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# Dysfunctional CD8<sup>+</sup> T cells in hepatitis B and C are characterized by a lack of antigen-specific T-bet induction

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**The transcription factor T-bet regulates the production of interferon- $\gamma$  and cytotoxic molecules in effector CD8 T cells, and its expression correlates with improved control of chronic viral infections. However, the role of T-bet in infections with differential outcome remains poorly defined. Here, we report that high expression of T-bet in virus-specific CD8 T cells during acute hepatitis B virus (HBV) and hepatitis C virus (HCV) infection was associated with spontaneous resolution, whereas T-bet deficiency was more characteristic of chronic evolving infection. T-bet strongly correlated with interferon- $\gamma$  production and proliferation of virus-specific CD8 T cells, and its induction by antigen and IL-2 stimulation partially restored functionality in previously dysfunctional T-bet-deficient CD8 T cells. However, restoration of a strong interferon- $\gamma$  response required additional stimulation with IL-12, which selectively induced the phosphorylation of STAT4 in T-bet<sup>+</sup> CD8 T cells. The observation that T-bet expression rendered CD8 T cells responsive to IL-12 suggests a stepwise mechanism of T cell activation in which T-bet facilitates the recruitment of additional transcription factors in the presence of key cytokines. These findings support a critical role of T-bet for viral clearance and suggest T-bet deficiency as an important mechanism behind chronic infection.**

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Abbreviations used: acHCV, acute chronic-evolving HCV; arHBV, acute resolving HBV; arHCV, acute resolving HCV; cHBV, chronic HBV; cHCV, chronic HCV; EBV, Epstein-Barr virus; Eomes, Eomesodermin; Flu, Influenza A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; PBSE, Pacific Blue succinimidyl ester; rHBV, long-term resolved HBV; rHCV, long-term resolved HCV; STAT4, signal transducer and activator of transcription 4.

The transcription factor T-bet (T-box expressed in T cells; Tbx21) is a crucial regulator of T cell immunity. It mediates the differentiation of CD4 T cells into Th1 cells and of CD8 T cells into Tc1 cells (Szabo et al., 2000; Mullen et al., 2001; Sullivan et al., 2003). In effector CD8 T cells, T-bet is an activator of interferon- $\gamma$  production and correlates with increased cytotoxic activity (Szabo et al., 2000; Cruz-Guilloty et al., 2009). A recent study has found that T-bet is highly expressed in HIV-specific CD8 T cells of HIV elite controllers who control viral load to very low levels without therapy (Hersperger et al., 2011). Correspondingly, its loss has been

observed in dysfunctional CD8 T cells of chronic HIV patients and in the murine LCMV model of chronic viral infection (Kao et al., 2011; Ribeiro-dos-Santos et al., 2012). Furthermore, it has been shown that T-bet and the homologous transcription factor Eomesodermin (Eomes) define two distinct states of virus-specific CD8 T cells and their balance plays an important role in the control of chronic viral infection (Paley et al., 2012). Interestingly, retroviral

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overexpression of T-bet prevented CD8 T cell exhaustion in chronic LCMV infection, demonstrating the therapeutic potential of T-bet modulation (Kao et al., 2011). However, the role of T-bet in human viral infections with dichotomous outcome remains to be determined. Because HIV and LCMV clone13 establish chronic infection in all infected subjects, other pathogens would be more suitable to dissect the differences between successful versus failing immune response during acute infection.

Human hepatitis B virus (HBV) and hepatitis C virus (HCV) infection can both either resolve spontaneously or establish chronic infection. Virus-specific CD8 T cells play a causal role in the clearance of both infections, as demonstrated by *in vivo* CD8 T cell depletion in the chimpanzee model where all subjects challenged with HBV or HCV developed chronic infection (Shoukry et al., 2003; Thimme et al., 2003). In chronic HBV and HCV infection, virus-specific CD8 T cells gradually lose their effector functions and become increasingly dysfunctional (Lechner et al., 2000a; Gruener et al., 2001; Boni et al., 2007). One hallmark of severe dysfunction is the lack of antigen-specific interferon- $\gamma$  production by T cells (Lechner et al., 2000b). Although the molecular mechanisms behind T cell dysfunction are the focus of intensive research (Bowen et al., 2004; von Hahn et al., 2007; Wherry, 2011) it is yet unknown how far impaired regulation of T-bet might be involved in the development of chronic HBV and HCV infection.

In this study, we determined the expression of T-bet in virus-specific CD8 T cells during acute HBV and HCV infection and examined its correlation with the clinical outcome. T-bet was highly expressed in spontaneously resolving but deficient in chronic-evolving infection. When we further characterized the functional correlates behind these differential expression patterns, we found a strong association of T-bet with antigen-specific proliferation and interferon- $\gamma$  production by virus-specific CD8 T cells. Induction of T-bet by antigen or IL-2 recovered antigen-specific proliferation but was not sufficient to restore interferon- $\gamma$  expression. However, restoration of a strong interferon- $\gamma$  response in previously dysfunctional CD8 T cells was achieved by additional stimulation with IL-12, which selectively induced phosphorylation of STAT4 (pSTAT4) in T-bet<sup>+</sup> CD8 T cells. This is consistent with previous findings that T-bet and STAT4 cooperate in the transcriptional control of interferon- $\gamma$  (Thieu et al., 2008). The observation that T-bet rendered CD8 T cells susceptible to IL-12 suggests a stepwise mechanism of T cell activation in which T-bet facilitates the recruitment of additional transcription factors in the presence of key cytokines, and thus contributes to the adjustment of an appropriate T cell response.

These findings indicate a critical role of T-bet for a successful CD8 T cell response against HBV and HCV infection and suggest that impaired induction of T-bet could be an important mechanism involved in CD8 T cell dysfunction during chronic viral infections.

## RESULTS

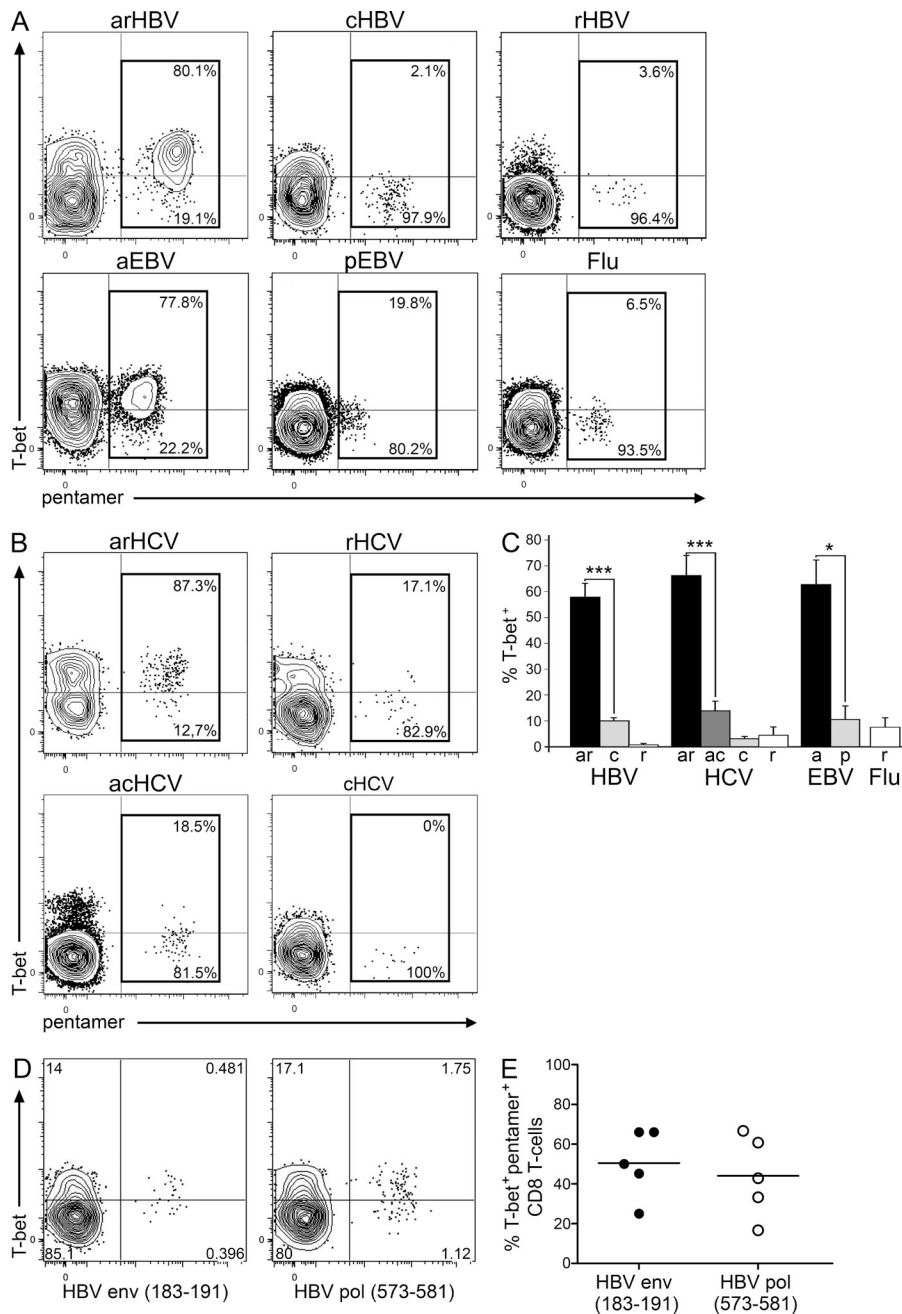
### T-bet is highly expressed during acute resolving HBV infection

