Reappraisal of the Relationship between the HIV-1-Protective Single-Nucleotide Polymorphism 35 Kilobases Upstream of the *HLA-C* Gene and Surface HLA-C Expression[▽]

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Previous studies have found an association between a single-nucleotide polymorphism 35 kb upstream of the *HLA-C* locus (−35 SNP), HLA-C expression, and HIV-1 set point viral loads. We show that the difference in HLA-C expression across −35 SNP genotypes can be attributed primarily to the very low expression of a single allelic product, HLA-Cw7, which is a common HLA type. We suggest that association of the −35 SNP and HIV-1 load manifests as a result of linkage disequilibrium of this polymorphism with both favorable and unfavorable HLA-C and -B alleles.

Infection with HIV-1 involves a complex interplay between virus and host. After the acute phase of infection, an equilibrium is reached between HIV-1 and the immune system, resulting in the decline of HIV-1 RNA levels to the widely variable viral set point, a known (though imperfect) predictor of time to progression to AIDS (36, 37). The early appearance of HIV-1-specific CD8 T cells and their rapid selection of escape mutants during the initial decline of viremia in primary infection imply that this T-cell response plays a central role in resolution of primary viremia and the long-term suppression of viral replication (21, 34).

The major histocompatibility complex (MHC), located on chromosome 6, contains the HLA class I and II genes, which are the most polymorphic loci in humans (9). HLA class I molecules play a significant role in the immune response against HIV-1 by presenting viral peptides to CD8 T cells (adaptive immunity) and by serving as ligands for killer cell immunoglobulin-like receptors (KIRs) expressed on natural killer (NK) cells (innate immunity). The central role of the MHC region in HIV-1 control was highlighted by a recent genomewide association study of variants that influence the control of viral set point (17, 18). In Caucasian cohorts, two single-nucleotide polymorphisms (SNPs) were identified that associated with the HIV-1 set point, and a third was associated with disease progression. All three SNPs were located in the MHC region on chromosome 6. These findings have been replicated with other independent Caucasian cohorts (10, 15, 31, 53, 55).

One minor allelic variant of an SNP (rs2395029) associated with lower viral set points is located in the *HLA complex P5* (*HCP5*) gene and is a tag for *HLA-B*5701* (17), which is associated with slow disease progression (2, 9, 25, 40, 42). The second most significant polymorphism (rs9264942), accounting for 6.5% of the variation in viral set point (18), is located 35 kb upstream of the *HLA-C* gene (−35 SNP), with the minor allele (*C*) associating with a lower set point than the major allele (*T*). The −35 SNP is also present in African-American populations but does not correlate with viral set point (47, 49), which suggests that the −35 SNP is a marker for another polymorphism and is not the causal SNP. Although *HLA-B*5701* is in linkage disequilibrium with the −35 SNP, both associate independently with the viral load set point (17, 18).

The −35 *C* variant was associated with higher *HLA-C* mRNA expression in Epstein-Barr virus (EBV)-transformed B-cell lines, suggesting an inverse correlation between *HLA-C* expression and viral load (51). However, the extreme polymorphism of *HLA-C* and posttranslational regulation of HLA class I molecules may complicate this relationship (23, 33). The −35 SNP is known to be in strong linkage disequilibrium with *HLA-C* alleles (*Cw*0102, -0202, -0302, -0501, -0602, -0801, -0802, -1202, -1203,* and *-1402* for the −35 *C* allele and *Cw*0303, -0304, -0401, -0701, -0702, -0704, -1502, -1504, -1505, -1506, -1601, -1602, -1604,* and *-1701* for the −35 *T* allele) (17, 52), and each type may have its own intrinsic level of expression. Therefore, protein expression studies are needed to resolve the question of whether the −35 SNP is associated with increased levels of surface HLA-C. Recently, it was reported that surface expression of HLA-C varies significantly across −35 SNP genotypes and that the *HLA-C* alleles that are in linkage disequilibrium with the −35 *C* allele are expressed at

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higher levels than those that are in linkage disequilibrium with the −35 T allele (52). However, interpretation of this study is complicated by the known cross-reaction with HLA-E of the antibody used for the study (DT9) (7). Our results show that when this cross-reaction is taken into account, the difference in the levels of HLA-C expressed at the cell surface between −35 SNP genotypes is actually greater than previously realized. However, we also show that nearly all of this difference can be accounted for by the particularly low expression of HLA-Cw7, which is in strong linkage disequilibrium with the −35 T allele. Although HLA-Cw7 has been shown to associate with more rapid progression of disease in HIV-1-infected patients (48), it cannot account fully for association of the −35 SNP with lower HIV-1 loads and slower progression to AIDS (17).

