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Apoptotic epitope-specific CD8$^+$ T cells and interferon signaling intersect in chronic hepatitis C virus infection

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Abbreviations*:* CIA, chronic immune activation; IFN-I, interferon type I; L, ligand; DCs, dendritic cells; ACTB, actin cytoplasmic 1; ROK, heterogeneous nuclear ribonucleoprotein; LAM1, lamin B1; MYH9, non-muscle myosin heavy chain 9; VIME, vimentin; PSA1, proteasome component C2; GDIS, rho GDP dissociation inhibitor 2; RLA, 60S acidic ribosomal protein P2; AEs, apoptotic epitopes; MHC, major histocompatibility complex; HCV, hepatitis C virus; MS, multiple sclerosis; c, chronic; DAAs, direct-acting antiviral agents; PD, programmed death; TIM-3, T cell immunoglobulin mucin-3; IFN, interferon; ISGs, interferon-stimulated genes; TNF, tumor necrosis factor; PBMCs, peripheral blood mononuclear cells; LILs, liver-infiltrating lymphocytes; ELISPOT, enzyme-linked immunospot; IL, interleukin; RT-PCR, reverse transcription polymerase chain reaction; NK, natural killer; mAb, monoclonal antibody; HDs, healthy donors; ALT, alanine aminotransferase; HAI, histological activity index; t, transcripts; PKR, protein kinase R; ds, double-stranded; MxA, myxovirus resistance A; pIFN/RIBA, pegylated IFN-α-ribavirin; TEM, effector memory; TEMRA, terminal effector memory RA; Eomes, Eomesodermin; T-bet, T-box transcription factor; TN, naïve T; TCM, central memory T; USP, ubiquitin specific protease 18; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; HBV, hepatitis B virus.

Abstract

CD8$^+$ T cells specific to caspase-cleaved antigens derived from apoptotic T cells represent a principal player in chronic immune activation (CIA). Here, we found that both apoptotic epitope (AE)-specific and hepatitis C virus (HCV)-specific CD8$^+$ T cells were mostly confined within the effector memory (EM) or terminally differentiated EM CD45RA$^+$ cell subsets expressing a dysfunctional T-helper-1-like signature program in chronic (c)HCV infection. However, AE-specific CD8$^+$ T cells produced tumor necrosis factor (TNF)-α and interleukin-2 at the intrahepatic level significantly more than HCV-specific CD8$^+$ T cells, despite both populations acquiring high levels of programmed death-1 receptor expression. Contextually, only AE-specific CD8$^+$ T cells correlated with both interferon-stimulated gene levels in T cells and hepatic fibrosis score. Taken together, these data suggest that AE-specific CD8$^+$ T cells can sustain CIA by their capacity to produce TNF-α and be resistant to inhibitory signals more than HCV-specific CD8$^+$ T cells in cHCV infection.

Introduction

The phenomenon of chronic immune activation (CIA) [1-3] is a hallmark of several chronic immune-mediated diseases and is attributed to polyclonal hyperactivation of B and T cells caused by both antigen-dependent (e.g. self-antigens) and antigen-independent (e.g. bystander activation) stimuli [1, 4]. Generally, CIA occurs subsequent to a primary immune response specific to persistent infections and, ultimately, amplifies chronic immunopathology [5, 6]. In HIV infection, CIA is closely linked to $CD4^+$ T cell apoptosis and depletion, sustains viral replication, and associates with progression toward AIDS [1, 2, 4]. Recent experimental evidence has demonstrated that a direct causal link exists between disease progression in persistent viral infections and chronic interferon type I (IFN-I) signaling, CIA, and increased expression of negative immune regulatory molecules and that blockade of IFN-I signaling diminishes CIA and immune suppression, ultimately redirecting the immune environment to enable control of infection [7, 8].

We previously proposed that the enormous number of apoptotic cells resulting from the rapid turnover of effector T cells during inflammatory diseases contributes to establishing CIA, on the basis of the following three pieces of evidence [2, 5, 9, 10]. First, apoptotic cells derived from activated T cells retain the expression of CD40 ligand (L) and, in contrast to $CD40L^-$ apoptotic cells (e.g. those derived from epithelial cells), can condition $CD40^+$ dendritic cells (DCs) to acquire high stimulatory capacities [11-13]. Second, apoptotic T cells that have been phagocytosed by DCs can release, within phagosomes, caspase-cleaved fragments, particularly from actin cytoplasmic 1 (ACTB), heterogeneous nuclear ribonucleoprotein (ROK), lamin B1 (LAM1), non-muscle myosin heavy chain 9

(MYH9), vimentin (VIME), proteasome component C2 (PSA1), rho GDP dissociation inhibitor 2 (GDIS), and 60S acidic ribosomal protein P2 (RLA). Third these fragments are efficiently translocated into cytosol, processed, and, ultimately, cross-presented as distinct epitopes (apoptotic epitopes [AEs]) on major histocompatibility complex (MHC) class I molecules to a wide repertoire of autoreactive AE-specific $CD8^+$ T cells [2, 5]. In chronic (c) HIV or acute hepatitis C virus (HCV) infections, the proportion of AE-specific $CD8^+$ T cells correlates with the proportion of circulating apoptotic $CD4^+$ T cells *in vivo* and with the disease progression, supporting the opinion that apoptotic T cells increase in relation to the level of T cell activation during the disease evolution and represent a preponderant source of the apoptotic antigens [2, 3]. The emergence and the maintenance of these responses contribute to intensify the CIA [1-3], not only in viral infections but also in autoimmune diseases such as multiple sclerosis (MS) [14] or rheumatoid arthritis [15], through their capacity to produce high levels of inflammatory cytokines in response to AEs. Therefore, CIA induced by $CD8^+$ T cell autoimmunity to AEs seems to be a general mechanism in both viral and autoimmune diseases.

To examine in-depth the multifaceted aspects of the amplification and spreading of CIA, we studied the dynamics of both AE-specific and virus-specific $CD8^+$ T cell responses in cHCV infection. Despite the recent availability of highly effective direct-acting antiviral agents (DAAs) providing complete viral clearance [16], HCV infection remains, to date, a unique example of a natural, extremely widespread infection that can be exploited to understand the intersecting mechanisms of chronic immunopathology. Studies on the natural history of HCV infection indicate that chronic evolution correlates with the acquisition of HCV-specific T cell dysfunction [17-23], which has been particularly associated with the expression of programmed

death (PD)-1 receptor and/or T cell immunoglobulin mucin-3 (TIM-3), inducing T cell exhaustion upon contact with the corresponding ligands [23-28].

