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Elevated LAG-3 on CD4$^+$ T cells negatively correlates with neutralizing antibody response during HCV infection

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ABSTRACT

Lymphocyte activation gene-3 (LAG-3), an inhibitory molecule, which has been shown co-expressed with multiple inhibitory receptors on CD8$^+$ T and natural killer (NK) cells and negatively regulates T and NK cell responses during hepatitis C virus (HCV) infection. However, whether LAG-3 is involved in the regulation of the antibody response remains unclear. This study aims to investigate the relationship of LAG-3 with neutralizing antibody (nAb) response during HCV infection. A total of 66 HCV-infected individuals and 36 sex- and age-matched healthy controls from a population of intravenous drug users were recruited. Circulating follicular helper T (cTfh) cells and LAG-3-expressing CD4$^+$ T cells, type 1 regulatory T (Tr1) cells, and regulatory T (Treg) cells were characterized by flow cytometry. Serum nAb response of HCV-infected individuals was determined using pseudoparticle neutralization assays. We found that HCV infection enhanced LAG-3 expression on CD4$^+$ T cells and exhibited regulatory T cell-like phenotype and inversely associated with the HCV nAb response. Further analysis showed that frequency of CXCR3$^+$ cTfh cells positively correlated with nAb response, however LAG-3$^+$ CD4$^+$ T cells inversely associated with CXCR3$^+$ cTfh cells. This study observed that LAG-3$^+$ CD4$^+$ T cells exhibit a regulatory cell phenotype and negatively associate with the HCV nAb response, implying that LAG-3 may be involved in the negative regulation of humoral immunity during HCV infection.

1. Introduction

Lymphocyte activation gene-3 (LAG-3) is a CD4-related transmembrane protein that binds to MHC class II molecules [1,2]. Once T and NK cells are activated, LAG-3 is selectively trafficked onto the cell surface from the intracellular space to limit T and natural killer (NK) responses [3,4]. LAG-3 was identified as an inhibitory receptor together with programmed cell death protein-1 (PD-1), T cell immunoglobulin mucin-3 (Tim-3) and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) [5,6]. LAG-3, along with other inhibitory receptors, is highly expressed on antigen-specific CD8$^+$ T cells during chronic virus infection or tumor-infiltrating CD8$^+$ T cells to induce CD8$^+$ T cell exhaustion, and blocking these inhibitory receptors significantly restores CD8$^+$ T cell functions [5–7]. During hepatitis C virus (HCV) infection, elevated LAG-3 expression on antigen-specific CD8$^+$ T cells contributes to HCV persistence [8,9].

In addition LAG-3 is also required for maximal regulatory T (Treg) cell function, and ectopic expression of LAG-3 is sufficient to confer regulatory activity [10]. Natural CD4$^+$ CD25$^+$ FoxP3$^+$ Treg cells express LAG-3 upon activation, which is significantly enhanced in the presence of effector cells, whereas CD4$^+$ CD25$^+$ FoxP3$^+$ Treg cells from LAG-3-deficient mice exhibit reduced regulatory activity [10]. The CD4$^+$ CD25$^+$ FoxP3$^+$ LAG-3$^+$ population is preferentially expanded among peripheral blood mononuclear cells (PBMCs), tumor-invaded lymphocytes and lymphocyte-infiltrating visceral metastasis of patients with cancer [11]. This population comprises functionally active cells that release the immunosuppressive cytokines IL-10 and TGF-β1 [10,11]. Moreover, the combined expression of LAG-3 and CD49b specifically identifies IL-10-producing type 1 regulatory T (Tr1) cells in mice and humans [12]. Tr1 cells have been proven to be important in maintaining immunological homeostasis and preventing T cell-mediated diseases [12,13]. Both natural Treg and inducible Tr1 cells are

Abbreviations: HCV, hepatitis C virus; Tr1 cell, type 1 regulatory T cell; Treg cell, regulatory T cell; Tfh cell, follicular helper T cell; LAG-3, lymphocyte activation gene-3; CXCR3, C-X-C motif chemokine receptor 3; nAb, neutralizing antibody

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expanded and contribute to HCV persistence or HCV recurrence after liver transplantation [14–16].

