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Research Article **Immunology**

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Defining the directionality and quality of influenza virus–specific CD8+ T cell cross-reactivity in individuals infected with hepatitis C virus

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Cross-reactivity of murine and recently human CD8+ T cells between different viral peptides, i.e., heterologous immunity, has been well characterized. However, the directionality and quality of these cross-reactions is critical in determining their biological importance. Herein we analyzed the response of human CD8+ T cells that recognize both a hepatitis C virus peptide (HCV-NS3) and a peptide derived from the influenza neuraminidase protein (Flu-NA). To detect the cross-reactive CD8+ T cells, we used peptide-MHC class I complexes (pMHCs) containing a new mutant form of MHC class I able to bind CD8 more strongly than normal MHC class I complexes. T cell responses against HCV-NS3 and Flu-NA peptide were undetectable in normal donors. In contrast, some responses against the Flu-NA peptide were identified in HCV+ donors who showed strong HCV-NS3–specific reactivity. The Flu-NA peptide was a weak agonist for CD8+ T cells in HCV+ individuals on the basis of novel pMHCs and functional assays. These data support the idea of cross-reactivity between the 2 peptides, but indicate that reactivity toward the Flu-NA peptide is highly CD8-dependent and occurs predominantly after priming during HCV infection. Our findings indicate the utility of the novel pMHCs in dissecting cross-reactivity and suggest that cross-reactivity between HCV and influenza is relatively weak. Further studies are needed to relate affinity and functionality of cross-reactive T cells.

Introduction

CD8+ T cells responding to virus-derived peptides play a critical role in host defense. It has long been recognized that such CD8+ and CD4+ T cells are able to react not only against one specific peptide ("index" or "consensus"), but also against variants of that peptide or "altered peptide ligands" (1). The quality of these cross-reactions is complex and dependent on the TCR affinity. The variant may act as a full agonist, which induces normal T cell activation, or only partially activates T cell functions (weak or partial agonist, or antagonist) (2, 3). These cross-reactivities have been explored in a number of model systems, through systematic mutation of the target peptide, but the cross-recognition of naturally arising viral variants (e.g., in HIV or hepatitis B virus) may also be affected if the mutations influence TCR binding (4, 5). The ability of viruses such as HCV and HIV to generate novel variants that evade cross-recognition by T cells is probably critical to their persistence.

CD8+ T cells, which are able to cross-recognize peptides from 2 distinct viruses, have also been detected in murine models — a phenomenon described as heterologous immunity (6, 7). The first description of this in humans was shown using an influenza virus peptide derived from neuraminidase 231-239 (Flu-NA) and an HCV CTL epitope from NS3 1073-1081 (HCV-NS3), both of which are HLA-A2–restricted (8). The neuraminidase 231-239 (Flu-NA) peptide (a previously unidentified epitope) was identified in a sequence homology search of the National Center for Biotechnology Information GenBank database. This study was based on prior observations that in vitro peptide stimulation with the HCV-NS3 epitope elicited detectable responses in chromium release assays in some healthy subjects with no history of HCV infection (9). Recently, the cross-reactivity of this HCV epitope with the Flu-NA peptide was analyzed in 2 patients with a severe clinical course of acute HCV infection; a dramatic expansion of CD8+ T cells focused on the HCV-NS3 epitope was found in both patients, but not in 3 other HLA-A2 patients with acute HCV infection in which only mild clinical symptoms developed (10). It has been proposed that influenza infection may prime CD8+ T cell populations that are cross-reactive with HCV and subsequently expand when they encounter this pathogen. However, the origin and memory status of the Flu-NA peptide–specific responses are not yet clear and are not uniformly observed (11); such cross-reactive cells have not been visualized directly ex vivo.

We wished to examine this cross-reactivity using novel multimeric peptide-MHC class I complex peptides ("tetramers" or pMHCs) for 2 reasons. First, such an approach allows determination of the ex vivo frequency of CD8+ T cells that recognize the Flu-NA peptide in healthy HLA-A2 individuals and to what extent they cross-react with the HCV-NS3 peptide. Importantly,

Nonstandard abbreviations used: pMHC, peptide-MHC class I complex.

Conflict of interest: The authors have declared that no conflict of interest exists.

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Table 1
Analysis of Flu matrix, Flu-NA, and HCV-NS3 responses within the CD8+ T cell population in healthy donors

Patient	Ex vivo staining			Magnetically enriched ex vivo staining			Staining after in vitro peptide restimulation		
	Flu Matrix	Flu-NA	HCV-NS3	Flu Matrix	Flu-NA	HCV-NS3	Flu Matrix	Flu-NA	HCV-NS3
HD1	0.03	0[A]	0	1.33	0	0	10.03	0	0
HD2	0.01	0	0	0	0	0	0.17	0	0
HD3	0.03	0	0	2.78	0	0	8.59	0	0
HD4	0.02	0	0	0.98	0	0	5.24	0	0
HD5	0.05	0	0	1.56	0	0	0.59	0	0
HD6	0.10	0	0	24.46	0	0	26.93	0	0
HD7	0.50	0	0	33.77	0	0	N/D	0	0
HD8	0.12	0	0	1.17	0	0	23.47	0	0
HD9	0.03	0	0	0.90	0	0	30.51	0	0
HD10	0	0	0	0	0	0	0	0	0
HD11	0	0	0	0	0	0	0	0	0
HD12	0	0	0	0	0	0	0	0	0
HD13	0	0	0	N/D	N/D	N/D	0	0	0
HD14	0	0	0	N/D	N/D	N/D	0	0	0
HD15	0	0	0	N/D	N/D	N/D	0	0	0
HD16	0	0	0	N/D	N/D	N/D	0	0	0
HD17	0	0	0	N/D	N/D	N/D	0	0	0
HD18	0	0	0	N/D	N/D	N/D	0	0	0
HD19	0	0	0	N/D	N/D	N/D	0	0	0
HD20	0	0	0	N/D	N/D	N/D	0	0	0

N/D, not done. [A]0 indicates a frequency of below 0.01%.

we wished to test the directionality of the cross-reactivity. Individuals infected with influenza but not HCV may prime CD8+ T cells that cross-react "forward" with an as yet unencountered HCV infection. Alternatively, CD8+ T cells primed by HCV may cross-react "backwards" with influenza, even if they were not primed in the original influenza infection. Understanding the order of the priming may be critical in interpreting the likely in vivo effects of such antiviral populations (12, 13).

Second, we wished to examine the quality of such cross-reactivity. Modulation of the relatively weak binding of the coreceptor CD8 to MHC can markedly influence the capacity of T cell complexes to bind a given MHC peptide (14, 15). We evaluated the use of a new mutant Class I construct in which the CD8 binding site had been mutated at position 115 of the MHC Class I heavy chain (Q115E) such that the avidity of the interaction with CD8 was slightly enhanced (CD8hi) (16, 17). We showed that the CD8hi reagent is able to reliably and specifically stain conventional antiviral T cell populations, but has a markedly enhanced capacity to detect weakly cross-reactive populations, as is the case in HCV/influenza cross-reactivity.

Results

Analysis of influenza responses in HCV– individuals. Direct ex vivo analysis showed that 9 of 20 HLA-A2+ subjects had a detectable CD8+ T cell response to the Flu matrix peptide using conventional pMHC staining (Table 1 and Figure 1). Magnetic bead enrichment (Table 1 and Figure 1) was used to test further whether low-level responses existed that could be detected ex vivo. This confirmed the previously detected responses in 8 of 9 cases, but showed no further low-level responses.