Acute HBV infection resolved spontaneously in all enrolled patients. Therefore, we would expect up-regulation of T-bet in HBV-specific CD8 T cells of these individuals, in case it plays an important role in viral clearance. *Ex vivo* expression of T-bet was determined by intracellular flow cytometry combined with MHC-I pentamers detecting HBV core 18–27 (c18–27)-specific CD8 T cells, which react with a major immunodominant epitope in MHC-I A0201 background. The frequencies of virus-specific CD8 T cells are shown in Table S1.

When we compared T-bet expression in patients with acute resolving HBV (arHBV), chronic HBV (cHBV) and resolved HBV (rHBV) infection, we found a significantly higher mean percentage of T-bet<sup>+</sup> c18–27-specific CD8 T cells during arHBV (57.9%) compared with cHBV (10%) and rHBV (0.7%; Fig. 1, A and C). Our subanalysis of anti-HBe<sup>+</sup> and anti-HBe<sup>-</sup> cHBV patients showed no significant difference in T-bet expression (unpublished data). In healthy controls T-bet<sup>+</sup> CD8 T cells comprised 10.3% (mean) of total CD8 T cells (unpublished data). Elevated T-bet expression during arHBV was confirmed for 2 additional HBV-specific epitopes (HBV envelope 183–191 and HBV polymerase 573–581; Fig. 1, D and E). To rule out nonspecific bystander up-regulation of T-bet in CD8 T cells, we determined its expression in non-HBV-specific CD8 T cells during acute HBV infection. We chose EBV-specific CD8 T cells because they are broadly detectable. There was no significant increase in T-bet<sup>+</sup> EBV-specific CD8 T cells (mean 10.2%; unpublished data). As a proof of principle, we determined antigen-specific T-bet up-regulation in EBV-specific CD8 T cells (EBV BMLF-1 259–267) during acute (aEBV) and latent persisting (pEBV) EBV infection. 62.7% (mean) of EBV-specific CD8 T cells were T-bet<sup>+</sup> in aEBV, whereas only 10.2% were T-bet<sup>+</sup> in pEBV. Additionally, we found a mean of 7.5% T-bet<sup>+</sup> CD8 T cells specific for Influenza A (Flu; Influenza A MP 58–66) in healthy controls with previously resolved infection, which served as control for a memory CD8 T cell response (Fig. 1, A and C).

### Strong T-bet expression in acute HCV infection correlates with spontaneous resolution

Whereas acute HBV infection in adults is almost universally cleared, acute HCV infection becomes chronic in the majority of cases (Wright and Lau, 1993; Lauer and Walker, 2001). In our study, 50% of the patients with acute HCV developed chronic infection. HCV-specific CD8 T cells were detected by HCV-specific pentamers covering several epitopes with different MHC-I backgrounds. T-bet expression was determined during the earliest available time points of acute HCV infection that was either cleared (arHCV) or became chronic (acHCV) later on. All analyzed patients were viremic at this time. Consistent with our results found in arHBV infection, frequencies of T-bet<sup>+</sup> HCV-specific CD8 T cells were



**Figure 1. HBV- and HCV-specific CD8 T cells express high amounts of T-bet during acute spontaneously resolving infection.** PBMCs were isolated from (A) patients with arHBV, cHBV, rHBV (top), aEBV, pEBV, or resolved Flu infection (bottom) and (B) patients with arHCV (top left), rHCV (top right), acHCV (bottom left), or cHCV infection (bottom right) and analyzed by flow cytometry. The outlined areas indicate the population of pentamer<sup>+</sup> CD8 T cells and the numbers indicate the percentage of T-bet<sup>+</sup> (above) and T-bet<sup>-</sup> (below) cells among total pentamer<sup>+</sup> CD8 T cells. (C) Quantification of ex vivo T-bet expression in virus-specific CD8 T cells of the subjects described in A and B. Bars represent the mean percentage of T-bet<sup>+</sup> pentamer<sup>+</sup> CD8 T cells among total pentamer<sup>+</sup> CD8 T cells of patients with arHBV ( $n = 19$ ), cHBV ( $n = 24$ ), rHBV ( $n = 5$ ), arHCV ( $n = 7$ ), acHCV ( $n = 7$ ), cHCV ( $n = 7$ ), rHCV ( $n = 7$ ), aEBV ( $n = 3$ ), pEBV ( $n = 6$ ), and Flu ( $n = 6$ ). Error bars indicate the SEM. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$  (Mann-Whitney- $U$  test). (D) Representative ex-vivo flow cytometry plots showing expression of T-bet in HBV envelope (183–191)-specific (left) and HBV polymerase (573–581)-specific CD8 T cells (right) of patients with arHBV. The numbers indicate the percentage of T-bet<sup>±</sup> and pentamer<sup>±</sup> CD8 T cells among total CD8 T cells. (E) Scatter dot plot showing the percentage of T-bet<sup>+</sup> envelope (183–191)-specific (filled circles;  $n = 5$ ) or polymerase (573–581)-specific (open circles;  $n = 5$ ) CD8 T cells among total pentamer<sup>+</sup> CD8 T cells of patients with arHBV. The data are representative of one experiment due to limited patient material.

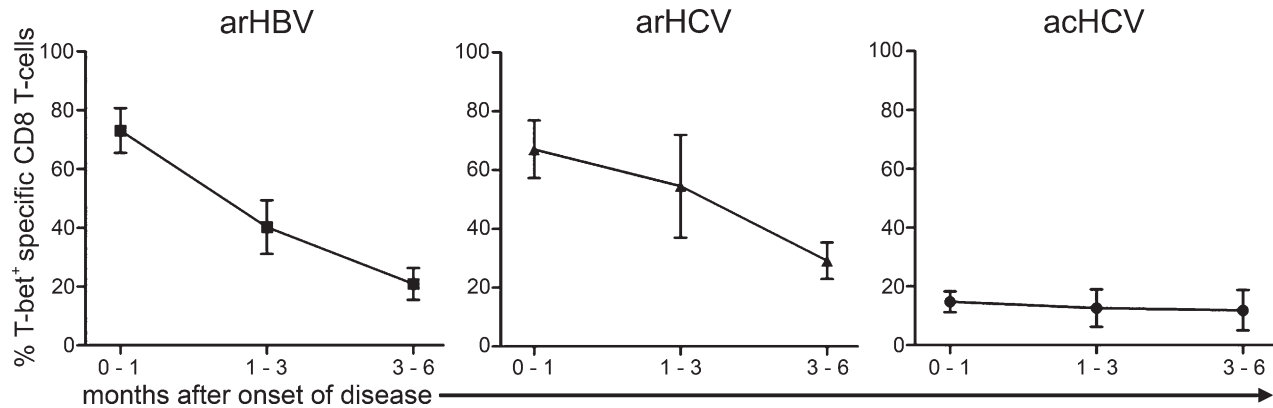
significantly higher in patients with spontaneous resolution of HCV infection (arHCV; mean, 66.3%) compared with patients with chronic-evolving infection (acHCV; mean, 14%; Fig. 1, B and C). The frequencies of T-bet<sup>+</sup> HCV-specific CD8 T cells in long-term chronic (cHCV; mean, 4.5%) and long-term resolved (rHCV; mean, 6.9%) HCV infection were comparable to those found in cHBV and rHBV.

