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Circulating follicular helper T cells presented distinctively different responses toward bacterial antigens in primary biliary cholangitis

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ABSTRACT

Primary biliary cholangitis (PBC) is a chronic and progressive cholestatic liver disease with unknown causes. The initiation of PBC is associated with bacterial infections and abnormal immune correlates, such as the presence of self-reactive anti-mitochondrial antibodies and shifted balance of T cell subsets. In particular, the $CD4^+CXCR5^+$ follicular helper T (Tfh) cells are highly activated in PBC patients and are significantly associated with PBC severity, but the underlying reasons are unknown. In this study, we found that the circulating $CD4^+CXCR5^+$ T cells were enriched with the interferon (IFN)-γ-secreting Th1-subtype and the interleukin (IL)-17-secreting Th17-subtype, but not the IL-4-secreting Th2 subtype. We further demonstrated that a host of microbial motifs, including Pam3CSK4, poly(I:C), LPS, imiquimod, and CpG, could significantly stimulate IFN-γ, IL-17, and/or IL-21 from circulating $CD4^+CXCR5^+$ T cells in PBC patients, especially in the presence of monocytes and B cells. Whole bacterial cells of *Escherichia coli*, *Novosphingobium aromaticivorans*, and *Mycobacterium gordonae*, could also potently stimulate IFN-γ, IL-17, and/or IL-21 production from circulating $CD4^+CXCR5^+$ T cells. But interestingly, while the whole cell could potently stimulate circulating $CD4^+CXCR5^+$ T cells from both healthy controls and PBC patients, the cell protein lysate could only potently stimulate circulating $CD4^+CXCR5^+$ T cells from PBC patients, but not those from healthy controls, suggesting that circulating $CD4^+CXCR5^+$ T cells in PBC patients had distinctive antigen-specificity from those in healthy individuals. Together, these data demonstrated that bacterial antigen stimulation is a potential source of aberrant Tfh cell activation in PBC patients.

1. Introduction

Primary biliary cholangitis (PBC) is an autoimmune liver disease characterized by slow and progressive destruction of small intrahepatic bile ducts [1]. The disease leads to the accumulation of bile and toxins in the liver combined with an inflamed immune system, which can cause liver damage, fibrosis, and cirrhosis. In earlier stages, PBC can be treated with ursodeoxycholic acid (UDCA), which has significantly extended the survival of PBC patients. But the effectiveness of UDCA is reduced in more advanced patients, whose only other option is liver transplant. Furthermore, for reasons not completely clear, 40% of PBC patients do not present a biochemical response to UDCA [2]. It is thought that in most cases, PBC is triggered by environmental conditions in genetically predisposed individuals. Population studies and genome-wide association studies tend to reveal associations between genes involved in immune functions and the risk of PBC. For example, studies in the North American and European population have identified strong links between certain HLA alleles and PBC, while other HLA alleles may confer protection [3–5]. Another study has demonstrated that genetic variations in the IL-12 signaling pathway are linked to PBC development [6]. In approximately 95% of PBC patients, anti-mitochondrial self-reactive antibodies are present in the serum and have been used as a diagnostic criterion [7]. Other autoantibodies, such as anti-Sp100 and anti-gp210 antibodies, can also occur in PBC patients [8]. Interestingly, autoantibodies may persist in the patients even after a liver transplant. An enrichment of NKT cells in the liver of PBC patients but not of healthy individuals is identified [9]. Together, these results suggest that many branches of the immune system are involved in the development of PBC, but the specific underlying mechanisms remain elusive.

Recently, it has been shown that the follicular helper T (Tfh) cells, a $CD4^+$ T cell subset with characteristic $CXCR5^+$ expression and primarily functions to help B cell germinal center (GC) reactions, can be involved in PBC development [10,11]. In GC, Tfh cells can receive

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chemotactic signals from the CXCR5-CXCL13 axis and locate the B cell follicles [12]. Through expressing several cell surface and secreted molecules, such as programmed death (PD)-1, CD40 ligand, SLAM family receptors and SLAM-associated protein (SAP), ICOS, interleukin (IL)-6, IL-10, and IL-21, Tfh cells enable T-B engagement and interactions, and promote B cell activation, proliferation, affinity maturation, and differentiation into antibody-producing plasmablasts/plasma cells [13,14]. In peripheral blood, the $CD4^+CXCR5^+$ T cells display similar functions as germinal center Tfh cells and promote B cell differentiation into antibody-secreting cells in vitro [15,16]. It was reported that PBC patients presented increased circulating Tfh cell frequency compared to autoimmune hepatitis patients and healthy controls, with higher IL-21 secretion and more potent ability to induce B cell maturation and autoantibody secretion [10]. Moreover, UDCA-nonresponders presented higher circulating Tfh cell frequency than UDCA-responders. Combined with the abundance and persistence of autoantibodies in PBC patients, these results suggest that aberrant Tfh cell activation and function contribute to PBC development.

