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This information is current as of December 8, 2011

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*J Immunol* 2010;185:7623-7632; Prepublished online 12 November 2010;

doi:10.4049/jimmunol.1001561

<http://www.jimmunol.org/content/185/12/7623>

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<b>Supplementary Data</b>	<a href="http://www.jimmunol.org/content/suppl/2010/11/12/jimmunol.1001561.DC1.html">http://www.jimmunol.org/content/suppl/2010/11/12/jimmunol.1001561.DC1.html</a>
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# Antibodies and Lentiviruses That Specifically Recognize a T Cell Epitope Derived from HIV-1 Nef Protein and Presented by HLA-C

Alon Herschhorn,<sup>\*,1</sup> Wayne A. Marasco,<sup>†</sup> and Amnon Hizi<sup>\*</sup>

HIV selectively downregulates HLA-A and -B from the surfaces of infected cells to avoid detection by the immune system. In contrast, the HLA-C molecules are highly resistant to this downregulation. High expression level of HLA-C on the cell surface, which correlates with a single nucleotide polymorphism, is also associated with lower viral loads and slower progression to AIDS. These findings strongly suggest that HIV-1-derived peptides are efficiently presented by HLA-C and trigger the elimination of infected cells. Accordingly, the ability to detect these HLA-C-peptide complexes may be used for therapeutic targeting of HIV-1-infected cells and for measuring effective presentation of vaccine candidates after immunization with HIV-1-related proteins or genes. However, low level of HLA-C expression on the cell surface has impeded the development of such complex-recognizing reagents. In this study, we describe the development of a high-affinity human Ab that specifically interacts, at low pM concentrations, with a conserved viral T cell epitope derived from HIV-1 Nef protein and presented by HLA-C. The human Ab selectively detects this complex on different cells and does not interact with a control complex that differed only in the presented peptide. Engineering lentiviruses to display this Ab endowed them with the same specificity as the Ab, whereas coexpressing the Ab and Fas ligand enables the lentiviruses to kill specifically Nef-presenting cells. Abs and pseudoviruses with such specificity are likely to be highly valuable as building blocks for specific targeting and killing of HIV-1-infected cells. *The Journal of Immunology*, 2010, 185: 7623–7632.

During the intracellular replication of HIV type 1, some viral proteins are cleaved into peptides in the cell cytoplasm. These peptides are actively transported into the endoplasmic reticulum, where they are bound by HLA class I molecules. The assembled HLA-peptide complexes are subsequently transported into the plasma membrane of the infected cell and can selectively mark the infected cells for an immune attack by specific CTLs (1, 2). Cleaved peptides derived from HIV-1 proteins can potentially be presented by either HLA-A, HLA-B, or HLA-C, which are expressed on virtually every nucleated cell in the human body (3). Presentation on the cell surface specifically labels HIV-1-infected cells and, therefore, can be further used for a selective detection and targeting of these cells. A similar approach was successfully used to analyze the expression level of HLA-A2, presenting a viral peptide derived from human T cell leukemia virus type 1 (4), or to direct a toxin-conjugated Ab to HLA-A2–

expressing cancer cells (5). However, applying this strategy to HIV-1-infected cells should take into account the ability of HIV-1 to interact with HLA complexes on the surfaces of HIV-infected cells (1, 2). HIV-1 evades the immune response by selectively downregulating HLA-A and HLA-B molecules from the infected cell surface (6, 7). Downregulation is very extensive (8), although not absolute, as evident from the selective pressure of HLA-B-restricted CTLs on HIV-1 adaptation (9, 10). In contrast, HIV-1 is unable to remove the HLA-C molecules from the cell surface as this will expose the infected cells to an attack by NK cells (6, 7). Accordingly, targeting viral peptides that are presented by HLA-C may be more effective than targeting other HLA-peptide complexes. This notion is further supported by the growing evidence for the key role of HLA-C in the development of the immune response to HIV-1. Despite the low physiological levels of HLA-C expression on the cell surface, relative to HLA-A and HLA-B, specific HLA-C-restricted CTLs may be as effective as the ones generated against other HLA-peptide complexes. Such responses can account for as much as 54% of the total CTL response in individuals, and these CTLs are functionally and phenotypically identical to the other HLA-restricted CTLs (11). In another study, 17% of the peptides that were associated with specific CTL responses were HLA-C restricted, whereas 22% were HLA-A restricted and 60% HLA-B restricted (10). A single HLA-C allele (HLA-C\*07:01) was also found to be the most frequent in presenting peptides derived from HIV-1 Nef protein (subtype A) (12). Moreover, a host genetic variant, located upstream of the HLA-C gene (–35C), correlated with increased expression of HLA-C and was associated with lower viremia and slower progression toward AIDS in recent studies (13, 14). Nevertheless, in some individuals that carry the protective variant, HIV-1 may still counteract this protection by enhancing Nef-mediated viral infectivity and by manipulating both T cell functions and Ag presentation (15). Notably, even when HIV-1 overcame this protection, there was no evidence for an increased

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Received for publication May 11, 2010. Accepted for publication October 8, 2010.

This work was performed in partial fulfillment of the requirements for a Ph.D. degree of A. Herschhorn. A. Herschhorn was supported by the Tel Aviv University President and Rector Scholarship for excellence. A. Hizi is the Incumbent of the Gregorio and Dora Shapira Chair for the Research of Malignancies.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: B-HLA, biotinylated HLA;  $\beta_2m$ ,  $\beta_2$ -microglobulin; MFI, mean fluorescence intensity; PV, pseudovirus; RT, reverse transcriptase; ScFv, single chain fragment variable.

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capability of Nef to downregulate HLA-C (15), indicating that it may be extremely difficult to downmodulate this HLA. Collectively, these findings indicate that presentation of HIV-1 peptides by HLA-C is highly effective. Accordingly, reagents that recognize these complexes may be very valuable for targeting HIV-1-infected cells or for assessing the presentation of viral peptides on an infected cell surface. However, reagents with such specificity are not yet available, and this is, at least in part, due to the requirement of such a molecule to have a very high affinity toward the HLA-C complexes to detect their naturally low levels on cell surfaces.

To meet this requirement, we have developed human Abs specific for HLA-C\*07:02 in complex with a highly conserved peptide derived from HIV-1 Nef protein. This complex is a preferable target because HLA-C\*07:02 is the most frequent allele within the HLA-C group in many human populations worldwide (16). In addition, HIV-1 Nef was reported to be the single most immunogenic protein of HIV-1, inducing CTL responses in 91.3% of individuals across four different human ethnicities (17). In a different study, Nef-associated responses were found to be the most dominant at 12 wk postinfection (18). These findings further suggest that in most individuals, Nef-derived peptides are properly processed and presented on the surfaces of infected cells.

An HIV-1 Nef-derived epitope presented by HLA-C\*07 was first identified as RRQDILDWVY (19). To test the conservation of this Nef-derived peptide, it was compared by us with the corresponding sequences of 661 HIV-1 isolates. Based on this analysis, the first Arg was replaced by a Lys residue and the second, C-terminal, Ile residue by a Val, generating a highly conserved peptide, designated Nef1 (Table I). Notably, the same Nef1 sequence was the consensus sequence in two independent reports that analyzed the variation of HIV-1 Nef in different patients (20, 21). Moreover, HLA-C\*07:02 presenting the specific Nef-derived peptide (that is identical to Nef1 but with an Arg instead of a Lys residue in the first position) is the only HLA complex that has ever been shown to resist specifically any downregulation from the cell surface by HIV-1 Nef (22). The Nef1 epitope was also reported to be within a frequently targeted and immunodominant region in different human ethnicities (17). Furthermore, a CTL response to a peptide that contained the Nef1 peptide was detected in acute HIV-1 infection in one of three studied patients, indicating that the target T cell epitope is available already early in the course of infection (23).

