD) is less likely as such severe speech problems are not typical of AD. A behavioral variant of frontotemporal dementia (bvFTD) is unlikely, as severe behavioral disturbances are missing apart from occasional impulsivity. Furthermore, her primary permanent speech disturbance argue against bvFTD. The other PPA subtypes are clinically unlikely and would have not been confirmed in the anterior hypometabolism in FDG-PET.

Having detected anti-GFAP antibodies could imply an autoimmune encephalitis. Accordingly, MRI suggested an encephalitis in the past with damage and atrophy in the hippocampi. However, her clinical phenotype and 18F-FDG-PET data are more likely to indicate a svPPA. Thus, it is likely that the GFAP antibodies are associated with svPPA. She has been given three cycles of steroids so far, but she has unfortunately failed to demonstrate any clinical improvement: her semantic paraphasia and word-finding difficulties persist. Long-term follow up is needed to monitor cognitive deficits that can be reversed or disease progression can be slowed at least.

DISCUSSION

Our case broadens on the one hand the clinical spectrum of GFAP astrocytopathy involving frontotemporal lobar degeneration in the form of svPPA as a phenotype. On the other hand, there is additional evidence that svPPA can be triggered or exacerbated by GFAP antibodies. The pathogenic role of GFAP antibodies remains incompletely understood although recent evidence indicates that CD8+T cells drive pathology in GFAP autoimmunity (16), moreover, the intracellular location of GFAP suggests that the role of GFAP antibodies is not pathogenic.

Our patient exhibited axonal neurodegeneration as well as elevated phosphorylated tau protein 181 and tau protein.

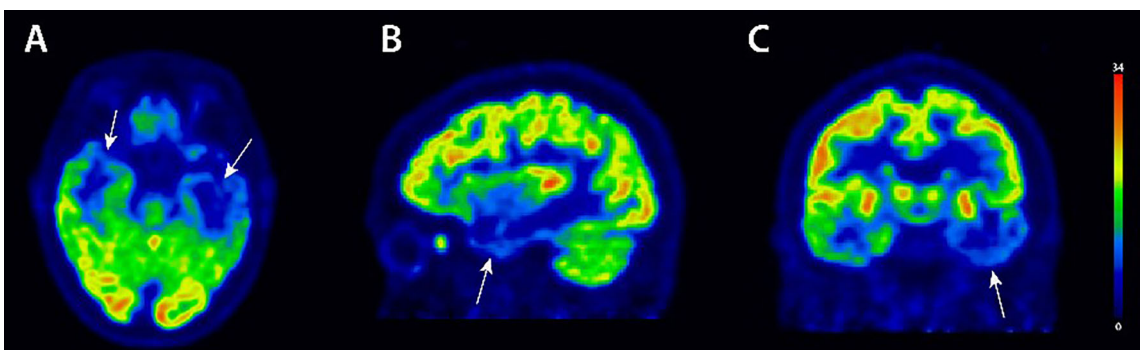

FIGURE 1 | 18F-FDG PET/CT imaging. 18F-FDG-PET images in axial **(A)**, sagittal **(B)** and coronal **(C)** view showing asymmetric hypometabolism in both temporal lobes (arrows), predominantly in the anterior left temporal lobe. 18F-FDG-PET/CT, 18F-fluorodeoxyglucose (18F-FDG) Positron Emission Tomography (PET)/computed tomography (CT) imaging.

However, it is not known whether GFAP antibodies lead to secondary neurodegeneration, or if they are an epiphenomenon unrelated to the neurodegeneration in our patient. Besides axonal degeneration, astrocytes might contribute to neurodegeneration. An animal study (17) demonstrated GFAP autoantibodies in cattle with bovine spongiform encephalopathy compared to healthy cattle, thus revealing a link between neurodegeneration and peripheral blood GFAP autoimmunity. A human study (18) suggested that GFAP autoantibodies of the IgG subtype are produced with one-week latency after the occurrence of a brain injury. Cell culture experiments (18) showed that GFAP autoantibodies can disrupt glial cell functioning by entering living astroglia cells, supporting the hypothesis of astroglial-derived neurodegeneration in our patient. In their study of 22 patients (19) with different clinical diagnosis ranging from meningoencephalomyelitis, encephalitis, ataxia to optic neuritis, GFAP autoantibodies were detected following a histopathological investigation of a leptomeningeal biopsy showing CD8+ T cells and macrophages. Their research group study reported that a combined T-cell and GFAP autoantibody dependent mechanism might induce neuroinflammation that could culminate in astroglial degeneration through still-unknown mechanisms. A recent study (20) reported the occurrence of black holes in the brain and spinal atrophy identified in patients with GFAP autoantibodies, underlying a neurodegenerative mechanism beyond autoimmunity. Thus, several mechanistic animal (17) and human studies (18–20) point towards astroglial damage in patients with GFAP autoantibodies.

An imaging marker was recently established to help diagnose svPPA, namely reduced metabolic activity in bilateral temporal lobes (21). Our patient partly fulfilled this type of metabolic pattern, as she revealed reduced bilateral metabolic activity in the temporal lobes. The same pattern of atrophy was also detected in structural neuroimaging, supporting the svPPA in our patient. MRI and 18F-FDG-PET data might also serve as valid markers of disease progression (21, 22). Recent findings suggest that the anterior temporal lobe degeneration in svPPA assessed *via* anterior temporal hypometabolism in FDG-PET, as in our patient, leads to a reorganized brain networks with dysfunctional semantic representations compensated by augmented perception (23). The cerebral metabolism is specific for each PPA variant, and correlates strongly with the PPA subtype (24), supporting the usefulness of FDG-PET for the differential diagnosis of PPA subtypes, as was the case for our patient. FDG-PET is known to be valuable for the early diagnosis of PPA subtypes (25) detecting synaptic dysfunction that appears earlier than atrophy (26). However, the MRI pattern did not clearly support our clinical diagnosis of svPPA, although the MRI pattern of anterior temporal lobe atrophy is known to be highly specific and sensitive among the other PPA subtypes (27).

In the absence of therapeutic options for frontotemporal dementia, our case reveals the need for neural autoantibody testing in such patients. We wish to draw attention to the recently reported case of patient with GFAP antibody-associated progressive dementia (28) who responded rapidly (and so well) to immunotherapy that led to a stabilization without further deterioration of her dementia.

There is no data that we are aware of on the efficacy of immunotherapy in such cases. We suspected an autoimmune etiology because of her repeatedly detected serum GFAP antibodies, and applied high-dose intravenous steroids. Previous trial data have suggested high responsiveness to steroids from patients with autoimmune dementia and autoantibody-associated cognitive impairment (29–32), which is why we presumed a benefit by applying steroids. She has not shown any neuropsychological improvement so far, but has only been given one cycle until now. Before her responsiveness can be assessed, we believe she must complete three more cycles of steroids. Large-scale and longitudinal studies are required to evaluate the benefit of immunotherapy in patients with neural autoantibody-associated dementia and subtypes like frontotemporal dementia. Our patient's immunopathology is still unclear, thus at present, we can only speculate that her dementia has an immunopathological basis. However, we do think that frontotemporal dementia and autoimmune diseases are interlinked. There is recent evidence that svPPA and frontotemporal dementia with progranulin mutation carriers are associated with non-thyroid autoimmune disease more often than AD patients or normal subjects are (33). This link is further supported by recent research that revealed some FTD related genes such as C9orf72 are also involved in neuroinflammatory processes [(34); for review see (35)]. It would therefore be worthwhile to investigate whether certain types of autoantibodies such as GFAP in svPPA reflect an underlying neuroinflammation responsible for initiating or maintaining neurodegeneration. The other conceivable possibility is that GFAP-associated dementia is an individual disease type that differs from classical svPPA. However, as its phenotype is so similar to svPPA that svPPA associated with GFAP antibody is the patient's most likely diagnosis, while the pathogenic relevance of the GFAP antibodies in this type of dementia remains thus far unclear.

Limitations of our report concern the serum (and not CSF-proof) of GFAP autoantibodies, as they are less accurate indicators of an autoimmune origin of svPPA. However, the repeated proofs of serum GFAP antibodies argue against a casual association. Furthermore, the diagnostic criteria of svPPA are clearly supported by the clinical and neuroimaging evidence we collected, so that there can be no doubt about the diagnosis. The strength of our study is the extensive neuropsychological investigation combined with neuroimaging techniques and our assessment of anti-neural autoantibodies.

Taken together, our report expands our knowledge of subtyping svPPA, and elaborates upon the disease entity of a frontotemporal dementia potentially caused by neuroinflammation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients provided her written informed consent to participate in this study. Written informed consent was obtained from the individual for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

NH, CBo and CBa wrote the manuscript. JW, KR, and WS revised the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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The Expression of Inflammasomes NLRP1 and NLRP3, Toll-Like Receptors, and Vitamin D Receptor in Synovial Fibroblasts From Patients With Different Types of Knee Arthritis

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Activated rheumatoid arthritis (RA) synovial fibroblasts (SFs) are among the most important cells promoting RA pathogenesis. They are considered active contributors to the initiation, progression, and perpetuation of the disease; therefore, early detection of RASF activation could advance contemporary diagnosis and adequate treatment of undifferentiated early inflammatory arthritis (EA). In this study, we investigated the expression of nucleotide-binding, oligomerization domain (NOD)-like receptor family, pyrin domain containing (NLRP)1, NLRP3 inflammasomes, Toll-like receptor (TLR)1, TLR2, TLR4, vitamin D receptor (VDR), and secretion of matrix metalloproteinases (MMPs) in SFs isolated from patients with RA, osteoarthritis (OA), EA, and control individuals (CN) after knee surgical intervention. C-reactive protein, general blood test, anticyclic citrullinated peptide (anti-CCP), rheumatoid factor (RF), and vitamin D (vitD) in patients' sera were performed. Cells were stimulated or not with 100 ng/ml tumor necrosis factor alpha (TNF-α) or/and 1 nM or/and 0.01 nM vitamin D3 for 72 h. The expression levels of *NLRP1*, *NLRP3*, *TLR1*, *TLR2*, *TLR4*, and *VDR* in all examined SFs were analyzed by quantitative real-time PCR (RT-qPCR). Additionally, the secretion of IL-1β by SFs and MMPs were determined by ELISA and Luminex technology. The expression of *NLRP3* was correlated with the levels of CRP, RF, and anti-CCP, suggesting its implication in SF inflammatory activation. In the TNF-α-stimulated SFs, a significantly lower expression of *NLRP3* and *TLR4* was observed in the RA group, compared with the other tested forms of arthritis. Moreover, upregulation of *NLRP3* expression by TNF-α alone or in combination with vitD3 was observed, further indicating involvement of NLRP3 in the inflammatory responses of SFs. Secretion of IL-1β was not detected in any sample, while TNF-α

upregulated the levels of secreted MMP-1, MMP-7, MMP-8, MMP-12, and MMP-13 in all patient groups. Attenuating effects of vitD on the expression of *NLRP3*, *TLR1*, and *TLR4* suggest potential protective effects of vitD on the inflammatory responses in SFs. However, longer studies may be needed to confirm or fully rule out the potential implication of vitD in SF activation in inflammatory arthritis. Both *VDR* and *NLRP3* in the TNF-α-stimulated SFs negatively correlated with the age of patients, suggesting potential age-related changes in the local inflammatory responses.

Keywords: arthritis (including rheumatoid arthritis), Toll-like receptor (TLR), vitamin D, metalproteinase, osteoarthristis, inflammasome NLRP, early arthritis (EA), VDR

1 INTRODUCTION

Onset of inflammatory arthritis is a rheumatic condition that has different outcomes, as reported in different cohort studies: up to 20%–60% of patients will resolve completely even without any treatment, 13%–54% of patients with undifferentiated arthritis (UA) will develop rheumatoid arthritis (RA), 21%–87% UA will persist after 1 year (1). In total, 0.5%–11% UA can be diagnosed with osteoarthritis (OA) (2). Treatment and outcomes of inflammatory rheumatic diseases improved after introduction of "early treatment" strategy and "target" treatment therapy (tumor necrosis factor alpha (TNF-α) inhibitors, interleukin-6 (IL-6) inhibitors, CD20 blockers, etc.) in rheumatology practice, but it still remains unknown, why not all patients receiving this treatment achieve full disease remission (3). Patients with positive rheumatoid factor (RF) and/or anticyclic citrullinated peptide (anti-CCP) and/or C-reactive protein (CRP) are known to be at increased risk of developing worse prognosis of erosive arthritis, the main clinical manifestation of which is synovitis (4). Anti-CCP is known to be one of most sensitive and specific RA diagnostic markers (sensitivity 92.70 [90.67–94.74] and specificity 79.93 [72.44–87.42]) (5). Nevertheless, it can be found positive in other diseases, for instance, approximately 5% of patients with psoriatic arthritis are anti-CCP positive (6, 7). In rare cases, anti-CCP can also be detected as positive in autoimmune hepatitis-1, biliary cirrhosis, hepatitis C virus-related disease, Sjögren's syndrome (SS), palindromic rheumatism, and systemic lupus erythematosus (SLE) (8, 9). The role of mentioned tests is important but not always enough for early diagnosis and treatment strategy decision-making and needs to be studied further (10). Still, there is no clear link between blood inflammatory and specific immunological laboratory tests and inflammatory processes in synovial fibroblasts (SFs) (11). Therefore, identifying the links between those parameters could lead to a prompt diagnosis of a disease and enable to introduce more successful personalized treatment of various types of early arthritis.

We and others have shown that the SF phenotype differs between forms of arthritis (12, 13). Activated rheumatoid arthritis synovial fibroblasts (RASFs) are among the most important cells promoting RA pathogenesis. They are considered active contributors to the initiation, progression, and perpetuation of the disease (14). In RA, SF transform into aggressive, proliferating RASFs, and along with immune cells, become an invasive, hyperplastic tissue known as pannus, which over time causes cartilage destruction and bone erosion (15). RASFs secrete high levels of proinflammatory cytokines, chemokines, and matrix-degrading enzymes that stimulate chronic inflammation and lead to progressive, irreversible damage of the diseased joint (16). TNF-α is one of the crucial mediators in the pathogenesis of arthritis, one of the main targets in biological therapy of RA. TNF-α rapidly induces production of matrix metalloproteinases (MMPs) in RASFs that directly contribute to degradation of cartilage components, including collagen type II and proteoglycan aggrecan (17).

Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), shown to detect distinct pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), may play a pivotal role in the modulation of joint tissue homeostasis (18) TLRs are predominantly expressed by immune cells; however, their expression has been determined in SFs as well (19, 20). Normally, activation of PPRs is important for adequate inflammatory response; however, if regulatory mechanisms fail, activation of TLRs can lead to uncontrolled local inflammation, trigger a pathological immune response, and lead to inflammatory or autoimmune disorders (21–25). The upregulated expression of TLR2, TLR3, TLR4, TLR5, and TLR7 was found in RASFs compared with those with OA (19) or patients without inflammatory arthritis (26–29). Activation of these receptors in immune cells leads to the upregulation of local

Abbreviations: Anti-CCP, anticyclic citrullinated peptide; AS, ankylosing spondylitis; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; B2M, beta-2 microglobulin; CIA, collagen-induced arthritis; CN, control; CRP, C-reactive protein; CU, chemiluminescent unit; DAMP, damage-associated molecular pattern; DMARD, disease-modifying antirheumatic drug; EA, undifferentiated early inflammatory arthritis; ELISA, enzyme-linked immunosorbent assay; GK, glucocorticosteroid; IL, interleukin; MMP, matrix metalloproteinase; MTX, methotrexate; NLR, NOD-like receptor; NLRP, NOD-like receptor family pyrin domain containing; NOD, nucleotide-binding oligomerization domain; OA, osteoarthritis; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cell; PRR, pattern recognition receptor; PsA, psoriatic arthritis; RA, rheumatoid arthritis; RASF, rheumatoid arthritis synovial fibroblast; RF, rheumatoid factor; RPS9, ribosomal protein S9; RTX, rituximab; SF, synovial fibroblast; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; SSc, systemic sclerosis; TLR, Toll-like receptor; TNF-α, tumor necrosis factor alpha; VDR, vitamin D receptor; VitD, vitamin D; VitD3, 1α,25-dihydroxy vitamin D3; UA, undifferentiated arthritis.

inflammatory reactions, including intracellular innate immune sensors nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), forming multiprotein complexes called inflammasomes, whose activation results in cell pyroptosis and generation of proinflammatory cytokines IL-1α, IL-1β, and IL-18 (30, 31).

NOD-like receptor family pyrin domain containing 1 (NLRP1) was recognized as the first protein from the NLR family to recruit an inflammasome (32), while the NLRP3 inflammasome is currently the most studied one. The oligomerization of NLRP3 inflammasome requires two signals. The first priming signal occurs through membrane receptors, such as TLRs, and *via* activation of the nuclear factor-κB (NF-κB) signaling pathway initiates transcription of *NLRP3* and in turn of *IL-1β* and *IL-18* genes. A second signal is required for activation of inflammasome complex assemblance from NLRP3, adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and pro-caspase-1 proteins (31). This may be triggered by diverse stimuli, for instance, extracellular ATP, K^+ and Ca^{2+} level changes, lysosomal destabilization, mitochondrial dysfunction, reactive oxygen species, uric acid crystals, etc. (31, 33–35). The exact mechanism of NLRP1 inflammasome activation is unknown, but it seems that the NLRP1 protein N-terminal domains must be degraded to promote inflammasome assembly (36). The formation of both mentioned inflammasomes activates caspase-1, which cleaves proinflammatory cytokine progenitor pro-IL-1β and pro-IL-18 into their biologically active forms (31). The activation of the NLRP3 inflammasome contributes to multiple autoimmune diseases, such as ankylosing spondylitis (AS), systemic sclerosis (SSc), SLE, (SS), and RA (31, 33). However, it remains unclear whether their activation might reflect outcomes of early arthritis and potentially become a diagnostic marker facilitating the differentiation of undifferentiated early inflammatory arthritis (EA) into RA, OA or other inflammatory arthritis.

Vitamin D (vitD) is a potential therapeutic molecule, which may prevent or treat inflammatory and possibly degenerative joint diseases (37–39). The active form of vitD binds to specific vitamin D receptors (VDRs), thus activating downstream signaling pathways. Upon vitD binding, VDR dimerizes with the retinoid X receptor to form a heterodimer and afterward attaches to vitD response elements in the promoter regions of various genes (40). In this way, the complex acts as a transcription factor that may regulate more than 900 genes (41). VDR expression has been described in a wide range of different cell types, such as in immune cells, keratinocytes, enterocytes, pancreatic endocrine cells, peripheral blood mononuclear cells (PBMC), etc. (42). Also, VDR expression has been detected in chondrocytes and synoviocytes from inflamed joints of RA subjects (43, 44), suggesting that in addition to the systemic control of the innate and adaptive immune responses, vitD may also be implicated in local joint inflammation (45). Moreover, direct interaction of VDR with NLRP3 at the posttranscriptional level has been demonstrated recently. VDR blocks the association of NLRP3 with BRCC3 deubiquitinase, thus inhibiting the deubiquitination of NLRP3 and afterward suppressing oligomerization and activation of the inflammasome complex (46). Therefore, in the present study, we were seeking to investigate the effect of 1α,25-dihydroxy vitamin D3 (vitD3) on gene expression of innate immune sensors TLRs and NLRPs in SFs of RA, OA, EA, and control individuals (CN).

The main aim of the present study was to investigate the expression of inflammasomes NLRP1 and NLRP3, TLR1, TLR2, TLR4, and VDR in SFs and to compare the data between different patient groups, following stimulation or not with TNF-α and vitD3. We hypothesized that TNF-α stimulates the expression of TLRs and, in turn, inflammasome activation of which further results in proinflammatory activities through the secretion of IL-1β. TNF-α also activates MMPs, which may lead to enhanced catabolic degradation of cartilage and bone. We have also investigated the potential of vitD3 to attenuate the effects of TNF-α and searched for the potential correlations of expression of the tested SF genes with their secretion levels of MMPs, patient age, serum levels of CRP, RF, anti-CCP, and vitD. In parallel, we were interested in investigating whether those processes are differently regulated in various types of arthritis (RA, OA, EA) and CN.

2 MATERIALS AND METHODS

2.1 Patients Cohort, Blood Tests, and Synovial Tissue Sample Collection

This study has been approved by the Vilnius Regional Biomedical Research Ethics Committee (Approval No. 158200-18/5-1037-533). Following study protocol, samples were obtained from adult patients (≥18 years) of synovium as remaining tissues after articular replacement surgery in the RA (n = 7) and OA (n = 4) cases, or during knee arthroscopic synovectomy performed for therapeutic purposes in EA (n = 4) cases, or patients who underwent arthroscopy due to meniscus or cruciate ligament traumatic tearing and had no signs of inflammatory arthritis, osteoarthritis, crystal arthropathies, or septic arthritis CN (n = 4) cases. All operations were performed by the three senior surgeons. Synovial tissue samples were collected during arthrotomic or arthroscopic knee surgeries under direct visualization. RA was diagnosed based on the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 RA classification criteria (4). OA was diagnosed according to ACR classification criteria for knee OA (47). Patients with inflammatory arthritis at least in one knee, arthritis duration <12 months, and at the moment of surgery, without compliance with the criteria for any inflammatory rheumatic disease were included into the EA group. Patients with other autoimmune diseases, acute inflammation, fever, thyroid disease, diabetes, malignancies, and severe liver and kidney diseases that might have had a huge impact on results were excluded from this study. CRP (elevated if >5 mg/l), general blood test, anti-CCP (positive if >10 U/ml), RF (positive if >30 U/ml), and vitD (normal range 75–100 nmol/l) blood tests were performed to all patients involved in this study. Synovial tissues removed and collected during

arthroscopy or surgeries were further processed for cell cultures and analysis.

2.2 Synovial Tissue and Cell Culture Preparation

Cells were isolated from synovial tissues as previously described (13). Briefly, mechanically minced synovial tissues were incubated overnight in Dulbecco's modified Eagle's medium (DMEM) (with 1 g/L D-glucose, sodium pyruvate, L-glutamine, phenol red, Invitrogen) in a humidified 5% CO_2 incubator at 37°C. After incubation, synovial tissues were digested with 0.1% collagenase (Type I, Biochrom, Cambridge, UK) at 37°C in a shaking mode overnight. Isolated cells were centrifuged at 400×*g* for 10 min and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Biochrom), 1% stock solution of penicillin (10,000 units/ml), streptomycin (10 mg/ml), and amphotericin B (0.025 mg/ml, Biological Industries, Haemek, Israel). Passages 2–4 SFs were plated into 25–cm^2 culture flasks in DMEM containing 10% FBS and stimulated or not for 72 h with or without 100 ng/ml TNF-α (Thermo Fisher Scientific, Waltham, MA, USA) (with additional stimulation after 36 h) and 1 or 0.01 nM of vitD3 (Sigma, St. Louis, MO, USA). At the end of the experimental stimulation, cell culture supernatants were collected under sterile conditions and stored at −80°C until further analysis; cells were lysed in RLT buffer (RNeasy kit, Qiagen, Hilden, Germany) and used for gene expression analysis.