Currently, it is unclear through which mechanisms Tfh cells become dysregulated in PBC patients. The hepatic microenvironment is enriched with food nutrients as well as toxins and microbial metabolites through a portal vein that carries blood from the intestinal tract to the liver. This influx of microbial metabolites could affect Th cell responses through T cell receptor (TCR)-dependent and TCR-independent mechanisms. First, the bacterial antigens could be presented by liver-resident macrophages on the MHC molecules to stimulate T cells with antigen-specific TCRs. Second, the microbial metabolites could activate antigen-presenting cells through pattern recognition receptors, such as the Toll-like receptors (TLRs), to stimulate inflammatory responses. And third, memory Th cells also express TLRs, through which the bacterial motifs could promote T cell activation and inflammation [17,18].

Based on previous findings, we hypothesized that the microbial metabolites from the hepatic microenvironment might cause aberrant Tfh activation and inflammation in PBC patients. To investigate our hypothesis, we collected circulating $CD4^+CXCR5^+$ T cells from PBC patients and examined their cytokine response toward a variety of bacterial antigens.

2. Methods

2.1. Patients and controls

A total of 26 female volunteers, including 16 PBC patients and 10 healthy individuals, were enrolled in Shanghai Jiao Tong University Affiliated Sixth People's Hospital. The diagnosis of PBC was based on the previously established criteria [1,19]. Patients with overlapping autoimmune hepatitis, hepatitis B/C infections, drug-induced liver disease, alcoholic liver disease, fatty liver disease, and/or diabetes were excluded from the study. All patients were newly diagnosed and treatment-naive. Clinical data were managed by DICAT system (Vancouver, Canada). Peripheral blood mononuclear cells (PBMCs) were harvested from each volunteer by leukapheresis. Written informed consent was obtained from every individual. The institutional ethics review board of Shanghai Jiao Tong University Affiliated Sixth People's Hospital approved the study.

2.2. Cell subset isolation

PBMCs were purified from leukapheresis samples by standard Ficoll-Paque centrifugation. Briefly, 15 mL leukapheresis sample was layered on top of 10 mL Ficoll-Paque PREMIUM (Sigma-Aldrich) and were centrifuged for 30 min at 400*g* without braking. The PBMC layer was aspirated and washed before use. Excess PBMCs were stored at − 80 °C in 10% DMSO and thawed in sterile RPMI 1640 supplemented with 15% FBS (Thermo Fisher) and 1% DNase (Sigma) before use. The thawed cells were then washed before use.

$CD4^+CXCR5^+$ T cells were isolated from PBMCs by fluorescence-activated cell sorting. Briefly, PBMCs were first treated with FcR blocker (FcX reagent, BioLegend) and then stained with fluorophore-conjugated anti-human CD3, CD4, and CXCR5 antibodies (all from BioLegend) for 30 min in dark on ice. The $CD3^+CD4^+CXCR5^+$ T cells were then washed and sorted in a FACSAria cytometer (BD).

Monocytes and B cells were isolated using EasySep Human Monocyte Enrichment kit and B cell Enrichment kit (both from Stemcell) following the manufacturer's instructions, with 96.4%–98.5% purity confirmed by CD14/CD16-positive staining for monocytes and CD19/CD20-staining for B cells.

All gradient centrifugation, staining and sorting were performed under aseptic conditions. After sorting, the cell viability was examined by dyeing with 0.4% Trypan Blue solution (Thermo Fisher), and only live cells were counted for inclusion in the experiments.