Our data show that the frequency of AE-specific (but not HCV-specific) $CD8^+$ T cells correlates with the evolution of cHCV infection and the level of various IFN-stimulated genes (ISGs), suggesting that ISGs can regulate AE-specific $CD8^+$ T cells in establishing CIA and that AE-specific $CD8^+$ T cells may ultimately amplify liver immunopathology, particularly by the production of tumor necrosis factor (TNF)-α.

Patients and methods

Please refer to the Supplementary Materials for more detailed descriptions.

Patients

Twenty HLA-$A2^+$ patients (11 males and 9 females) with cHCV infection were enrolled in this study for diagnostic evaluation and follow-up assessment. The clinical characteristics of the patients included are shown in **Supplementary Table 1**.

Samples and processing

The isolation of peripheral blood mononuclear cells (PBMCs) and liver-infiltrating lymphocytes (LILs) and the detection of T cell apoptosis are detailed in Supplementary Materials.

Synthetic peptides

The descriptions of procedures and lists of synthetic peptides (**Supplementary Tables 2 and 3**) are reported in Supplementary Materials.

Ex vivo *and* in vitro *assays*

The methods for assays of enzyme-linked immunospot (ELISPOT), PD-1-dependent inhibitory function, RNA purification, and real-time reverse transcription

polymerase chain reaction (RT-PCR) of ISGs are described in Supplementary Materials.

Flow cytometry

The descriptions of procedures for T cell surface or intracellular staining and for functional assays as well as a list of antibodies or dextramers used for flow cytometry are reported in Supplementary Materials (**Supplementary Table 4**). Monocytes, NK cells, B cells, and dead cells were excluded by staining with anti-CD14, -CD16, -CD19, -CD56 monoclonal antibodies (mAbs) and Fixable Viability Dye eFluor® 780 (dump channel).

Statistics

The collected data underwent statistical analysis with GraphPad Prism® 6 software (GraphPad Software). Comparisons between healthy donors (HDs) and patients and between patients with different responses to therapy were analyzed with the Mann-Whitney test. Comparisons between patients at different time points were analyzed with the Wilcoxon matched-pairs signed-rank test. Correlations between tests and clinical data were analyzed with linear regression analysis or the Spearman rank coefficient test. In all tests, $p < 0.05$ was considered statistically significant.

Results

HCV- and AE-specific CD8$^+$ T cells parallel in patients with cHCV infection

The capacity to form IFN-γ-spots (as detected by ELISPOT assay) in response to both HCV epitope and AE pools by CD8$^+$ T cells was significantly higher and wider in cHCV patients than in HDs (**Supplementary Fig. 1A,B**). However, despite the multispecificity of these IFN-γ-producing CD8$^+$ T cells in cHCV patients, they appeared at very low frequencies. No correlation was found between the ELISPOT-

IFN-γ responses to HCV epitopes or AEs and various clinical markers of hepatitis activity tested (alanine aminotransferase [ALT], viral load, and histological activity index [HAI] score). Thus, we enumerated directly *ex vivo* the entire HCV- or AE-specific $CD8^+$ T cell populations, irrespective of their function or differentiation phase, by using both dextramers of HLA-A*0201 molecules complexed to $MYH9_{478-486}$, $MYH9_{741-749}$, $VIME_{78-87}$, $VIME_{225-233}$, or $ACTB_{266-274}$ peptide, and dextramers of HLA-A*0201 molecules complexed to HCV-$NS3_{1073-1082}$, -$NS3_{1406-1415}$, -$NS4b_{1807-1817}$, -$NS5b_{2727-2736}$, or -$Core_{132-140}$ peptide (**Fig. 1A**), in combination with anti-CD8 mAb. Circulating frequencies of these cells were quantified with gating strategy indicated allowing to exclude dead cells, monocytes, NK cells and B cells within the dump channel including Fixable Viability Dye eFluor780 together with anti-CD14, anti-CD16, anti-CD56, and anti-CD19 mAbs conjugated to APCeFluor780 **(Supplementary Fig. 2A)**. Control dextramers complexed to a non-natural irrelevant peptide (ALIAPVHAV) were unable to stain $CD8^+$ T cells, in all samples tested **(Fig. 1A and Supplementary Fig. 2B):** these analyses represented the basis on which we set up $dextramer^+$ cell gates of antigen-specific CD8 T cells, allowing to exclude cells with unspecific staining. Interestingly, 20 $HLA\text{-}A2^+$ cHCV patients (including the 14 studied for the ELISPOT assay) (**Supplementary Table 1**) presented parallel frequencies of peripheral AE- or HCV-specific ($dextramer^+$) $CD8^+$ T cells that were significantly higher than in 13 HDs studied, calculated both as a single dextramer percentage (**Fig. 1B**) and as the sum of dextramer percentages by a single patient or HD (**Fig. 1C**). It is to note the relatively high frequency of HCV-specific $CD8^+$ T cells in some HDs (**Fig. 1B**), likely due to the cross-reactivity between $NS3_{1073-82}$ and influenza virus-$neuraminidase_{231-239}$ epitopes known to increase both response and frequency of these $CD8^+$ T cells [29, 30]. In addition, AE-specific (but not HCV-specific [not shown]) $CD8^+$ T cells

positively correlated with the “HAI staging” (i.e. the HAI of liver fibrosis) (**Fig. 1D**), but not with ALT or viral load (data not shown). A considerable proportion of both HCV-specific and AE-specific $CD8^+$ T cells tested, particularly expressed CD95 and (in a lesser degree) the late activation marker HLA-DR, instead of the early activation marker CD69, indicating that they are long-term experienced memory T cells, as expected in a chronic infection setting (**Fig. 2A**). Interestingly, $dextramer^-$ $CD8^+$ T cells from cHCV patients expressed an activation phenotype similar to the $dextramer^+CD8^+$ T cells (**Fig. 2A**). Activated ($HLA\text{-}DR^+$) HCV-specific (but not AE-specific [not shown]) $CD8^+$ T cells positively correlated with ALT (representing a reliable marker of hepatocyte lysis) (**Fig. 2B**), which suggests that they are involved in the lysis of hepatocytes expressing the viral epitopes. In addition, a notable proportion of both AE-specific and HCV-specific $CD8^+$ T cells expressed the proliferation marker Ki67 (in contrast to the $dextramer^-$ cells), suggesting that they likely proliferated in response to the relevant antigenic epitopes *in vivo* (**Fig. 2C**)**.** In support of this possibility, both the HCV- and AE-specific $CD8^+$ T cells tested were capable of degranulation (as detected by CD107a upregulation) in response to the relevant peptides *ex vivo* (**Fig. 2D**), thereby clearly validating their antigen specificity.

