Original research

Single-cell RNA sequencing reveals intrahepatic and peripheral immune characteristics related to disease phases in HBV-infected patients

Chao Zhang,1 Jiesheng Li,2,3 Yongqian Cheng,1 Fanping Meng,1 Jin-Wen Song,1 Xing Fan,1 Hongtao Fan,2 Jing Li,1 Yu-Long Fu,1 Ming-Ju Zhou,1 Wei Hu,1 Si-Yu Wang,1 Yuan-Jie Fu,1 Ji-Yuan Zhang,1 Ruo-Nan Xu,1 Ming Shi,1 Xueda Hu,2 Zemin Zhang,2,3 Xianwen Ren,2,4 Fu-Sheng Wang1

ABSTRACT

Objective A comprehensive immune landscape for HBV infection is pivotal to achieve HBV cure.

Design We performed single-cell RNA sequencing of 243 000 cells from 46 paired liver and blood samples of 23 individuals, including six immune tolerant, 5 immune active (IA), 3 acute recovery (AR), 3 chronic resolved and 6 HBV-free healthy controls (HCs). Flow cytometry and histological assays were applied in a second HBV cohort for validation.

Results Both IA and AR were characterised by high levels of intrahepatic exhausted CD8+ T (Tex) cells. In IA, Tex cells were mainly derived from liver-resident GZMK+ effector memory T cells and self-expansion. By contrast, peripheral CX3CR1+ effector T cells and GZMK+ effector memory T cells were the main source of Tex cells in AR. In IA but not AR, significant cell–cell interactions were observed between Tex cells and regulatory CD4+ T cells, as well as between Tex and FCGR3A+ macrophages. Such interactions were potentially mediated through human leukocyte antigen class I molecules together with their receptors CANX and LILRBs, respectively, contributing to the dysfunction of antiviral immune responses. By contrast, CX3CR1+GNYL+ central memory CD8+ T cells were concurrently expanded in both liver and blood of AR, providing a potential surrogate marker for viral resolution. In clinic, intrahepatic Tex cells were positively correlated with serum alanine aminotransferase levels and histological grading scores.

Conclusion Our study dissects the coordinated immune responses for different HBV infection phases and provides a rich resource for fully understanding immunopathogenesis and developing effective therapeutic strategies.

INTRODUCTION

More than 280 million people worldwide are chronically infected with HBV, resulting in 700 000 deaths a year from cirrhosis and hepatocellular carcinoma. Only a minority of patients with chronic HBV (CHB) can achieve functional cure by current treatment regimens.

We delineated a comprehensive landscape of immune cells across different hepatitis B stages at single-cell resolution. Exhausted CD8+ T cells were preferentially expanded in immune active (IA) and acute recovery patients with different dynamics. Network analysis of cell–cell interactions and histological validations revealed that exhausted CD8+ T cells exhibited significant interactions with regulatory CD4+ T cells and FCGR3A+ macrophages in immune tolerant (IT) and IA patients, potentially mediating by HLA class I molecules together with their receptors CANX and leucocyte immunoglobulin-like receptor, respectively.

What is already known on this subject?

More than 280 million people worldwide are chronically infected with HBV, resulting in >700 000 deaths a year from cirrhosis and hepatocellular carcinoma. Only a minority of patients with chronic HBV (CHB) can achieve functional cure by current treatment regimens.

What are the new findings?

We delineated a comprehensive landscape of immune cells across different hepatitis B stages at single-cell resolution. Exhausted CD8+ T cells were preferentially expanded in immune active (IA) and acute recovery patients with different dynamics. Network analysis of cell–cell interactions and histological validations revealed that exhausted CD8+ T cells exhibited significant interactions with regulatory CD4+ T cells and FCGR3A+ macrophages in immune tolerant (IT) and IA patients, potentially mediating by HLA class I molecules together with their receptors CANX and leucocyte immunoglobulin-like receptor, respectively.

Significance of this study

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immunosuppressive hepatic microenvironments in CHB. The natural history of CHB follows several clinical phases with distinct levels of viral replication and dynamics in liver disease progression. The immune tolerant (IT) phase is characterised by high-serum HBV DNA but normal serum alanine aminotransferase (ALT) and mostly normal liver histology. It is still unknown how the host precisely establishes and maintains immunological ignorance to viral replication in this phase. The immune active (IA) phase is featured by liver necroinflammation, fibrosis and fluctuating serum ALT level. During the IA phase, the host immune response is thought to be responsible for liver pathogenesis. Only a minority of patients with CHB can achieve HBsAg seroclearance (defined as chronic resolved (CR)), either spontaneously or by antiviral treatment. However, the underlying immunological mechanisms related to the establishment, maintenance and transition of distinct HBV phases remain elusive.

Although many immunological insights into HBV pathogenesis have been made by studying peripheral blood, it has come to light that tissue-resident immune subsets play vital roles in frontline immunosurveillance in the liver. For example, the liver is enriched with mucosal-associated invariant T (MAIT) cells, natural killer (NK) cells, and unique tissue-resident CD8+ T-cell subsets. Due to the infeasibility of liver sampling in most HBV-infected patients, limited information can be accessed. However, single-cell RNA sequencing (scRNA-seq) technologies have greatly advanced our understanding of liver biology and diseases. By dissecting the cellular heterogeneity, novel insights have been made into liver zonation and regeneration.

Multiple novel cell subtypes related to the pathological processes of HCC, liver fibrosis and metabolic-associated fatty liver disease have also been identified by scRNA-seq. A comprehensive view of the intrahepatic immune responses to HBV infection by using scRNA-seq is still lacking.

To get an unbiased and comprehensive understanding of the intrahepatic immunological features and connections with disease status in HBV-infected patients, we applied scRNA-seq and constructed a comprehensive landscape of immune cells in liver and blood across different hepatitis B stages (IT, IA, AR, CR and healthy controls (HCs)). The expansion of exhausted CD8+ T (T ex) cells was observed in IA and AR phases, with different sources and cellular interactions. Our data highlight the interaction network of T cells and macrophages that contribute to immune failure during HBV persistence, which may be used to guide the development of immunotherapy.

**RESULTS**

**Single-cell immune landscape of HBV-infected individuals**

To better understand the heterogeneity within and across individuals infected with HBV, we applied scRNA-seq to characterise the transcriptomic status of intrahepatic immune cells as well as the peripheral blood mononuclear cells (PBMCs) from individuals in IT, IA, AR and CR phases (figure 1A and online supplemental figure 1A). As a control, we profiled both peripheral blood and normal liver tissues obtained from liver transplant donors (HCs). Detailed clinical and pathological information, including time since HBV diagnosis, HBsAg level, HBV DNA level, ALT and AST, is provided in table 1 and online supplemental table 1.