#### T-bet deficiency in early acute HCV precedes chronic-evolving infection

To characterize the duration of T-bet up-regulation and fluctuations in its expression levels in virus-specific CD8

T cells we performed longitudinal measurements of T-bet expression in 5 patients with arHBV, 4 patients with acHCV, and 4 patients with arHCV. This kinetics profile contained data from 3 different time points within 6 mo after symptom onset. The first time point ( $t_1$ ) was defined as the earliest available patient sample obtained within 1 mo after onset of acute symptoms. The following analyzed time points were 1–3 mo ( $t_2$ ) and 3–6 mo ( $t_3$ ) after symptom onset, respectively. In arHBV, we observed a high percentage of T-bet<sup>+</sup> c18–27-specific CD8 T cells at  $t_1$  (mean, 73.1%), which gradually decreased to 40.3% at  $t_2$  and 21.8% at  $t_3$  (Fig. 2, left). In arHCV infection, we found a similar pattern as seen





**Figure 2. T-bet expression is lost early in acute chronic-evolving HCV infection.** Percentage of T-bet<sup>+</sup> pentamer<sup>+</sup> CD8 T cells among total pentamer<sup>+</sup> CD8 T cells of patients with arHBV ( $n = 5$ ; left), arHCV ( $n = 4$ ; middle), and acHCV ( $n = 4$ ; right) was determined by ex vivo flow cytometry at the indicated time points. Data show the mean percentages and are representative of one experiment due to limited patient material. Error bars indicate SEM.

in HBV-specific CD8 T cells during arHBV (mean  $t_1 = 67.1\%$ ;  $t_2 = 54.5\%$ ; and  $t_3 = 29.2\%$ ) with high initial expression of T-bet in HCV-specific CD8 T cells, which slowly decreased over the 6 mo of follow up (Fig. 2, middle). In contrast, in acHCV T-bet expression was low right from the beginning (mean:  $t_1 = 14.8\%$ ;  $t_2 = 12.6\%$ , and  $t_3 = 11.9\%$ ; Fig. 2, right). The 4 patients with acHCV were further differentiated into partial ( $n = 3$ ) versus no controllers of viremia ( $n = 1$ ) according to the evolution of viral load between  $t_1$  and  $t_3$  (Table 1). No correlation was found between T-bet or Eomes expression and quality of viral control. However, studies on larger cohorts would be required to confirm this observation.

#### Eomes and T-bet can be coexpressed and define different subsets of virus-specific CD8 T cells

As recent literature has reported that Eomes, which bears strong homology with T-bet, can compensate for T-bet deficiency in CD8 T cells of T-bet<sup>-/-</sup> mice, we investigated if Eomes is associated with a successful immune response in human HBV and HCV infection as well (Intlekofer et al., 2005). Furthermore, one study has demonstrated that the balance of two subsets of CD8 T cells, T-bet (high) and Eomes (high), respectively, is important for the control of murine LCMV and chronic HCV infection (Paley et al., 2012). Therefore, we examined the co-expression of T-bet and Eomes in virus-specific CD8 T cells under several conditions (Fig. 3, A and D). We found that Eomes<sup>+</sup>/T-bet<sup>-</sup> CD8 T cells did not show any significant difference between arHBV (10%, mean), cHBV (8.9%), arHCV (3%),

acHCV (10.5%), and cHCV (8.7%). In contrast, T-bet<sup>+</sup>/Eomes<sup>-</sup> cells, which comprised the majority of virus-specific CD8 T cells, were significantly more frequent during arHBV (mean, 38.5%) and arHCV (54.6%) as compared with cHBV (9.2%), acHCV (11.7%), and cHCV (13.4%). Interestingly, we consistently observed a population of Eomes<sup>+</sup>/T-bet<sup>+</sup> cells, which were significantly increased in arHBV (19.4%, mean) and arHCV (11.7%) compared with cHBV (0.8%), acHCV (2.3%), and cHCV (1.1%).

#### T-bet and PD-1 are coexpressed during acute but not in chronic HBV or HCV infection

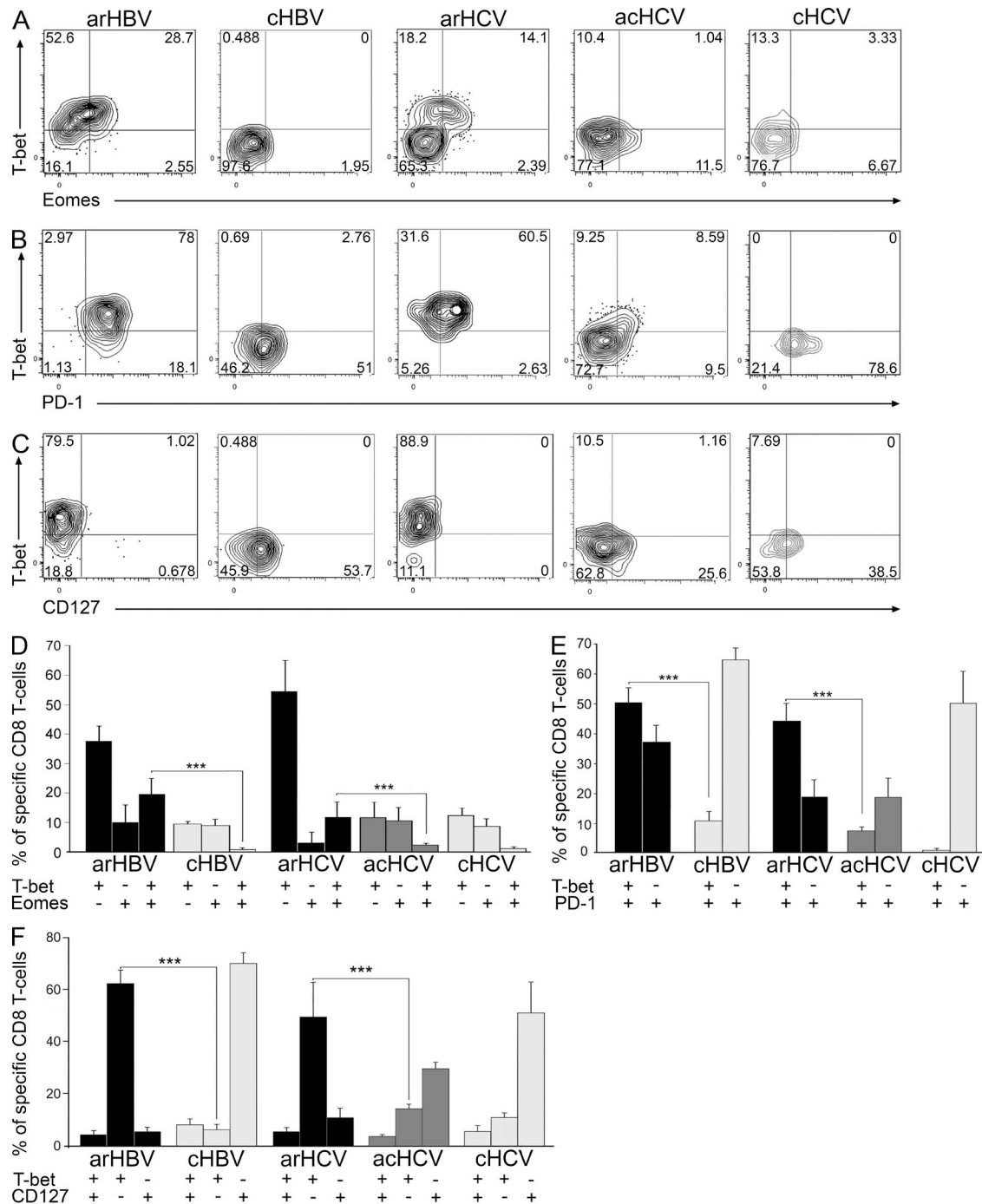
PD-1 is the hallmark inhibitory receptor found on exhausted CD8 T cells. As recent publications have shown that T-bet can suppress PD-1 we examined the coexpression of both factors in HBV and HCV infection ex vivo. T-bet and PD-1 are highly coexpressed in virus-specific CD8 T cells during resolving arHBV (mean 51.2%) and arHCV (45%), whereas the frequencies of T-bet<sup>+</sup>/PD-1<sup>+</sup> virus-specific CD8 T cells were significantly lower in cHBV (11%), acHCV (7.6%), and cHCV (0.7%; Fig. 3, B and E). In contrast, the mean percentage of PD-1<sup>+</sup>/T-bet<sup>-</sup> virus-specific CD8 T cells was highest in cHBV (65.7%) and cHCV (50.9%) as compared with acute arHBV (37.7%), arHCV (19.2%), and acHCV (19%) infection.