In contrast, no response was seen when the Flu-NA or HCV-NS3 pMHCs were used in either of these ex vivo assays (refolded around the genotype 1a or 1b peptide). The enrichment assay has a sensitivity of approximately 0.001% of CD8+ T cells according to previous studies (18, 19). Ex vivo IFN-γ ELISPOT assays were also performed in healthy donors, which showed responses to the Flu matrix peptide but not to the Flu-NA peptide or HCV-NS3 peptide (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI33082DS1).

To further test whether Flu-NA responses were present, but at very low frequencies, PBMCs were restimulated with peptides (as described above), and the presence of expanded peptide-specific CD8+ T cells was determined by pMHC staining. Again, the Flu matrix peptide was able to stimulate a distinct population of peptide-specific CD8+ T cells in 9 of 20 cases, which confirmed the ex vivo data. In contrast, the Flu-NA and HCV-NS3 (both genotypes 1a and 1b) peptides were unable to stimulate a peptide-specific CD8+ T cell population. In addition, we were unable to detect cells that cross-recognized HCV-NS3 after restimulation using the Flu-NA peptide (Table 1 and Figure 1). None of 10 non–HLA-A2+ subjects tested showed any positive responses in any of the above assays.

Overall, therefore, about half of the healthy adult HLA-A2+ donors had a detectable CD8+ T cell response to the immunodominant Flu matrix epitope. We found no evidence of detectable ex vivo responses to the Flu-NA epitope, or to HCV-derived peptides, in those with a positive or negative Flu matrix response. We also failed to detect such responses after in vitro peptide stimulation.

Analysis of influenza reactivity in HCV+ donors. We next addressed the question of whether Flu-NA–specific responses could be detected in the context of HCV infection in a cohort of HLA-A2+ HCV+ donors. We first studied the ex vivo responses to HCV-NS3 (peptide 1073-81) in 11 donors, of whom 8 had a detectable response to this peptide. Ten of these donors also had a response to the Flu matrix peptide. However, in this group, no responses to the Flu-NA peptide were detectable ex vivo using conventional tet-

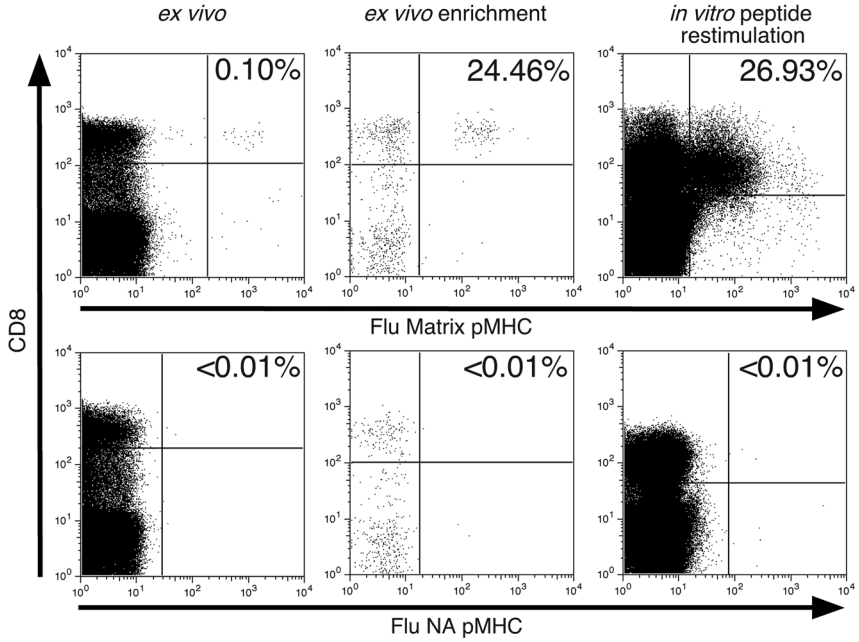

Figure 1
Staining for HCV-NS3 responses and Flu-NA responses in healthy donors. Representative density plots of ex vivo pMHC staining (left), ex vivo MHC Class I pMHC and magnetic bead enrichment (middle), and in vitro peptide restimulation of PBMCs followed by pMHC staining (right). Top row shows an individual with positive Flu matrix responses, and the bottom row shows the equivalent result with Flu-NA. The frequency of the pMHC-positive cells in the $CD8^+$ population is shown in the top right portion of each plot.

ramer staining techniques. Additionally, ex vivo IFN-γ ELISPOT assays also failed to reveal populations of Flu-NA–reactive cells (Supplemental Figure 1).

We next analyzed a series of long-term T cell lines that had been established from this cohort. First, responses to the control Flu matrix peptide were readily generated using this technique, as in normal donors (9 of 10 tested). Nine of these 11 donors also showed responses to the HCV-NS3 after restimulation in vitro. In 8 of these 9 cases, the responses were very strong; up to 85% of $CD8^+$ T cells within the culture stained for the specific HCV tetramer (Figure 2A). We used pMHC staining to test whether these lines were able to cross-recognize the Flu-NA peptide. Interestingly, under these conditions, we did observe some Flu-NA–specific reactivity in 7 of 11 patients tested (Figure 2, B and C). The number of cells responding to the Flu-NA peptide was significantly lower than the number of cells with HCV reactivity (Figure 2C; Wilcoxon signed-rank test; $P = 0.008$). Additionally, the staining characteristics of the Flu-NA pMHC-positive cells showed only very weak positivity, without the emergence of a clearly distinct population, unlike the staining observed using the HCV-NS3 pMHCs. Using simultaneous staining with the 2 pMHCs, Flu-NA–positive cells were typically double positive for both Flu-NA and HCV-NS3 (Figure 2B), and a subpopulation of the HCV-specific cells were capable of binding the Flu-NA pMHC to low levels. Typically, those cells with the brightest staining, and thus the highest binding levels of the HCV-NS3 tetramer, were those that were seen to bind the Flu-NA tetramer to some extent.

The previous data indicated that, after in vitro expansion, a subpopulation of HCV-specific T cells was able to partially recognize Flu-NA. To analyze these in vitro phenomena further, we tested whether we could trigger HCV-specific T cells to proliferate and generate lines after stimulation with the Flu-NA peptide. The results were quite different in that only very-low-frequency HCV-NS3–specific populations could be generated under otherwise identical stimulation conditions (Figure 2B). The responses were above the detection limit after in vitro stimulation in 4 of 9 patients, but all at very low frequencies (0.1%–1%), using conventional MHC multimer staining. Each of these responses showed some binding of pMHCs refolded with the Flu-NA peptide used to stimulate, which overall were of a similar magnitude to the HCV-NS3 responses. The HCV-NS3–stimulated lines showed only a very low level of staining intensity, without the appearance of a distinct population.

Analysis of the affinity of HCV-NS3– and Flu-NA–specific responses. These data imply that the Flu-NA response is only detectable as a cross-reactive response in the context of previous HCV infection in T cell populations expanded in vitro, after restimulation with the HCV-NS3 peptide. We hypothesized, therefore, that the HCV-primed population cross-reacted with the Flu-NA peptide, but that this was an infrequent and low-affinity interaction, as previously encountered in mice (7). To address this directly we created pMHCs in which the CD8 binding site on the MHC Class I α2 domain had been modified, as previously described (16, 17). The constructs, containing a mutation leading to enhanced CD8 binding ($CD8^{hi}$), will additionally bind $CD8^+$ T cells with TCRs of low affinity and high CD8 dependence, which may not be readily visualized using a conventional stain.

