Analysis of CD161 expression on human CD8$^+$ T cells defines a distinct functional subset with tissue-homing properties

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CD8$^+$ T lymphocytes play a key role in host defense, in particular against important persistent viruses, although the critical functional properties of such cells in tissue are not fully defined. We have previously observed that CD8$^+$ T cells specific for tissue-localized viruses such as hepatitis C virus express high levels of the C-type lectin CD161. To explore the significance of this, we examined CD8$^+$CD161$^+$ T cells in healthy donors and those with hepatitis C virus and defined a population of CD8$^+$ T cells with distinct homing and functional properties. These cells express high levels of CD161 and a pattern of molecules consistent with type 17 differentiation, including cytokines (e.g., IL-17, IL-22), transcription factors (e.g., retinoic acid-related orphan receptor γ-t, $P = 6 \times 10^{-9}$; RUNX2, $P = 0.004$), cytokine receptors (e.g., IL-23R, $P = 2 \times 10^{-7}$; IL-18 receptor, $P = 4 \times 10^{-6}$), and chemokine receptors (e.g., CCR6, $P = 3 \times 10^{-8}$; CXCR6, $P = 3 \times 10^{-7}$; CCR2, $P = 4 \times 10^{-7}$). CD161$^+$CD8$^+$ T cells were markedly enriched in tissue samples and coexpressed IL-17 with high levels of IFN-γ and/or IL-22. The levels of polyfunctional cells in tissue was most marked in those with mild disease ($P = 0.0006$). These data define a T cell lineage that is present already in cord blood and represents as many as one in six circulating CD8$^+$ T cells in normal humans and a substantial fraction of tissue-infiltrating CD8$^+$ T cells in chronic inflammation. Such cells play a role in the pathogenesis of chronic hepatitis and arthritis and potentially in other infectious and inflammatory diseases of man.

hepatitis | hepatitis C virus | IL-17 | IL-22 | arthritis

The CD8$^+$ T lymphocyte response against human virus infections has been under intense scrutiny since the emergence of HIV and hepatitis C virus (HCV) as global health threats in the 1980s (1). In the past decade, the emergence of MHC class I peptide tetramer technology allowed, for the first time, detailed ex vivo analysis of the T cell responses to such infections, and comparisons of frequency and also phenotype. A common differentiation pathway for antiviral responses has been proposed (2), although it has been difficult to link differences in cellular phenotype to functions, and more importantly to clinical outcomes.

HCV infects nearly 200 million people globally and sets up persistent hepatitis leading over time to a high burden of liver failure and cancer (3). The outcome of infection is related to the CD4$^+$ and CD8$^+$ T cell response, as indicated by a wealth of studies involving host genetics, chimpanzee model infections, and human correlative analyses (4). In those in which persistence is established, CD8$^+$ T cell responses are weak or undetectable in blood, although they may be enriched in the liver (5). A number of potential viral mechanisms for evasion of CD8$^+$ T cell responses have been proposed, although most are common to all persistent virus models (including HIV and lymphocytic choriomeningitis virus in the mouse). These include escape in immunodominant peptides, immune exhaustion, induction of regulatory responses, and loss of CD4$^+$ T cell help (1). However, an alternative explanation is that this outcome reflects the induction of a CD8$^+$ T cell response of a distinct lineage as a result of the localized tissue expression of the virus.

We previously observed expression of CD161 (NKRP1A) (6) on circulating virus-specific T cells in patients with hepatitis B and C, and the levels were higher on the tissue-infiltrating populations of such cells (7). Such expression was not found on T cells specific for most other viruses tested, including HIV, CMV, and EBV. CD161 expression therefore represents a unique potential marker of CD8$^+$ T cells that might potentially differentiate responses targeting specific organs, e.g., the liver. This idea is supported by the close association between CD161 and CXCR6 expression in peripheral blood CD8$^+$ T cells (7). CXCR6 is a chemokine receptor, which binds CXCL16, a chemokine that is constitutively expressed in organs such liver and respiratory tract (8), although it can be induced in other inflamed tissues such as joints (9). Modulation of CXCR6 signaling in murine models disrupts normal tissue immunosurveillance and impairs local immune responses (10–12).

We therefore tested the hypothesis that CD161 expression might define a subset of CD8$^+$ T cells with a distinct differentiation program, leading to a novel phenotype, homing, and function.

Results

CD161^{++} CD8$^+$T Cell Populations Contain the T_C17 Subset. We first examined CD161$^+$CD8$^+$CD3$^+$ T cell populations from healthy donors. We observed two populations of CD161$^+$CD8$^+$CD3$^+$ T cells based on the staining intensity of CD161 (13), CD161^{bright} (CD161^{++}) cells (15% of total CD8 T cells), and CD161^{mid} (CD161$^+$) cells (10% of CD8$^+$ T cells; Fig. 1*A*). After PMA/ionomycin stimulation, CD161^{++} CD8$^+$ T cells secreted IFN-γ and high levels of TNFα (Fig. 1*B*).

