Received: 7 May 2018 | Revised: 8 October 2018 | Accepted: 9 October 2018

DOI: 10.1111/cas.13836

ORIGINAL ARTICLE

WILEY Cancer Science

Functionally impaired follicular helper T cells induce regulatory B cells and CD14$^+$ human leukocyte antigen-DR$^-$ cell differentiation in non-small cell lung cancer

Liannv Qiu[1] | Qinhua Yu[1] | Yonglie Zhou[1] | Sujie Zheng[1] | Jiaojiao Tao[2] | Qian Jiang[3] | Guorong Yuan[4]

[1]Department of Clinical Laboratory, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, Hangzhou, China

[2]School of Laboratory Medicine and Life Science, Wenzhou Medical University, Wenzhou, China

[3]College of Medical Technology, Zhejiang Chinese Medical University, Hangzhou, China

[4]Department of Oncology, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, Hangzhou, China

Correspondence
Liannv Qiu, Department of Clinical Laboratory, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, Hangzhou, China.
Email: qlv2012@126.com

Funding information
Zhejiang Provincial Natural Science Fund, Grant/Award Number: No: Y18H200014; Zhejiang Provincial Program for the Cultivation of High-level Innovative Health Talents, Grant/Award Number: No: 2012

Non-small cell lung cancer (NSCLC) represents one of the most common and aggressive cancers worldwide, as it typically displays irreversible progression and poor prognosis. Interaction between programmed death 1 (PD-1) and its ligand, PD-L1, plays important roles in tumor immunology. Follicular helper T (Tfh) cells have characteristically high PD-1 expression; thus, in the present study, we investigated the role of circulating Tfh cells and their correlation with disease-free survival after tumor resection in NSCLC. We found significantly higher number of Tfh cells but lower serum interleukin (IL)-21 levels in NSCLC patients, especially in those with advanced stage (III and IV), indicating that the function of Tfh cells to produce IL-21 was impaired. Further analysis showed that the increase in Tfh cells was attributable to an expansion of the PD-1$^+$-Tfh2 and PD-1$^+$-Tfh17 subtypes. Functional analysis showed that Tfh cells from NSCLC patients induced the differentiation of regulatory B cells and CD14$^+$ human leukocyte antigen (HLA)-DR$^-$ cells. Interestingly, the number of Tfh1 subtypes in NSCLC patients was negatively correlated with disease-free survival after tumor resection. In short, the high number and abnormal function of Tfh cells could cause further immunosuppression and lead to tumor development in NSCLC. Rescuing Tfh functions therefore represents a potential therapeutic strategy in NSCLC.

KEYWORDS
CD14$^+$ HLA-DR$^-$, Follicular helper T cell, interleukin-21, non-small cell lung cancer, regulatory B cell

1 | INTRODUCTION

Lung cancer is the leading cause of cancer-related death worldwide.[1] Non-small cell lung cancer (NSCLC) accounts for approximately 75% of lung cancers, is usually diagnosed at advanced stages and has a 5-year survival rate of <5%.[2] Targeted therapy improves the survival rate for advanced NSCLC, although this only benefits a subset of patients.[3]

Studies have shown that the interaction between programmed death 1 (PD-1) and its ligand, PD-L1, inhibit immune responses in tumors. PD-1/PD-L1 checkpoint inhibitors show impressive antitumor activity in NSCLC.[4-8] The recently defined follicular helper T (Tfh) cells express characteristically high PD-1, which is a common mediator of immunosuppression in multiple cancers,[9,10] but it is not clear whether Tfh cells can also inhibit immune responses in NSCLC.

Follicular helper T cells coexpress high levels of surface C-X-C motif chemokine receptor 5 (CXCR5) and inducible costimulator (ICOS), with Bcl-6 as their distinguishing transcription factor and interleukin (IL)-21 as their characteristic cytokine.[11-13] Both peripheral circulating and germinal center (GC) Tfh cells support B-cell activation, expansion and differentiation into plasma cells and memory B cells in GC, thereby promoting B-cell immunity.[13-15] Human circulating Tfh cells are divided into three major functional subtypes (Tfh1, Tfh2, and Tfh17),[16] with skewing to Tfh2 and Tfh17 subtypes observed in autoimmune disease and several tumors.[17,18]

Recent studies have suggested that Tfh cells and IL-21 regulate humoral immunity and cell-mediated antiviral/tumor responses in some infectious diseases and tumors.[19,20] Breast cancer patients with higher Tfh cell infiltrates in the tumor show better survival over those with lower Tfh cell infiltration.[21] However, we do not know whether changes in circulating Tfh cells in NSCLC are associated with tumor development, progression and, eventually, clinical prognosis.

In the present study, we aimed to investigate the levels of circulating Tfh cells in the peripheral blood of newly diagnosed NSCLC patients to explore their role in the pathogenesis of NSCLC and to show their predictive value for disease-free survival.

2 | MATERIALS AND METHODS

2.1 | Patients

From June 2015 to March 2017, 57 newly diagnosed NSCLC patients (34 males and 23 females; median age 60.48 years; range 38 to 81 years) at Zhejiang Provincial People's Hospital before any systemic or local treatment was given were enrolled in the present study. Of all 57 NSCLC patients, 14, 12, 15, and 16 were classified into stages I, II, III and IV, respectively, based on criteria described in the seventh edition International Association for the Study of Lung Cancer (IASLC) TNM staging system. Detailed clinical characteristics of NSCLC patients are shown in Table 1. We also recruited 20 age-matched healthy subjects (HS) from the physical examination department. The study was approved by the Zhejiang Provincial People's Hospital Review Board. All methods were carried out in accordance with the 1975 Declaration of Helsinki. All tumor specimens in the pathological analysis were obtained with informed consent with approval by the ethics committee of our hospital.

2.2 | Cell isolation

Peripheral blood mononuclear cells were isolated from blood samples using a Ficoll-Hypaque density gradient centrifugation method. Serum samples were obtained by centrifugation at 1500 *g* for 10 minutes and were immediately stored at −80°C. Serum IL-21 was assessed using ELISA (eBioscience, San Diego, CA, USA).

$CD4^+CXCR5^+ICOS^+PD\text{-}1^+$ Tfh cells, $CD19^+IgD^+$ naive B cells, and $CD14^+HLA\text{-}DR^-$ cells from six HS and six NSCLC patients were purified using a FACSAria III Aria cell sorter (Becton Dickinson, Sparks, MD, USA) based on the expression of CD4, CXCR5, ICOS, and PD-1 or CD19 and IgD or CD14 and HLA-DR. Cell purity was confirmed to be >95% by flow cytometry.

TABLE 1 Clinical characteristics of NSCLC patients and HS

	NSCLC	HS
Gender	57	20
Male	34	12
Female	23	8
Years (range)	60.48 ± 12.46	60.36 ± 12.07
>60	29	9
≤60	28	11
Histology		
Adenocarcinoma	31	NA
Squamous	12	NA
Other types	14	NA
TNM stage		
I	14	NA
II	12	NA
III	15	NA
IV	16	NA
PD-L1, n		
Positive	25	NA
Negative	32	NA
White blood cell ($\times10^9$/L)	6.95 ± 2.31	6.00 ± 0.97
Lymphocyte ($\times10^9$/L)	1.76 ± 0.52	2.21 ± 0.50

HS, healthy subjects; NA, not applicable; NSCLC, non-small cell lung cancer; PD-L1, programmed death ligand 1.