Divergent functional capacity of peripheral and intrahepatic AE-specific or HCV-specific $CD8^+$ T cells

Both AE-specific and HCV-specific $CD8^+$ T cell populations were mostly represented by effector memory (TEM) T cells ($CCR7^-CD45RA^-$) and by terminal effector memory RA (TEMRA) T cells ($CCR7^-CD45RA^+$) containing significantly higher percentages of cells co-expressing both the nuclear transcription factors Eomesodermin and the transcription factor T-box ($Eomes^+/T\text{-}bet^+$) than $Eomes^+/T\text{-}bet^-$ cells (**Fig. 3A-C**). As a control, CCR7, CD45RA, EOMES, and T-bet gates in

the total CD8 population from cHCV patients are reported (**Supplementary Fig. 3**). As expected, Eomes and T-bet were barely represented in the naïve T (TN) or central memory T (TCM) cell populations within both AE-specific and HCV-specific $CD8^+$ T cells (**Fig. 3B**). Despite the percentage of $PD\text{-}1^+$ cells was nearly similar within the total AE-specific and HCV-specific $CD8^+$ T cells (**Supplementary Fig. 4A**), the $Eomes^+/T\text{-}bet^-$ subset within both AE-specific and HCV-specific $CD8^+$ T cell populations significantly expressed more PD-1 than did the $Eomes^+/T\text{-}bet^+$ subset, providing evidence that $Eomes^+/T\text{-}bet^+$ cells are mostly $PD\text{-}1^-$ (**Fig. 4A,B**) [31, 32]. The different HCV genotypes of our patients seem to not influence PD-1 expression on the basis of the following observations: a) no significant difference in PD-1 expression by HCV-specific $CD8^+$ T cells was observed between HCV genotype 1- and genotype 2- or 3-infected individuals (**Supplementary Fig. 4B**); b) PD-1 was significantly (or tended to be) less expressed by $Eomes^+/T\text{-}bet^+$ than by $Eomes^+/T\text{-}bet^-$ cells in both the patient populations (**Supplementary Fig. 4C**). However, despite the fact that the majority of both AE-specific and HCV-specific $CD8^+$ TEM or TEMRA cell populations expressed T-bet, a major transcription factor mediating IFN-γ production [33], they produced very low levels of IFN-γ (as we had also determined with the ELISPOT assay [see **Supplementary Fig. 1A,B**]) in the periphery (**Fig. 5A-C**). To investigate if IFN-γ-producing cells migrated into the inflamed tissue to perform their effector functions, we studied the phenotype and function of LILs isolated from the liver biopsies of cHCV patients. Interestingly, both $CD8^+$ LIL subsets produced significantly higher levels of TNF-α in response to the related peptides *ex vivo* than the counterparts in the periphery (**Fig. 5A-C**). Notably, AE-specific $CD8^+$ LILs were significantly more efficient than HCV-specific $CD8^+$ LILs in performing this function (**Supplementary Fig. 5**), and produced significantly higher levels of IL-2 than the peripheral counterparts (**Fig. 5A-C**)**.** Interestingly, AE-

specific $CD8^+$ LILs producing TNF-α frequency parallels, despite in a non-significant fashion, the total HAI (grading + staging) score (**Fig. 5D**). In addition, both AE-specific (**Fig. 6A**) and HCV-specific (data not shown) $CD8^+$ TEM cells expressing HLA-DR, CD69, or PD-1 accumulated more within the LILs than in the periphery in all patients tested (**Fig. 6A-C**). Similarly, the dextramer$^-$$CD8^+$ T cells with TEM phenotype and expressing the various activation markers analyzed were more accumulated in the liver than in the periphery (**Fig. 6B,C**). Antigen-specific $CD8^+$ LILs increased their capacity to produce TNF-α and IL-2 upon one round of stimulation with autologous irradiated PBMCs in the presence of the relevant peptide for 10 days in IL-2-conditioned medium *in vitro*, confirming that they are mainly represented within LIL-derived memory T cells (**Supplementary Fig. 6**). Nevertheless, PD-1 provided inhibitory signals, as evidenced by the improved capacity to produce IFN-γ by both peripheral AE- and HCV-specific $CD8^+$ T cells (expressing PD-1) in the presence of a blocking antibody specific to PD-L1 *in vitro* (**Supplementary Fig. 7**).

AE-specific $CD8^+$ T cells, CIA, and ISGs

To provide evidence of the interaction between CIA and IFN-I signaling [7, 8] in cHCV infection, we correlated the frequency of AE- or HCV-specific $CD8^+$ T cells with the expression of the following ISG transcripts (t) in T cells from both cHCV patients and HDs: protein kinase R (PKR) – an IFN-inducible double-stranded RNA (dsRNA) activated protein kinase [34]; myxovirus resistance A (MxA) – an IFN-I-induced protein expressed in various cell types and known for its antiviral activity against orthomyxoviruses [35]; and ISG15 – an IFN-I-inducible ubiquitin-like protein conjugated with many intracellular substrates via ISGylation [36]. We evaluated the baseline and the day (d)30 time point (after 1 month of weekly administration of pegylated IFN-α ribavirin [pIFN/RIBA]), representing a more chronic IFN-I exposure

(and thus chronic IFN-I signaling) and corresponding to 2 days after the fourth (weekly) IFN administration. The ISGt levels significantly increased following pIFN/RIBA therapy, indicating that T cells are susceptible to the IFN-I signaling *in vivo* (**Fig. 7A**). Both PKRt and MxAt levels directly correlated with the percentage of AE-specific (but not HCV-specific [not shown]) $CD8^+$ T cells in cHCV patients at baseline (**Fig. 7**) and after 1 month of therapy (data not shown), supporting previous evidence showing an association between sustained ISG expression (including MxA and PKR) and CIA in persistent infections [7, 8, 37]. By contrast, ISG15 inversely correlated with AE-specific $CD8^+$ T cell frequency (**Fig. 7**), consistent with the recent evidence showing a strict association of ISG15 deficiency with increased immunopathology [38].