#### Mutually exclusive expression of T-bet and CD127

In the murine LCMV infection model, it has been shown that T-bet promotes the differentiation of effector and effector-memory CD8 T cells at the cost of central-memory cells by repression of IL-7R $\alpha$  (CD127; Intlekofer et al., 2007). We investigated if T-bet can repress CD127 expression on virus-specific CD8 T cells during HBV and HCV infection, as this could affect the generation of memory CD8 T cells. T-bet<sup>+</sup>/CD127<sup>+</sup> virus-specific CD8 T cells were rare (means: arHBV, 3.7%; cHBV, 7.7%; arHCV, 4.9%; acHCV, 3.1%; and cHCV, 5.9%). The T-bet<sup>+</sup>/CD127<sup>-</sup> population showed higher frequencies in resolving (means: arHBV, 61.8%; arHCV, 48.9%) as compared with chronic-evolving infections (cHBV, 5.8%;

**Table 1.** Kinetics of viral load in acute chronic-evolving HCV infection

Patient	$t_1$ viral load [IU/ml]	$t_3$ viral load [IU/ml]
P1	1,300	14
P2	2,980	15
P3	110,000	150
P4	66,000	9,350,000



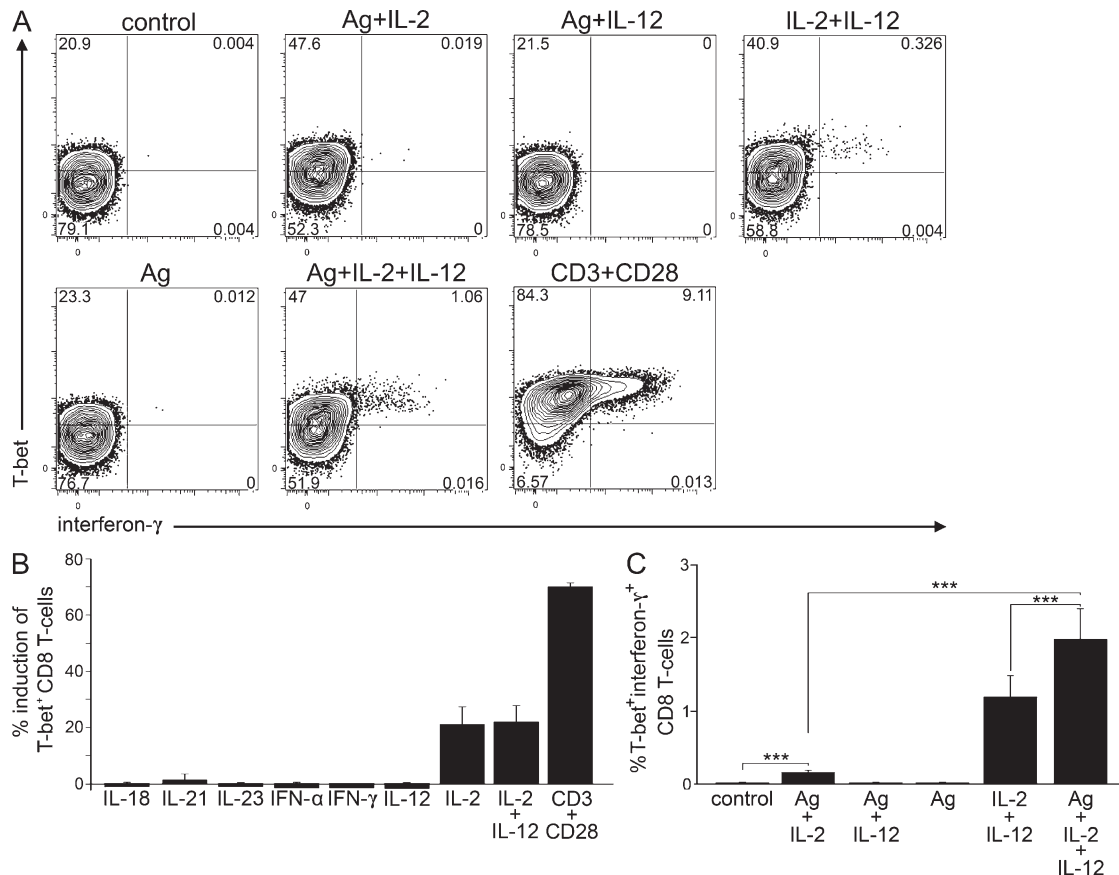
**Figure 3. Expression of T-bet is associated with distinct phenotypes of virus-specific CD8 T cells.** Ex vivo expression of T-bet, Eomes, PD-1, and CD127 in PBMCs from patients with acute HBV or HCV infection was analyzed by flow cytometry on the earliest available samples obtained within 3 wk of acute symptom onset. The data on cHBV and cHCV patients were obtained at any time point during chronic infection. (A) Contour plots show ex vivo coexpression of T-bet and Eomes in virus-specific CD8 T cells of patients with arHBV, cHBV, arHCV, acHCV, and cHCV infection. The numbers indicate the percentage of T-bet<sup>+</sup>/Eomes<sup>-</sup> and T-bet<sup>-</sup>/Eomes<sup>+</sup> CD8 T cells among pentamer<sup>+</sup> CD8 T cells. (B) Representative contour plots of T-bet and PD-1 coexpression as described in (A). (C) Representative contour plots of T-bet and CD127 coexpression as described in (A). (D) Mean percentage of pentamer<sup>+</sup> CD8 T cells with a T-bet<sup>+</sup>/Eomes<sup>-</sup>, T-bet<sup>-</sup>/Eomes<sup>+</sup>, and T-bet<sup>+</sup>/Eomes<sup>+</sup> phenotype as determined by flow cytometry. Data were obtained from arHBV ( $n = 19$ ), cHBV ( $n = 24$ ), arHCV ( $n = 7$ ), acHCV ( $n = 7$ ), and cHCV ( $n = 7$ ) patients. (E) Mean percentage of pentamer<sup>+</sup> CD8 T cells with T-bet<sup>+</sup>/PD-1<sup>+</sup> and T-bet<sup>-</sup>/PD-1<sup>+</sup> phenotype found in the patients described in (D). (F) Mean percentage of T-bet<sup>+</sup>/CD127<sup>+</sup>, T-bet<sup>-</sup>/CD127<sup>+</sup>, and T-bet<sup>+</sup>/CD127<sup>-</sup> analogous to D. All error bars indicate SEM. \*\*\*,  $P < 0.001$  (Mann-Whitney- $U$  test). Data are representative of one experiment due to limited patient material.

acHCV, 13.5%; cHCV, 10.8%). On the contrary, T-bet<sup>-</sup>/CD127<sup>+</sup> cells were more frequent in chronic-evolving (means: cHBV, 69.8%; acHCV, 29.5%; cHCV, 50.4%) than in resolving infection (arHBV, 4.8%; arHCV, 10.4%; Fig. 3, C and F).

#### Induction of T-bet by IL-2 facilitates antigen-specific interferon- $\gamma$ production in HBV-specific CD8 T cells in cooperation with IL-12

To examine the effects of T-bet on CD8 T cell functionality, we compared its coexpression with interferon- $\gamma$  and perforin in CD8 T cells of healthy controls, which were nonspecifically activated by TCR-stimulation with CD3<sup>+</sup>CD28. The vast majority of interferon- $\gamma$ <sup>-</sup> and perforin-producing stimulated CD8 T cells were T-bet<sup>+</sup> (unpublished data). Next, we addressed the question of whether specific stimulation with antigen (c18-27) or cytokines described as T-bet inducers in

literature (Lighvani et al., 2001; Ylikoski et al., 2005) could increase T-bet and interferon- $\gamma$  expression in virus-specific CD8 T cells of chronic HBV patients. We tested several cytokines in different concentrations (see Materials and methods) for their potential to induce T-bet in CD8 T cells (Fig. 4 B). The only cytokine that significantly induced T-bet was IL-2. Therefore, we wanted to examine if T-bet induction by IL-2 was directly coupled with interferon- $\gamma$  production. We found a significant but weak increase of T-bet<sup>+</sup>/interferon- $\gamma$ <sup>+</sup> CD8 T cells after stimulation with IL-2+c18-27 (mean, 0.154%) compared with stimulation with c18-27 antigen alone (mean, 0.012%) or the control (mean, 0.008%). However, we observed that stimulation with IL-2+IL-12+c18-27 resulted in a much stronger increase of T-bet<sup>+</sup>/interferon- $\gamma$ <sup>+</sup> CD8 T cells (mean 1.981%), whereas IL-12+c18-27 was not able to induce the same effect in the absence of IL-2 (0.014%). IL-2+IL-12 induced considerable background stimulation (1.191%; Fig. 4, A and C).