It has recently been shown independently that CD161 is highly expressed on human CD4$^+$ T helper cells of the type 17 lineage

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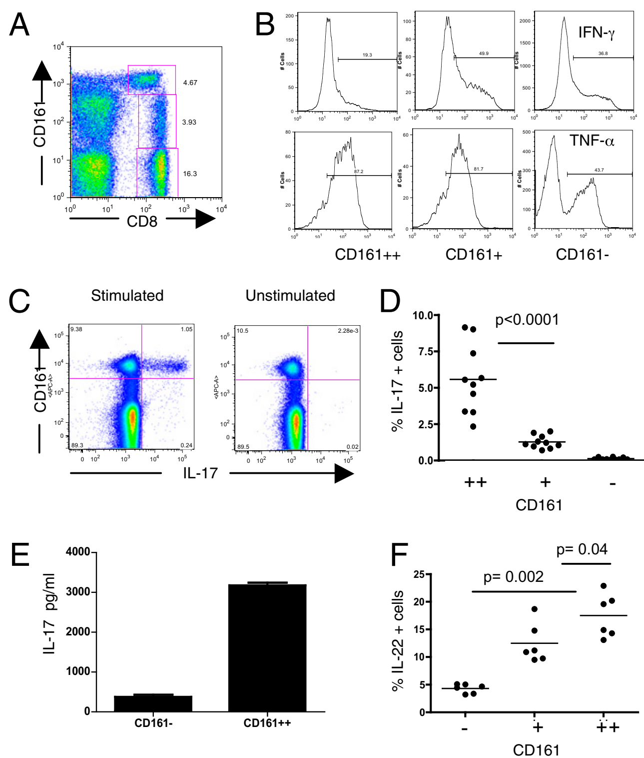

Fig. 1. Function of CD161-expressing CD8⁺ T cells. (*A*) FACS profile of CD161 expression on CD3⁺CD8⁺ T cells in healthy donors. Cells within the live lymphocyte CD3⁺ gate are shown. The gates shown here were also the gates used for sorting (*B*). Panels show PMA/ionomycin–stimulated expression of IFN-γ (*Upper*) and TNF-α (*Lower*) according to CD161 expression, using gates as indicated in Fig 1*A*. Unstimulated controls are shown in Fig. S1*D*. (*C*) IL-17 production in PMA/ionomycin–stimulated cells (gated as in Fig 1*A*). (*D*) Group data from 10 healthy control subjects showing percentage of IL-17 production in CD161⁺⁺, CD161⁺, and CD161⁻ CD8⁺ T cells. (*E*). Secretion of IL-17 as measured by ELISA after stimulation of sorted cells. ICS is shown below for comparison. One representative experiment of two is shown. (*F*) Expression of IL-22 in healthy donors. Healthy donor PBMCs were stimulated as in Fig 1*B* and IL-22 secretion determined in CD161⁺⁺ cells. The percentage of cells staining positive is indicated.

(T_H17) (14, 15). T_H17 cells are defined by secretion of the cytokine IL-17 (16) and play an important role in tissue inflammation. T_H17 cells are characterized by, among others, the expression of the transcription factor retinoic acid–related orphan receptor γ-t (RORγt) (17, 18), the IL-23 receptor (IL23R) (19) and the chemokine receptor CCR6 (20). IL-17 is secreted not only by T_H17 cells but may also be produced by several other cell types, including $CD8^+$ T cells in mouse (21) and man (22). We therefore tested for IL-17 secretion in stimulated $CD8^+$ T cells and observed striking populations of IL-17–secreting $CD8^+$ T cells restricted to the $CD161^{++}$ T cell subset (Fig. 1*C*), consistent across all donors (Fig. 1*D*; $P < 0.0001$). IL-17 secretion by $CD161^{++}CD8^+$ T cells was confirmed by analysis of supernatants in sorted, stimulated cells (Fig. 1*E*).

IL-17–secreting human T lymphocytes may also cosecrete IFN-γ (14, 15, 22). $CD161^{++}$ $CD8^+$ T cells showed three populations of IFN-γ^+, IL-17^+, and IFN-γ/IL-17–cosecreting cells (Fig. S1*A*). Among $CD4^+$ T cells, IL-17 secretion and IFN-γ/IL-17 cosecretion was also associated with CD161 expression (although a $CD161^{++}$ population is not distinct; Fig S1 *B* and *C*) (14, 15). In these assays, IL-17 was produced by 4.5% of $CD161^{++}$ $CD8^+$ T cells in healthy blood, (range, 2.1% to 7.1%). This compares to 5.5% of $CD161^+$ $CD4^+$ T cells [range, 2.6% to 8.9%; *P* value not significant (NS); Fig. S1*C*].