In addition, the longitudinal information for AR and CR patients is shown in online supplemental figure 1B, C. The sampling timepoints for AR patients were at the convalescent stage, that is, less than 6 months after initial diagnosis with rapid decrease of HBsAg (online supplemental figure 1B, table 1 and online supplemental table 1). The CR patients were diagnosed with a history of CHB and HBsAg loss, either spontaneously or by antiviral treatment (online supplemental figure 1C, table 1 and online supplemental table 1).

After quality control and filtering (online supplemental figure 2A, B, and online supplemental table 2), we obtained 243,000 transcriptomes of single cells from 23 individuals. Eight major cell clusters were identified, including CD4+ T cells (CD3D+CD4+), CD8+ T cells (CD3D+CD8A+), γδ T cells (CD3D+TRDC+), NKT-like cells (CD3E+KLRF1+), NK cells (GLNY+NKG7+), B cells (MZB1+CD79A+), plasma B cells (MZB1+CD38+) and myeloid cells (LYZ+CD14+) (figure 1B). In total, we identified 10 subclusters for CD4+ T cells, 12 for CD8+ T cells, 9 of γδ T cells, 1 of NKT-like cells, 7 of NK cells, 7 of B cells, 3 of plasma B cells and 11 of myeloid cells (figure 1B).

Based on top differentially expressed genes (DEGs), we identified unique markers for each of the 60 clusters (figure 1C and online supplemental table 3).

The cell clusters showed different tissue origins and enrichment in disease stages (figure 1D, online supplemental figure 3A, B, and online supplemental table 2). To investigate the distinctive immune profiles of different disease phases, we analysed the immune cell composition in each of the individuals. We found that both CD4+ and CD8+ T cells were enriched in the liver of IA and AR phases, both of which are characterised by elevated levels of liver inflammation. Myeloid cells showed preferential enrichment in the liver of IT and IA phases, indicating altered myeloid compartments during chronic HBV infection. γδ T cells and B cells were increased in the liver of CR phase, implicating potential long-term effects of chronic infection on these cells similar to resolved HCV infection.

Expansion of T cells and loss of MAIT cells in HBV-infected patients

Further clustering of CD8+ T cells yielded 12 subclusters, including naïve (CD8T_c01-LEF1), central memory T (Tcm) (CD8T_c02-SELL, CD8T_c09-ACT1N1 and CD8T_c10-BACH2); recently activated effector memory or effector T (Temra) (CD8T_c03-CX3CR1); effector memory
Hepatology (CD8\textsubscript{T}_{c04}-GZMK-SIRPG, CD8\textsubscript{T}_{c05}-GZMK-GPR183, CD8\textsubscript{T}_{c06}-GZMK-IFNG and CD8\textsubscript{T}_{c07}-GZMK-PDCD1); exhausted (CD8\textsubscript{T}_{c08}-LAYN); MAIT (CD8\textsubscript{T}_{c11}-SLC4A10); and a proliferating subset (CD8\textsubscript{T}_{c12}-MKI67) (figure 2A, online supplemental figure 4A and online supplemental table 3).

By examining signature genes defined previously,\textsuperscript{24} we observed distinct functional status for each CD8\textsuperscript{+} T-cell subset, with the highest exhaustion score for CD8\textsubscript{T}_{c08}-LAYN (Tex), high inflammatory scores in effector memory subsets and MAIT, the highest cytotoxic score for CD8\textsubscript{T}_{c03}-CX3CR1 (Temra), and high naive scores in CD8\textsubscript{T}_{c01}-LEF1 and two central memory subsets (CD8\textsubscript{T}_{c02}-SELL and CD8\textsubscript{T}_{c09}-ACTN1) (online supplemental figure 4B and online supplemental table 4).

To understand the potential developmental transitions of CD8\textsuperscript{+} T-cell clusters, we applied partition-based graph abstraction (PAGA) analysis to construct the developmental trajectories of 12 CD8\textsuperscript{+} T clusters.\textsuperscript{25} CD8\textsubscript{T}_{c11}-SLC4A10 cells stood out as a distinct population outside of the trajectory, consistent with

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**Figure 1** Single-cell transcriptome map of immune cells in liver and blood. (A) An experimental scheme diagram of the overall study design. Hepatic immune cells and paired PBMCs were collected and subjected for cell barcoding. The cDNA libraries of 5′-mRNA expression, TCR and BCR were constructed independently, followed by high-throughput sequencing and downstream analyses. (B) UMAP plots of the 243,000 single cells from 23 individuals, including 8 major clusters and 60 subclusters. (C) Gene expression heatmap in each cell cluster. EXP, z-score normalised mean expression. (D) UMAP plots of major cell clusters showing tissue distribution and stage distribution. (E) Tissue prevalence of major cell clusters estimated by Ro/e score. AR, acute recovery; BCR, B-cell receptor; cDNA, complementary DNA; CR, chronic resolved; HC, healthy control; IA, immune active; IT, immune tolerant; NK, natural killer; PBMC, peripheral blood mononuclear cell; TCR, T-cell receptor; UMAP, uniform manifold approximation and projection.
the annotation of MAIT cells. For the other CD8+ T-cell subsets, the Tax (CD8T_c08-LAYN) and Temra (CD8T_c03-CX3CR1) subsets were characterised as two different branches (figure 2B). Such developmental features for Tax and Temra were further supported by RNA velocity analysis (figure 2C).36

Further, each subset showed distinct tissue preference: CD8T_c01-LEF1, CD8T_c02-SELL, CD8T_c03-CX3CR1, CD8T_c05-GZMK-GPR183 and CD8T_c09-ACTN1 clusters were mainly derived from blood, while CD8T_c04-GZMK-SIRPG, CD8T_c06-GZMK-IFNG, CD8T_c07-GZMK-PDCD1, CD8T_c08-LAYN, CD8T_c10-BACH2 and CD8T_c11-SLCA410 clusters were mainly found in the liver (figure 2D,E). As expected, when compared with blood-enriched subsets, these liver-enriched cell subsets expressed higher levels of liver-resident markers, such as CXCR6, CXCR3, CD69 and CD1103.15 20 27 28 and exhibited a GZMBlow IL-2high phenotype29 (online supplemental figure 4C).

We further compared the transcriptional features of each cluster between liver and blood. Interestingly, we identified a series of commonly upregulated genes in the liver among subsets, with CXCR4 being the most frequent (8 times among 12 subsets, CXCR6 and CD103 being the most frequent (8 times among 12 subsets, online supplemental figure 4B). It was reported that CXCR4 was expressed at a high level on intrahepatic HBV-specific T cells,30 and the CXCL12/CXCR4 pathway was involved in the recruitment and retention of immune cells in the liver during chronic HBV infection.31 Further studies are needed to clarify the significance of CXCR4 for liver residency.