LAG-3-expressing $CD4^+$ T cells, including Tr1 and Treg cells, not only interfere with the conventional helper T (Th) cell response but also interact with Tfh cells, thereby impacting humoral immunity [7,17]. Tfh cells are a $CD4^+$ T cell subset specialized to regulate the types of antibodies produced in the germinal center (GC) [18,19]. Tfh cell differentiation is primarily controlled by $Foxp3^+$ follicular regulatory T (Tfr) cells through CTLA-4 in the GC [20–23]. Recently, several studies indicated that LAG-3 expressing $CD4^+$ T cells may as negative regulator involving modulation of humoral immunity responses. Such as blocking PD-L1 and LAG-3 restores $CD4^+$ T cell functions; amplifies the number of Tfh cells, GC B cells and plasmablasts; and enhances protective antibody production during *Plasmodium falciparum* infection in mice [7]. Plasmodium infection also induces Tr1 cell expansion, which limits Tfh cell accumulation and restricts antimalarial humoral immunity [24]. Liver Tr1 cells inactivate both Tfh cells and GC B cells by secreting IL-10, resulting in impaired GC formation and anti-HBV antibody production in HBV-carrier mice [17]. More recently, LAG-3 expressing on plasma cell identified as natural regulatory plasma cell [25]. These studies suggest that LAG-3 may be involved in the regulation of humoral immunity partially by interfering with Tfh cell differentiation during infection.

cTfh cells from individuals with HCV infection promote B cell antibody production in vitro and intrahepatic HCV antigen-specific $CD4^+$ T cells exhibit Tfh properties associated with antiviral antibody production [26,27]. However, whether LAG-3 is involved in the regulation of nAb responses during HCV infection remains unclear. In this study, we show that LAG-3 expression is elevated during HCV infection and endows cells with a regulatory T cell-like phenotype and negative associate with HCV nAb response.

2. Materials and methods

2.1. Participants and sampling

In total, 2367 intravenous drug users (IDUs) were recruited from 2015 to 2017 in Chenzhou, Hunan Province, China. Each participant was interviewed using a structured questionnaire to collect demographic data and environmental exposure history. The study was approved by the Ethics Committee of The First People's Hospital of Chenzhou (No. 2015002) according to the Declaration of Helsinki. All participants enrolled in the study provided written informed consent.

All individuals were subjected to anti-HCV IgG, anti-human immunodeficiency virus (HIV) IgG, anti-hepatitis D virus (HDV) IgG and hepatitis B virus surface antigen (HBsAg) serum testing to determine the infectious status and to exclude other viral infections. Finally, 66 HCV-infected antiviral treatment-naive individuals and 36 healthy controls were enrolled into this study. As shown in Table 1, HCV-infected individuals and healthy controls were age- and sex-matched. HCV-infected individuals showed higher alanine transaminase (ALT) and aspartate transaminase (AST) levels ($P = 0.089$ and $P = 0.029$, respectively).

Table 1
Clinical information of HCV infected individuals.

	Healthy control (n = 36)	HCV infected individuals (n = 66)	*P* Value[a]
Age(years)	37.44(31.4–44.23)	39.93(34.10–43.34)	0.530
Sex (Male/Female)	36/0	61/5	0.158
ALT(U/L)	22.90(15.4–33.3)	25.20(17.30–65.25)	0.089
AST(U/L)	19.50(12.95–26.50)	25.4(17.4–40.45)	0.029*
HCV RNA ($\times 10^4$copies/ml)		3.65(0.60–8.32)	
HCV genotype (1b/3a/3b/6a/N.D)	–	3/12/12/30/9	

[a] Mann-Whitney *U* test was used for age and ALT/AST comparison, Fisher's exact test was used for sex distribution between two groups.

* $P < 0.05$ was consider significant between two groups. Data present as median and interquartile range. N.D, not determinant.

2.2. Serological tests

HBsAg, anti-HCV IgG, anti-HDV IgG, and anti-HIV IgG were detected using commercial enzyme-linked immunosorbent assay (ELISA) kits (Wantai Biological Pharmacy, Beijing, China) according to the manufacturer's instructions. HCV viral loads were quantitatively determined by qPCR using a commercially available Nucleic Acid Diagnostic kit (Sansure Biotech, Changsha, China).

PBMCs were isolated by using SepMate tubes (Stem cell Technologies, Vancouver, Canada) and Ficoll density gradient centrifugation (GE Healthcare Bio Sciences AB, Uppsala, Sweden) from fresh anticoagulant-containing blood within 12 h of collection according to the manufacturer's protocol. Isolated PBMCs and serum were stored in liquid nitrogen or a −80 °C refrigerator until further analysis.