We also showed that $CD161^{+/++}$ $CD8^+$ T cell populations secrete high levels of IL-22, a cytokine involved in tissue repair and epithelial defense (23) and linked to a Th17 phenotype (Fig. 1*F*).

Expression and Phenotypic Analysis of CD161⁺⁺ CD8⁺ T Cells. To define the detailed characteristics of the $CD161^{++}$ $CD8^+$ T cell subset, we performed expression profiling of sorted lymphocytes ex vivo (Fig. 2 and Table S1). Differential gene expression among $CD161^{++}$, $CD161^+$, and $CD161^-$ cells was assessed for the three possible group comparisons. We identified a number of up-regulated genes relating to type 17 cell differentiation. First, RORγt (RORC)—the key transcription factor—was the most highly discriminatory transcript comparing $CD161^{++}$ and $CD161^-$ cells (5.88 $\log_2$ fold change; adjusted $P = 5.78 \times 10^{-9}$; Fig. 2*A*). Comparing $CD161^{++}$ versus $CD161^+$, RORγt was expressed as the second most significant differential signal (3.91 $\log_2$ fold change; adjusted $P = 1.87 \times 10^{-6}$). Elevated expression was confirmed by qRT-PCR, and RORγt staining was observed using flow cytometry (Fig. 2*B*) and immunofluorescence on sorted $CD161^{++}$ cells (Fig. 2*C*).

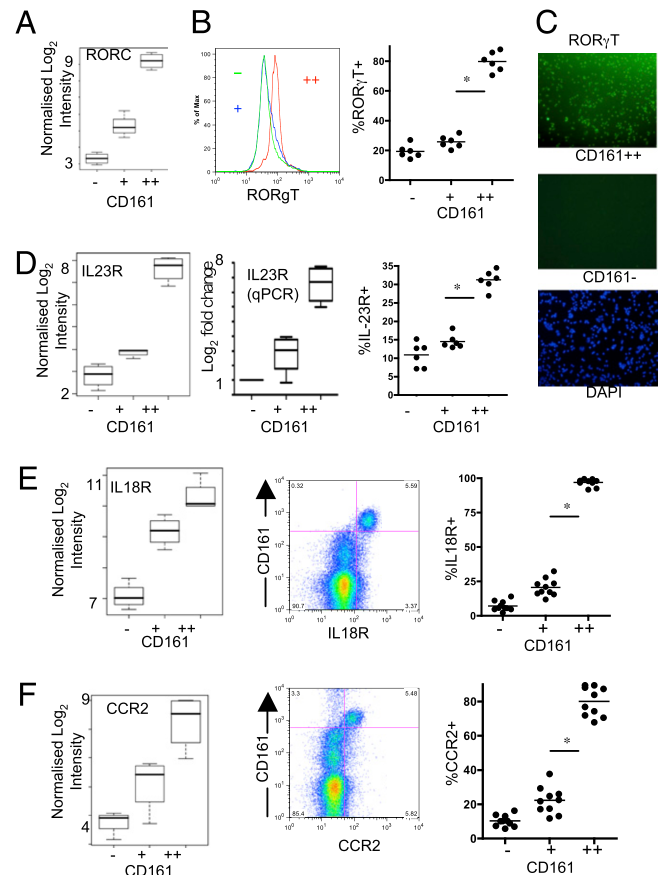

Fig. 2. Phenotype of CD161⁺⁺ cells (*A*) Relative expression of levels of RORC (RORγt) in CD161⁻, CD161⁺, and CD161⁺⁺ sorted CD8⁺ T cells (shown as –, ⁺, and ⁺⁺). Data are derived from the RNA expression analyses after normalization among the 12 samples ($n = 4$ per group). (*B*) Expression of RORγT by flow cytometry in CD161⁺⁺, CD161⁺, and CD161⁻ CD3⁺CD8⁺ T cells. Representative histogram plot (*Left*) and combined data (*Right*); $*P = 0.002$. (*C*) Expression of RORγT by immunofluorescence of sorted CD161⁺⁺ cells (*Upper*) and CD161⁻ cells (*Lower*); DAPI staining is shown for CD161⁻ cells. Magnification: ×200. (*D*) Expression of IL23R by gene array (*Left*), qRT-PCR (*Center*), and flow cytometry (*Right*). Combined staining from six healthy donors is shown. (*E*) IL-18 receptor (IL18R) expression on CD161⁺⁺ cells: gene expression (*Left*), example flow cytometry (*Center*), and group flow cytometry data (*Right*). $*P < 0.0001$. (*F*) CCR2 expression on CD161⁺⁺ cells displayed as in Fig 1*E*.