2.3 RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

RNA was extracted with RNeasy Mini Spin columns (Qiagen) according to the manufacturer's instructions, and RNA concentration and purity were measured with the SpectraMax® i3 (Molecular Devices, San Jose, CA, USA) spectrophotometer. Before synthesizing the first complementary DNA strand, RNA samples were treated with DNase I (Thermo Fisher Scientific) and cDNA synthesis was performed with the Maxima®First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer protocols. qPCRs were performed using the Maxima® Probe qPCR Master Mix (2×) (Thermo Fisher Scientific) and AriaMx real-time PCR system (Agilent Technologies, Santa Clara, CA, USA). The TaqMan® Gene Expression Assays (Applied Biosystems, Waltham, MA, USA) for 8 genes were used for gene expression analysis, using primers as indicated in **Table 1**. The qPCR reaction volume was 25 µl with 0.5 µl of 20× Taqman® Gene Expression Assay mix. All reactions were run in triplicates. Cycle conditions were as follows: initial denaturation step for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C for denaturation and 60 s for annealing and extension. Each RNA sample was controlled for genomic DNA contamination by reactions without reverse transcriptase (RT), and reagent contamination was checked by the reactions without template (NTC). Relative gene expression quantification was calculated using $2^{-\Delta CT\times1,000}$ method. The geometric mean of two reference genes—RPS9 and B2M—was used to normalize gene expression. qPCR data were analyzed using AriaMx (Agilent Technologies) software.

TABLE 1 | The TaqMan Gene Expression Assays used for gene expression analysis.

Gene Assay ID	Encoded Protein
RPS9 Hs02339424_m1	40S ribosomal protein S9
B2M Hs00984230_m1	Beta-2 microglobulin
TLR-1 Hs00413978_m1	Toll-like receptor 1
TLR-2 Hs02621280_s1	Toll-like receptor 2
TLR-4 Hs00152939_m1	Toll-like receptor 4
VDR Hs01045843_m1	Vitamin D receptor
NLRP1 Hs00248187_m1	PYD domain-containing protein 1
*NLRP3*Hs00918082_m1	PYD domain-containing protein 3

2.4 Detection of Secreted Proteins by Luminex and ELISA Assays

Analysis of IL-1β concentration in nondiluted supernatants was performed using commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA); levels of MMP-1, MMP-7, MMP-8, MMP-12, and MMP-13 were measured using Luminex Technology and ProcartaPlex Human MMP-Panel 5 plex panel (Affymetrix, eBioscience, San Diego, CA, USA), according to manufacturer's instruction. Cell culture medium was used for background normalization.

2.5 Statistical Analysis

The descriptive statistics (mean values ± standard deviation, median, and lower and upper ranges) were applied for data analysis. The variables were tested for normality using the Kolmogorov–Smirnov test. After evaluating the sample size, nonparametric statistics small sample size has been applied. For the comparisons between the groups, medians were compared using the Mann-Whitney *U* test. The related samples were compared using a nonparametric Wilcoxon signed-rank test. Correlations between all cohort data were calculated using Spearman's nonparametric correlation test. *p*-values less than 0.05 were considered statistically significant. Data were analyzed using Graphpad Prism v. 9 and SPSS version 20 (IBM Corp, Armonk, NY, USA) software.

3 RESULTS

3.1 Patient Enrollment and Characterization

A total of 19 patients (7 in RA, 4 in OA, 4 in EA, 4 in CN group) were included in the study after informed consent was signed. Mean age was 53.1 years (± 11.9), and 14 (73.7%) were females. No differences in age or sex were detected between different pathology patient groups. In total, 10 (52.6%) patients were RF positive and 9 (47%) were anti-CCP positive. All RA patients were anti-CCP and RF positive, EA patients were either RF or/and anti-CCP positive, and all OA and CN patients were negative for both anti-CCP and RF. RA group had the highest value of CRP ($p < 0.05$). Normal vitD level in 4 (21.05%) patients' sera was found (**Table 2**). In all study cohort ($n = 19$) patients, RF showed correlation with anti-CCP (r −0.869, $p < 0.000$), CRP

TABLE 2 | Baseline demographic, laboratory tests, and treatment history characteristics.

Characteristic	All Patients *N* = 19	EA *N* = 4	RA *N* = 7	OA *N* = 4	CN *N* = 4
Females (*N* (%)]	14 (73.7%)	2 (50%)	6 (85.7%)	4(100%)	2 (50%)
Age (years: mean (SD)]	53.1 (11.9)	40.5 (7.4)	62.29 (7.3)	60.75 (2.87)	42.0 (5.1)
Anti-CCP [positive *N* (%)]	9 (47%)	2 (50%)	7 (100%)	0	0
Anti-CCP [(CU), median]	12.6	70.7*	1,610.5*	–	–
Anti-CCP (min-max)	4.1–2,776.8	4.6–199.6	12.6–2,776.8	–	–
RF [positive *N* (%)]	10 (52.6%)	3 (75%)	7 (100%)	0	0
RF [(IU/ml) median]	23.2	102.8	110.1	–	–
RF [(IU/ml) min-max]	20.0–1,221.5	20.0– 597.6	23.2–1,221.5	–	–
CRB [(mg/l), mean (SD)]	7.1 (7.5)	3.5 (3.9)	13.9 (7.9)	2.2 (3.1)	3.6 (3.3)
CRB [(mg/l) median]	4.8	2.6**	17.1**	0.9**	2.97**
CRB [(mg/l), min-max]	0.1–22.3	0.63–9.12	1.5–22.3	0.1–6.7	0.98–7.8
VitD (nmol/l)					
Normal result (%)	4 (21.05%)	1 (25.0%)	2 (28.6%)	1 (25%)	0 (0%)
Mean (SD)	51.14 (29.2)	54.4 (30.8)	49.99 (37.1)	61.01 (29.6)	39.97 (15.2)
Ever DMARD treatment [*N* (%)]	7 (36.8)	1 (25)	7 (100)	0	0
Ever used TNF-α [*N* (%)]	3 (15.8)	0	3 (42.9)	0	0
Ever used RTX [*N* (%)]	2 (10.5)	0	2 (28.6)	0	0
Ever used MTX [*N* (%)]	4 (21.1)	1 (25)	3 (42.9)	0	0
GK	7 (36.8)	0	7 (100)	0	0

*EA, early arthritis; RA, rheumatoid arthritis; OA, osteoarthritis; CN, control group; SD, standard deviation; Anti-CCP, anticyclic citrullinated peptide; CU, chemiluminescent units; CRP, C-reactive protein; RF, rheumatoid factor; DMARD, disease-modifying antirheumatic drugs; TNF-α, tumor necrosis factor alpha; RTX, rituximab; MTX, methotrexat; GK, glucocorticosteroid. *p < 0.05 (RA compared with EA group); **p < 0.05 (RA compared with EA, OA, and CN groups). CRP (elevated if >5 mg/l), general blood test, anti-CCP (positive if >10 U/ml), RF (positive if >30 U/ml), and vitD (normal range 75–100 nmol/l).*

(r −0.628, p −0.004), as well as anti-CCP and CRP (r −0.539, $p <$ 0.017). No correlation was found between age and serum levels of RF, anti-CCP, CRP, and vitD.

All patients in the RA group had a history of disease-modifying antirheumatic drug (DMARD) treatment; one patient in the EA group was treated with methotrexate [following early arthritis treatment EULAR strategy (48)] (**Table 2**).

3.2 Synovial Fibroblast Analysis

The expression of *VDR*, *TLR1*, *TLR2*, *TLR4*, *NLRP1*, and *NLRP3* inflammasome genes and secretion of MMP-1, MMP-7, MMP-8, MMP-12, and MMP-13 in SF cultures and after stimulation at different vitD3 doses (0.01 or 1 nM) or TNF-α 100 ng/ml alone or their combination was measured in all patients. Data were analyzed between the related nonstimulated and stimulated samples in the whole study cohort (**Tables 3**–**5**) and compared between the different pathology patient groups (**Figures 1**–**3**).

3.2.1 Expression of *NLRP1* and *NLRP3* Inflammasomes and *VDR* Gene in Synovial Fibroblasts

Expression of *NLRP1*, *NLRP3*, and *VDR* genes was detected in SFs of all patients enrolled into the study (**Table 3**); the levels were similar in all tested pathology groups without stimulation (**Figure 1**). Stimulation with TNF-α and/or 0.01 or 1 nM of vitD3 had no effect on the expression of *NLRP1* and *VDR* in neither whole cohort nor in either group separately. However, under stimulation with TNF-α or TNF-α and 0.01 nM vitD3, the expression of the *NLRP3* was higher in the whole study cohort. Under stimulation with TNF-α alone, expression of the *NLRP3* gene tended to increase in all pathology groups, except RA. Consequently, stimulation with TNF-α resulted in a significantly higher expression of *NLRP3* in CN, OA, and EA patient groups, as compared with the RA group. In the presence of vitD3,

TABLE 3 | Effects of the stimulation with TNF-α and VitD on the expression of NLRP1 and NLRP3 inflammasomes and *VDR* genes in synovial fibroblast cultures of study individuals (*N* = 16–18).

Analyzed Gene	Stimulation	Relative Transcript Level [median (range)]
NLRP1	Nonstimulated	1.23 [0.01–6.76]
	TNF-α 100 ng/ml	1.04 [0.05–10.43]
	1 nM vitD3	1.17 [0.29–7.47]
	0.01 nM vitD3	1.55 [0.31–9.03]
	1 nM vitD3 TNF-α 100 ng/ml	0.87 [0.18–8.57]
	0.01 nM vitD3 TNF-α 100 ng/ml	0.7 [0.06–8.7]
NLRP3	Nonstimulated	0.08 [0.01–0.96]*
	TNF-α 100 ng/ml	0.13 [0.01–5.01]*
	1 nM vitD3	0.08 [0.01–0.76]
	0.01 nM vitD3	0.09 [0.01–0.66]
	1 nM vitD3 TNF-α 100 ng/ml	0.09 [0.01–5.95]
	0.01 nM vitD3 TNF-α 100 ng/ml	0.11 [0.02–3.42]*
VDR	Nonstimulated	21.86 [1.77–58.76]
	TNF-α 100 ng/ml	26.17[0.74–107.66]
	1 nM vitD3	24.32 [3.61–56.33]
	0.01 nM vitD3	25.9 [3.46–60.66]
	1 nM vitD3 TNF-α 100 ng/ml	29.4 [3.3–89.67]
	0.01 nM vitD3 TNF-α 100 ng/ml	29.47 [0.82–72.63]

*NLRP, NOD-like receptor family, pyrin domain containing; VDR, vitamin D receptor; TNF-α, tumor necrosis factor alpha; vitD3, 1α,25-dihydroxy vitamin D3. Nonparametric Wilcoxon signed-rank test: *p-value <0.05 (compared with nonstimulated and stimulated-related samples).*

TABLE 4 | Effects of the stimulation with TNF-α and VitD on the expression of *TLR1*, *TLR2*, and *TLR4* genes in synovial fibroblast cultures of study individuals (*N* = 17–19).

Analyzed Gene	Stimulation	Relative Transcript Level [median (range)]
TLR1	Nonstimulated	1.87 [0.41–7.97]*
	TNF-α 100 ng/ml	2.85 [0.08–18.32]*,‡
	1 nM vitD3	1.73 [0.49–5.60]
	0.01 nM vitD3	1.79 [0.5–11.65]
	1 nM vitD3 TNF-α 100 ng/ml	2.86 [0.46–13.91]*
	0.01 nM vitD3 TNF-α 100 ng/ml	2.23 [0.43–11.99]*,‡
TLR2	Nonstimulated	0.03 [0.003–3.00]*
	TNF-α 100 ng/ml	1.64 [0.32–13.54]*
	1 nM vitD3	0.03 [0.01–4.99]
	0.01 nM vitD3	0.01 [0.01–6.49]
	1 nM vitD3 TNF-α 100 ng/ml	1.47 [0.02–13.30]*
	0.01 nM vitD3 TNF-α 100 ng/ml	1.55 [0.3–8.81]*
TLR4	Nonstimulated	11.52 [2.67–272.82]*
	TNF-α 100 ng/ml	5.84 [0.43–156.4]*,‡
	1 nM vitD3	11.7 [1.68–130.88]
	0.01 nM vitD3	15.45 [1.69–110.73]
	1 nM vitD3 TNF-α 100 ng/ml	5.46 [1.5–155.41]*
	0.01 nM vitD3 TNF-α 100 ng/ml	5.12 [0.47–142.23]*,‡

*TLR, Toll-like receptor; TNF-α, tumor necrosis factor alpha; vitD3, 1α,25-dihydroxy vitamin D3. Nonparametric Wilcoxon signed-rank test: *p-value <0.05 (compare nonstimulated and stimulated related samples); ‡p-value <0.05 (compare stimulated TNF-α 100 ng/ml and stimulated TNF-α 100 ng/ml with 0.001 nMvitD3-related samples).*

stimulation with TNF-α resulted in similar although somewhat less-expressed differences between the patient groups. *VDR* was similarly expressed in all the groups of this study.

3.2.2 Expression of *TLR1*, *TLR2*, and *TLR4* Genes in Synovial Fibroblasts

Expression of *TLR1*, *TLR2*, and *TLR4* genes was detected in SFs of all patients enrolled into the study. Stimulation with TNF-α alone or in combination with vitD3 (at doses 0.01 or 1 nM) resulted in increase of *TLR1* and *TLR2* and decrease of *TLR4* expression related samples in all study cohort ($p < 0.05$). Stimulation with 0.01 or 1 nM vitD3 alone did not affect *TLR1*, *TLR2*, and *TLR4* gene expression, but related sample analysis revealed that 0.01 nM vitD3 attenuated effects of TNF-α on *TLR1* and *TLR4* gene expression ($p < 0.05$) (**Table 4**).

Further expression of *TLR1*, *TLR2*, and *TLR4* genes was analyzed in SFs of different pathology patient groups. Without stimulation, the expression of *TLR2* gene was significantly higher in the RA, as compared with the EA patients group, while the expression of *TLR4* was 3.9- and 3.5-fold lower in the RA, as compared with the EA and OA groups, respectively. The differences between OA and RA groups were also observed in the presence of 1 nM vitD3 stimulation. Somewhat similar results were obtained for *TLR1* gene expression; however, the differences between the groups did not reach the level of statistical significance, except between the EA and RA groups under stimulation with vitD3. Seventy-two-hour cell stimulation with TNF-α resulted in significant upregulation of *TLR2* gene expression in the EA, OA, and RA groups. Similar effects were observed in both, at the absence or the presence of vitD3 stimulation (**Figure 2**).

3.2.3 Secretion of MMPs and IL-1β by Synovial Fibroblasts

Secretion of MMP-1, MMP-7, MMP-8, MMP-12, and MMP-13 was analyzed in SFs of all 19 patients enrolled into the study in related sample analysis. After stimulation with TNF-α, secretion

TABLE 5 | The levels of MMP-1, MMP-7, MMP-8, MMP-12, and MMP-13 secretion in supernatants of synovial fibroblast cultures of the studied cohort (*N* = 19).

MMP	Stimulation	Median [Range] (pg/ml)
MMP-1	Nonstimulated	586.18 [177.32–5,567.35]***
	TNF-α 100 ng/ml	43632.13 [6,666.88–118,020.06]***
	1 nM vitD3	815.26 [347.74–6876.54]
	1 nM vitD3 TNF-α 100 ng/ml	41103.14 [5,184.93–112,105.89]***
MMP-7	Nonstimulated	0.00 [0.00–198.92]***
	TNF-α 100 ng/ml	245.55 [0.00–1,126.93]***
	1 nM vitD3	0.00 [0.00–172.45]
	1 nM vitD3 TNF-α 100 ng/ml	241.4 [52.87–1,649.88]***
MMP-8	Nonstimulated	58.68 [0.00–303.18]***
	TNF-α 100 ng/ml	906.86 [275.33–1,605.19]***
	1 nM vitD3	58.68 [0.00–343.53]
	1 nM vitD3 TNF-α 100 ng/ml	821.58 [296.3–2,082.79]***
MMP-12	Nonstimulated	162.73 [0.00–1,842.93]**
	TNF-α 100 ng/ml	743.13 [302.63–2,078.4]**
	1 nM vitD3	0.00 [0.00–675.1]
	1 nM vitD3 TNF-α 100 ng/ml	847.49 [302.63–2,689.64]**
MMP-13	Nonstimulated	33.08 [0.00–993.27]***
	TNF-α 100 ng/ml	328.7 [54.31–2,173.26]***
	1 nM vitD3	28.1 [0.00–722.72]
	1 nM vitD3 TNF-α 100 ng/ml	292.51 [22.64–3,217.84]***

*MMP, matrix metalloproteinase; TNF-α, tumor necrosis factor alpha; vitD3, 1α,25-dihydroxy vitamin D3. Nonparametric Wilcoxon signed-rank test: *p-value < 0.05; **p-values < 0.01; ***p < 0.001 (comparison of MMP secretion levels (Luminex technology) in 72 h nonstimulated and stimulated related samples).*

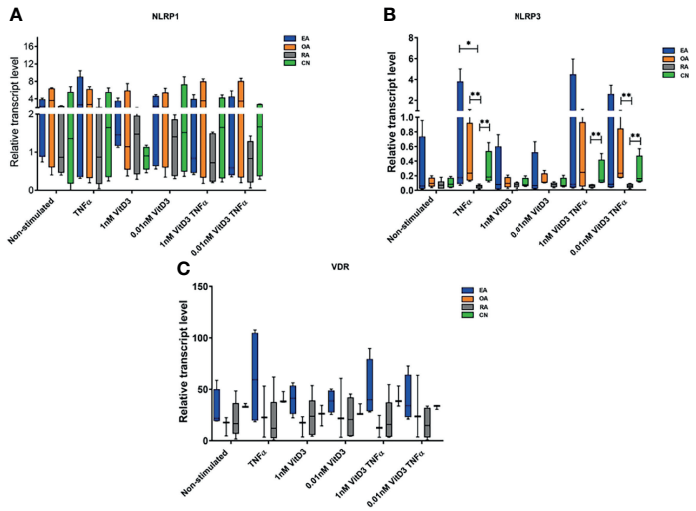

FIGURE 1 | Relative NOD-like receptor family, pyrin domain containing (NLRP)1 **(A)** and NLRP3 **(B)** inflammasome and vitamin D receptor (VDR) **(C)** gene expression levels. Synovial fibroblasts from patients with undifferentiated early inflammatory arthritis (EA), osteoarthritis (OA), rheumatoid arthritis (RA), and control individuals (CN) were cultured for 72 h with or without stimulation with 100 ng/ml tumor necrosis factor α (TNF-α), 1 or 0.01 nM 1α,25-dihydroxy vitamin D3 (vitD3), or their combination. The relative gene expression quantification was calculated using $2^{-\Delta CT \times 1,000}$ methodology. Geometric means of two control genes, 40S ribosomal protein S9 (RPS9) and beta-2 microglobulin (B2M) were used to normalize gene expression. The box length represents the interquartile range with a median. The whiskers represent the minimum and maximum data values. *$p < 0.05$; **$p < 0.01$.

of all MMPs was statistically significantly increased. A similar effect was observed after stimulation with TNF-α and 1 nM vitD3. Difference between stimulation with TNF-α alone or TNF-α in combination with 1 nM vitD3 MMP secretion was not significant in the related sample analysis. Stimulation with 1 nM vitD3 alone had no effect on MMP level (**Table 5**).

Secretion of MMPs without and under stimulation with TNF-α, 1 nM vitD3, and TNF-α with 1 nM vitD3 was further analyzed in different pathology patient group SFs (**Figure 3**). No differences were confirmed in patient groups in all MMP secretions in SFs without stimulation. Stimulation with 1 nM vitD3 alone had no influence on expression of all tested MMPs in

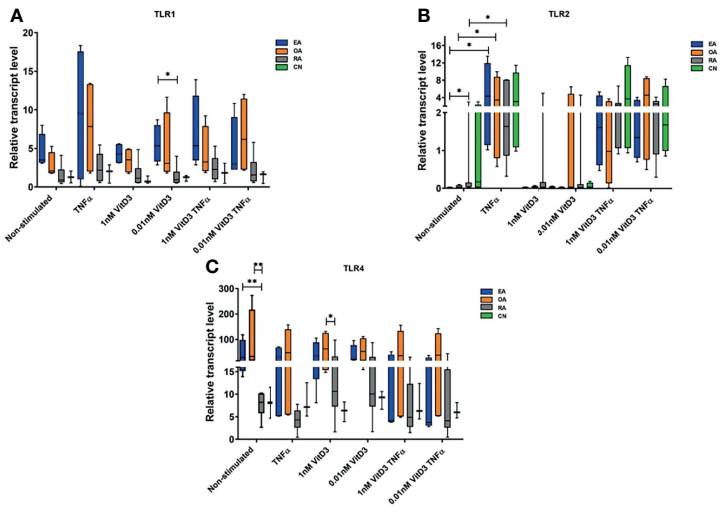

FIGURE 2 | Relative gene expression levels of Toll-like receptor (TLR)1 **(A)**, TLR2 **(B)**, and TLR4 **(C)**. Synovial fibroblasts from patients with undifferentiated early inflammatory arthritis (EA), osteoarthritis (OA), rheumatoid arthritis (RA), and control individuals (CN) were cultured for 72 h with or without stimulation with 100 ng/ml tumor necrosis factor α (TNF-α), 1 or 0.01 nM 1α,25-dihydroxy vitamin D3 (vitD3), or their combination. The relative gene expression was calculated using $2^{-\Delta CT \times 1,000}$ method. Geometric means of two control genes, 40S ribosomal protein S9 (RPS9), and beta-2 microglobulin (B2M) were used to normalize gene expression. The box length represents the interquartile range with a median. The whiskers represent the minimum and maximum data values. *$p < 0.05$; **$p < 0.01$.