MATERIALS AND METHODS

Subjects. Peripheral blood mononuclear cells (PBMC) from healthy volunteers of European descent were isolated from whole blood by Ficoll-Hypaque gradient centrifugation. Written informed consent was obtained from all volunteers under appropriate university regulations. Cryopreserved PBMC from 25 HIV-1-infected subjects of European descent were obtained from the CHAVI 001 and SCOPE (22) cohorts. All experiments on HIV-infected samples were approved by the Oxford Tropical Research Ethics Committee.

−35 SNP genotyping. Genomic DNAs from the healthy volunteers were genotyped for the −35 SNP by PCRs using a common forward primer (5′-GGGTG GTGCCAAGTATGAG-3′) and alternative allele-specific reverse primers (5′-A GAAAGTCCCACAGTGCCT**A**-3′ and 5′-AGAAAGTCCCACAGTGCCT**G**-3′). Genotyping reaction mixtures also contained control primers that amplify a constant region in the *DRB1* locus (43). Thermocycling conditions were as follows: 96°C for 1 min; 5 cycles of 96°C for 25 s, 70°C for 45 s, and 72°C for 45 s; 21 cycles of 96°C for 25 s, 65°C for 50 s, and 72°C for 45 s; and 4 cycles of 96°C for 25 s, 55°C for 1 min, and 72°C for 2 min. Products were resolved in 2% agarose gels and visualized under UV with ethidium bromide. This PCR assay was validated by direct sequencing of a PCR product spanning the −35 SNP for the first 100 samples typed.

Monoclonal antibodies and flow cytometry. MEM-E/06 and MEM-E/08 were obtained from Santa Cruz; anti-human HLA-E (3D12), anti-human HLA-A,B,C (W6/32), and a mouse IgG2b kappa isotype control (clone MPC-11) were obtained from BioLegend; CD3-PerCP (clone SK7) was obtained from Becton Dickinson; a mouse IgG2b-fluorescein isothiocyanate (FITC) (clone MOPC-141) isotype control and mouse anti-human MHC class I (W6/32)–FITC were obtained from Sigma; and rat anti-mouse kappa (clone 187.1)–FITC was obtained from Southern Biotech. Monoclonal antibody DT9 was affinity purified on protein A-Sepharose beads (Sigma-Aldrich) from a hybridoma supernatant by standard procedures and conjugated using a FluoroTag FITC conjugation kit (Sigma).

For all cell surface staining, 1 million thawed PBMC were washed once with phosphate-buffered saline (PBS; Sigma-Aldrich) and then stained with Live/ Dead stain (Invitrogen) per the manufacturer's instructions. For direct antibody staining, cells were then incubated on ice for 15 min with appropriate antibodies. For indirect antibody staining, cells were incubated with primary antibodies (15 min on ice), free secondary antibody binding sites were blocked with mouse serum (Southern Biotech), and then cells were incubated with secondary antibody and anti-CD3–PerCP (15 min on ice). Cells were washed twice between each step with flow cytometry buffer (PBS containing 0.5% bovine serum albumin [BSA]). After being stained, cells were washed again, fixed with Cell Fix buffer (Becton Dickinson), and analyzed using a CyAn flow cytometer and FlowJo software (Treestar, Inc.).