The 12 CD8+ T-cell subsets showed stage heterogeneity (figure 2F and online supplemental figure 5A, B). Among CD8+ T subsets, the MAIT population decreased significantly in the liver of all four groups of HBV-infected patients, with that in the IA group being the lowest (figure 2G). The results are consistent with a prior study reporting the loss of hepatic MAIT cells in patients with HBV-related HCC.31 Strikingly, the frequency of MAIT cells in CR failed to restore to the level of HC (figure 2G), although these patients had achieved HBsAg loss for more than 8 months, suggesting that the loss of intrahepatic MAIT induced by HBV infection is long-lasting. Notably, the frequencies of CD8T_c08-LAYN cells increased in IT, IA and AR patients (figure 2H), of which CD8T_c08-LAYN cells from IA patients also displayed the highest exhaustion score (figure 2H). Notably, CD8T_c08-LAYN cells from IA patients showed higher expression of exhaustion genes (such as LAG3 and LAYN), while AR patients had higher expression of cytotoxic genes (such as GZMH, GZMA, GZMB and GZMK) (online supplemental figure 6A), suggesting a cytotoxic CD8+ T-cell response might contribute to control of HBV infection.

Since exhaustion markers are also upregulated in CD8+ T cells by T-cell receptor (TCR)-induced activation, it is also possible that CD8T_c08-LAYN cells from AR are activated rather than exhausted, reminiscent of that observed in patients with COVID-19.32 As for transcriptional level, CD8T_c08-LAYN cells from AR displayed a typical exhausted state, which is featured by high expression of inhibitory markers (TOX, HAVCR2, LAG3, LAYN, PDCD1 and TIGIT) and low expression of effector markers (TBX21 and IFNG) (online supplemental figure 6B). Moreover, the phenotypes of exhausted CD8+ T cells in AR were also validated by flow cytometry (online supplemental figure 6C, D). Therefore, CD8T_c08-LAYN cells represent the exhausted cluster in AR, as well as in other groups.

To further understand the changes of CD8+ T-cell subsets among liver immune cells, we also compared the abundance of each CD8+ T-cell cluster among liver CD45+ cells across stages (online supplemental figure 5C), which confirmed the dramatic loss of MAIT cells and significant expansion of CD8+ T cells in IA and AR patients (online supplemental figure 5A). Taken together, these results indicated that exhausted CD8+ T cells in the liver were expanded in both chronic (IT and IA) and acute (AR) HBV-infected patients.

**Dynamics differences of Tex cells between IA and AR patients** Since we also captured TCR sequences from scRNA-seq (online supplemental table 5), this enabled us to trace the dynamics of T cells, such as clonal expansion (expa), migration (migr) and developmental transition (tran), by previously developed bioinformatics metrics, that is, single T-cell analysis by RNA sequencing and TCR tracking (STARTRAC) indices (figure 2I).

The STARTRAC-expa index revealed that CD8T_c03-CX3CR1 and CD8T_c08-LAYN displayed a high degree of clonal expansion, mainly in the AR and IA stages (online supplemental figure 7A). Of note, CD8T_c03-CX3CR1 was more expanded in AR patients, whereas CD8T_c08-LAYN was more expanded in IA patients (figure 2J).

STARTRAC-tran analysis indicated that CD8T_c08-LAYN was highly associated with GZMK+ subsets, including CD8T_c07-GZMK-PDCD1, CD8T_c04-GZMK-SIRPG and CD8T_c06-GZMK-IFNG (online supplemental figure 7B, C). Notably, CD8T_c08-LAYN in IA was more closely linked to CD8T_c06-GZMK-IFNG but less closely to CD8T_c03-CX3CR1 compared with that in AR (figure 2K), suggesting different sources of exhausted CD8+ T cells in IA and AR patients.

Overall, CD8T_c08-LAYN displayed modest mobility as revealed by the STARTRAC-migr analysis (online supplemental figure 7D), similar to the observation in tumours.33 However, exhausted T cells in AR patients exhibited higher STARTRAC-migr scores when compared with that in IA patients (figure 2L).

**Table 1** Patient information

| Group        | Immune tolerant | Immune activation | AR | CR | HC |
|--------------|-----------------|-------------------|----|----|----|
| Cases        | 6               | 5                 | 3  | 3  | 6  |
| Age (years)  | 34 (21–55)      | 29 (25–45)        | 37 (28–45) | 47 (45–49) | 52 (33–66) |
| Sex (M/F)    | 4/2             | 4/1               | 2/1 | 3/0 | 4/2 |
| Time since HBV diagnosis (years) | 14.7 (1.5–27.0) | 20 (10–20) | <0.5 | 10 (1–20) | NA |
| HBsAg level (IU/mL) | 40636 (5652–69 050) | 7881 (833.2–32 970.0) | 783 (0–1293) | <0.05 | NA |
| HBV DNA level (log10 IU/mL) | 8.328 (8–8.88) | 6.761 (5.29–8) | 0.9783 (0–2.935) | LDL | NA |
| ALT (U/L)    | 36.1 (20–45.7)  | 232.2 (60–424)    | 108 (25–192) | 18.67 (13–22) | 20.8 (11–31) |
| AST (U/L)    | 28.7 (19–42)    | 91.2 (34–129)     | 44 (21–58) | 19 (16–24) | 19 (15–27) |

ALT, alanine aminotransferase; AR, acute recovery; AST, aspartate transaminase; CR, chronic resolved; HC, healthy control; LDL, lower detection limit; M, male; NA, not applicable.
Moreover, we found that the cells that shared the same TCR clonal type with liver T ex cells were mainly CD8T_c03- CX3CR1 and CD8T_c04- GZMK- SIRPG subsets (online supplemental figure 7E).

Furthermore, RNA velocity analysis of CD8+ T-cell clusters also corroborated the strong self-expansion of CD8+ T ex in IA patients and the transition of liver T em to CD8+ T ex in IA together with the transition of blood T em to CD8+ T ex in AR patients (online supplemental figure 8A, B). The different dynamics of Tex in IA and AR patients suggest that the transition of CD8+ T cells between liver and blood might facilitate viral clearance, implicating vital roles of factors that affect the function and differentiation of CD8+ T cells on HBV infection.