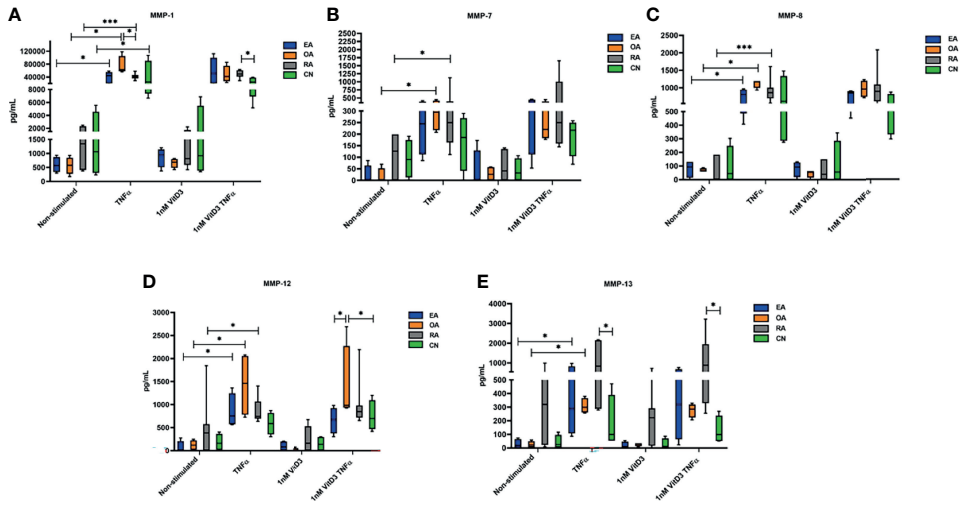

FIGURE 3 | Secretion of matrix metalloproteinases (MMP)-1 **(A)**, MMP-7 **(B)**, MMP-8 **(C)**, MMP-12 **(D)**, and MMP-13 **(E)**, determined by Luminex technology in culture supernatants of synovial fibroblasts from patients with undifferentiated early inflammatory arthritis (EA), osteoarthritis (OA), rheumatoid arthritis (RA), and control individuals (CN) after 72 h stimulation with 100 ng/ml tumor necrosis factor α (TNF-α) or 1 nM 1α,25-dihydroxy vitamin D3 (vitD3) or in supernatants of cell culture cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% antibiotics and 10% fetal bovine serum (control). The box length represents the interquartile range with a median. The whiskers represent the minimum and maximum data values. *$p < 0.05$; ***$p < 0.001$.

different pathology SFs. Following stimulation with TNF-α, the levels of MMP-1 were significantly increased in all tested groups (EA, OA, RA, CN). MMP-1 secretion was higher in SFs of the OA group, as compared with the RA ($p < 0.05$). Levels of MMP-7 were upregulated in the OA ($p < 0.05$) and RA ($p < 0.05$) groups, and levels of MMP-8 and MMP-12 were upregulated in the EA, OA, and RA groups ($p < 0.05$). Under stimulation with TNF-α, the secretion of MMP-13 increased in all groups, although the increase was statistically significant only in the EA and OA groups. Furthermore, under stimulation with TNF-α, the levels of MMP-13 were statistically higher in the RA, as compared with the CN group. Stimulation with 1 nM vitD3 alone had no effect on levels of MMPs in SFs of different pathology patient groups. Combined stimulation with 1 nM vitD3 and TNF-α had relatively similar effects on the expression of MMPs to those of stimulation with TNF-α alone.

Secretion of IL-1β, the cytokine regulated through activation of inflammasome, was also analyzed. No traces of IL-1β were detected in supernatants of either tested group even after stimulation with TNF-α, although the ELISA test was chosen with sensitivity as low as 1 pg/ml.

3.3 Analysis of Correlation Between Characteristics of Synovial Fibroblasts and Patient Age, Serum Levels of CRP, RF, anti-CCP, and vitD

Correlations between *NLRP1*, *NLRP3*, *TLR1*, *TLR3*, *TLR4*, and *VDR* gene expression levels, MMP-1, MMP-7, MMP-8, and MMP-12 secretion levels in nonstimulated and stimulated with TNF-α SF samples, patient age, serum levels of CRP, RF, anti-CCP, and vitD were analyzed in the whole study cohort. Confirmed significant correlations are presented in **Table 6**. Correlations between serum levels of CRP, anti-CCP, RF, and vitD are described in Section 3.1. No correlations were detected between VitD and tested gene expression in SF and MMP secretion levels.

In statistical analysis, patient age had negative correlations with *VDR* and *NLRP3* gene expression levels in TNF-α-stimulated samples (**Table 6**).

NLRP1 gene expression levels have any correlation with serum laboratory tests. *NLRP1* gene expression in the nonstimulated and stimulated with TNF-α SF samples correlate with the expression of *TLR1*, *TLR2*, *TLR4*, and *VDR* in TNF-α in stimulated and *TLR4* nonstimulated with TNF-α SF samples (**Table 6**).

NLRP3 gene expression levels correlate with serum anti-CCP and RF level in nonstimulated samples, whereas TNF-α-stimulated SF samples correlate with serum anti-CCP and CRP level. *NLRP3* gene expression levels in nonstimulated samples do not present statistically significant correlation with other analyzed gene expression levels. After stimulation with TNF-α, *NLRP3* gene expression levels correlate with gene expression levels in *TLR4* in nonstimulated and stimulated with TNF-α and *VDR* in stimulated with TNF-α SF samples (**Table 6**).

VDR gene expression levels have any correlation with serum laboratory tests. *VDR* and *TLR4* gene expression levels correlate in nonstimulated SF samples. *VDR* gene expression levels in stimulated with TNF-α SF samples correlate with the *TLR1*, *TLR2*, *TLR 4*, *NLRP1*, and *NLRP3* gene expression levels (**Table 6**).

In the correlation analysis between *TLR* gene expression levels and serum laboratory tests, only *TLR4* gene expression in nonstimulated samples correlates with serum anti-CCP levels. In the correlation analysis between *TLR* gene expression and

TABLE 6 | Analysis of correlation between characteristics of synovial fibroblasts and patient age, serum levels of CRP, RF, anti-CCP, and vitD in whole patient cohort[a].

	MMP-12 TNF-α	MMP-13 NS	MMP-13 TNF-α	Other MMPs***	*VDR* TNF-α	*VDR* NS	*TLR4* TNF-α	*TLR4* NS	*TLR2* TNF-α	*TLR2* NS	*TLR1* TNF-α	*TLR1* NS	*NLRP3* TNF-α	*NLRP3* NS	*NLRP1* TNF-α	*NLRP1* NS
Age					–0.594*								–0.516*			
Anti-CCP								–0.583*					–0.746**	–0.498*		
RF														0.518*		
VitD																
CRP		0.472*	0.644*										–0.606**			
***NLRP1* NS**					0.593*		0.579*	0.615*	0.647**		0.594*				0.911**	
***NLRP1* TNF-α**					0.689**		0.703**	0.571*	0.723**		0.618*					
***NLRP3* NS**													0.482*			
***NLRP3* TNF-α**					0.561*		0.703**	0.609*								
***TLR1* NS**								0.752**								
***TLR1* TNF-α**					0.807**		0.600*	0.733**	0.662**							
***TLR2* NS**								0.525*								
***TLR2* TNF-α**					0.774**		0.532*									
***TLR4* NS**	0.487*				0.632*	0.647**	0.637**									
***TLR4* TNF-α**					0.739**											
***VDR* NS**					0.644**											
***VDR* TNF-α**																

[a]*Only statistically significant results are presented after whole study cohort (19 patients) data correlation analysis.*

*Anti-CCP, anti-cyclic citrullinated peptides; RF, rheumatoid factor; VitD, vitamin D; CRP, C-reactive protein; NS, nonstimulated; TNF-α, after stimulation with 100 ng/ml TNF-α; NLRP, NOD-like receptor family pyrin domain containing; TLR, Toll-like receptor; VDR, vitamin D receptor; MMP, metalloproteinases. *p ≤ 0.05; **p ≤ 0.01; ***Other MMP (MMP-1, MMP-7, MMP-8) did not correlate with TLRs and NLRPs.*

MMP secretion levels, only one correlation was confirmed between *TLR4* gene expression in nonstimulated and MMP-12 stimulated with TNF-α samples. Correlation analysis between *TLRs*, *NLRPs*, and *VDR* in nonstimulated and stimulated with TNF-α SF samples gene expression level is described above in this section. There were also multiple correlations between expressions of different *TLRs* (**Table 6**).

No significant correlations between MMP secretion levels and *NLRPs* and *VDR* gene expression levels were confirmed. Only MMP-13 stimulated with TNF-α had a weak correlation with *TLR4* in nonstimulated SFs. In the correlation analysis between MMP secretion levels and serum laboratory tests, only MMP-13 secretion in nonstimulated SF samples correlated with serum CRP levels (**Table 6**).

4 DISCUSSION

Long-term acute inflammation-related phenotypic changes in RASFs lead to pannus formation and increased cartilage and bone tissue degradation (12, 49). Activated RASFs promote inflammation of the synovial tissue and thus maintain the autoimmune process by expressing adhesion molecules, secreting inflammatory cytokines and matrix-degrading enzymes (50). Therefore, development of the inflammatory joint disease was shown to depend not only on external factors but also on changes in functional properties of SF (51). Pathological reactions might arise from the inappropriate response of the receptor, for instance, TLR or NLRP, due to their particular genetic background or to the inappropriate quantity or quality of ligands (52).

Inflammasomes are functionally involved in inflammatory responses of multiple cell types, including fibroblasts from different origins, i.e., gingival, lung, liver, heart, skin, and cancer-associated fibroblasts (53–55). In the present study, we aimed to investigate if inflammasomes were associated with the inflammatory responses of SFs during different forms of arthritis. TNF-α is one of the essential mediators of inflammation in RA (56, 57), so we ought to better understand how it contributes to the aggressive phenotype of RASFs. In the present study, the cells were stimulated with 100 ng TNF-α as suggested in previous publications (58, 59). VDR plays an important role in limiting the inflammatory phenotype in a mouse model of RA (60) and was shown to block the activation of inflammasomes (46). Therefore, we hypothesized that vitD signaling pathway can attenuate inflammatory activation of RASFs through modulation of inflammasome activation pathway. The present study determined the expression of both *NLRP1* and *NLRP3* in

SFs of all patients involved, suggesting their potential role in the synovial tissue.

Although very little data exist, NLRP1 inflammasome is likely to be implicated in the pathogenesis of RA. For instance, inhibition of P2X4 receptor and 11β-hydroxysteroid dehydrogenase 1 was shown to attenuate activation of inflammasome NLRP1 and expression of IL-1β in both collagen-induced arthritis (CIA) model and synovial cells of patients with RA (61, 62). However, we found no statistically significant difference in *NLRP1* gene expression between the tested groups in the present study. Stimulation with neither TNF-α nor vitD3 had any effect on the *NLRP1* inflammasome expression in SFs of all the analyzed groups, which suggests that those factors do not directly regulate NLRP1 activation. However, gene correlation analysis of all patients involved in this study showed that the *NLRP1* in both nonstimulated or TNF-α-stimulated cells is positively correlated with *TLR1*, *TLR2*, *TLR4*, and *VDR* gene expression in TNF-α-stimulated cells. These data suggest that these genes are implicated in synovial responses together though.

NLRP3 reacts to a diverse set of PAMPs and DAMPs, which may contribute to the etiopathogenesis of inflammatory disease through inflammasome activation (63). Increased expression and function of NLRP3 inflammasome were previously reported in the peripheral blood cells of RA patients (63). TNF-α acts as a priming signal, which induces expression of NLRP3 mRNA and IL-1β mRNA in RASFs (64). The expression of synovial NLRP3 was positively correlated with clinical and radiographic arthritis scores in the CIA model in mice (65); however, the synovial tissue, which was analyzed in that study, contains several cell types. In the present study, we were focused on the role of NLRP3 exclusively in SFs, which are considered the aggressive drivers of local inflammation in the joint. In our study, the expression of *NLRP3* was correlated with the levels of CRP, RF, and anti-CCP, suggesting its implication in SF inflammatory activation. Moreover, significant upregulation of *NLRP3* expression by TNF-α alone or TNF-α in combination with 0.01 nM vitD3 was observed, further indicating involvement of NLRP3 in the inflammatory responses of SFs. Although the combination of TNF-α with 1 nM of vitD3 resulted in somewhat upregulation of *NLRP3* expression, the change was not significant, suggesting that higher dose of vitD3 might have played a protective role.

The lower *NLRP3* expression after TNF-α stimulation in the RA patient group suggests an imbalance of this system activation in the acute inflammatory state. However, it is complicated to evaluate whether this dysregulation is a consequence of a long-term inflammation, and medication prescribed to RA patients or rather patients with this imbalance are more prone to develop RA. In other cells from patients with RA, monocyte, neutrophil, and inflammasome activation results in IL-1β and IL-18 secretion (65, 66). Therefore, we were seeking to determine if altered NLRP3 activation in TNF-α-stimulated SFs of the RA group results in functional responses. Although there are some data on IL-1β secretion by SF (67), we found no traces of its presence in supernatants of SFs. These data suggest that the inflammasome complex may not have assembled in our cells, even though *NLRP3* gene expression was observed. This is in agreement with the previously reported data, where in fibroblast-like synoviocytes from patients with RA function, the inflammasome complex was not formed even though NLRP3 mRNA was detected (68). The explanation could be that the signal received through membrane receptor activation is sufficient to upregulate the expression of the *NLRP3* gene, but protein expression and additional cellular or molecular stimulation is required for further inflammasome complex assembly and afterward inflammasome-activated proinflammatory cytokine secretion (31). Various molecules, such as crystals, and fragments of apoptotic cells, which might act as potential contributors to NLRP3 inflammasome activation *via* the second signal, are detected in the OA- or RA-affected joint (34, 35). However, the lack of these molecules in our *in vitro* experimental setting could have led to the absence of IL-1β secretion by SFs; therefore, further research should be conducted to investigate the effects of complex NLRP3 formation in SFs. Noteworthy, even after the formation of the inflammasome complex, IL-1β secretion may be impaired due to inefficient *IL-1β* gene expression and translation or caspase-1 activation.

It has been demonstrated that interaction between vitD and TLRs could have a mutual form, and activation of TLRs may affect the expression of the *VDR* gene in direct and indirect manners. For instance, stimulation of human monocytes by appropriate TLR1/2 ligands leads to increased expression of VDR and CYP27B1 (VD-activating enzyme 1α-hydroxylase) (69). Also, TLR1/2 ligands increased the expression and functions of VDR in human monocytes (70) and increased antimicrobial functions of human macrophages in a vitD-dependent manner (71). Our study revealed correlations of *VDR* expression with TLRs and inflammasomes *NLRP1* and *NLRP3*, particularly under stimulation with TNF-α. The moderate positive correlation between *VDR* and *NLRP3* after stimulation with TNF-α indicates that VDR may inhibit NLRP3 inflammasome assembly eventually at the protein level. Furthermore, both *VDR* and *NLRP3* in the TNF-α-stimulated SFs negatively correlated with the age of patients, suggesting potential age-related changes in inflammatory responses.

Our results demonstrate that *TLR1*, *TLR2*, and *TLR4* gene expression are characteristic for SFs and were detected in all analyzed patient groups. Furthermore, we found higher levels of *TLR2* in RA as compared with EA, which only partially corresponds to the results of previous studies, where significantly higher TLR2 mRNA levels were demonstrated in both early and long-standing RA (72–75). In the present study, TNF-α significantly upregulated *TLR2* gene expression in the EA, OA, and RA groups but not in the CN group. However, *TLR2* gene expression levels in unstimulated RA and OA samples were similar. Also, only the expression of the *TLR2* gene was significantly higher in the RA than in the EA patients group but not the expression of *TLR1* or *TLR4*. Although TNF-α stimulated *TLR1* expression, differences between the stimulated and nonstimulated samples reached a significance level only when *TLR1* gene expression was compared in the related samples of the whole cohort. Such differences seem to be associated with a small number of patients in the groups divided by diagnoses.

Higher expression levels of *TLR4* were found in the OA and EA groups compared with the RA group, which is controversial to the data of recent studies where *TLR4* expression was statistically significantly higher in the RA group than OA (75, 76). On the other hand, not all studies uniformly confirm the association of TLR4 with RA pathogenesis. For instance, similar expression levels of *TLR1* and *TLR4* genes in RA and OA were reported (77). Also, no differences in the baseline expression levels of *TLR2*, *TLR4*, and *TLR9* were observed (73). In addition, *TLR4* gene expression was upregulated by any of the stimuli used. Moreover, a slight, but reproducible decrease in *TLR4* gene expression was observed after stimulation with IL-1β, TNF-α, and synthetic bacterial lipopeptide (sBLP) (73). Similar results were found in the present study, where TNF-α significantly reduced *TLR4* expression (**Table 4**). Reasons for these discrepancies might be differences in the patient cohorts examined and might be related to clinically active disease duration or to variations in the level of inflammation *in vivo* due to prolonged RA treatment. Noteworthy, upregulated expression of TLR4 is not only specific for RA. TLR4 is implicated in many other inflammatory diseases such as psoriasis, inflammatory bowel disease, and SLE. Also, all TLRs, including TLR1 and TLR4, are present and upregulated in OA cartilage and synovium, particularly in the lower limbs such as knee and hip cartilage (25, 73, 77, 78). In the present study, the expression of the *TLR4* gene in the OA group was significantly higher than in the RA group. Similar results were determined for *TLR1* expression. Previous studies have shown that in the OA synovial fluid, TLR4 is also present in soluble form, and it is associated with the OA severity as well (79, 80). The fact that TLR4 and TLR1 may be involved in the pathogenesis not only of RA but also of other forms of arthritis, including OA, is also indicated by the correlation analysis between clinical parameters and TLR gene expression. In the present study, a statistically significant negative correlation was determined between anti-CCP and *TLR4* expression levels, potentially due to a separation of patients with early or long arthritis duration.

It has been shown that vitD has a dual effect on the expression and functions of TLRs in immune and nonimmune cells; however, the effect of vitD has been mainly studied in cells of the immune system. For instance, several investigations approved the roles of vitD in the downregulation of *TLR2* and *TLR4* in human pathologic proinflammatory conditions. For instance, vitD decreased expression of *TLR2* and *TLR4* on the monocytes of the patients suffering from type 2 diabetes mellitus and latent autoimmune diabetes (81). It has also been reported that calcitriol decreased *TLR2* and *TLR4* expression and downstream proinflammatory cytokines in human keratinocytes (82). Similar results were observed examining the effect of vitD on *TLR2* (83, 84) and *TLR4* (83, 85, 86) expression. On the other hand, the opposite effect of vitD has also been shown. For instance, increased TLR2/1-dependent expression of cathelicidin against *Mycobacterium tuberculosis* was demonstrated (87). Increased expression and functions of TLR2 (88), TLR3 (89), and TLR8 (90) by vitD to protect injuries from infections have also been documented. Despite the abundance of studies, we were unable to find previous data on the effect of vitD on SFs. In our study, vitD-attenuated TNF-α modulated expression of *TLR1* and *TLR4* and show similar modulation tendency for *NLRP3*. It may likely be related to a short period of stimulation. SFs with vitD3 were stimulated for only 3 days. Also, the preventive effect of the vitD could be more substantial if cells at first would be stimulated with vitD3 and then with TNF-α.

We were also seeking to identify associations of altered *NLRP3* expression with other functional activities of SFs. The activation of TLRs initiates the production of destructive tissue enzymes, as metalloproteinases, the main factors involved in the degradation of bone and cartilage during RA. Previous studies have demonstrated that MMP-1 secretion was higher in the RA (including early RA) groups compared with the OA or non-RA (other inflammatory arthritis like psoriatic arthritis (PsA) and AS) (91). Another study showed no differences in MMP-1 secretion between the OA patients and healthy controls (92). In the present study, the stimulation with TNF-α resulted in higher levels of MMP-1 in the OA, as compared with the RA group. MMP-13 is the most prominent MMP in RA (93). We also found that MMP-13 secretion was highest in the RA group as compared with the other groups.

No effect of vitD on MMP-1 secretion in SFs of the RA patients has been previously reported (94). On the other hand, in rat articular cartilage model, low vitD diet resulted in elevated MMP-13 levels, while vitD supplementation significantly decreased MMP-13 levels (95). In the OA cartilage, the effect of vitD3 alone on MMP-1 production was hardly evident, but when TNF-α was added, more marked responses were observed (96). We have also demonstrated that stimulation with TNF-α has increased the secretion of all MMPs in all groups, whereas vitD3 had no significant effects on their levels.

Taken together, we determined the expression of both *NLRP1* and *NLRP3* in all tested samples of SFs. Stimulation with TNF-α showed no effects on the *NLRP1*, whereas significantly upregulated the expression of *NLRP3*. When comparing different forms of arthritis, significantly lower expression of *NLRP3* under stimulation with TNF-α was observed in the RA group compared with all other tested groups, suggesting potential contribution of altered NLRP3 activation to the etiopathogenesis of advanced RA. The differences in expression of *NLRP3* and *TLR4* in EA and RA highlight the importance of long-lasting inflammation or potentially long-lasting treatment on the activation of this pathway. Our data do not confirm the functional outcomes of NLRP3 activation in SFs, i.e., IL-1β secretion or association with levels of MMPs, although we cannot rule out that the drawbacks of the *in vitro* system, i.e., lack of secondary signal may have led to the inefficient formation of inflammasome complex and in turn lack of functional signaling. Attenuating effects of vitD on the expression of *NLRP3*, *TLR1*, and *TLR4* in SFs suggest potential protective effects of vitD on the inflammatory responses. In contrast, it may be worth to investigate longer or preventive stimulation, prior to the application of TNF-α, which could have revealed even more evident effects of vitD. Noteworthy, both *VDR* and *NLRP3* in the TNF-α-stimulated SFs negatively correlated with the age of patients, suggesting potential age-related changes in inflammatory responses.