**Figure 4. Antigen-specific interferon- $\gamma$  production correlates with T-bet and can be restored by IL-2+IL-12 co-stimulation.** PBMCs of patients with cHBV ( $n = 11$ ) were cultured for 3 d in culture medium (control) containing either HBV-c18-27 antigen (Ag), c18-27 antigen+IL-2 (Ag+IL-2), c18-27 antigen+IL-12 (Ag+IL-12), IL-2+IL-12, c18-27 antigen+IL-2+IL-12 (Ag+IL-2+IL-12), or CD3+CD28. On day 3 antigen-treated groups were restimulated with antigen and all groups were incubated for 6 h in the presence of Brefeldin A before intracellular flow cytometry was performed. (A) Expression of T-bet and interferon- $\gamma$  by CD8 T cells. The numbers indicate the percentage of T-bet<sup>+</sup>/<sup>-</sup> and interferon- $\gamma$ <sup>+</sup>/<sup>-</sup> CD8 T cells among total CD8 T cells. (B) Mean induction of T-bet by treatment with the respective cytokines. Induction was defined as the percentage of T-bet<sup>+</sup> CD8 T cells after stimulation subtracted by the percentage of T-bet<sup>+</sup> CD8 T cells in unstimulated controls. Error bars represent the SEM. (C) Mean percentage of T-bet<sup>+</sup> interferon- $\gamma$ <sup>+</sup> CD8 T cells after stimulation. Error bars indicate the SEM. Data are representative of one experiment due to limited patient material. \*\*\*,  $P < 0.001$  (Mann-Whitney- $U$  test).

### High expression of T-bet is associated with strong antigen-specific proliferation

Antigen-specific proliferation of CD8 T cells is key to the generation of sufficient amounts of effector cells for control of the virus. We analyzed the correlation between T-bet expression and expansion of epitope-specific CD8 T cells upon stimulation. PBMCs of patients with chronic HBV were stimulated with either antigen c18-27 and/or cytokines IL-2 and IL-12 for 7 d as described in the Materials and methods. The expansion of c18-27-specific CD8 T cells and the correlation between T-bet expression and proliferation was determined by flow cytometry with the proliferation marker Pacific Blue succinimidyl ester (PBSE). The strongest expansion of c18-27-specific CD8 T cells was observed in the groups stimulated with antigen c18-27 (mean, 0.7% of total CD8 T cells), IL-2<sup>+</sup>c18-27 (mean, 1.83%), and IL-2<sup>+</sup>IL-12<sup>+</sup>c18-27 (mean, 1.93%), which was a significant induction compared with controls (mean, 0.1%). Of note, IL-12 did not significantly increase proliferation when added to IL-2<sup>+</sup>Ag. PBMCs stimulated with IL-2 (mean, 0.1%), IL-12 (mean, 0.09%), or IL-2<sup>+</sup>IL-12 (mean, 0.15%) showed no increased frequencies of virus-specific CD8 T cells (Fig. 5, A and B). Next, we divided proliferating PBSE- and c18-27-specific CD8 T cells in a T-bet<sup>+</sup> and a T-bet<sup>-</sup> subset to determine if T-bet was preferentially expressed in proliferating cells. We found significantly higher frequencies of T-bet<sup>+</sup> CD8 T cells in the groups with strong proliferation, whereas T-bet<sup>-</sup> CD8 T cells showed low frequencies (Ag mean: T-bet<sup>+</sup>, 0.29%; T-bet<sup>-</sup>, 0.09%; IL-2<sup>+</sup>Ag T-bet<sup>+</sup>, 1.12%; T-bet<sup>-</sup>, 0.07%; and IL-2<sup>+</sup>IL-12<sup>+</sup>Ag T-bet<sup>+</sup>, 1.04%; T-bet<sup>-</sup>, 0.05%; Fig. 5, C and D). Interestingly, we observed strong induction of T-bet<sup>+</sup> virus-specific CD8 T cells in the groups stimulated with antigen c18-27 (mean, 82.6% of specific cells) or IL-2 (mean, 56.2%) compared with IL-12 (mean, 12.5%), and control (mean, 8.5%; unpublished data).

### IL-12 selectively induces phosphorylation of STAT4 in T-bet<sup>+</sup> CD8 T cells

We sought to determine the mechanism behind the much stronger induction of interferon- $\gamma$  after additional stimulation with IL-12. STAT4 is of crucial importance for signal transduction by the IL-12 receptor and is known to cooperate with T-bet in the regulation of the interferon- $\gamma$  gene (Thieu et al., 2008). Therefore, we investigated if phosphorylation of STAT4 might be a possible explanation. PBMCs were either pretreated with IL-2 to induce T-bet or left unstimulated as control. On day 3, cells were restimulated with either IL-12 which should induce STAT4 phosphorylation or IL-2 as negative control. We found that IL-12 preferentially induced pSTAT4 in T-bet<sup>+</sup> CD8 T cells (mean, 2.6%) as compared with T-bet<sup>-</sup> cells (0.38%; Fig. 6, A and B). IL-2 restimulation minimally induced pSTAT4 in T-bet<sup>+</sup> (0.3%) and T-bet<sup>-</sup> CD8 T cells (0.34%) compared with controls (T-bet<sup>+</sup>, 0.03%; T-bet<sup>-</sup>, 0.05%).

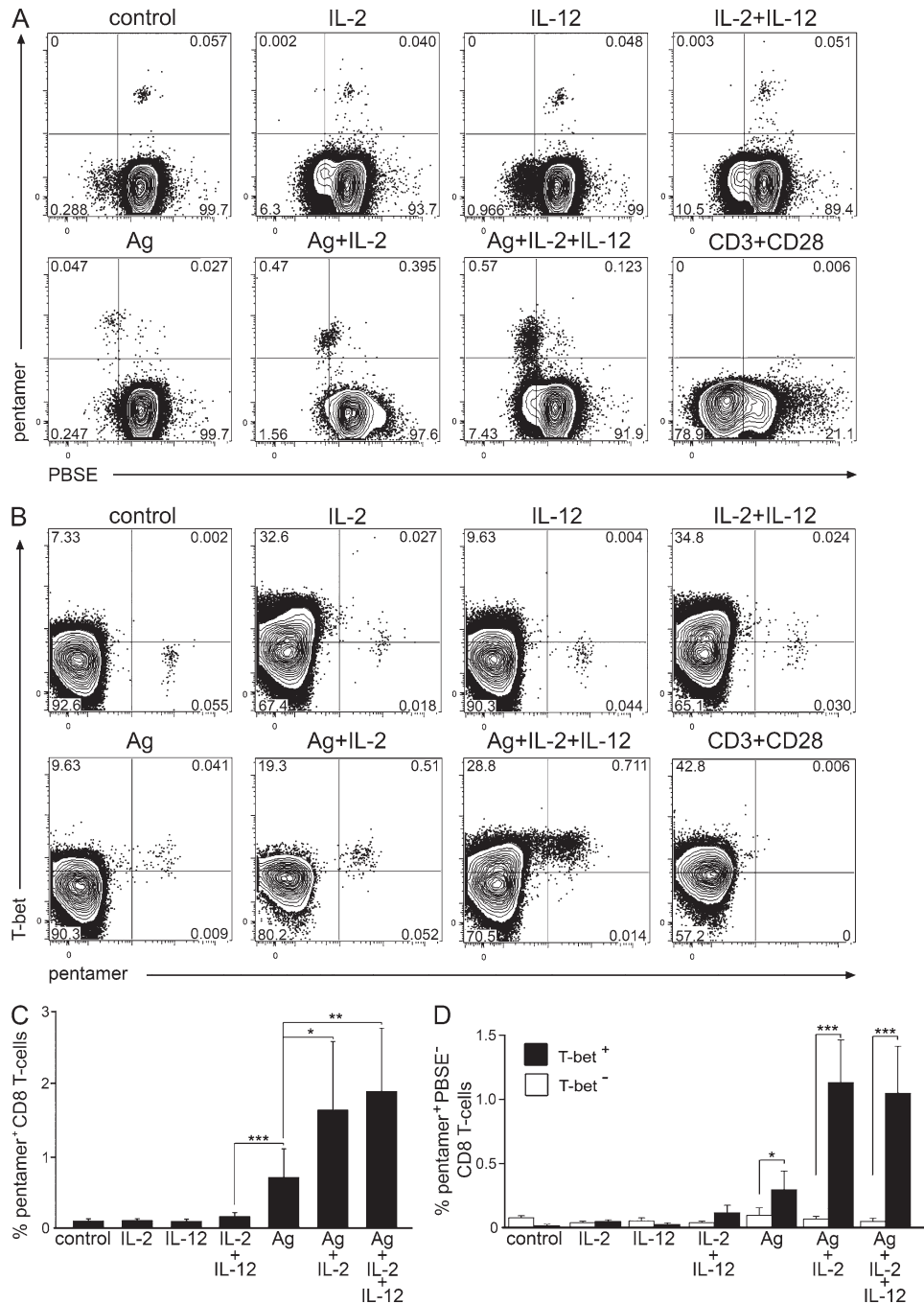
### DISCUSSION

In this study, we investigated in how far T-bet is involved in the early events during acute infection, which are critical for viral clearance. Although previous studies have discovered the