2.3. Flow cytometry

For flow cytometry analysis, recovered PBMCs were allowed to rest in 10% FBS, 1% pen/strep, and L-glutamine RPMI-1640 medium overnight at 5% CO_2 in a 37 °C incubator. Dead cells were excluded from all samples by using LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Thermo Fisher Scientific, Waltham, MA, USA), and the samples were treated with Fc Block (BioLegend, San Diego, CA, USA) before staining. For cell surface staining, 1×10^6/mL PBMCs were stained with a titrated amount of antibodies at 4 °C for 30 min. For characterization of transcription factor expression, a Foxp3 Transcription Factor Staining Buffer Kit (eBioscience, San Diego, CA, USA) was used for cell permeabilization and transcription factor antibody staining. The antibodies used were PE-Cy5 mouse anti-human CD25 (M-A251), PE-Cy7 mouse anti-human CD45RA (HI100), PE mouse anti-human CXCR3 PE (1C6), FITC mouse anti-human CD45RA (HI100), and BUV 737 mouse anti-human CD4 (SK3) from BD Biosciences (Franklin Lake, NJ, USA); APC-Cy7 mouse anti-human CD3 (SK7) and PE mouse anti-human CD49b (P1E6-C5) from BioLegend (San Diego, CA, USA); FITC mouse anti-human LAG-3 (17B4) from LifeSpan Bioscience (Seattle, WA, USA); and PE-eFluor 610 mouse anti-human CXCR5 (MU5UBEE) and PE-eFluor 610 rat anti-human Foxp3 (PCH101) from Thermo Fisher Scientific (Waltham, MA, USA). In this study, the $CD4^+$ $CD25^+$ $Foxp3^+$ $CD4^+$ T subset was identified as Treg cells, and the $CD4^+$ $CD45RA^-$ $LAG\text{-}3^+$ $CD49b^+$ $CD4^+$ T subset was identified as Tr1 cells. Cell population gating was performed based on mean fluorescence intensity "minus one" (FMO) and unstained controls. Samples were analyzed on a MoFlo XDP flow cytometer (Beckman Coulter, Brea, CA, USA) immediately after staining. All subsequent analyses were performed with FlowJo 10.0 software (Tree Star, San Carlos, CA, USA).

2.4. Neutralization assay

HCV pseudo particles (HCVpp) carrying a luciferase gene reporter were generated by cotransfecting HEK-293 cells with one of 6 subtypes of HCV E1E2 protein-encoding plasmids (genotype 1a: strain H77c; genotype 1b: strain HC-J4; genotype 2a: strain J6; genotype 3a: strain S52; genotype 4a: strain ED43; or genotype 5a: strain SA13) mixed with diluted serum to infect Huh7.5 cells, and the efficiency of neutralizing antibodies was detected in each serum sample. For HCV-neutralization test, HCVpp were mixed with diluted serum (dilutions: 1:100, 1:400, 1:1600, 1:6400), incubated in 5% CO_2 for 1 h at 37 °C and used to infect Huh7.5 cells for 4 h. Then, culture supernatants were removed, and fresh medium was added for 3 days of culture. Luciferase activities of the Huh7.5 cells were measured using a luciferase assay system (Promega, Madison, WI, USA) to assess the reduction in infectivity. In

this study, the nAb response was depicted as ranks combining neutralization titer and breadth as previously described [28].

2.5. Statistical analysis

All the results are presented as the median and interquartile range. The Mann-Whitney *U* test was used for comparison between two independent samples, and paired *t* tests were used to analyze paired samples. Fisher's exact test was used for the determination of the distribution of sex between groups. Spearman's rank correlation coefficient was used to evaluate the relationship between two variables. Significance was set at $P < 0.05$. All statistical calculations were performed with either SPSS version 19.0 (Chicago, IL, USA) or Prism 7 (GraphPad, La Jolla, CA, USA).

3. Results

3.1. LAG-3$^+$ CD4$^+$ T cells increased and exhibited regulatory T cell-like phenotype during HCV infection

To determine the potential role of LAG-3 in antibody response during HCV infection, we first compared LAG-3 expression on CD4$^+$ T cells between HCV-infected individuals (n = 66) and healthy controls (n = 36) based on the gating strategy (Fig. 1A). LAG-3 expression on CD4$^+$ T cells was significantly enhanced during HCV infection relative to that of healthy controls ($P = 0.011$), albeit at extremely low levels (Fig. 1B). Due to LAG-3 also usually expressed on some regulatory T cell subset such as Tr1, Treg involving in negative regulation of immune response [11,29]. Thus, we compared the difference in CD49b, FoxP3 and CD25 expression, which represent Tr1 cell or Treg cell markers, between LAG-3$^+$ CD4$^+$ T cells and LAG-3$^-$ CD4$^+$ T cells from HCV-infected individuals. Phenotypically, LAG-3$^+$ CD4$^+$ T cells express relatively higher levels of CD49b and FoxP3 but not CD25 relative to LAG-3- CD4$^+$ T cells during HCV infection ($P < 0.001$ and $P < 0.001$, respectively) (Fig. 1C).