## Materials and Methods

### Cell lines, media, and peptides

B lymphocytes (721.221) were maintained in RPMI 1640 medium, complemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U penicillin, and 100 µg/ml streptomycin. Jurkat T lymphocytes were maintained in the same medium supplemented by 2 mM sodium pyruvate. 293T (human embryonic kidney) cells were maintained in DMEM high-glucose medium containing 10% FCS, 100 U penicillin, and 100 µg/ml streptomycin. The peptides used in this study were Nef1 (residues 105–115 of HIV-1 Nef), KRQDILDWVY (22); gp120 (residues 45–55 of HIV-1 Env), VYGVVWVWKEA (24); Conpep, KYFDEHYEY (25); and Nef2 (residues 90–97 of HIV-1 Nef), FLKEKGGL (26). Nef1 and gp120 were synthesized by M. Fridkin (Weizmann Institute of Science, Rehovot, Israel), Conpep by Genemed Synthesis (San Antonio, TX), and Nef2 by GL Biochem (Shanghai, China).

### Preparation of HLA-peptide complexes

All complexes contained three components: HLA H chain,  $\beta_2$ -microglobulin ( $\beta_2$ m), and a peptide derived from either a specific HIV-1 protein or an unrelated cellular protein. They were prepared according to National Institute of Allergy and Infectious Diseases Tetramer Core Facility protocols ([http://research.yerkes.emory.edu/tetramer\\_core/](http://research.yerkes.emory.edu/tetramer_core/)) with several modifications described later. The plasmids that carry the genes for HLA-C\*07:02 H chain and  $\beta_2$ m and original protocols for refolding of biotinylated complexes were a gift from Dr. K. Maenaka (Kyushu University, Fukuoka,

Japan). Each plasmid was transformed into BL-21 bacteria that were later induced with 1 mM isopropyl  $\beta$ -D-thiogalactoside for 4 h for expression of the corresponding protein as inclusion bodies. These were then purified and dissolved in 8 M urea.  $\beta_2$ m was refolded in 200 ml refolding buffer, concentrated, dialyzed against borate buffer pH 8, and biotinylated with sulfo-NHS-LS-biotin, according to the manufacturer's instructions (Pierce, Rockford, IL). The biotinylated and refolded  $\beta_2$ m was added to 200 ml refolding buffer along with denatured HLA-C\*07:02 H chain and 10 µM of a specific peptide. The solution was incubated at  $\sim 10^\circ\text{C}$  for 48 h with repeated pulses of the H chain every  $\sim 12$  h and then concentrated with an Amicon stirred cell (Millipore, Billerica, MA) equipped with 5-kDa membrane. Concentration products were separated on gel filtration column (S-200; GE Healthcare, Piscataway, NJ), and fractions were analyzed by SDS-PAGE. Those that corresponded with the m.w. of the HLA-peptide complex, which contained equal molar amounts of H chain and  $\beta_2$ m, were pooled, dialyzed against PBS, and stored at  $-80^\circ\text{C}$ . Control complexes labeled with biotin were prepared using the same procedure, with the exception that HLA-B\*08:01-Nef2 was biotinylated in the carboxyl terminus of the B8 H chain using the BirA enzyme (Avidity, Aurora, CO). Other control MHC complexes, which were not labeled with biotin and presented either Conpep or gp120 peptides, were prepared in a similar manner but without the biotinylation step.

### Production of soluble Abs

Periplasmic fractions of selected clones were prepared as previously described (27). The DNA encoding for C3-single chain fragment variable (ScFv) was subcloned into an expression vector that added a six-histidine tag to its carboxyl terminus. C3-ScFv was purified from the periplasmic fraction on a Ni-NTA column as previously described (28). C3-IgG was expressed by transfecting 293T cells with pLEX-C3L2AH plasmid (see later) using the calcium phosphate method (29). The medium containing C3-IgG was collected 48 or 72 h later, and the C3-IgG concentration was determined by ELISA using human IgG as standard.

### ELISA

ELISA was performed after immobilizing specified biotin-HLA complexes through NeutrAvidin (Pierce) onto immunosorb 96-well plates (Nunc, Roskilde, Denmark), as previously described (27). Detection of pseudoviruses (PVs) that present different Abs was done with equal amount of PV-C3 or PV-Control, according to the measured PV-associate reverse transcriptase (RT) activity, as previously described (30), after solubilizing the PVs with 1% Triton X-100. This was followed by secondary Abs. The amount of biotin-HLA complex was measured with the W6/32 Ab (mouse IgG anti-human HLA-A, -B, and -C; eBioscience, San Diego, CA).

### Plasmids construction

*pLEX-C3L2AH (for C3-IgG expression).* The DNA sequences encoding for C3 variable L and H chains were PCR-amplified and cloned in several steps to generate the full-length human C3-IgG1 gene in pLEX-IRES-GFP (constructed by replacing the puromycin gene by the GFP gene in pLEX-MCS [Open Biosystems, Huntsville, AL]). The final IgG1-encoding gene contained the C3 L chain linked to a furin cleavage site, then to the 2A self-processing element (31) and followed by the C3 H chain. This allowed the expression of the C3-IgG1 as a single polypeptide that is cleaved into the H and L chains during translation with an equimolar ratio of the two chains (31).

*pLEX-C\*07:02.* HLA-C\*07:02 gene was subcloned from a pCDNA-based vector into XhoI/BamHI restriction sites in pLEX-MCS vector.

*pCDNA3.1-C3scFvFc-CD28-gp41 (C3 surface display).* The DNA sequence of C3-ScFv was subcloned into SfiI/NotI restriction sites in pCDNA3.1-PS11scFvFc-CD28-gp41 (32).

*piG2-FasL.* The Fas ligand gene was PCR-amplified from pDNR-Dual-FasL (received from Harvard Institute of Proteomics, Boston, MA [clone HsCD00004915]) with primers that added restriction sites, six-histidine tags, and the HIV-1 packaging signal (residues 706–713 of the gp41 protein: NRVRRQGS) (32) and then cloned into pLeGO-ig2. All PCR-amplified sequences were fully sequenced to verify the correct DNA sequence.

### PV preparation and infection

PVs were prepared by co-transfection of 293T cells with three plasmids as previously described (33). For stable expression of the HLA-C\*07:02 in 721.221 cells, the PVs were prepared using 10 µg pLEX-C\*07:02, which is transcribed into mRNA containing an encapsidation signal and the C\*07:02-coding sequences, 10 µg pCMV $\Delta$ 8.2Gagpol (34), which encodes for HIV-1 proteins (except for Env), and 3 µg pVSV-G, which supplies an

envelope protein able to infect a wide variety of cells. The virions were allowed to infect 721.221 cells at high multiplicity of infection in the presence of 5.6 µg/ml polybrene. After 72 h, the cells were further sorted twice for high level of C\*07:02 expression after labeling with W6/32 mAb and FITC-conjugated secondary Ab, using FACSaria (BD Biosciences, San Jose, CA). Sorted cells were maintained in a medium containing 4 µg/ml puromycin to avoid genetic reversions. PV-C3 was prepared by co-transfecting 293T cells with 10 µg pCMVΔ8.2Gagpol, 7.5 µg pLeGO-iG2 (35), and 15 µg pCDNA3.1-C3scFvFc-CD28-gp41. Control PVs were prepared similarly, but with pCDNA3.1-PS11scFvFc-CD28-gp41, encoding an unrelated control Ab instead of the C3 Ab. PV-C3-FasL was prepared as PV-C3 but with 4 µg piG2-FasL plasmid replacing the pLeGO-iG2 plasmid. In this case, apoptosis of some 293T cells was observed during preparation, but the cells could still support PV production as determined by binding of the PV to Nef1-expressing cells and by RT activity assay, which measured the titer of the generated PV.