2.3. Cell culture and ELISpot

Purified $CD4^+CXCR5^+$ T cells and monocytes/B cells (if present) were cultured at 1:1 ratio in sterile RPMI 1640 supplemented with 15% FBS. For $CD4^+CXCR5^+$ T cells alone culture, cells were incubated at 5×10^5 cells per mL, while for $CD4^+CXCR5^+$ T cells + monocyte/B cell cultures, cells were incubated at 1×10^6 cells per mL. The Human IFN-γ, IL-17, and IL-21 ELISpot kits (all from MABTECH) were used following manufacturer's instructions. Briefly, plates were coated cytokine capture antibodies overnight at 4 °C, washed thoroughly, and blocked with culture medium for 1 h at room temperature. Cell suspension were then added with 10 × sequential dilutions such that the most concentrated well contained 1×10^5 $CD4^+CXCR5^+$ T cells and the least concentrated well contained 1×10^3 $CD4^+CXCR5^+$ T cells. Anti-human CD3/CD28 (BioLegend), PMA/ionomycin (Sigma-Aldrich), TLR ligands (Invivogen), or bacterial antigens, were then added. Cells were incubated in 5% CO_2 100% humidity at 37 °C. For experiment in Fig. 1, stimulation was performed directly in the ELISpot plates for 6 h. For experiments in Figs. 2 and 3, stimulation was performed in a separate 96-well plate for 12–24 h, as indicated per experiment. The monocytes and B cells were then removed by EasySep Human CD14 Positive Selection and Human CD19 Positive Selection kits (Stemcell) according to the manufacturer's instructions. The remaining $CD4^+CXCR5^+$ T cells were then plated in the ELISpot plates for 6 h. After incubation, the plates were thoroughly washed, and were sequentially treated with biotinylated detection antibodies, streptavidin-ALP, and substrate solution. Positive spots were counted using the CTL ImmunoSpot system. An average of triplicate experiments from every PBC or HC donor was calculated and presented in figures.

2.4. Bacteria processing

Escherichia coli (Seattle 1946), *Novosphingobium aromaticivorans* (SMCC F199), and *Mycobacterium gordonae* (TMC 1324) were purchased from ATCC and grown to an OD of 0.5 at 600 nm. Bacterial cells were then washed and resuspended in sterile PBS. For heat-killing, bacterial cells were placed in 60 °C water bath for 30 min. For protein lysate extraction, cells were pelleted at 5000*g* for 10 min, and were then treated with B-PER Bacterial Protein Extraction Kit (Thermo Scientific) following manufacturer's instructions. Briefly, 2 μL lysozyme, 2 μL DNase I, and cOmplete EDTA-free protease inhibitor cocktail (Sigma-Aldrich) were added to each mL of B-PER reagent, which was then added cell pellet at 4 mL per gram. The mixture was incubated for 15 min in room temperature. The lysate was then centrifuged at 15,000*g* for 5 min and the supernatant was aspirated. For stimulation, the lysates were added such that the bacterial protein concentration was at 2 μg/mL.