**Increased intrahepatic regulatory CD4+ T cells in IT and IA patients**

A total of 10 CD4+ T-cell clusters were identified, including 3 naive (CD4T_c01-LEF1, CD4T_c05-CCR7 and CD4T_c06-IF144L), central memory (CD4T_c02-ANX2A), effector (CD4T_c03-GNYL), 2 regulatory (CD4T_c04-FOXP3 and CD4T_c09-CTLA4), transient memory (CD4T_c07-TIMP1), effector memory (CD4T_c08-GZMK) and Th1-like (CD4T_c10-CXCL13) T cells (figure 3A, 3B, 3C).

**Figure 2** Immunological features of CD8+ T-cell subsets. (A) UMAP plots of CD8+ T-cell subsets. (B) PAGA analysis showing the potential developmental connectivity between different CD8+ T-cell subsets. (C) RNA velocity analysis showing the transition potential among CD8+ T-cell subsets. (D) UMAP plots of CD8+ T-cell subsets showing tissue distribution. (E) Tissue prevalence of CD8+ T-cell subsets estimated by Ro/e score. (F) UMAP plots of CD8+ T-cell subsets showing stage distribution. (G) Box plots showing the proportions of MAIT cells in liver CD8+ T cells across stages. One-sided unpaired Wilcoxon test. (H) Box plots showing the proportions and violin plots showing the exhaustion scores of Tex cells across stages. One-sided unpaired Wilcoxon test. (I) Clonal expansion and state transition of CD8+ T-cell subsets estimated by STARTRAC analysis based on all patients. (J) Box plots showing the expansion levels (STARTRAC-expa) of Temra and Tex subsets in IA and AR stages. One-sided unpaired Wilcoxon test. (K) Box plots showing the transition scores (STARTRAC-tran) between Tex and CD8T_c06-GZMK-IFNG and CD8T_c03−CX3CR1 in IA and AR stages. One-sided unpaired Wilcoxon test. (L) Box plots showing the migration scores (STARTRAC-migr) of the Tex subset between blood and liver in IA and AR stages. One-sided unpaired Wilcoxon test. AR, acute recovery; CR, chronic resolved; HC, healthy control; IA, immune active; IT, immune tolerant; MAIT, mucosal-associated invariant T; PAGA, partition-based graph abstraction; STARTRAC, single T-cell analysis by RNA sequencing and TCR tracking; Temra, recently activated effector memory or effector T; Tex, exhausted CD8+ T; UMAP, uniform manifold approximation and projection.
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Figure 3  Immunological features of CD4+ T-cell subsets and interaction with CD8+ T cells. (A) The UMAP plots of CD4+ T-cell subsets. (B) PAGA analysis showing the potential developmental connectivity between different CD4+ T-cell subsets. (C) RNA velocity analysis showing the transition potential among CD4+ T-cell subsets. (D) The UMAP plots of CD4+ T-cell subsets showing tissue distribution. (E) Tissue prevalence of CD4+ T-cell subsets estimated by Ro/e score. (F) The UMAP plots of CD4+ T-cell subsets showing stage distribution. (G) Box plots showing the proportions of CXCL13+ CD4+ T cells in liver CD4+ T cells across stages. One-sided unpaired Wilcoxon test. (H) Box plots showing the proportions and violin plots showing the regulatory scores of liver-resident Treg cells across stages. One-sided unpaired Wilcoxon test. AR, acute recovery; CR, chronic resolved; HC, healthy control; IA, immune active; IT, immune tolerant; PAGA, partition-based graph abstraction; Treg, regulatory T-cell; UMAP, uniform manifold approximation and projection.

online supplemental figure 9A and online supplemental table 3). PAGA trajectory analysis revealed four different fates of CD4+ T cells, with CD4T_c02-ANXA2, CD4T_c03-GNLY, CD4T_c04-FOX3 and CD4T_c06-TIMP1 as different ends (figure 3B). The developmental direction towards the effector and regulatory subset was also informed by RNA velocity analysis (figure 3C). The developmental trajectory was correlated with the functional scores in various cell clusters, with CD4T_c08-CTLA4 cluster being the most exhausted and CD4T_c03-GNLY cluster being the most cytotoxic (online supplemental figure 9B). Tissue preference analysis revealed that CD4T_c05-CCR7, CD4T_c07-TIMP1, CD4T_c08-GZMK, CD4T_c09-CTLA4 and CD4T_c10-CXCL13 accumulated in the liver, and the rest of the five subsets were mainly found in blood (figure 3D,E).

We then compared the fractions of CD4+ T-cell subsets among CD4+ T cells (figure 3F and online supplemental figure 10A, B) or liver CD45+ cells (online supplemental figure 10C) across different groups. Among these CD4+ T subsets, the CD4T_c10-CXCL13 subset was enriched in AR patients in both liver CD4+ T cells (figure 3G) and liver CD45+ immune cells (online supplemental feature 10C). CD4T_c10-CXCL13 cells exhibited transcriptional features of T-follicular helper cells (PDCD1, ICOS, TIGIT, TNFRSF4 and CXCL13, MAF) (online supplemental figure 9A and online supplemental table 3), implicating the potential role of this subset in enabling protective B-cell immune responses. Moreover, CD4T_c09-CTLA4, referred to as the liver-resident regulatory T-cell (Treg) subset, was increased in both IT and IA patients among liver CD4+ T cells (figure 3H) or liver CD45+ immune cells (online supplemental feature 10C). In addition, regulatory effector score analysis of CD4T_c09-CTLA4 cells among stages revealed high levels of regulatory effector function in IT and IA patients (figure 3H). These results suggested that immune regulation by Treg cells might be associated with immune tolerance and viral persistence in CHB.

Potential cell–cell interactions between Treg and Tex cells

Using cellular spatial organisation mapper (CSOmap), a bioinformatics tool to infer spatial organisation of tissues and molecular determinants of cellular interaction, we estimated the cellular interaction potentials between CD4+ T-cell and CD8+ T-cell subsets in a computationally constructed pseudo-space. Significant interactions were found between Treg (CD4T_c09-CTLA4) and Tex (CD8T_c08-LAYN) in IT and IA patients (figure 4A and online supplemental table 6). Notably, Tex and Treg were expanded coordinately in the liver of IT and IA patients.
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We further confirmed the adjacent spatial relationship between T ex (CD8+PD-1+) and T reg (CD4+FOXP3+) cells in IT and IA patients by multicolour immunohistochemistry (IHC) staining. We then compared the strength of potential ligand–receptor (L–R) pairs contributing to T reg–CD8 T ex interaction in IT and IA patients. We found that several L–R pairs, CD44_VIM, HLA-B_CANX, TNFRSF1A_LTB and CD74_APP, showed higher interaction potentials in the IT phase, while HLA-A_APLP2, KLRD1_HLA and KLRD1_HLA-E showed higher interaction potentials in the IA phase (figure 4C,D). It was recently reported that the HLA-B_CANX axis is able to restrain antitumor immunity of T cells. Vimentin, encoded by the VIM gene, is a key