### Flow cytometry

About 10<sup>6</sup> sorted cells were washed twice with RPMI 1640 without serum and divided into two sets: one incubated with 100 µM Nef1 peptide and the other with 100 µM control peptide (Conpep). ScFv binding was tested by incubating with the cells for 30–60 min, washing the cells, and incubating with 1 µl mouse IgG anti-C-myc mAb (as C-myc is incorporated at the C terminus of all ScFvs). Cells were washed, incubated with fluorescein-conjugated goat anti-mouse IgG Abs for 20 min, washed twice, and analyzed with FACSsort (BD Biosciences). Binding of C3-IgG and PV particles were tested by incubating them with each set of cells (preincubated with either Nef1 or control peptide) for 40 min. The cells were washed, incubated with 1 µl PE-conjugated donkey anti-human IgG or 1.2 µl DyLight649-conjugated donkey anti-human IgG Abs for 15 min, and then further washed twice. The cells were analyzed with FACSCalibur (BD Biosciences). All secondary Abs were Fab<sub>2</sub> fragments from Jackson ImmunoResearch Laboratories (West Grove, PA). Washes were performed with PBS containing 5% FCS, and incubations were done on ice.

### Confocal microscope

Binding was tested essentially as described for the flow cytometry, but only DyLight649-conjugated secondary Abs were used to label the C3-IgG or PV-C3. The last step was followed by fixation with 1% paraformaldehyde, and the cells were cyto-spun for 5 min onto a microscope slide, mounted with a drop of antifade solution, and covered with a coverslip. Images were acquired with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Germany).

## Results

### Screening phage display libraries

To isolate specific Abs against the recombinant HLA-C\*07:02 presenting the Nef1 peptide (designated HLA-C\*07:02–Nef1) (Table I), we assembled a biotin-tagged complex. For this purpose, β<sub>2</sub>m protein was refolded and chemically tagged with biotin. The tagged β<sub>2</sub>m protein was then incubated with a denatured, recombinant HLA-C\*07:02 H chain and the Nef1 peptide to allow complex formation. The resulting complex was purified, and the presence of correctly folded HLA-C\*07:02–Nef1 was confirmed by both ELISA and dot blot assays using the W6/32 Ab (Supplemental Fig. 1). The biotinylated complex was next used for selection of three phage libraries. These libraries display human ScFv Abs on the surfaces of the phages. The first two libraries, designated Metha1 and Metha2, were constructed from 2.75 × 10<sup>8</sup> B cells that were isolated from

the peripheral blood of 57 nonimmunized human volunteers. The Metha1 library was constructed through the random assembly of 2.75 × 10<sup>8</sup> V<sub>H</sub> genes with 1.6 × 10<sup>6</sup> V-κ and 1.6 × 10<sup>6</sup> V-λ genes and yielded 1.2 × 10<sup>10</sup> member library. The Metha2 library contained random combinations of the V<sub>H</sub> genes with only the V-λ genes and consisted of 1.5 × 10<sup>10</sup> members (36). The third phage library, Griffin1, was a semisynthetic library of 1.2 × 10<sup>9</sup> different clones and was based on the lox library (37). This library contained the vast majority of V gene segments used *in vivo* as building blocks, and these were further randomly mutated at the CDR 3 (CDR3) loop to increase the diversity of the library.

Selection of the three phage libraries was directed to isolate Abs against specific epitopes of the presented Nef1 peptide and possibly nearby HLA-C\*07:02 residues. To select for such a specificity toward the Nef1-presenting complex, we applied a subtraction step (5) by incubating the phages with two different complexes: biotin–HLA-C\*07:02–Nef1 and an excess of control, nonbiotinylated HLA-C\*07:02–gp120. The excess nonbiotinylated HLA-C\*07:02–gp120 was used as a trap for binders against epitopes common to all HLA-C\*07:02 complexes, and streptavidin-coated magnetic beads were applied to retrieve the phages bound to biotin–HLA-C\*07:02–Nef1. After four rounds of panning against each library, the phage populations were tested by ELISA with the two complexes. Phages selected from both Metha1 and Metha2 libraries showed enrichment of binders specific to the biotin–HLA-C\*07:02–Nef1 complex and a much lower binding to the control complex (Fig. 1). In contrast, phages selected from Griffin1 library showed comparable binding to both complexes and probably contained only binders to common epitopes on the HLA-C\*07:02 molecule (Fig. 1). Based on this analysis, the phage populations from the Metha libraries were then screened to identify monoclonal ScFv Abs expressed from single clones. This resulted in identifying four unique clones that were isolated for further evaluations: C3, A4, D6, and E9.

### Soluble ScFvs

To test the fine specificity of the soluble ScFvs, they were tested against three different biotinylated complexes: the biotinylated HLA (B-HLA)-C\*07:02–Nef1 and two control complexes. The first control complex was B-HLA-C\*07:02–Conpep, which differs from the previous complex by presenting an unrelated peptide (Conpep). Conpep is derived from human cyclin-dependent kinase interacting protein and was found as a natural C\*07:02 ligand of human 721.221 cells (25). Therefore, Conpep serves as an ideal control for an endogenous peptide that is naturally processed and presented by HLA-C\*07:02 on human cells (hence, Abs interacting with this complex may bind normal cells without any specificity toward HIV-infected cells). The second control complex used was B-HLA-B\*08:01–Nef2, which presented a second unrelated peptide derived from HIV-1 Nef on a different recombinant HLA molecule (HLA-B\*08:01). When the identified Abs were tested against the different HLA complexes, C3 and E9 ScFvs showed

Table I. Identity of the residues in the sequence of Nef1 peptide and their prevalence in 661 HIV-1 isolates

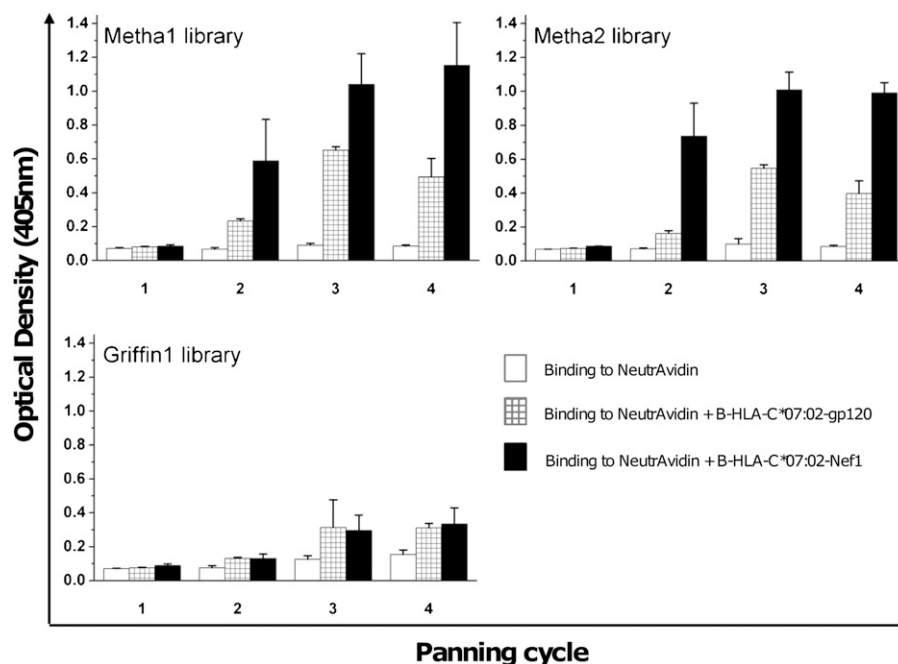
Nef1 Peptide (Derived from Nef Protein Residues 105–115)											
Amino acids	K	R	Q	D (D/E) <sup>a</sup>	I	L	D	L	W	V	Y
Identity (%)	73.7	99.1	94.1	62.9 (99.1)	98.9	99.4	98.8	98.5	97.9	84.4	97.1

