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CXCR5⁺ CD4⁺ T Follicular Helper Cells Participate in the Pathogenesis of Primary Biliary Cirrhosis

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Abstract

There is increasing interest in the role of T follicular helper (Tfh) cells in autoimmunity from the perspective of both their role in breach of tolerance and their effects on the natural history of disease progression. Indeed, the critical role of Tfh cells in autoimmunity is further highlighted based on their location in the germinal center (GC), a pathogenic hot spot for development of autoreactivity. To address the role of Tfh cells in primary biliary cirrhosis (PBC), we comprehensively evaluated the immunobiology of CXCR5⁺CD4⁺ Tfh cells in 69 patients with PBC, including a nested subgroup of 16 autoimmune hepatitis (AIH) and 20 healthy controls (HC), followed for one year. We report herein several key observations. Firstly, there was an increased frequency of circulating Tfh cells in patients with PBC compared to AIH ($p < 0.05$) and HC ($p < 0.01$). Second, the function of circulating Tfh cells from PBC patients, including IL-21 production ($p < 0.05$), the ability to promote B cell maturation and autoantibody production, were

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greater than HC. Third, the frequency of these cells was significantly decreased in UDCA responders compared to UDCA-treated non-responders, in both cross-sectional ($p = 0.023$) and longitudinal studies ($p = 0.036$), respectively. Indeed, similar increases of Tfh cells were noted in liver and spleen. In conclusion, these results significantly extend our understanding of lymphoid subpopulations in PBC and their relative role in disease expression. Our data also provide a novel biomarker for evaluation of the effectiveness of new therapeutic approaches.

Keywords

Primary biliary cirrhosis; T follicular helper cells; ursodeoxycholic acid; plasma B cells

Primary biliary cirrhosis (PBC) remains a difficult-to-treat clinical liver disease. Indeed, despite major advances in defining the immunological features involved in the loss of tolerance, there remains a paucity of data on the biological effector mechanisms of biliary damage (1, 2). In general, PBC is characterized by the presence of the most highly directed and specific autoantibody in human immunopathology: the anti-mitochondrial antibody, and the presence of a high frequency of antigen specific autoreactive CD4 and CD8 T cells (3–6). Although we have postulated that PBC is a multi-hit disease, involving both innate and adaptive immunity, the relationships between specific T cell subpopulations and the natural history of the disease have been enigmatic (7, 8).

Recent data have implied that the germinal center (GC) may be a pathogenic hot spot for production of autoantibodies in autoimmune diseases (9). In particular, T follicular helper (Tfh) cells are located in GCs, where they primarily drive B cells to differentiate into memory B cells and form antibody-producing plasma cells (10). Tfh cells function through the signal transcription factors B-cell lymphoma 6 (Bcl-6), CD40 ligand, chemokine C-X-C receptor 5 (CXCR5), programmed cell death protein-1 (PD-1) and the cytokines IL-21, IL-6 and IL-10. Several studies have demonstrated that Tfh cells are expanded in the peripheral blood of human and murine models of systemic autoimmune diseases, including rheumatoid arthritis (11), systemic lupus erythematosus (SLE) (12), primary Sjogren's syndrome (13), autoimmune thyroid diseases (14) and autoimmune hepatitis (15, 16). CXCR5 has recently been identified as a risk locus for PBC (17), which further highlights the potential relationships of Tfh cells and the pathogenesis of PBC. To address these issues, we comprehensively evaluated Tfh cell number, frequency and function in patients with PBC and controls. We report that patients with PBC manifest not only increased Tfh cells *in vivo*, but also that their frequency correlates with increased B cell activation, disease severity and responsiveness to ursodeoxycholic acid (UDCA) therapy. These data have broad implications for our understanding of the immunopathogenesis of PBC and may serve as a biomarker for monitoring new therapeutic tools in PBC.

Materials and Methods

Patients and samples

One hundred and five subjects, including 69 patients with PBC, 16 patients with AIH and 20 healthy controls (HC), were enrolled in this study from January 2011 to April 2014 in

Beijing 302 hospital. The diagnosis of PBC and AIH (Supplemental Table 2) was based on established criteria and excluded any AIH-PBC overlap in both groups (18–20). There were no co-existing liver diseases nor other autoimmune diseases in these patients, including hepatitis B, hepatitis C, drug-induced liver disease, alcoholic disease, non-alcoholic fatty liver disease, or diabetes. Of the 69 PBC patients, there were 41 newly diagnosed patients who were UDCA treatment-naïve. The remaining 28/69 patients were previously diagnosed with PBC and had completed more than 12 months of UDCA treatment (according to the weight-based dose, 13–15 mg/kg/day) before sampling and were classified as either responders (n = 14) or non-responders (n = 14), according to the Paris definition (21). The Institutional Committee for Human Research of Beijing 302 Hospital approved this study and written informed consent was obtained from each subject.

Blood was collected for immunological analysis from 35 patients from the 41 UDCA treatment-naïve PBC patients; 6/41 declined to provide blood samples but did approve the use of excess tissue from liver biopsies. These 35 UDCA treatment-naïve patients were further categorized into non-cirrhotic (n = 18/35), cirrhotic (n = 8/35) and decompensated cirrhotic patients (n = 9/35) who were defined by the presence of liver dysfunction and portal hypertension with Child–Pugh class B or C cirrhosis (22, 23) (Table 1).

Paraffin-embedded liver tissues were available either from ultrasound-guided needle liver biopsies or resection from 24 of these 41 PBC patients. Based on Ludwig's classification, these 24 patients were divided into stage I (n = 6), stage II (n = 6), stage III (n = 6) and stage IV (n = 6), respectively (24). Healthy liver tissues were collected from five donors whose livers were subsequently used for liver transplantation. Spleen samples were obtained from three PBC patients with portal hypertension and three patients with splenic injury (healthy controls) who underwent splenectomy.