As shown in Fig. 1, LAG-3$^+$ CD4$^+$ T cells express a high level of CD49b and Foxp3 during HCV infection. Thus, we investigated the frequencies of Tr1 (CD45RA$^-$ LAG-3$^+$ CD49b$^+$ CD4$^+$ T) and Treg (CD25$^+$ FoxP3$^+$ CD4$^+$ T) cells during HCV infection (Fig. 2A). Compared with healthy controls, HCV-infected individuals show increased frequency of Tr1 cells ($P = 0.019$); although there is no significant enhancement in Treg cells in our cohort ($P = 0.305$), the frequency of LAG-3$^+$ Treg cells was significantly increased during HCV infection ($P < 0.001$) (Fig. 2B–D). Of note, among these LAG-3 expressing CD4$^+$ T cells, more than 95% LAG-3$^+$ CD4 T cells are conventional CD4 T cells (CD4$^+$ FoxP3$^-$ CXCR5$^-$), only a small proportional LAG-3 distributed on circulating Treg cells (CD4$^+$ CD25$^+$ FoxP3$^+$ CXCR5-), Tfh cells (CD4$^+$ CXCR5$^+$ FoxP3-), and Tfr cells (CD4$^+$ CXCR5$^+$ FoxP3$^+$) (Supplementary Fig. 1). These findings suggest that HCV infection promotes LAG-3 expression on CD4$^+$ T cells and endows LAG-3

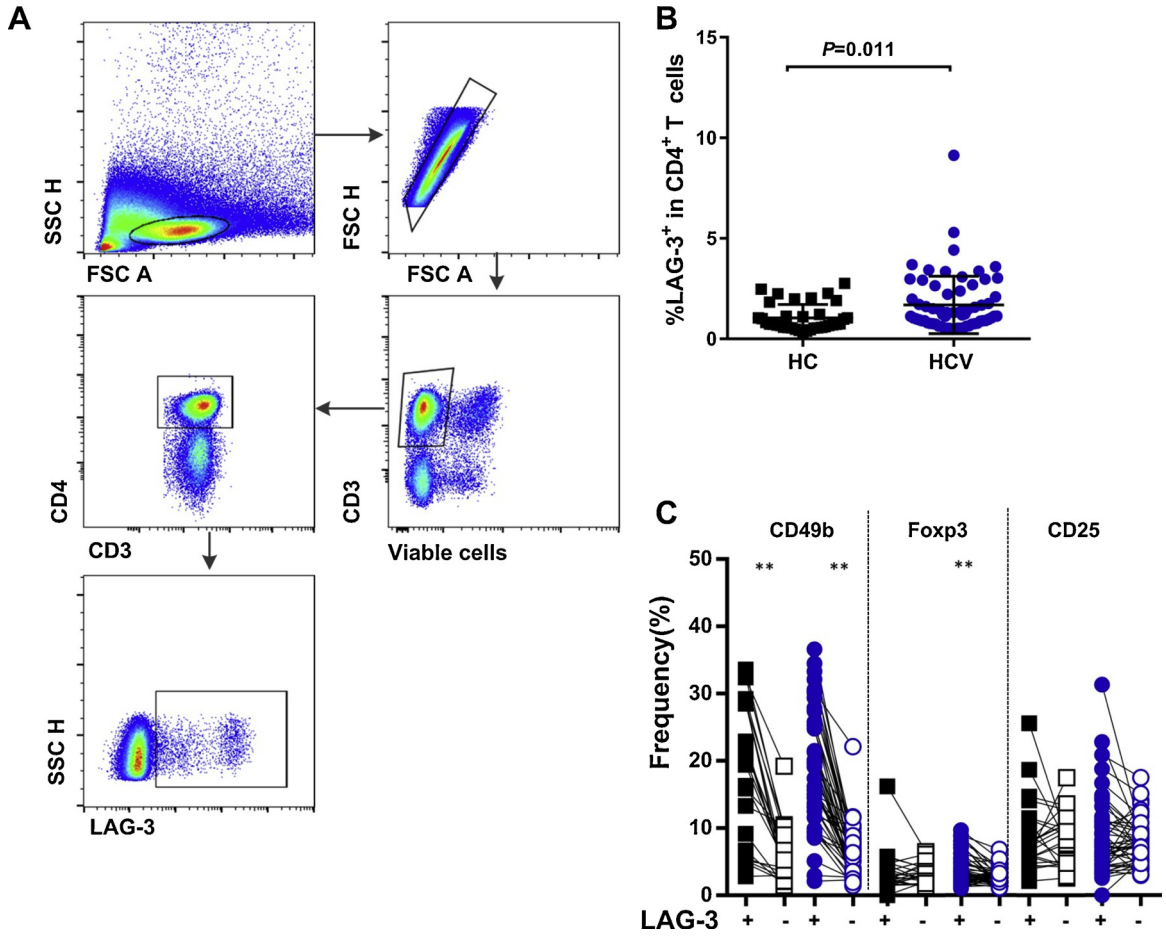

Fig. 1. CD4$^+$ T cells exhibit elevated LAG-3 expression and a regulatory T cell-like phenotype during HCV infection. (A) Representative flow cytometry plots of the gating strategy for CD4$^+$ LAG-3$^+$ T cells. Gating based on the mean fluorescence intensity "minus one" (FMO). (B) Comparison of LAG-3 expression on CD4$^+$ T cells between HCV-infected individuals (n = 66 circle) and healthy controls (n = 36 square). (C) Comparison of CD25, CD49b, and Foxp3 expression between CD4$^+$ LAG-3$^+$ T cells and CD4$^+$ LAG-3$^-$ T cells from HCV-infected individuals (n = 49) and healthy controls (n = 24). All data collected represent 3 independent experiments. Data represent the median and interquartile range. The Mann-Whitney *U* test was used for two different groups comparison. Paired *t* test was used for paired groups comparison. $^*P < 0.05$ and $^{**}P < 0.001$.