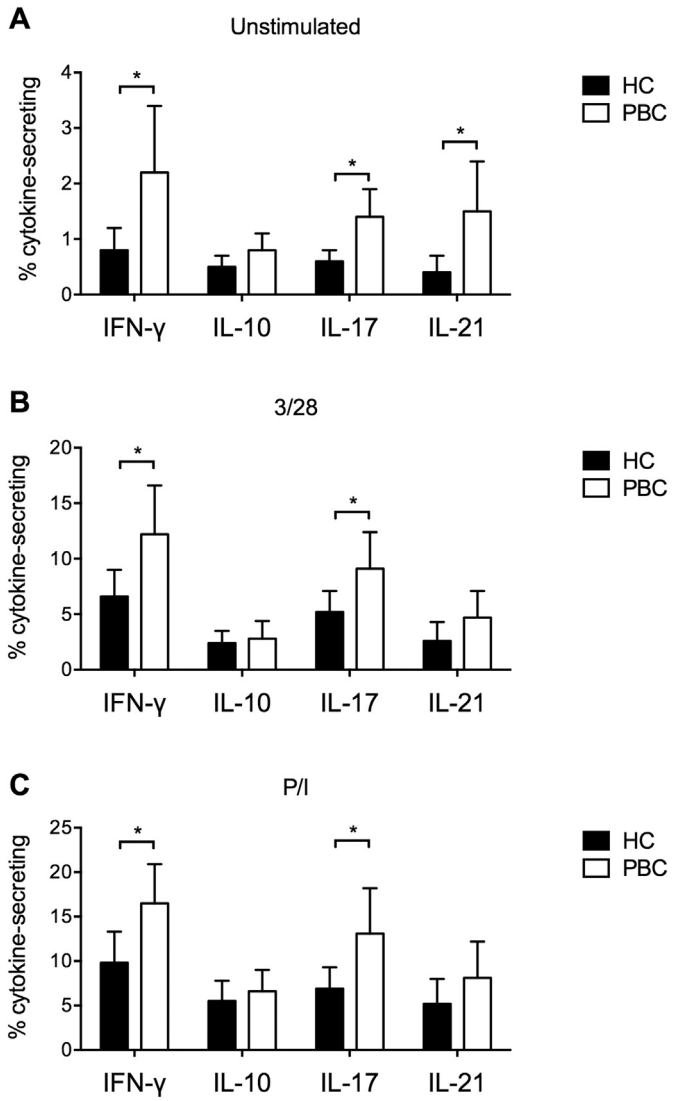

Fig. 1. $CD4^+CXCR5^+$ T cells in PBC patients demonstrated upregulated cytokine secretion.
Freshly isolated peripheral blood $CD4^+CXCR5^+$ T cells were treated with (A) unstimulated medium, with (B) anti-CD3 and anti-CD28 monoclonal antibodies (3/28), 1 μg/mL each, or with (C) 50 ng/mL PMA and 1 μg/mL ionomycin (P/I), for 6 h. The IFN-γ-, IL-10-, IL-17-, and IL-21-secreting cell frequencies were examined by ELISpot. All experiments were performed in triplicates and a pooled average of each group is shown. N = 10 for the healthy control (HC) group and 16 for the PBC group. Differences between HC and PBC patients were examined by Mann-Whitney test. *$P < 0.05$.

2.5. Statistical analyses

All statistical tests were performed in PRISM software, with $P < 0.05$ considered significant.

3. Results

3.1. Circulating $CD4^+$ $CXCR5^+$ T cells from PBC patients are enriched with IFN-γ- and IL-17-producing cells

We first compared the cytokine secretion by circulating $CD4^+CXCR5^+$ T cells in PBC patients and in healthy controls (HCs) immediately after isolation. $CD4^+CXCR5^+$ T cells were sorted from fresh PBMCs and were incubated in unstimulated culture media, with anti-CD3/CD28, or with PMA/ionomycin for 12 h. The frequencies of IFN-γ-, IL-10-, IL-17-, or IL-21-secreting cells were measured by ELISpot. Directly ex vivo, the frequencies of IFN-γ-, IL-17- and IL-21-secreting $CD4^+CXCR5^+$ T cells were significantly higher in PBC patients than in HC (Fig. 1A). After CD3/CD28 or PMA/ionomycin stimulation, the frequencies of IFN-γ- and IL-17-secreting $CD4^+CXCR5^+$ T cells were significantly higher in PBC patients than in HC (Fig. 1B and C).

3.2. TLR ligands promote circulating $CD4^+$ $CXCR5^+$ T cell activation

The higher IFN-γ, IL-17, and IL-21 secretion directly ex vivo and higher IFN-γ and IL-17 secretion following stimulation suggested an elevated activation status in circulating $CD4^+CXCR5^+$ T cells and a dysregulated circulating $CD4^+CXCR5^+$ T cell subset balance in PBC patients. Since $CD4^+CXCR5^+$ T cells could potently infiltrate the liver in PBC patients [10], and the liver is enriched with bacterial metabolites, we thought that microbial metabolites in the liver environment might contribute to the Tfh inflammation in PBC patients. First, we examined the effects of a series of TLR ligands, including Pam3CSK4, poly(I:C), LPS, imiquimod, and CpG, on $CD4^+CXCR5^+$ T cells sorted from thawed PBMCs. We found that in HCs, the presence of poly(I:C), LPS, and CpG in pure $CD4^+CXCR5^+$ T cell cultures could significantly elevate the frequency of IFN-γ-secreting $CD4^+CXCR5^+$ T cells (Fig. 2A), while the presence of CpG could also significantly increase the frequency of IL-17-secreting and IL-21-secreting $CD4^+CXCR5^+$ T cells (Fig. 2B and C). In pure $CD4^+CXCR5^+$ T cell cultures of PBC patients, Pam3CSK4, LPS, imiquimod, and CpG significantly increased the frequency of IFN-γ-secreting $CD4^+CXCR5^+$ T cells (Fig. 2A) and CpG significantly increased the frequency of IL-17-secreting and IL-21-secreting $CD4^+CXCR5^+$ T cells (Fig. 2B and C), similar to the observation in HCs. The presence of monocytes or B cells further elevated the frequencies of IFN-γ-, IL-17- and IL-21-secreting $CD4^+CXCR5^+$ T cells in both HC and PBC individuals to varying degrees. These data suggested that certain TLR ligands could directly stimulate circulating $CD4^+CXCR5^+$ T cells to increase IFN-γ, IL-17 and IL-21 production, whereas monocytes and B cells could further amplify the effect of TLR ligands.