Fluorescence antibodies and flow cytometry

Fluorescein isothiocyanate (FITC)-conjugated anti-CD38, Alexa 488-anti-CXCR5, Alexa F-647-anti-CXCR5, phycoerythrin (PE)-conjugated anti-IL-21, PE-Cy7-conjugated anti-CD4, allophycocyanin (APC)-conjugated anti-IL-6 and FITC-conjugated anti-IFN- γ were purchased from BD Bioscience and BD Pharmingen™ (San Diego, CA, USA). FITC-conjugated anti-CD3, PE-conjugated anti-ICOS, PE-conjugated anti-IL-17a, peridinin chlorophyll protein (PerCP)-conjugated anti-CD4 and anti-CD8 and APC-conjugated anti-CD279 (PD-1) were purchased from eBioscience (San Diego, CA, USA); and APC-Cy7-conjugated anti-CD3 and anti-CD27, PE-conjugated anti-CD24, PE-Cy7-conjugated anti-CD19 and PerCP-Cy5.5-conjugated anti-IgD were purchased from BioLegend (San Diego, CA, USA). Peripheral blood mononuclear cells (PBMCs) were isolated and phenotypic analysis performed using optimal concentrations of mAbs according to standard protocols (25, 26). Aliquots of cells were also utilized for flow cytometry and FlowJo software analysis (Tristar Inc, San Carlos, CA, USA). At least 50,000 events per sample were analyzed.

Cell purification and stimulation

1×10^7 PBMCs were first stained with optimal concentrations of PerCP-anti-CD4, Alexa F-647-anti-CXCR5 and PE-Cy7-anti-CD19 at RT for 30 min, followed by washing with PBS. A FACS Aria II cell sorter was further used to purify CXCR5⁺CD4⁺, CXCR5⁺CD4⁻ cells and CD19⁺ B cells (27). The purity of sorted cell populations was more than 90%.

Enzyme-linked immunosorbent assays

A human IgG and IgM ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX) measured total IgG and IgM concentrations in culture supernatants. The line immunoassay (LIA) using the Euroimmune Test System (Euroimmune, Lubeck, Germany) detected AMA (Anti-M2-3E ELISA kit was used for further confirmation), and antinuclear antibodies (ANA) including Sp100 and Gp210. Indirect immunofluorescence (IIF), using the Euroimmune Test System (Euroimmune), was used to additionally detect AMA titers.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded 4- μ M tissue sections. Sections were incubated with optimal concentrations of primary monoclonal antibodies, including anti-CD38, anti-CD20, anti-CD4, anti-PD-1 (R&D, AF1086, Abington, UK) and anti-Bcl-6 (Santa Cruz biotechnology, sc-858, Santa Cruz, CA, USA) for 1 hr at RT and then incubated with biotinylated goat anti-rat, goat-rabbit and biotinylated rabbit anti-mouse antibody (Zhongshan Goldenbridge Biotech, Beijing, China), respectively. The avidin-biotin-peroxidase system with 3-amino-9-ethyl-carbazole (AEC) (red color) or Vector blue (blue color) as substrates was used to perform double staining. Based on the double immunostaining, PD-1 and Bcl-6 double positive cells in liver tissue, and CXCR5 and CD4 double positive cells in the spleen were identified as Tfh cells. The absolute number of Tfh cells was independently counted by two pathologists from five representative high-power microscopic fields (400 magnification) relative to the area most involved by the inflammatory infiltrate (28).

Quantitation of Tfh cell percentages and correlation analysis

Quantitation of Tfh cell percentages—Quantitation of peripheral Tfh cell percentages was performed in 35 treatment-naïve PBC patients, 28 UDCA-treatment patients (14 UDCA responders and 14 UDCA non-responders), 16 treatment naïve AIH patients and 20 HCs. Peripheral Tfh cells were defined as CXCR5⁺CD4⁺ T cells. Among the subjects with available peripheral blood, 20 treatment-naïve PBC patients, 16 AIH and 10 HCs were further analyzed for PD-1 and ICOS expression within the CXCR5⁺CD4⁺ Tfh cells.

Correlation of peripheral Tfh cells with disease severity

The correlations between peripheral CXCR5⁺CD4⁺ T cell percentages and AMAs, and clinical status were performed in the 35/41 UDCA naïve patients. In addition, the correlations between the peripheral CXCR5⁺CD4⁺ T cell percentages and diseases stages indicated by liver tissue evaluation in 20 treatment-naïve patients (eight stage I, five stage II, four stage III and three stage IV) were investigated.

Production of IL-21 by Tfh cells

To evaluate the IL-21 secreting function of CXCR5⁺CD4⁺ T cells, PBMCs were stimulated by PMA/ionomycin for 6 hours and the percentages of IL-21-producing CXCR5⁺CD4⁺ T cells analyzed between treatment-naïve PBC patients (n = 24) and HCs (n = 10), or CXCR5⁺CD4⁺ T cells and CXCR5⁻CD4⁺ T cells (n = 24), or UDCA responders (n = 12) and UDCA non-responders (n = 8). Finally, the correlations between the percentage of IL-21⁺CXCR5⁺CD4⁺ T cells and CXCR5⁺CD4⁺T cells were analyzed.

Correlation of CXCR5⁺ CD4⁺ Tfh cells and B cell phenotype and function

To evaluate the correlation between CXCR5⁺ CD4⁺ T cells and relevant B cell subsets, 16 treatment-naïve PBC patients were studied; another tube of PBMC was used for B cell phenotype studies by addition of PE-Cy7-anti-CD19, PE-anti-CD24, FITC-anti-CD38, and APC-Cy7-CD27. The correlation between the percentage of total B cells (SSC/ CD19) or plasma cells (CD24⁻CD38⁺⁺CD27⁺CD19⁺) within the CD19⁺B cells and the frequency of CXCR5⁺CD4⁺ T cells were studied separately. In addition, another B cell subset staining panel (PE-Cy7-anti-CD19, APC-Cy7-anti-CD27, Percp-anti-IgD and FITC-anti-CD38), which divided B cells into IgD⁺CD27⁻ naïve B cells, IgD⁺CD27⁺ marginal zone like B cells and IgD⁻CD27⁺ class-switched memory B cells and plasmablasts (29), was used to analyze B cell differentiation states after co-culture with CXCR5⁺CD4⁺ T cells.