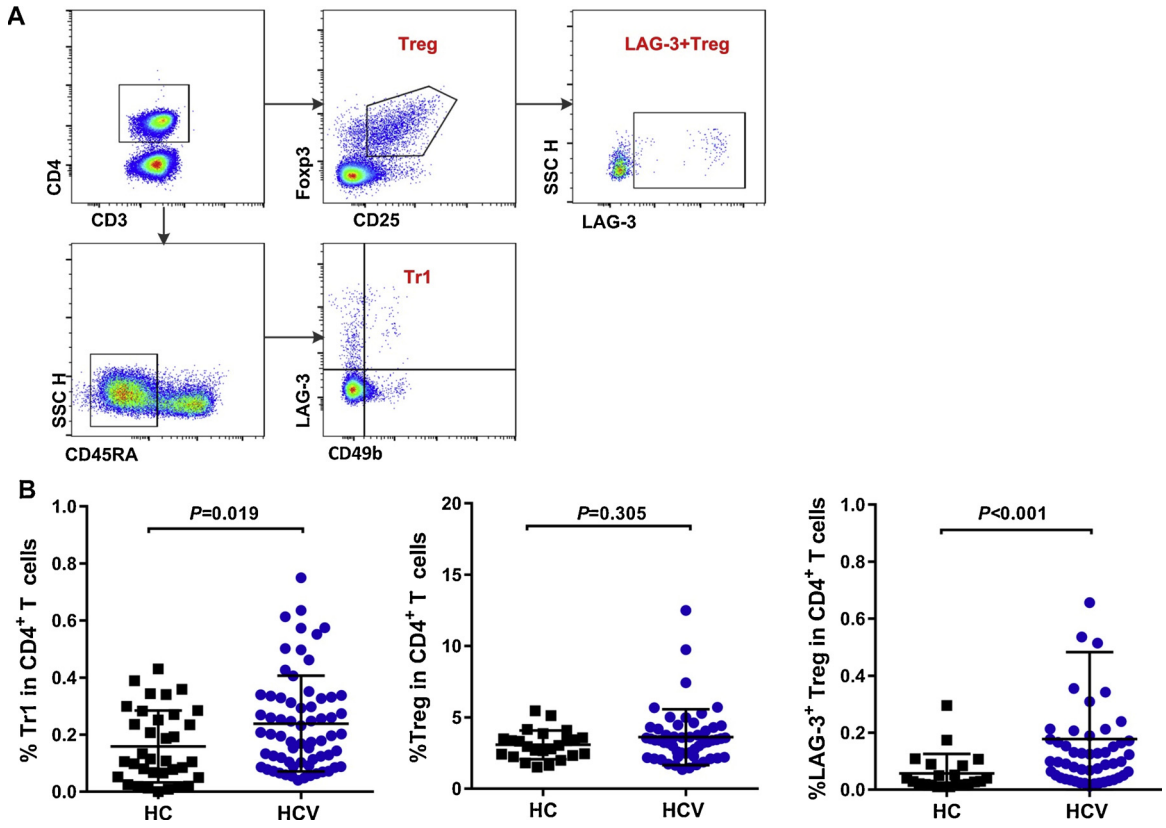

Fig. 2. Comparison of the frequency of Tr1 cells, Treg cells and LAG-3$^+$ Treg cells between HCV-infected individuals and healthy controls. (A) Representative flow cytometry plots of the gating strategy for type 1 regulatory T (Tr1), regulatory T (Treg), and LAG-3$^+$ Treg cell subsets among CD4$^+$ T cells. Gating based on mean fluorescence intensity "minus one" (FMO). Frequency of Tr1 cells(HCV n = 66, HC n = 36)(B), Treg cells(HCV n = 53, HC n = 24)(C), and LAG-3$^+$ Treg cells(HCV n = 51, HC n = 22)(D) among CD4$^+$ T cells from healthy controls and HCV-infected individuals. All data collected represent 3 independent experiments. Data represent the median and interquartile range. The Mann-Whitney *U* test was used for data analysis.

expressing CD4$^+$ T cell with a regulatory T cell-like phenotype.