3.3. PBC patients and HCs respond differently toward bacterial antigens

A number of studies have identified that certain bacterial pathogens, including *Escherichia coli*, *Novosphingobium aromaticivorans*, and *Mycobacterium gordonae*, are associated with PBC initiation [20–22]. The precise mechanism through which these pathogens might induce PBC is yet unclear, but most hypotheses suggest that molecular mimicry between bacterial proteins and self-antigens might play an important role. Here, we investigated whether circulating $CD4^+CXCR5^+$ T cells could be activated by bacterial antigens presented by monocytes/macrophages. Whole bacteria killed by heating, or bacterial cell whole protein lysates, were used to stimulate $CD4^+CXCR5^+$ T cells from HCs and PBC patients in the presence of autologous monocytes. In general, we found that whole bacterial cells stimulated higher frequencies of IFN-γ-, IL-17-, and IL-21-secreting $CD4^+CXCR5^+$ T cells than the whole protein lysates. Moreover, following whole bacterial cell stimulation, the frequencies of IFN-γ-, IL-17-, and IL-21-secreting $CD4^+CXCR5^+$ T cells from HCs and PBC patients were comparable, with the exception that *E. coli*-stimulated higher frequencies of IFN-γ-secreting $CD4^+CXCR5^+$ T cells in PBC patients than in HCs (Fig. 3A). In striking contrast, the HCs and PBC patients demonstrated significantly different cytokine secretion patterns when stimulated with bacterial cell protein lysates. After *E. coli* protein lysate stimulation, the frequencies of IFN-γ- and IL-21-secreting $CD4^+CXCR5^+$ T cells were significantly higher in PBC patients than in HCs (Fig. 3A). A minor subset of HC patients did not respond to *E. coli* lysate, while all PBC patients responded to *E. coli* lysate. Similarly, after *N. aromaticivorans* protein lysate stimulation, the frequencies of IFN-γ-, IL-17-, and IL-21-expressing $CD4^+CXCR5^+$ T cells were significantly higher in PBC patients than in HCs (Fig. 3B). The majority of HCs did not respond to *N. aromaticivorans* lysate, while