Co-culture of Tfh cells and B cells

The isolated CXCR5⁺CD4⁺ or CXCR5⁺CD4⁻ T cells from five treatment-naïve PBC patients or four healthy controls were incubated with allogeneic CD19⁺ B cells from healthy controls in a 96-well plate, at a ratio of 1:1 in the presence of SEB (100ng/ml) in RPMI 1640 complete medium containing 10% FCS only or with rIL-21R-Fc (10 µg/ml; R&D Systems, Minneapolis, MN) or rIL-21 (20 ng/ml; Peprotech, Rocky Hill, NJ, USA) (30, 31). After 7–8 days of culture, cells were harvested to evaluate surface phenotypes and supernatants were collected to detect IgM and IgG (32).

Follow-up and outcome measures

All of the 41 treatment-naïve patients received standard UDCA therapy at a daily dose of 13 to 15 mg/kg of body weight; while 18 of the patients with stage I or II, or those who had clinical features without decompensation, declined participation in our subsequent 12-month-period prospective study. At the onset of UDCA treatment, and at 6 and 12 months post treatment, peripheral blood was collected at each visit and CXCR5⁺CD4⁺ Tfh cell percentages were analyzed and correlated with their 1-year UDCA efficacy data.

Statistical analysis

All statistics were performed using SPSS version 17.0 software and data presented as mean values ± standard deviations. Multiple comparisons were made between the different groups with the Kruskal-Wallis H nonparametric test. Comparisons between various individuals were performed with a Mann-Whitney *U* test, whereas comparisons between the same individual were performed with Wilcoxon's matched-pairs test. The relationship between

two variables was evaluated using the Spearman rank correlation test. For all analyses, a two-sided P value < 0.05 was considered to be significant.

Results

Tfh cells are significantly enriched in PBC patients in vivo

The frequencies of peripheral CXCR5⁺CD4⁺ T cells were first analyzed using flow cytometry. As shown in Figure 1A, the percentage of CXCR5⁺ CD4⁺ T cells in peripheral blood of PBC patients was significantly higher than that in AIH ($17.8 \pm 5.3\%$ vs. $13.67 \pm 4.9\%$, $p < 0.05$) and HCs ($17.8 \pm 5.3\%$ vs. $9.9 \pm 3.1\%$, $p < 0.01$). Further analysis indicated that these CXCR5⁺ CD4⁺ T cells also expressed PD-1 and ICOS. In particular, the percentages of PD-1^{high}CXCR5⁺CD4⁺ T cells among CD4 T cells were higher in PBC ($n = 20$) than in AIH ($n = 16$) and HCs ($n = 10$) (both $p < 0.01$). However, no significant difference in frequency of ICOS^{high}CXCR5⁺CD4⁺ T cells was found between PBC and AIH patients, although both of them exhibited higher levels than the counterpart in HC. In addition, there were no gender-specific differences in the percentages of Tfh cells between male and female patients, although the PBC cohort was predominantly female (Supplemental Fig 5).

We then investigated the distribution of hepatic (PD-1⁺ and Bcl-6⁺ double positive) and splenic (CD4⁺ and CXCR5⁺ double positive) Tfh cells using immunohistochemical double staining. PD-1⁺ and Bcl-6⁺ positive Tfh cells were absent in healthy donor liver. In contrast, more PD-1⁺ and Bcl-6⁺ cells accumulated around the damaged interlobular bile ducts in PBC with chronic non-suppurative destructive cholangitis (CNSDC) ($p < 0.05$). A significant proportion of PBC displayed high numbers of Tfh cells in a lymph follicle-like structure close to damaged bile ducts, which is consistent with a permissive environment for Tfh generation ($p < 0.01$) (Fig 1 B). CD4, CD20 (total B cells) and CD38 (plasma B cells) were also detected. CD4 T and B cells co-located with Tfh cells around the bile ducts. Splenic Tfh cells localized in the T-B cells zone in HCs, whereas these cells moved to GC-bearing B-cell follicles in PBC; the splenic tissue was derived only from decompensated cirrhotics patients (ie variceal bleeding leading to splenectomy) (Supplemental Fig 1).

Tfh cells were positively correlated with disease severity

Positive correlations were found between circulating CXCR5⁺CD4⁺ T cells and PBC specific parameters (including ALP, $r = 0.427$, $p < 0.05$; IgM, $r = 0.399$, $p < 0.05$) in treatment-naïve patients (Supplemental Fig 2). We also further found that the frequencies of CXCR5⁺CD4⁺ T cells were higher in AMA⁺ patients than those in AMA⁻ PBC ($18.4 \pm 5.3\%$ vs. $13.9 \pm 2.8\%$, $p < 0.05$) (Fig 2 A, Supplemental Table 1). The frequency of CXCR5⁺CD4⁺ T cells in the non-cirrhotic group was significantly lower than the cirrhotic ($p < 0.01$) and decompensated cirrhotic groups ($p < 0.01$). The levels of CXCR5⁺CD4⁺ T cells were higher in patients in stages II, III, IV than with stage I disease ($11.21 \pm 4.14\%$ vs. $17.8 \pm 6.09\%$, $p < 0.05$; vs. $19.75 \pm 3.75\%$, $p < 0.05$; vs. $20.7 \pm 1.20\%$, $p < 0.05$, respectively) (Fig 2 C). However, there was no significant difference in the frequency of CXCR5⁺CD4⁺ T cells between the cirrhotic and decompensated cirrhotic groups (Fig 2 B).

IL-21-producing CXCR5⁺CD4⁺ T cells in PBC

As shown in Fig 3 A, the levels of IL-21⁺ CXCR5⁺CD4⁺ T cells in PBC were significantly higher than those in HCs ($11.99 \pm 6.87\%$ vs. $2.77 \pm 1.74\%$, $p < 0.05$). In addition, CXCR5⁺CD4⁺ T cells also produced higher levels of IFN- γ ($p < 0.01$) and IL-17 ($p < 0.05$) in PBC compared with HCs, although IL-6 demonstrated an increasing trend compared with HCs. In contrast to CXCR5⁺CD4⁺ T cells, which predominantly produced IFN- γ , CXCR5⁺CD4⁺ T cells produced more IL-21 than IL-6 ($p < 0.01$) and IL-17 ($p < 0.01$) after PMA/ionomycin stimulation (Fig 3 B). In addition, we also noted that patients with higher levels of CXCR5⁺CD4⁺ T cells generated more IL-21 ($r = 0.449$, $p = 0.028$) after stimulation with PMA/ionomycin (Fig 3 C).