We also observed clear up-regulation of relevant chemokine receptors: IL23R was up-regulated in the gene array (confirmed by qRT-PCR and flow cytometry; Fig. 2*D*) and also IL-18 receptor, shown to be coordinately important in type 17 differentiation (20, 24) (Fig. 2*E*). Chemokine receptors CCR6 (20) and CCR2 (25), both associated with IL-17–secreting T cells, were up-regulated at the RNA and protein level (Fig. 2*F* and Fig. S2*A*). Expression of the chemokine receptor CXCR6 was also tightly linked to $CD161^{++}$ (but not $CD161^{+}$ $CD161^{+}$) expression on $CD8^{+}$ T cells (Fig. S2*B*). CXCR6 is of specific interest for tissue homing T cells through triggering via locally expressed CXCL16 (7–10).

A number of other genes potentially contributing to type 17 differentiation, including the transcription factors RORα (18) ($\log_2$ fold change, 1.6; adjusted $P = 0.02$) and RUNX2 (26) ($\log_2$ fold change, 1.3; adjusted $P = 0.004$) were up-regulated; elevated RUNX2 expression was confirmed by flow cytometry (mean fluorescence intensity, 988 vs. 392; $P = 0.03$). Additionally, CYP1B1 (downstream of the aryl hydrocarbon receptor) was overexpressed in $CD161^{++}$ cells ($\log_2$ fold change, 1.9; adjusted $P = 0.007$) (27).

We also observed down-regulation of specific molecules in $CD161^{++}$ cells. This included reduction in the expression of CXCR3 in $CD161^{++}CD8^{+}$ T cells (Fig S2*C*); additionally CCR7 was low in both $CD161^{+}$ and $CD161^{++}$ subsets (Fig S2*D*). We also observed low expression of granzyme B and perforin (Fig S3 *A* and *B*). Degranulation, as assessed by CD107a up-regulation, was seen in this population, although the level of CD107a staining was lower than in $CD161^{+}$ cells (Fig S3*C*). A similar phenotype of T_C17 cells with low levels of cytolytic granules and limited cytolytic capacity has been recently reported in the mouse (21).

Further analysis of memory and maturation markers in healthy donors was consistent with $CD161^{++}$ cells representing an "effector" memory population (7, 13), with a distinct phenotype that was not "exhausted" or "terminally differentiated" ($PD\text{-}1^{lo}$, $CD127^{hi}$, $KLRG1^{hi}$, $CD45R0^{+}$, $CD27^{hi}$, $CD28^{hi}$; Figs. S3 and S4).

We confirmed that the populations studied represented conventional TCR-αβ T cells, rather than TCR-γδ T cells or natural killer (NK) T cells (Fig. S5): we have previously shown that conventional CD1d-restricted NKT cells in man constitute only a very small fraction (<0.1%) of total $CD3^{+}$ T cells in blood and typically <0.5% in liver, and most are $CD8^{-}$ (28, 29). Additionally, although the $CD161^{++}$ subset showed reduced CD8 expression, they were $CD4^{-}$ (Fig. S5*D*).

Enrichment of $CD161^{++}$ T_C17 Cells in the Liver During Chronic Hepatitis C. To address the question of whether IL-17–secreting $CD8^{+}$ T cells (T_C17) are involved in the immunopathogenesis of tissue-localized persistent virus infection, we examined the frequency of T_C17 cells in $CD8^{+}$ populations, initially expanded from blood and liver of chronically HCV-infected patients (30), upon stimulation with PMA/ionomycin (Fig. 3). In this well established method, $CD8^{+}$ T cells are stimulated using anti-CD3 and feeder cells for 3 weeks in vitro with supplementary IL-2 but without antigen. Exactly the same procedure is performed in parallel using peripheral blood mononuclear cells (PBMCs) and liver-infiltrating lymphocytes (LILs; *SI Methods*). We found a marked enrichment of such Tc17 cells in liver compared with blood ($P < 0.0001$; Fig. 3 *A* and *B*). This enrichment was much more significant than that of total IFN-γ–secreting cells (P = NS; Fig. 3*A* and *B*). Intrahepatic IL-17–secreting $CD8^{+}$ T cells expressed high levels of CD161 (Fig. 3*C*).

Because in vitro expansion can modulate the functions of T cells and could have been responsible for induction of a Tc17 phenotype, we analyzed these populations ex vivo by using liver explant tissue. Similarly, substantial populations of $CD8^{+}CD161^{++}$ T cells secreting IL-17 were found in liver-infiltrating lymphocytes tested directly ex vivo after PMA/ionomycin stimulation (Fig. S6). Among these cells, substantial IFN-γ/IL-17 coexpression was also observed: we noted that the highest levels of IFN-γ were seen among the dual IFN-γ/IL-17–secreting cell subset compared with IFN-γ monosecretors (Fig. S6*B*; $P = 0.006$). Enrichment of $CD161^{++}$ $CD8^{+}$ T cells in the liver was associated with substantial reductions of the same populations in peripheral blood, and was not seen for $CD161^{+}$ cells, suggestive of specific redistribution in vivo (Fig. S6 *C* and *D*).