Figure 4  Multicolour IHC staining of Treg and Tex cells in the liver. (A) CSOmap analysis showing the interaction between CD8+ T-cell and CD4+ T-cell subsets. (B) Multicolour IHC staining in the liver across HC, IT, IA and AR groups. One representative image for each group is shown. White dotted arrows indicate Tex cells, white solid arrows indicated Tregs, yellow solid arrows indicated CD4 + PD1+ cells. scale bar, 50 µm. (C) Box plots showing the contributions of indicated L–R pairs to interaction affinity between Tex and Treg in IT and IA stages. one-sided unpaired Wilcoxon test. (D) Bubble heatmap showing the selected L–R pairs between Treg and Tex cells. AR, acute recovery; CR, chronic resolved; CSOmap, cellular spatial organisation mapper; HC, healthy control; IA, immune active; IHC, immunohistochemistry; IT, immune tolerant; L–R, ligand–receptor; Tex, exhausted CD8+ T; Treg, regulatory T-cell.
metabolic and functional controller of Treg activity, and CD44 immune checkpoint controls CD8+ T-cell activation. Moreover, the HLA-E KLRL1 signal is involved in mediating T-cell dysfunction and viral persistence in chronic HBV and HCV infections. Together, these data provide valuable information for functional verification in future studies regarding immune pathogenesis as well as therapeutic attempts for CHB.

### Intrahepatic myeloid cells and their interactions with T cells in HBV-infected patients

Myeloid cells were clustered into 11 cell subsets belonging to four common lineages (dendritic cells (DCs), macrophages, megakaryocytes and monocytes) based on canonical cell markers (figure 5A, online supplemental figure 12A, B, and online supplemental table 3). DCs were further divided into two classical cDC subsets (CLEC9A+ and CLEC10A+) and a mature cDC subset (C2L2-LAMP3), a proliferating subset (cDC2-MKI67) and pDC. All the five DC subsets were mainly found in the liver (figure 5B, C) and were enriched in IT patients (figure 5D, E), suggesting these cells might be driven by the high level of viral replication in IT patients. Macrophages were further divided into macro_c01-C1QC, macro_c02-NLRP3, and macro_c03-FCGR3A. Similar to DCs, macrophage subsets were also mainly distributed in the liver (figure 5B, C). Among the five patient groups, macro_c01-C1QC and macro_c03-FCGR3A showed significant expansion among liver CD45+ cells of IT patients (figure 5F).

To understand the origins of the macrophage subsets, we defined scores regarding Kupffer-derived source (Kupffer score) or monocyte-derived macrophages (MoMF score) based on reported markers (online supplemental table 4). Expression analyses based on genes associated with Kupffer/monocyte origin did not fully explain the heterogeneity of macrophages (figure 5G, online supplemental figure 12C, D, and online supplemental table 4). Further comparison of the DEGs between macrophage populations revealed that macro_c01-C1QC expressed high levels complement C1Q genes and class II HLA molecules, suggesting capacities of phagocytosis (figure 5H) and antigen presentation in this cluster (online supplemental figure 12E and online supplemental table 4). Macro_c02-NLRP3 expressed high levels of proinflammatory genes, such as S100 genes, IL1B, VCAN and LYZ (online supplemental figure 12E and online supplemental table 3) and was featured by a high proinflammatory score (figure 5H). Macro_c03-FCGR3A expressed high levels of antiviral ISGs (IFITM2, IPITM3, SPN, WARS and CTSC) and immunoregulatory genes (SOD1, MT2A, LST1, LYN, MTSS1, FCEAL1, LILRB2 and LILRAP) (online supplemental figure 12E and online supplemental table 3), exhibiting a high proinflammatory score and high ability to inhibit T-cell activation (figure 5H).

To dissect the potential crosstalk of macrophages and T cells, we used CSOmap to infer cell–cell interaction between macrophage subsets and T cells. Strikingly, CSOmap analysis revealed that macro_c03-FCGR3A dominated the interaction with T cells in two chronic phases (IT and IA), and the intensity of such interaction decreased in two resolved phases (AR and CR), while macro_c02-NLRP3 dominated the interaction with T cells in resolved phases (figure 5I). By examining L-R pair interactions between macro_c03-FCGR3A and CD8+ T cells, several L-R pairs between the HLA class I molecules (HLA-I) and leucocyte immunoglobulin-like receptor (LILR) family exhibited strong potential interaction (figure 5J). Consistently, we observed the enrichment of LILRB2-positive macrophages with adjacent locations to T cells in the liver of IT and IA patients (figure 6). These potential interactions might be functional, considering previous findings that LILRB1 and LILRB2 can facilitate immune evasion in the tumour microenvironment by competing with CD8+ T cells for HLA-I binding as well as by recruiting inhibitory molecules through their immunoreceptor tyrosine-based inhibitory receptor motif. Taken together, these results indicate a central role of FCGR3A+ macrophages in modulating T-cell responses in CHB, potentially through HLA-I LILRB signalling.

### Correlations of liver immune subsets with clinical parameters

To understand the relationships of hepatic immune subsets with clinical parameters in HBV-infected patients, we applied correlation analysis of cell frequencies with the levels of HBsAg, HBV DNA, ALT, AST, and histological grade and stage among all the 17 HBV-infected patients. Age and time since diagnosis were also included in the analysis to set aside factors that are affected by these two parameters (online supplemental figure 13).

Interestingly, the levels of ALT showed negative correlation with the frequencies of MAIT cell and positive correlations with the frequencies of CD8+ Treg and CD4+ CD10- CXCL13 in the liver (figure 7A and online supplemental figure 13), suggesting these cell subsets could be the key executors or responders of liver injury. Moreover, the frequencies of NK_c03-PTGDS and pDC in liver CD45+ cells also negatively correlated with ALT levels (online supplemental figure 13). Consistently, we observed a similar pattern for the correlations of AST levels with hepatic immune subsets compared with that of ALT (online supplemental figure 13).

Similar to ALT, a negative correlation for MAIT cell and a positive correlation for CD8+ Treg were observed with liver histological grading scores of liver biopsies (figure 7B). In addition, histological grade positively correlated with the frequencies of CD8T_c05-GZMK-GPR183 and negatively with plasmaB_c01-SDC1 and two myeloid subsets (cDC1 and macro_c03-FCGR3A) (figure 7B and online supplemental figure 13). In contrast, the histological stage only showed positive correlation with the frequencies of CD8T_c05-GZMK-GPR183 in liver CD45+ cells (online supplemental figure 13).