Circulating CXCR5⁺CD4⁺ T cells correlated with B cell proportions and function

Circulating CXCR5⁺CD4⁺ T cells induce naïve B cells to differentiate into plasmablasts and promote class switching. The link between Tfh cells and IgM or AMA prompted us to test for an association between circulating Tfh cells and B cells. Although there was no significant correlation between circulating levels of CXCR5⁺CD4⁺ T cells and the proportion of autologous B cells ($r = 0.363$, $p = 0.167$), the levels of CXCR5⁺CD4⁺ T cells were significantly associated with levels of plasma B cells (defined as CD24⁻CD38⁺⁺CD27⁺CD19⁺ B cells) in PBC ($r = 0.513$, $p = 0.041$, $n = 16$) (Fig 4 A).

We then studied the influence of expanded Tfh cells on B cells in PBC. Circulating Tfh cells were sorted and co-cultured with allogeneic B cells from healthy donors. To mimic the antigen-specific interaction between T and B cells, SEB was added to the culture. As shown in Fig 4 B, total B cells significantly increased in the co-culture systems comprising patient's CXCR5⁺CD4⁺ T (pTfh) and allogeneic B cells from healthy controls (h'B) compared with that of HCs' CXCR5⁺CD4⁺ T (hTfh) and allogeneic B cells from HCs (h'B) ($59.2 \pm 6.7\%$ vs. $36.5 \pm 7.2\%$, $p < 0.05$) and other groups. B cells may be divided into IgD⁺CD27⁻ naïve B cells, IgD⁺CD27⁺ marginal zone-like B cells, IgD⁻CD27⁺ class-switched memory B cells and plasmablasts (29). We noted that pTfh could promote the differentiation of B cells and induce more class-switched memory B cells and plasmablasts than hTfh cells ($p < 0.05$). Similar results were also found when Tfh derived from PBC patients or HC were co-cultured in the presence of allogeneic patients' B cells (Supplemental Fig 3). In contrast, CXCR5⁺CD4⁺ T cells derived from PBC (pTfh-) or healthy controls (hTfh-), did not significantly promote B cell differentiation.

Sequentially, we investigated helper function of CXCR5⁺CD4⁺ T cells on B cells (Fig 4 C). Co-culture experiments demonstrated that CXCR5⁺CD4⁺ T cells from PBC had a greater potential to promote production of IgG (180.3 ± 22.6 ng/ml vs. 56.6 ± 12.0 ng/ml, $p < 0.01$) and IgM (267.6 ± 47.5 ng/ml vs. 94.2 ± 21.8 ng/ml, $p < 0.01$) compared with CXCR5⁺CD4⁺ T cells from HCs. We further observed that the addition of rIL-21 (20 ng/ml) led to enhanced IgG production (290.3 ± 71.9 ng/ml vs. 180.3 ± 22.6 ng/ml, $p < 0.05$) and IgM (763.0 ± 141.8 vs. 267.6 ± 47.5 ng/ml, $p < 0.05$), respectively. Blockade of IL-21 in the co-cultures with soluble rIL-21R-Fc resulted in decreased IgG production (119.9 ± 39.6 ng/ml vs. 180.3 ± 22.6 ng/ml, $p < 0.05$) and IgM (143.5 ± 33.7 ng/ml vs. 267.6 ± 47.5 ng/ml, $p < 0.05$).

0.05). These results suggest that CXCR5⁺CD4⁺ T cells from PBC have an increased ability to promote immunoglobulin production which, to some extent, is dependent on IL-21.

A decrease in circulating Tfh cells is associated with response to UDCA

Levels of CXCR5⁺CD4⁺ T cells were significantly lower in UDCA responders than those in non-responders ($p = 0.023$, Fig 5 A). Further, there was also a decreased trend of IL-21 production by CXCR5⁺CD4⁺ T cells from UDCA responders ($n = 12$) compared with that from UDCA non-responders ($n = 8$) ($p = 0.103$, Fig 5. B). Consistent with this data, the percentages of circulating CXCR5⁺CD4⁺ T cells were also reduced at 12 months compared with entry data ($p < 0.05$). In contrast, no significant change in circulating CXCR5⁺CD4⁺ T cells was observed in the non-responder group during the 1-year follow-up period (Fig 5 C). In addition, UDCA has a dose-dependent effect on Tfh cells *in vitro*, which partially supports the thesis that it has immunomodulatory function (Supplemental Fig 4).

Discussion

Primary biliary cirrhosis has a number of well-defined abnormalities in lymphoid subpopulations. Firstly, there is a multi-lineage loss of tolerance to the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2), including the presence of autoreactive CD4 (4) and CD8 (3) cells and a highly directed B cell response (33, 34). There is also evidence for defects in innate immunity and the growing recognition that the pathogenesis of disease may involve different lineages of cells at different stages of disease activity, including relative contributions of innate or bystander cells (34–36). Interestingly, the ability to define subpopulations of T cells has contributed to our understanding of immunological interactions, some of which may promote excessive immune responses.

In the present study, we provide evidence of an increased number of circulating CXCR5⁺CD4⁺ T cells in PBC. The origins of peripheral circulating Tfh cells is still not clear. Some studies reported that the circulating Tfh cells shared similar phenotypic and functional properties with GC-Tfh cells in the follicles, indicating circulating Tfh cells derived from GC-Tfh cells and shuffling between peripheral blood and lymphoid tissues (9). More importantly, circulating (CD4⁺/CXCR5⁺) Tfh cells constitute a subset of memory Tfh cells persisting for a long time in blood (37). Upon subsequent antigenic stimulation, these memory cells may quickly form Tfh cells and promote GC responses (38). Uncontrolled generation of circulating Tfh cells may reflect GC dysregulation and play an important role in amplifying autoreactive B cells, promoting pathogenic autoantibody production, the onset of clinical symptoms, continued immune responsiveness, and eventually irreversible tissue damage (39). Our data further reflect the thesis that these cells are actively involved in autoimmune cholangitis, promoting the skewing of B cells towards antibody-secreting plasma cells.