2.3 | Flow cytometry analysis

The following cell surface antibodies were used: PerCP-CD3 (clone SK7; BD Biosciences, San Diego, CA, USA), PC7-CD4 (clone 13B8.2; Beckman Coulter, Marseille cedex, France), Alexa Fluor 488-CXCR5 (clone RF8B2; BD Biosciences), APC-ICOS (clone ISA-3; BD Biosciences), PerCP-cy7-PD-1 (clone H12.1; BD Biosciences), APC-CXCR3 (clone IC6; BD Biosciences), PerCP-cy5.5-CCR6 (clone 11A9; BD Biosciences), FITC-CD19 (clone J4.119; Beckman Coulter) and PE-CD14 (clone RMO52; Beckman Coulter). After cells were incubated with cell surface antibodies for 30 minutes at 4°C in the dark, they were washed with PBS and then analyzed by flow cytometer. $CD4^+CXCR5^+ICOS^+PD\text{-}1^+$ Tfh cells were identified based on ICOS and PD-1 expression after cells were gated on $CD3^+CD4^+CXCR5^+$ (Figure S1). Tfh subtypes were determined according to CXCR3 and CCR6 expression after cells were gated on $CD3^+CD4^+CXCR5^+$ (Figure S1) and the PD-1 expression of the three subtypes was further analyzed.

For the detection of intracellular cytokines following cell surface staining, cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) and then stained using PE-IL-10 (clone JES5-19F1; BD Biosciences) and PE-cy7-TGF-β (clone TW4-9E7; BD Biosciences) or Alexa Fluor 488-TNF-α (clone MAb11; BD Biosciences). Stained cells were then analyzed using a FACS Canto II flow cytometer and Diva software (Becton Dickinson). All staining was carried out according to the manufacturer's protocol.

2.4 | Function analyses of Tfh cells

Isolated Tfh cells (1.5×10^4) were cultured either alone or 1:1 with purified $CD19^+IgD^+$ cells (1.5×10^4) in complete RPMI 1640 containing L-glutamine, $NaHCO_3$, 10% FCS and penicillin/streptomycin (100 U/mL) in 96-well U-bottom plates in the presence of 2 μg/mL Staphylococcal Exterotoxin B (SEB) for 72 hours, with PIB (phorbol-12-myristate-13-acetate + ionomycin + brefeldin A) added in the last 5 hours, as described elsewhere. Cells were then stained with FITC-CD19, permeabilized, stained intracellularly with PE-IL-10 and PE-cy7-transforming growth factor beta (TGF-β) and analyzed by flow cytometry. The supernatant was harvested for IL-10 and TGF-β detection.

Isolated Tfh cells (1.5×10^4) were cultured either alone or 1:1 with purified $CD14^+HLA\text{-}DR^-$ cells (1.5×10^4) in complete RPMI 1640 containing L-glutamine, $NaHCO_3$, 10% FCS and penicillin/streptomycin (100 U/mL) in 96-well U-bottom plates for 72 hours, with PIB added in the last 5 hours as described elsewhere. Cells were then stained with PE-CD14, permeabilized, stained intracellularly with Alexa Fluor 488-tumor necrosis factor (TNF)-α and analyzed by flow cytometry. Supernatant TNF-α levels were examined by ELISA.

2.5 | Enzyme-linked immunosorbent assay

Human IL-21, IL-10, TGF-β and TNF-α ELISA Ready-Set-Go Kits (eBioscience) were used to examine cytokine levels following instructions provided by the manufacturer.

2.6 | Immunohistochemistry for PD-L1

All measurements for PD-L1 were obtained according to the immunohistochemistry (IHC) protocols provided by the manufacturers. All IHC results were checked independently by two pathologists. The cutoff for PD-L1 expression on tumor cells (Dako, 22C3, Copenhagen, Denmark; approved by the FDA) was equal to or more than 50% staining.

2.7 | Statistical analysis

Statistical analysis was carried out with GraphPad Prism 5.01 software (GraphPad Software Inc., San Diego, CA, USA). The statistical tests used for data analysis included the Mann-Whitney *U* test and the Pearson test for correlation analysis. Quantitative data are presented as the mean values ± standard deviations (SD). Differences were considered to be statistically significant at values of $P < .05$ and $P < .01$.

3 | RESULTS

3.1 | Elevated numbers of Tfh cells and skewing to PD-1^+-Tfh2 and PD-1^+-Tfh17 subtypes in NSCLC patients

Compared to HS, a significantly higher frequency and number of circulating $CD4^+PD\text{-}1^+$, Tfh cells were observed in NSCLC patients (Figure 1A,B), but there was no significant difference in the frequency and number of $CD4^+CXCR5^+$ cells between the two groups (Figure 1A,B). Because Tfh cells can be divided into Tfh1 ($CXCR3^+CCR6^-$), Tfh2 ($CXCR3^-CCR6^-$) and Tfh17 ($CXCR3^-CCR6^+$) subtypes (Figure 1C,D), we further compared the prevalence of these three subtypes in NSCLC patients. Tfh2 and Tfh17 subtypes were significantly elevated in NSCLC patients, whereas Tfh1 subtypes were decreased in NSCLC patients (Figure 1C,D).

We also investigated PD-1 expression of three Tfh subtypes in HS and NSCLC patients and found higher mean fluorescence intensity (MFI) of PD-1 in Tfh1 than in Tfh2 and Tfh17, and higher MFI of PD-1 in Tfh1 in NSCLC patients than in HS (Figure 1E,F). However, we further compared PD-1 expression of three Tfh subtypes in NSCLC patients with those in HS and found that the frequency and the number of PD-1^+-Tfh2 and PD-1^+-Tfh17 in NSCLC patients were higher than those in HS (Figure 1G). These data suggest that circulating Tfh cells, especially PD-1^+-Tfh2 and PD-1^+-Tfh17 subtypes, expand in NSCLC and indicate a potential involvement of Tfh cells in NSCLC.

Non-small cell lung cancer patients can be classified into stage I, stage II, stage III and stage IV according to the TNM classification system, with severity increasing from stage I to stage IV. Thus, we further analyzed the numbers of circulating Tfh cells in NSCLC patients with different stages. Our data showed lower numbers of $CD4^+PD\text{-}1^+$ (Figure 1I), Tfh cells (Figure 1J), Tfh2 (Figure 1L) and Tfh17 subtypes (Figure 1M) but a higher number of Tfh1 subtypes (Figure 1K) in NSCLC patients with early stage (I and II) relative to those with advanced stage (III and IV); however, there was no significant difference in the number of $CD4^+CXCR5^+$ cells in NSCLC with different stages (Figure 1H).

3.2 | Decreased serum IL-21 levels and its negative association with circulating Tfh cells in NSCLC patients

Interleukin-21 is produced mainly by Tfh cells. Although the number of Tfh cells was elevated in NSCLC patients, in the present study, we observed decreased serum IL-21 levels (Figure 2A), especially in those with advanced stage (III and IV; Figure 2B). Moreover, IL-21 levels were significantly and negatively correlated with the number of circulating Tfh cells in NSCLC patients (Figure 2C). We further analyzed IL-21 levels relative to Tfh subtypes and found that the number of Tfh17 subtypes was negatively correlated with IL-21 levels (Figure 2F), whereas the number of Tfh1 and Tfh2 subtypes were not correlated with IL-21 levels in NSCLC patients (Figure 2D,E). These results indicated that the function of Tfh cells to produce IL-21 could be impaired in NSCLC patients.