The sequences that correspond with this T cell epitope were retrieved for all sequences available in the Los Alamos HIV database (<http://www.hiv.lanl.gov/>) and exported to an Excel (Microsoft, Redmond, WA) worksheet. The frequency of each residue at each position was then calculated.

<sup>a</sup>At this position, 62.9% of the sequences contained an Asp residue, whereas 99.1% of the sequences contained either an Asp or a Glu residue at this position.



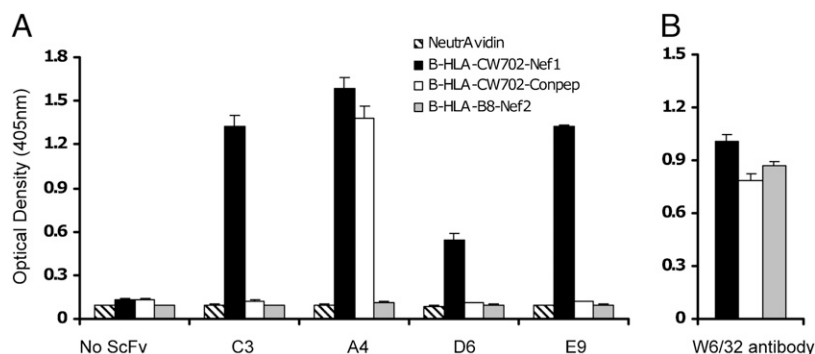
**FIGURE 1.** Polyclonal phage ELISA using the phage populations that was performed after four panning cycles. Selection of Metha1, Metha2, and Griffin1 phage display libraries against biotinylated Ag was performed as previously described (5, 28), but washes in all panning cycles were done 10–12 times with PBS containing 2% skim dry milk and 0.1% Tween 20 and then twice with PBS. From the second round of selection, about a 2-fold excess of unrelated and nonlabeled HLA control complex (C\*07:02–gp120) was incubated, along with the C\*07:02–Nef1 complex, during the binding step of the phages (5). This was done to specifically enrich for phages specific against epitopes formed by the Nef1 peptide and HLA protein interface. Bulk phage populations were tested for binding to the biotin–HLA–C\*07:02–Nef1 complex as well to the control complex (biotin–HLA–C\*07:02–gp120) and to NeutrAvidin.



comparable binding efficiencies to B-HLA-C\*07:02–Nef1. Notably, both Abs did not interact with either of the two control complexes, indicating a high specificity toward the Nef1-presenting complex (Fig. 2A). The D6 ScFv showed a similar reactivity, albeit reacting to a lower extent with the B-HLA-C\*07:02–Nef1 complex. In contrast, A4 bound the two B-HLA-C\*07:02 complexes, but not the B-HLA-B8–Nef2 complex, suggesting that it recognizes an epitope found exclusively on the H chain of the C\*07:02 molecule. All three complexes were present at comparable amounts, as determined from the binding of W6/32 Ab (Fig. 2B). The genes encoding for all four ScFvs were subsequently sequenced and compared with known variable germline Ab-encoding genes (Supplemental Table I).

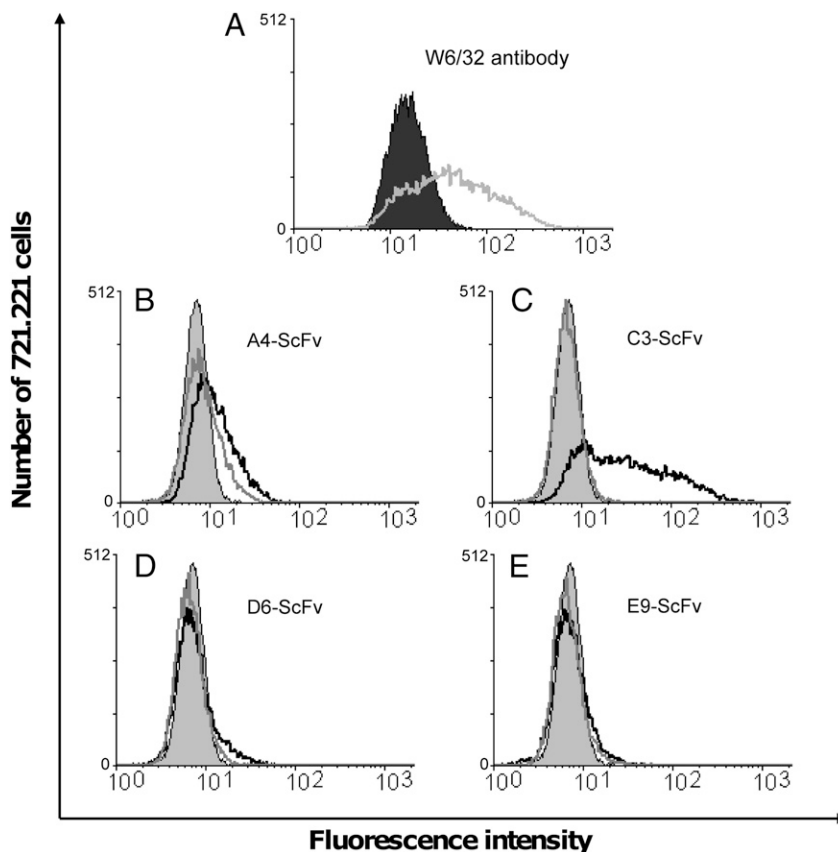
The four ScFvs were next tested for their ability to bind cells presenting the HLA-C\*07:02–Nef1 complex. Binding was assessed with 721.221 human B lymphocytes that overexpress the HLA-C\*07:02 complex on their surfaces after a lentivirus-mediated gene transfer of the HLA-C\*07:02 allele. These cells, designated 721-C\*07:02, exclusively express the HLA-C\*07:02 allele, as non-infected 721.221 lymphocytes are characterized by a complete absence of HLA-A, -B, and -C expression on their surfaces (38). The

expression of HLA-C\*07:02 on these cells was further verified with the W6/32 Ab (Fig. 3A). Of the four tested ScFvs, C3 showed the strongest and most specific binding to 721-C\*07:02 lymphocytes that were preincubated with Nef1 peptide (Fig. 3C); hence, it was studied further. In flow cytometry, C3-ScFv bound specifically 721.221 lymphocytes that presented the HLA-C\*07:02–Nef1 complex in a dose-dependent manner over a wide concentration range (Fig. 4A). Specific binding could be detected from as low as ~5 nM ScFv, whereas a minor nonspecific binding to lymphocytes that were preincubated with Conpep was observed at only high concentrations (Fig. 4A, 4C). The binding of W6/32 to cells presenting each peptide confirmed that both complexes were presented at comparable levels on both sets of cells (Fig. 4E). Because addition of exogenous peptides was reported to increase cell surface expression of the HLA class I molecules (39), the extent of binding of W6/32 was also measured for each set of cells at a very low concentration of each peptide (table in Fig. 4E). When the signal from high peptide concentration was compared with the signal from the low peptide concentration, a shift of ~25 mean fluorescence intensity units was observed for both the Conpep and Nef1 peptides, confirming that these two peptides were actually bound and effec-



**FIGURE 2.** Binding of monoclonal ScFv Abs to recombinant HLA complexes, as detected by ELISA. *A*, Bacterial-periplasmic fractions, which contain the specific soluble ScFvs, were tested against NeutrAvidin or three different biotinylated HLA complexes that were previously immobilized through NeutrAvidin, as specified. Although the initial panning was done with the HLA-C\*07:02 complex, containing the gp120-derived peptide as control, further experiments showed that the complex did not fold very efficiently and, therefore, the gp120-derived peptide was replaced in further experiments by Conpep. *B*, All immobilized HLA complexes were also detected with the W6/32 Ab to assess their amounts in the reactions.