Characterization of monoclonal antibodies. LABScreen single-antigen class I Combi beads (One Lambda), each coated with a single HLA allotype (46), were incubated with DT9, 3D12, MEM-E/06, MEM-E/08, and W6/32 antibodies for 30 min at room temperature, washed 3 times with PBS, incubated at room temperature for 30 min with goat anti-mouse IgG–R-phycoerythrin (R-PE) (Invitrogen), washed 3 times as described before, and analyzed on a LABScan 100 flow analyzer.

The specificity of the monoclonal antibody panel for HLA-E was tested by binding to HLA-E on transfected 721.221 AEH cells (29), a gift from V. Braud (University of Nice).

RESULTS

Determining the specificity and applicability of HLA-C and HLA-E antibodies. The study of individual surface HLA molecules is complicated by the scarcity of allele-specific antibodies. The monoclonal antibody DT9 (7), originally raised against MHC class I proteins from cottontop tamarins, is the only available HLA-C-specific antibody that does not extensively cross-react with HLA-A or -B alleles. However, immunoprecipitation and one-dimensional isoelectric focusing (1D-IEF) studies confirmed that it also recognizes the nonclassical HLA-E protein (6, 7). A recent study showing a correlation between surface HLA-C expression and the −35 SNP discounted cross-reaction of DT9 with HLA-E, as it was shown that the HLA-E-specific antibody MEM-E/08 stains a cell line transfected with HLA-E but does not stain PBMC (52). However, it has previously been reported that MEM-E/08 cannot be used to detect HLA-E on PBMC by flow cytometry (32). Therefore, we decided to reexamine cell surface expression of HLA-E by PBMC to better understand DT9 staining.

In addition to MEM-E/08, we tested two other commercially available antibodies that are nominally specific for HLA-E (MEM-E/06 and 3D12) (29, 38, 44), using 220.221 cells transfected with HLA-E. As can be seen in Fig. 1A, the levels of staining with all of these antibodies were similar to that with DT9, confirming that they all recognize HLA-E, at least as expressed by transfected cells. When we used these antibodies to stain PBMC from healthy volunteers, however, we saw marked differences in the levels of staining. Consistent with previous reports (32, 52), we did not see staining of PBMC with MEM-E/08 (Fig. 1B). In contrast, the level of staining seen with MEM-E/06 varied considerably from donor to donor, suggesting that this antibody may cross-react with other HLA proteins. Finally, we observed fairly constant levels of staining of PBMC with 3D12.

The possibility that MEM-E/06 cross-reacts with other class I proteins prompted us to test the specificity of all of the antibodies used. To do this, we employed the LABScreen system, a Luminex assay that allows detection of antibody cross-reactions with 97 common HLA class I alleles spanning HLAs A, B, and C. In addition to testing the three HLA-E antibodies, we also tested DT9. Since there can be considerable bead-to-bead differences in the amount of HLA protein present on the individual antigen beads (S. Fuggle and S. Page, personal communication), we used binding of the pan-class I antibody W6/32 (3) to normalize the results. While DT9 was highly specific for HLA-C and cross-reacted only with the rare HLA-A*8001 and -B*7301 alleles (Fig. 2A), confirming previous findings (52), MEM-E/06 did indeed show extensive cross-reactions with many HLA-A, -B, and -C proteins (Fig. 2B), as previously reported (32, 38), and there was good correlation with the levels of PBMC staining described above (data not shown). Cross-reaction of MEM-E/06 with HLA-A*0301 but not with HLA-A*0201 was confirmed by immunoprecipitation and 1D-IEF, using 220.221 cells transfected with these HLAs (data not shown). Much less cross-reaction was seen for both MEM-E/08 and 3D12 (Fig. 2C and D), and for 3D12, the level of binding seen with the beads did not translate into cross-reaction with these HLAs at the cell surface.