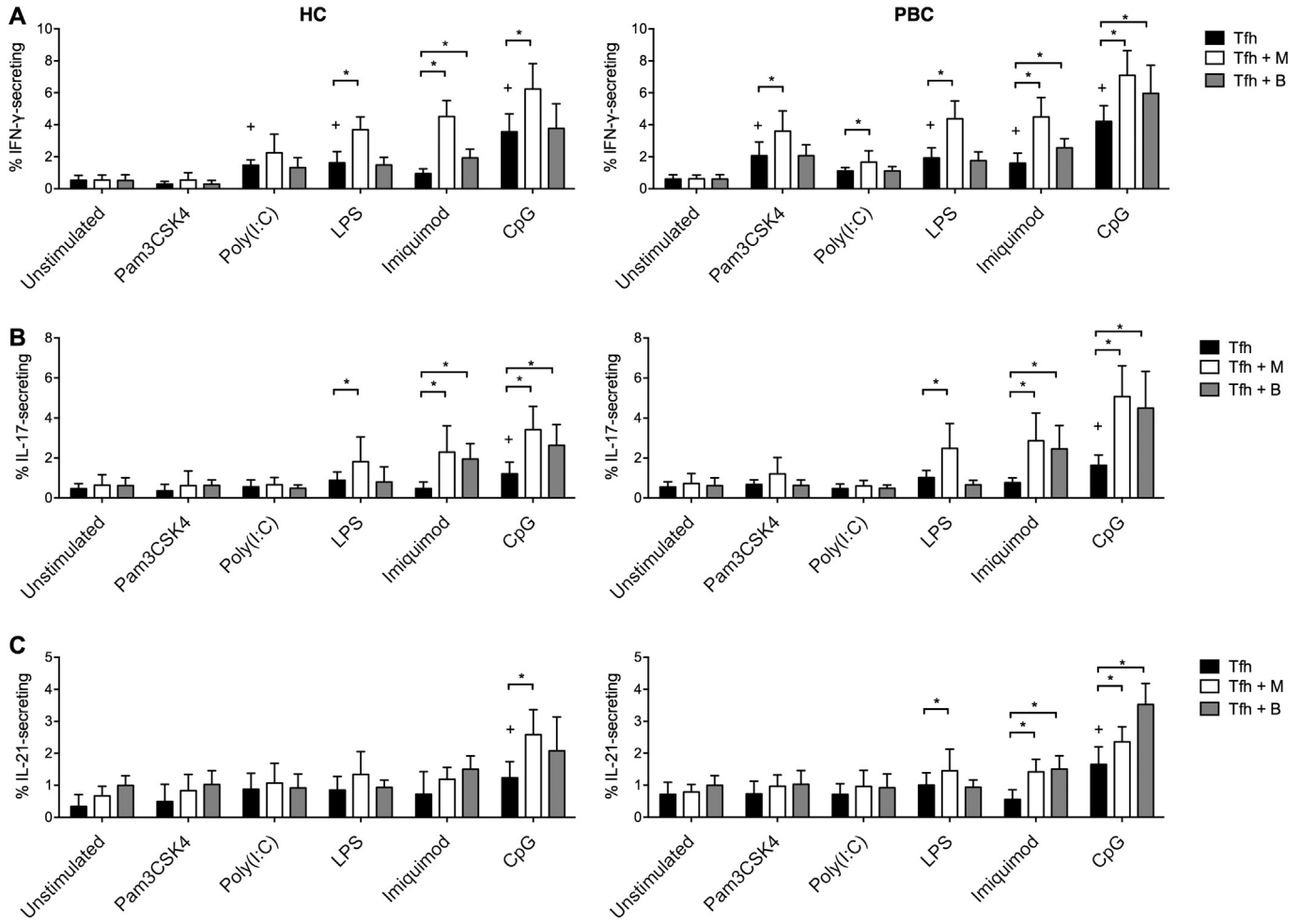

Fig. 2. TLR ligands could directly and indirectly elevate the cytokine secretion by $CD4^+$ $CXCR5^+$ T cells from HCs and from PBC patients.
$CD4^+$ $CXCR5^+$ T cells were sorted from thawed PBMCs and were incubated alone (Tfh), with autologous monocytes (Tfh + M), or with autologous B cells (Tfh + B). Various TLR ligands were added for 12 h, and the frequencies of (A) IFN-γ-secreting, (B) IL-17-secreting, and (C) IL-21-secreting cells were examined by ELISpot. For Tfh + M and Tfh + B experiments, the monocytes and B cells were removed before ELISpot by magnetic sorting. All experiments were performed in triplicates and a pooled average of each group is shown. N = 10 for the healthy control (HC) group and 16 for the PBC group. Statistical differences were tested by two-way ANOVA followed by Dunnett's test. Differences between TLR ligand-stimulated Tfh cells and unstimulated Tfh cells were denoted by plus (+) signs, while differences between Tfh, Tfh + M, and Tfh + B conditions were denoted by asterisks (*). +/*P < 0.05.

a minor subset of PBC patients did not respond to *N. aromaticivorans* lysate. In term of *M. gordonae*, only a minority of PBC patients presented significant cytokine response toward *M. gordonae* lysates (Fig. 3C). Together, these data demonstrated that the circulating $CD4^+$ $CXCR5^+$ T cells from PBC patients and HCs presented significantly different response patterns toward bacterial antigens.

4. Discussion

PBC is a progressive cholestatic liver disease primarily affecting middle-aged women [2], and the underlying mechanisms of PBC are still unclear to this date. The association between PBC with various infectious agents, microbial organisms, certain HLA alleles, and autoimmune antibodies suggest that dysregulated inflammation plays a crucial role in the pathogenesis of PBC. In particular, the Tfh cells are thought to be involved because of the following features: first, the $ICOS^{high}$ and PD-1^{high} $CD4^+$ $CXCR5^+$ T cells are significantly enriched in the peripheral blood of PBC patients, and the intrahepatic double-expression of Bcl-6 (Tfh lineage transcription factor) and PD-1 are enriched in PBC but not HC liver; second, Tfh cells from PBC patients demonstrate high activation status and functionality, and actively promote the secretion of antibodies from autologous B cells; and third, decrease in Tfh frequency is associated with better response to UDCA [10]. These results have placed Tfh cells at the center of the dysregulation in humoral responses in PBC patients, but very little is understood about the activation mechanism of Tfh cells.

In the present study, several mechanisms through which bacteria may stimulate the circulating Tfh cells were examined in PBC patients. We observed that a few TLR ligands could directly activate circulating $CD4^+$ $CXCR5^+$ T cells in both HC and PBC subjects. This activation was possibly through the TLRs expressed on the $CD4^+$ $CXCR5^+$ T cells in an antigen-independent manner, since it could happen in the absence of antigen-presenting cells. When monocytes and B cells were present, the TLR ligands demonstrated more potent activation of circulating $CD4^+$ $CXCR5^+$ T cells. This monocyte and B cell-mediated enhancement of inflammation may occur through indirect mechanisms. The monocytes and B cells, upon receiving TLR ligand stimulation, could upregulate the expression of cytokines and costimulatory molecules, which then increases the inflammation of circulating $CD4^+$ $CXCR5^+$ T cells. However, since no bacterial protein antigen was added, this monocyte and B cell-mediated enhancement likely did not involve antigen-specific mechanisms. Subsequently, we examined the response of circulating $CD4^+$ $CXCR5^+$ T cells to specific bacterial antigens, including the whole bacterial cells and the protein lysates. In this case, antigen-specific mechanisms are likely involved due to two reasons. First, the specific protein antigens from each bacterial strain and the antigen-presenting monocytes were both present; and second, the circulating $CD4^+$ $CXCR5^+$ T cells from HC subjects could be potently