**FIGURE 3.** Flow cytometric analysis of the binding of ScFv Abs to cells presenting the HLA-C\*07:02–Nef1 complex or a control complex. *A*, Binding of the W6/32 Ab to noninfected 721.221 cells (filled black histogram, negative control) or to the 721.221 cells stably expressing the HLA-C\*07:02 gene after lentiviral-mediated gene transfer (gray histogram, positive control for HLA-C\*07:02 expression). *B–E*, Binding of specified ScFv Abs to 721.221 cells expressing HLA-C\*07:02 after a prior incubation with Nef1 peptide (black) or with the Conpep peptide (dark gray). Filled gray histograms represent the binding of the secondary Abs to these cells (anti-C-myc followed by FITC-conjugated goat anti-mouse IgG Ab) that are used as baseline.



tively presented by the HLA-C\*07:02 molecule on these cells. Notably, incubation of Nef1-presenting lymphocytes with 505 nM C3-ScFv has resulted in a similar binding pattern as that of W6/32, suggesting that C3-ScFv bound all available molecules on the cells (Fig. 4*A*, 4*E*). Binding of C3-ScFv to Nef1-presenting lymphocytes correlated also with the density of the Nef1 peptide on these cells (Fig. 4*B*, 4*D*), demonstrating once again that the binding is specific to this complex of HLA-C\*07:02 with the Nef1 peptide. In all cases, there was no significant cross-reactivity with Conpep-presenting lymphocytes (and only very minor binding could be detected at high peptide concentrations).

#### C3-IgG Ab

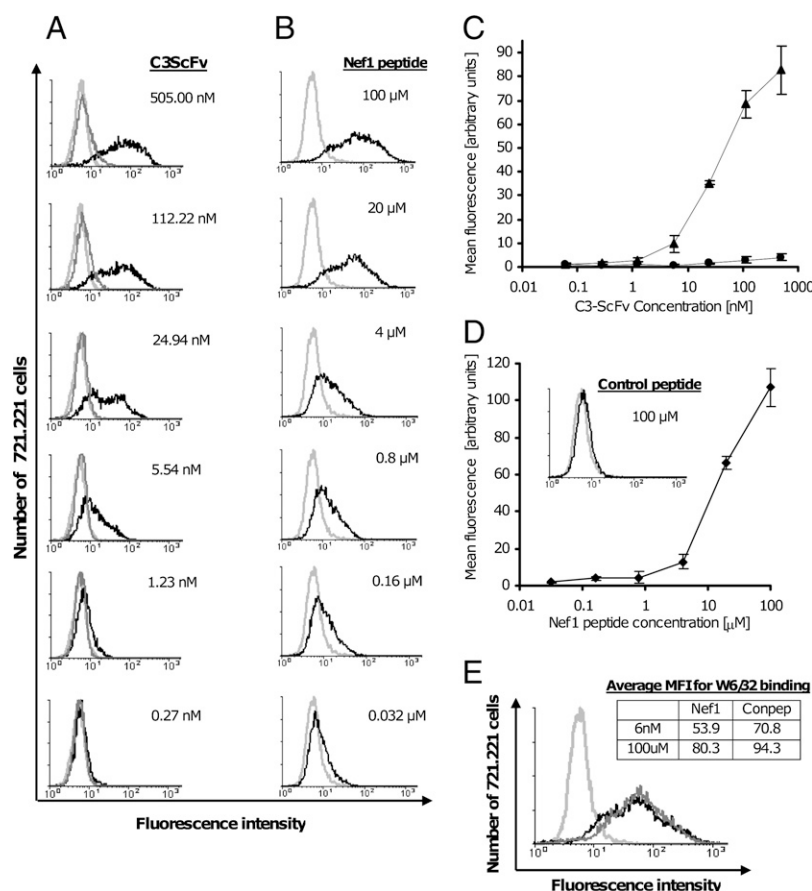
C3-ScFv was further tested for its ability to identify the Nef1 peptide presented on Jurkat cells, which are T lymphocytes that carry the human HLA-C\*07:02 allele. These cells constitutively express on their surfaces low levels of this complex, as opposed to the overexpression of HLA-C\*07:02 by the 721-C\*07:02 cells. Unexpectedly, binding to Nef1-presenting Jurkat cells could not be detected by C3-ScFv, and this was probably due to the low levels of the HLA-C\*07:02 complex on these cells (data not shown). In an attempt to improve its binding ability, C3-ScFv was converted into a full-length human IgG molecule, which retains two identical Ag binding sites for its target. For efficient expression, the 2A self-processing peptide was used to facilitate equimolar expression of the L and H chains (see *Materials and Methods* and Ref. 31), and this resulted in high expression level of the C3-IgG in 293T cells.

C3-IgG molecule showed an exceptionally strong binding and could easily detect C\*07:02–Nef1 on different types of cells. Nef1-presenting B lymphocytes, which overexpress the HLA-C\*07:02 allele, could be detected at as low as 10 pM C3-IgG, whereas Jurkat T cells, which express lower levels of endogenous HLA-C\*07:02,

were detected at a slightly higher concentration of ~66 pM (Fig. 5*B*, 5*C*). C3-IgG could also bind Lck cells, which are human B lymphocytes expressing the HLA-C\*07:02 from an endogenous gene, after they were pulsed with Nef1 peptide (data not shown). The binding efficiency of C3-IgG was dose-dependent in all instances (Fig. 5*B*, 5*C*), and no significant binding was detected to B lymphocytes that express a different HLA (HLA-B8) and were pulsed with Nef1 peptide (Fig. 5*A*). To validate the specificity of C3-IgG, its binding to Nef1-presenting cells (either 721-C\*07:02 or Jurkat) was tested in the presence of recombinant soluble HLA-C\*07:02 complexes (Fig. 5*D*). C3-IgG binding to the cells was drastically reduced when the soluble HLA-C\*07:02–Nef1 complex was added to the reaction as a competitor, thus confirming the binding specificity. As expected, the presence of the control HLA-C\*07:02–Conpep did not significantly affect the extent of binding. Specific binding of C3-IgG was also detected by confocal microscopy (Fig. 5*E*, 5*F*). In this method, single Nef1-presenting lymphocytes (either 721-C\*07:02 or Jurkat cells) displayed a staining ring that is typical of membrane binding of C3-IgG, whereas lymphocytes pulsed with the Conpep showed merely a background signal.