importance of T-bet in HIV and LCMV clone 13 that universally establish chronic infection, we assessed its role in human HBV and HCV infection that allowed us the direct comparison of successful versus failing CD8 T cell response against the same pathogen (Hersperger et al., 2011; Kao et al., 2011; Ribeiro-dos-Santos et al., 2012). Our most important finding is that expression of T-bet in virus-specific CD8 T cells was strongly associated with clearance of acute HCV infection. HCV-specific CD8 T cells in acute chronic-evolving HCV infection, though broadly detectable, failed to clear HCV infection and were deficient in T-bet expression. In acute HBV infection, increased expression levels of T-bet were observed in patients who later resolved the disease, whereas expression was lost in dysfunctional CD8 T cells in spite of viral persistence during long-term chronic infection. As acute HBV infection rarely takes chronic course in adults, we were not able to confirm T-bet deficiency in HBV-specific CD8 T cells of such patients. Therefore, further studies will be required to establish a definitive association between T-bet expression and clinical course of HBV infection. All patients who later controlled viral replication had high expression of T-bet in virus-specific CD8 T cells, and we confirmed the same pattern in acute and latent persisting EBV infection. These data provide further evidence for a central role of T-bet for a successful virus-specific CD8 T cell response in self-limiting human viral infections whereas T-bet deficiency was associated with chronic infection. Several studies have provided insights into the mechanisms behind T-bet deficiency in chronic infections. In the murine LCMV infection model, antigen persistence and high viral load lead to reduced T-bet levels in CD8 T cells, possibly by impaired T cell receptor signaling (Kao et al., 2011). Direct inhibition of CD8 T cell signaling by interaction with viral proteins can occur as well. For example, HCV core protein inhibits proliferation and interferon- $\gamma$  production of HCV-specific T cells by blocking their C1q complement receptor (Kittlesen et al., 2000). Generation of escape mutations can also lead to reduced T cell receptor stimulation (Chang et al., 1997). Interestingly, we did not observe *ex vivo* expression of T-bet and Eomes on HCV-specific CD4 T cells of patients with acute HCV infection, which warrants further investigation (Fig. S1). Of note, T-bet was readily inducible in CD4 T cells under Th1 culture conditions.

Our longitudinal measurements demonstrate that T-bet expression levels are stable over longer time spans and are not affected by rapid fluctuations. In acute resolving HBV and HCV infection, the highest expression of T-bet was observed at the earliest available time points and remained elevated compared with controls for at least 4–6 mo. In acute chronic-evolving HCV infection, however, T-bet remained low at all analyzed time points. Although very early loss of previously induced T-bet might be one explanation, our observations of T-bet kinetics in resolving infection suggest that this elevation should be detectable for several months. As this was not the case, an alternative explanation could be that T-bet is only weakly induced and thus lost at earlier time points or is not induced at all.

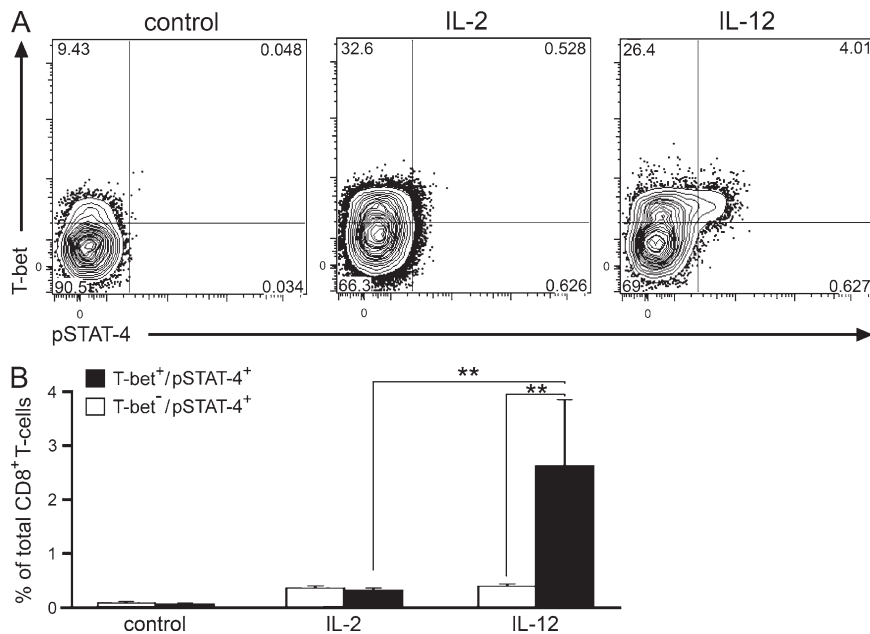




**Figure 5. Induction of T-bet by IL-2 and antigen is associated with antigen-specific proliferation.** PBMCs of patients with cHBV ( $n = 10$ ) were labeled with the proliferation marker PBSE and cultured for 7 d in culture medium in the presence or absence of HBV-c18-27 antigen (Ag), IL-2, or IL-12. Antigen c18-27 was added on day 0, and cytokines were administered on day 4 in the designated groups. Negative controls were cultured in medium without further supplements, while positive controls were stimulated with CD3+CD28. On day 7, cells were stained for flow cytometry. (A) Frequencies of pentamer<sup>+</sup> CD8 T cells among total CD8 T cells and their PBSE labeling intensity. (B) Expression of T-bet and the frequency of pentamer<sup>+</sup> CD8 T cells after stimulation. (C) Mean percentage of c18-27-specific CD8 T cells among total CD8 T cells. (D) Mean percentage of PBSE<sup>-</sup>/T-bet<sup>-</sup> (white) and mean percentage of PBSE<sup>+</sup>/T-bet<sup>+</sup> specific CD8 T cells (black) among total CD8 T cells. Error bars represent the SEM. Data are representative of one experiment due to limited patient material. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (Mann-Whitney- $U$  test).

Eomes can trigger antigen-specific interferon- $\gamma$  production in CD8<sup>+</sup> T cells of T-bet<sup>-/-</sup> mice and, together with T-bet, it defines different subsets of virus-specific CD8 T cells

that play an important role in the control of chronic viral infection (Pearce et al., 2003; Paley et al., 2012). As Eomes might compensate for the lack of T-bet in HBV and HCV infection,



**Figure 6. IL-12 selectively induces STAT4 phosphorylation in T-bet<sup>+</sup> CD8 T cells.** PBMCs of patients with cHBV ( $n = 7$ ) were cultured for 3 d with either IL-2 or medium as control. On day 3 cells were restimulated for 20 min with either IL-2 or IL-12. Controls were left unstimulated. Cells were then analyzed by flow cytometry. (A) Coexpression of T-bet and pSTAT4 in CD8 T cells of an unstimulated control (left), PBMCs cultured with IL-2 and restimulated with IL-2 (middle), and PBMCs cultured in IL-2 and restimulated with IL-12 (right). Representative plots demonstrate the percentage of T-bet<sup>+</sup> and pSTAT4<sup>+</sup> CD8 T cells among total CD8 T cells. (B) Mean frequency of T-bet<sup>-</sup> pSTAT4<sup>+</sup> (white) and mean frequency of T-bet<sup>+</sup> pSTAT4<sup>+</sup> (black) CD8 T cells among total CD8 T cells after stimulation with the respective cytokines. Error bars represent the SEM. Data are representative of one experiment due to limited patient material. \*\*,  $P < 0.01$  (Mann-Whitney- $U$  test).

we further analyzed its coexpression with T-bet in virus-specific CD8 T cells under different conditions. In contrast to the predominant T-bet<sup>+</sup> Eomes<sup>-</sup> population, which was significantly more frequent in acute resolving versus chronic-evolving infection and correlated with viral clearance, we did not observe any up-regulation of Eomes<sup>+</sup> T-bet<sup>-</sup> CD8 T cells in acute resolving infection. Of note, this was true for patients with cHBV and cHCV as well, which differs from previous studies that have suggested up-regulation of Eomes in exhausted cells of patients with chronic infection (Paley et al., 2012). This might be explained by the fact that we focused on HBV- and HCV-specific peripheral blood lymphocytes, whereas the cited study analyzed HCV-specific intrahepatic lymphocytes or LCMV-specific lymphocytes in murine infection. Furthermore, a subset of T-bet<sup>+</sup> Eomes<sup>+</sup> cells was consistently detectable and showed significantly elevated frequencies in resolving versus chronic-evolving infection, as described for the T-bet<sup>+</sup> Eomes<sup>-</sup> subset. The finding that Eomes was preferentially expressed in T-bet<sup>+</sup> CD8 T cells supports the notion of a dominant role of T-bet for a functional CD8 T cell response during acute infection.