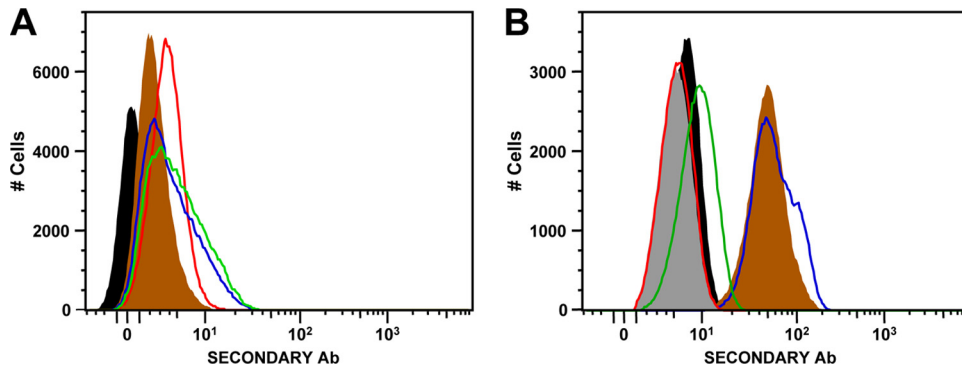Although at present we cannot rule out cross-reaction with

FIG. 1. Staining of 220.221AEH cells and PBMC with DT9 and HLA-E-specific antibodies. (A) Staining of the 220.221AEH cell line, showing unstained cells (filled black area) and cells stained with MEM-E/06 (filled brown area), MEM-E/08 (red line), 3D12 (green line), and DT9 (blue line). (B) Representative staining of CD3^{+} lymphocytes (gating on appropriate forward and side scatter, live cells, and CD3 expression) from a healthy volunteer (HLA-A2, -B35, -B44, -Cw4, and -Cw5), showing unstained cells (filled black area) and cells stained with MEM-E/06 (filled brown area), MEM-E/08 (red line), 3D12 (green line), DT9 (blue line), and an IgG2b isotype control (filled gray area).

other nonclassical HLA proteins, it appears that 3D12 is indeed specific for HLA-E and that HLA-E is present on the surfaces of cells at levels detectable by flow cytometry. It is not clear why MEM-E/08 stains HLA-E-transfected cells and not PBMC, but one possible explanation is that this antibody is actually specific for a misfolded HLA heavy chain which may be present at increased levels on the surfaces of transfected cells.

DT9 staining of PBMC from healthy and HIV-1-infected subjects. In the absence of a truly HLA-C-specific antibody, DT9 (which recognizes both HLA-C and HLA-E) was used to stain cryopreserved PBMC from 28 HIV-1-uninfected subjects selected on the basis of homozygosity of their −35 SNP genotype (both *TT* and *CC*) and the absence of the HLA alleles (*A*8001* and *B*7301*) that cross-react with DT9. There was a significantly higher level of DT9 staining of lymphocytes (CD3-
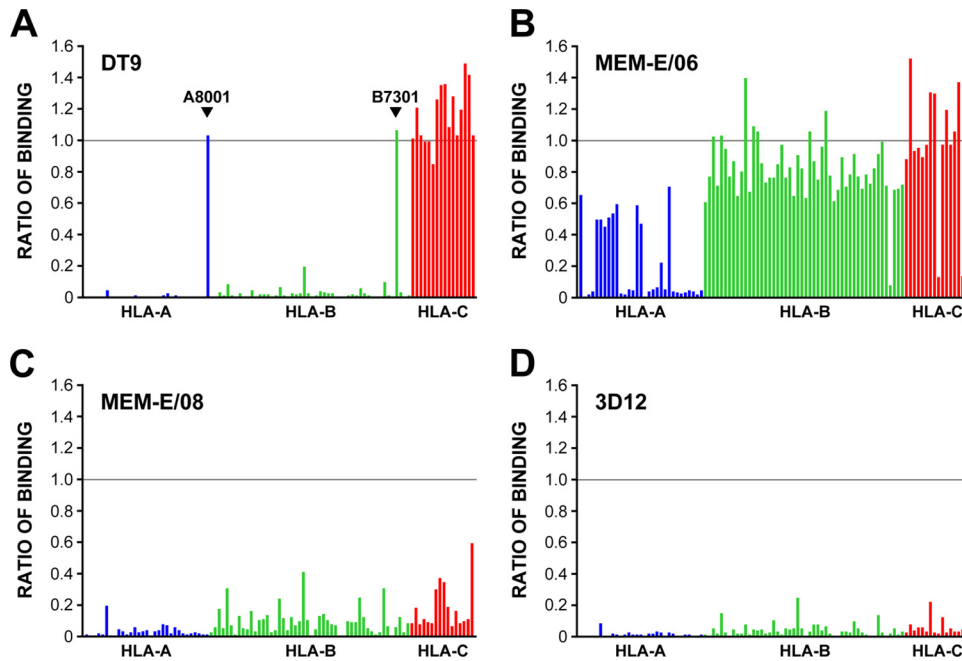