Discussion

Our findings suggest that AE-specific $CD8^+$ T cells play an important role in establishing CIA and disease progression in cHCV infection. The finding that both antigen-specific (AE- or HCV-specific) dextramer$^+$$CD8^+$ T cells and dextramer$^-$ $CD8^+$ T cells expressed an activation phenotype (particularly when they migrate into inflamed liver), suggests that a global (polyclonal) T cell activation including both activated AE-specific and HCV-specific $CD8^+$ T cells contribute to CIA development during cHCV infection. Virus-specific and AE-specific $CD8^+$ T cells may favor the recruitment of independent $CD8^+$ T cell populations and likely of inflammatory innate immune cells, which contribute to magnify CIA via the production of a storm of cytokines (including IFNs and TNFs) ([39, 40]. The evidence showing that activated (HLA-DR^+) AE-specific (but not HCV-specific) $CD8^+$ T cells correlated with the liver fibrosis score suggests that they may be involved in amplifying liver inflammation, ultimately driving the progression to fibrosis likely via the production of multiple

cytokines including TNF-α [2, 5]. These data are consistent with the results showing that the frequency of AE-specific $CD8^+$ LILs producing TNF-α (that was produced by AE-specific significantly more than by HCV-specific $CD8^+$ T cells in the liver) parallels the HAI score (including “grading” or “inflammatory index” and “staging” or “fibrosis index”), supporting thus the possibility that they can contribute to mediate immunopathology ultimately leading to fibrosis likely via the activation of hepatic stellate cells [41].

The possibility that AE-specific $CD8^+$ T cells may contribute to CIA development is emphasized by the correlation between their frequency and some ISGt levels. The direct correlation between AE-specific $CD8^+$ T cell frequency and MxA or PKR is consistent with previous experimental data showing that sustained expression of ISGs (including MxA and PKR) is associated with CIA development and disease progression in persistent infections and that blockade of IFN-I signaling diminishes CIA and facilitates clearance of the persistent infection [7, 8, 37, 42]. On the contrary, our observation of an inverse correlation between ISG15 and AE-specific $CD8^+$ T cell frequency suggests that ISG15 counterbalances MxA and PKR functions, ultimately helping to limit chronic inflammation [38]. In support of this hypothesis, recent data indicate that inherited ISG15 deficiency in humans paradoxically associates with increased IFN-I immunity via the destabilization of ubiquitin specific protease 18 (USP18; itself an ISG), which is a negative regulator of IFN-I responses [38]. Collectively, our findings support the idea that the balance between pro-inflammatory (e.g. MxA and PKR) and anti-inflammatory (e.g. ISG15/USP8 axis) ISGs may contribute to establishing a status of low-grade/long-lasting CIA in cHCV infection. Studies are required to screen additional ISGs [43] for possible correlations with the generation of CIA in a larger cohort of cHCV patients.

Recently, the division of virus-specific $CD8^+$ T cells into $Eomes^+/T\text{-}bet^-$ and $Eomes^+/T\text{-}bet^+$ cells has been validated to discriminate dysfunctional and functional cells, respectively, in various chronic viral infections [22, 44], on the basis of previous experimental data showing that T-bet expression in memory T cells is associated with the control of chronic infection through down-regulation of PD-1 [31, 32] and that Eomes is associated with memory and with the control of chronic infection when it is co-expressed with T-bet [32]. We found that the majority of both AE-specific and HCV-specific $CD8^+$ T cells (in both the periphery and the inflamed livers) were confined within TEM and TEMRA cell subsets displaying a dysfunctional Th1-like program because they produced very low or undetectable amounts of IFN-γ, despite the notable expression of the related transcription factor T-bet. However, both HCV-specific and, to a significantly higher extent, AE-specific $CD8^+$ T cells increased their capacity to produce TNF-α and IL-2 in response to the relevant antigenic peptides when they migrated into the inflamed liver, despite a concomitant increase in PD-1 expression on these cells. The dichotomy between the poor IFN-γ or IL-17 production and the notable TNF-α production by intrahepatic $CD8^+$ T cells may be determined by the possibility that these cells have lost the polyfunctional capacity that was previously observed in patients resolving acute HCV infection [3], likely due to the progressive establishment of the “T cell exhaustion” process in chronic infections [28]. In the present study, AE-specific $CD8^+$ LILs were more efficient than virus-specific LILs in producing TNF-α. This finding is likely because AE-specific $CD8^+$ T cells interact with tissue-resident antigen-presenting cells (APCs) that cross-present apoptotic T cells and deliver stimulatory (in addition to inhibitory) signals, whereas HCV-specific $CD8^+$ T cells target infected hepatocytes that simultaneously express viral antigenic stimuli and various inhibitory (but not stimulatory) receptor ligands.

Our data differ substantially from those of recent studies showing that the majority of virus-specific $CD8^+$ T cells is dysfunctional and associated with low T-bet expression in chronic hepatitis B virus (HBV) and HCV infections [22], as well as chronic HIV infection [44]. These contradictory findings may reflect the heterogeneity of these patients, thus underscoring the need to verify whether a genetic background predisposing to viral persistence harbors variants that may foster these differences. These discrepancies may also be attributed to the different functional assays (e.g. in terms of cytokines produced by T cells) of peripheral and tissue-recruited lymphocytes that were performed in the various studies. The finding that TNF-α- or IL-2-producing cells were mainly recruited to the liver compartment may explain why they were visualized to a lesser extent in the periphery. In addition, we cannot exclude that some of the discrepancies could be the consequence of a mismatch of the HCV-epitope sequence in the autologous virus as a result of mutational escape of the epitope that may profoundly change the virus-specific $CD8^+$ T cell phenotype including the PD-1 expression. Further studies are required to ascertain the potential impact of such epitope mismatches on these differences. Here we propose that both intrahepatic HCV-specific and AE-specific $CD8^+$ T cells be defined as semi-functional cells because they can carry out certain functions but not others: they have the ability to produce IL-2 and, in particular, TNF-α (a potent inflammatory cytokine even contributing to hepatocellular carcinoma promotion [45, 46], but they do not have polyfunctional capacities, including the production of high levels of IFN-γ or IL-17 in response to the relevant epitopes. Our results are consistent with reports proposing that intrahepatic virus-specific $CD8^+$ T cells are not completely exhausted in cHCV infection [47] and that $PD\text{-}1^+$ T cells in chronic infections or tumors conserve a crucial level of responses that is instrumental in controlling pathogen or tumor spread and in causing minimal tissue damage [48].

Under these conditions, the persistence of functionally competent AE-specific $CD8^+$ T cells would strongly promote CIA and immunopathology, particularly via their notable production of TNF-α. In this context, it should be taken into account that the application of immunotherapy inhibiting PD-1/PD-L1 interaction, despite potentially capable to improve anti-viral immune responses [49] [50], may also increase CIA when $PD\text{-}1^+$ AE-specific CD8 T cells are disengaged from the PD-1-dependent control. Nevertheless, this therapy is limited in cHCV infection by the recent development of highly effective DAAs providing complete HCV eradication [16]. Future studies will be planned to ascertain the possibility that DAAs that elicit complete HCV eradication may, as a consequence, reduce/eliminate the immune cell activation that is represented by the substrate-inducing AE-specific T cells [2, 5].