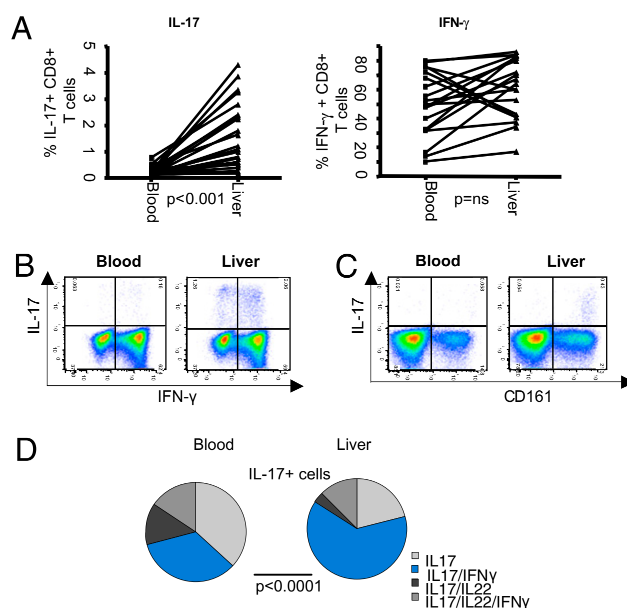

Fig. 3. Peripheral and intrahepatic distribution of IL-17–secreting $CD8^{+}$ T cells during chronic HCV infection. (*A*) Analysis of intracellular production of IL-17 and IFN-γ after stimulation with PMA/ionomycin in $CD8^{+}$ T cells expanded from PBMCs and livers of HCV-infected patients. (*B*) FACS plot from one representative subject showing the distribution of IL-17– and IFN-γ–secreting $CD8^{+}$ T cells in blood and liver. (*C*) Representative FACS plot showing CD161 expression on peripheral and intrahepatic IL-17–secreting $CD8^{+}$ T cells. (*D*) Polyfunctionality of $IL17^{+}$ cells: Percentages of IL-17–secreting, IL-17/IFN-γ–secreting, IL-17/IL-22–secreting, and IL-17/IL-22/IFN-γ–secreting cells among the liver and blood-derived IL-17–producing $CD8^{+}$ T cell populations.

Analysis of IL-22, IFN-γ Secretion, and IL-17 Secretion in Tissue-Homing Cells. IL-22 is also highly expressed by $CD161^{+}$ cells (Fig. 1*F*). We analyzed coexpression of IL-22 and IFN-γ with IL-17 in T_C17 populations expanded from blood and liver of chronically HCV infected patients as described earlier (Fig. 3*D*). IL-22 was coexpressed with IL-17 in a substantial proportion of cells, especially in those derived from liver. The majority of $IL\text{-}22^{+}$ cells also expressed IFN-γ. Comparing the fractions of cells coexpressing IL-22 and IFN-γ among T_C17 populations identified in blood and liver, the following features were noted: a significant intrahepatic enrichment of IL-17/IFN-γ dual secretors ($P = 0.0005$) and a reduction in intrahepatic IL-17 monosecretors ($P = 0.02$). Triple IL-17/IL-22/IFN-γ secretors were present in similar proportions in blood and liver.

Antigen-specific $CD8^{+}$ T cells constitute only only a fraction of the total $CD8^{+}$ T cell infiltrate during chronic hepatitis (5, 30). We therefore asked whether we could observe HCV-specific $CD8^{+}$ T cell populations in liver-infiltrating $CD8^{+}$ T cell populations using a modification of previously established methods to define IFN-γ–secreting populations (*Methods*). As described earlier, $CD8^{+}$ T cells from liver and peripheral blood were expanded using nonspecific stimulation only for 3 weeks, supported by IL-2 supplementation. Expanded T cell populations were then tested against HCV-derived peptides to assess cytokine release. Analysis using

IL-17 ELISpots and an overlapping peptide set spanning the HCV genome revealed that IL-17–secreting, antigen-specific $CD8^+$ T cells directed against diverse peptides were present in the liver. Fig. S7*A* shows data from LILs from four patients in whom this was performed. T cell responses against peptides from across the entire HCV genome were observed. Similar results were obtained from blood-derived cells treated in parallel, although the frequencies of antigen-specific $CD8^+$ T cells were slightly lower than in the liver ($P = 0.04$; Fig. S7*B*). Blood-derived HCV-specific T_H17 cells have been recently reported (31).