The contribution of Tfh cells to autoimmune diseases was initially studied in murine models that were noted to have expanded Tfh populations (40, 41). Subsequent data in humans with SLE, rheumatoid arthritis and autoimmune thyroid disease also noted a correlation of Tfh levels with not only disease severity, but also with titers of autoantibodies and frequency of circulating GC B cells (11, 12, 14, 39, 42–44). Our study highlight these data but, more

importantly for PBC, potentially provide a novel biomarker to monitor disease activity, levels of circulating Tfh cells. Our data also may potentially distinguish non cirrhotic from cirrhotic patients. Clearly, however, further studies must be done in other liver diseases to further dissect this observation and, in particular, its correlation with advanced fibrosis. Interestingly, there are a higher percentage of Tfh cells in AMA positive versus AMA negative patients, suggesting that this cell population may be specifically involved in the production of autoantibodies. However, we should emphasize that our data is limited in the evaluation of AMA negative patients and will require further study.

We propose that Tfh cells promote the progression of PBC in part by regulating B cell activation, and we provide three pieces of data to support this thesis. First, the levels of circulating CXCR5⁺CD4⁺ T cells positively correlate with the frequency of circulating plasma cells, but not with total B cells. Indeed, it has been reported that CXCR5⁺CD4⁺ T lymphocytes reflect the circulating memory compartment of relevant Tfh cells involved in the germinal center (37, 39, 45). Second, circulating Tfh cells from PBC have a greater ability to promote maturation and antibody production of B cells compared with normal controls. Third, the addition of IL-21 to the co-culture of Tfh and B cells markedly promotes IgM and IgG production, whereas blockade of IL-21 significantly reduced antibody production. These results suggested that circulating Tfh cells induce and maintain autoantibody production through IL-21.

There are several limitations associated with the present study. Firstly, although Tfh cell expansion has been reported to be associated with other autoimmune diseases, the specific phenotype remains unclear. Recently, Tfh cells have been defined as CXCR5⁺CD4⁺, ICOS^{hi}CXCR5⁺CD4⁺ and PD-1^{hi}ICOS^{hi}CXCR5⁺CD4⁺ (11, 37, 39) as well as PD-1⁺CXCR3⁻CXCR5⁺CD4⁺ T cells basing on gene and cytokine profiling which appears to be closer to the true GC Tfh cell population (46). Tfh cells defined on the basis of these new phenotypes should be further investigated. Secondly, we note previous data that PDC-E2 specific T cells (CD4⁺ T (4), and CD8⁺ T (3)) and B cells exist in the blood and liver of patients with PBC. It remains to be defined whether there are any antigen-specific Tfh cells in PBC; however, antigen specific Tfh cells reactive with GP120 or the core protein GAG are found in HIV-infected patients (47) or, in the case of chronic hepatitis B, reactive with rHBcAg 1-183 or rHBcAg 1-149 (48). Future studies should take advantage of overlapping peptide pools corresponding to PDC-E2 to detect whether there is a Tfh-specific recognition motif. Thirdly, although we noted the accumulation of Tfh cells around damaged interlobular bile ducts in the liver and their location in GC-bearing B cell follicles in the spleen, further studies should address a cross-sectional analysis of patients at different stages of the disease. We also noted that the number of subjects in our UDCA study is relatively small and during long-term therapy, UDCA appeared not to alter the proportion of Tfh cells in non-responders. These comments notwithstanding, our results reflect the existence of continuously activated and expanded Tfh cells in PBC. We suggest that Tfh cells interact with localized B cells to form lymphoid follicle-like structures that promote B cells to differentiate into plasmablasts, a key factor associated with the pathogenesis of PBC. These data extend our understanding of the mechanisms of PBC and provide a potential new therapeutic target. Finally, we suggest that qualitative and quantitative studies of Tfh cells may reflect a new biomarker to monitor disease activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PBC	primary biliary cirrhosis
Tfh	follicular helper T cells
CXCR5	Chemokine C-X-C receptor
Bcl-6	B-cell lymphoma 6
PD-1	programmed cell death protein-1
ALP	alkaline phosphatase
AMA	antimitochondrial antibodies
UDCA	ursodeoxycholic acid
ANA	antinuclear antibodies
GC	germinal center
SEB	staphylococcal enterotoxin B
SLE	systemic lupus erythematosus
AIH	autoimmune hepatitis
TBIL	total bilirubin
ALB	albumin
AST	aspartate aminotransferase
GGT	g-glutamyltransferase
ALT	alanine aminotransferase
ULN	upper limit of normal
FITC	Fluorescein isothiocyanate
PE	phycoerythrin
APC	allophycocyanin
PerCP	peridinin chlorophyll protein
PBMC	Peripheral blood mononuclear cells
PMA	phorbol-12-myristate-13-acetate

LIA	line immunoassay
IIF	Indirect immunofluorescence
AEC	3-amino-9-ethyl-carbazole
pTfh	patient's CXCR5 ⁺ CD4 ⁺ T
hTfh	healthy controls' CXCR5 ⁺ CD4 ⁺ T
h'B	allogeneic B cells from healthy control
pTfh-	CXCR5 ⁻ CD4 ⁺ T cells derived from patient
hTfh-	CXCR5 ⁻ CD4 ⁺ T cells derived from healthy controls
HC	healthy control
PDC-E2	E2 subunit of the pyruvate dehydrogenase complex