In conclusion, we raise the hypothesis that better understanding of inflammasome activation in synovial fibroblasts and possible effects of vitamin D could help to modulate outcomes of early and advanced arthritis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Vilnius Regional Bioethics Committee (Approval No. 158200-18/5-1037-533, date of approval 2018-05-08). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RS, SS, GK, NP, and VT: patient selection and enrolment into the study, clinical data and blood sample collection, and data analysis and interpretation. EB, SS, and IB: study conception. RS, JD, and SM: cell culture experiments, sample data analysis, and interpretation. RS, JD, SM, EB, and SS manuscript preparation. All authors: manuscript editing. AV and IB: critical revision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.767512/full#supplementary-material

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The Role of IgG4 in Autoimmunity and Rheumatic Diseases

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The distinguishing of the IgG4-related disease (IgG4-RD) from among other rheumatic diseases has brought attention to the IgG4 subclass of immunoglobulins. It is the least numerous subclass among immunoglobulins G. In general, IgG4 is considered to be non-inflammatory and tolerance inducing, due to its unique structure. However, in IgG4-RD this antibody plays a pathogenic role in activation of the fibrinogenesis and of the inflammatory process; there are also suggestions that it may be a marker of an abnormal inflammatory response. The importance of IgG4 for the pathogenesis of allergic diseases, with a vital role of its ratio to immunoglobulin E (IgE/IgG4 ratio), has been known for years. The role of IgG4 in the course and pathogenesis of rheumatic diseases is still being researched and is not yet fully understood. Increased IgG4 levels have been revealed in rheumatoid arthritis, although no clear link between this phenomenon and disease activity has been demonstrated. There are articles on the potential importance of IgG4 concentration (of both elevated and decreased serum levels) in Sjogren's syndrome. Additionally, anti-nuclear IgG4 antibody significant titers have been detected in SLE patients, and it has been suggested that the effect of these antibodies on complement consumption and the production of proinflammatory cytokines may play a role in inhibiting the progression of SLE. IgG4 plays a role in autoimmune diseases other than rheumatic diseases, such as pemphigus, bullous pemphigoid, idiopathic membranous glomerulonephritis, or myasthenia gravis, but also in helmints infections. Research shows the importance of IgG4 in malignancy of neoplasms. Melanoma cells are known to stimulate IgG4 production through a modified Th2-based inflammatory response. The role of this immunoglobulin in cholangiocarcinoma is also considered as possible. The aim of this review article is to discuss the current knowledge of IgG4 not only from the perspective of the IgG4-RD but also from a point of view of other autoimmune diseases with particular emphasis on rheumatic diseases.

Keywords: immunoglobulin G4, rheumatic diseases, autoimmune diseases, malignancy, allergic diseases

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Abbreviations: ANCA, anti-neutrophilic cytoplasmic antibodies; c-ANCA, cytoplasmic anti-neutrophilic cytoplasmic antibodies; p-ANCA, perinuclear anti-neutrophilic cytoplasmic antibodies; CIDP, demyelinating polyradiculoneuropathy; CH, constant domain; FAE, fab arm exchange; FcγR, Fc gamma receptor; VL, light chain variable domain; VH, heavy chain variable domain; IL, interleukin; IgG4 ARD, immunoglobulin G4 autoimmune-related disease; IgG4 RD, immunoglobulin G4-related disease; MuSK-MG, muscle-specific kinase myasthenia gravis; TTP, thrombotic thrombocytopenic purpura; EGPA, eosinophilic granulomatosis with polyangiitis; GPA, granulomatosis with polyangitis; EGPA, eosinophilic granulomatosis with polyangiitis.

INTRODUCTION

IgG4 is a subclass of immunoglobulin G. Its role in the inflammation is still being defined, as the significance of its anti-inflammatory activity and tolerance-inducing properties is counteracted by its pathogenic features present in recently identified IgG4-dependent diseases. IgG4 is the least numerous of four IgG subclasses and accounts for only about 5% of IgG immunoglobulins in the human body. Its unique properties, dissimilar to other IgGs, are the lack of influence on the classical complement component pathway and, through a property called "fab-arm exchange" (FAE), inhibition of the formation of large immune complexes (1).

These specific qualities of IgG4 are related to its varied affinity to specific Fc gamma receptors (FcγRs); in fact, IgG4 has no affinity to the receptors FcγRIIIa and FcγRIIIb (1, 2).

IgG4 may mimic rheumatoid factor (RF) activity by interacting with other immunoglobulins G, although IgG4 binds to their constant domains, while a classical RF acts *via* variable domains (3, 4). Zack et al. (3) also found that in rheumatoid arthritis (RA) RF IgG represents mainly the IgG4 class.

IgG4 blocking of the binding of IgG1 to C1q and thus inhibiting the activity of IgG1 underlie anti-inflammatory properties of IgG4 (5, 6).

Over the years, these properties of IgG4 have been analyzed, revealing its vital role in a number of autoimmune diseases, including pemphigus, bullous pemphigoid, idiopathic membranous glomerulonephritis, or myasthenia gravis, as well as its association with parasitic infections (7). The importance of IgG4 in the course of some neoplasms, e.g., cholangiocarcinoma or melanoma, was also noted (8). However, it was the establishment of IgG4-related (IgG4-RD) diseases that caused a wider interest in this subclass of immunoglobulins among scientists, immunologists and clinicians of many specialties. The aim of this article is to discuss, from the clinical point of view, the currently known importance of IgG4, especially in rheumatic diseases.

STRUCTURAL AND PHYSIOLOGICAL DIFFERENCE OF IgG4 FROM OTHER IMMUNOGLOBULINS G

Immunoglobulins G are important in secondary immune response, particularly in response during infections and allergy. Four subclasses of immunoglobulin G have different abilities and affinity to type of FcR receptor, as well as the possibilities for activating the complement pathways.

Focusing on IgG4, its molecule is composed of two heavy chains (with three specific constant domains: CH1, CH2, CH3, and one variable VH domain) and two light chains (consisting of two domains: one constant—CL and one variable—VL). The antigen-binding region of IgG4 consists of both constant (CL) and variable (VL) domains of the light chain and the heavy chain, a variable domain of the heavy chain (VL) and one of its constant domains (CH1). The IgG molecule is bonded by disulfide bridges inside the heavy and light chains and between heavy chains in the hinge region. IgG4 can dissociate into two halves, each consisting of one heavy and one light chain. Such semi-molecules are able to join corresponding semi-molecules deriving from other IgG4—this unique property being called "Fab-arm exchange" (FAE). A bispecific antibody with two antigen-binding sites is thus generated. This mechanism is possible due to the replacement of proline for a serine at position 331 ("P331"; binding of the protein C1q) and a proline for a serine in the hinge region at position 228 (P228S) compared to IgG (9, 10). The FAE phenomenon is still not fully understood, as it has not been unequivocally established how this phenomenon is being regulated. It has been observed that glutathione (GSH) can initiate FAE *in vitro*, while *in vivo* it can be caused by certain drugs (e.g., natalizumab, humanized monoclonal antibody against alpha-4 (α4) integrin) and it can run spontaneously (6). **Figure 1** shows the general principle of the FAE.

This phenomenon causes high variability of IgG4 that generally prevents it from forming immune complexes under chronic antigen stimulation, which possibly plays a role in inhibiting inflammatory reactions. However, the formation of immune complexes with IgG4 seems likely under certain conditions—this phenomenon being described in RA, IgG4-RD, and membranous nephropathy (11, 12). Its occurrence is attributed to the fact that the Fc region of IgG4 may react with other IgG through the Fc–Fc interaction. This phenomenon is not fully understood, and it is possible only in a solid phase of other immunoglobulins.

Apart from FAE mechanism, IgG4 does not have the ability of bivalent antigen binding, suggesting that it should not play an important role in the context of inducing autoimmunity. However, IgG4 is associated with various autoimmune diseases in which it is thought to have diverse range of functions—as it is discussed further below.

Immunoglobulins G bind FcγRs by its crystallizable fragment Fc; FcγRs and these receptors are on most effector cells of the immune system such as monocytes, macrophages, NK cells, mast cells, eosinophils, neutrophils, basophils, dendritic cells, and platelets (13, 14). These receptors are also responsible for various cell responses (activation and/or inhibition) and interactions in the immune system. In **Table 1**, the overview of the main types and roles of FcγRs and its affinity of IgGs especially IgG4 are presented.

IgG4 presents affinity to FcγRI, FcγRIIa, and FcγRIIIav, low affinity to FcγRIIb/c, and no affinity to FcγRIIIaF and FcγRIIIb receptors. All immunoglobulins G, including IgG4, present high affinity to the FcγRn receptor. This receptor is responsible for the transport of IgG across the intestinal mucosa, placenta, and mammary gland, and due to this activity, the balance of IgG and albumin in the body remains stable. These functions of FcγRn depend on the intracellular signal transduction and activation caused by the combination of its extracellular domain and the IgG Fc domain (15, 16). As it has no affinity to FcγRIIIb and has low affinity to C1q, it cannot

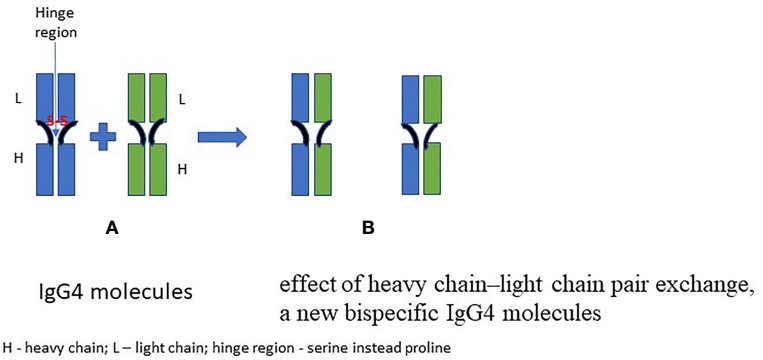

FIGURE 1 | A schematic presentation of the FAE phenomenon (6, 9).

TABLE 1 | The main types and roles of FcγRs and its affinity of IgGs (13–17).

Fcγ receptor/subclasses binding	Cells expression	Role	IgG4 affinity
FcγRI IgG1, IgG3, IgG4	Monocytes, macrophages, dendritic cells, neutrophils, mast cells	Activation	**
FcγRIIaH IgG1,/IgG2/, IgG3, IgG4 FcγRIIaR IgG1,/IgG2/, IgG3, IgG4	Neutrophils, monocytes, macrophages, dendritic cells, basophils, eosinophils	Activation/inhibition	** **
FcγRIIb/c IgG1,/IgG2/, IgG3, IgG4	B cells, dendritic cells, mast cells, basophils/NK cells, monocytes, macrophages, neutrophils	Inhibition/activation	*
FcγRIIIaF IgG1, IgG2, IgG3, FcγRIIIaV IgG1, IgG2, IgG3, IgG4	NK cells/monocytes, platelets, macrophages	Activation/inhibition	- **
FcγRIIIb IgG1, IgG3	Neutrophils, eosinophils, basophils	Activation	-
FcγRn (bind its ligand in pH <6) IgG1,/IgG2/, IgG3, IgG4	Monocytes, macrophages, neutrophils, dendritic cells, endothelial cells, epithelial cells	Recycling transport uptake	***

*-no affinity; **moderate affinity; ***strong affinity.*

activate the classic complement pathway. It also inhibits the binding of C1q to IgG1, thus inhibiting the activity of this immunoglobulin. IgG4 is considered as a "blocking antibody" due to its ability to compete for the same epitopes with other immunoglobulins, e.g., IgE, thus preventing IgE-dependent allergic responses. The lack of ability to form immune complexes does not activate the classical complement cascade, so it does not stimulate antigen presentation.

The regulation of IgG4 production is closely associated with that of IgG1. The production of both these immunoglobulins is stimulated by Th2 lymphocytes *via* release of cytokines (IL- 4, IL-13) in the course of chronic exposure to the antigen (10–13). What is interesting is that IL-10, which stimulates IgG4 secretion, is produced not only by regulatory T lymphocytes but also by regulatory B lymphocytes, which themselves produce IgG4. This mechanism plays an important role in the immune tolerance and anti-inflammatory effect of IgG4 (10, 18).

To consider clinical effects of IgG4 presence, it is necessary to point out its properties, which could underlie the role it may play in certain diseases.

The main features of IgG4 antibodies are summarized in **Table 2**.

Diseases, in which IgG4 involvement is of clinical significance, can be basically divided into three groups: those in which it has a pathogenic role, those in which its effects are considered to be protective and finally a group in which the role of IgG4 is still debatable and needs a further investigation. **Table 3** presents a list of diseases or main clinical problems and the role assigned to IgG4 in them (1, 4, 10).

IgG4 AUTOIMMUNE-RELATED DISEASES

In the majority of autoimmune diseases, a type III hypersensitivity is involved, in which circulating IgG, IgM, and IgA bind soluble antigen and may create immune complexes. Complement activation and activation of immune cells (Fc receptors) cause a cellular cytotoxicity, opsonization, phagocytosis, cell damage, and inflammatory process. As it has been described above, the unique structure of IgG4 causes that this antibody is an exception from this pathophysiological picture. Still, this does not rule out this antibody

TABLE 2 | Immunoglobulin G4 main characteristic (2, 11).

Immunoglobulin G4	
Basic features	Molecular mass 146 kDa
	Half-life of IgG4 molecule—21 days
	Serum abundance maximum 5% of total IgG
	Placenta transfer
Immunological features	High affinity to receptor FcγRI
	Lower affinity to FcγRII
	Response to proteins (++), polysaccharides (+/-)
	No response to allergens
	No complement component 1q (C1q) binding
	Immunologically inert
	Functionally monovalent
Serum concentration	Elevation in chronic exposure to the antigen
	Elevation in immune tolerance state
	Elevation in chronic inflammatory process (also in asymptomatic infection)

TABLE 3 | The role of IgG4 (1, 4, 10).

Role	**Protective**	**Pathogenic**	**Not fully understood**
Disease	Allergic (inhibition of hypersensitivity) – Beekeepers – Laboratory workers – Allergen specific immunotherapy	IgG-4 ARD – MuSK-myasthenia gravis – Pemphigus foliaceus – Pemphigus vulgaris – Thrombotic thrombocytopenic purpura	IgG-4—RD See **Table 4**
	Helmints infection (inhibition of IgE dependent hypersensitivity reactions)	Helmints infection (persistence of infection) Cancer e.g., melanoma, cholangiocarcinoma (disadvantageous suppression)	Rheumatoid arthritis

IgG4-ARD, autoimmune related diseases; IgG4 RD, IgG4-related diseases.

from being a causative agent of some autoimmune diseases. There are autoimmune diseases, in which autoantibodies consisted mainly of the IgG4 subclass of immunoglobulins G (IgG4-AID). These include diseases such as pemphigus vulgaris and foliaceus (19), thrombotic thrombocytopenic purpura (TTP) (20), muscle-specific kinase myasthenia gravis (MuSK-MG) (21), primary membranous nephropathy (22), Goodpasture syndrome (23), and chronic inflammatory demyelinated polyradiculoneuropathy (CIDP) (24). A number of autoantigens have been confirmed as a target for IgG4 antibodies; for example, in CIDIP the autoantigen is neurofascin-155 (NF155), with anti-NF155 IgG4 antibodies which were found in this disease (25, 26).

The diagnosis of IgG4-AID is based on the presence of antigen-specific autoantibodies with combination of specific clinical symptoms. In contrast to this, the diagnosis of IgG4-related diseases (IgG4-RD) is based on the presence of elevated serum concentrations of IgG4, pseudotumors, infiltrations of IgG4-positive plasma cells, fibrosis, and obliterative phlebitis (27).

In the 50's, Witebsky postulated (28) that to identify an autoimmune disease three conditions have to be met: 1) recognition of an autoimmune response (autoantibody or cell-mediated), 2) identification of a corresponding autoantigen, and 3) induction of an analogous autoimmune response in experimental models, causing diseases in experimental animals. IgG4 ARD can be divided into three classes, based on the role IgG4 plays in the processes responsible for meeting Witebsky's conditions. Class I includes diseases, in which two or all three Witebsky's conditions are met. The pathogenicity of IgG4 antibodies can be proved by the transfer of these antibodies and their pathogenic action *in vitro* (28, 29). Class II includes disease, in which there is only indirect evidence of the pathogenicity of antibodies, e.g., the correlation of the IgG4 concentration with the severity of the disease or of the IgG4 presence with the occurrence of a specific phenomenon in the course of the disease. Class III diseases are those in which the role of IgG4 antibodies is not yet proven and their significance for the pathologic process has not been excluded (29).

In MuSK-MG, pemphigus foliaceus and vulgaris, thrombotic thrombocytopenic purpura (TTP), and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), it was concluded that IgG4 is involved in the processes responsible for meeting at least two of Witebski's condition (27).

Less certain, although not devoid of evidence, is the effect of this antibody on the development and severity of diseases such as membranous nephropathy (proteinuria and nephrotic syndrome with immune deposits predominantly with IgG4) and AIDP (acute idiopathic demyelinating polyneuropathy) as well as anti-LGI1 autoimmune encephalitis and Morvan's syndrome (peripheral nerve hyperexcitability, autonomic instability, encephalopathy). In this group, IgG4 takes part in mechanisms, which result in meeting first of Witebsky's conditions (28, 29).

TABLE 4 | The list of IgG4-RD (52–55).

Organ/system	Disease	Comments
Pancreas	Autoimmune pancreatitis	AIP type 1
Liver	IgG4 RD hepatitis	Hepatic mass, icterus
Bill ducts	IgG4-RD cholangitis	Often accompanied by AIP
Salivary glands	Mikulicz's disease	Tumor, pain, swelling, mouth dryness
	Kuttner's tumor	Attention: differential diagnosis of Sjogren's syndrome
Lungs	Pseudotumor, interstitial pneumonia, or pleuritis	Dyspnea, cough, hemoptysis, pleural effusion, deterioration of exercise tolerance, hypoxia (decrease in oxygen saturation)
Retroperitoneum	Retroperitoneal fibrosis	2 types Ureter obliteration Hydronephrosis Leg edema
Mesentery	Sclerosing mesenteritis	Uncharacteristic complaints—pain, flatulence Changes in CT imaging
Organ of sight	Orbital pseudotumor, chronic sclerosing dacryoadenitis, eosinophilic angiocentric fibrosis, idiopathic orbital inflammation	Pain,
Thyroid	Riedel's thyroiditis	Asymptomatic or hypothyroidism, dyspnea, dysphagia, dysphonia neck pain,
Endocrine system other than thyroid glands	Hypophysitis	Pituitary inflammatory disorder with pituitary dysfunction (anterior and/or posterior) with diabetes insipidus (DI) and/or other endocrinopathy development.
Kidneys	Tubulointerstitial nephritis	Proteinuria, hematuria, hypocomplementemia, increased creatinine (chronic or acute renal failure)
Vessels	Aortitis, periaortitis, abdominal aneurism	May be associated with retroperitoneal fibrosis
Heart	Cardiac muscle infiltration/cardiac mass	Intra-cardiac mass may be asymptomatic; on auscultation heart murmurs due to an intra-cardiac obstructing mass, sinoatrial disturbances requiring pacemaker
Nervous system	Related hypophysitis, IgG4-related pachymeningitis	Headache, symptoms of spinal compression, radiculopathies
Prostate and male genitalia	Prostatitis, orchitis	Pain, pollakisuria, benign prostatic hyperplasia
Skin	Primary: Cutaneous plasmacytosis, pseudolymphoma, and angiolymphoid hyperplasia with eosinophilia Mikulicz disease (inflammation of lacrimal and salivary glands) Secondary: Psoriasis-like lesions Unspecified maculopapular or erythematous lesions Hypergammaglobulinemia, purpura, and urticarial vasculitis Ischemic digit	Primary: Histopathology: Marked lymphocyte and plasmacyte infiltration, IgG4+/IgG+ >40% No of IgG4+ cells per high-power field >10. primary Secondary: Plasmacyte infiltration with IgG4+/IgG+ >40% and/or perivascular IgG4 deposition

The weakest data link IgG4 with, e.g., such rare diseases as Goodpasture syndrome (antibodies against type IV collagen) or Iglon 5 parasomnia (Iglon-5 Ag). In this group, there is a proof of a presence of IgG4 autoantibodies, but there is no proof of their involvement in the pathological process (10).

What matters in the context of the topic under discussion in autoimmune diseases of the central and peripheral nervous system with importance of CASPR2, CASPR1, LGI1, or neurofascin 155 antibodies is that the shared feature is the predominance of antigen-specific antibodies of the IgG4 subclass (30, 31).

What can be seen are the main areas of the IgG4 ARD which are the skin, kidneys, and the central nervous and peripheral systems.

IgG-4-RELATED DISEASES

The IgG4-related diseases form a still growing group of fibroinflammatory diseases with special clinical features such as pseudotumors, storiform fibrosis, obliterative phlebitis, and organ damage. Their main histopathological feature is a presence of infiltrations by IgG4-producing plasma cells and eosinophils.

Apart from the above described diagnostic criteria, there has been suggestion by Hubers et al. (32) that the presence of A11-specific IgG4 antibodies might play a role of a specific marker for IgG4-RD. Their formation attenuated IgG1-mediated pro-inflammatory autoreactivity against annexin A11 in patients with IgG4-RD (32). This result is contrary to what might be assumed in an inflammatory disease, such as IgG4-RD, and may confirm the potentially anti-inflammatory role of IgG4 in IgG-RD.

Storiform Fibrosis

The inflammatory infiltrates which include IgG4-positive plasma cells probably activate myofibroblasts and lead to a storiform fibrosis, characteristic for IgG4-RD. It can be assumed that the inflammatory process with the activation of B cells and activation of fibrosis (myofibroblasts) may proceed simultaneously. Changes in the histopathological image can also be expected over time—from the predominance of cellular infiltration to a weakly cellular image with dominant fibrosis (33). Della-Torre et al. (34) found that B cells from IgG4-RD patients produced the pro-fibrotic molecule PDGF-B and stimulated collagen

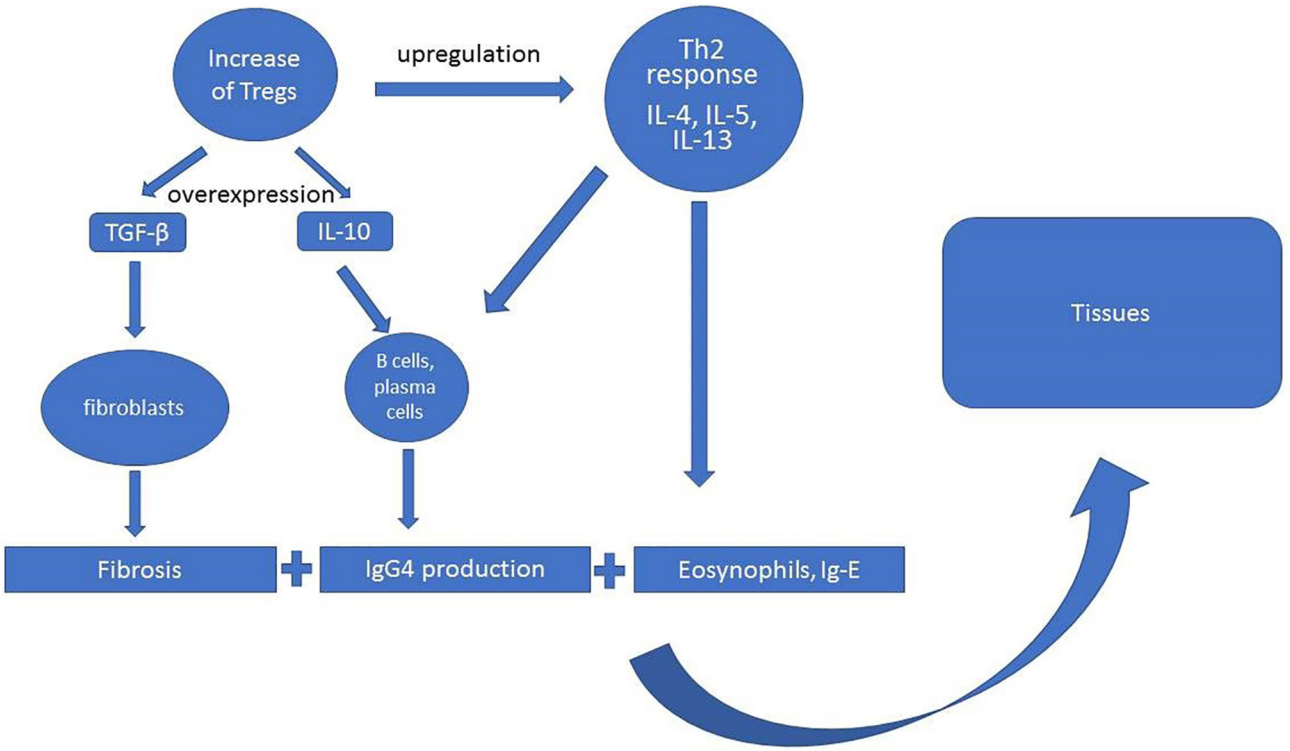

FIGURE 2 | Outline of IgG4-related disease pathogenesis (55, 58, 59).

production by fibroblasts. These B cells also expressed enzymes such as LOXL2, which are involved in extracellular matrix remodeling. Also, these authors presented that B cells produced chemotactic factors CCL-4, CCL-5, and CCL-11 and induced the production of these same chemokines by activated fibroblasts. In a cited work, the authors concluded that plasmablasts expressed intrinsic pro-fibrotic properties (34).