3.3 | Follicular helper T cells from NSCLC patients induce differentiation of regulatory B cells and $CD14^+HLA\text{-}DR^-$ cells

We know that interaction of PD-1 and PD-L1 inhibits immune responses in tumors. Our previous study found high numbers of

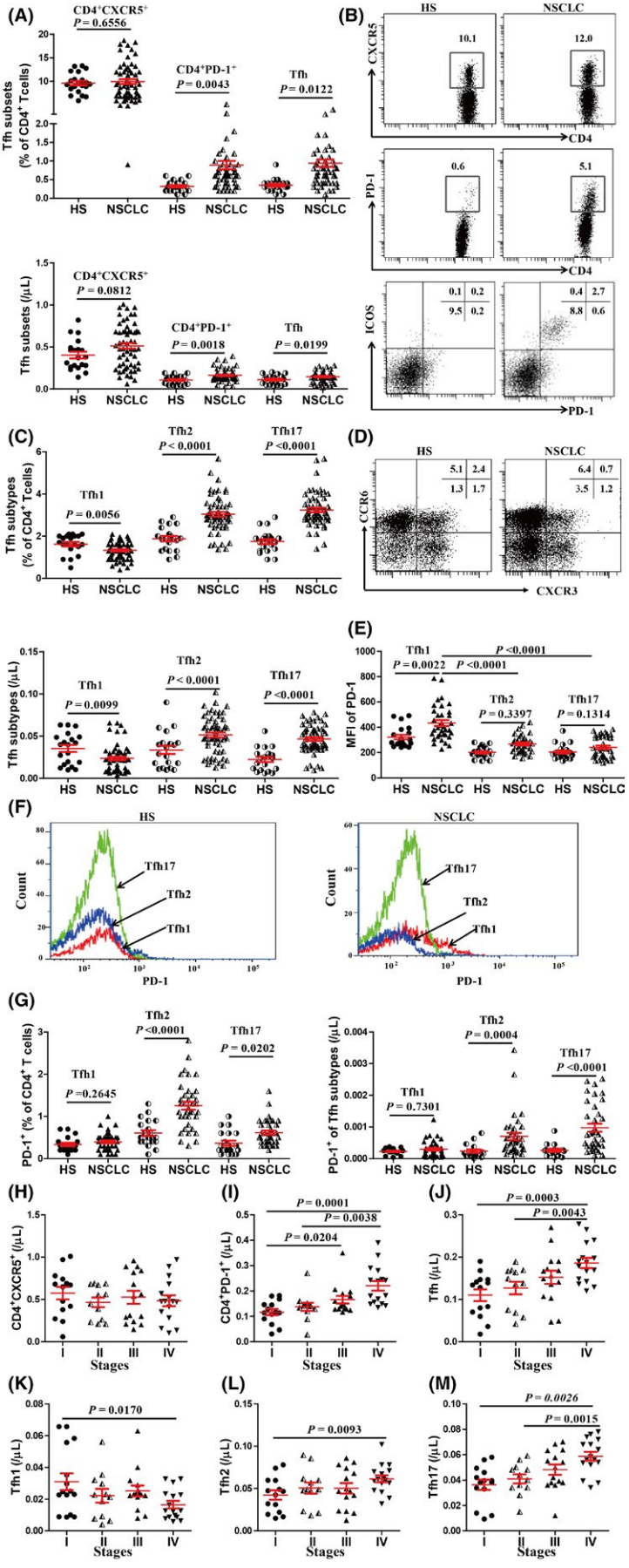
(A)
CD4+CXCR5+
P = 0.6556
CD4+PD-1+
P = 0.0043
Tfh
P = 0.0122
Tfh subsets (% of CD4+ Tcells)
HS NSCLC HS NSCLC HS NSCLC
CD4+CXCR5+
P = 0.0812
CD4+PD-1+
P = 0.0018
Tfh
P = 0.0199
Tfh subsets (/μL)
(B)
HS
NSCLC
10.1
12.0
CXCR5
CD4
0.6
5.1
PD-1
CD4
0.1 0.2
9.5 0.2
0.4 2.7
8.8 0.6
ICOS
PD-1
(C)
Tfh1
P = 0.0056
Tfh2
P < 0.0001
Tfh17
P <0.0001
Tfh subtypes (% of CD4+ Tcells)
HS NSCLC HS NSCLC HS NSCLC
(D)
HS
NSCLC
5.1 2.4
1.3 1.7
6.4 0.7
3.5 1.2
CCR6
CXCR3
(E)
Tfh1
P = 0.0099
Tfh2
P < 0.0001
Tfh17
P <0.0001
Tfh subtypes (/μL)
HS NSCLC HS NSCLC HS NSCLC
Tfh1
P = 0.0022
P <0.0001
Tfh2
P = 0.3397
Tfh17
P = 0.1314
MFI of PD-1
HS NSCLC HS NSCLC HS NSCLC
(F)
HS
NSCLC
Tfh17
Tfh2
Tfh1
Count
PD-1
(G)
Tfh1
P =0.2645
Tfh2
P <0.0001
Tfh17
P = 0.0202
PD-1+ (% of CD4+ T cells)
HS NSCLC HS NSCLC HS NSCLC
Tfh1
P = 0.7301
Tfh2
P = 0.0004
Tfh17
P <0.0001
PD-1+ of Tfh subtypes (/μL)
HS NSCLC HS NSCLC HS NSCLC
(H)
CD4+CXCR5+ (/μL)
Stages
(I)
P = 0.0001
P = 0.0038
P = 0.0204
CD4+PD-1+ (/μL)
Stages
(J)
P = 0.0003
P = 0.0043
Tfh (/μL)
Stages
(K)
P = 0.0170
Tfh1 (/μL)
Stages
(L)
P = 0.0093
Tfh2 (/μL)
Stages
(M)
P = 0.0026
P = 0.0015
Tfh17 (/μL)
Stages

FIGURE 1 Elevated follicular helper T (Tfh) cells and skew to Tfh1 and Tfh17 subtypes in non-small cell lung cancer (NSCLC) patients. A, Frequency in $CD4^+$ T cells and number of $CD4^+CXCR5^+$, $CD4^+PD\text{-}1^+$ and Tfh cells in healthy subjects (HS) (n = 20) and NSCLC patients (n = 57) are shown in the top and bottom panels, respectively. B, Representative dot plots of $CD4^+CXCR5^+$ (top panel), $CD4^+PD\text{-}1^+$ (middle panel), Tfh cells (bottom panel) from one HS and one NSCLC patient are shown. Numbers indicate frequency of cells among $CD4^+$ T cells. C, Frequency in $CD4^+$ T cells and number of Tfh1, Tfh2 and Tfh17 subtypes are shown in the top and bottom panels, respectively. D, Representative dot plots of Tfh1, Tfh2 and Tfh17 subtypes from one HS and one NSCLC patient are shown. E, Mean fluorescence intensity (MFI) of $PD\text{-}1^+$ in Tfh1, Tfh2 and Tfh17 subtypes is shown. F, Representative flow cytometric plots of PD-1 in Tfh1, Tfh2 and Tfh17 subtypes from one HS and NSCLC patient are shown in the left and right panels, respectively. G, Frequency in $CD4^+$ T cells and number of PD-1 in Tfh1, Tfh2 and Tfh17 are shown in the left and right panels, respectively. H-M, Differences in the number of $CD4^+CXCR5^+$ (H), $CD4^+PD\text{-}1^+$ (I), Tfh (J), Tfh1 (K), Tfh2 (L) and Tfh17 subtypes (M) in NSCLC patients with different stages. CXCR5, C-X-C motif chemokine receptor 5; PD-1, programmed death 1