We initially validated the use of such a construct in a series of donors with HLA-A2–restricted responses to immunodominant peptides derived from EBV and CMV. Donors ($n = 40$) in whom the response to CMV pp65 was detected using a conventional pMHC had equivalent responses to the same peptide presented in the $CD8^{hi}$ pMHC (Figure 3, A and C). Similar results were obtained for EBV ($n = 24$) (Figure 3, A and C). In contrast, we did not observe any staining with the $CD8^{hi}$ constructs in individuals in whom conventional pMHC staining was negative (Figure 3B). These data are consistent with previous observations that such constructs, with subtle modifications in CD8 binding, retain specificity and sensitivity. Indeed, staining with the $CD8^{hi}$ construct did not reveal any new populations with apparently low TCR affinity. The latter result also demonstrates that all the EBV- or CMV-specific cells identified ex vivo were of sufficient

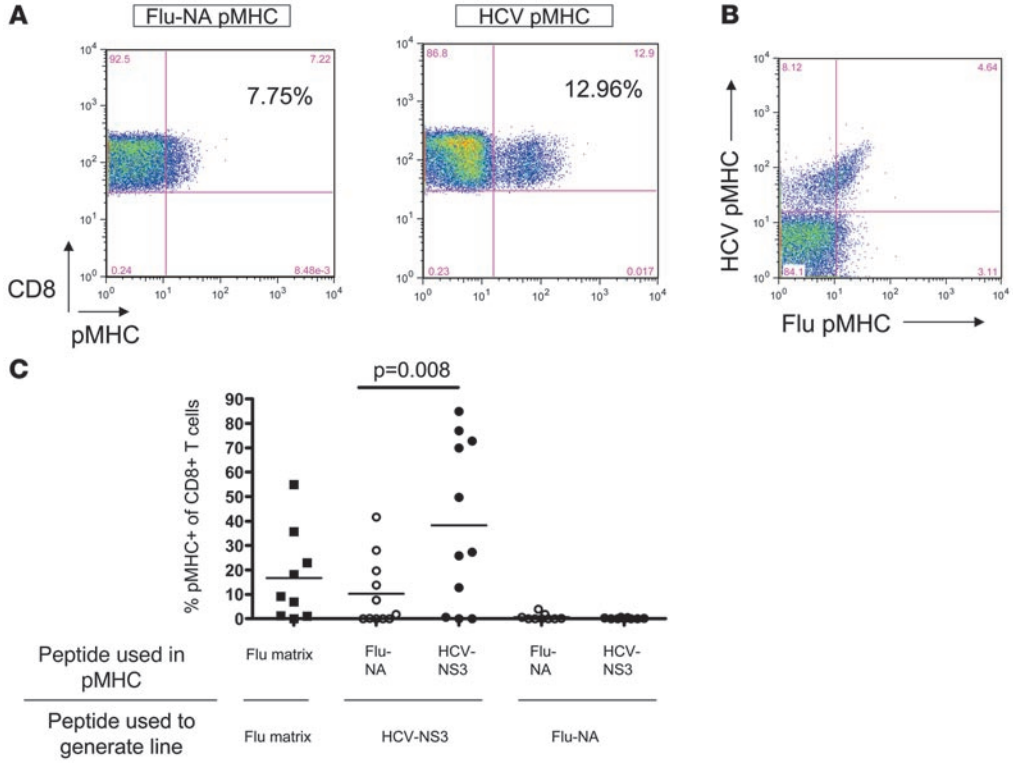

Figure 2
Conventional pMHC staining for HCV-NS3 responses and Flu-NA responses in lines derived from HCV+ donors. Long-term T cell lines were established from HCV+ donors (as indicated in Table 2). Staining for these lines was performed using normal pMHCs refolded around the HCV-NS3 peptide or the Flu-NA peptide. (**A**) Representative staining of lymphocytes showing CD8+ tetramer–positive populations. A population staining weakly with the Flu-NA pMHC is observed. (**B**) Gating on CD8+ lymphocytes, the Flu-NA pMHC-positive population lies among the HCV pMHC-positive cells, particularly evident among those cells with the highest level of staining for the HCV-NS3 pMHC. (**C**) Combined data from T cell lines from the cohort. Control lines were generated using the Flu matrix peptide and then tested with the identical peptide. Test lines were either stimulated with the HCV-NS3 peptide or the Flu-NA peptide and then analyzed with either pMHC. The percentage of CD8+ cells for each pMHC is indicated. Analysis was by Wilcoxon paired test.

affinity to bind a conventional pMHC, and no additional populations of very low affinity cells were found.

In the context of HCV-NS3/Flu-NA cross-reactivity, these constructs proved to be highly informative. We created new short-term CD8+ T cell lines expanded in vitro from patients with HCV that responded to the NS3 peptide (Figure 4). We showed that lines specific for HCV-NS3 stained with both the normal and CD8hi pMHCs when refolded with the HCV-NS3 peptide, although most intensely with the latter (mean fluorescence intensity: 4707 compared with 1122). By comparison, identical constructs refolded around the Flu-NA peptide showed only borderline staining with the normal pMHC and very clear staining with the CD8hi pMHC (17% compared with 0.23%) (Figure 4A). These data strongly imply that the affinity of the HCV-specific cells in these lines is higher for the autologous peptide than for the Flu-NA peptide and that the response to the latter is highly CD8 dependent. Very similar findings were observed using lines stimulated with the Flu-NA peptide (Figure 4B). We observed an equivalent marked increase in staining using the Flu-NA CD8hi tetramer in all patients studied and, where measurable, an increase in mean fluorescence intensity (Figure 4C).

Attempts to compare the functionality of these HCV-stimulated lines showed that, although pMHC staining for Flu-NA can be observed, it was difficult to detect IFN-γ after stimulation with the same peptide, even at high concentrations (Figure 5, A and B). By comparison, the lines generated cytokine readily after stimulation with the HCV-NS3 peptide. Similar results were obtained using an ELISPOT assay (Figure 5A) and an intracellular cytokine stain (Figure 5B).

To explore the relationship between functional assays and tetramer binding, we tested how further modifications within the peptide sequence might affect both staining and functional response of HCV-NS3–specific lines. We therefore analyzed the T cell responses to peptide variants of the HCV-NS3 epitope sequence using variants derived from genotype 1 and genotype 4 (Figure 6, A and B). We constructed pMHCs using these peptides, refolded in each case with not only the normal and CD8hi constructs as done previously, but also with a CD8lo construct. This molecule contains mutations in the α3 domain that abrogate CD8/MHC Class I interactions; thus, these complexes are bound only by CD8-independent T cells with high-affinity TCRs (16, 20). Virus-specific T cells were detected using the CD8hi, normal, and CD8lo pMHC constructs for the genotype 4 variant, but only the CD8hi and normal pMHCs for the genotype 1 variant (Figure 6A). This was paralleled by enhanced functional responses using the genotype 4 variant in ELISPOT assays (Fig-