References

1. Kaplan MM, Gershwin ME. Primary biliary cirrhosis. *N Engl J Med.* 2005; 353:1261–1273. [PubMed: 16177252]
2. Selmi C, Bowlus CL, Gershwin ME, Coppel RL. Primary biliary cirrhosis. *Lancet.* 2011; 377:1600–1609. [PubMed: 21529926]
3. Kita H, Lian ZX, Van de Water J, He XS, Matsumura S, Kaplan M, Luketic V, et al. Identification of HLA-A2-restricted CD8(+) cytotoxic T cell responses in primary biliary cirrhosis: T cell activation is augmented by immune complexes cross-presented by dendritic cells. *J Exp Med.* 2002; 195:113–123. [PubMed: 11781370]
4. Shimoda S, Nakamura M, Ishibashi H, Hayashida K, Niho Y. HLA DRB4 0101-restricted immunodominant T cell autoepitope of pyruvate dehydrogenase complex in primary biliary cirrhosis: evidence of molecular mimicry in human autoimmune diseases. *J Exp Med.* 1995; 181:1835–1845. [PubMed: 7536796]
5. Takahashi T, Miura T, Nakamura J, Yamada S, Miura T, Yanagi M, Matsuda Y, et al. Plasma cells and the chronic nonsuppurative destructive cholangitis of primary biliary cirrhosis. *Hepatology.* 2012; 55:846–855. [PubMed: 22031474]
6. Gershwin ME, Mackay IR, Sturgess A, Coppel RL. Identification and specificity of a cDNA encoding the 70 kd mitochondrial antigen recognized in primary biliary cirrhosis. *J Immunol.* 1987; 138:3525–3531. [PubMed: 3571977]
7. Jin Q, Moritoki Y, Lleo A, Tsuneyama K, Invernizzi P, Moritoki H, Kikuchi K, et al. Comparative analysis of portal cell infiltrates in antimitochondrial autoantibody-positive versus antimitochondrial autoantibody-negative primary biliary cirrhosis. *Hepatology.* 2012; 55:1495–1506. [PubMed: 22135136]
8. Yang CY, Ma X, Tsuneyama K, Huang S, Takahashi T, Chalasani NP, Bowlus CL, et al. IL-12/Th1 and IL-23/Th17 biliary microenvironment in primary biliary cirrhosis: Implications for therapy. *Hepatology.* 2014; 59:1944–1953. [PubMed: 24375552]
9. Zhang X, Ing S, Fraser A, Chen M, Khan O, Zakem J, Davis W, et al. Follicular helper T cells: new insights into mechanisms of autoimmune diseases. *Ochsner J.* 2013; 13:131–139. [PubMed: 23531878]
10. Cannons JL, Lu KT, Schwartzberg PL. T follicular helper cell diversity and plasticity. *Trends Immunol.* 2013; 34:200–207. [PubMed: 23395212]
11. Ma J, Zhu C, Ma B, Tian J, Baidoo SE, Mao C, Wu W, et al. Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis. *Clin Dev Immunol.* 2012; 2012:827480. [PubMed: 22649468]

12. Feng X, Wang D, Chen J, Lu L, Hua B, Li X, Tsao BP, et al. Inhibition of aberrant circulating Tfh cell proportions by corticosteroids in patients with systemic lupus erythematosus. *PLoS One*. 2012; 7:e51982. [PubMed: 23284839]
13. Szabo K, Papp G, Barath S, Gyimesi E, Szanto A, Zeher M. Follicular helper T cells may play an important role in the severity of primary Sjogren's syndrome. *Clin Immunol*. 2013; 147:95–104. [PubMed: 23578551]
14. Zhu C, Ma J, Liu Y, Tong J, Tian J, Chen J, Tang X, et al. Increased frequency of follicular helper T cells in patients with autoimmune thyroid disease. *J Clin Endocrinol Metab*. 2012; 97:943–950. [PubMed: 22188745]
15. Aoki N, Kido M, Iwamoto S, Nishiura H, Maruoka R, Tanaka J, Watanabe T, et al. Dysregulated generation of follicular helper T cells in the spleen triggers fatal autoimmune hepatitis in mice. *Gastroenterology*. 2011; 140:1322–1333. e1321–1325. [PubMed: 21237169]
16. Ma L, Qin J, Ji H, Zhao P, Jiang Y. Tfh and plasma cells are correlated with hypergammaglobulinaemia in patients with autoimmune hepatitis. *Liver Int*. 2013
17. Mells GF, Floyd JA, Morley KI, Cordell HJ, Franklin CS, Shin SY, Heneghan MA, et al. Genome-wide association study identifies 12 new susceptibility loci for primary biliary cirrhosis. *Nat Genet*. 2011; 43:329–332. [PubMed: 21399635]
18. Lindor KD, Gershwin ME, Poupon R, Kaplan M, Bergasa NV, Heathcote EJ. American Association for Study of Liver D. Primary biliary cirrhosis. *Hepatology*. 2009; 50:291–308. [PubMed: 19554543]
19. Alvarez F, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, Chapman RW, et al. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol*. 1999; 31:929–938. [PubMed: 10580593]
20. Manns MP, Czaja AJ, Gorham JD, Krawitt EL, Mieli-Vergani G, Vergani D, Vierling JM, et al. Diagnosis and management of autoimmune hepatitis. *Hepatology*. 2010; 51:2193–2213. [PubMed: 20513004]
21. Corpechot C, Abenavoli L, Rabahi N, Chretien Y, Andreani T, Johanet C, Chazouilleres O, et al. Biochemical response to ursodeoxycholic acid and long-term prognosis in primary biliary cirrhosis. *Hepatology*. 2008; 48:871–877. [PubMed: 18752324]
22. Kuiper EM, Hansen BE, de Vries RA, den Ouden-Muller JW, van Ditzhuijsen TJ, Haagsma EB, Houben MH, et al. Improved prognosis of patients with primary biliary cirrhosis that have a biochemical response to ursodeoxycholic acid. *Gastroenterology*. 2009; 136:1281–1287. [PubMed: 19208346]
23. Shi TY, Zhang LN, Chen H, Wang L, Shen M, Zhang X, Zhang FC. Risk factors for hepatic decompensation in patients with primary biliary cirrhosis. *World J Gastroenterol*. 2013; 19:1111–1118. [PubMed: 23467321]
24. Ludwig J, Dickson ER, McDonald GS. Staging of chronic nonsuppurative destructive cholangitis (syndrome of primary biliary cirrhosis). *Virchows Arch A Pathol Anat Histol*. 1978; 379:103–112. [PubMed: 150690]
25. Fu J, Zhang Z, Zhou L, Qi Z, Xing S, Lv J, Shi J, et al. Impairment of CD4+ cytotoxic T cells predicts poor survival and high recurrence rates in patients with hepatocellular carcinoma. *Hepatology*. 2013; 58:139–149. [PubMed: 22961630]
26. Zhang Z, Zhang S, Zou Z, Shi J, Zhao J, Fan R, Qin E, et al. Hypercytolytic activity of hepatic natural killer cells correlates with liver injury in chronic hepatitis B patients. *Hepatology*. 2011; 53:73–85. [PubMed: 21254163]
27. Zhang JY, Zhang Z, Lin F, Zou ZS, Xu RN, Jin L, Fu JL, et al. Interleukin-17-producing CD4(+) T cells increase with severity of liver damage in patients with chronic hepatitis B. *Hepatology*. 2010; 51:81–91. [PubMed: 19842207]
28. Tripodo C, Petta S, Guarnotta C, Pipitone R, Cabibi D, Colombo MP, Craxi A. Liver follicular helper T-cells predict the achievement of virological response following interferon-based treatment in HCV-infected patients. *Antivir Ther*. 2012; 17:111–118. [PubMed: 22267475]
29. Deneys V, Mazzon AM, Marques JL, Benoit H, De Bruyere M. Reference values for peripheral blood B-lymphocyte subpopulations: a basis for multiparametric immunophenotyping of abnormal lymphocytes. *J Immunol Methods*. 2001; 253:23–36. [PubMed: 11384666]