Obliterative Phlebitis and Arteritis

In IgG4-RD, obliteration of venous vessels with their inflammation, i.e., lymphoplasmacytic infiltration of their walls, is found. The occlusion of these vessels without inflammation in the vascular wall is not a clue for the diagnosis of IgG4-RD (35). Arteries are less prone to inflammation in IgG4-RD. Their inflammation, when it occurs, is not necrotic, which is important in the differential diagnosis. Arteritis may be accompanied by retroperitoneal fibrosis, lung, and heart involvement or AIP.

According to the classification criteria for IgG4-RD, the ratio of IgG4-positive plasma cells to total IgG plasma cells should be over 40% (27). The group includes autoimmune pancreatitis (AIP), IgG4 kidney disease, or IgG4-related retroperitoneum fibrosis (Ormond's disease). The updates of the American College of Rheumatology (ACR) and the European League Against Rheumatism (Eular) classification criteria, which were simultaneously published in 2020, take into account entry criteria ("characteristic clinical or radiologic involvement of a typical organ, e.g., pancreas, salivary glands, bile ducts, orbits, kidney, lung, aorta, retroperitoneum, pachymeninges, or thyroid gland [Riedel's thyroiditis] OR pathologic evidence of an inflammatory process accompanied by a lymphoplasmacytic infiltrate of uncertain etiology in one of these same organs") and exclusion criteria. The presence of the specific features has assigned certain point scores to it. If the entry criteria are met, no exclusion criterion is present and the total point score is ≥20 the Ig-G4 RD may be diagnosed.

Recent publications suggest candidates for potential new biomarkers in Ig-G4 RD, such as serum and tissue IgG2 or soluble IL-2 receptor (sIL2R)—for inflammation intensity and cc–chemokine ligand 18 (CCL18) for fibrosis (36). The IgG4+ plasmablast level was also highlighted as a potential biomarker useful for diagnosis, assessment during the course of the disease, and efficacy of treatment (37, 38). It has been demonstrated that the overexpression of TLR-7 in IgG4-RD stimulates macrophages 2 to produce IL-33 which in turn stimulates Th2 response (39), which suggests that the TLR-7 level might be also considered as a biomarker of the activation of Th2 pathway and of inflammatory response.

It was found that in AIP associated with the IgG4-RD, the serum concentration of laminin 511-E8 was significantly higher in comparison with healthy controls (40). Another study revealed a similar phenomenon concerning serum galectin-3 (41). What is important is that galectin-3 plays a significant role in the fibrosis (42) in IgG4-RD, as well as in an idiopathic pulmonary fibrosis (IPF) (43). The novel small-molecule TD139, an inhibitor of Gal-3, is currently investigated in I/II phase clinical trials in the treatment of patients with IPF (43).

The introduction of a concept of IgG-4 RD means that some diseases were treated until now as one uniform entity, such as autoimmune pancreatitis (AIP); a subtype concerning IgG-4 involvement was distinguished. In case of AIP, AIP-1 is a subtype regarded as IgG-4 RD. AIP 1 is characterized by lymphoplasmacytic sclerosing pancreatitis (LPSP) in histopathological assessment, with storiform fibrosis, IgG4-positive plasma cell infiltrations, and presence of other Ig-G4 RD features, including extraintestitial symptoms (44). In case of some diseases, such as the extraperitoneal fibrosis idiopathic type (Ormond's disease), no official IgG4-RD subtype was distinguished, with both IgG4-RD and disease with no involvement of IgG4 having the same clinical picture (44–46). The primary Sjogren's syndrome (pSS)—an autoimmune and inflammatory disease in which B-lymphocyte hyperreactivity, enlargement of salivary glands, the presence of autoantibodies, and mononuclear cell infiltration are the predominant features—can be confused with IgG4 RD. As it turns out, some cases initially recognized as a former Mikulicz's disease, which is a pSS with a chronic salivary enlargement, without antibodies to ribonucleoproteins most characteristic for pSS (47)—such as anti-Sjögren's-syndrome-related antigen A autoantibodies (anti SSA/RoAb) and anti-Sjögren's syndrome-related antigen B antibodies (anti-SSB/La)—are now recognized as instances of IgG4-related disease (IgG4-related plasmacytic exocrinopathy) (48). The SSA/Ro antigen is a ribonucleoprotein complex containing hY-RNAs and two proteins differing molecular mass—Ro 52 and Ro 60 kD. The SSB/La antigen consists of protein of 48-kD mass (48). The Ro protein has been identified as an E3 ligase, which regulates negatively cytokine production induced by the IFNγ pathway, while the La protein works as a transcription termination factor of the RNA polymerase III transcripts (49).

The history of the discovery of IgG4 RD begun since the first description of the association of the elevated serum concentration of IgG4 with clinical features of autoimmune pancreatitis (AIP), by Hamano et al. in 2001. These observations and conclusions were confirmed and extended by other researchers primarily from Japan (50, 51). However, as mentioned above after analysis of clinical features of IgG4 RD (52), it was found that the image assigned to Sjogren's syndrome, described by Polish physician Jan Mikulicz Radecki in 1888, also corresponds to an IgG4-dependent disease. Currently, the list of IgG4-RD is still being extended; it is presented in **Table 4**.

The scheme of the pathogenesis of IgG4-RD assumes the increased activity of T regulatory cells (Treg) with overexpression of IL-10, transforming growth factor β (TGF-β) production, and upregulation of Th2 response, in which predominant roles play interleukins 4, 5, and 13 (IL-4, IL-5, Il-13) (**Figure 2**). The activity of these cytokines translates into specific pathophysiological phenomena such as fibrosis (TGF-β), activation of B cells and plasma cells to IgG4 production (IL-10, IL-4, IL-5), and eosinophilia (Il-5, Il-4, Il-13) (30, 38). However, current ACR/EULAR classification criteria for IgG4-RD peripheral eosinophilia above 3,000/μl are considered as exclusion criteria depending on the clinical picture (27).

Presently, the knowledge of IgG4-RD is still expanding and the list of these diseases is constantly being supplemented. Several questions concerning IgG4-RD still remain to be answered, such as the following:

- is the presence of an increased amount of IgG4 in the patients' sera a result of the activation of their production as an anti-inflammatory antibody in response to other factors, e.g., environmental such as infections, trauma, and other inflammatory processes (e.g., the inflammatory theory of atherosclerosis)? (56, 57)
- does the possibility of IgG4 complement activation other than the classic pathways (the mannose binding lectin pathway) better explain the role of this antibody in IgG4-RD?
- does IgG4 play a pathogenic role in IgG4-RD or is its increased concentration only an epiphenomenon related to other immunological processes taking place in IgG4-RD?
- does the effectiveness of therapies directed against B cells (anti-CD-20 and anti-CD-19) in IgG4-RD derive from the results they have on the IgG4 overproduction or from other effects of the depletion of B cell populations?

OTHER RHEUMATIC DISEASES AND IgG4

Rheumatoid Arthritis

In recent years, the importance of the IgG4 subclass in RA has been raised. There was an increase in the concentration of this immunoglobulin in RA patients compared to the control group. Among antibodies to cyclic citrullinated peptides (ACPA), IgG4 levels exceeded IgG2 and IgG3, and among immunoglobulins G belonging to the class of rheumatoid factor (RF), IgG4 levels were second only to IgG1. It is assumed that the persistent autoimmune stimulation in the course of active RA gives a boost to the production of IgG4 (60, 61). This observation was confirmed, as Yu et al. (62) found in 30.3% of studied RA patients (n= 433) elevated serum levels of IgG4. The infiltration of IgG+ plasma cells in IgG4 RD is a crucial feature of this disease, but was also described in synovium in the course of RA (63). Some reports suggest that the serum level of IgG4 ACPA may serve as a biomarker for monitoring the response of RA patients to therapy (61). Engelman et al. (64) demonstrated a significant decrease in IgG4 ACPA levels in responders to treatment, regardless of the treatment type (methotrexate as well as biologics). Carbone et al. (65) showed that blocking IL-6 by tocilizumab also reduces IgG4 anti-CCP antibodies, but such an effect was not shown on IgG1 anti-CCP antibodies. This may indicate the influence of IL-6 on the production of IgG4 anti-CCP antibodies in RA.

Sjögren's Syndrome

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease in which under certain conditions, the tolerance is broken and B cells are stimulated depending on and independently of the activity of T lymphocytes, which causes their hyperreactivity and the production of antibodies mainly for ribonucleoproteins: anti-SSA (Ro52 + Ro60) and SSB/La. A further consequence of these events is the accumulation of mononuclear cells in infiltrates in the exocrine glands, which leads to their damage and clinical symptoms, such as

dryness (changes in the secretion of, among others, tears, pancreatic juice, saliva) (66). This disease may be mild, but it can also be severe with involvement of organs and systems, including vasculitis and nerve damage. Phenomena such as hypocomplementemia, deficiencies of erythrocytes, platelets, lymphocytes, and the presence of cryoglobulins with cryoglobulinemic purpura are some of the possible immunological deviations in its course. Inflammation or enlargement of the salivary glands is quite common in pSS, with or without symptoms of dryness. Over the years, also another entity with a similar course, i.e., Mikulicz's disease, was treated as a subgroup of patients with Sjögren's syndrome. It is currently known, that in fact its discoverer Jan Mikulicz Radecki dealt with IgG4 RD (67). In IgG4 RD, sialadenitis and dacryoadenitis may also be a manifestation of this disease and in some studies in these patients the serum IFN-γ/IL-4 level was higher than in those with pSS (48).

There are contradictory reports on IgG4 in pSS; an increased concentration of this immunoglobulin in serum was reported in some of pSS patients. Mavgrani et al. (68) noted that this group had some features of IgG4-dependent disease. Another study shows a decrease in serum IgG4 concentrations in pSS patients, especially in those with high immune activity and C4 hypocomplementemia (69).

ANCA-Associated Vasculitis

There are reports about increased IgG4 levels in some vasculitides, especially in eosinophilic granulomatosis with polyangiitis (EGPA)—formerly known as Churg-Strauss syndrome—and in granulomatosis with polyangiitis (GPA), associated with anti-neutrophil cytoplasmic antibodies (c-ANCA).

In EGPA with eosinophilia and other allergic features such as asthma, allergic rhinitis, and sinusitis, the increase in IgG4 levels can be explained as being analogous to allergic diseases. However, misdiagnoses of IgG4 RD are also possible. EGPA represents necrotizing vasculitis affecting medium-sized small vessels, while in IgG4-RD an involvement of larger vessels, such as the aorta, is expected. In case of doubts in the diagnosis, these diseases will be differentiated mainly by histopathology. The presence of ANCA antibodies may also point against IgG4-RD (70). Differentiation of GPA and IgG4-RD may be more difficult, and biopsy may not always bring unambiguous results. In a biopsy, especially from the orbit or sinus area, IgG4-positive plasmatic cells may be present in the GPA (71). The main distractor is the presence of ANCA antibodies in GPA. Increased serum IgG4 level is also seen in some cases of vasculitis; however, the IgG4/total IgG ratio is generally higher in IgG4-RD (72). In some cases, there is also the possibility of the IgG4-RD-vasculitis overlapping syndrome. Interestingly in GPA, IgG4-ANCA antibodies were detected, which have the ability to activate primed neutrophils (73).

Systemic Lupus Erythematosus

The function of IgG4 antibodies in systemic lupus erythematosus (SLE) is still being discussed. Significant anti-nuclear IgG4 antibody titers (ANA-IgG4) have been detected in SLE patients, and it has been suggested that the effect of these antibodies on complement consumption and the production of proinflammatory cytokines may play a role in inhibiting the progression of SLE. The presence of anti-dsDNA IgG4 antibodies was also reported and was associated with skin lesions and kidney involvement in patients with SLE (74). Pan et al. (75) demonstrated in an *in vitro* constructed study using IgG4 healthy controls and IgG4 antibodies from SLE patients that the latter can inhibit complement consumption by the activity of autoantibody–autoantigen immune complexes (ICs). The authors concluded that SLE IgG4 inhibited the activation of the alternative pathway by other subclasses of autoreactive IgGs (75).

Helmints Infection

The helmints infection is a dynamic pathophysiological state. At the start of the infection, Th1 response dominates. At later stages, Th1/Th2 responses become more balanced, while in chronic condition Th2 response with eosinophilia and increased concentration of IgG4 and Il-10 dominates. In the chronic infection, the downregulation of Th1-cell, Th17 cell response, and amplification of Th2 cell response occurs. Therefore, due to the activity of the abovementioned cytokines, the increased secretion of IgG4 can also be expected. Indeed, in parasitic infections, increased concentrations of IgG4 and IgE are found. A strong anti-parasite IgE response is associated with the resistance to infection (1, 3). The role of IgG4 in helmints infection is still analyzed. Essentially, two possible mechanisms are proposed that can lead to the inhibition of IgE-mediated immunity and resistance to helmints (74, 76). One is associated with the inhibition of a switch in plasma cell production from IgM *via* IgG4 to IgE. In this case, the inhibition of that switch at the IgG4 level disturbs the ratio of IgG4 to IgE in favor of the former. The second proposed mechanism assumes a competition between IgG4 (with low affinity for Fcγ receptors) and IgE for epitopes on the helmints surface or in a close environment. The similar situation is described in reactions to allergens. "Blocking antibody" activity explains the finding that IgE-mediated hypersensitivity reactions are rare in patients with chronic parasitic infections (76).

Allergic Diseases

Allergic diseases as well as IgG4-RD have the same immunological features including predominant Th2 response with elevated IgG4 and IgE and eosinophilia. In allergic diseases, the balance between IgG4 and IgE depends on the activity of Th2 cells and of IL-4 and IL-10. Interleukin-4 stimulates the production of both IgG4 and IgE, while IL-10 only of IgG4. The higher the concentration of allergen-specific IgG4, the greater the tolerance to the allergen and the greater the inhibition of the allergic reaction. Hence, the immune tolerance state should be characterized by a high IgG4-to-IgE ratio (IgG4/IgE). As more often the ratio of IgE to IgG4 (IgE/IgG4) is used, its lower value indicates higher tolerance to the allergen (77, 78).

The ability of IgG4 to compete with IgE for the same epitopes in this case prevents the IgE-dependent acute reaction with degranulation of mast cells and release of histamine and

bradykinin. In IgG4-RD, the presence of allergy, atopy, eosinophilia, and increased serum levels of IgE and IgE-positive mast cells in lymphoid, biliary, and pancreatic tissues is quite often described (79). Higher serum levels of IgE in IgG4-RD may indicate a relapse and, together with the increased concentration of IgG4, can be taken into account as a predictive factor (78). However, Della Torre et al. (80) presented different results of investigation concerning atopy in IgG4-RD patients. This study revealed that the majority of patients were non-atopic; atopy was confirmed in 33% of patients. The authors concluded that the prevalence of atopy in this group was similar to that of the general population. This indicates that this issue requires further investigation.

In allergic diseases, specific immunotherapy (SIT) relies on the administration of allergen extracts to produce the emergence of tolerance to those allergens. This method implies an impact on Treg cells and switching of allergen-specific B-cells toward IgG4 production (81). This causes an induction of Tregs, reduction in specific IgE levels, induction of IgG4 (blocking) antibodies, and shift from Th2 to Th1 response.

MALIGNANCIES AND IgG4

The IgG4 serum level may be elevated in IgG4-RD but may also be within a normal range—the latter does not exclude the diagnosis of IgG4-RD. On the other hand, the elevation of IgG4 may be observed in case of malignancy (82).

It is not uncommon that a pseudotumor lesion in an organ is treated as neoplastic, when in fact it is IgG4-RD. Therefore, in the differential diagnosis, IgG4-RD as a cancer mimicker should be taken under consideration. In 2.6% of patients with suspected pancreatic cancer, in whom the surgery was performed, AIP was a final diagnosis (83). In both clinical situations, biopsy and histopathological examination are essential for proper diagnosis and treatment (82).

In some malignancies, tumor cells escape host control, by inducing the switch of the production of autoantibodies toward IgG4, thus taking the advantage of the protective properties of IgG4. Such a phenomenon has been demonstrated for a number of neoplasms, including melanoma, extrahepatic cholangiocarcinoma, pancreatic cancer, or glioblastoma (55). The knowledge about cancer immunology is constantly growing. It has been found that in melanoma or cholangiocarcinoma, the concentration of tumor-specific IgG4 increases and the tumor cells stimulate their production. Tumor modifies the Th2 response and the activity of cytokines, especially IL-4 and IL-10. The increase in the activity of IgG4 with IgG1-inhibiting properties thus inhibits the antitumor effect of IgG1 and the activation of macrophages and in this way inhibits the death of the tumor cell (1, 26).

In a Chinese study presented in 2020, researchers found increased incidence of malignancy in patients with IgG4-RD. The authors pointed to risk factors, such as autoimmune pancreatitis, but also highlighted potential protective role of other phenomena, such as eosinophilia (8). Also, other studies reported malignancies in IgG4-RD patients; however, opposite conclusions are being presented as well. Hirano et al. (84) tested 113 of IgG4-RD patients and concluded that IgG4-RD is not associated with an increased incidence of total malignancies with comparison to the general population.

Another interesting conclusion presented by Wallace et al. (85) is that patients with IgG4-RD more often have a history of malignancy prior to their diagnosis. The most common malignancy in this studied group was prostate cancer and lymphoma. The authors suggested that the treatment of malignancy may also be a risk factor to the development of IgG4-RD or there are similar risk factors for both clinical situations.

An interesting research and conclusions were presented by Zhou et al. (86) in a study of 60 patients diagnosed with IgG4. The study revealed that as many as 60% (n = 38) were initially suspected of cancer, which resulted in greater interventions in 14 patients. General symptoms (e.g., fever, weight loss, fatigue), the presence of pseudotumors/infiltrations in various areas such as the abdominal cavity, salivary glands, or pleura, presence of lymphadenopathy, and increased inflammatory activity in laboratory tests led to the diagnostics being focused on proliferative processes. Oncological vigilance is important in any case with similar symptoms, but taking into account the possibility of the presence of an IgG4-related disease is necessary in order to avoid unnecessary burdensome procedures and inappropriate treatment. The simultaneous occurrence of IgG4 disease and a malignancy is also most commonly with lymphomas, such as marginal zone lymphoma (MZL), mucosa associated lymphoid tissue lymphoma (MALT), and a non-Hodgkin lymphoma. There were also reports of IgG4-RD concurrent with pancreatic cancer and colon carcinoma (87).

To summarize, increased serum levels of IgG4 may in some instances may be useful in the differentiation of clinical conditions. Maślińska et al. reported (69) the lowering of serum concentration of IgG4 below the established normal range in patients with pSS and suggested that it may be also a marker of this autoimmune disease. It was pointed out that C4 hypocomplementemia found in a significant number of pSS patients may have an impact on the reduction of IgG4 concentration and is also an indicator of disease activity taken into account in the ESSDAI assessment (88).

There is also an increase in the concentration of IgG4 in chronic inflammation, which takes place, for example, in active RA (89). It can be assumed that such an increase is important as an expression of the organism's attempt to inhibit the inflammatory process, with IgG4 playing a positive role as an inhibitory antibody (4). Some authors also concluded that the elevation of IgG4 serum levels in RA concerns a distinct subtype of patients RA with a worse course of the disease and a poorer response to the treatment with classical disease-modifying drugs (cDMARDs) such as methotrexate and leflunomide (90). Umekita et al. (91) described the case with arthropathy with infiltrate IgG4-positive plasma cells in the synovium without any other organ involvement, and the authors argued that the image did not correspond to RA. This topic seems debatable. In the IgG4 RD, arthritis with synovial hyperplasia and infiltration of IgG4+ plasma cells may occur.

An interesting work was recently published by Olejarz et al. (92) showing that in Graves' orbitopathy (GO), the serum concentration of IgG4 is increased, especially in patients diagnosed at a younger age and with a more severe course of GO with a higher Clinical Activity Score (CAS). The authors suggested that the diagnosis of GO with elevated IgG4 serum concentration in patients with previously established diagnosis of Graves' disease, as well as the differential diagnosis of conditions such as IgG4 RD or overlapping syndrome, should be taken into account.

From the clinical point of view, other diseases located in the eyeball and the periocular area such as orbital pseudotumor, orbital neoplasms (malignant as lymphomas or benign), infections, orbital myositis, sarcoidosis, and inflammatory orbitopathy in the course of vasculitides especially granulomatosis with polyangiitis (GPA) should be considered in the differential diagnosis. The above considerations indicate the scale of difficulties in the proper diagnosis of IgG4-RD in everyday clinical practice.