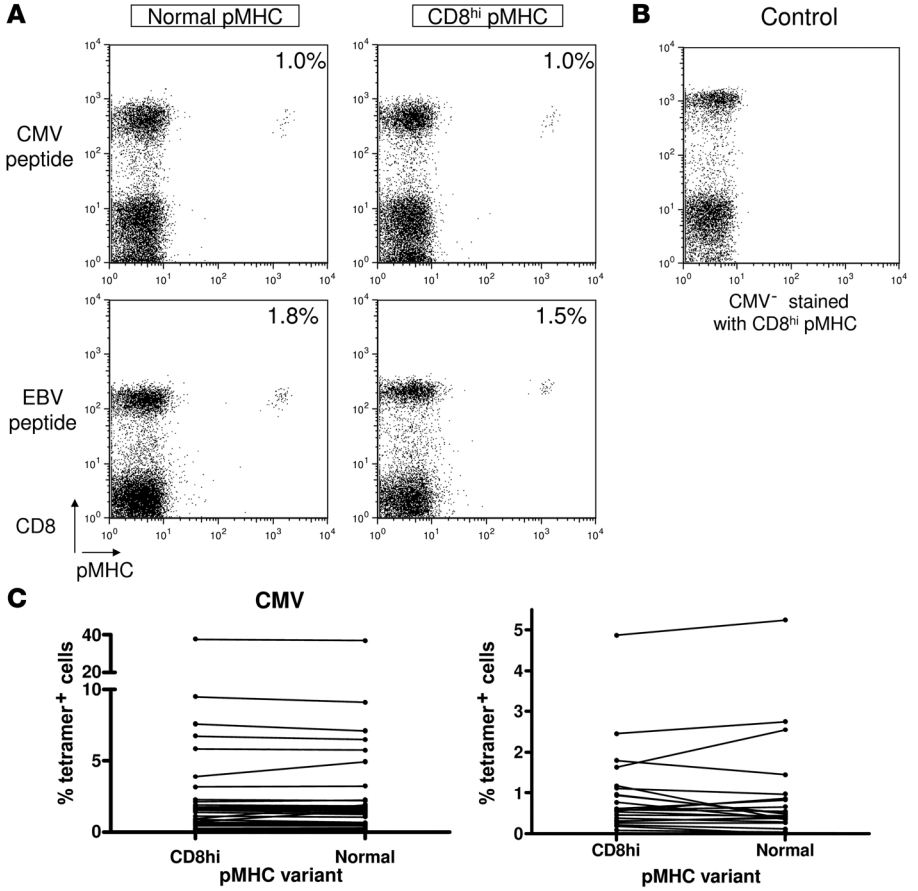

Figure 3
Analysis of T cell reactivity against control antigens using the CD8^{hi} pMHC. (**A**) Examples of staining using a conventional pMHC (left) and a CD8^{hi} pMHC (right) ex vivo in the same individuals. Top: staining using pMHCs containing a CMV-derived peptide; bottom: pMHCs containing an EBV-derived peptide as described in Methods. (**B**) Example of staining of cells from a control subject (CMV^{-}) using the CMV CD8^{hi} pMHC. Similar results were obtained using the EBV pMHC. (**C**) Group data comparing the frequency of cells stained with the CMV and EBV tetramers (n = 40 and 24 individuals, respectively). The median values were not significantly different using a Wilcoxon paired test.

ure 6B). These data indicate that a correlation exists between functional assays and the staining pattern using the 3 pMHCs as well as establish a hierarchy whereby high-affinity T cells (e.g., HCV-NS3 genotype 4 specific, which are CD8 independent) bind all 3 constructs, intermediate cells (genotype 1 specific, which are CD8 dependent) fail to bind the CD8^{lo}, and very-low-affinity cells (Flu-NA specific) bind only the CD8^{hi} pMHC. Further examples of binding to the CD8^{lo} tetramer and enhanced functionality are shown in Supplemental Figure 2.

As a further control, we constructed normal, CD8^{hi}, and CD8^{lo} pMHCs for another independent epitope from HCV (ALYDVVTKL, NS5b 2594-2602). HCV-NS5b–specific T cell lines showed binding to the CD8^{lo}, the normal, and the CD8^{hi} HCV-NS5b constructs (Figure 6C). These staining patterns are consistent with the functional response to the HCV-NS5b epitope (half maximal stimulation in IFN-γ ELISPOT at 0.02 μM), compared with the relatively low-affinity HCV-NS3 response (half maximal stimulation at 2 μM), and are consistent with previous data (21).

Dual staining of T cells using CD8^{hi} pMHCs. Finally we reanalyzed the cross-reactivity between HCV-NS3 and Flu-NA at a single cell level by costaining the short-term expanded lines and ex vivo PBMCs with CD8^{hi} pMHCs created with either HCV-NS3 or Flu-NA peptides and tagging them with different fluorochromes. We first observed, in expanded lines, a subpopulation of the HCV-NS3–specific cells stained positive with the Flu-NA pMHC (Figure 7A), as previously observed using the conventional construct (Figure 2B). However, such Flu-NA–specific populations were very difficult to discern ex vivo, even though HCV-NS3–specific populations were readily detectable (Figure 7B). We concluded that the frequency of Flu-NA cross-reactive cells, even as measured using the CD8^{hi} pMHC, was very low ex vivo, but this population can be readily expanded to measurable levels after in vitro restimulation with the HCV-NS3 peptide. As a comparison, ex vivo responses using the HCV-NS3 genotype 4 normal and CD8^{hi} tetramers were equivalent in the 5 donors tested (data not shown).

Discussion

The ability of CD8^{+} T cells to cross-react to some extent with diverse peptides is central to their normal role in disease control and potentially in immunopathology (6). Selection within the thymus on self-derived peptides implies that a degree of flexible recognition must occur, although downstream signaling may be highly tuned (22). Broadly, in terms of infection, there are 2 important situations in which the cross-reactive nature of the TCR becomes most crucial. First, in the context of a variable virus, if mutation occurs within epitopes, the ability of the TCRs within the existing antiviral peptide–specific response to recognize novel variants will determine the success or failure of viral control. It is recognized in this context that the flexibility of the response in terms of generating new TCRs is not always the same as a response that sees the new variant without previous priming by the index peptide (12, 23).

The second category of cross-reactivity that we considered here is related, but the peptide and variants were derived from different viruses. This was originally observed in the mouse (7), and previous work has shown evidence of cross-reactivity between the HCV-NS3 and Flu-NA peptides (8, 10) and between HCV and other