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Figure legends

Fig. 1. AE-specific and HCV-specific dextramer$^+$CD8$^+$ T cells parallel in cHCV infection. (A) Flow cytometry analyses of dextramer$^+$CD8$^+$ T cells specific to the indicated AEs (left) or HCV epitopes (right) in a patient and an HD. The gates of dextramer$^+$ antigen-specific CD8 T cells were set up on the basis of the control dextramers complexed to a non-natural irrelevant peptide (Dextr neg) that were unable to stain CD8$^+$ T cells. (B) Percentage of AE- (left) or HCV-specific (right) dextramer$^+$CD8$^+$ T cell populations in 13 HDs and 20 patients (each symbol represents a single dextramer$^+$CD8$^+$ T cell percentage, and the grey symbols represent dextramer$^+$CD8$^+$ T cell specific to NS3$_{1073\text{-}82}$). (C) Sum of percentages of AE- (left) or HCV-specific (right) dextramer$^+$CD8$^+$ T cell populations in 13 HDs and 20 patients (each symbol represents a single patient). Statistical analysis was performed with the Mann-Whitney test. ****p <0.00001. (D) Correlation between the sum of percentages of the various AE-specific CD8$^+$ T cell populations and HAI staging. Statistical analysis was performed with the Spearman correlation. *p <0.01.

Fig. 2. Proliferation and antigen-specific validation of HCV-specific or AE-specific CD8$^+$ T cells. (A) Mean of percentages of the indicated markers in AE- and HCV-specific (dextramer$^+$) CD8$^+$ T cells from 9 cHCV-patients (left graphs), and representative flow cytometry analysis in a cHCV-patient (right graph). (B) Correlation between the mean of percentages of HLA-DR$^+$ cells in HCV-specific CD8$^+$ T cells and ALT levels. Statistical analysis was performed with the Spearman correlation; *p <0.01. (C) Mean of percentages of Ki67$^+$ cells in AE- or HCV-specific CD8$^+$ T cells, compared with dextramer$^-$ (Dxtr$^-$) cells, from 10 cHCV patients (left

graph), and representative flow cytometry analysis (right graph). Statistical analysis was performed with the Wilcoxon matched-pairs test (the continuous lines link Dextramer$^-$CD8$^+$ cells and AE-specific CD8$^+$ T cells; the dotted lines link Dextramer$^-$ CD8$^+$ cells and HCV-specific CD8$^+$ T cells). *p <0.01; **p <0.001. (D) Representative flow cytometry analysis of CD107a$^+$ cells in AE- or HCV-specific CD8$^+$ T cells before and after stimulation with the relevant peptide from 1 of 4 patients.

Fig. 3. Preponderant expression of Eomes and T-bet in AE-specific and HCV-specific CD8$^+$ TEM or TEMRA cells. (A) Representative flow cytometry analysis of TN, TCM, TEM, or TEMRA cells in AE- or HCV-specific dextramer$^+$CD8$^+$ T cells from a patient. (B) Representative flow cytometry analysis of cells expressing T-bet and/or Eomes within TN, TCM, TEM, or TEMRA cells present in AE-CD8$^+$ T cells or HCV-CD8$^+$ T cells from a cHCV patient. (C) Mean of percentages of T-bet$^-$/Eomes$^+$ and T-bet$^+$/Eomes$^+$ cells within TEM or TEMRA cells present in AE-specific (top graph) or HCV-specific (bottom graph) CD8$^+$ T cells from 10 patients. Statistical analysis was performed with the Wilcoxon matched-pairs test. *p <0.01.

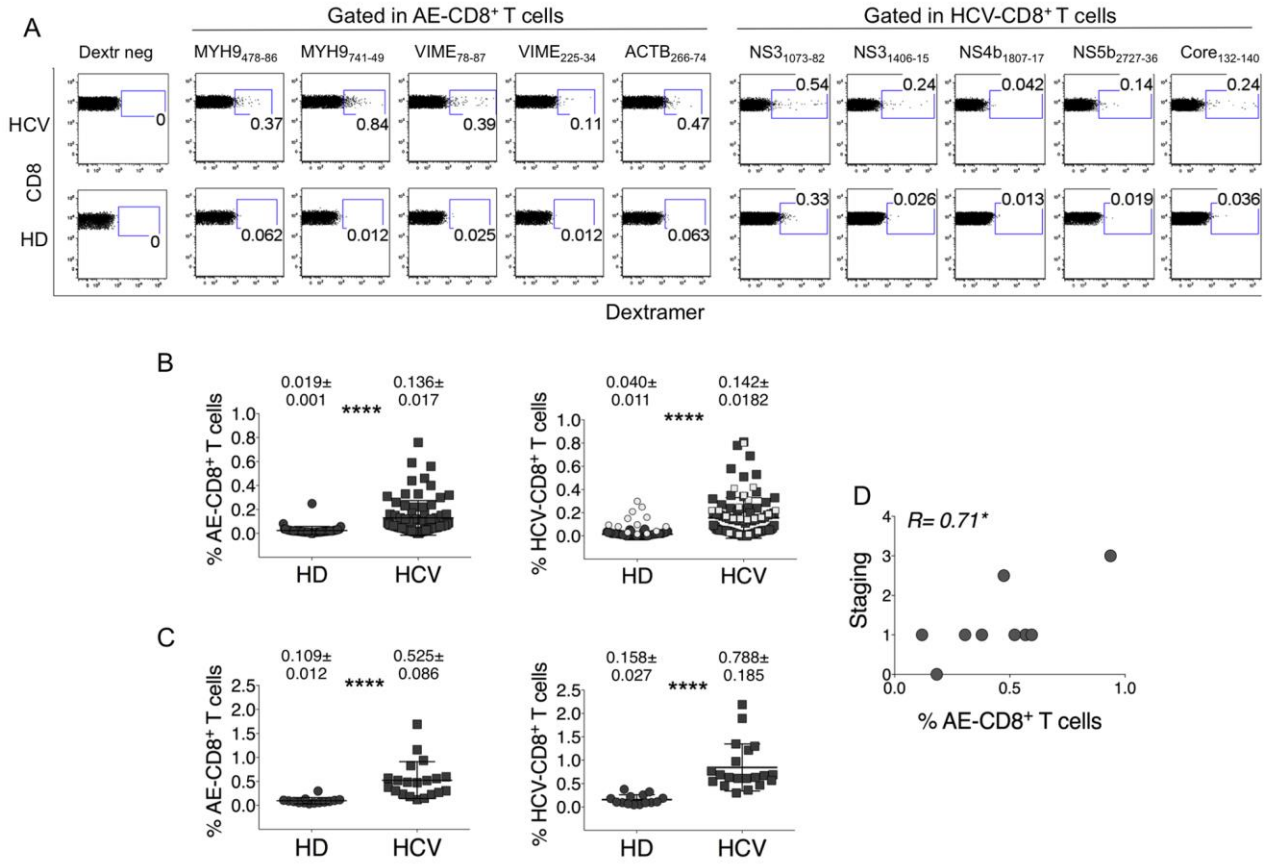
Fig. 4. Prevalent expression of PD-1 in Eomes$^+$/T-bet$^-$ subset of AE-specific or HCV-specific CD8$^+$ T cells. (A) Representative flow cytometry analysis of PD-1$^+$ cells within the Eomes$^+$/T-bet$^-$ or Eomes$^+$/T-bet$^+$ cells present in AE- or HCV-specific CD8$^+$ T cells from a patient. (B) Mean of percentages of PD-1$^+$ cells within the Eomes$^+$/T-bet$^-$ or Eomes$^+$/T-bet$^+$ cells present in AE-specific (empty bars) or HCV-specific (filled bars) dextramer$^+$CD8$^+$ T cells from 10 patients. Statistical analysis was performed with the Wilcoxon matched-pairs test**.** *p <0.01; **p <0.001.