IgG4 antibodies found a place in immunotherapy, as their specific anti-inflammatory and protective abilities make them an advantageous subclass in the design of therapeutic antibodies, especially when there is a need to block other antibodies. The interesting therapeutical option is a pembrolizumab-humanized anti-receptor monoclonal antibody programmed cell death-1 (PD-1) which consists of IgG4/kappa isotype with stabilizing modification of the Fc region sequence. This monoclonal antibody found use in therapy of several neoplasms, among others melanoma, cervical cancer, esophageal cancer, colorectal cancer, and triple-negative breast cancer) (93). In June 2020, the Food and Drug Administration (FDA) granted accelerated approval to pembrolizumab for the treatment of patients with unresectable or metastatic tumor mutational burden-high solid tumors, which have progressed following prior treatment and who have no satisfactory alternative treatment options (94). This approval concerns both adult and pediatric patients.

Another interesting drug is nivolumab—a fully human immunoglobulin G4 and a PD-1 immune checkpoint inhibitor antibody. It has demonstrated efficacy in the treatment of patients with advanced non-small-cell lung cancer (NSCLC) as well as melanoma, urothelial cancer, renal cell carcinoma (RCC), or malignant pleural mesothelioma (MPM) (95). Nivolumab was also approved by FDA for the treatment of patients with completely resected esophageal or gastroesophageal junction (GEJ) cancer with residual pathologic disease, who have received neoadjuvant chemoradiotherapy, as well as for patients with metastatic gastric cancer and esophageal adenocarcinoma in combination with chemotherapy (96). The efficacy of nivolumab (anti-PD-1) in combination with ipilimumab (anti-CTLA-4) in the treatment of melanoma or metastatic or recurrent non-small cell lung cancer (NSCLC), in the latter case also with platinum-doublet chemotherapy, has also been demonstrated (97).

The disbalance between IgG4 and other classes or subclasses of immunoglobulins, depending on how it is shaped and in which disease it occurs, may lead to various outcomes tilting the clinical picture toward inhibition of inflammation or its activation, e.g., in allergic diseases where the IgG4 role is strongly inhibitory and protective, in IgG4 RD where IgG4 is an indicator of inflammation and uncontrolled fibrosis (however, the overall role of IgG4 *per se* in IgG-RD is unclear) and in neoplasms, in which the pathogenic role of IgG4 is overwhelming.

Particularly, the interest in their importance in IgG4-related diseases has increased, but no specific IgG4 antibodies have been identified in them, and in the affected organs, infiltrates are composed of many different cells apart from plasma cells that produce this immunoglobulin, and not all patients also present high levels of IgG4 in the serum.

CONCLUDING REMARKS

Immunoglobulin G4 may play both pathogenic and protective role (as a blocking antibody with anti-inflammatory properties) in the human body and disbalance between this and other immunoglobulins may be, depending on circumstances, interpreted in different ways, e.g., in malignances and allergic diseases. The elevation of IgG4 levels by itself is not always an indication of an ongoing pathologic process, as the role of IgG4 is very diverse. The unique properties of this immunoglobulin, such as FAE and its influence on the other immunoglobulins, most likely determine such a variability of its impact. The mere increase in the concentration of IgG4 in the patient's serum without taking into account all the accompanying phenomena and tests, including histopathological ones, cannot form the basis for the diagnosis of a specific disease. The recent establishment of IgG4-RD has brought IgG4 into the spotlight and raised interest in its involvement in rheumatic and autoimmune diseases. As we learn more about IgG4, the need for taking its presence into consideration during diagnostic and therapeutic process will grow.

AUTHOR CONTRIBUTIONS

MM—first author, author for correspondence, concept of the work, writing, literature review, final acceptance. JD-C—writing, literature review, final acceptance. MJ—writing, literature review, final acceptance.

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The role of virus infections in Sjögren's syndrome

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Primary Sjögren's syndrome (pSS) is an autoimmune disease with a clinical picture of not only mainly exocrine gland involvement, with dryness symptoms, but also internal organ and systems involvement. The epithelial damage and releasing of antigens, which, in some circumstances, become autoantigens, underlay the pathogenesis of pSS. The activation of autoimmune processes in pSS leads to the hyperactivation of B cells with autoantibody production and other immunological phenomena such as hypergammaglobulinemia, production of cryoglobulins, or formation of extra-nodal lymphoid tissue. Among the risk factors for the development of this disease are viral infections, which themselves can activate autoimmune reactions and influence the host's immune response. It is known that viruses, through various mechanisms, can influence the immune system and initiate autoimmune reactions. These mechanisms include molecular mimicry, bystander activation, production of superantigens—proteins encoded by viruses—or a programming to produce viral cytokines similar to host cytokines such as, e.g., interleukin-10. Of particular importance for pSS are viruses which not only, as expected, activate the interferon pathway but also play a particular role, directly or indirectly, in B cell activation or present tropism to organs also targeted in the course of pSS. This article is an attempt to present the current knowledge of the influence specific viruses have on the development and course of pSS.

KEYWORDS

Sjögren's syndrome, herpesviruses, retroviruses, hepatitis viruses, autoimmunity, viral infections

Abbreviations: APRIL, a proliferation-inducing ligand; BAFF, B cell-activating factor; CMV, cytomegalovirus; C-X-C motif, chemokine; CXCL, ligand; EBV, Epstein–Barr virus; GCs, germinal centers; HHV, human herpes virus; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; HTLV, human T-lymphotropic virus; IgG-RD, IgG4 related disease; IRF5, interferon regulatory factor 5; IRGs, interferon response genes; IL, interleukin; MSGB, minor salivary gland biopsy; pB19, parvovirus B-19; RA, rheumatoid arthritis; RF, rheumatoid factor; STAT4, signal transducer activator of transcription 4; SAGs, superantigens; SLE, systemic lupus erythematosus; TRIM, tripartite motif; TNF, tumor necrosis factor.

Introduction

In many rheumatic diseases, the influence of viral infections is considered as a potential triggering factor for the activation of an autoimmune process. Several viruses are suggested to have an effect on autoimmunity—among them are Epstein–Barr virus (EBV) and human herpes virus-6 (HHV-6), hepatitis C (HCV) and B (HBV) viruses, enteroviruses, influenza A virus, human immunodeficiency virus (HIV), or viruses of Flaviviridae family (e.g., Zika and dengue viruses) (1). In the case of some viruses, the mechanism of their influence on autoimmunity is uncertain as the autoimmune reaction is triggered by the reactivation of a virus that previously remained in a latent state (e.g., polyomavirus JC virus). Some of the viruses involved in autoimmune processes infect exclusively humans, with a virus targeting only specific host cells (e.g., B cells)—e.g., EBV. Environmental factors, including viral infections, lead to the development of the autoimmune reaction by essentially causing a breakdown of autotolerance, which triggers the production of autoantibodies and the development of specific clinical phenomena. Viruses influence the immunity through mechanisms of molecular mimicry, generation of superantigens, apoptosis, necrosis, clearance deficiency, and bystander activation (1, 2).

Primary Sjögren's syndrome (pSS), also known as "autoimmune epithelitis", is a model autoimmune disease, in which damage to the epithelial barrier, release of autoantigens, and the development of a cascade of autoimmune reactions constitute the core of the problem. The epithelial damage and the activation of B cells are essential for the activation of the immune system in pSS. These elements and the whole cascade of autoimmune phenomena can be stimulated at various stages by environmental factors, including viral infections. The aim of this article is to discuss the influence of viral infections on the development and course of pSS.

Primary Sjögren's syndrome

Primary Sjögren's syndrome is an autoimmune disease with inflammatory infiltration, with mononuclear cells affecting primarily the exocrine glands, epithelium damage, and hyperreactivity of B cells with autoantibodies against ribonucleoproteins: anti-SSA/Ro and anti-SSB/La production (3, 4).

The main clinical feature of pSS is mucosal dryness of the mouth, eyes, vagina, and bronchial tree, which occurs due to the involvement of the exocrine glands. Dryness symptoms may occur late in the course of the disease, leading to the undetected development of pSS for a long period of time, with the disease expressed initially through chronic fatigue, joint pain, or formation of stones in the kidneys, gallbladder, or salivary glands. Primary Sjögren's syndrome, as a systemic disease, may progress with the involvement of internal organs manifesting itself as an interstitial lung disease, autoimmune cholangitis, hepatitis, vasculitis in the form of skin lesions (livedo reticularis and palpable purpura), and peripheral or central nervous system dysfunction. This often leads to the patient being referred to various specialists before the pSS diagnosis is eventually established (5). An active HCV infection (often asymptomatic in the area of hepatic lesions) may take the form suggesting a systemic disease—including pSS. Therefore, exclusion of HCV infection is an indispensable part of the differential diagnosis of pSS, as reflected in the pSS diagnostic criteria (5).

The main histopathologic feature of pSS is the presence of mononuclear cell infiltrations in the salivary glands and often in other internal organs as well. Infiltrations include dendritic cells (DC), CD 4+, and plasma cells (6). Cell clusters can form germinal centers (GC) and ectopic lymphoid tissue, the emergence of which increases the risk of developing lymphomas (mainly B cell) (6).

The pathogenesis of pSS is thought to consist in the damage of the epithelium and the release of antigens (7), with pSS being sometimes described as "autoimmune epithelitis". The autoantigens Ro52, Ro60, and La (48 kD) are associated with pSS, but the mechanism of the breakdown of the immune tolerance remains unclear. Apoptosis is suggested to cause the presentation of these autoantigens to the immune system (8). Subsequently, the innate immune system response starts. The activation of Toll-like receptors (TLR) and the stimulation of DC and macrophages trigger, in turn, the release of interferons (IFNs), the stimulation of T cells (especially CD 4+), and the production of B cell-stimulating factors—such as BAFF and a proliferation-inducing ligand (APRIL) (7). The hyperactivation of B cells is key to this disease, as it leads to hypergammaglobulinemia and the production of anti-ribonucleoprotein antibodies (anti-SSA/Ro and SSB/La antibodies). The anti-SSA/Ro antibodies recognize two cellular proteins, which have a molecular weight of 52 and 60 kD and are therefore named anti-Ro52 and anti-Ro60, respectively (8, 9). Those autoantibodies do not cross-react with each other. They are also located in various cell regions: Ro60 in the nucleus and nucleolus and Ro52 in the cytoplasm. They are also encoded by two different genes: located on chromosome 19 for Ro60 and on chromosome 11 for Ro52 (10). It turned out that Ro60 exists as a small cytoplasmic conserved protein which binds RNA (hY-RNA) (11). Ro52 was discovered (in 1988) to be a part of the SSA complex. Later, it was identified as an E3 ubiquitin ligase (12) and a protein belonging to the tripartite motif (TRIM) protein family (13). Anti-SSA/Ro antibodies, particularly anti-Ro (52 kD), are detected in several autoimmune diseases, especially in SLE, RA, idiopathic inflammatory myopathy, dermatomyositis, and systemic sclerosis (14). Importantly, Ro52 is a protein, the production of which is induced by viral infections through the stimulation of TLRs and of the interferon I pathway (IFN I) (15).

It is suggested that anti-Ro60 is more specific for pSS than anti-Ro52 (16). The SSB/La protein is a transcription–termination factor for RNA polymerase III. The anti-SSB/La antibodies commonly occur along with anti-SSA/Ro antibodies; they are present in some pSS patients and in SLE patients. Their presence is associated with neutropenia and cryoglobulinemia. In maternal SLE the presence of anti-SSB/La and anti-SSA/Ro antibodies is associated with disorders of the heart conduction system during fetal development and, after birth, in neonatal lupus (17, 18).

The tropism of some viruses to exocrine glands, such as salivary glands (e.g., EBV) may cause the development of dryness symptoms in the course of the infection, requiring the differentiation of such phenomena from pSS. At the same time, the same viruses may also contribute to the development of pSS.

Recognized genetic risk factors for the development of pSS include the presence of certain human leukocyte antigen (HLA) system genotypes (HLA-B8, HLA-DW3, HLA-DR3, and DRw52) and a polymorphism of genes for interferon regulatory factor 5 (IRF5) and signal transducer and activator of transcription 4 (STAT4) [which are both gene activators of the immune response of the type I of interferon (IFN) system], CXCR5 (cell surface protein found in antibody-producing B cells), TNIP1 (binding partner for TNFAIP3—protein which plays a role in limiting inflammation), IL12A (part of this protein activates T cells and natural killer cells), and BLK (tyrosine protein kinase, which activates B cells) (19–22). The epigenetic phenomena (the influence of environmental factors on human genes through the mechanisms of DNA methylation and histone modification of non-coding RNA) (23), which result in the modification of the interferon pathway, are also considered as risk factors for pSS development. Such changes may occur in pSS in peripheral blood cells, serum, and saliva (24). These processes are reversible and represent a potential target for new therapies being developed (25).

The estrogen imbalance is considered as a hormonal risk factor in pSS. In genetically predisposed individuals, the decrease or lack of estrogens may lead to autoimmunity development and dry eye syndrome and may result in a more severe course of the disease—which was confirmed both in animal studies and in clinical observations (26, 27).

Principles of primary Sjögren's syndrome diagnosis

The current classification criteria for pSS, employed in the diagnosis of this disease, were published in 2016 (5). The features that are most important for pSS diagnosis include confirmation of dryness of eyes/mouth, presence of the SS-A/Ro antibodies, and presence of typical infiltrates consisting of mononuclear cells in the assessment of histopathological material from labial minor salivary gland biopsy (MSGB). The latter must meet the so-called focus score (at least one focus present with a minimum of 50 mononuclear cells in a 4-mm^2 biopsy section). The patient must also be checked by a clinician for the presence of exclusion criteria, which include irradiation of the head and neck, active HCV infection, diagnosis of IgG4-related disease (IgG-RD), and presence of lymphoma, making the diagnosis impossible. In Table 1, the scoring system for the current main pSS classification criteria is presented. According to the classification criteria, pSS diagnosis requires a total score of ≥4 points (5, 28).

Viral infections and pSS—potential mechanisms of virus influence on pSS development

Infections, especially viral ones, play a crucial role in pSS pathophysiology. Several mechanisms found to be important for the effect of viruses on pSS are presented below.

Molecular mimicry

Molecular mimicry is indicated as the mechanism of adaptation by a pathogen to avoid immune detection by mimicking a host antigen for which immune tolerance exists. It facilitates virus penetration into the host's cells, utilizing the host's immune tolerance to self-antigens (29). An example of molecular mimicry is the sequence homology (87%) between the 222–229 region of the major linear B cell epitope of Ro60 kD

TABLE 1 Main items and scoring in the recent pSS classification criteria (acc. 5).

Parameter	Scoring (points)	Comment
Schirmer's test ≤5 mm/5 min in at least one eye	1	Without using artificial saliva
or	1	Using lissamine green and fluorescein staining
ocular staining score (OSS) ≥5	1	Lissamine green or Bengal rose staining
or		
van Bijsterveld's score ≥4) in at least one eye		
Unstimulated salivary flow ≤0.1 mL/min	1	Without using artificial saliva
Presence of anti-SSA/Ro antibodies	3	Without division into Ro52 and Ro60
Focus score ≥1 foci/4 mm2	3	Important representative

autoantigen (pep216-232) and Coxsackie virus 2B protein (pepCoxs). In the Stathopoulou study (30), a possible cross-reaction in pSS patients between the antibodies to the Ro60 kD autoantigen and the homologous pepCoxs was suggested. Such a close homology may, on one hand, cause the tolerance for the "aggressor", but on the other hand, it may lead to the breakdown of self-tolerance and the formation of antibodies that also react with the host's own antigens (30).

We distinguish a direct structural mimicry relying on the similarity of antigens and causing a reaction against self-antigens (31), a functional mimicry, where different antigens share common surface topologies with respect to a shape or chemical structure, as well as where the flexible paratope accommodates dissimilar antigens by adjusting the structural features according to antigenic epitopes reflecting functional equivalence in the absence of structural correlation (32), and a genetic mimicry, where viral genes are functional and structural homologs of important genes of the immune response (33).

The achieved similarity of the pathogen to the host may elicit an immune response against the antigenic determinants present in both the host and the pathogen. The consequence of the autoimmune response is the activation of autoreactive T or B cells and the destruction of host cells and tissues by the host's own effector mechanisms, such as the system of phagocytic cells and/or the complement system.

Table 2 shows examples of the similarity of viral and human antigens and the potential diseases associated therewith.

Bystander activation

Bystander activation is an interesting problem of viruses inducing a non-specific autoimmune response, which results in the creation of an inflammatory environment with cytokine and chemokine production. In susceptible—genetically predisposed—subjects, viruses activate antigen-presenting cells (APC), especially DC, and auto-reactive naïve T cells, which may, in turn, stimulate different immune cells for migration to the infected area (34). During infection, naïve CD8+ cells release tumor necrosis factor (TNF) and IFNγ and produce cytotoxins (perforins and granzymes), in this way inducing apoptosis. Another way leading to the destruction of infected cells is Fas/FasL interaction. FasL protein, which is a type II transmembrane protein that belongs to the TNF family, is expressed on the activated CD8+ T cell surface. Its binding to the Fas receptor (also known as tumor necrosis factor receptor superfamily member 6—TNFRSF6) on the target cell surface is a signal for the caspases' cascade activation and cell death (34, 35). As a side effect of bystander activation, damage to healthy cells occurs, with a release of self-antigens and the activation of autoimmune reactions. In a healthy, not genetically predisposed, organism, such situation is self-limiting. However, in the case of a dysfunction of the immune system (e.g., immunosuppression, defects of the immune system, and persistent infection), the eradication of immune cells targeting self-antigens—as a prevention of self-antigen antibody production—becomes inefficient.

Epitope spreading and cryptic antigens

Tissue damage, oxidative stress, and cell death lead to the release of antigens that can become targets for the organism's own immune system. In inflammation, APC cells can process the released epitopes in such way that, instead of being sequestered, they become immunogenic—leading to the breakdown of self-tolerance and the activity of self-reactive

TABLE 2 Examples of molecular mimicry between viral and human antigens (31–33).

Viral antigen	Human antigen	Associated rheumatic disease or autoimmune disease	Organ/systems affected
Epstein–Barr EBV nuclear antigen (protein)-EBNA	DNA, RNP ribonucleoprotein antigen Ro and La antigens in the nuclear and cytoplasmic ribonucleins	Sjogren syndrome Systemic lupus erythematosus	Skin, mucous and serous membranes, joints, kidneys, central system nervous, blood, exocrine glands
EBV Epstein–Barr nuclear protein 1	Epidermal keratin, collagen type II, actin	Rheumatoid arthritis	joints structures
EBV virus polymerase	Human basic myelin protein	Sclerosis multiplex	Central nervous system
Encephalomyocarditis virus Coxsackievirus B3 (VP1 protein)	Histidyl tRNA synthetase Myosin, tropomyosin, vimentin	Polymyositis myocarditis	Muscles
Epstein–Barr nuclear antigen (EBNA1)	ribonucleoproteins Ro (SSA)	Sjogren's syndrome	Exocrine glands Epithelium
Viral EBER-1 and EBER-2	Anti SS-B/La antibodies	Sjogren's syndrome	Exocrine glands (salivary glands) Epithelium

Tlymphocytes. This phenomenon is based on the epitope-specific immune response directed against foreign or self-proteins/antigens, which may be spreading to subdominant or even cryptic epitopes of those proteins/antigens (1, 36). In the course of a viral infection, more self-antigens may be released, triggering the *de novo* activation of auto-reactive cells, which also target other epitopes.

Superantigen theory

The role of superantigens (SAGs) in the mechanism of autoimmunity is controversial. The SAGs are proteins produced mainly by bacteria but may also be produced by virus-infected cells. Superantigens can bind to the TCR receptor regardless of their specificity, causing the activation of T cells (37). Viral superantigens may activate non-specifically to any particular epitope immune response with the activation of a large number of T cells, which produce IFNγ, and with the stimulation of B cells. In the 1990s, the B cell-stimulating SAGs were already discovered, able to activate B cells without cross-talk with T cells. Recently, these mechanisms have been better understood, mainly through the analysis of the influence of bacterial infections (38).

In the context of a discussion about the role of autoimmune processes and immune defense in pSS pathogenesis, the impact of SAG should be considered as a potential source of the direct activation of receptors and a possible factor in the hyperstimulation of the immune system. Although the role of SAGs cannot be unequivocally considered as a source of the autoimmune process, this mechanism of inducing an immune reaction should not be omitted when discussing the role of infections in pSS (39, 40).

The influence of some superantigens (proteins, toxins, etc.) should be taken into account in the search for therapeutic and diagnostic options based on the use of protein engineering methods (41).

In Figure 1, an outline of the possible action of viruses in the pathogenesis of pSS is presented.

Viral cytokines

Viruses can influence the functioning of the host immune system to their advantage by the expression of viral cytokines (virokines), chemokines, and proteins similar in structure to those of the host. Viral cytokines help to create progeny virions and increase the chance of survival of the virus in the host organism (37). Brown et al. (42) first discovered in 1985 that the 140-residue polypeptide encoded by one of the early genes of vaccinia virus (VAV) is closely related to the human epidermal growth factor (EGF) and type I transforming growth factor (TGF). The researchers pointed out that the production of EGF-like growth factor (viral-EGF or vEGF) by virus-infected cells may be the cause of proliferative diseases caused by this family of Poxviruses, such as e.g., rabbit (shope) fibroma virus, Yaba (monkey) tumor virus, and molluscum contagiosum virus. This topic has been explored over the years, especially since the VAV virus belongs to the same family as the smallpox virus. The effect of vEGF inhibition on the migration of infected cells

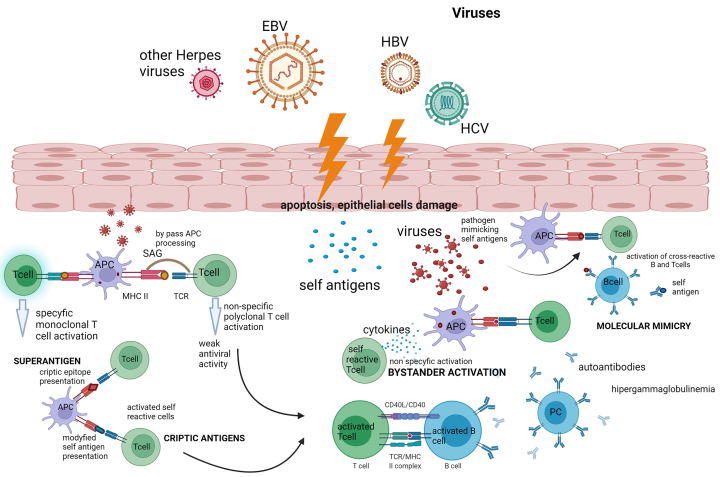

FIGURE 1
Outline of the possible action of viruses in the pathogenesis of pSS. The presented diagram shows the possible paths of activation of the autoimmune process by viruses such as bystander activation and molecular mimicry and the action of the virus as a superantigen—which are described in full in the text of the article. Created with BioRender.com. APC, antigen-presenting cell; EBV, Epstein–Barr virus; HCV, hepatitis C virus; HBV, hepatitis C virus; PC, plasmatic cell; SAG, superantigen.

was investigated as a method for preventing the spread of the virus (43). Although a number of viral cytokines, chemokines, receptors, and binding proteins (among others, vEGF, vβ-NGF, vTNFR, and vCXC1) have been identified over the years, their significance for autoimmune diseases and autoimmunity is still debated (37).