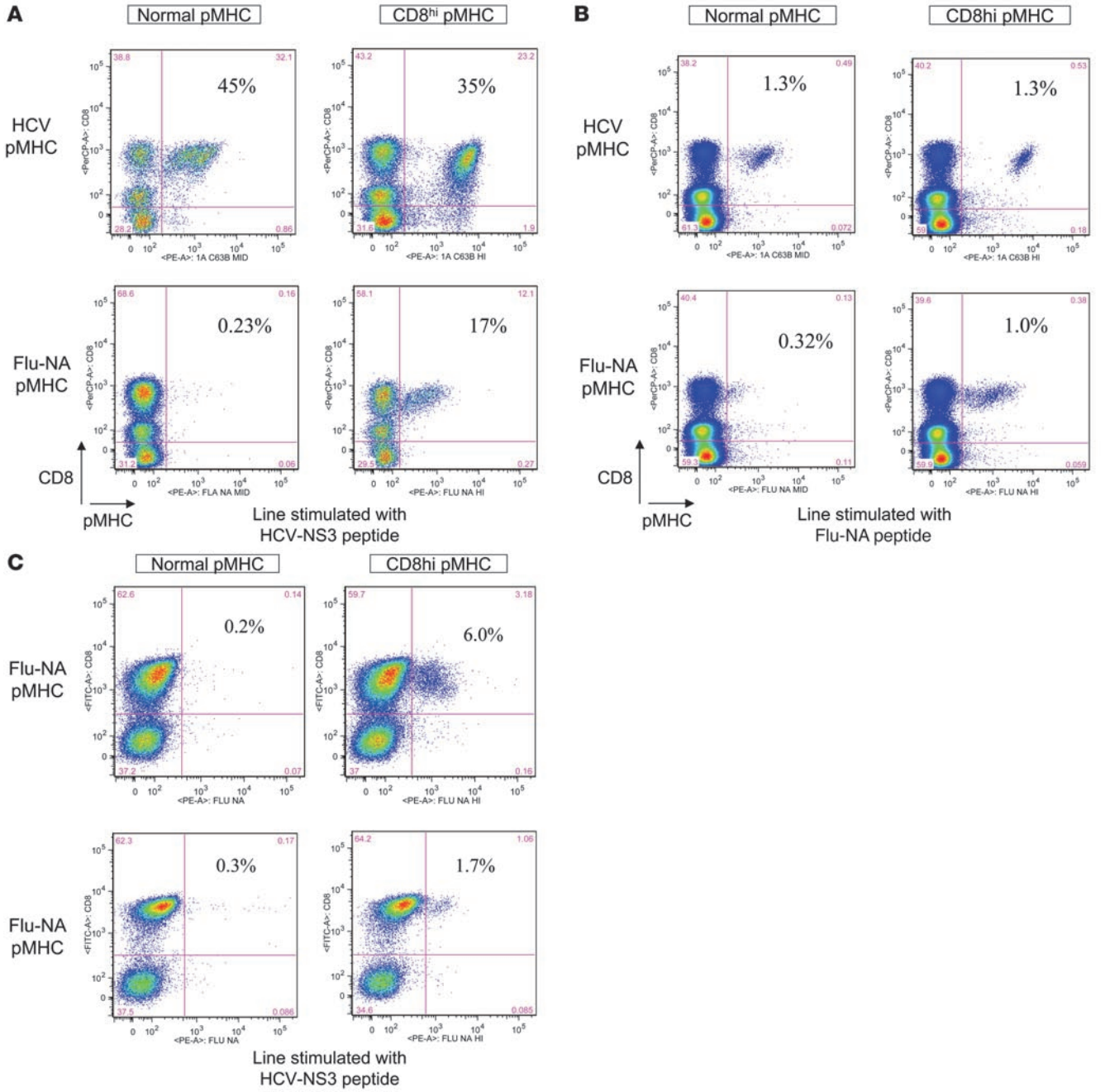

Figure 4

Analysis of T cell cross-reactivity between HCV-NS3 and Flu-NA using CD8^{hi} pMHCs. (**A**) A short-term T cell line was stimulated from HCV^{+} donor 01-07 using the HCV-NS3 peptide (as described in Methods). The line was then stained with conventional or CD8^{hi} pMHCs refolded with HCV-NS3 peptide (top) or Flu-NA peptide (bottom) as indicated. The percentage of CD8^{+} T cells stained with the pMHCs is indicated in each case. (**B**) A short-term T cell line was stimulated from donor 1144 using the Flu-NA peptide. The line was then stained with conventional or CD8^{hi} pMHCs refolded with HCV-NS3 peptide (top) or Flu-NA peptide (bottom) as in **A**. The percentage of CD8^{+} T cells stained with the pMHCs is indicated in each case. The mean fluorescence intensities for the HCV tetramers are 1173 and 4464, and those for the Flu-NA tetramer are 500 and 1171. (**C**) Similar results for Flu-NA pMHC staining are shown for 2 of 4 donors tested (donor 949: top; donor 111: bottom) in lines restimulated initially with HCV-NS3.

viruses (11). In the present study, we were interested in defining the nature of this cross-reactivity, particularly its direction and affinity. Because influenza is likely to be encountered by most individuals before contact with HCV (except in the rare cases of mother-to child transmission), we use the term forward cross-reactivity for the cross-reactivity between Flu-NA primed T cells and HCV-NS3. However, we found little evidence for responses to the Flu-NA peptide in the healthy non-HCV^{+} individuals analyzed in this study. The reverse process — i.e., generation of a Flu-NA–specific response after encounter with HCV, does, however, appear to occur. The