One recent study has shown that the expression of PD-1, the hallmark inhibitory receptor of exhausted CD8 T cells, can be repressed by T-bet in chronic murine LCMV infection. Retroviral overexpression of T-bet in CD8 T cells led to a sustained antiviral CD8 T cell response and down-regulation of several inhibitory surface receptors (Kao et al., 2011). In another study, induction of T-bet by third signal cytokines decreases PD-1 levels in chronic HBV infection and restored interferon- $\gamma$  production (Schurich et al., 2013). We found that T-bet and PD-1 are highly coexpressed in acute infection but later on T-bet is lost while PD-1 levels remain high. The lack of T-bet might facilitate the high expression of PD-1 on dysfunctional CD8 T cells during chronic infection.

However, as T-bet was not expressed at later stages of infection in our analyzed samples we could not assess possible suppressive effects on PD-1 ex vivo.

A successful immune response also depends on the balanced differentiation of CD8 T cells into terminally differentiated effector CD8 T cells and self-renewing central-memory CD8 T cells. Former studies demonstrated that T-bet drives effector CD8 T cell differentiation at the expense of central-memory cells. Our finding that T-bet and CD127 are expressed in a mutually exclusive way fits into this hypothesis. It remains to be determined how far T-bet expression during acute infection could impair the differentiation of memory CD8 T cells. Of note, T-bet was down-regulated in memory CD8 T cells of patients with resolved HBV, HCV, and Flu infection. This could be explained by a lack of TCR stimulation after antigen elimination. Another possible explanation is that T-bet<sup>+</sup> CD8 T cells are short-lived effector cells and are lost over time (Intlekofer et al., 2005; Joshi et al., 2007). In summary, the factor that shows the strongest correlation with viral clearance is expression of T-bet. Although significant, the correlation of T-bet<sup>+</sup> Eomes<sup>+</sup> or T-bet<sup>+</sup> PD-1<sup>+</sup> HCV-specific CD8 T cells and viral clearance was less pronounced. Interestingly, CD127 expression on virus-specific CD8 T cells during acute HCV infection has shown a negative correlation with viral clearance.

Because our data suggested that the association of T-bet with clearance of infection is not just a secondary phenomenon but causally involved in the mechanisms behind a successful immune response, we further investigated the effects of T-bet on proliferation and interferon- $\gamma$  production of virus-specific CD8 T cells. We observed that virtually all interferon- $\gamma$ -producing CD8 T cells expressed T-bet as well, whereas T-bet<sup>-</sup> expressing CD8 T cells did not necessarily produce interferon- $\gamma$ . We tested several cytokines and cytokine combinations, including

interferon- $\alpha$ , interferon- $\gamma$ , IL-2, and IL-12, which have been used for induction of T-bet in recent literature and observed the most striking effect on T-bet induction when stimulating with IL-2 (Lighvani et al., 2001; Ylikoski et al., 2005). Subsequent experiments revealed a similar effect by stimulation with antigen, which had an additive effect when combined with IL-2. It was surprising that antigen-stimulated proliferation in exhausted CD8 T cells. However, it has been previously shown that exhausted T cells can proliferate extensively upon antigen exposure without producing cytokines (Shin et al., 2007). This fits with our experience that T cells require antigen for *in vitro* expansion. Although IL-2 plays an important role for *in vitro* expansion, our results suggest that antigen itself can trigger proliferation to some extent, too. We used low doses of antigen because higher doses can induce anergy. In addition, antigen concentration was kept constant. This is different from the *in vivo* setting, where antigen levels are fluctuating and can reach much higher levels as used in our experiments. Induction of T-bet by IL-2 and/or antigen strongly induced proliferation of HBV-specific CD8 T cells but did not induce interferon- $\gamma$ . Restoration of a strong interferon- $\gamma$  response required additional stimulation with IL-12, which could be explained by the finding that T-bet can induce the IL-12R $\beta$ 2 receptor, and thus make T cells more susceptible to IL-12 (Liao et al., 2011). We sought to clarify the mechanism behind the enhanced IFN- $\gamma$  induction in IL-12-stimulated CD8 T cells and examined if STAT4, which is known as specific signal transducer of the IL-12 receptor, might be involved. Interestingly, we found that IL-12 selectively induced STAT4 phosphorylation in T-bet<sup>+</sup> CD8 T cells, which is consistent with previous studies that describe cooperation of T-bet and pSTAT4 in the induction of IFN- $\gamma$  (Thieu et al., 2008). IL-12 single treatment induced neither T-bet up-regulation nor interferon- $\gamma$  secretion or proliferation. We observed background stimulation of CD8 T cells in the group stimulated with IL-2+IL-12 but without antigen, which can be explained in part by TCR-independent reactivation of CD8 T cells by cytokines, as described for memory CD8 T cells (Raué et al., 2013). One recent study has found that IL-12 induces T-bet and interferon- $\gamma$  in CD8 T cells of patients with chronic HBV infection (Schurich et al., 2013). However, as IL-2 was universally added to cell culture medium in the cited study, considering our observations, we suggest that the observed effects occur only under combined treatment with both cytokines. This is important, as the synergistic effects of both cytokines allowed us a 200-fold reduction of the effective IL-12 dose, thus making it more approachable for potential clinical trials. Although previous trials with single cytokine treatment of chronic HBV and HCV infection found no significant improvement of the clinical outcome (Pardo et al., 1997; Artillo et al., 1998; Zeuzem et al., 1999; Carreño et al., 2000), a combination treatment with reduced cytokine doses might be more promising.

Viral clearance requires a balanced interplay of virus-specific CD4 T cells, CD8 T cells and APC. The various patterns of viral replication seen during acute infection could

result from impairment at different levels of this regulatory network during its adjustment. Loss of HCV-specific CD4 T cell responses and IL-2 production as consistently observed in patients developing chronic infection is one mechanism that could interfere with proper induction of T-bet in CD8 T cells (Diepolder et al., 1995; Gerlach et al., 1999; Urbani et al., 2006). Furthermore, several studies have reported that HCV core protein disturbs APC maturation during acute HCV infection, leading to decreased levels of IL-12, which could contribute to loss of interferon- $\gamma$  production (Auffermann-Gretzinger et al., 2001; Eisen-Vandervelde et al., 2004).

Our data provide for the first time evidence for the central role of T-bet in self-limiting human viral infections and for deficient T-bet induction in virus-specific CD8 T cells as a mechanism associated with viral persistence. T-bet induction by IL-2 and co-stimulation with IL-12 restored function in previously exhausted virus-specific CD8 T cells and could be a potential target for future therapies.

## MATERIALS AND METHODS

**Study subjects.** Peripheral blood was obtained from patients and controls at the University Hospital Munich. We examined HBV-specific CD8 T cell responses in patients with MHC-I A0201 background and arHBV, cHBV, or rHBV infection. The HCV-specific CD8 T cell responses were analyzed in patients with arHCV, acHCV, cHCV, and rHCV HCV infection. HCV patients had MHC-I backgrounds A0101, A0201, A0301, B0701, or B3501. All HCV genotypes were included. Patients with acute EBV infection and healthy subjects served as controls. The patient characteristics are summarized in Table 2.

**Ethics statement.** This study was conducted in conformity with the ethical guidelines of the Declaration of Helsinki. Written informed consent was obtained from all patients. Approval for this study was obtained from the Institutional Review Board of the medical faculty of the Ludwig-Maximilians-University (Munich).