FIG. 2. Characterization of monoclonal antibodies DT9, 3D12, MEM-E/06, and MEM-E/08 against 97 common HLA class I allotypes. Binding was determined for DT9 (A), MEM-E/06 (B), MEM-E/08 (C), and 3D12 (D), using beads coated with recombinant HLA-A (blue), -B (green), and -C (red) proteins, and normalized relative to the binding of W6/32 (the horizontal line corresponds to the level of binding seen with W6/32). Values shown represent the averages for three runs; the maximum standard errors of the means were 0.06 (DT9), 0.05 (MEM-E/06), 0.012 (MEM-E/08), and 0.01 (3D12). The order of the HLA allotypes tested (along the *x* axis) was as follows: A0101, -0201, -0203, -0206, -0301, -1101, -1102, -2301, -2402, -2403, -2501, -2601, -2901, -2902, -3001, -3002, -3101, -3201, -3301, -3303, -3401, -3402, -3601, -4301, -6601, -6602, -6801, -6802, -6901, and -7401, B8001, -0702, -0801, -1301, -1302, -1401, -1402, -1501, -1502, -1503, -1510, -1511, -1512, -1513, -1516, -1801, -2705, -2708, -3501, -3701, -3801, -3901, -4001, -4002, -4006, -4101, -4201, -4402, -4403, -4501, -4601, -4701, -4801, -4901, -5001, -5101, -5102, -5201, -5301, -5401, -5501, -5601, -5701, -5703, -5801, -5901, -6701, -7301, -7801, -8101, and -8201, and Cw0102, -0202, -0302, -0303, -0304, -0401, -0501, -0602, -0702, -0801, -1203, -1402, -1502, -1601, -1701, and -1802.
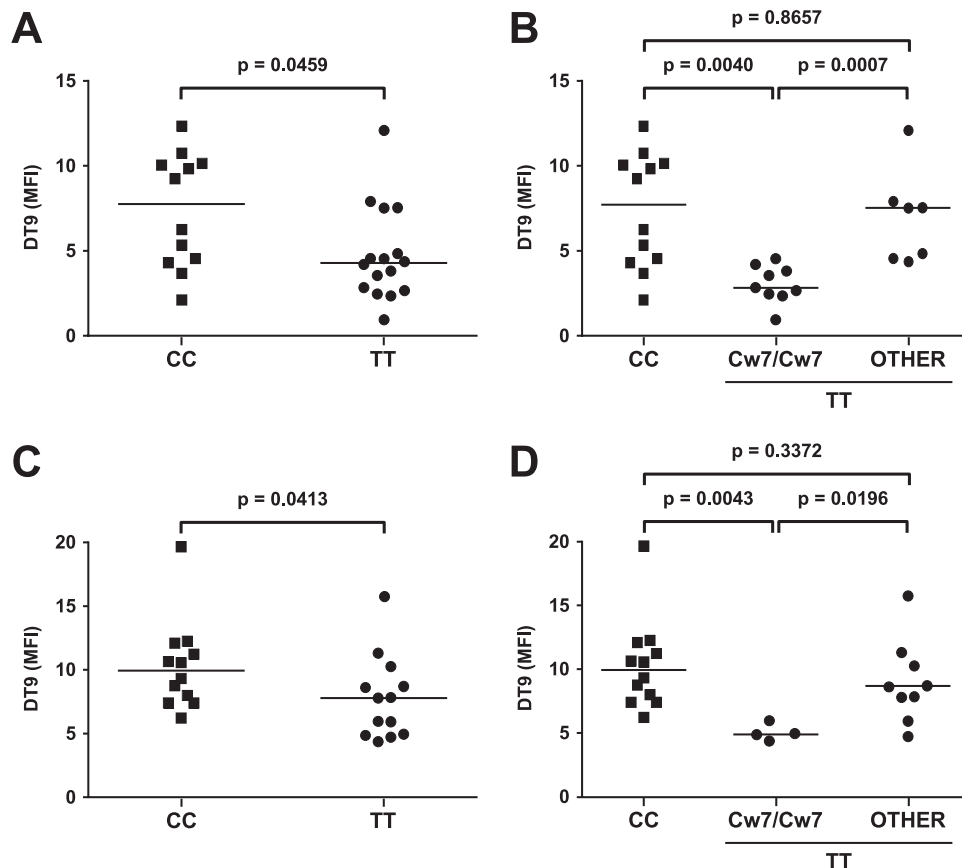
FIG. 3. Direct DT9 staining of live CD3$^+$ T lymphocytes from -35 SNP homozygotes. PBMC from HIV-1-uninfected (A and B) and chronically HIV-1-infected (C and D) subjects were stained with DT9. DT9 median fluorescence intensities (MFI; y axis) were corrected for background autofluorescence of unstained cells. -35 SNP genotypes are indicated on the x axis. In panels B and D, "other" refers to -35 *TT* individuals who were not homozygous for HLA-Cw7. *P* values were calculated using the Mann-Whitney test.