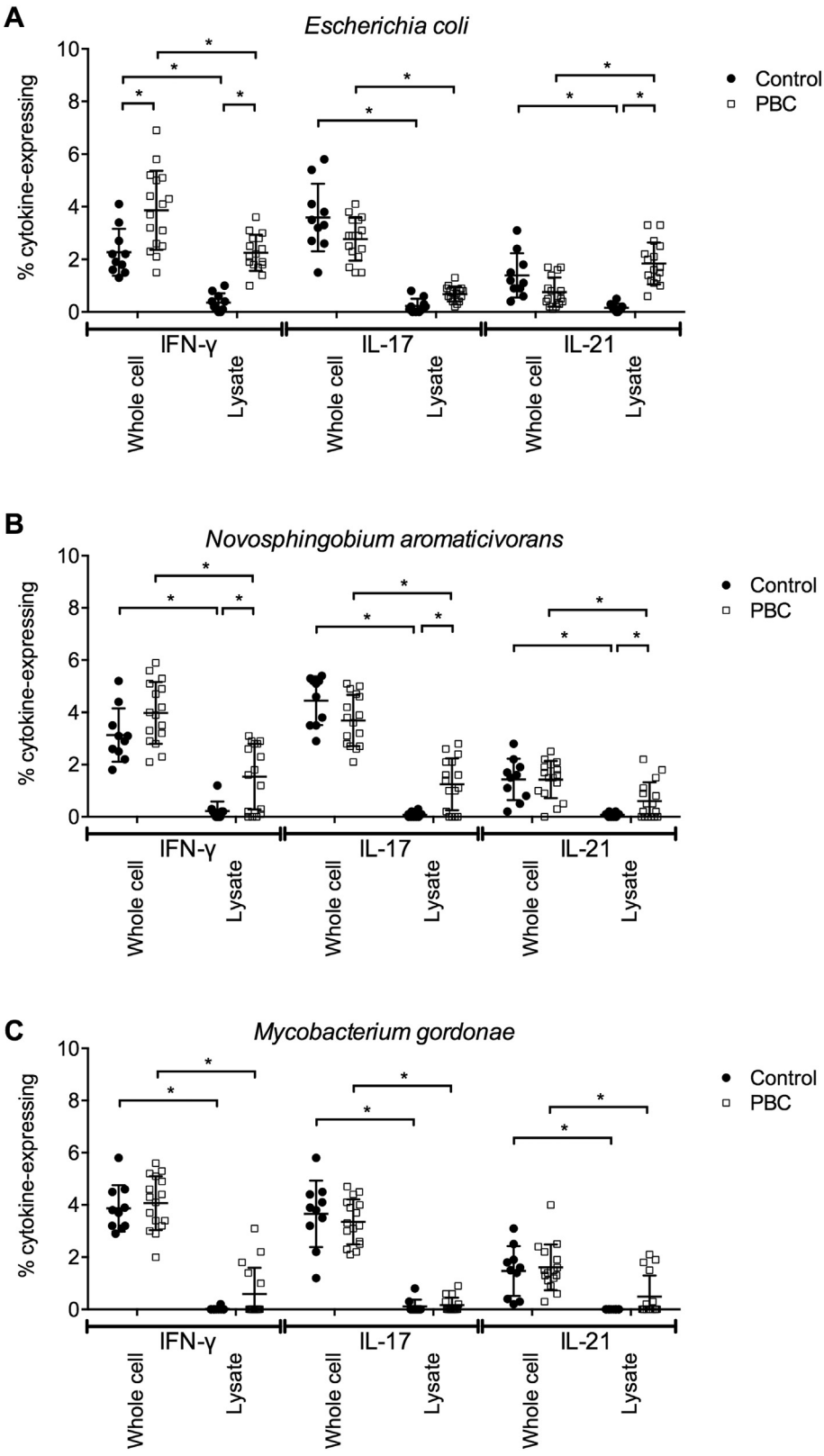

Fig. 3. Whole bacteria and bacterial proteins stimulated Tfh cells in healthy and PBC patients.
Healthy and PBC $CD4^+CXCR5^+$ T cells were sorted from thawed PBMCs and were incubated with autologous monocytes, with heat-killed whole bacterial cells or protein lysates for 24 h. The frequencies of IFN-γ-, IL-17-, and IL-21-secreting $CD4^+CXCR5^+$ T cells were examined by ELISpot. The monocytes were removed before ELISpot by magnetic sorting. The bacteria used are (A) *Escherichia coli*, (B) *Novosphingobium aromaticivorans*, and (C) *Mycobacterium gordonae*. All experiments were performed in triplicates. N = 10 for the healthy control (HC) group and 16 for the PBC group. Data were examined by two-way ANOVA followed by Tukey's multiple comparisons test. *P < 0.05.

stimulated by TLR ligands but not by bacterial protein lysates, suggesting that the latter stimulation method did not involve typical TLR ligands. Antigen-independent mechanisms still likely played a major role in whole cell stimulations because the bacterial cell membrane contained a variety of TLR ligands. The difference between $CD4^+CXCR5^+$ T cell response toward whole cells and $CD4^+CXCR5^+$ T cell response toward lysates therefore could come from two sources. First, in the lysates, the intracellular protein antigens were exposed to the extracellular milieu in experiments, while in whole cells, these antigens were enclosed within the bacterial membrane and were not in contact with immune cell surface molecules. And second, the whole cells contained additional cell membrane components, such as

peptidoglycan and LPS, that could directly stimulate the TLRs, while in the lysates, those components might be modified or lost during the lytic process.

We showed that the IFN-γ-secreting Th1 subset and the IL-17-secreting Th17 subset of circulating $CD4^+CXCR5^+$ T cells, but not the IL-4-secreting Th2 subset, were enriched in PBC patients. Th1 and Th17 responses were previously implicated in numerous autoimmune diseases, including PBC [23]. Th17-type Tfh cells were also shown to be the most potent cell in inducing antibody production from naive B cells [16]. IFN-γ, on the other hand, is a potent IgG2a class switch factor, and is possibly responsible for the elevated IgG production by B cells in PBC patients [10,24]. The expression of IFN-γ and IL-17 were potently supported by the treatment of circulating $CD4^+CXCR5^+$ T with TLR ligands, especially in the presence