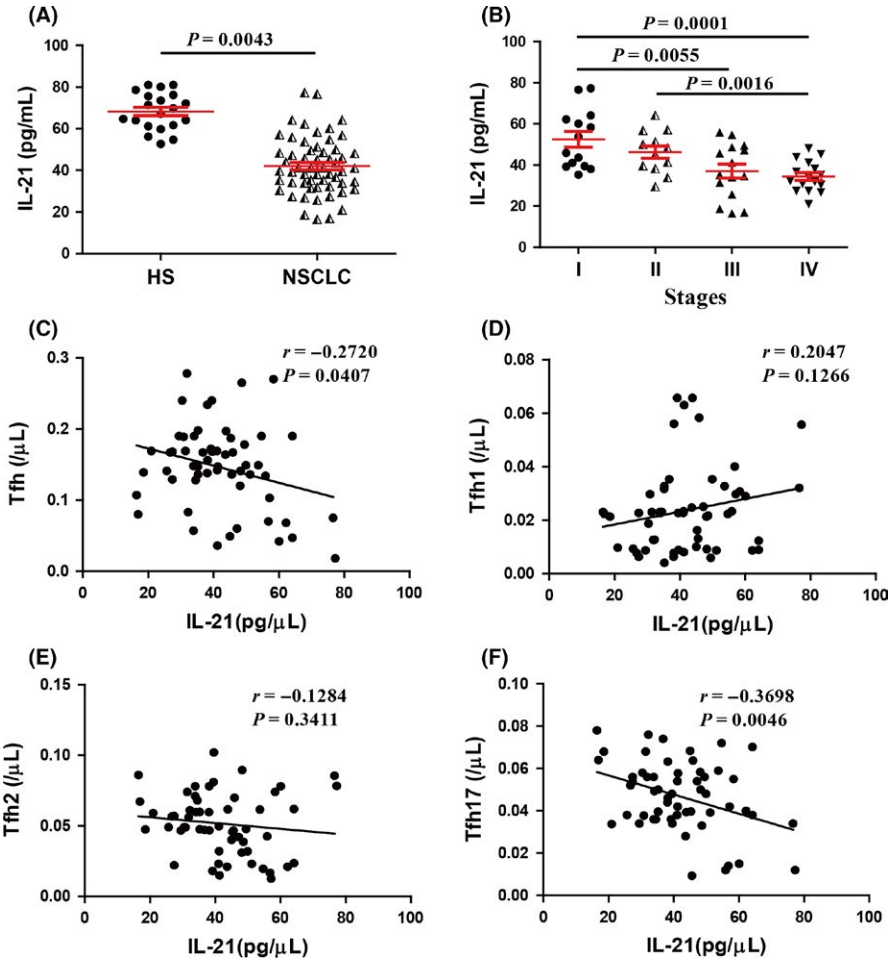

FIGURE 2 Correlation of serum interleukin (IL)-21 levels with circulating follicular helper T (Tfh) cells in non-small cell lung cancer (NSCLC) patients. A, Serum IL-21 levels were measured for healthy subjects (HS) (n = 20) and NSCLC patients (n = 57). B, IL-21 levels in NSCLC patients with different stages. C-F, Correlation of IL-21 levels with the number of Tfh, Tfh1, Tfh2 and Tfh17 subtypes in NSCLC patients, respectively

$IL\text{-}10^+$ regulatory B cells and $CD14^+HLA\text{-}DR^{low/-}$ myeloid-derived suppressor cells (MDSC) in NSCLC patients[22] and, thus, in the present study, we investigated the functional effect of Tfh cells on $CD19^+IgD^+$ naïve B cells and $CD14^+HLA\text{-}DR^-$ cells. Tfh cells were sorted from six HS and six NSCLC patients and were cocultured 1:1 with autologous $CD19^+IgD^+$ naïve B cells in the presence of SEB. After 72 hours, we observed a slightly higher frequency of IL-10- and TGF-β-producing $CD19^+$ B cells (Figure 3A-C) and supernatant IL-10 and TGF-β levels (Figure 3D,E) when cocultured with Tfh cells compared to $CD19^+IgD^+$ cells alone from NSCLC patients.

Similarly, after coculture with Tfh cells from NSCLC patients, a higher, statistically significant frequency of TNF-α-producing $CD14^+HLA\text{-}DR^-$ cells (Figure 3F,G) and supernatant TNF-α levels (Figure 3H) was observed when compared to $CD14^+HLA\text{-}DR^-$ cells alone. However, coculture with Tfh cells from HS had no significant effect on the cytokine production of $CD19^+IgD^+$ naïve B cells and $CD14^+HLA\text{-}DR^-$ cells (Figure 3).

3.4 | High expression of PD-L1 on tumor cells is significantly correlated with high numbers of circulating Tfh cells in NSCLC patients

Non-small cell lung cancer is known to upregulate PD-L1 expression, and high PD-L1 expression is an independent predictor of poor prognosis.[23,24] In the present study, we found that 32 NSCLC samples (56.1%) stained negative for PD-L1, and 25 NSCLC samples (43.9%) stained positive for PD-L1 (Figure 4A,B).