3.2. LAG-3-biased CD4$^+$ T cells are negatively associated with the HCV nAb response

As shown in Figs. 1 and 2, HCV infection promotes LAG-3 expression on CD4$^+$ T cells and induces a regulatory T cell-like phenotype. To address the relationship between LAG-3-biased CD4$^+$ T cells and the HCV nAb response, we assessed the serum nAb response using an HCVpp assay (genotypes 1–5) in individuals infected with HCV. NAb responses were determined by combining neutralization titer and breadth and are presented as ranks (Supplementary Table 1). Interestingly, the frequencies of LAG-3$^+$ CD4$^+$ T cells exhibited a significant negative correlation with the HCV nAb response during HCV infection (R = -0.404, P = 0.002) (Fig. 3A). LAG-3$^+$ subset Tr1 cells and LAG-3$^+$ Treg cells but not Treg cells also exhibited an inverse association with the HCV nAb response during HCV infection (R = -0.468, P < 0.001; R = -0.489, P < 0.001; and R = 0.111, P = 0.486, respectively) (Fig. 3B–D). These results indicated that LAG-3-biased CD4$^+$ T cells may be involved in the negative regulation of humoral immunity during HCV infection.

3.3. LAG-3-biased CD4$^+$ T cells are negatively correlated with CXCR3$^+$ cTfh cells, which are positively associated with the HCV nAb response during HCV infection

As shown above, LAG-3-biased CD4$^+$ T cells were negatively correlated with the HCV nAb response during HCV infection. In contrast,

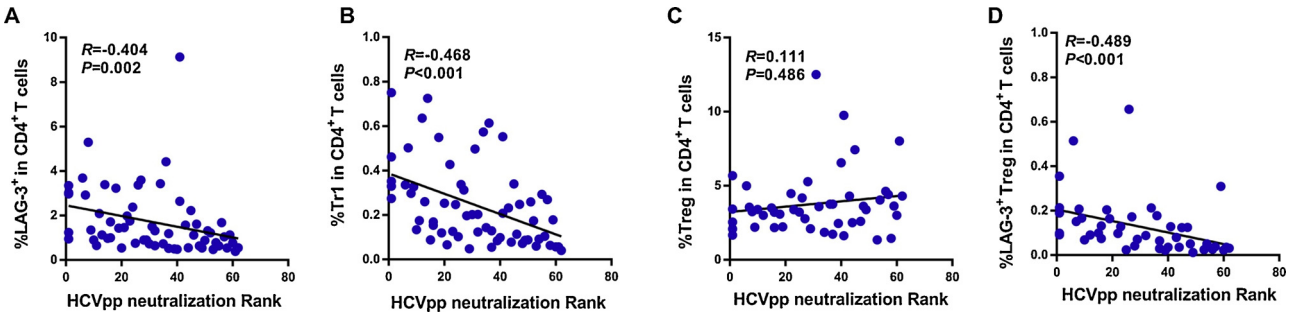

Fig. 3. Frequency of LAG-3$^+$ CD4$^+$ T cells, Tr1 cells and LAG-3$^+$ Treg cells is inversely associated with the HCV nAb response. Correlation of LAG-3$^+$ CD4$^+$ T cells (n = 58) (A), Tr1 cells (n = 43) (B), Treg cells (n = 45) (C), and LAG-3$^+$ Treg cells (n = 45) (D) with the HCV nAb response. Spearman's correlations were used for data analysis; R and P values are depicted in the upper left corner of each graph.

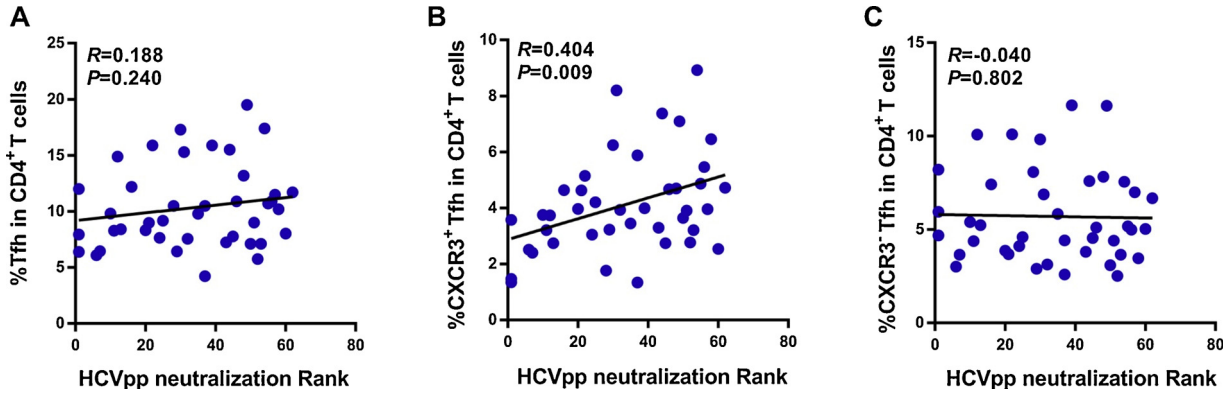

Fig. 4. The frequency of circulating CXCR3$^+$ cTfh cells is positively correlated with the HCV nAb response. Correlation of cTfh cells (n = 41) (A), CXCR3$^+$ cTfh cells (n = 41) (B), and CXCR3$^-$ cTfh cells (n = 41) (C) with the HCV nAb response. Spearman's correlations were used for data analysis; *R* and *P* values are depicted in the upper left corner of each graph.