We then assessed the relationship of intrahepatic immune subsets with levels of serum HBsAg and serum HBV DNA. Serum HBsAg levels positively correlated with the frequencies of macro_c03-FCGR3A, macro_c02-NLRP3, cDC-LAMP3 and cDC-MKI67 (figure 7C). Moreover, HBV DNA levels showed a positive correlation with the frequencies of NK_c07-MKI67, CD4+ CD8+ GZMK and pDC in liver CD45+ cells (online supplemental figure 13).

Taken together, these results suggest that the massive HBV DNA and HBsAg in IT patients might be related to the alterations in the myeloid compartment, whereas liver injury in IA and AR patients is likely closely associated with T-cell dysfunction. This notion is consistent with recent findings that HBsAg has minimal impact on virus-specific or global T cells in humans or animal models.

To dissect if there were coordinated immune changes between blood and liver, we applied correlation analysis for all identified immune subsets between blood and liver in 17 HBV-infected patients. Among 60 subsets, four subsets (CD8T_c03-CX3CR1, CD8T_c09-ACTN1, CD8T_c12-MKI67 and plasmaB_c03-MKI67) showed significant correlations between blood and liver (figure 7D and online supplemental table 7). Intrahepatic immune cell subsets with significant alterations across stages, such as MAIT, Treg and CD8+ T cells, could barely be found in the blood (figure 7D). Notably,

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the frequencies of both CD8T_c03-CX3CR1 and CD8T_c09-ACTN1 showed positive correlations between blood and liver (figure 7D). Both CD8T_c03-CX3CR1 (resembles of Temra) and CD8T_c09-ACTN1 (a Tcm subset) cells showed similar functional features, including low exhaustion and high cytotoxicity levels (figure 7E and online supplemental figure 7A, B). Circulating CX3CR1+CD8+ T cells were recently identified as a predictor for the response of checkpoint therapy in cancer.44 By using an additional validation cohort (online supplemental table 1), we confirmed that CX3CR1+GNLY+ Tcm cells (potential surrogates...
for CD8T_c09-ACTN1 cells) preferentially expanded in the blood of AR patients (figure 7F and online supplemental figure 14). The possible roles of the CX3CR1+GNLY+ CD8+ Tcm cells in the cure of HBV should be investigated.

**DISCUSSION**

In this study, we employed scRNA-seq to generate a comprehensive immune landscape of both hepatic and peripheral immune microenvironment at single-cell resolution in patients with acute or chronic HBV infection. The findings will not only get more insights into further understanding of HBV pathogenesis at different phases of HBV infection in humans but also help the discovery of novel immune targets or the development of novel therapeutic strategies for the clinical cure of CHB.

Our data provide an unbiased illustration of the immunological hallmarks for HBV-infected patients (online supplemental figure 15). Specifically, IT patients were characterised by a decrease of MAIT cells and increases of Treg and Tex in the liver. These changes became more pronounced in IA patients and were associated with liver inflammation. Notably, Tex, Treg and FCGR3A+ macrophage subsets showed strong interactions in the IT and IA phases. These alterations might collectively contribute to immune tolerance and viral persistence. As for acute HBV infection, AR patients also showed expansion of Tex but with different sources compared with IA patients. Importantly, we found that such immune alterations in the liver can hardly be simultaneously observed in the blood, highlighting the requirement of evaluating immunological changes compartmentalised at the site of infection. Several recent studies have suggested the use of fine-needle aspirates and flow cytometry to analyse the local immune cells in the liver, which showed good clinical manoeuvrability. Our data provide an in-depth insight into liver immunology and might be an essential resource for drug discovery in the future.

Current guidelines recommend observation rather than active treatment for IT patients. However, it is becoming realised that there might be profound immune alterations at least in some IT patients, which can subsequently link to disease progression. For example, both NK cells and T cells are functionally impaired in IT patients. Our study incisively strengthened this notion and highlighted the coordinated dysregulation of MAIT, Treg, Tex and myeloid subsets in the liver of IT patients, which preceded the elevation of aminotransferases and histological alterations. For this reason, we recommend taking the immunological parameters into account for the decision to start antiviral therapy in the IT phase.

Clear differences in immune features between IA and AR phases were unveiled in this study, representing 'inadequate response' and 'successful response', respectively. First, the sources of expanded liver Tex cells in IA and AR phases were different. Specifically, intrahepatic Tex cells in AR but not IA were observed to be derived from peripheral CX3CR1+ Temra cells. Moreover, AR exhibited stronger expansion of CX3CR1+GNLY+ Tcm cells than IA, which were consistently observed in both liver and blood, suggesting that CX3CR1+GNLY+ Tcm cells may serve as a candidate marker for prediction of HBV clearance. Second, Tex cells in IA and AR displayed different patterns of cellular interaction. In particular, Tex showed more significant interaction with Treg and FCGR3A+ macrophage in IA than that in AR, suggesting potential roles of these cell–cell communications in shaping CD8+ T-cell dysfunction and viral persistence in CHB. Furthermore, several L-R pairs, such as HLA-B_CANX and RPS19_C5aR1, were potentially involved in mediating the interactions of Tex cells with Treg and/or FCGR3A+ macrophages, respectively. Third, the CD4T_c09-CXCL13 subset was preferentially expanded in AR. CD4T_c09-CXCL13 is referred to a CXCL13-producing PD-1hiCXCR5− (Tfh-like or peripheral helper) CD4+ T-cell cluster, which was first described in synovial tissues of rheumatoid arthritis, promoting the formation of tertiary lymphoid-like structures and B-cell responses. Taken together, the different immune features underlying the active hepatitis of IA and AR might be leveraged to the understanding of active hepatitis in various clinical settings, including on-treatment flares, treatment withdrawal flares, reactivation flares with immunosuppressive therapy, etc, and further to optimal management.

In summary, our study revealed the intrahepatic landscape of immune cells across different phases of HBV infection.
Despite the limitations from the cross-sectional design with small sample size and lack of hepatocytes and CD45 negative non-parenchymal cells, our data will serve as a rich resource to gain a deeper understanding of immune responses in HBV infection and might provide valuable insights for the development of novel immunotherapeutic strategies for HBV cure.