30. Ma CS, Avery DT, Chan A, Batten M, Bustamante J, Boisson-Dupuis S, Arkwright PD, et al. Functional STAT3 deficiency compromises the generation of human T follicular helper cells. *Blood*. 2012; 119:3997–4008. [PubMed: 22403255]
31. Ma CS, Hare NJ, Nichols KE, Dupre L, Andolfi G, Roncarolo MG, Adelstein S, et al. Impaired humoral immunity in X-linked lymphoproliferative disease is associated with defective IL-10 production by CD4+ T cells. *J Clin Invest*. 2005; 115:1049–1059. [PubMed: 15761493]
32. Chevalier N, Jarrossay D, Ho E, Avery DT, Ma CS, Yu D, Sallusto F, et al. CXCR5 expressing human central memory CD4 T cells and their relevance for humoral immune responses. *J Immunol*. 2011; 186:5556–5568. [PubMed: 21471443]
33. Nakamura M, Ishibashi H, Matsui M, Shimoda S, Hayashida K, Koike K, Niho Y. Peripheral B lymphocyte repertoire to mitochondrial antigen in primary biliary cirrhosis--positive correlation between the disease activity and the frequency of circulating B lymphocytes specific for pyruvate dehydrogenase complex. *Autoimmunity*. 1995; 21:253–262. [PubMed: 8852516]
34. Chuang YH, Lian ZX, Tsuneyama K, Chiang BL, Ansari AA, Coppel RL, Gershwin ME. Increased killing activity and decreased cytokine production in NK cells in patients with primary biliary cirrhosis. *J Autoimmun*. 2006; 26:232–240. [PubMed: 16730427]
35. Kita H, Naidenko OV, Kronenberg M, Ansari AA, Rogers P, He XS, Koning F, et al. Quantitation and phenotypic analysis of natural killer T cells in primary biliary cirrhosis using a human CD1d tetramer. *Gastroenterology*. 2002; 123:1031–1043. [PubMed: 12360465]
36. Zhao J, Zhao S, Zhou G, Liang L, Guo X, Mao P, Zhou X, et al. Altered biliary epithelial cell and monocyte responses to lipopolysaccharide as a TLR ligand in patients with primary biliary cirrhosis. *Scand J Gastroenterol*. 2011; 46:485–494. [PubMed: 21275501]
37. Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, Foucat E, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity*. 2011; 34:108–121. [PubMed: 21215658]
38. Papp G, Szabo K, Szekanecz Z, Zeher M. Follicular helper T cells in autoimmune diseases. *Rheumatology (Oxford)*. 2014
39. Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, Manku H, et al. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum*. 2010; 62:234–244. [PubMed: 20039395]
40. Linterman MA, Rigby RJ, Wong RK, Yu D, Brink R, Cannons JL, Schwartzberg PL, et al. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med*. 2009; 206:561–576. [PubMed: 19221396]
41. Vinuesa CG, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, Yu D, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature*. 2005; 435:452–458. [PubMed: 15917799]
42. Hutloff A, Buchner K, Reiter K, Baelde HJ, Odendahl M, Jacobi A, Dorner T, et al. Involvement of inducible costimulator in the exaggerated memory B cell and plasma cell generation in systemic lupus erythematosus. *Arthritis Rheum*. 2004; 50:3211–3220. [PubMed: 15476242]
43. Liu R, Wu Q, Su D, Che N, Chen H, Geng L, Chen J, et al. A regulatory effect of IL-21 on T follicular helper-like cell and B cell in rheumatoid arthritis. *Arthritis Res Ther*. 2012; 14:R255. [PubMed: 23176102]
44. Terrier B, Costedoat-Chalumeau N, Garrido M, Geri G, Rosenzweig M, Musset L, Klatzmann D, et al. Interleukin 21 correlates with T cell and B cell subset alterations in systemic lupus erythematosus. *J Rheumatol*. 2012; 39:1819–1828. [PubMed: 22859347]
45. Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev*. 2009; 229:152–172. [PubMed: 19426221]
46. Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, Su LF, et al. Human circulating PD-(+)/CXCR3(-)CXCR5(+) memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity*. 2013; 39:758–769. [PubMed: 24035365]

47. Lindqvist M, van Lunzen J, Soghoian DZ, Kuhl BD, Ranasinghe S, Kranias G, Flanders MD, et al. Expansion of HIV-specific T follicular helper cells in chronic HIV infection. *J Clin Invest*. 2012; 122:3271–3280. [PubMed: 22922259]
48. Li Y, Ma S, Tang L, Li Y, Wang W, Huang X, Lai Q, et al. Circulating chemokine (C-X-C Motif) receptor 5(+) CD4(+) T cells benefit hepatitis B e antigen seroconversion through IL-21 in patients with chronic hepatitis B virus infection. *Hepatology*. 2013; 58:1277–1286. [PubMed: 23703545]