Tfh cells are a CD4$^+$ T cell subset specialized in regulating the types of antibodies produced in the GC [18,19]. cTfh cells have been identified as counterparts of GC Tfh cells and can support T-dependent B cell maturation and antibody response [30]. Thus, we addressed the relationship between cTfh cells with nAb and LAG-3-biased CD4$^+$ T cells during HCV infection. To this end, the relationship between cTfh cells and their subsets with the HCV nAb response was analyzed (Supplementary Fig. 2), and the frequency of CXCR3$^+$ cTfh cells exhibited a positive association with the HCV nAb response during HCV infection ($R = 0.404$, $P = 0.009$); there were no correlations between cTfh cells or CXCR3$^-$ cTfh cells with the HCV nAb response (Fig. 4). These findings may indicate that circulating CXCR3$^+$ cTfh cells play an important role in the HCV nAb response, which is consistent with several studies reporting that CXCR3-biased cTfh cells contribute to antibody titer or breadth in infection or vaccination [31,32]. We also demonstrated that CXCR3$^+$ cTfh cells from HCV-infected individuals can efficiently support antigen-specific memory B cell expansion in vitro and as a major contributor to HCV nAb response during HCV infection (Jian Zhang, et al, submitted).

As expected, the frequency of LAG-3$^+$ CD4$^+$ T cells was negatively associated with the frequency of CXCR3$^+$ cTfh cells during HCV infection (R=-0.390, $P = 0.009$) (Fig. 5A). Similarly, Tr1 cells and LAG-3$^+$ Treg cells but not Treg cells also exhibited an inverse correlation with CXCR3$^+$ cTfh cells during HCV infection (R=-0.290, $P = 0.056$; R=-0.384, $P = 0.040$; and $R = 0.138$, $P = 0.461$, respectively) (Fig. 5B and D). There was no association between LAG-3$^+$ CD4$^+$ T cells, Tr1 cells or LAG-3$^+$ Treg cells and cTfh cells or CXCR3$^-$ cTfh cells during HCV infection (Supplementary Table 2). These results may partially explain why LAG-3$^+$ CD4$^+$ T cells, Tr1 cells, and LAG-3$^+$ Treg cells are inversely correlated with the HCV nAb response during HCV infection.

4. Discussion

In this study, we show that HCV infection induce LAG-3 expression on CD4$^+$ T cells which endows cells with a regulatory T cell-like phenotype during HCV infection. The frequency of LAG-3$^+$ CD4$^+$ T cells was negatively correlated with CXCR3$^+$ cTfh cells, which are the major contributors to the HCV nAb response during HCV infection. This finding suggests that LAG-3 may act as a negative regulator involving in the regulation of the nAb response during HCV infection.

LAG-3 has been identified as an inhibitor of T cell overactivation [5]. HCV infection induces LAG-3 expression on antigen-specific CD8$^+$ T cells [8]. Here, we found that LAG-3 expression was also enhanced, albeit at extremely low levels, on CD4$^+$ T cells. LAG-3$^+$ CD4$^+$ T cells show regulatory T cell-like phenotype with higher CD49b and FoxP3 expression than that in LAG-3$^-$ CD4$^+$ T cells; in particular, they resemble Tr1 cells and Treg cells during HCV infection. Tr1 cells are required to maintain peripheral tolerance and to regulate the antibody response in GC reactions [17,24]. Here, we analyzed the relationship between elevated LAG-3 on CD4$^+$ T cells and the HCV nAb response. Interestingly, LAG-3$^+$ CD4$^+$ T cells exhibit an inverse correlation with the HCV nAb response, as well as Tr1 cells. Moreover, we determined that LAG-3$^+$ Treg, but not Treg cells were also negatively associated with the HCV nAb response. These results indicated that LAG-3 may play an important role in mediating humoral immunity regulation which is consistent with the report that PD-1 and LAG-3 blockade significantly enhances the antibody response during *Plasmodium falciparum* infection in mice [7].