#### Lentiviruses expressing the C3 Ab

To explore further the ability to detect Nef1-presenting cells, we engineered HIV-1–based lentiviruses that were pseudotyped with the C3 Ab (designated PV-C3). Displaying this Ab on the virus surface was facilitated by fusing C3 to the trans membrane portion of CD28 and incorporating the putative HIV-1 packaging signal at the C terminus of the fused protein (32) (see *Materials and Methods*). In this format, multiple C3 molecules are displayed on the viral particle, enabling simultaneous interactions with more than one cellular HLA-C\*07:02–Nef1 complex and increasing the total avidity of the displayed C3 molecules. When the binding



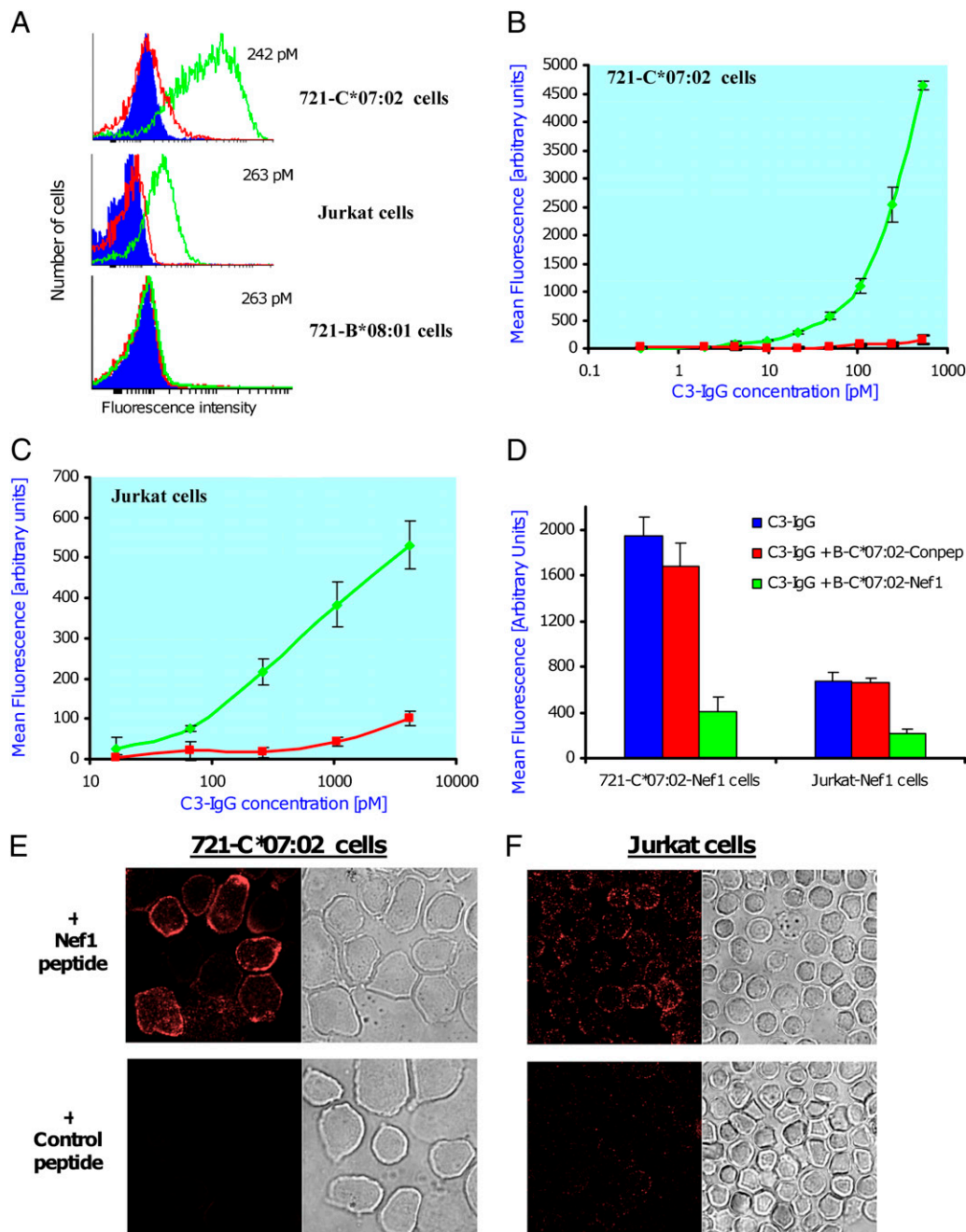
**FIGURE 4.** Binding of C3-ScFv to 721.221 cells presenting either the HLA-C\*07:02–Nef1 complex or a control complex. *A*, Flow cytometric analysis of the binding of increasing concentrations of the purified C3-ScFv Ab to cells preincubated with 100  $\mu$ M Nef1 (black histograms). Binding of the secondary Abs used (anti-C-myc, followed by FITC-conjugated goat anti-mouse IgG Ab that was used as a baseline) is displayed as light-gray histograms, and binding of the specified concentration of C3 to 721-C\*07:02 cells, preincubated with the control peptide (Conpep), is displayed as dark-gray histograms. Each histogram exhibits a representative result of three experiments. *B*, Flow cytometric analysis of the binding of C3-ScFv to cells preincubated with increasing concentrations of Nef1 peptide. Light-gray histograms represent binding of the secondary Abs used (as in *A*), and black histograms represent binding of 505 nM C3-ScFv to cells preincubated with varying concentrations of Nef1 peptide. Each histogram displays a representative result of two (for binding of the secondary Ab) or three (for binding to cells loaded with control or Nef1 peptide) experiments. *C* and *D*, The results from *A* and *B* were used to generate a dose-response curve for the binding of C3-ScFv. Experimental data represent average fluorescence  $\pm$  SD for each concentration,  $n = 3$ . The inset in *D* shows the binding of 505 nM C3-ScFv to cells preincubated with 100  $\mu$ M Conpep. *E*, The level of HLA-C\*07:02 molecules on the surfaces of the two different sets of 721.221 cells used (preincubated with either 100  $\mu$ M Conpep or 100  $\mu$ M Nef1) was measured by flow cytometry with W6/32 Ab. The inset table compares the mean fluorescence intensity (MFI, arbitrary units) for binding of W6/32 to cells incubated with either 6 nM or 100  $\mu$ M of each peptide.

ability of the lentiviral particles was tested, PV-C3 specifically detected the recombinant HLA-C\*07:02–Nef1 complex, with no significant binding to the HLA-C\*07:02–Conpep control complex (Fig. 6A) (40). In contrast, a control PV that displayed an unrelated Ab [specific to cyclin T1 (32)] did not react with both Nef1 and control peptide-presenting complexes. In flow cytometry, PV-C3 showed a similar pattern of binding to the Nef1-presenting Jurkat cells as that of the parental C3-IgG, with a wide range of binding and high specificity (Fig. 6B). Minor binding to Conpep-presenting lymphocytes could be detected only at high peptide concentrations. The specific binding of PV-C3 to the HLA-C\*07:02–Nef1 complex was further supported by detecting simultaneously the binding of PV-C3 and W6/32 to Nef1-presenting B lymphocytes in two different assays (Fig. 6C, 6D). Confocal microscopy has demonstrated that both PV-C3 and W6/32 Ab were colocalized to the cell membrane surface with some low intracellular localization of W6/32 that was probably due to a minor internalization (Fig. 6C). Flow cytometric analysis further showed a direct correlation between the extent of PV-C3 binding and the binding of W6/32 to the lymphocytes (Fig. 6D). In both analyses, simultaneous incubation of

Conpep-presenting lymphocytes with PV-C3 and W6/32 resulted in the binding of only the latter Ab to the cells.