Fig 1. Increased frequency of follicular helper T (Tfh) cells in primary biliary cirrhosis

(A) Comparison of the frequencies of total circulating Tfh in PBC, AIH and healthy controls (HCs). PBMC from PBC (n = 35), AIH (n = 16) and HCs (n = 20) were stained with labeled antibodies. Representative expressions of CXCR5⁺CD4⁺ (and ICOS^{high} CXCR5⁺CD4⁺ or PD-1^{high} CXCR5⁺CD4⁺) versus CD4 expression were detected by flow cytometry. Representative dot plots are shown in the right panel. (B) Intrahepatic double positive Bcl-6 (blue) and PD-1 (red) cells were found around chronic non-suppurative destructive cholangitis (CNSDC) in PBC (n = 24), but not in HCs. The white arrow indicates the Bcl-6⁺PD-1⁺ Tfh cells.

Fig 2. Peripheral Tfh cells correlate with disease severity

(A) Higher frequencies of circulating Tfh cells were found in AMA⁺ PBC (n = 29) than in AMA⁻ patients (n = 6), $p < 0.05$. (B) There was a higher frequency of circulating Tfh cells based on disease severity. (C) Circulating Tfh cells were higher in late stages (II, III and IV) compared with early stage (I). Horizontal lines illustrate the median percentiles. P values are shown.

Fig 3. Cytokine production by CXCR5⁺CD4⁺ T cells

(A) Representative results reflect cytokine production by CXCR5⁺CD4⁺ T cells (upper part). CXCR5⁺CD4⁺ T cells release more IL-21, IFN- and IL-17 in PBC (n = 24) than in HCs (n = 10) (lower part) when stimulated with PMA/ionomycin. *: $p < 0.05$; **: $p < 0.01$, respectively. (B) CXCR5⁺CD4⁺ T cells produced higher levels of IL-21 than IL-6 and IL-17 compared with CXCR5⁻CD4⁺ T cells; while, CXCR5⁻CD4⁺ T cells predominantly produced IFN- γ . PBMCs from 24 patients were analyzed. P values are shown. (C) Correlation between the frequency of CXCR5⁺CD4⁺ T cells and IL-21 secretion from 24 PBC patients when stimulated with PMA/ionomycin ($p < 0.05$).

Fig 4. Increased Tfh cells correlate with plasma cell proportions and affect B cell function in PBC

(A) Percentages of CXCR5⁺CD4⁺ T cells correlated with the percentage of CD24⁻CD38⁺⁺CD27⁺CD19⁺ plasma cells in 16 treatment-naïve PBC patients ($r = 0.513$, $p < 0.05$), but were not correlated with total B cells. (B) Patient's CXCR5⁺CD4⁺ T (pTfh) promoted more proliferation and differentiation of allogeneic B cells compared with that of HCs' CXCR5⁺CD4⁺ T (hTfh) and CXCR5⁺CD4⁺ T cells derived from PBC (pTfh⁻) or healthy controls (hTfh⁻). *: $p < 0.05$; **: $p < 0.01$, respectively. (C) CXCR5⁺CD4⁺ T fh from PBC (pTfh) promoted higher production of IgG and IgM compared with CXCR5⁺CD4⁺ T fh from HCs (hTfh). Exogenous IL-21 or blockade of IL-21 with soluble rIL-21R-Fc resulted in significantly increased or decreased immunoglobulin production by co-cultured B cells, respectively. Five PBC patients and four HCs were studied. Representative data are from three independent experiments.

Fig 5. Decreased proportion of circulating Tfh cells is detected in UDCA responders

(A) The frequency of CXCR5⁺CD4⁺ T cells in UDCA responders (n = 14) was significantly lower than that of UDCA non-responders (n = 14). $p < 0.05$ (B) Comparison of IL-21 secretion by CXCR5⁺CD4⁺ T cells between UDCA responders (n = 12) and non-responders (n = 8). There was a decreased trend of IL-21 production by CXCR5⁺CD4⁺ T cells from UDCA responders compared with those from UDCA non-responders. (C) After 1 year of follow-up, there were 10 UDCA responders and 8 non-responders. The percentages of circulating CXCR5⁺CD4⁺ T cells were reduced at 12 months compared with entry data in the UDCA responders group. In contrast, no significant changes were observed in the non-responders group during the 1-year follow-up period. P values are shown.

Table 1

Clinical features of PBC patients included in the study

	PBC (n = 69)		AIH (n = 16)	HC (n = 20)
	Naïve (n = 41)	UDCA treatment (n = 28)		
Age	53 (28–74)	55 (21–71)	48 (30–67)	48.5 (24–70)
Gender (F/M)	31/10	26/2	14/2	12/8
ALT (U/L)	59 (9–378)	30 (14–182)	99.5 (19–581)	19.5 (9–57)
AST (U/L)	86 (17–272)	64.5 (22–334)	158.5 (34–652)	23 (13–50)
ALP (U/L)	292 (91–962)	228 (92–701)	153 (47–882)	88 (56–149)
GGT (U/L)	290 (22–1484)	123.5 (14–845)	73.5 (21–498)	34.5 (20–51)
TBIL (μmol/L)	26.1 (6.3–597)	21.9 (3.5–122.5)	37.7 (2.1–446.1)	12.3 (4.2–21)
Albumin (g/L)	35 (23–62)	34 (21–43)	31 (20–37)	43 (32–51)
AMA (+) / (-)	34/7	25/3	-	-
UDCA response (R/N)	-	14/14	-	-
Clinical stage				
Non-cirrhotic	18	9	6	-
Cirrhotic	8	10	5	-
Decompensated cirrhotic	9	8	5	-
Peripheral blood usage	35	28	16	20
Liver Biopsy usage	26	-	-	5
Stage I	8	-	-	-
Stage II	6	-	-	-
Stage III	6	-	-	-
Stage IV	6	-	-	-

F/M, Female and Male; R/N, responders and non-responders.