Neutralizing antibodies play an important role during HCV infection, as a robust early induction of the appropriate neutralizing antibodies during acute infection contribute to spontaneous HCV clearance and the partial prevention of reinfection [33–35]. During chronic HCV infection, broadly neutralizing antibodies are associated with improved

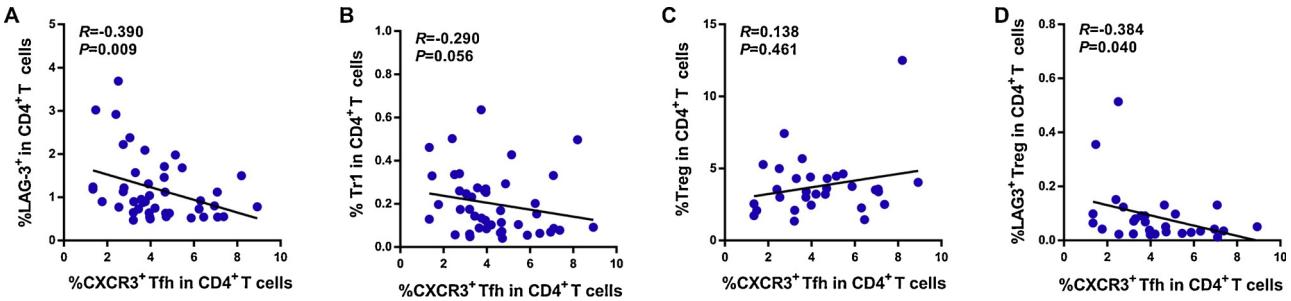

Fig. 5. Relationship of LAG-3 biased CD4$^+$ T cell subsets with CXCR3$^+$ cTfh cells during HCV infection. Correlations of LAG-3$^+$ CD4$^+$ T cells (n = 44) (A), Tr1 cells (n = 31) (B), Treg cells (n = 31) (C), and LAG-3$^+$ Treg cells (n = 29) (D) with CXCR3$^+$ cTfh cells in HCV-infected individuals. Spearman's correlations were used for data analysis; *R* and *P* values are depicted in the upper left corner of each graph.

clinical parameters, and higher titers of neutralizing antibodies also promote the natural resolution of chronic HCV infection [28,36,37]. Early studies showed that cTfh cells can support memory B cell differentiation and antibody production, although HCV infection partially impaired IL-21 production by cTfh cells [26]. Here, we further found that circulating $CXCR3^+$ cTfh cells positively correlated with the HCV nAb response. We also observed that $CXCR3^+$ cTfh cells efficiently support HCV E2 antigen-specific B cell expansion in vitro (Jian Zhang, et al, in submission). Raziorrouh B. et al recently reported that HCV NS4-specific $CD4^+$ T cells from patients with acute HCV infection express markers of Tfh cells and secrete interleukin-21 in response to HCV exposure; moreover these cells express high levels of chemokine receptor CXCR3, which positively correlates with anti−HCV NS4 antibodies [27].

$CXCR3^+$ cTfh cells positively correlated with the HCV nAb response; In contrast, LAG-3^+ $CD4^+$ T cells inversely associated with the HCV nAb response during HCV infection. Whether LAG-3^+ $CD4^+$ T cells as regulatory T-like cells regulate the cTfh cell response remains to be determined. We showed that LAG-3 biased subsets, including LAG-3^+ $CD4^+$ T cells, Tr1 cells and LAG-3^+ Treg cells but not Treg cells, were significantly inversely associated with $CXCR3^+$ cTfh cells. Several reports have suggested that LAG-3^+ $CD4^+$ T cells typically express significantly higher levels of FoxP3, and CTLA-4 and higher levels of IL-10 and TGF-beta than LAG-3^- $CD4^+$ T cells, endowing these cells with regulatory properties [10,29,38]. In fact, HIV-1-infected individuals with broadly neutralizing antibodies exhibit a higher frequency of circulating memory Tfh cells, a lower frequency of Treg cells, and a higher PD-1 expression on Treg cells than HIV-1-infected individuals without broadly neutralizing antibodies [39,40]. The tight regulation of and the balance between Tfh and Treg cells during infection work to maintain appropriate antibody production to avoid autoimmunity.

Although we show here that LAG-3^+ $CD4^+$ T cells may act as regulatory T-like cells involved in the regulation of the HCV nAb response, many issues remain unclear. First, whether LAG-3-biased subsets directly inhibit the Tfh response and the possible mechanisms need to be further investigated. Second, Tfr cells expressing CTLA-4 inhibit Tfh cells differentiation [22,23], and whether LAG-3 also plays a similar role in regulating the Tfh cell response. Tfr cells act as major antibody response regulators and are also induced during HCV infection [41]. To better understand the negative regulation of the HCV nAb response, the impact of Tr1, Tfr and Treg cells on the HCV nAb response needs to be further characterized.

In summary, our results suggest that LAG-3 may act as one of the negative factors involved in the regulation of the nAb response during HCV infection. This finding will help further the understanding of the mechanisms of HCV persistence and improve vaccination regimens via these immunologic perturbations.

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Authors' contributions

Xiaowang Qu, Jian Zhang and Wenpei Liu contributed to the study design. Jian Zhang, Ting Xie, Liyan Huang, Bo Wen, Ping Tang, Yabin Hu, Kun Jin, Ziyan Liu and Ling Niu performed the experiments and analyzed the data. Jian Zhang, Wenpei Liu and Xiaowang Qu prepared the manuscript. All authors reviewed the manuscript.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.06.003.

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