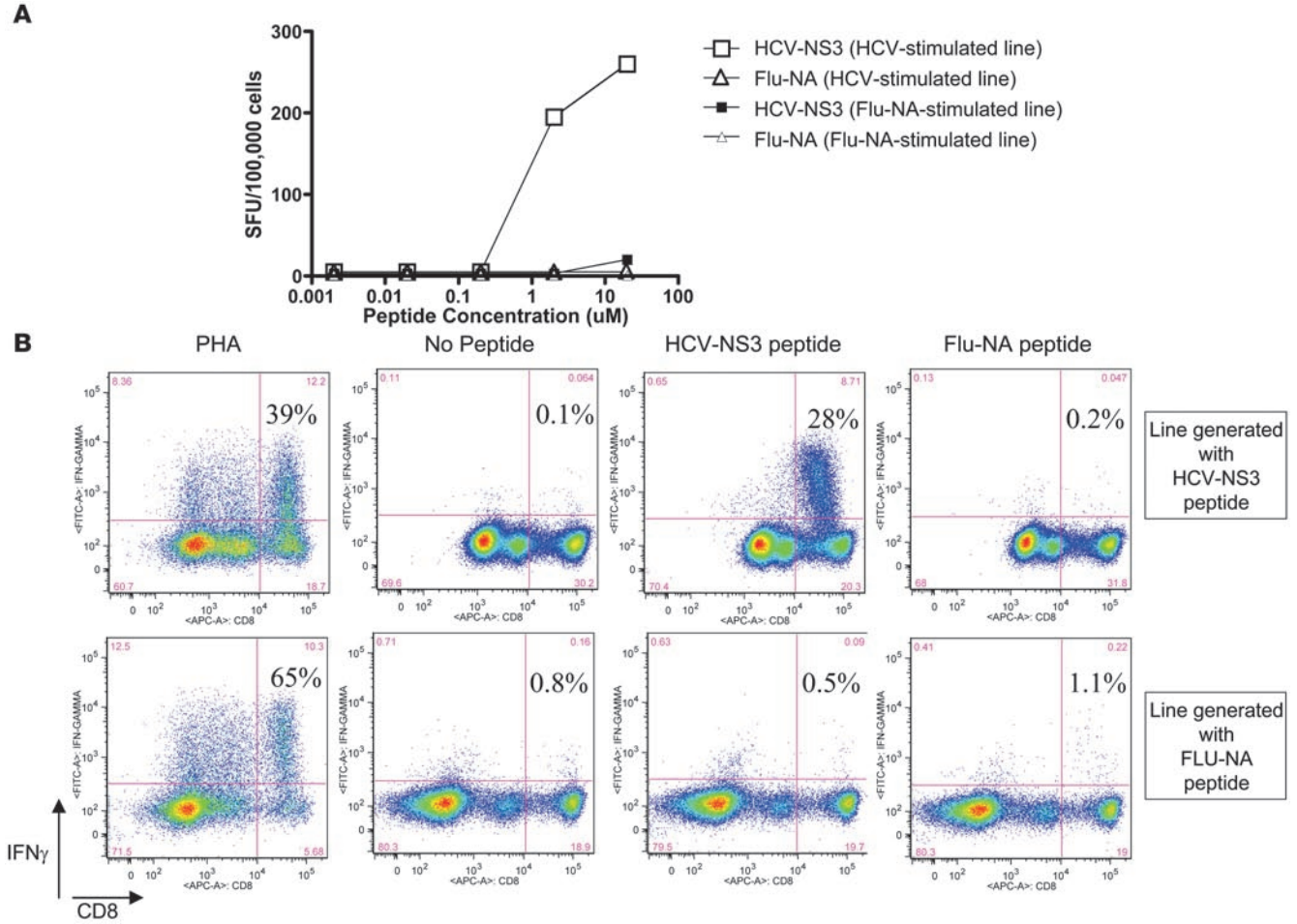

Figure 5

Functional assays of T cell lines from HCV+ donors. (**A**) Short-term T cell line from donor 1144 was generated after restimulation with the HCV-NS3 peptide or the Flu–NA peptide. The lines were tested in an IFN-γ ELISPOT assay (**A**) and intracellular cytokine staining assay (**B**). Responses were similar in a line from donor 554.

nature of this was of some interest because we observed relatively weak Flu-NA responses in the face of expanded HCV responses. We provided evidence that the interaction between HCV-specific $CD8^+$ T cells and the Flu-NA peptide is of low affinity. This evidence is based on functional analysis and the novel analysis using pMHCs with mutated CD8 binding sites. It was predicted, using a peptide library, that $CD8^{hi}$ tetramers, because of their enhanced CD8 binding, could cross-react more strongly with a wider range of altered peptide ligands than could conventional constructs (16). This appears to be the case here. Importantly, in the case of the control studies, there was a very strong relationship between the presence of a response, as defined using conventional Class I constructs, and staining with the $CD8^{hi}$ tetramers. In other words, the addition of the enhanced binding site did not lead to a loss of specificity. This was clearly shown in the case of EBV and CMV tetramers, for which enhancement of CD8 binding does not lead to the visualization of previously undetected responder populations ex vivo.

We examined herein the relationship between tetramer staining patterns and functional assays in 2 ways. First, we showed that the peptide variant of the HCV-NS3 epitope, generated using the genotype 4 sequence, is recognized at consistently lower peptide concentrations than is the genotype 1 version. This was associated with the ability to bind the $CD8^{lo}$ pMHC, for which CD8 binding is abrogated and which is bound by high-affinity T cells, which was seen only with the genotype 4 version. Second, an alternative epitope derived from HCV-NS5b was studied. We previously identified this as a high-affinity response (21), which was temporally associated with the resolution of acute infection. Again, this high-affinity response showed, in addition to binding the normal and $CD8^{hi}$ pMHC, conserved binding of the $CD8^{lo}$ tetramer. These data are consistent with biochemical studies using a range of altered peptide ligands, which have correlated binding of modified pMHCs with the threshold for T cell stimulation (17, 24).

Interestingly, in these combined tetramer staining/functional studies of HCV-NS3–stimulated lines, it was noted that the level of IFN-γ release was higher in response to the modified genotype 4 peptide, despite the fact that the frequencies of the genotype 4–specific cells were lower using conventional tetramer staining (Figure 6, A and B). It is well recognized that the frequency of $CD8^+$ T cells releasing IFN-γ in response to antigen (e.g., in ELISPOT) is often lower than the equivalent frequency of tetramer-staining cells (10, 21). The high avidity cells, which release IFN-γ more readily in

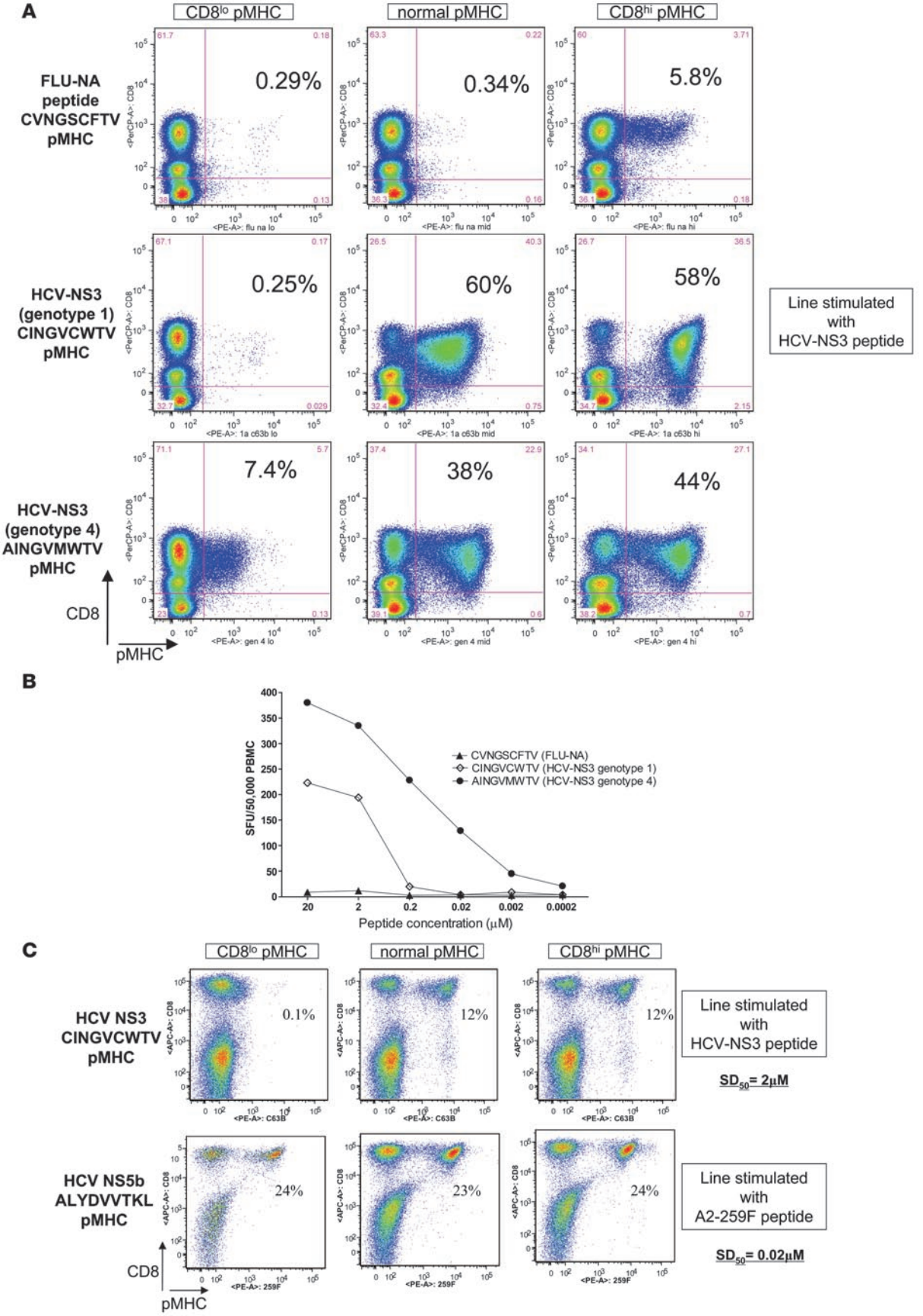
A
CD8lo pMHC
normal pMHC
CD8hi pMHC
FLU-NA peptide CVNGSCFTV pMHC
0.29%
0.34%
5.8%
HCV-NS3 (genotype 1) CINGVCWTV pMHC
0.25%
60%
58%
Line stimulated with HCV-NS3 peptide
HCV-NS3 (genotype 4) AINGVMWTV pMHC
7.4%
38%
44%
CD8
pMHC
B
SFU/50,000 PBMC
CVNGSCFTV (FLU-NA)
CINGVCWTV (HCV-NS3 genotype 1)
AINGVMWTV (HCV-NS3 genotype 4)
Peptide concentration (μM)
C
CD8lo pMHC
normal pMHC
CD8hi pMHC
HCV NS3 CINGVCWTV pMHC
0.1%
12%
12%
Line stimulated with HCV-NS3 peptide
SD50= 2μM
HCV NS5b ALYDVVTKL pMHC
24%
23%
24%
Line stimulated with A2-259F peptide
SD50= 0.02μM
CD8
pMHC