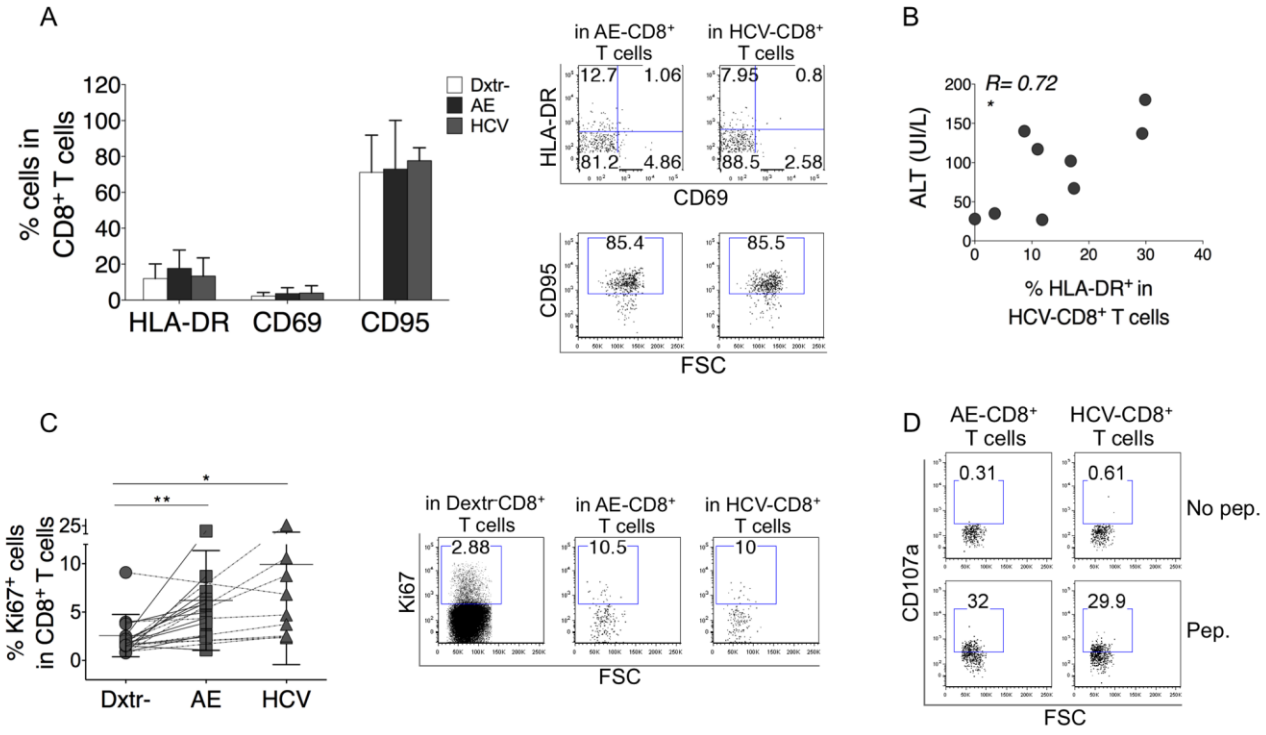
Fig. 5. Differential functions of peripheral and liver-infiltrating AE-specific or HCV-specific CD8$^+$ T cells. Flow cytometry analyses of the indicated cytokines by AE-specific (A) or HCV-specific (B) CD8$^+$ T cells, stained with the pool of the related dextramers, in PBMCs or LILs of a patient, stimulated (or not) with the pool of the relevant peptides. Dot plot analyses are gated on CD8$^+$dextramer$^+$ cells and show percentages of cytokine-producing cells. (C) Percentage of cells producing the indicated cytokines in AE-specific (left graph) or HCV-specific (right graph) CD8$^+$ T cells in response to the related peptides from PBMCs or LILs of 13 and 5 patients, respectively. Data are represented with background (no stimulation) subtracted. Statistical analysis was performed with the Wilcoxon matched-pairs test**.** ***p* <0.001; ****p* <0.0001; *****p* <0.00001. (D) Relationship between HAI and percentage of AE-specific CD8$^+$ T cells producing TNF-α.