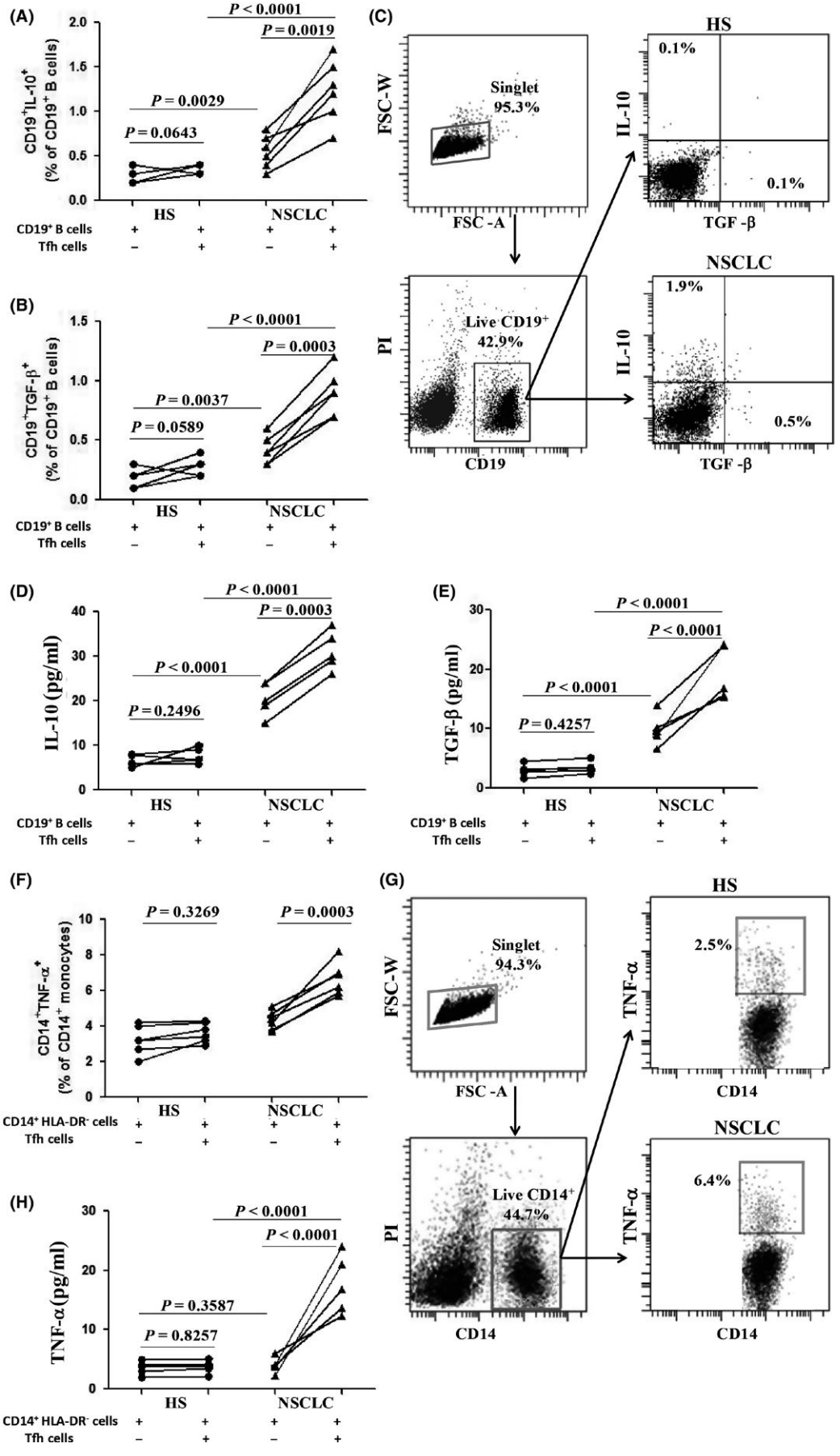
(A)
CD19+IL-10+ (% of CD19+ B cells)
P < 0.0001
P = 0.0019
P = 0.0029
P = 0.0643
HS
NSCLC
CD19+ B cells
Tfh cells
(B)
CD19+TGF-β+ (% of CD19+ B cells)
P < 0.0001
P = 0.0003
P = 0.0037
P = 0.0589
(C)
HS
Singlet 95.3%
FSC-W
FSC-A
IL-10
TGF-β
0.1%
0.1%
NSCLC
Live CD19+ 42.9%
PI
CD19
1.9%
0.5%
(D)
IL-10 (pg/ml)
P < 0.0001
P = 0.0003
P < 0.0001
P = 0.2496
(E)
TGF-β (pg/ml)
P < 0.0001
P < 0.0001
P < 0.0001
P = 0.4257
(F)
CD14+TNF-α+ (% of CD14+ monocytes)
P = 0.3269
P = 0.0003
CD14+ HLA-DR- cells
Tfh cells
(G)
Singlet 94.3%
TNF-α
CD14
2.5%
Live CD14+ 44.7%
6.4%
(H)
TNF-α (pg/ml)
P < 0.0001
P < 0.0001
P = 0.3587
P = 0.8257

FIGURE 3 Follicular helper T (Tfh) cells from non-small cell lung cancer (NSCLC) patients induce differentiation of regulatory B cells and $CD14^+HLA\text{-}DR^-$ cells. FACS-sorted Tfh cells were cultured 1:1 with $CD19^+IgD^+$ naïve B cells or $CD14^+HLA\text{-}DR^-$ monocytes from healthy subjects (HS) (n = 6) and NSCLC patients (n = 6). Intracellular levels of interleukin (IL)-10 and transforming growth factor (TGF)-β among $CD19^+$ B cells and tumor necrosis factor (TNF)-α among $CD14^+$ monocytes were measured by flow cytometry. Supernatant cytokines were measured by ELISA. A-B, Graphs show the frequency of $CD19^+IL\text{-}10^+$ and $CD19^+TGF\text{-}\beta^+$ before or after $CD19^+IgD^+$ naïve B cells cocultured with Tfh cells. C, Representative dot plots show the gating strategy and the frequency of live $CD19^+$ B cells, $CD19^+CD10^+$ and $CD19^+TGF\text{-}\beta^+$ from a single HS (top panel) and NSCLC patient (bottom panel). All the B cell subsets were analyzed after gating on live $CD19^+$ cells. D-E, Supernatant IL-10 and TGF-β levels from HS and NSCLC patients. F, Graphs show the frequency of $CD14^+TNF\text{-}\alpha^+$ before and after $CD14^+HLA\text{-}DR^-$ monocytes cocultured with Tfh cells. G, Representative dot plots show the gating strategy and the frequency of live $CD14^+$ cells, $CD14^+TNF\text{-}\alpha^+$ from a single HS (top panel) and NSCLC patient (bottom panel). $CD14^+TNF\text{-}\alpha^+$ were analyzed after gating on live $CD14^+$ cells. H, Supernatant TNF-α levels from HS and NSCLC patients. HLA-DR, human leukocyte antigen-DR