Figure 6
Analysis of function and pMHC staining patterns of HCV lines using a variant peptide. (**A**) A short-term cell line was generated from donor 1144, as in Figure 5, by restimulation with HCV-NS3 and then stained using 3 different pMHC constructs ($CD8^{hi}$, normal, and $CD8^{lo}$) refolded around the Flu-NA peptide (top), HCV-NS3 peptide genotype 1 variant (middle), or genotype 4 variant (lower). (**B**) ELISPOT assay results from a T cell line derived from donor 1144 after incubation with HCV-NS3 genotype 1 peptide, genotype 4 peptide, or Flu-NA peptide at the concentrations shown. As previously observed, little response was seen against the Flu-NA peptide, but an enhanced response with the genotype 4 variant was observed, consistent with the enhanced binding to the $CD8^{lo}$ construct. (**C**) pMHC staining of T cell lines from HCV^+ donors specific for 2 separate epitopes. $CD8^{hi}$, normal, and $CD8^{lo}$ pMHC constructs were refolded using HCV-NS3 as done previously and HCV A2-2594-2602 ALYDVVTKL derived from NS5b. Preserved staining of the HCV-NS5b line was observed using $CD8^{lo}$ pMHCs, and staining was not enhanced with the $CD8^{hi}$ construct, consistent with a population of largely high-affinity, CD8-independent T cells. These data indicate that the failure of the T cells specific to the HCV-NS3 genotype 1 epitope CINGSCWTV to bind $CD8^{lo}$ pMHCs (as also seen in Figure 6A) is not universal in HCV and that the response to Flu-NA, which is only clearly seen with the $CD8^{hi}$ construct, is atypical. We previously reported that the HCV-NS5b peptide has a 50% maximal stimulation concentration (SD_{50}) 2 logs lower than that of the NS3 epitope (21). This is consistent with peptide titration ELISPOT data from these lines, as illustrated.

response to peptide, would be expected to give a relatively greater response in such an assay. We propose that the binding to the $CD8^{lo}$ tetramer (seen with the genotype 4 but not with the genotype 1 peptide) reveals the cells of higher functionality — consistent with the IFN-γ release assay. This may well be relevant to a number of other settings in which mismatches between function and tetramer staining are seen using conventional tetramers and may be resolved potentially using the modified tetramers illustrated here.

Our results extend the observations of others, who first identified this cross-reactivity and also showed cross-reactive responses during fulminant hepatitis (8, 10). In the latter setting, as in our case, the level of functional response to the Flu-NA peptide was much lower than that to HCV (approximately 1%–5% of the HCV response), which is consistent with low-affinity cells primed largely with the HCV-NS3 peptide. Therefore, it seems unlikely that such T cells contribute substantially to the emergence of the dominant HCV-NS3–specific response, but rather they are a consequence of such a response. It remains unclear why the HCV-NS3 (1073-81) epitope attracts apparently dominant responses in many patients who are $HLA\text{-}A2^+$ (25, 26); therefore, the overall effect of this response in controlling infection in both genotype 1 and genotype 4 HCV needs further investigation. The composition of the peptide containing 2 cysteines might influence its biology, both in vivo and in vitro. However, because both the Flu-NA and HCV-NS3 peptides are similar in this respect, this is unlikely to explain the differences seen across the experiments.

The findings in this study also indicate that the frequency of Flu-NA–specific responses in healthy donors is very low, as assessed using

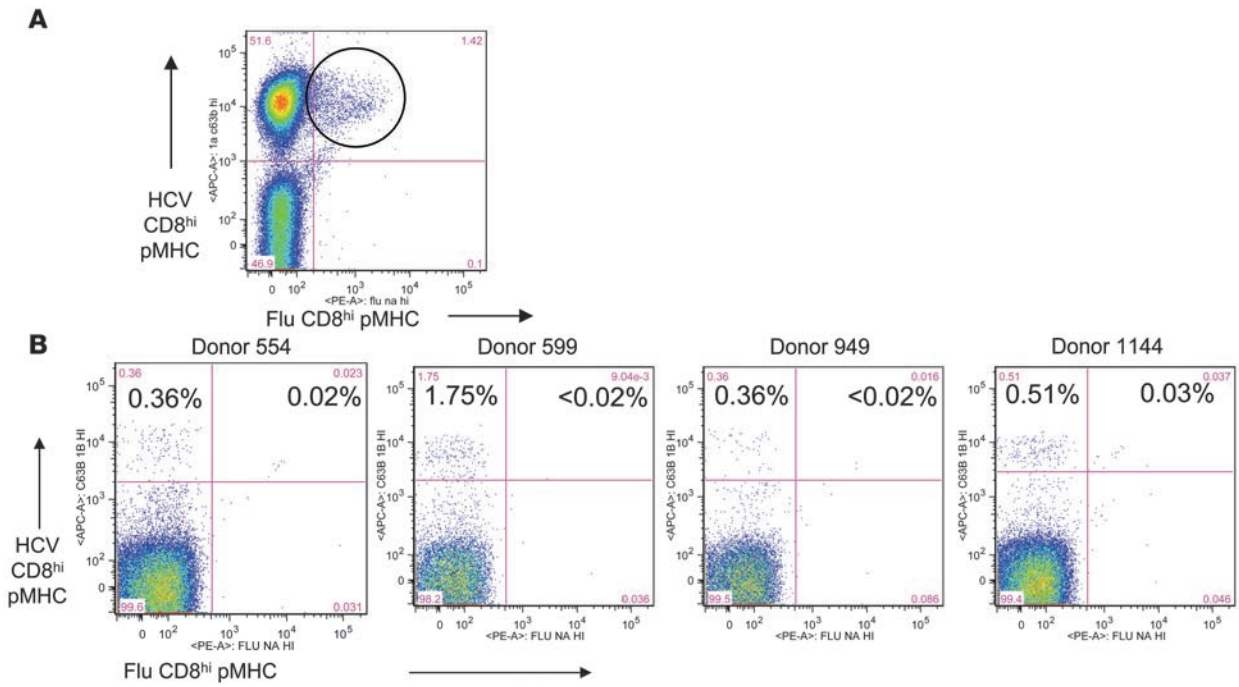

Figure 7
Combination staining for HCV-NS3 and Flu-NA responses with $CD8^{hi}$ pMHCs ex vivo. (**A**) A short-term line was established from donor 1144 and stained with $CD8^{hi}$ pMHCs refolded with HCV-NS3 peptide (fluorochrome label APC) or Flu-NA (fluorochrome label PE) together with anti-CD8. Dual staining profile, gating on live $CD8^+$ T cells, after costaining with both HCV and Flu-NA pMHCs. The Flu-NA pMHC-positive cells shown were all positive for HCV-NS3 tetramer staining (circled). (**B**) Ex vivo analyses. An identical staining protocol as for **B** was performed ex vivo on PBMCs from 4 donors. Only minimal Flu-NA staining is seen in each case (plots gated on $CD8^+$ T cells).