METHODS

Study subjects

The present study recruited patients with CHB and patients with resolved HBV infection from the Fifth Medical Centre of Chinese PLA General Hospital. Patients were classified into different clinical phases of HBV infection according to the European Association for the Study of the Liver guideline of

Figure 7  Associations between intrahepatic immune features and clinical parameters in HBV-infected patients. (A) Correlations of the compositions of cell subsets in liver CD45+ cells with ALT levels in HBV-infected patients. (B) Correlations of the compositions of cell subsets in liver CD45+ cells with histological grading of liver biopsies in HBV-infected patients. (C) Correlations of the compositions of cell subsets in liver CD45+ cells with serum HBsAg levels in HBV-infected patients. (D) Correlations of immune subsets between blood and liver. (E) Violin plots showing the expression levels of indicated genes in each CD8+ T-cell subset. (F) Dot plots showing the frequencies of CX3CR1+GNY+ Temra or Tcm cells in CD8+ T cells from blood across stages. One-sided unpaired Wilcoxon test. ALT, alanine aminotransferase; AR, acute recovery; CR, chronic resolved; HBsAg, HBV surface antigen; HC, healthy control; IA, immune active; IT, immune tolerant; PBMC, peripheral blood mononuclear cell; Tcm, central memory T; Temra, recently activated effector memory or effector T.
acceptable for liver transplantation from the Department of obtained from livers procured from deceased donors deemed significant alcohol intake or history of HCC. Control liver tissue was primary biliary cholangitis, non-alcoholic steatohepatitis, significant alcohol intake or history of HCC. Control liver tissue was

Sample collection and cell isolation

PBMCs were isolated using the HISTOPAQUE-1077 (Sigma-Aldrich) solution according to the manufacturer’s instructions. Briefly, 2mL fresh peripheral blood was collected prior to surgery in EDTA anticoagulant tubes and subsequently layered onto HISTOPAQUE-1077. After centrifugation, immune cells remained at the plasma-HISTOPAQUE-1077 interface and were transferred to a new tube, with two washes using phosphate-buffered saline (PBS) (Gibco). Cells were treated with a red blood cell lysis buffer (Miltenyi Biotec) on ice for 1~2 min. After being washed twice, the cell pellets were resuspended in the fluorescence-activated cell sorting (FACS) buffer (PBS supplemented with 2% fetal bovine serum (FBS)) at the approximate concentration of 10^6 cells/mL.

Liver tissue samples were obtained through ultrasound-guided percutaneous liver biopsy with 21G needle and treated as previously described. Briefly, tissues were cut into small pieces in the RPMI-1640 medium (Invitrogen) with 10% FBS (ScienCell), and enzymatically digested with MACS tumour dissociation kit (Miltenyi Biotec) for 60 min on a rotor at 37°C, according to the manufacturer’s instructions. Cells were then stained with APC anti-human CD45 antibody (clone HI30, BioLegend) and 7-AAD (ThermoFisher). Live CD45+ cells were sorted into 0.2 mL low saline (PBS) (Gibco). Cells were treated with a red blood cell lysis buffer (Miltenyi Biotec) on ice for 1~2 min. After being washed twice, the cell pellets were resuspended in the fluorescence-activated cell sorting (FACS) buffer (PBS supplemented with 2% fetal bovine serum (FBS)) at the approximate concentration of 10^6 cells/mL.

Library preparation and sequencing (10x Genomics)

Library construction was performed using the 10X Chromium Single-cell 3’ and VDJ Library Construction Kits V1.1 (10x Genomics, Pleasanton) as per the manufacturer’s protocol. Purified libraries were analysed by an Illumina Hiseq 4000 sequencer with 150bp paired-end reads.

Single-cell RNA-seq data processing

The Cell Ranger toolkit V3.1.0 provided by 10x Genomics was applied to align reads and generate the gene-cell unique molecular identifier (UMI) matrix against the reference genome GRCh38 downloaded from 10x Genomics official website. For each cell, we quantified the number of genes and UMIs and kept high-quality cells with 600~10 000 genes detected and no more than 10% of mitochondrial gene counts.

Unsupervised clustering analysis and identification of signature genes

To comprehensively assign cell clusters, we performed three rounds of clustering for most of the cell types. Each round of clustering starts from a raw count matrix. Using Scanpy V1.4.3, we normalised raw counts and transformed these into logarithmic scales. Highly variable genes were selected using the function sc.pp.highly_variable_genes with the parameter ‘batch_key’ set as patient ID. Principal component analysis (PCA) matrix was then calculated and the top 50 PCA components were used to build a batch balanced k nearest neighbour graph using the BBKNN algorithm. With the batch effects adjusted, cell clusters were identified by Leiden algorithm. DEGs were identified using the Scanpy function sc.tl.rank_genes_groups with the parameter ‘use_raw=True’ based on clusters.

Based on the DEGs for each cluster, the first round of clustering identified major cell types, such as T cells, NK cells, B cells, myeloid cells and non-immune cells. An additional round of clustering was performed within each major cell type. The cells of each major cell type were extracted and clustered at a relatively large resolution (2~10). The resulting clusters with more than 10% of cells with doublet scores larger than 0.5 (defined by Scrublet) were flagged and further examined by the expression of canonical markers. The cells from the flagged cluster would be identified as doublet if they expressed more than one set of canonical markers. Doublets and non-immune cells were then excluded, resulting in 243 000 cells for further analysis. A third round of clustering was then performed to identify the subtypes of cell, following a similar process as the first round within each major type of cells. For cDC2 cells, a fourth round of clustering was also performed to investigate the detailed status.

Dimensionality reduction

For visualisation, the dimensionality of each dataset was further reduced using either the Barnes-Hut t-distributed stochastic neighbour embedding or uniform manifold approximation and projection (UMAP) with Seurat functions RunTSNE and RunUMAP on R and scanpy toolkits with functions sc.pl.umap and sc.pl.tsne on Python. The principle components used to calculate the embedding were the same as those used for clustering.

TCR/B-cell receptor (BCR) analysis

The TCR/BCR-seq sequencing data were processed using the Cell Ranger vdj pipeline with GRCh38 as reference. In all TCR/BCR contigs assembled, we first discarded the low-confidence, non-productive or those UMIs<2. For T cells with two or more α or β chains assembled, the α–β pair showing the highest expression level (UMI) was defined as the dominant α–β pair in the corresponding T cell. If two or more cells had identical dominant α–β pairs, the dominant α–β pairs were identified as clonal TCRs, and these T cells were identified as clonal T cells. To integrate TCR results with the gene expression data, the TCR-based analysis was performed only for cells that were identified as T cells.

A similar protocol was applied to identify BCR clones. For B cells with two or more heavy or light (kappa or lambda) chains assembled, the heavy-light chain with the highest expression level (UMI) was defined as the dominant heavy-light pair.