Tc17 Cells and Disease Pathogenesis. We finally addressed whether these cells with a unique functional profile, and enriched at the site of infection, played a potential role in disease pathogenesis. For this analysis we performed studies on cell populations taken directly ex vivo from liver and stimulated directly with PMA/ionomycin. Interestingly, the clinical disease score of such patients was not correlated with the frequency of intrahepatic IFN-γ–monosecreting cells, but was inversely correlated with the fraction of intrahepatic IL-17/IFN-γ $CD8^+$ T cells ($r = -0.5$, $P = 0.01$; Fig. 4*A Upper*). There was no ex vivo correlation with viral load and no correlation with liver inflammation as measured by alanine aminotransferase levels.

A reproducible inverse correlation with clinical disease score was observed in a separate set of patients by analysis of intrahepatic cell populations that had been nonspecifically expanded in vitro (Fig. 4*A Lower*; $r = 0.57$, $P = 0.0006$). Thus, it appears—in two independent populations tested separately—that the presence of IL-17/IFN-γ dual producing $CD161^{++}$ T_C17 populations within liver tissue is linked to the control of HCV disease progression in vivo above and beyond any antiviral effects of these cells. However, it is not possible to determine from such studies whether this link is causal.

Diverse Biologic Roles of $CD161^{++}$ $CD8^+$ T Cells. As $CD161^{++}$ Tc17 cells are found at high frequencies in healthy normal donors, and express chemokine receptors (e.g., CCR6 and CXCR6) linked to homing to diverse organs (9, 32, 33), we tested the hypothesis that they represent a stereotypical $CD8^+$ T cell response homing to such tissues.

First, to analyze nonviral inflammation confined to the liver, we performed analyses of T_C17 cells in blood- and liver-derived lymphocytes expanded from patients with nonalcoholic steatohepatitis. Here, as expected, T_C17 populations were also enriched within liver infiltrates, at frequencies similar to those seen in HCV ($P = 0.03$; Fig. S8*A*). Second, we analyzed responses homing to the upper respiratory tract. CCL20, the ligand for CCR6, is constitutively expressed by tonsillar epithelium (as well as liver) (34). Samples from tonsillar tissue showed clear populations of $CD161^{++}$ Tc17 cells, enriched compared with blood ($P = 0.04$; Fig. S8*B*). As in liver, all Tc17 cell populations were high in CD161: up to 20% of $CD161^+$ $CD8^+$ T cells in such tissue were IL-17–secreting. We also analyzed a separate epithelial tissue: kidney derived from two healthy donors. $CD8^+$ T lymphocytes from these organs showed elevated levels of $CD161^+$ $CD8^+$ T cells (Fig. S8*C*), which specifically contained the IL-17–secreting fraction (mean, 1.6%). Third, to analyze the origins of such populations, we analyzed human cord blood. This revealed that the prototypical $CD161^{++}$ $CD8^+$ T cell population is already clearly present among naive cell populations ($CD45RA^+$; Fig. S8*D*). Interestingly, although naive, these cells already express CCR6, i.e., with potential to home to tissue (Fig. S8*D*). Finally, we analyzed joint inflammation because we hypothesized CXCL16/CXCR6 interactions might recruit $CD161^{++}$ cells to this site (9). We examined inflammatory infiltrates from donors with psoriatic and rheumatoid arthritis and showed a striking enrichment of $CD161^{++}CD8^+$ T cells in these infiltrates (mean, 19.2% vs. 5.6%; $P = 0.02$; Fig. 4 *B* and *C*). These $CD161^{++}$ T cells expressed high levels of IL-17 in the joint (median, 10%; Fig. 4*D*), elevated compared with $CD161^{+/-}$ populations ($P = 0.004$).