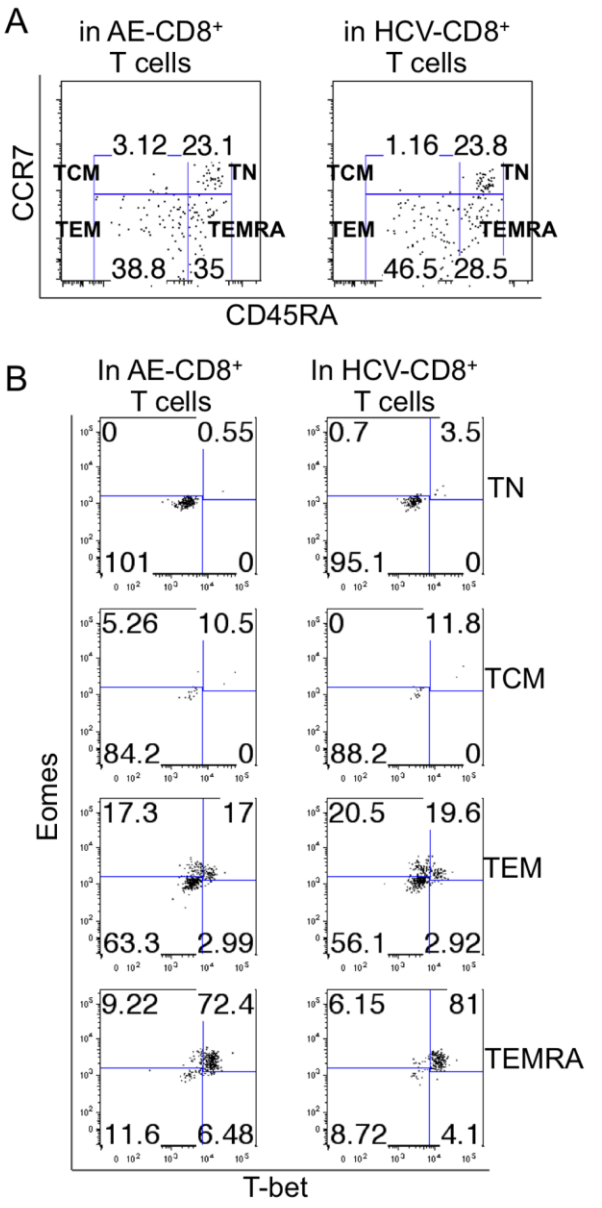
Fig. 6. Compartmentalization of activated dextramer$^+$AE-specific or dextramer$^-$CD8$^+$ T cells with effector phenotype in inflamed liver. (A) Percentage of AE-specific CD8$^+$ T cells in PBMCs or LILs from the 5 patients providing sufficient amounts of LILs to perform both functional (see Fig. 5) and phenotype assays (left graph), and representative flow cytometry analysis (right graph). Statistical analysis was performed with the Wilcoxon matched-pairs test. **p* <0.01. (B) Percentages of TN, TCM, TEM, TEMRA cells in AE-specific dextramer$^+$ or dextramer$^-$CD8$^+$ T cells from PBMCs or LILs of 4 patients tested (left graph), and representative flow cytometry analysis (right graph). Statistical analysis was performed with the Wilcoxon matched-pairs test. **p* <0.01. (C) Percentages of HLA-DR$^+$, CD69$^+$, PD-1$^+$, CD95$^+$ cells in AE-specific or dextramer$^-$CD8$^+$ T cells from PBMCs or LILs of 4 patients analyzed (left graph), and representative flow

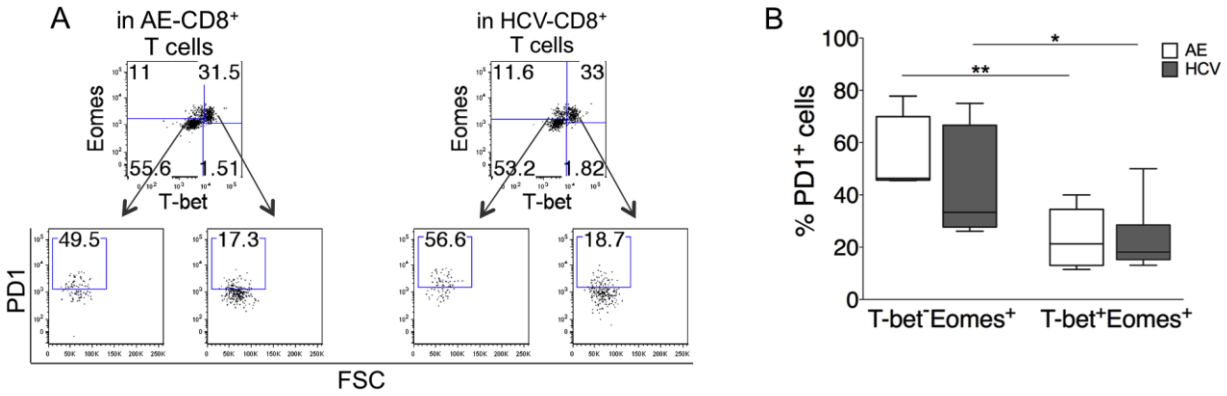
cytometry analysis (right graph). Statistical analysis was performed with the Wilcoxon matched-pairs test. *p <0.01.

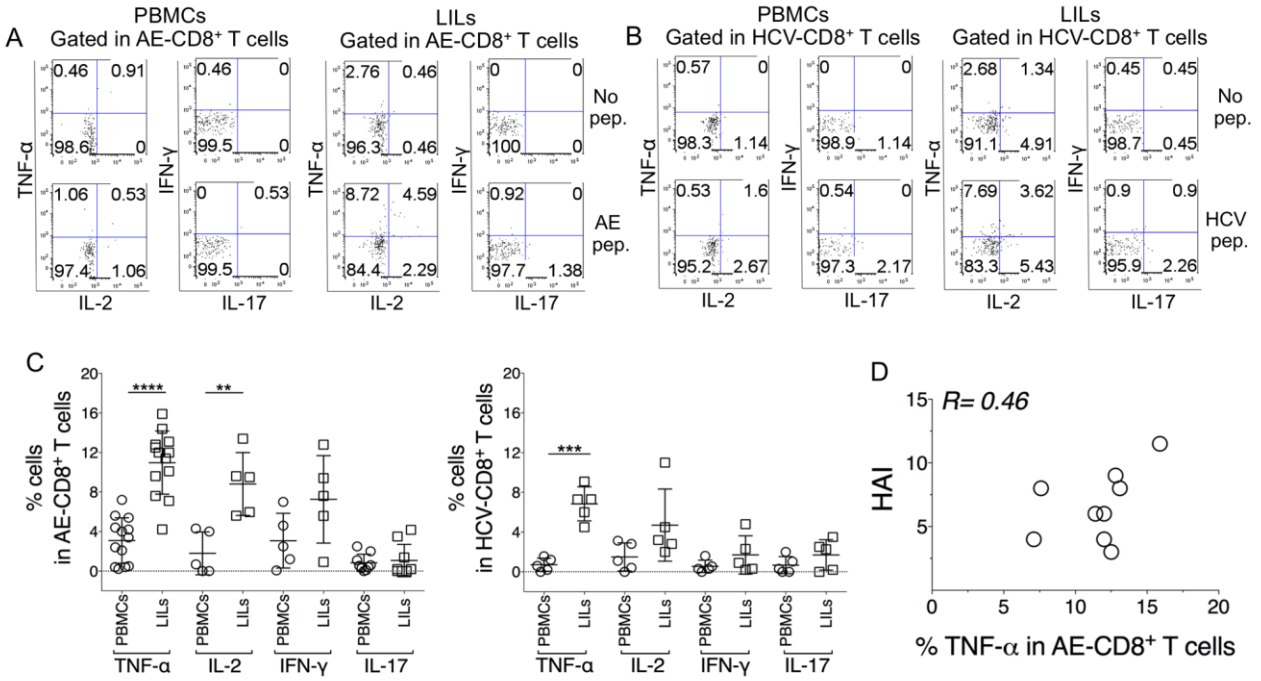
Fig. 7. Correlation between AE-specific CD8$^+$ T cells and ISGs. (A) Levels of PKR (left graph), MxA (central graph), and ISG15 (right graph) in T cells of patients at baseline (time [T]0) and after 1 month (T1) of IFN-based therapy. Statistical analysis was performed with the Wilcoxon matched-pairs test. *p <0.01. (B) Correlations between the sum of percentages of AE-specific (dextramer$^+$) CD8$^+$ T cells and PKR (left graph), MxA (central graph), or ISG15 (right graph) levels in T cells at baseline. Statistical analyses in all panels were performed with the Spearman correlation. *p <0.01.