#### *Lentiviruses coexpressing C3 and Fas ligand*

The ability to pseudotype virions enabled us also to coexpress both Fas ligand (41) and C3 Ab on the surface of the same virion particle. The rationale behind such display was that the Fas ligand may potentially interact with a FasR on the target cells and consequently induce apoptosis of these cells. Therefore, the combined presentation endowed the PV-C3–FasL particle with both a recognizing ability (by the C3 Ab) and a killing capacity (by the Fas ligand) that are vital components for directing specific cell killing activity. When tested, PV-C3–FasL showed similar binding specificity as PV-C3 (data not shown). Interaction of PV-C3–FasL with Nef1-presenting Jurkat cells resulted in a significant and specific (though relatively low) apoptosis of these cells, leading to their elimination. PV-C3–FasL killed between ~6 and 19% of the Nef-presenting cells in a concentration-dependent manner (Fig. 6E). Nevertheless, at high PV-C3 concentrations, the higher killing of Nef-presenting cells was accompanied by some nonspecific killing of Conpep-



**FIGURE 5.** Specific binding of C3-IgG to different cells that present the HLA-C\*07:02–Nef1 complex. *A*, Flow cytometric analysis of the binding of C3-IgG to three different types of cells preincubated with Nef1 (green), Conpep (red), or secondary Ab (blue). *B*, The flow cytometric binding results of C3-IgG to B lymphocytes stably expressing the HLA-C\*07:02 were used to generate a dose-response curve for the binding of C3-IgG. Experimental data represent average fluorescence  $\pm$  SD for each concentration,  $n = 3$ . *C*, Similar to *B*, but the binding was tested with Jurkat cells (T lymphocytes) that express only low, endogenous levels of HLA-C\*07:02. Color codes are as in *B*. *D*, Binding of 290 pM C3-IgG to 721-C\*07:02–Nef1 cells (*left*) or 4.2 nM C3-IgG to Jurkat cells presenting Nef1 (*right*) in the absence or presence of similar amounts of correctly refolded (determined by ELISA with W6/32 Ab), different B-HLA-C\*07:02 complexes. *E* and *F*, Detection of C3-IgG binding to HLA-C\*07:02–Nef1 presenting cells by confocal microscopy (original magnification  $\times 63$ ; digital scan zoom  $\times 2$ ). Binding of 4.4 nM C3-IgG to B lymphocytes (*E*) or Jurkat cells (*F*), which present either Nef1 or control peptide by HLA-C\*07:02, was visualized by a confocal microscope after detection with DyLight649-conjugated secondary Abs. In each panel, the *left side* shows the emission observed above 640 nm, and the *right side* shows the cells under phase contrast setting.

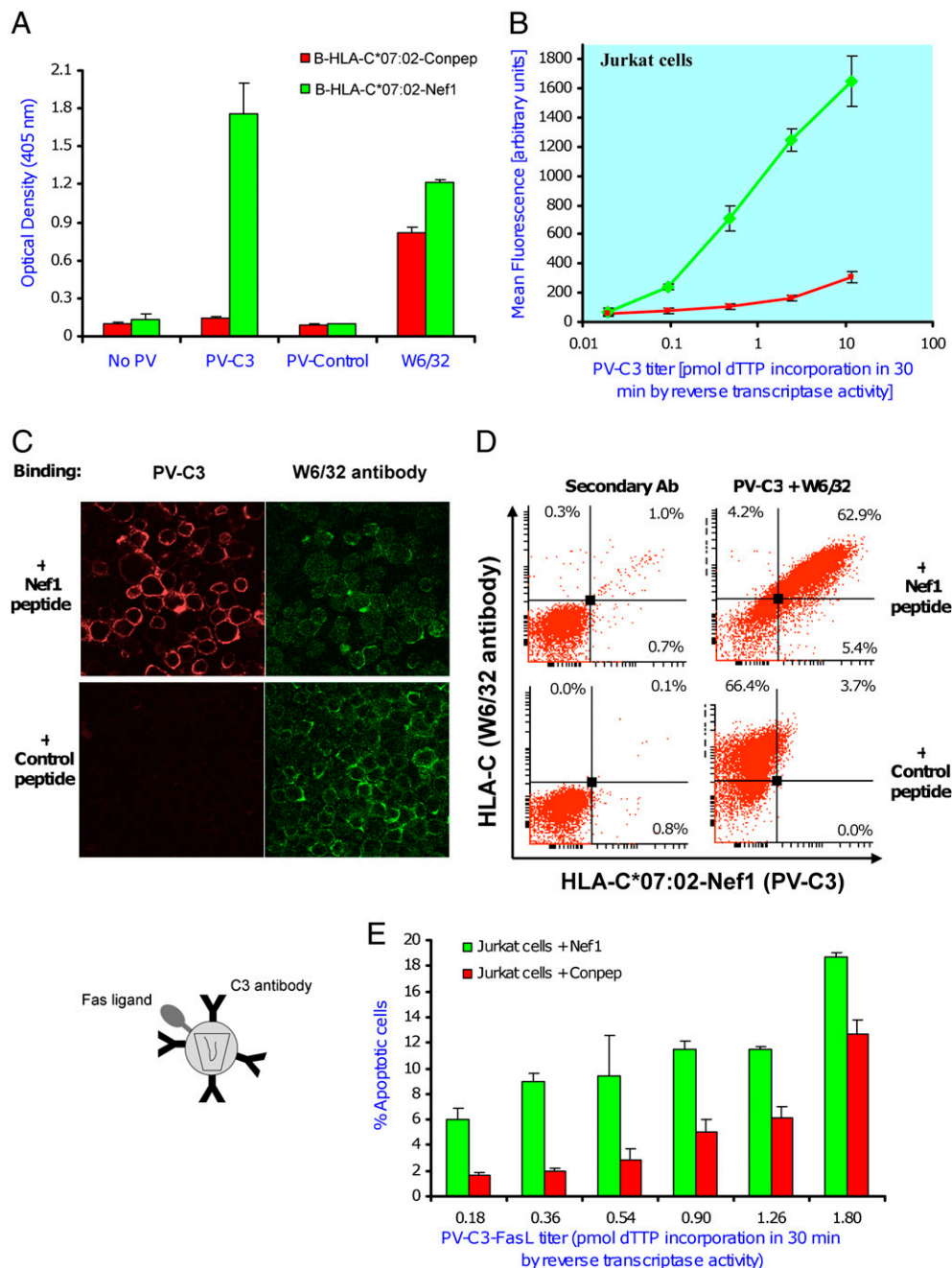
presenting cells (yet, killing of Nef-presenting cells was always higher).