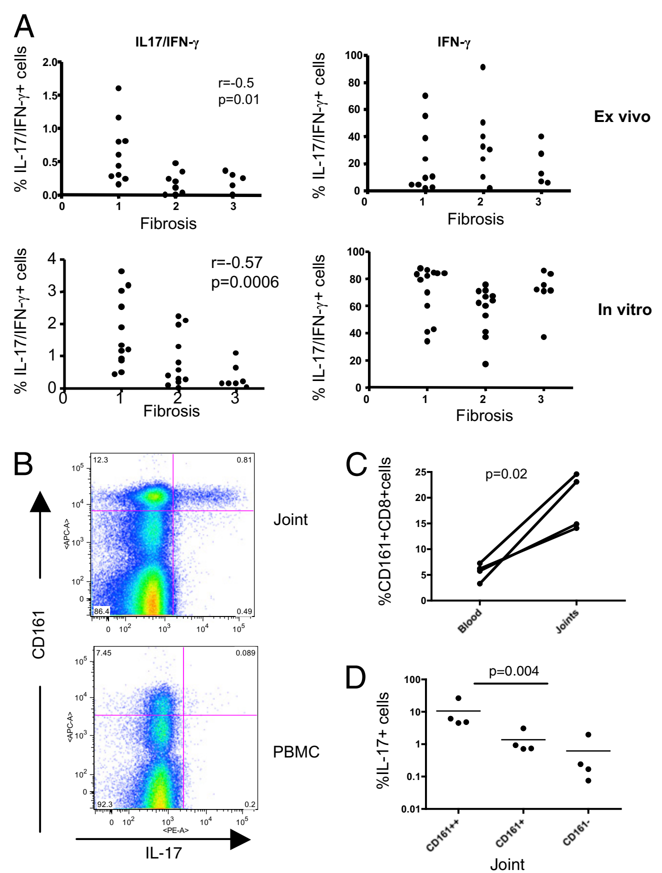

Fig. 4. Frequencies of tissue-homing T_C17 cells. (*A*) Correlations between the frequency of intrahepatic IFN-γ– or IL-17/IFN-γ–producing $CD8^+$ T cells and clinicopathologic disease score (Metavir) in HCV patients. Increasing Metavir score relates to increasing levels of intrahepatic fibrosis, with 4 being the most severe (i.e., cirrhosis). Each dot represents one patient. (*Upper*) Cytokines are analyzed on ex vivo–derived cells (using PMA/ionomycin stimulation); (*Lower*) Cells are analyzed in a separate set of patients after nonspecific in vitro expansion followed by PMA/ionomycin stimulation as in Fig 3 (Spearman correlation). (*B*) CD161-expressing $CD8^+$ T cells from an inflamed joint secreting IL-17 after PMA/ionomycin stimulation. One representative example (psoriatic arthritis) is shown (PBMCs below). (*C*) Group data from four donors ($n = 2$ psoriatic, $n = 1$ rheumatoid, $n = 1$ reactive arthritis; paired *t* test). (*D*) Relative secretion of IL-17 in $CD161^{++}$ versus $CD161^{+/-}$ cells in joints (Mann-Whitney test for ++ vs. +/−).

Taken together, these data suggest that $CD161^{++}$ $CD8^+$ T cell populations represent a prototypical response to tissue-localized infection/inflammation in major organs including, but not exclusive to, the liver. The presence of $CD161^{++}CCR6^+$ $CD8^+$ T cells among a naive T cell population in cord blood reflects the capacity for this response to be primed in a variety of nonlymphoid organs.

Discussion

Taken together, this study identifies a unique population of human IL-17–secreting $CD161^{++}$ $CD8^+$ T cells (T_C17 cells) present in normal individuals, which share common differentiation patterns with T_H17 cells, including key transcription factors, chemokine receptors, and cytokine receptors. We show that such cells are an important component of the tissue-homing populations at diverse sites. T_C17 cells have been recently independently described in healthy human donor blood (22), and in those studies, the link with CCR6 expression, as with $CD4^+$ T_H17 cells, was confirmed (20, 35). However, it is clear from our investigation that T_C17 cells identified through functional assays represent a subset of the larger fraction of $CD161^{++}$ $CD8^+$ T

cells found in normal humans and even in cord blood. As these cells in adults express RORγt and IL23R—both critical steps in differentiation (17–19, 26, 36)—we propose that the $CD161^{++}$ subset are polarized toward the type 17 lineage, even as naive cells, but may require other signals in vivo, e.g., through the aryl hydrocarbon receptor (27), to develop IL-17 and/or IL-22 secretion. The overexpression of RUNX2, another transcription factor, is consistent with a previously described interaction between this protein (as well as RUNX1) and RORγt, which leads to up-regulation of IL-17 (26).

Studies in Tbet- and Eomes-deficient mice have recently indicated that type 17 $CD8^{+}$ T cells occur under certain polarized conditions, and suggested that they may play a role in viral infection (37). In the lymphocytic choriomeningitis virus model used, cytolysis through perforin and granzyme B is critical to control of viremia; in contrast, in human HBV and HCV infection, there is good in vivo and in vitro evidence that IFN-γ secretion plays a dominant antiviral role (38, 39). The environment of specific organs may favor noncytolytic clearance mechanisms to avoid severe immunopathology (38). A recent report also shows a protective effect of T_C17 in a murine influenza challenge (21), driven by noncytolytic mechanisms. Overall, the protective or pathogenic role in any given clinical setting is likely to depend on the tissue concerned, the degree of viral cytopathic effect, and the chronicity of infection.