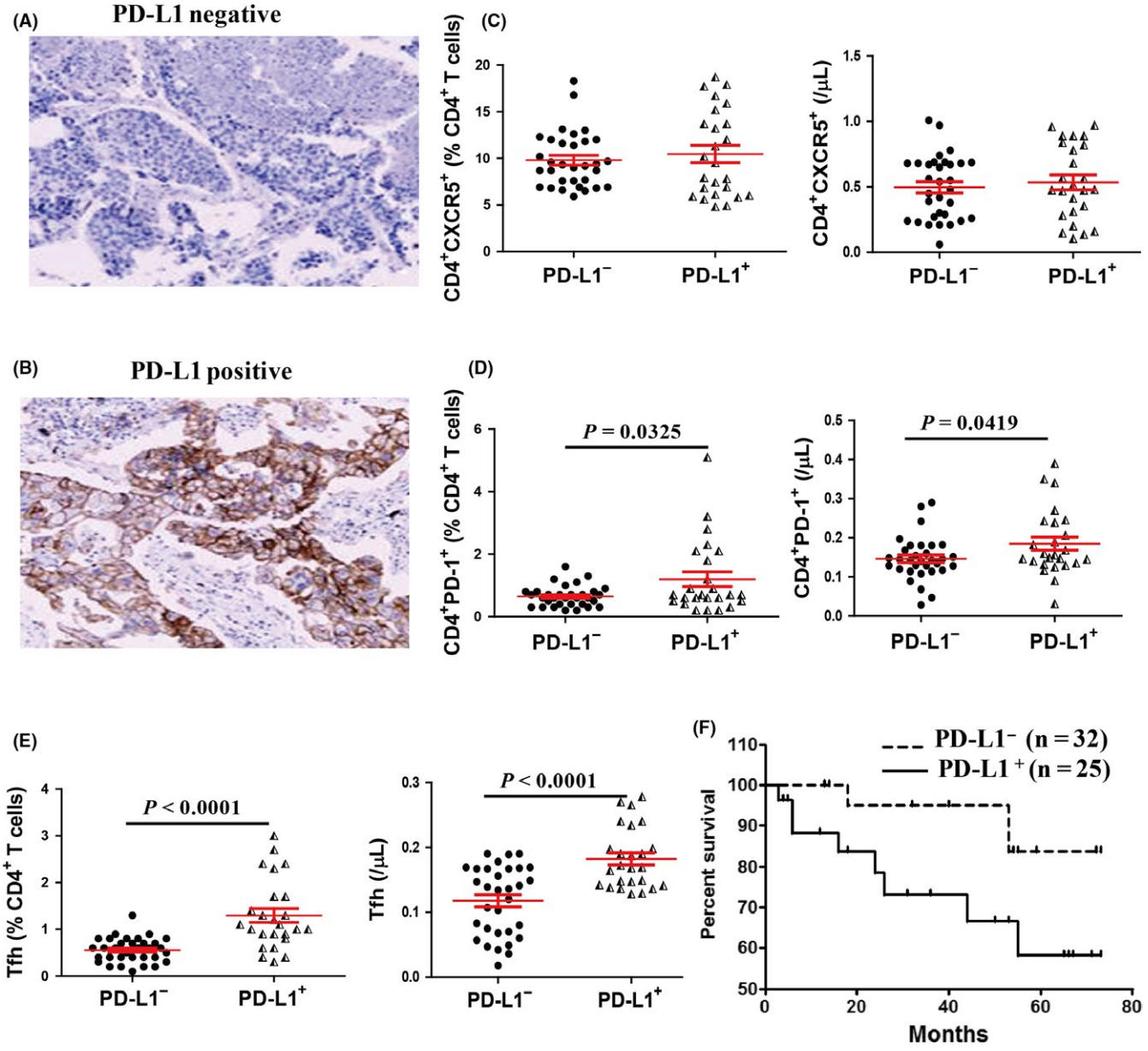

FIGURE 4 Non-small cell lung cancer (NSCLC) tumor cell programmed death ligand 1 (PD-L1) expression and its association with circulating follicular helper T (Tfh) cells. A, Negative cell surface staining (brown) and B, positive PD-L1 staining are shown for NSCLC tumor cells (20×). C-E, Frequency and number of circulating $CD4^+CXCR5^+$, $CD4^+PD\text{-}1^+$ and Tfh cells between patients with PD-L1-negative and -positive tumors are shown in the left and right panels, respectively. F, Disease-free survival in patients with PD-L1-negative and -positive tumors. CXCR5, C-X-C motif chemokine receptor 5; PD-1, programmed death 1

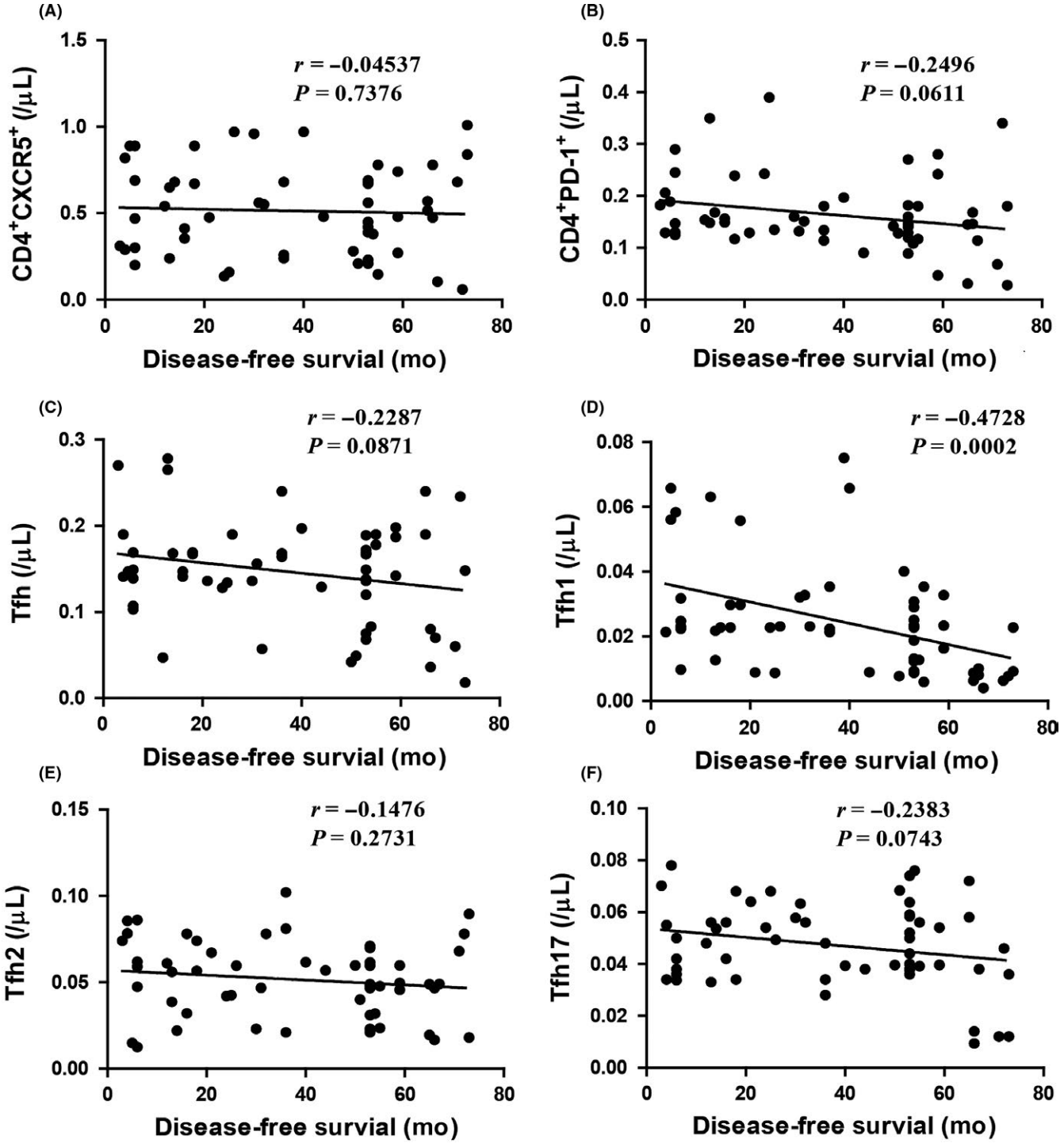

FIGURE 5 Correlation between follicular helper T (Tfh) cells and disease-free survival. A-F, Correlations between the number of $CD4^+CXCR5^+$, $CD4^+PD\text{-}1^+$, Tfh cells, Tfh1, Tfh2, and Tfh17 subtypes with disease-free survival

PD-L1-positive NSCLC tumor cells were significantly correlated with high numbers of $CD4^+PD\text{-}1^+$ (Figure 4D) and Tfh cells (Figure 4E), but there was no significant correlation with $CD4^+CXCR5^+$ (Figure 4C) and clinicopathological factors (data not shown). As previously reported, we also found that NSCLC patients with PD-L1-negative tumor cells had longer disease-free survival after tumor resection (Figure 4F).