## Discussion

We have developed reagents that can selectively detect the HLA-C presenting a peptide derived from HIV-1 Nef on the surfaces of human cells. HLA-C\*07:02–Nef1 complex represents an opti-

mized target for targeting HIV-1-infected cells. The HLA-C\*07:02 allele is frequent in the population, and the Nef1 peptide is highly conserved. Notably, a Nef1 peptide was also reported to bind and be presented by HLA-C\*07:01 (18, 23) (Los Alamos HIV database at <http://www.hiv.lanl.gov/>), which differ in only two amino acid residues from HLA-C\*07:02. Accordingly, HLA-C\*07:01–Nef1 may also be recognized by the C3 Ab, and this





**FIGURE 6.** Functional presentation of C3 Ab and the Fas ligand on lentiviruses. *A*, Equal amounts of PV-C3 or PV-Control (0.069 pmol dTTP/30 min of RT activity) were tested for binding recombinant B-HLA-C\*07:02–Nef1 or B-HLA-C\*07:02–Concep complexes in ELISA. W6/32 Ab was used to measure to amount of the complexes in the reaction. *B*, Dose-response curve of flow cytometric analysis for the binding of different amounts of PV-C3 (measured by RT activity, as specified) to Jurkat cells preincubated with Nef1 (green) or with Concep (red). *C*, Specific binding of PV-C3 to the HLA-C\*07:02–Nef1 presented on B lymphocytes was tested by simultaneously incubating the cells with PV-C3 and the W6/32 Ab. Binding of PV-C3 was detected with DyLight649-conjugated secondary Abs, and binding of W6/32 Ab to HLA-C\*07:02 was detected with fluorescein-conjugated secondary Abs. Emission pattern of the two fluorophores was recorded with a confocal microscope (original magnification  $\times 63$ ). *D*, The cells from *A* were analyzed by flow cytometry, and the results are shown as a dot plot of the emission of each cell at 661 nm (*x*-axis, DyLight649) and 530 nm (*y*-axis, fluorescein). The percentages of cells from the total population are displayed in each quadrant (except the *lower left*). Measurements are displayed by the Cyflogic software (CyFlo, Turku, Finland). *E*, *Left panel*, A schematic presentation of the PV-C3–FasL particle. *Right panel*, The killing effect of PV-C3–FasL on Nef1-presenting Jurkat cells (green) or Concep-presenting Jurkat cells (red) was tested by incubating the different cells with PV-C3–FasL particles for 16 h at 37°C. Apoptosis was recorded by measuring the loss of mitochondrial membrane potential with the cell-permeable green-fluorescent lipophilic dye DiOC<sub>6</sub> as previously described (40).

ability has to be tested. Such recognition will expand the use of the C3 Ab, as within the HLA-C group both HLA-C\*07:01 and HLA-C\*07:02 alleles are dominant in many human populations worldwide. Nef1 peptide was identified based on various reports and after an exhaustive alignment with different HIV-1 isolates.

Direct evidence shows that HLA-C\*07:02 presenting this peptide explicitly resists any downregulation of the complex by HIV-1 Nef, and cells presenting this complex retain susceptibility to a specific CTL attack (22). Additional reports demonstrated that only a limited number of mutations have been observed in this peptide

[only V114I or D108E in early stage of infection and either K105R/Q or Q107R in a more elaborated study (23, 42, 43)] making it an excellent candidate for targeting.

Screening Metha1, Metha2, and Griffin1 phage display libraries resulted in the identification of significant binders only in the first two libraries. Both Metha libraries have a richer repertoire of Abs and contain at least 10 times more members than the Griffin1 library ( $1.2 \times 10^{10}$  and  $1.5 \times 10^{10}$  versus  $1.2 \times 10^9$  different clones), so in these libraries there was originally a higher probability to find anti-HLA-C\*07:02–Nef1 Abs. Our screening strategy was directed to identify and isolate ScFv Abs against a limited spatial region in the whole HLA-C\*07:02–Nef1 complex. This task was much more complex than identifying Abs against a whole protein, as in this case the desired Abs have to bind to a limited number of epitopes that are formed in the interface of the peptide and nearby residues in the H chain of the HLA protein. To achieve this aim, the successful panning was carried out with a mix of a biotinylated C\*07:02–Nef1 complex and a nonbiotinylated HLA-C\*07:02 control complex, presenting a gp120-derived peptide (as bait for Abs against undesired epitopes). It is important to note that although this complex was prepared in a similar manner as the HLA-C\*07:02–Nef1 (with identical concentrations of all components), later experiments showed that there were some difficulties in refolding it correctly, as a lower yield of refolded complex was observed in these preparations in comparison with that of the HLA-C\*07:02–Nef1. Nevertheless, using excess of the control complex still allowed us to isolate four unique ScFv Abs: three of them showed specific binding to the recombinant HLA-C–Nef1 complex. The fourth ScFv, A4, reacted most probably with an epitope found exclusively on the HLA-C molecule (as it reacted with similar complex that presented a control peptide), demonstrating that the selection scheme was not absolutely selective for HLA-C–Nef1 complex. Within the three specific ScFvs, C3 showed the strongest binding to cells expressing HLA-C\*07:02–Nef1. The inability of the other two Abs to detect the HLA-C\*07:02–Nef1 on cells can result from different potential reasons. D6 may have low affinity that does not enable it to detect the cell-expressed complex under the experimental conditions used, whereas E9 may bind an epitope that is either modified or inaccessible during presentation on cells.

Conversion of C3-ScFV to C3-IgG substantially increased the binding capacity of the molecule. The enhanced ability of C3-IgG to detect the HLA-C\*07:02–Nef1 on surfaces of cells was demonstrated with several cell lines and by using different techniques. According to the data presented, C3-IgG has a notably high affinity to the HLA-C\*07:02–Nef1 as it detects, at picomolar concentration, low levels of the complex on the surfaces of cells. Our study also shows that complexes of HLA-C presenting different peptides can be readily detected and visualized with high-affinity Abs. To our knowledge, this is the first report on the ability to detect and target specifically HLA-C–presenting complexes by Abs and Ab-presenting PVs. These reagents can be further applied to several fields of study. Such binders, used as either Abs or PVs with TCR-like specificity, are likely to be highly valuable in developing vaccines against HIV-1 infection. For example, these Abs may enable one to assess the level of HLA–peptide complexes presented after immunizing with HIV-1-related proteins or genes. Accordingly, they can be used to select and optimize the candidates for effective vaccinations. The Ab reagents may also be useful in tracking in the infected cells the transport of HLA-C complexes that display viral peptides. This can allow the precise characterization of the dynamics of viral peptide presentation. More importantly, as epitomized by coexpressing C3 and Fas ligand on PVs (Fig. 6E), Abs and PVs with TCR-like specificity are likely to be highly valuable

as building blocks for specific targeting and killing of HIV-1-infected cells.

## Acknowledgments

We thank the following researchers from Tel Aviv University (Tel Aviv, Israel): E. Bacharach for the pseudovirus system, I. Benhar for the IgG-expression plasmids, Z. Fishelson for the Jurkat cell line and DiOC<sub>6</sub> dye, and M. Shaharabany and Y. Oshri for help with setting up the confocal and flow cytometric experiments. We also thank R. Taube from Ben Gurion University (Beer-Sheva, Israel) for the pCDNA3.1-PS11scFvFc-CD28-gp41 plasmid, O. Mandelboim from the Hebrew University (Jerusalem, Israel) for the 721.221 cells, K. Maenaka from Kyushu University (Japan) for the pET plasmids for bacterial expression of HLA-C\*07:02 and  $\beta_2m$  proteins, K. Breckpot from the Medical School of Vrije Universiteit Brussel (Belgium) for the plasmid for expressing the HLA-C\*07:02 protein in eukaryotic cells, K. Weber from the University Medical Center Hamburg (Germany) for the lentiviral plasmid pLeGO-iG2, T. Dong and S.L. Rowland-Jones from the Medical Research Council (Oxford, U.K.) for the plasmid for bacterial expression of HLA-B\*08:01, G. Winter from the Medical Research Council (Cambridge, U.K.) for the Griffin1 phage display library, the Harvard Institute of Proteomics for the plasmid that carries the Fas ligand, and the International Cell and Gene Bank at the Fred Hutchinson Cancer Research Center (Seattle, WA) for the Lck cells.

## Disclosures

The authors have no financial conflicts of interest.

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