As far as HCV is concerned, the overall effect of $CD161^{-/+/++}$ populations in disease outcome and the role of antigen-specific versus antigen-nonspecific cells in tissue infiltrates still need to be defined; however, a potentially important association between $CD161^{+}$ Tc17 subsets and limited disease progression is reproducibly observed. If the effect is causal, the mechanism for this is not yet clear, even though the association was robust and reproducible. An alternative explanation for our findings, however, is that such functionality declines as disease progresses; further longitudinal studies are required to address this. Loss of functionality of $CD8^{+}$ T cells in the liver of HCV is known to occur, although we were unable to address this directly in this study.

More generally, $CD161^{++}$ $CD8^{+}$ T cells in vivo could possess critical functions in diverse tissues as a result of their specific tissue-homing and chemokine receptor expression (e.g., CXCR6 and CCR6). CCL20 (the ligand for CCR6) is constitutively highly expressed in the liver and tonsillar epithelia—consistent with the results shown—although it may be up-regulated at many peripheral sites during inflammation, as is CXCR6 (9, 34). We also show very striking enrichment of $CD161^{++}$Tc17 cells in inflamed joints from patients with autoimmune arthritis, in which they may play an important role in disease pathogenesis either directly or through recruitment of other cell types.

A key function is likely to be elevated secretion of cytoprotective IL-22 (33), which has specific protective activity in those tissues expressing IL22R (e.g., liver, gut, kidney) (23). Their action in such organs could also be indirect via activity of other inflammatory infiltrating cells (as appears to be the case in the murine lung) (21). Although IFN-γ is antiviral, IL-17 and IL-22 are not thought to have direct antiviral effects (40), but the latter can induce secretion of antibacterial peptides at epithelial surfaces (32).

Although culture conditions could have affected the results from the cytokine assays, we were able to robustly demonstrate IL-17/IFN-γ–coproducing cells in LILs studied directly ex vivo and thus it is clear the populations are detectable without further cytokine or proliferation-induced artifact. Currently, the ex vivo antigen-specific T cell populations were too infrequent to assay reliably by using flow cytometry for cosecretion of IFN-γ/IL-17, and further work, together with MHC class I peptide tetramer staining, is required to define their full functionality.

Studies are also required to define the mechanisms leading to the origin of the naïve $CD161^{++}$ T cells, their priming, and their role in healthy humans. The presence of "preconditioned" naive $CD8^{+}$ T cells with a distinct chemokine receptor profile ($CCR6^{+}$) is a striking result of this study and suggests that the transcriptional program associated with CD161 is of general significance. In this context, the roles of the known CD161 ligands [LLT-1 (41) and PILAR (42)] are not yet defined. CD161 triggering may inhibit lymphocyte activation (41, 43), although its impact on T_C17 functions has not been examined.

Finally, in addition to the data presented indicating enrichment of $CD161^{+}$ T cells in liver, upper respiratory tract, kidney, and joint, the finding of $CXCR6^{+}$ and/or $CCR6^{+}$ T cells in other organs suggest that the answers to such questions may be of widespread interest immunologically (44–46). Interestingly, polymorphisms in CD161 itself are implicated in multiple sclerosis (47). We therefore speculate that further definition of the lineage may provide novel therapeutic avenues in a number of important human diseases..

Methods

Chronically HCV-infected patients ($n = 81$), patients with nonviral hepatitis ($n = 12$), and healthy normal donors ($n = 15$) were enrolled in this study after informed consent and after appropriate ethical review in the authors' three institutions. The patient characteristics of the liver biopsy study cohorts (in Freiburg, Germany) are summarized in Table S2. PBMCs, liver cells, and liver-derived cell lines were prepared as described previously (30). Lymphocyte staining was performed on whole blood as described previously (7), using antibodies as indicated in *Methods*. Intracellular cytokine staining after PMA/ionomycin stimulation was performed as described previously (7, 30). Cells were analyzed on an LSRII machine, FACSCanto, or FACSCalibur machine (BD Biosciences) using FlowJo software. For immunologic studies, Spearman rank correlation and unpaired and paired Student *t* tests were used. For cell sorting experiments PBMCs (1×10^8) from four healthy donors were stained with CD161, CD3, and CD8 and sorted to >95% purity on a MoFlo MLS cell sorter (Dako). Three sorted CD8 cell subsets from each donor (12 in total) were lysed and homogenized using a QIAshredder spin column (Qiagen). Total RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized and cRNA was amplified and purified using Illumina TotalPrep RNA amplification kit (Ambion). cRNA was hybridized to Illumina Sentrix Human Whole Genome 6 BeadChips (Illumina) and scanned with an Illumina BeadArray Reader. Details of the downstream analysis are included in *SI Methods*. Antiviral assays were performed using the HCV replicon system as previously described (48), using human recombinant IFN-γ (Biomol) or IL-17 (Immunotools). After 48 h of incubation at 37 °C, luciferase activity was assessed by using the Steady-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions and a Lumat LB 9507 (Berthold). Further details are available in *SI Methods*.

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