Table 2
Patient information and ex vivo percentage of pMHC-positive cells within the CD8+ T cell population in individuals acutely infected with HCV

Patient code	Mode	HCV status	Genotype	Ex vivo pMHC staining		
				Flu matrix	Flu-NA	HCV-NS3
554	Co	Chronic	1	0.04	0[A]	0.63
949	Ho	Chronic	1	0.02	0	0.65
393	Ho	Resolved	1	0	0	0
1036	Co	Resolved	N/D	0.04	0	0.1
1144	Co	Resolved	1	0.08	0	0.47
111	Ho	Chronic	1	0.31	0	0.14
1524	Co	Resolved	1	0.19	0	0
320	Co	Resolved	1	0.72	0	0.088
475	Ho	Resolved	N/D	0.087	0	0.1
599	Co	Resolved	1	0.17	0	0.38
903	Ho	Chronic	3	0.7	0	0

Ho, hospital acquired (e.g., surgery, transfusion); Co, community acquired. [A]0 indicates a frequency of below 0.01%.

a range of techniques in donors from both Europe and the United States. This contrasts with the findings of some studies (8), but is consistent with others (11, 27). One potential reason for the different results, aside from methodologic differences, is the variability of the epitope among circulating influenza strains. The sequence is relatively maintained among N1 strains, apart from a V/I substitution at positions 2 and 9, although it is typically mutated at several residues in N2 strains, which have cocirculated (consensus CINGTCTVV) (28, 29) (Supplemental Figure 3). Apart from potential effects on TCR interactions, both the V9I substitution and the changes in the N2 consensus are predicted to have major effects on HLA binding, increasing the dissociation rate by around 10-fold (30).

The Flu-NA peptide was identified through a database screen for potential cross-reactive epitopes based on the HCV sequence; this contrasts with other studies of influenza-dependent heterologous immunity, which have examined established epitopes, e.g., from the matrix (31). These findings, together with the relatively low frequency and very low affinity of this response, are hard to reconcile with a major role in forward cross-reactivity between influenza and HCV and are more consistent with a weak backwards cross-reactivity after HCV exposure. Further studies to determine what influence prior antigenic exposure has on the subsequent T cell response to HCV may yet reveal alternative cross-reactive responses.

In conclusion, the use of pMHCs has been of value in dissecting these cross-reactive responses, allowing ultrasensitive analysis ex vivo, analysis of dual responsiveness on a single cell, and finally, a novel analysis of affinity. It is clear that cross-reactivity can occur, and indeed must occur to some degree, but the direction of cross-reactivity, which is readily discernible in this case, plays a key role in determining the biological importance. Our evidence suggests that the biological relevance of the cross-reactivity shown here is likely to be low in the typical case. Further evaluation of the affinity and functionality of T cells directed against peptide variants both within and between infections is required to provide a clearer picture of the importance of such cross-reactivities in human disease.

Methods

Patients. Written informed consent was obtained from each patient, and the study protocol conformed with the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval from the ethics committee at the John Radcliffe Hospital, Oxford, and the Massachusetts General Hospital. Blood from 30 healthy HCV-unexposed individuals (healthy donors) was used for this study, of whom 20 were identified as HLA-A2 (healthy donors 1–20 in Table 1) and 10 were used as HLA-A2$^-$controls. The HCV$^+$ patients studied included a cohort of HLA-A2$^+$ donors recruited with acute HCV infection. Details of the acute patients included are shown in Table 2. For analysis of CMV- and EBV-specific responses in non-HCV$^+$ donors, a high throughput population screening approach was used as previously described (32). All subjects were HLA typed by PCR–single strand conformation polymorphism or using an HLA A2–FITC antibody (One Lambda) and subsequent fluorescence activated cell sorting analysis.

Cells. PBMCs were obtained from whole blood by density gradient centrifugation over Lymphoprep (Nycomed) or Histopaque (Sigma-Aldrich) and used immediately or frozen for future analysis.

Peptides. The peptides used were obtained from Research Genetics: HLA-A2–restricted HCV genotype 1a NS3 peptide 1073-1081 (CINGVCWTV), HLA-A2–restricted HCV genotype 1b NS3 peptide 1073-1081 (CVNGVCWTV), HLA-A2–restricted Flu-NA peptide 231-239 (CVNGSCFTV), HLA-A2–restricted HCV-NS5b peptide (ALYDVVTKL) (21), HLA-A2–restricted Flu matrix peptide 58-66 (M_{58}) (GILGFVFLT), CMV pp65 peptide (NLVPMVATV) (33), and EBV BMLF peptide (GLCTLVANL) (34). Peptides were used to produce MHC Class I tetramers as previously described (21). Additionally, we created MHC peptide tetramers using modified HLA-A2 constructs as previously described. The CD8^{hi} constructs contain point mutations at the CD8 binding site in the α2 domain Q115E. These constructs increase Kd by a factor of 1.5-fold as confirmed by Biacore measurements, whereas the CD8^{lo} constructs used in later experiments contain mutations in the a3 domain DT227/228KA, which abrogate CD8 binding (16, 17). For the initial experiments (as illustrated in Figure 1), pentamers for the HLA-A2–restricted Flu-NA and the Flu matrix peptides were purchased (ProImmune) and showed staining patterns equivalent to those of conventional tetramers. All MHC Class I multimers (tetramers and pentamers) were PE- or APC-conjugated. For simplicity, all peptide MHC constructs are referred to as pMHCs in the text and figures.

Tetramer staining. CD8$^+$ T cells were stained ex vivo and after in vitro restimulation as previously described (21, 35, 36). pMHC enrichment was performed as previously described (18, 37). A standard calculation was used to determine the input-specific T cell frequency after measurement of the percentage input CD8$^+$ T cells in 1 of 9 of the preenriched sample. This has been shown to be accurate at very-low T cell frequencies (18, 19). Flow cytometric analysis was performed on a FACSCalibur (BD — Biosciences), and CellQuest software (BD — Biosciences) was used for the analysis.

Short-term T cell lines. Restimulation of PBMCs in healthy volunteers was performed in 48-well plates as previously described, and the percentage of pMHC-specific CD8$^+$ T cells was determined at 21 days (38). A similar protocol was used for T cell lines in HCV-infected patients. In all cases, lines were supplemented at 3-day intervals with IL-2. Longer-term lines were maintained in HCV$^+$ individuals by repetitive stimulation using IL-2, autologous feeders, and phytohemagglutinin (Sigma-Aldrich).

Functional assays. IFN-γ ELISPOT assays were used to determine the functionality of identified responses in a limited set of patients. ELISPOT assay

was performed using PBMCs or in vitro lines as responder cells, exactly as previously described (36). Intracellular cytokine staining for IFN-γ was performed on expanded cell lines as previously described (36, 38).

Statistics. Nonparametric paired 2-tailed tests (Wilcoxon) were used for comparison throughout.

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