TCR clonal information and the cell cluster annotations were then used to perform the STARTRAC analysis as previously described. In brief, the degree of clonal expansion, migration and transition of T-cell clusters were determined using three STARTRAC indices, STARTRAC-expa, STARTRAC-migr and
Cell–cell interaction analysis by CSOmap
CSOmap was used to construct a three-dimensional (3D) pseudo space and calculate the cell–cell interaction based on our scRNA-seq data. For each sample, CSOmap was applied individually to reconstruct the cell spatial organisation based on the corresponding scRNA-seq data. Then, a distance matrix among cells was calculated based on the 3D coordinates generated by CSOmap. The shorter the distance of a pair of cells was, the closer the cells were located in the 3D space. A connection matrix was further constructed for cell clusters, of which the connections of a specific pair of cell clusters was defined as the number of cell pairs that belonged to the specific cell clusters with their distance smaller than the cut-off estimated by CSOmap according to the median number of cell neighbours. For a cluster pair, normalised smaller than the cut-off estimated by CSOmap according to the pairs that belonged to the specific cell clusters with their distance a specific pair of cell clusters was defined as the number of cell was calculated based on the 3D coordinates generated by CSOmap. The shorter the distance of a pair of cells was, the closer the cells were located in the 3D space. A connection matrix was further constructed for cell clusters, of which the connections of a specific pair of cell clusters was defined as the number of cell pairs that belonged to the specific cell clusters with their distance smaller than the cut-off estimated by CSOmap according to the median number of cell neighbours. For a cluster pair, normalised connection was further calculated by dividing its corresponding connection value by the product of their respective cell numbers and then multiplying 10000 to eradicate the potential biases resulting from cell numbers of different cell clusters. Meanwhile, to highlight the key L-R pairs underlying the cellular interactions, we also examined the contribution of each L-R pair to the cell–cell affinity estimation with the function provided by CSOmap. During the analysis, both MATLAB and R version (https://github.com/lijxug/CSOmapR) of CSOmap were used.

Definition of functional scores
To illustrate the functional properties of each cell type, we collected sets of genes from the literature and calculated functional scores for each cell. For T cells, exhaustion-related and naiveness-related genes were defined as in Guo et al.; effector genes were extracted from Herbst et al.; inflammatory genes were previously defined in Spranger et al.; and regulatory genes were from Zemmour et al. As for macrophages, the genes for Kupffer score and MoMF score were adapted from Guillot et al. and genes for phagocytosis and proinflammatory were extracted from. Each score was defined as the mean expression of function-related genes (normalised by z-score). The genes for each score are listed in online supplemental table 4.

Tissue enrichment analysis
To quantify the enrichment of immune cell types across different tissues, we compared the observed and expected cell numbers for each cluster in each tissue according to the following formula as we previously described. Ro/e=(observed/expected), where the expected cell numbers of immune cell clusters in a given tissue were calculated from the Chi-square test. We assumed that one cluster was enriched in a specific tissue if Ro/e>1. Fisher exact tests were applied and p-values were adjusted using Bonferroni method to acquire the significance levels in online supplemental table 3.

Correlations between proportion of cell types and clinical features
We investigate the relationship between proportions of cell types and clinical features by fitting linear models. For each cell type and each clinical feature, a linear model was fitted with the proportion of cell type as the predictor and the values of the clinical feature as the response variable using R function lm. Cell types with the output coefficient ‘Pr(>t)’ <0.05 were considered significant.

Flow cytometric analysis
For phenotypic staining in figure 6E, PBMCs were stained extracellularly using primary antibodies for specific detection of surface markers for 30 min at 4°C. The cells were then washed with FACS buffer. For subsequent intracellular staining, the cells were permeabised, fixed and stained using the eBioscience Permeabilization/Fixation Kit according to the manufacturer’s instructions. The cells were then incubated for 30 min at 4°C with antibodies for specific detection of intracellular antigens. Information for the antibodies and reagents used is listed in online supplemental table 8. Samples were analysed by flow cytometry using a BD Canto II flow cytometer (BD Biosciences) with the FlowJo software (BD Biosciences).

For spectrum flow cytometric analysis in online supplemental figure 6B, C, cryopreserved PBMCs were thawed and incubated in RPMI 1640 media containing 10% FBS at 37°C for 3 hours resting. PBMCs were then washed twice in PBS and incubated in Fc block and True Stain Monocyte Blocker for 5 min at room temperature. Cells were stained with surface antibodies for 15 min at 4°C and intracellular antibodies for 30 min at 4°C with Brilliant Buffer Plus (eBioscience) after fixation and permeabilisation for 30 min at room temperature. The information for antibodies is listed in online supplemental table 8. Data were acquired on a five-laser Aurora spectral flow cytometer (Cytek) then analysed using FlowJo software V.10.8.1 (BD Biosciences). For UMAP generation and visualisation as in online supplemental figure 6B, FCS files from one IA, one AR and one CR patients were used for downstream analysis. First, the FlowAI plugin was used to remove aberrant events. CD8+ T cells in each sample were randomly down-sampled to 100 000 cells using the DownSample plugin, and all the downsampled CD8+ T cells were concatenated into a single FCS file. The UMAP plugin was used to calculate the UMAP coordinates (with 15 neighbours, metric=euclidian and minimum distance=0.5 as default parameters). Then the FlowSOM plugin was used in parallel on the same dataset to create a self-organising map (using n=12 clusters as default parameter). Cell populations were annotated according to conventional gating strategy and colour coded as indicated.

Multicolour IHC
Fresh tissues were fixed in 4% neutral buffer formalin and embedded in paraffin. Multiplex immunofluorescence staining was performed using PANO 7-plex IHC Kit (cat#0004100100, Panovue) according to the manufacturer’s protocol. In brief, tissue sections (4 µm) were melted at 60°C for 1 hour followed by deparaffinisation and rehydration. Heat-mediated antigen retrieval was performed in citrate acid buffer (pH 6.0) using microwave incubation. After incubation with blocking buffer at room temperature for 10 min, the sections were incubated with different primary antibodies, including CD4 (ZM0418, Zsbio), CD8 (CST70306, CST), FOXP3 (BLG320202, BioLegend), PD-1 (CST43248, CST), CD68 (CST76437, CST), LILRB2 (AF2078, R&D) and HLA-B (17260–1AP, ProteinTech). Sections were incubated for 10 min at room temperature with an anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody. The stained signals were further amplified using tyramide signal amplification reagents (0004100100, Panovue). Finally, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The stained slides were scanned with the Mantra System (PerkinElmer).

Author affiliations
1Senior Department of Infectious Diseases, The Fifth Medical Center of Chinese PLA General Hospital, Beijing, China
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ORCID iDs
Jin-Wen Song http://orcid.org/0000-0002-8582-8012
Fu-Sheng Wang http://orcid.org/0000-0002-8043-6685

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