3.5 | Negative correlation between Tfh1 subtypes with disease-free survival

Thus, we determined whether Tfh cells with high PD-1 expression were also predictive of survival time in NSCLC. We analyzed the correlation between Tfh cells and disease-free survival after tumor resection. Interestingly, the number of Tfh1 subtypes

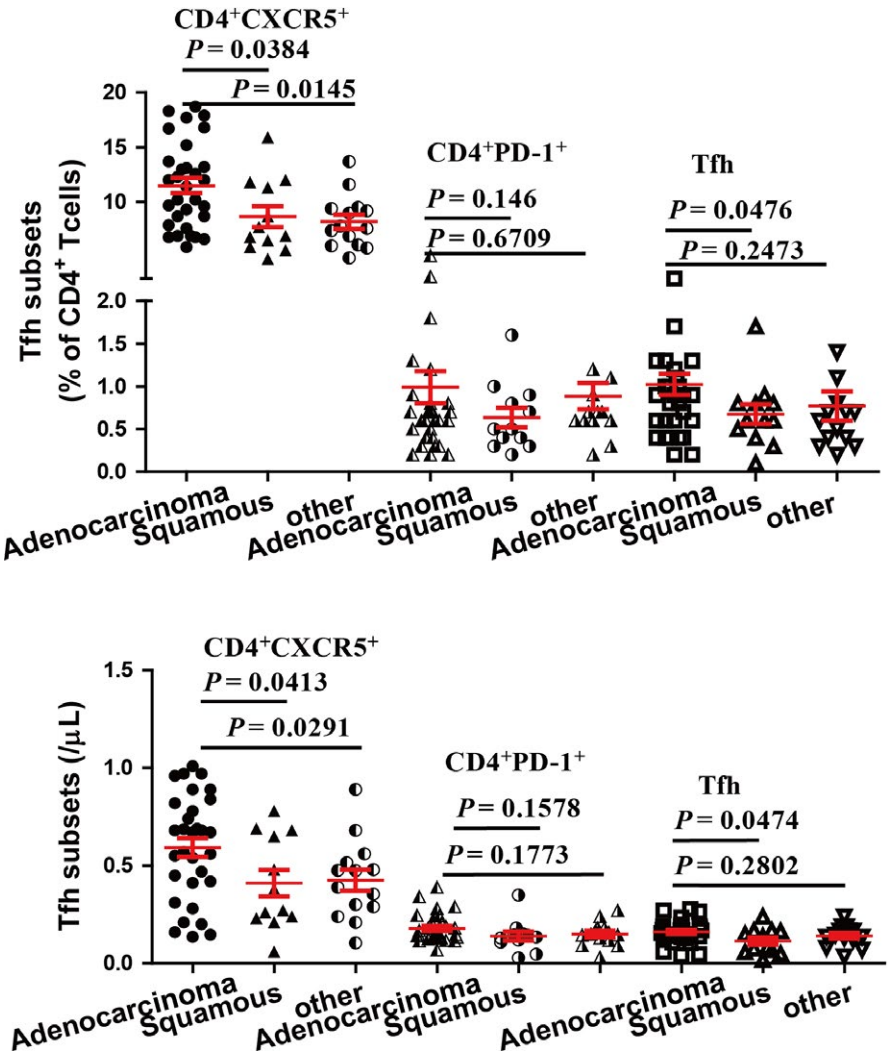

FIGURE 6 Circulating follicular helper T (Tfh) cells in non-small cell lung cancer patients with different histological subtypes. Frequency in $CD4^+$ T cells and number of $CD4^+CXCR5^+$, $CD4^+PD\text{-}1^+$, and Tfh cells are shown in the top and bottom panels, respectively. CXCR5, C-X-C motif chemokine receptor 5; PD-1, programmed death 1

was positively correlated with disease-free survival of NSCLC patients (Figure 5D). However, no significant association was found between $CD4^+CXCR5^+$, $CD4^+PD\text{-}1^+$, Tfh cells, Tfh2, or Tf17 subtypes and disease-free survival of NSCLC patients (Figure 5A,B,C,E,F).

3.6 | Circulating Tfh cells in NSCLC patients with different histological subtypes

Non-small cell lung cancer comprises several subtypes based on histological examination in which adenocarcinoma and squamous cell carcinoma are the two major subtypes. We investigated the levels of circulating $CD4^+CXCR5^+$, $CD4^+PD\text{-}1^+$ and Tfh cells in these NSCLC histological subtypes. Our data showed that the frequency and the number of circulating $CD4^+CXCR5^+$ and Tfh cells were higher in adenocarcinoma than in squamous cell carcinoma or in the other types, but the level of $CD4^+PD\text{-}1^+$ was no different in the three histological subtypes (Figure 6). However, the level of circulating $CD4^+CXCR5^+$, $CD4^+PD\text{-}1^+$ and Tfh cells was not significantly different between squamous cell carcinoma and other types.

4 | DISCUSSION

Follicular helper T cells represent a distinct $CD4^+$ T subset that helps B cells in GC differentiate into long-lived memory B and plasma cells.[13-15] Studies in several tumors have shown that Tfh cells have quantitative or functional abnormalities.[25-27] In the present study, we investigated circulating Tfh cells in newly diagnosed NSCLC patients and identified the expansion of circulating $CD4^+PD\text{-}1^+$ and Tfh cells, especially in those with advanced stage (III and IV). We also observed a great skewing toward PD-1^+-Tfh2 and PD-1^+-Tfh17 subtypes in NSCLC. In addition, we found increased circulating $CD4^+PD\text{-}1^+$ and Tfh cells in adenocarcinoma than in squamous cell carcinoma or other types, which suggested different histological subtypes of NSCLC might have different pathways of tumorigenesis.

Several studies have confirmed the antitumor role of IL-21 in different tumors.[28,29] IL-21 is the characteristic cytokine produced by Tfh cells. De Leur et al[30] have shown that Tfh2 and Tfh17, but not Tfh1, could effectively induce naive B cells to produce immunoglobulins through secreting of IL-21. Here, we found decreased IL-21 levels and a negative correlation between IL-21 and Tfh and Tfh17 cells, indicating that IL-21 production of Tfh17 cells could be greatly

impaired in NSCLC. GC and circulating Tfh cells can stimulate the production of antibodies through the expression of IL-10 and IL-21.[31] Ma[32] reported that Tfh cells from NSCLC patients were defective at helping naive B cells induce IgM, IgG, and IgA production. Tfh cells do not effectively produce IL-21, which further does not effectively stimulate the production of antibodies. Overall, our data indicate that Tfh cells from NSCLC patients have antitumor potential but are functionally impaired.

To further clarify the role of Tfh cells in NSCLC, we analyzed the functional effect of Tfh cells on $CD19^+IgD^+$ naïve B cells and $CD14^+HLA\text{-}DR^-$ cells. Our results showed that Tfh cells from NSCLC patients promoted the release of TGF-α and IL-10 from $CD19^+$ B cells and the release of TNF-α from $CD14^+$ cells. Increased PD-1^+-Tfh2 and PD-1^+-Tfh17 subtypes in NSCLC could inhibit immune response through interaction with PD-L1. The high number and abnormal function of Tfh cells induce the differentiation of regulatory B cells and $CD14^+HLA\text{-}DR^-$ cells, which could cause further immunosuppression and lead to tumor development and even metastasis.

High PD-L1 expression in NSCLC was an independent predictor of poor prognosis.[33] We found that high expression of PD-L1 in tumor cells is significantly correlated with a high number of circulating Tfh cells in NSCLC patients. NSCLC patients with PD-L1-negative tumor cells had longer disease-free survival, as reported in other studies.[23,24] Gu-Trantien et al[25] found that when Tfh cell infiltration in breast cancer patients was greater, the prognosis of patients was better. Additionally, impaired function of Tfh cells is associated with hepatocellular carcinoma progression.[34,35] Thus, Tfh cells are likely to be associated with prognosis of tumor. Interestingly, in the present study, we found that the number of Tfh1 subtypes was negatively correlated with disease-free survival after tumor resection, which may be related to immunosuppression induced by high PD-1 expression in Tfh1 subtypes. However, our data were based on a relatively small number of patients. For this reason, we were unable to reliably assess the predictive utility of Tfh1 subtypes on NSCLC patient survival and will expand our samples and conduct a larger study.