Among cytokines attributed to viruses, the role of vIL-10 has been implicated in pSS pathogenesis. As for other cytokines, such as IL-6 or IL-17, involved in the course of viral infections, there is currently insufficient data on the role that their viral equivalents play in the course of pSS.

IL-10 is an immunomodulatory cytokine that inhibits the immune response and the activity of proinflammatory cytokines. The homolog of this cytokine, IL-10- (v), is produced as a result of EBV activity, enabling EBV to escape the host's response and avoid elimination through inflammation (37, 44). It has been shown that this cytokine can be produced as a result of the activity of other viruses, such as cytomegalovirus. IL-10 (v) sequences are slightly different (two introns) from human IL-10, but IL-10 (v) still possesses immunosuppressive properties (45).

Zhu et al. (46) showed, using a mouse model of Sjogren's syndrome, the activity of adenovirus-mediated vIL-10 gene transduction (vIL-10 encoded by EBV) in tears and the impact of IL-10 on the clinical features of dry eye syndrome and the lymphocytic infiltration associated with the experimental autoimmune lacrimal gland inflammation.

Viral interleukin-6 (vIL-6) encoded by human herpes virus 8 (HHV8) is only partially identical to the human pro-inflammatory cytokine IL-6 (25% of homology). HHV8 activity is detected, *e.g.*, in patients with multifocal Castleman's disease, Kaposi's sarcoma, and HIV infection (47). There are studies showing the role of IL-6 in pSS, revealing the increased levels of IL-6, IL-17A, and nitrogen oxide in the serum and saliva of pSS patients compared to the healthy control group (48) and the correlation of IL-6 levels with the extent of mononuclear cell infiltration in salivary and lacrimal glands. Fujimura et al. (49) showed that IL-6 induces REG Iα (regenerating gene) transcription in salivary ductal cells through the activation of signal transduction and activation of transcription 3 (STAT3) and binding to the consensus sequence of REG Iα promoter in ductal epithelial cells. The authors concluded that the IL-6/STAT pathway may play a role in the pathogenesis of pSS. Although it can be assumed that viral IL-6 may also potentially play a role in autoimmune diseases, not only in the context of HHV8 infection and Kaposi sarcoma, there are no certain data on vIL-6 in pSS.

The role of the virus homolog of IL-17 (vIL-17) is not yet fully understood. It has been found to be encoded by herpesvirus saimiri (HVS13) and has affinity to T cells. vIL-17 possibly has a beneficial effect on virus survival (50). Interleukin-17 itself is a cytokine that can stimulate T cell proliferation and the secretion of pro-inflammatory cytokine-IL-6, granulocyte colony-stimulating factor, TNF, and chemokines (the chemokine C-X-C motif ligand) such as CXCL1, CXCL2, and CCL20 as well as acute-phase reactants—therefore promoting inflammation (50, 51). Studies in patients with pSS have shown the presence of IL17 and its activating cytokine IL-23 in the lymphocytic infiltrates of the exocrine glands and increased levels of circulating IL17 in the serum and saliva of these patients (52). Knowing the influence of IL-17 on pSS progression and some clinical features of this disease, it can be assumed that vIL-17 may also play a role in both pSS and SS-like disease development.

To sum up, viral cytokine-induced inflammation may promote immune dysregulation or intensify the activity of autoreactive lymphocytes, but there are currently no studies clearly demonstrating the causal role of viral cytokines in autoimmunity.

The role of Herpesviride

Herpesviruses (HV) constitute a virus family infecting mammals (including humans), birds, reptiles, fish, and oysters. The human herpes viruses are identified in the current taxonomy with symbols numbered from HHV1 to HHV-8; some of them are allocated to subfamilies (α, β, and γ) (45). Humans play the role of a host for herpes simplex virus types 1 and 2 (HSV-1 and 2) and varicella-zoster virus (VZV; HHV-3)—which belong to the α-HV subfamily, cytomegalovirus (CMV; HHV-5), human herpes virus 6 and 7 (HHV-6 and HHV-7)—which belong to β-HV subfamily and Epstein–Barr virus (HHV-4) and Kaposi sarcoma herpes virus (HHV-8)—from the γ-HV subfamily.

Viruses from this family, *e.g.*, EBV, CMV, or VZV, may stay in a latent phase and reactivate. Although the role of reactivation or increased antibody levels is not explicitly causal for pSS, those phenomena take part in creating immunologic differences between pSS patients and controls (53).

Epstein–Barr virus

The Epstein–Barr virus (HHV-4) is a DNA virus primarily attacking human B cells, presenting predilection to the salivary glands. It is known that EBV binds to CD21 receptor on the surface of B cells through the viral envelope glycoprotein gp350, while glycoprotein gp42 binds to a human leukocyte antigen as a co-receptor (54, 55). However, other cells without CD21 expression, such as epithelial cells, may also be infected by EBV. Epstein–Barr virus was found in some T cells in T cell lymphoma as well as in epithelial cells in nasopharyngeal and gastric carcinomas (56). It has been shown that, in case of asymptomatic virus carriers and in chronic infection, EBV is mainly confined to B lymphocytes, although it has been suggested that epithelial cells may be the site of EBV

replication and amplification rather than the location of the virus in its latent form (57). The establishment of sites of EBV activity is still a subject of research.

It was suggested that, in the pathogenesis of pSS, EBV infection plays an important role in the development of the autoimmune process, which leads to B cell hyperreactivity and may result in the immortalization of B cells. Immortalization is one of the mechanisms that allow EBV to survive in an infected cell and avoid elimination by inhibiting the apoptosis of these cells (58). *In vitro* studies confirmed that peripheral blood resting B cells are transformed by EBV to proliferating B-lymphoblastoid cell lines (LCLs). This is possible thanks to viral proteins, especially EBNA-2. These proteins contribute to the continuous proliferation of B lymphocytes arrested in the lymphoblastoid phase of differentiation and block the transition of the virus to the lytic cycle in most infected cells (4). These LCLs were initially considered as immortal cells that do not become tumorigenic, but it was shown that LCLs are, in fact, mortal (as cells with normal diploid karyotypes and shortening telomeres) (59). Some LCLs, however, become really immortalized because of a strong telomerase activity, aneuploidy, downregulation and mutation of some genes, and modulation of apoptosis. The immortalization may develop into the tumorigenesis of LCLs (60).

Epstein–Barr virus infection status was studied in a large group of patients with multiple sclerosis (MS), which is an immunologically mediated demyelinating disease affecting the central nervous system. Bjornevik et al. (61) investigated 955 subjects (military personnel) diagnosed with MS during their period of service, and 801 MS cases were available to assess EBV infection status; 1,566 controls were also tested. Over the course of 10 years, the researchers collected three samples from each subject for the analysis of EBV infection status, and ultimately only one of the MS-diagnosed subjects remained uninfected, while 35 of them seroconverted from a negative to a positive result before the onset of MS. This study revealed a high rate of seroconversion in the MS group (97%) in comparison with non-MS subjects (57%). There was no confirmation of such a strong association for a similarly transmitted cytomegalovirus (62). Attempts were made to associate an EBV infection with the risk of MS development, but no such association was found. The results of these studies may hold important clues for further research on the effects of EBV on the development of autoimmune diseases.

A persistent EBV infection may cause damage to the salivary glands, resulting in symptoms of dryness. EBV can persist in the human organism in a latent form, allowing the virus to hide in the host cell and reactivate under favorable conditions. In order to survive unnoticed, the virus uses various methods to hide its markers, so that it escapes the control of the host's immune system. Thanks to the discussed phenomenon of molecular mimicry (see Table 2), production of viral IL-10, T cell costimulatory gene CD70 overexpression, or impaired EBV-specific T cell response, EBV modulates host response as well as stimulates autoantibody production. Interestingly, EBV infection can exist in different cells both in the lytic and the latent forms simultaneously (63).

The detection of antibodies against specific virus proteins and the PCR confirmation of the presence of EBV-DNA may be employed by clinicians to establish the status of EBV infection. The more routine methods of determining the infection status include detection of antibodies against nuclear antigen-1 (EBNA-1), early antigen (EA), viral capsid antigen (VCA-IgM and VCA-IgG), noncoding RNA protein (EBERs) and, in certain indications—mainly in patients after transplantation with a suspected post-transplant lymphoproliferative disease (PTLD)—a PCR testing for the presence of EBV-DNA.

Depending on the stage of infection, the lab test results differ. In a recent infection, antibodies against EA, VCA-IgG, and VCA-IgM can be found, and the PCR test for the presence of EBV-DNA is positive. In the case of infection with reactivation, antibodies against EBNA-1, EA, VCA (IgM and IgG classes of immunoglobulin) can be detected. The past infection results in the presence of VCA-IgG (+) with a less probable presence of VCA-IgM (+/-) and EBNA-1 IgG (+/-). In post-transplant lymphoproliferative disease, PCR EBV-DNA is positive and EBER antibodies can be detected, with the presence of other antibodies being less likely (44).

Epstein–Barr virus not only promotes the development of autoimmunity but may also lead to the development of malignancies, especially of lymphoma (55). The higher incidence of EBV reactivation in pSS patients and the expression of HLADR antigens on salivary epithelial cells in this group as well as the increased levels of EBV antigens in infiltrating lymphocytes lead to the conclusion that pSS development is influenced by EBV. Several studies revealed that circulating B cells harboring EBV were present in pSS patients (64). Other evidence came from studies of the saliva of pSS patients, in which it was proved that it activates target genes for AhR (aryl hydrocarbon receptor) and BZLF1 (trans-activator protein—an EBV protein which takes part in switching of the infection phase from latent to lytic). CYP1A1 (the first gene to be transcribed during EBV replication) and Zp130 (synthetic peptide related to ZEBRA—Z Epstein–Barr replication activator) genes were also activated by the pSS saliva (65, 66). A correlation between the levels of anti-La/SSB in the sera and AhR activity in the saliva of SS patients was also found (66).

Cytomegalovirus

Cytomegalovirus (HHV-5 or CMV) is a common virus (infecting 45–99% of the adult population) usually responsible for mild flu-like or mononucleosis-like symptoms in the general population (67). However, in immunocompromised subjects, it

may lead to autoimmune disorders and even the occurrence of severe complications, such as pneumonia, retinitis, hepatitis, meningitis, or gastroenteritis (68, 69).

Following primary exposure, CMV has the ability to establish lifelong presence and latent infection (remains in CD34+ myeloid progenitors). Many cells possess receptors for the virus, one of them being the epidermal growth factor receptor (EGFR), which is important for binding, signaling, and host cell entry (70, 71). The analysis of the effect of CMV in pSS patients is difficult, given its very high prevalence. Infection by CMV emerges as a clinical problem mainly at the time of immunosuppression when CMV may become reactivated, and the course of the infection may be severe. Takizawa et al. (72) presented their work on a large group of patients with rheumatic diseases (RD; n = 7,377) who were examined for the development of CMV infection. The infection was confirmed in 151 RD patients, of whom most had a SLE diagnosis (n = 74), while only 2.7% (n = 4) had a pSS diagnosis. The authors concluded that patients with SLE, dermatomyositis, and microscopic polyangiitis were more prone to CMV disease than patients with other RDs. As the above-mentioned data shows, the role of CMV in the development of pSS itself is not fully understood. The reactivation and the symptoms of CMV infection in pSS patients may be associated with the use of more or less aggressive immunosuppression, as demonstrated by Takizawa et al. in their research (72).

The salivary glands are also a target for CMV, with parotiditis and sialadenitis being described in the course of this viral infection. The saliva of infected subjects is the source of the virus material. Symptoms suggesting pSS may then occur; however, the classification criteria for pSS are not met.

Varicella-zoster virus

Primary Sjogren's syndrome as well as other autoimmune diseases are considered as risk factors for the reactivation of varicella-zoster virus and for the occurrence of shingles symptoms in comparison to the general population, which was confirmed in several studies (73). In VZV infection, the varicella-autoantibody syndrome was described (74). The transient presence of antiphospholipid and coagulation protein autoantibodies in VZV infection in children was observed, but the influence of this phenomenon on thrombotic complications was not confirmed.

In the study of Chakarvarty et al. (75), the odds ratio for VZV infection was 1.03 (95% CI: 1.02–1.05) for each year of age, and the risk of VZV infection was at 1.8 (95% CI: 1.2–2.8) for pSS and at 2.7 (1.7–4.3) for SLE patients. In the group of SLE patients, Ro positivity was predictive of an earlier onset of VZV, which, however, was not observed in the group of pSS patients. The authors concluded that, in pSS, there is an increased risk of reactivation of VZV infection compared to healthy individuals but less frequently than in SLE or in the group of elderly patients. Anti-Ro seropositivity, but not a concentration of the studied cytokines (bLyS, IFN alpha and gamma, IP-10, CxCL-13, E-selectin, and MIP-1 beta), was associated with the development of VZV infection in both groups.

Roseoloviruses

Roseoloviruses also belong to the Herpesviride family and affect more than 90% of the general population. This data applies to the seropositivity or latent infection phase, as a majority of affected subjects are asymptomatic (76). Roseoloviruses are lymphotropic beta-herpes viruses. Human roseoloviruses include human herpes virus-6 (HHV-6A and HHV-6B) and human herpes virus- 7 (HHV-7). The variant of HHV-6 (HHV-B) is more frequent than HHV-7. These viruses mostly affect children and cause known diseases such as febrile infant infections, sometimes with rash (roseola infantum). Infections with those viruses are self-limiting, but viruses can stay in both active and latent forms. The infection can persist for a whole life. Viruses may replicate in peripheral blood mononuclear cells. These viruses may also locate in the salivary glands, and viral DNA can be detected in the saliva by the use of PCR (77). It has been proven that the reactivation of an infection with roseoloviruses may occur in an immunocompromised person. The link between central nervous system diseases such as multiple sclerosis and encephalitis leads to this genus of viruses being intensively studied (78, 79).

Ranger-Rogez et al. (80) indicated in their study higher titers of HHV-6 antibodies in the group of pSS patients than in healthy subjects. The study on RA patients with or without SS revealed that, in both groups, there was a significantly increased frequency of latent viral infection (sevenfold higher, p = 0.018 for HHV-6) compared to normal controls—interestingly only in cells isolated from saliva (81). More up-to-date research has been done on SLE, concluding that HHV-6 infection may contribute to the development of this autoimmune disease, but it is also noted that the autoimmune disease may cause the reactivation of human roseoloviruses (82). The research performed on animal models, mainly non-primates, e.g., macaques or marmosets, tried to establish the relationship between the development of AIDS or neurological diseases and an infection with roseoloviruses. Currently, mouse models, such as mice, naturally resistant to herpes virus infection or transgenic mouse models will allow for a better understanding of the role of HHV-6 infection in humans (83). In the work of Bigley et al. (84), it has been shown in a mouse model that a neonatal infection with murine roseolovirus related to human roseoloviruses causes autoimmune gastritis in adult subjects, the production of a number of autoantibodies, and an increase of thymocyte apoptosis at the negative selection stage. This is the first study which provides direct evidence that a roseolovirus

infection can induce autoimmunity and the production of pSS-associated autoantibodies (anti-SSA/Ro and anti-SSB/La).

Undoubtedly, this topic has not been exhausted, and the role of roseoloviruses in pSS should be re-investigated and described in more detail.

Retroviruses

Viruses, which were later assigned to the Retroviridae family, have been the subject of research since the beginning of the 20th century. In the 1970s, the attention of clinicians in the southern islands of Japan and in the USA was drawn to the emergence of numerous leukemia cases from mature T lymphocytes, and research led to the isolation of the human T cell leukemia virus (HTLV-I) (85, 86). In 1982, another virus which causes hairy cell leukemia—HTLV-II with 70% genomic homology to HTLV-1—was identified (87). The discovery of the viral reverse transcriptase (RTs) which has DNA polymerase and rnase H activity (88) was crucial for the understanding of the mechanisms of replication of retroviruses. In 1983, human immunodeficiency virus (HIV) was isolated in 1983 by a group of researchers under the direction of Luc Montagnier (89), who based their work on the previous research of Robert C. Gallo on HTLV-1 (90).

Human T lymphotropic virus type 1

Although the prevalence of HTLV-1 virus in some parts of the world is high, only about 5% of people develop symptomatic infection (91). Adult T cell leukemia (ATL) and HTLV-1-associated myelopathy (HAM) have been directly associated with HTLV-1 infection.

Studies conducted on transgenic mice have proved that subjects infected with retroviruses develop autoimmune diseases such as Sjögren's syndrome, polymyositis, or rheumatoid arthritis more often than the general population (92). The HTLV-1 virus equipped with the tax and bZIP gene (HBZ) infects salivary gland cells, leading to the increase of the concentration levels of inflammatory factors, e.g., ICAM-1, IP-10-, and chemokines, such as RANTES (regulated on activation, normal T cell expressed and secreted) (93). In the course of HTLV-I infection, B cells are inhibited, and the production of autoreactive antibodies is reduced (94).

Greene at al (95). confirmed the involvement of the lacrimal and salivary glands resembling Sjögren's syndrome in HTLV-1 tax transgenic mice. Mariette et al. (96) also described the presence of the tax retrovirus HTLV-1 gene in the salivary gland cells of patients with pSS. Nakamura et al. (97) noticed that, in patients with pSS and HAM, the titer of SSA/Ro antibodies was significantly lower compared to patients with pSS without HTLV-1 infection despite the lacrimal gland involvement being more severe in this group. An interesting observation was that the degree of mononuclear cell infiltration, assessed as focus score in the biopsy of the MSGB in both groups, was similar (98).

Terada et al. (99) pointed out that, among seropositive patients with pSS, antibodies against HTLV-1 in the IgA class are commonly found in saliva. In a subsequent study, the authors noted that, in HTLV-1-positive patients with pSS, there is a lesser destruction of the salivary glands than in HTLV-1-negative patients (94). Infection with HTLV-1 may inhibit cell apoptosis and enhance proliferating signals, which may explain the less intense destruction (100).

Magnetic resonance imaging of salivary glands in the group of patients with HTLV-1-associated myelopathy did not show typical changes for pSS, but the salivary flow rate was similar in both groups (101). In Brazil, 129 HTLV-1-seropositive patients were screened for meeting the criteria for pSS. Many of them had clinical symptoms of eye and mouth dryness (46 cases of dry mouth, 18 dry eyes, eight with confirmed decreased saliva secretion, and two with salivation disorders), but only one patient had antibodies characteristic for pSS (102). A biopsy of the minor salivary glands was performed in six patients of that group, revealing infiltrates with mononuclear cells characteristic for pSS (103). Analyzing the above-mentioned data, it seems that the HTLV-1 virus can act in two ways: in some patients, it stimulates an autoreactive response and causes the development of full-blown pSS, but it can also induce a non-specific inflammation of the salivary glands as manifested by excessive dryness. Moreover, in HTLV-1-positive patients, a smaller number of ectopic GC and a low expression of CXCL13 in mononuclear cells infiltrating the glands are observed compared to pSS patients without HTLV-1 infection (98).

Similar with other retroviruses, HTLV-1 primarily has an affinity to TCD4 + lymphocytes but can also infect other cells, e.g., salivary duct epithelial cells, which attract T lymphocytes in patients with pSS by secreting interferon-induced 10-kD protein (CXCL10) and monokines (CXCL9) (103). It is suggested that the change of the inflammatory environment in the epithelial cells of the salivary gland ducts and the significant transmigration potential of CD4+ T cells infected with HTLV-1 retrovirus may lead to the development of pSS in these patients. Recent studies have also focused on the expression of TCR3 receptor binding to viral RNA on salivary gland epithelial cells (103). The relationship between pSS and HTLV-1 may be further illustrated by studies on the role of HTLV-1 basic leucine zipper (HBZ) gene expression. An increased expression of this gene and induction of the chronic inflammatory process through Foxp3 were shown in the salivary gland tissue of patients with both ATL and Sjögren's syndrome. This may indicate the connection of these diseases (103).

In summary, it seems that HTLV-1 virus, by infecting salivary gland epithelial cells, changes their cellular functions, which may induce the development of pSS as demonstrated by Nakamura et al. (103). Lee et al. (104) confirmed HTLV-1

presence in the labial salivary glands of patients with pSS and suggested that it can be a special clinical subgroup.

Human immunodeficiency virus

The human immunodeficiency virus has an affinity to CD4+ cells but also affects macrophages and dendritic cells (105). The viral envelope glycoprotein gp120 is recognized by CD4+ molecule, which has a greater affinity for it than for its dedicated ligand, the MHCII molecule. The virus combines its genetic material with the material of the cell and goes into the latent phase. Even in the latent state, it maintains a high level of replication in the lymph nodes and lymphoid cells of the digestive system (106).

Subsequent studies have tried to explain the mechanisms of autoimmune disorders caused by HIV. The destruction of CD4+ cells and the activation of autoantigens and cytotoxic cells were taken into account. The phenomenon of molecular mimicry with the gp41 antigen and polyclonal activation of B lymphocytes has also been described (107). For many years, it has been pointed out that, in a population of HIV-positive patients, there is an increased frequency of symptoms that may correspond to pSS. In the study by Kordosis et al. (108) published in the 1990s, it was noted that, among 77 HIV-positive patients, 26 had symptoms of dry eyes and/or mouth. Furthermore, 14 patients from this group had MSGB performed, among which six had monocellular infiltrates meeting the criteria of focus score in pSS and four had mucoid degeneration of the stroma. However, in the immunohistochemistry assessment, CD8+ cells dominated in the infiltrate in contrast to pSS, where mainly CD4+ are observed. Interestingly, none of the studied patients had anti-SSA/Ro or SSB/La antibodies, but all of them had hypergammaglobulinemia (108). Many years of observations have led to the identification of a disease entity in HIV-infected patients called diffuse infiltrative lymphocytosis syndrome (DILS) with a clinical picture similar to Sjögren's syndrome. The criteria of DILS diagnosis, established in 1995, are as follows: a confirmation of HIV infection (positive serology), bilateral salivary gland enlargement or xerostomia, persistence of symptoms for 6 months or more, and histologic confirmation of salivary or lacrimal gland lymphocytic infiltration without granulomatosis or neoplastic involvement (fulfillment of all criteria is required to make a diagnosis) (109). In the course of DILS development, symptoms such as bilateral painless parotid and lachrymal gland enlargement—sometimes even with severe sicca symptoms—as well as lymphocytic interstitial pneumonitis, hepatitis, myositis, lymphadenopathy, polyneuropathy, and aseptic meningitis may occur. The main immunological feature of DILS is the proliferation of CD8+ T cells, with infiltrations containing these cells affecting multiple organs. DILS is suggested to reflect an over-response to HIV by the host. During HIV infection, marginal zone B cell MALT lymphomas were described, which also resemble pSS and confirm the possibility of a chronic stimulation of these cells by an infection, leading to the loss of control of multiplication and to lymphoproliferation. It was also shown that subjects with dryness syndrome and HIV have higher serum levels of interferon gamma (110).