**Diagnostic criteria.** Acute hepatitis was defined as acute onset of nonspecific Flu-like symptoms and jaundice in previously healthy persons with peak GPT elevation 10 times above the upper limit of normal. Acute HBV was confirmed by concomitant detection of HBsAg, HBV-DNA, or anti-HBc-IgM and acute HCV by detection of HCV-RNA or seroconversion of anti-HCV. Other possible causes of acute hepatitis, like autoimmune hepatitis, alcoholic liver disease, or toxins were excluded. Resolution of acute hepatitis B was confirmed by seroconversion of anti-HBs. Acute-resolving HCV was defined as spontaneous loss of initially detectable HCV-RNA, which remained negative for at least 12 mo. In chronic-evolving acute HCV infection, HCV-RNA remained detectable for longer than 6 mo after symptom onset. Resolved HCV was defined as a previously cleared HCV infection without detection of HCV-RNA for at least 12 mo before enrollment. Diagnosis of chronic HCV infection was based on elevated serum GPT levels for at least 6 mo and the consistent detection of HCV-RNA. Chronic HBV was defined by detection of HBV-DNA or HBsAg for more than 6 mo. Acute EBV was diagnosed by acute clinical symptoms, typical hematologic findings, and detection of EBV-DNA.

**Isolation of PBMCs.** Human PBMCs were isolated from heparinized blood by Ficoll-Paque density-gradient centrifugation as described earlier and were either analyzed directly or cryopreserved (Perlmann et al., 1976).

**HLA typing.** DNA was extracted from PBMCs with the QIAamp DNA Blood Mini kit (QIAGEN) following the manufacturer's instructions. HLA typing was performed as described previously (Witt et al., 2002).

**Table 2.** Patient characteristics

Group	<i>n</i>	Female /male	Age (mean)	GPT(U/l; mean)	Viral load (mean)	HCV GT1	Anti-HBe ± n.d.
arHBV	19	6/13	38.4	2,567	19.6 × 10 <sup>6</sup> cop/ml	-	8 6 5
cHBV	24	7/17	44.6	59.5	18.3 × 10 <sup>3</sup> cop/ml	-	13 5 6
aHCV	14	6/8	53.5	984	6.9 × 10 <sup>6</sup> IU/ml	9	-
cHCV	7	3/4	44.8	81.9	0.8 × 10 <sup>6</sup> IU/ml	n.d.	-
rHBV	5	0/5	47.4	normal	negative	-	-
rHCV	7	3/4	53.3	normal	negative	n.d.	-
aEBV	3	2/1	44.3	n.d.	negative	-	-
healthy	9	8/1	39.1	normal	negative	-	-

**Antibody reagents and viability dyes.** The following antibodies were used for flow cytometry: FITC anti-T-bet (clone 4B10), Pacific Blue anti-CD45RA (HI100), APC-H7 anti-interferon- $\gamma$  (4S.B3), and Pacific Blue anti-CD57 (HCD57; BioLegend); APC anti-Eomes(WD1928) and eFluor780 anti-CD127 (eBioRDR5; eBioscience); APC-H7 anti-CD27 (M-T271), APC anti-PD-1 (MIH4), Alexa Fluor 647 anti-STAT4 (pY693; 38/pStat4), PerCP anti-CD3 (SK7), V500 anti-CD8 (SK1), V450 anti-CD8 (RPA-T8), PerCP anti-CD14 (M $\phi$ P9), and PerCP anti-CD19 (SJ25C1; BD). 7-AAD (BD) was used as viability dye in ex vivo stainings, whereas FVD eFluor450 (eBioscience) was used for fixed cells.

**Synthetic peptides, pentamers, and cytokines.** The following MHC-I pentamers were used for the detection of epitope-specific CD8 T cells: HBV core antigen 18–27 FLPSDFPSPV, HBV polymerase 573–581 FLVSLGIHL, HBV envelope 183–191 FLLTRILTI, HCV-NS3 1406–1415 KLVALGINAV, EBV BMLF-1 259–267 GLCTLVAML, and Flu MP 58–66 GILGFVFTL (all with MHC-I A0201 background); HCV-NS3 1436–1444 ATDALMTGF (HLA-A0101); HCV-NS5 2588–2596 RVCEKMALY (HLA-A0301); HCV core 41–49 GPRLGVRAT (HLA-B0701) and HCV-NS3 1359–1367 HPNIE-EVAL (HLA-B3501). All MHC-I pentamers were PE-labeled and obtained from Prolimmune. All corresponding peptides were synthesized by EMC micro-collections and used for the in vitro stimulation of specific CD8 T cells. For stimulation experiments, recombinant human IL-2 (R&D Systems) and IL-12 p70 (eBioscience) were used. The following cytokines were screened for induction of T-bet in CD8 T cells: IL-18 (10 ng/ml), IL-21 (25 ng/ml), IL-23 (50 ng/ml), interferon- $\alpha$  (2,000 IU/ml), interferon- $\gamma$  (2,000 IU/ml; eBioscience).

**PBMC stimulation.** PBMCs were cultured for 3 d in RPMI 1640 medium (Invitrogen) containing 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml of penicillin, 100  $\mu$ g of streptomycin/ml and 5% human AB serum. In the cytokine-stimulated groups, IL-2 (20 IU/ml) and/or IL-12 (50 pg/ml) were added on day 0. Antigen-stimulated groups received 5  $\mu$ g/ml antigen on day 0 and were restimulated with the same dose of antigen on day 3. On day 3, cells were incubated for 6 h under stimulating conditions in the presence of Brefeldin A (eBioscience). After stimulation, cells were prepared for flow cytometry by intracellular cytokine staining.

**PBSE proliferation assay.** 500,000 PBMCs per condition were stained in 120  $\mu$ M PBSE (Life Technologies). Cells were distributed on a 96-well round-bottomed cell culture plate in 100  $\mu$ l of culture medium and were cultured in the presence or absence of antigen (5  $\mu$ g/ml) for 7 d. On day 4, IL-2 (20 IU/ml) and/or IL-12 (50pg/ml) were added. On day 7, cells were collected and stained for flow cytometry.

**Flow cytometry.** 2–3 × 10<sup>6</sup> PBMCs were stained with MHC-I pentamers according to the manufacturer's instructions. After staining with viability dyes and antibodies specific for surface markers, cells were fixed with intracellular fixation buffer and permeabilized with permeabilization buffer (both from eBioscience). After the permeabilization step, cells were stained with intracellular markers (T-bet, Eomes, and interferon- $\gamma$ ). For ex vivo staining, pentamer<sup>+</sup>

cells were enriched by anti-PE MACS-beads, directed against PE-labeled pentamers. Calculation of pentamer<sup>+</sup> CD8 T cells was performed as previously described (Lucas et al., 2007). In brief, samples were divided in a preenrichment probe (10% of sample cells), which was used to determine the input number of CD8 T cells and a post-enrichment probe (90% of sample cells), which was run through miniMACS MS separation columns (Miltenyi Biotec). Frequencies of pentamer<sup>+</sup> CD8 T cells were calculated by dividing the absolute number of pentamer<sup>+</sup> CD8 T cells from the postenrichment probe by the number of CD8 T cells in the preenrichment probe × 9. Samples were acquired on a FACSCanto II flow cytometer (BD). Data were analyzed with FlowJo 9.6.1. software (Tree Star). Gating strategy excluded monocytes (CD14<sup>+</sup>), B-lymphocytes (CD19<sup>+</sup>), and dead cells (7-AAD<sup>+</sup>) by a dump channel.

**Detection of phosphorylated STAT4 by intracellular phospho-flow cytometry.** 2 × 10<sup>6</sup> PBMCs per well were incubated for 3 d with either IL-2 or medium as control. On day 3, cells were stimulated for 20 min with either IL-2 or IL-12. After fixation and permeabilization with Perm buffer III (BD) cells were stained with anti-pSTAT4 and anti-T-bet before analysis by flow cytometry.

**Statistical analysis.** Statistical analysis was performed with Prism 5.0 software (GraphPad) and included 2-sided Wilcoxon matched pairs test and Mann-Whitney-U test for unpaired samples. Statistical significance was defined as P < 0.05.

**Online supplemental material.** Table S1 shows the ex vivo frequencies of pentamer<sup>+</sup> CD8 T cells of the patients described in Fig. 1. Fig. S1 demonstrates flow cytometry plots and gating procedure of CD4 tetramer stainings of 2 patients with acute HCV (central plots). Online supplemental material <http://www.jem.org/cgi/content/full/jem.20131333/DC1>.

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Author contributions: P.D. Kurktschiev, B. Raziorrouh, W. Schraut, H.M. Diepolder, and N.H. Gruener designed the experiments and analyzed the data; M. Spannagl and A. Dick did the HLA typing; B. Bengsch and R. Thimme performed analysis of cHCV patients and critically reviewed the manuscript; M. Wächter, M. Backmund, R. Zachoval, and G. Denk provided patient samples and clinical information; M.C. Jung and J. Haas provided patient samples and clinical information and critically reviewed the manuscript; and P.D. Kurktschiev and N.H. Gruener wrote the manuscript.

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