Based on our results, it is possible that elevated Tfh cells cause further immunosuppression in NSCLC. Whether rescuing Tfh functions can induce better B-cell responses and improve antitumor immunity in NSCLC is still unknown and will require further animal experiments and clinical trials for elucidation.

ACKNOWLEDGMENTS

This study was partly supported by the Zhejiang Provincial Natural Science Fund (No: Y18H200014), and the Zhejiang Provincial Program for the Cultivation of High-level Innovative Health Talents (No: 2012).

CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

ORCID

Liannv Qiu http://orcid.org/0000-0001-9853-8391

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics. *CA Cancer J Clin*. 2016;66(1):7-30.
2. Aupérin A, Le Péchoux C, Rolland E, et al. Meta-analysis of concomitant versus sequential radiochemotherapy in locally advanced non-small-cell lung cancer. *J Clin Oncol*. 2010;28(13):2181-2190.
3. Bradley JD, Paulus R, Komaki R, et al. Standard-dose versus high-dose conformal radiotherapy with concurrent and consolidation carboplatin plus paclitaxel with or without cetuximab for patients with stage IIIA or IIIB non-small-cell lung cancer (RTOG 0617). *Lancet Oncol*. 2015;16(2):187-199.
4. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. 2008;26(1):677-704.
5. Mu CY, Huang JA, Chen Y, Chen C, Zhang XG. High expression of PD-L1 in lung cancer may contribute to poor prognosis and tumor cells immune escape through suppressing tumor infiltrating dendritic cells maturation. *Med Oncol*. 2011;28(3):682-688.
6. Herbst RS, Baas P, Kim DW, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. *Lancet*. 2016;387(10027):1540-1550.
7. Fehrenbacher L, Spira A, Ballinger M, et al. Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2 randomised controlled trial. *Lancet*. 2016;387(10030):1837-1846.
8. Reck M, Rodríguez-Abreu D, Robinson AG, et al. Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. *N Engl J Med*. 2016;375(19):1823-1833.
9. Gettinger S, Herbst RS. B7-H1/PD-1 blockade therapy in non-small cell lung cancer: current status and future direction. *Cancer J*. 2014;20(4):281-289.
10. McDermott DF, Atkins MB. PD-1 as a potential target in cancer therapy. *Cancer Med*. 2013;2(5):662-673.
11. Ma CS, Deenick EK, Batten M, Tangye SG. The origins, function, and regulation of T follicular helper cells. *J Exp Med*. 2012;209(7):1241-1253.
12. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol*. 2011;29(1):621-663.
13. Tangye SG, Ma CS, Brink R, Deenick EK. The good, the bad and the ugly – TFH cells in human health and disease. *Nat Rev Immunol*. 2013;13(6):412-426.
14. Eivazi S, Bagheri S, Hashemzadeh MS, et al. Development of T follicular helper cells and their role in disease and immune system. *Biomed Pharmacother*. 2016;84:1668-1678.
15. Crotty S. A brief history of T cell help to B cells. *Nat Rev Immunol*. 2015;15(3):185-189.
16. Ueno H, Banchereau J, Vinuesa CG. Pathophysiology of T follicular helper cells in humans and mice. *Nat Immunol*. 2015;16(2):142-152.
17. Linterman MA, Hill DL. Can follicular helper T cells be targeted to improve vaccine efficacy? *F1000Res*. 2016;5.
18. Chung Y, Tanaka S, Chu F, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med*. 2011;17(8):983-988.
19. Amé-Thomas P, Le Priol J, Yssel H, et al. Characterization of intratumoral follicular helper T cells in follicular lymphoma: role in the survival of malignant B cells. *Leukemia*. 2012;26(5):1053-1063.
20. Kaltenmeier C, Gawanbacht A, Beyer T, et al. $CD4^+$ T cell-derived IL-21 and deprivation of CD40 signaling favor the in vivo development of granzyme B-expressing regulatory B cells in HIV patients. *J Immunol*. 2015;194(8):3768-3777.

21. Gu-Trantien C, Migliori E, Buisseret L, et al. CXCL13-producing TFH cells link immune suppression and adaptive memory in human breast cancer. *JCI Insight*. 2017;2(11):e91487.
22. Liu JL, Wang H, Yu QH, et al. Aberrant frequency of IL-10-producing B cells and its association with Treg and MDSC cells in Non Small Cell Lung Carcinoma patients. *Hum Immunol*. 2016;77(1):84-89.
23. Fujimoto D, Uehara K, Sato Y, et al. Alteration of PD-L1 expression and its prognostic impact after concurrent chemoradiation therapy in non-small cell lung cancer patients. *Sci Rep*. 2017;7(1):11373.
24. He YY, Rozeboom L, Rivard CJ, et al. PD-1, PD-L1 protein expression in non-small cell lung cancer and their relationship with tumor-infiltrating lymphocytes. *Med Sci Monit*. 2017;23:1208-1216.
25. Gu-Trantien C, Loi S, Garaud S, et al. CD4$^+$ follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest*. 2013;123(7):2873-2892.
26. Morita R, Schmitt N, Bentebibel SE, 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.
27. Vinuesa CG, Cook MC. Blood relatives of follicular helper T cells. *Immunity*. 2011;34:10-12.
28. Andorsky DJ, Timmerman JM. Interleukin-21: biology and application to cancer therapy. *Expert Opin Biol Ther*. 2008;8(9):1295-1307.
29. Sivakumar PV, Foster DC, Clegg CH. Interleukin-21 is a T-helper cytokine that regulates humoral immunity and cell-mediated anti-tumour responses. *Immunology*. 2004;112(2):177-182.
30. De Leur K, Dor FJMF, Dieterich M, van der Laan LJW, Hendriks RW, Baan CC. IL-21 receptor antagonist inhibits differentiation of B cells toward plasmablasts upon alloantigen stimulation. *Front Immunol*. 2017;8:306.
31. Zhang H, Yue R, Zhao P, et al. Proinflammatory follicular helper T cells promote immunoglobulin G secretion, suppress regulatory B cell development, and correlate with worse clinical outcomes in gastric cancer. *Tumour Biol*. 2017;39(6):1010428317705747.
32. Ma QY, Huang DY, Zhang HJ, Chen J, Miller W, Chen XF. Function of follicular helper T cell is impaired and correlates with survival time in non-small cell lung cancer. *Int Immunopharmacol*. 2016;41:1-7.
33. Cooper WA, Tran T, Vilain RE, et al. PD-L1 expression is a favorable prognostic factor in early stage non-small cell carcinoma. *Lung Cancer*. 2015;89(2):181-188.
34. Jia Y, Zeng Z, Li Y, et al. Impaired function of CD4 + T follicular helper (Tfh) cells associated with hepatocellular carcinoma progression. *PLoS ONE*. 2015;10:e0117458.
35. Zhou ZQ, Tong DN, Guan J, et al. Follicular helper T cell exhaustion induced by PD-L1 expression in hepatocellular carcinoma results in impaired cytokine expression and B cell help, and is associated with advanced tumor stages. *Am J Transl Res*. 2016;8(7):2926-2936.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Qiu L, Yu Q, Zhou Y, et al. Functionally impaired follicular helper T cells induce regulatory B cells and CD14$^+$ human leukocyte antigen-DR$^-$ cell differentiation in non-small cell lung cancer. *Cancer Sci*. 2018;109:3751–3761. https://doi.org/10.1111/cas.13836