Interferon gamma (IFNγ) plays an important role in antiviral defense. Its production clearly grows in the early and acute phase of HIV infection, and in the later stages of the disease, IFNγ, together with other inflammatory cytokines, is involved in persistent immune activation (111). The activity of IFNγ exacerbates the effects of HIV and plays a role in AIDS development. Interferon gamma and type I IFNs may also promote the IFN signature by the upregulation of IFN response genes (IRGs) in pSS (111).

HIV infection can be seen mainly as an element in the differential diagnosis of Sjogren's syndrome—especially now, thanks to highly active antiretroviral therapy. As HIV infection has become a chronic disease and its mortality has significantly decreased, it can be encountered more often in medical practice. The prevalence of DILS currently is significantly reduced (110).

The hepatitis viruses

Hepatitis C virus

Hepatitis C virus is a small single-stranded RNA virus, a member of the hepacivirus genus in the Flaviviridae family. This virus is a recognized cause of hepatitis, a risk factor for cirrhosis, liver tumor, and other cancers as well as lymphoma development. The hepatitis C virus presents not only tropism to hepatic cells but also sialo- and lymphotropism (112). For decades, there was an ongoing discussion on whether HCV infection is a cause of pSS or only a mimicker of this disease due to the symptoms of dryness, arthralgias, and other symptoms similar to the clinical picture of pSS (113). Hepatitis C virus infection, especially the chronic one, is associated with extrahepatic disease manifestations, stimulation of B-cell activity by BAFF, and autoimmune phenomena, including emerging cryoglobulins and immune complexes, leading to vasculitis, polyneuropathy, lymphoproliferation, and, as recently suggested, endocrine manifestations such as thyroid autoimmunity or diabetes mellitus type 2. Among autoantibodies, ANA and RFs are quite common, but others, e.g., anti-GM1 ganglioside and anti-sulfatide, may also be observed (114). Virus *per se*, especially in a chronic infection, may be a cause of focal sialoadenitis, with usual mild dryness symptoms (even in 50% cases) and inflammatory infiltration of mononuclear cells in the histopathological assessment being observed (115).

In the context of the impact of HCV on the development of pSS, the available meta-analyses and individual studies focus on the population of patients with the presence of HCV-RNA and HCV antibodies, and the authors indicate an SS-like disease more often than pSS (113). In both diseases, pSS and chronic

HCV infection, there is an increased risk of lymphoma (especially B cell lymphomas) development with the predominance of changes in the target organs—salivary glands in pSS and salivary glands and liver in HCV (114). Dryness symptoms and sialadenitis associated with hepatitis C infection are also linked to the increased risk of lymphoma development similar to that in pSS (115). To conclude, the immunological activity of HCV virus, its tropism to the salivary glands, and the possibility of induction of a pSS-like disease hinder the diagnostic process of patients with suspected pSS. The influence of a previous infection of HCV on the development of pSS has been discussed for years; it has even been suggested to be treated as a subtype of pSS (113). Eventually, HCV viremia and active infection were established as the exclusion criteria in pSS diagnosis.

An active HCV infection may be a multisymptomatic and multisystem disease with chronic fatigue, arthralgia, general symptoms (such as fever, weight loss, and loss of appetite), skin changes, such as Raynaud's phenomenon, livedo reticularis, or purpura (the latter most often associated with vasculitis in the course of cryoglobulinemia). Symptoms related to the gastrointestinal tract, resulting directly from the damage to the hepatic cells, do not have to be dominant or evidently present at the beginning of an HCV infection. The involvement of the salivary glands, with their enlargement and dryness, may also be a symptom of an HCV infection due to the predilection of this virus to lachrymal and salivary glands epithelial cells—which was confirmed by the detection of HCV-RNA in infected patients' saliva. In the course of HCV, dryness is usually mild. Ramos-Casals (116) proposed the term "SS secondary to HCV", but it seems inadequate, as in the case of HCV we are dealing with a wide range of phenomena, which depend on the stage of the infection (active, persistent, or past infection) and vary from pSS-like symptoms mimicking or accompanying pSS to the stimulation of the immune process, which may, in fact, play a role in pSS development.

Dengue virus

According to Chang et al. (117), an infection with a dengue virus (DV), another virus from the Flaviviridae family, is lowering the risk of pSS development. The authors presented results demonstrating that the incidence rate of pSS was lower in the DV cohort than in the non-DV cohort (0.51 vs. 1.47), with HR of 0.30 (95% CI: 0.13– 0.67), matching age, gender, and residence (117).

Hepatitis B virus

The role of HBV infection in the pathogenesis of Sjogren's syndrome seems quite unclear. In Spain, Marcos et al. (118) conducted studies on a large population of patients with Sjögren's syndrome. The study focused on the prevalence of HBV infection in this population and the clinical and laboratory evaluation of these patients. The presence of HBsAg was detected in five out of 603 patients with Sjogren's syndrome. Hepatitis B virus and HCV co-infection were not found. The main symptoms observed in the group with HBV infection were dryness and arthralgia or arthritis; the dominant positive laboratory tests were RF and ANA. However, cryoglobulinemia was not observed in contrast to HCV infection and pSS, where the presence of cryoglobulins is not rare. The authors stated in conclusion that the incidence of HBV infection among patients with pSS was found to be similar with that of the general population.

Similar research in Taiwan was carried out by Chen et al. (119), and their observations also confirmed higher RF concentrations in patients with the presence of HBsAg. Additionally, attention was drawn to a lower lung involvement rate in comparison with other pSS patients (119).

Another study conducted with a large group of pSS patients, including over 9,500 subjects, assessed the prevalence of hepatitis B and C infections in this group. It was estimated that, in the group with chronic HBV infection, the risk of developing Sjogren's syndrome is much lower (OR 1.25, 95%CI = 0.95–1.24) than in the case of HCV infection, which is associated with the risk of SS (OR = 2.49, 95% CI = 2.16–2.86) (120).

Ram et al. (120) suggested that HBV infection may even play a protective role in relation w autoimmune diseases. In another study, Ramos-Casals et al. (121) investigated the frequency and symptoms of liver involvement in 475 patients with pSS. Among 129 patients with liver involvement, only one had a confirmed HBV infection. The presence of HCV in the cited study was detected much more often, as in other studies (121).

For years, there was a high prevalence of HBV infection in Taiwan, and about 20% of the general population had HBV seroprevalence (the differences were mainly due to age and gender). Therefore, Taiwan became particularly interesting for studying the influence of HBV on the development of other diseases, including autoimmune diseases (122). The compulsory vaccination program has certainly improved the situation in the last 30 years (since 1984). Tung et al. (123) identified 26,147 adults diagnosed with HBV infection and chose 3,268 patients who, at some point, received nucleotide therapy (treated group) and compared them with the second untreated group (n = 13,072) in terms of pSS development. The authors found that the risk of pSS development was significantly lower in the treated group [15-year cumulative incidence, 2.4%; 95% confidence interval (CI), 1.4–3.7%] than in the second group (7.1%; 95% CI, 2.5–15.2%) (p = 0.015). The authors concluded that the obtained data points to the positive impact of the treatment of HBV infection with nucleotides on lowering the risk of Sjögren's syndrome development, which reversely may indicate some role of this viral infection in the pSS pathogenesis (123).

Although there are reports that HBV infection may be a protective factor against the development of SLE, MS, and DB1 (statistical significance in comparison with healthy controls, $p < 0.05$), there is no sufficient evidence to establish the same for pSS (no statistical significance, $n = 82$, 6.1 vs. 10.7%, healthy controls) (120). The authors argue that a low percentage of antibodies to the core antigen (HBcAg) in patients with SLE, MS, and T1D in comparison to healthy controls may suggest that the infection with some viruses, in this case HBV, may have a protective effect (120).

Hepatitis delta virus

Interesting observations were presented by Weller M et al. (124), who showed an increased presence of hepatitis delta virus (HDV) in 50% of the assessed pSS patients. The presence of the HDV antigen was confirmed in the minor salivary gland tissues. Significantly, patients with the presence of HDV in the minor salivary glands did not have detectable hepatitis B virus surface antigen (HBsAg) or antibodies to HBV or HDV. This is interesting, taking into account that HDV is strongly associated with HBV and depends on this virus in terms of release, replication, and transmission. The presence of HDV may be a prognostic factor for the course of this dual infection (125). In the presented results, the expression of HDV antigens was associated with a decrease in stimulated salivary flow, the production of autoantibodies, and the intensification of focal lymphocytic infiltrates in the tissue of minor salivary glands (124).

Hepatitis G virus

The genome of this RNA virus is similar in its organization to HCV virus; the sexual transmission of this virus was suggested and confirmed by some studies on homosexuals, sex workers, and homosexual patients with a coexisting HIV infection (126, 127). This virus infects peripheral blood mononuclear cells, mainly in B and T cells and the bone marrow. The primary replication of HGV in hepatocytes is under discussion as HGV hepatitis usually (75%) proceeds without an increase in liver enzymes (128)

In 1998, Font et al. (129) presented the results of a study on 100 pSS Spanish patients and concluded that, in this population, the prevalence of HGV infection is low (seroprevalence confirmed only 4 versus 3% in blood donor group). In this study, patients with confirmed HGV did not differ from those without infection; in one case, the increase in liver enzymes was most likely due to the coexistence of HCV infection (129). It seems that this virus does not significantly contribute to the development of autoimmune diseases, including pSS, and its co-presence with other viruses may even be a protective factor, as has been noticed in the case of HBV.

Hepatitis E virus

This is an RNA virus not only with hepatotropism but also with extrahepatic manifestations, e.g., in the intestine, kidneys, neurons, and lymph nodes. The seroprevalence of hepatitis E virus (HEV) IgG depends on the geographical region, the assay used, and the study cohort (age, ethnicity, and eating habits, especially a swine meat diet). Infection with this virus is rarely studied and detected in Europe, which may lead to its underestimation. Hartl et al. (130) showed the high anti-HEV IgG seroprevalence in the south of France and the lowest level in Scotland. Some of those infected may not have symptoms or may have mild transient unspecific symptoms, e.g., fatigue, nausea, itching, rare jaundice, and changes in laboratory test, such as elevation of liver enzymes. Some data suggests that the infection with a genotype 3 (GT3) of this virus is associated with extrahepatic manifestations and autoimmune diseases (131). Replication of this virus in the PBMC was confirmed in the acute HEV infection (132). During the HEV infection, a mix of cryoglobulinemia and cryoglobulinemic vasculitis may develop similarly as in the course of HCV. Fraticelli et al. (133) described the case of a pSS patient with cryoglobulinemic vasculitis and nervous system involvement treated with rituximab and mycophenolate mofetil, who developed symptoms of hepatitis. The authors described successive hypotheses concerning the cause of the active hepatitis, including autoimmune hepatitis, and the associated treatment modifications, up to taking into account the HEV infection and, after confirming this, the application of a targeted therapy (133). The conclusion that was drawn from this is that an unexplained hepatitis in immunosuppressed patients treated for systemic autoimmune diseases should be investigated for a possible HEV infection (132). In the current literature, there are studies describing the association of HEV with mixed cryoglobulinemia (134, 135), which may also be important in the context of pSS, but at the moment there are no broader studies on its relation to pSS itself.

Coxsackie

Coxsackie virus is a single-stranded RNA virus which belongs to the Picornaviridae family. Coxsackie viruses are divided into two main groups: Coxsackie A and B. Diseases caused by Coxsackie A are quite common in childhood with mild symptoms or an asymptomatic course such as, e.g., hand, foot, and mouth disease or herpangina. Different serotypes of Coxsackie B viruses can cause muscle, heart, and nervous system infections.

The B4 Coxsackie viruses (CB4) and C1 (CB1) serotypes are being researched as potential risk factors for developing autoimmune diseases. Their role in diabetes mellitus type 1, by showing their destructive activity on beta cells on Langerhans cells and pancreas islets, has been confirmed (136). Triantafyllopoulou et al. (137) demonstrated a relationship between Coxsackie virus

infection and pSS, presenting the evidence of minor salivary gland cell infection (due to the presence of main antigenic capsid protein VP1) in pSS patients and not finding such an infection in patients with secondary SS, controls with other rheumatic diseases without secondary SS and healthy volunteers. However, these data should be carefully analyzed due to the small number of the study groups (pSS: $n = 12$, sSS: $n = 13$, control: $n = 14$ with rheumatic diseases, and $n = 2$ healthy volunteers). A cross-reaction between antibodies to the major epitope of Ro60 kD autoantigen and a homologous peptide of Coxsackie virus 2B protein, as mentioned previously, was also shown (30). Gottenberg et al. (138), however, did not confirm the relationship between the infection with Coxsackie virus and Sjogren's syndrome by examining the presence of viral material in MSGB. It should be noted that this study was carried out on a small group of patients with pSS.

Parvovirus B-19

Due to the high seroprevalence of parvovirus B19 (p19V) IgG antibodies in the general population, estimated as 40–60% in children and adult population (even higher in elderly people—up to 75–85%), it was also taken into account as a potential environmental factor influencing the pathogenesis of pSS and other autoimmune diseases (139, 140). This virus shows tropism to human bone marrow.

De Stefano et al. (141) studied the presence of p19V DNA in the MSGB of patients with pSS and in healthy volunteers (all participants agreed to have MSGB, including the healthy controls). At the same time, the status of antibodies to p19V was assessed in these two groups. B19DNA was found in the smaller salivary glands in both groups; there was no association between the presence of viral DNA and the intensity of infiltration in pSS patients. Therefore, no relationship between pB19 infection and pSS was demonstrated, and the presence of viral genetic material was considered random.

By contrast, Ramos-Casals et al. (142) focused on the clinical significance and the immune picture of patients with pSS and pB19 parvoviral infection. Eighty patients diagnosed with pSS were examined for the presence of antibodies and genetic material of this virus. In 35% of them, a past infection with parvovirus was confirmed, and none had an active viremia. An interesting observation was the more frequent finding of leukopenia and thrombocytopenia in past p19V-infected patients in this group (142). Thrombocytopenia and leukopenia occurring during B19 infection are linked to the cytotoxicity of the parvovirus B19, which is directly related to the cytotoxicity of its non-structural protein (NS1) (143, 144). Several studies from the 1980s confirmed the effect of NS1 on bone marrow cells (144–146). This protein is also associated with arthritis and persistent B19 infection, which was also proved in animal models (146). Parvovirus infection was also assessed as a factor influencing lymphoproliferative processes in patients with Sjögren's syndrome. Ultimately, however, no such direct relationship was proven (147).

Based on the observations so far, it seems that pB19 does not directly affect the pathogenesis of pSS despite its importance in RA or SLE.

SARS CoV-2 virus

At present, no broader conclusions can be drawn about the risk of developing pSS after a SARS-CoV-2 infection, although it is known that xerostomia may also be a prodromal symptom of COVID-19. This clinical observation applies to patients with acute infection of a limited duration time (148). In the coming years, it will probably be possible to assess to what extent the SARS-CoV-2 virus may affect the development of pSS or cause symptoms similar to this autoimmune disease. An interesting multicenter observation revealed that, among pSS patients affected with SARS-CoV-2 virus, 57% remain symptomatic after 5 months. It was estimated that the risk of post-COVID-19 syndrome in hospitalized pSS patients is eight times higher than in non-hospitalized patients. The increased CRP levels and the use of hydroxychloroquine in the treatment of pSS were considered as risk factors for post-COVID-19 syndrome in this group (148).

In Table 3, the main viruses with their targeted cells and main diseases with which they are associated are presented. Whereas Table 4 compares the clinical features of pSS and HCV, HIV and CMV infection.

The effectiveness of anti-viral therapies in the treatment of autoimmune diseases is also being debated. Dreyfuss (151) suggests that drugs such as acyclovir (inhibitor of herpes virus DNA polymerase) or raltegravir (inhibitor of the activity of HIV-1 integrase), which act by inhibiting the activity of the virus, may also have a positive effect on the inhibition of autoimmune diseases such as RA, SLE, or MS. An example of a research regarding this problem is a work by Friedman et al. (152) lovir in patients with MS, although no statistically significant relationship has been proven between such treatment and the clinical improvement or changes in the MRI scans of patients. There are also analyses showing the benefits of anti-viral therapies in autoimmune diseases. Further interesting results were presented by Bech et al. (153) in the study of the efficacy of valacyclovir in patients with MS. Although they did not show improvement in the whole group with relapsing–remitting MS, a reduction in the number of relapses and a decrease in active changes in MRI were observed in the subgroup with very high disease activity. There are descriptions of positive therapeutic effects of anti-viral therapy (zidovudine) in combination with immunosuppressive drugs in the treatment of myelopathy in patients with HTLV-1 infection and Sjogren's syndrome confirmation (154).

TABLE 3 Main viruses involved in the pathogenesis of pSS.

Virus	Genetic material	Targeted cells	Circumstances of activity and main diseases
EBV	DNA	B cells	Sialadenitis Reactivation in immunocompromised subjects—PTLD, hepatitis Mononucleosis Nasopharyngeal cancer Burkitt's lymphoma Oral hairy leukoplakia Hodgkin disease (non-related and related to HIV)
CMV	DNA	Epithelial cells, endothelial cells, fibroblasts, and smooth muscle cells	Mononucleosis-like illness Severe complications in immunocompromised subjects including patients with AIDS: CMV-organ disease (retinitis, esophagitis, colitis, pneumonitis, hepatitis)
HTLV-1	RNA	T cells	Adult T cell leukemia, HTLV-1-associated myelopathy
HIV	RNA	T cells, macrophages, and dendritic cells	Flu-like symptoms (acute infection) asymptomatic stage (chronic infection) AIDS with opportunistic infections
HCV	RNA	Hepatocytes, dendritic cells, and B cells	Sialadenitis, chronic hepatitis, cirrhosis, hepatocellular carcinoma, extrahepatic manifestations (organs and systems involvement)
HBV	DNA	Hepatocytes	Acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma, Acute necrotizing vasculitis —polyarteritis nodosa (circulating immune complexes)* Other extrahepatic manifestations (organ and system involvement)
pB19	DNA	EPCs	In children: erythema infectiosum, thrombocytopenia, ITP In adults: arthralgia, arthritis, anemia, neutropenia, thrombocytopenia

AIDS, acquired immune deficiency syndrome; EBV, Epstein–Barr virus; EPCs, erythroid progenitor cells; CMV, cytomegalovirus; ITP, idiopathic thrombocytopenic purpura; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HTLV, human T-lymphotropic virus; pB19, parvovirus-19; PTLD, post-transplant lymphoproliferative disease.

TABLE 4 Differences between pSS, HCV, HIV and CMV (3, 7, 77, 85, 124, 126, 149, 150).

	Sjögren's syndrome	HCV	HIV	CMV
Age	40–50	All	All/young adults	Young/adults
Gender	F>M	All	M>F	Heterosexual transmission F>M Homosexual transmission M>F
	Dryness mild to severe	Dryness usually mild, medium	Dryness even severe (DILS)	Mouth dryness Possibility of HIV coinfections
Antibodies	Anti-SSA/Ro, anti SSB/La ANAs	Anti-HCV Ab presence of cryoglobulins; presence of other autoantibodies is rare, but when Abs are present, they are usually at low levels; ANAs are in low titers	Anti-HIV Ab; presence of other autoantibodies is rare, but when Abs are present, they are usually at low levels; ANAs are in low titers	Anti-CMV Anti-LKM (autoimmune hepatitis) ANAs may be present in low titer
RF IgM	May be highly positive ++/+++	+	+	+
Histopathology	Mainly CD4+	CD4+, CD20+	CD 8+	CD 34+,
Target cells	B cells	Hepatocytes, dendritic cells, and B cells	CD4+	Epithelial cells, endothelial cells, fibroblasts, and smooth muscle cells
Target organ	Exocrine glands Extraglandular organ and system involvement	Liver, salivary glands	Lymphoid tissue	Lymph nodes, central nervous system, liver, bone marrow (coagulopathy, hemophagocytosis)
Organ/system involvement	Pulmonary, nervous system, kidneys, gastrointestinal tract (liver)	Gastrointestinal tract, liver	Musculoskeletal, nervous system, pulmonary, gastrointestinal, skin	Upper respiratory tract, pulmonary, gastrointestinal (liver, spleen)
Possible connection with oncology	MZBL (marginal zone B cell lymphoma); MALT	Hepatocellular carcinoma	Kaposi Lymphoma Others depending on opportunistic infection HPV—cervical, anal, oropharyngeal, penile, vaginal, and vulvar cancer EBV—non-Hodgkin and Hodgkin lymphoma HBV/HCV—hepatocellular carcinoma	Breast and gastric cancer Glioblastoma

HPV, human papilloma virus; EBV, Epstein–Barr virus; HBV, hepatitis B virus; HCV, hepatitis C virus.

The above-described examples show the potential usefulness of anti-viral agents in autoimmune diseases; however, this topic requires a separate extensive study reaching beyond the scope of this article.

Conclusions

The role of viral infections in Sjogren's syndrome is a subject of ongoing research, but currently it is possible to demonstrate their impact on pSS with a high degree of certainty only in the case of some of the investigated viruses.

The Epstein–Barr virus is suggested as the one most involved in the pathogenesis of pSS. The HCV virus plays a dual role in pSS—on one hand acting as a factor promoting the pSS development (past infection) and on the other as an active HCV infection that constitutes an exclusion criterion for the diagnosis of pSS.

At present, the knowledge of the role of retroviruses such as HTLV-1 in the development of pSS is also increasing. The SARS-CoV-2 pandemic may lead to the expansion of our knowledge on the impact that viruses exert on the development of autoimmune diseases, including pSS.

Author contributions

MM: concept of the work, literature collection, writing, and acceptance of the final manuscript. KK-G: literature collection, writing, and acceptance of the final manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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