of autologous monocytes and B cells, suggesting that monocytes and B cells presented a role in exacerbating Tfh activation in PBC patients. Also, we found that the Tfh cells from HC patients and PBC patients presented similar levels of cytokine secretion after stimulation with whole bacteria. However, when the bacterial cells were lysed such that the internal antigens were directly exposed, the circulating $CD4^+CXCR5^+$ T cells from PBC patients demonstrated significantly elevated activation, characterized by potent IFN-γ, IL-17, and IL-21 secretion, while the circulating $CD4^+CXCR5^+$ T cells from HCs did not respond. After stimulation of *Escherichia coli*, a common intestinal bacterium, Tfh cells from all PBC patients responded potently to the lysate, while Tfh cells from HCs did not. These results suggested that Tfh cells from PBC patients presented different specificity compared to HCs. Molecular mimicry between the host protein pyruvate dehydrogenase complex subunit E2 (PDC-E2) and bacterial proteins from *Escherichia coli*, *Novosphingobium aromaticivorans*, and *Mycobacterium gordonae* is hypothesized to be the main mechanism through which infections with these pathogens could initiate PBC.

There remain many questions to be explored by future studies. First, given that TLR ligands could stimulate circulating Tfh cells from both HC and PBC individuals, why only the Tfh cells in PBC subjects were dysregulated? It was recently shown that the gut microbiome was altered in PBC individuals [25,26]. Thus, the Tfh cells in PBC patients possibly received stimulation from a set of bacteria different from those in HCs. Whether this supposition is true in vivo still requires further examinations. Previous studies also demonstrated that TLR ligands could activate memory $CD4^+$ T cells and that TLR expression on $CD4^+$ T cells was dependent on prior TCR activation [17,18]. Given that the $CD4^+CXCR5^+$ T cells are enriched with memory cells [16], it is therefore possible that the circulating $CD4^+CXCR5^+$ T cells in PBC patients presented varied TLR expression profile, resulting in varied responses toward TLR ligands. This possibility should also be examined in further experiments.

In conclusion, this study demonstrated an abnormal response toward bacterial internal antigens in circulating $CD4^+CXCR5^+$ T cells from PBC patients, which could contribute to autoimmune reactions.

Conflict of interest

None.

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