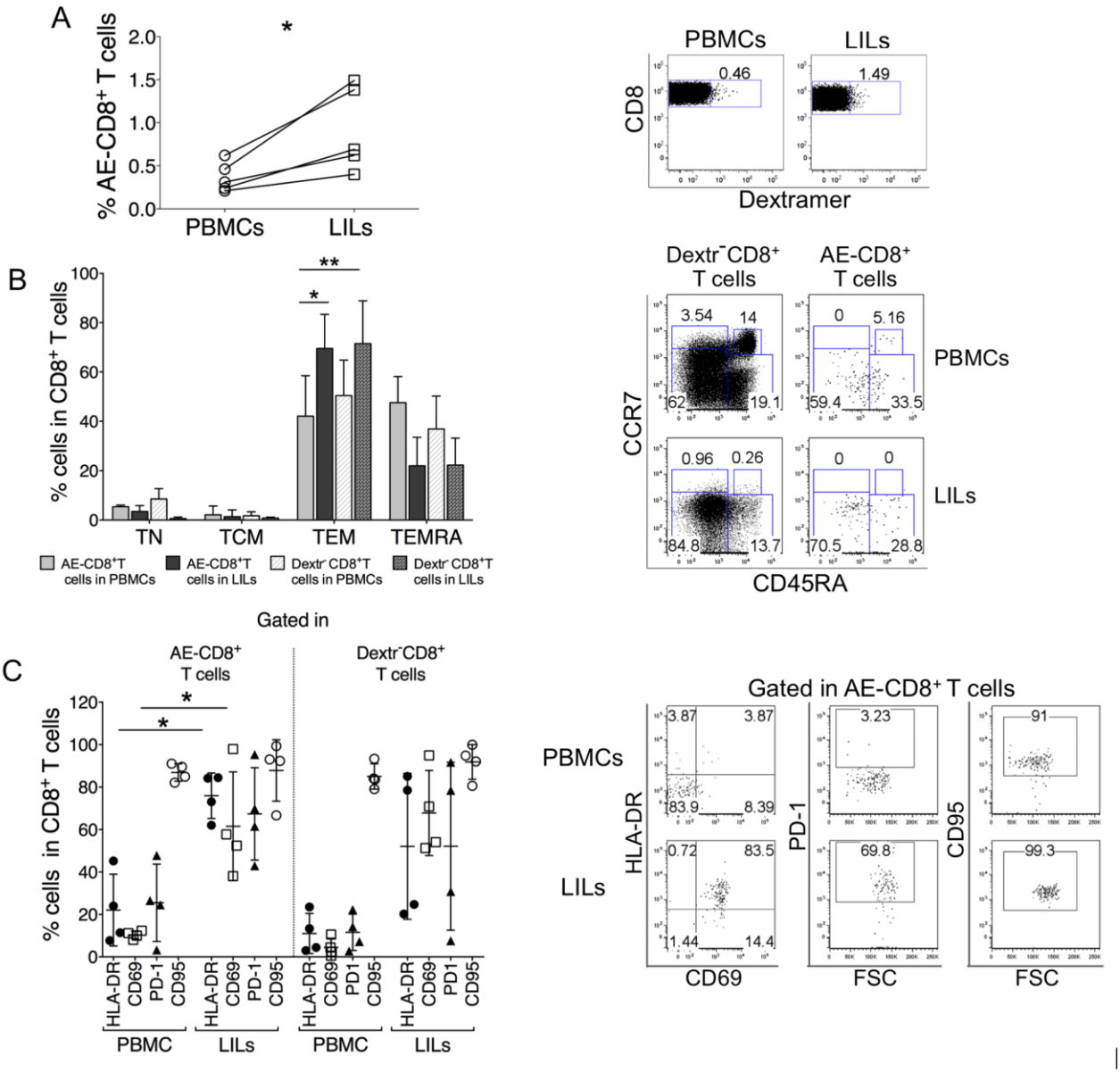












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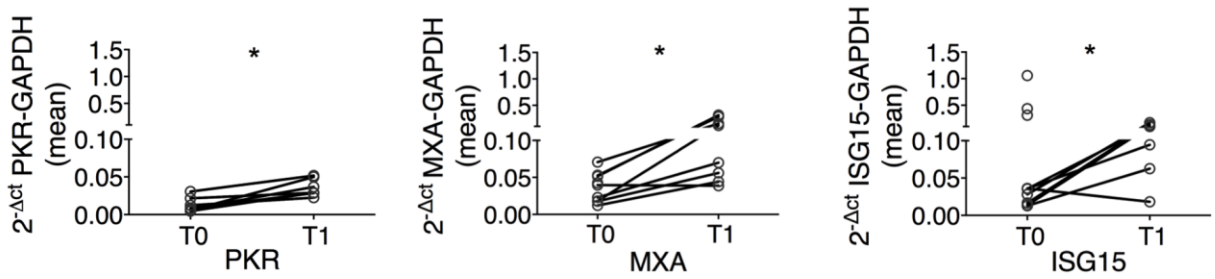

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