positive cells) from *CC* subjects than of those from *TT* subjects ($P = 0.0459$; Mann-Whitney test) (Fig. 3A). When the *TT* subjects were subdivided based on their HLA-C alleles, DT9 staining of *TT* subjects who were homozygous for *HLA-Cw*07* was significantly lower than that for both *CC* subjects, who were all *HLA-Cw*07* negative ($P = 0.004$), and *TT* subjects who were either heterozygous for or did not have *HLA-Cw*07* ($P = 0.0007$) (Fig. 3B). There was no significant difference in the DT9 staining of PBMC from *TT* subjects who were not homozygous for *HLA-Cw*07* and *CC* subjects. These findings imply that low HLA-Cw7 levels could account for the differences in HLA-C expression previously described as high levels associated with the -35 *CC* genotype.

Lymphocytes from 25 HIV-1-infected subjects were also stained with DT9. As seen before, DT9 staining of *CC* subjects was just significantly higher than staining of *TT* subjects ($P = 0.0413$) (Fig. 3C), and again, this difference in staining was even more striking with *TT* subjects who were also homozygous for *HLA-Cw*07* (Fig. 3D) ($P = 0.0043$). Furthermore, there was no significant difference in median fluorescence intensity values comparing HIV-1-uninfected and -infected subjects, suggesting that, at least in chronically infected subjects, HIV-1 does not upregulate expression of the HLA-C protein.

Quantifying HLA-C on the cell surface. In order to determine the contribution of HLA-C to DT9 binding, saturating amounts of DT9 and 3D12 were used to stain lymphocytes from HIV-1-uninfected subjects in indirect antibody binding assays (Fig. 4 and 5). As with direct DT9 staining, the low levels of staining observed in these indirect binding assays are consistent with cell surface levels of HLA-C protein being 10-fold less than those of HLA-A and -B proteins (50). Despite the consistently low staining with 3D12, there was a significant difference in median fluorescence intensity between unstained cells and cells stained with 3D12 ($P < 0.0001$; Mann-Whitney test), confirming surface expression of HLA-E (Fig. 5A), and when subjects were grouped by -35 SNP genotype, there was no significant difference in 3D12 binding (Fig. 5B). As seen before, differences in DT9 staining in this indirect staining assay were driven by the low levels of binding for *TT* individuals who were homozygous for *HLA-Cw*07* (Fig. 5C).

There was no statistically significant correlation between 3D12 and DT9 binding (Spearman test; $r = 0.3495$; $P = 0.0583$) (Fig. 5D). These data imply that the differences seen in DT9 staining are the result of differences in HLA-C binding alone. Furthermore, the constant contribution of surface HLA-E to DT9 binding means that the difference in
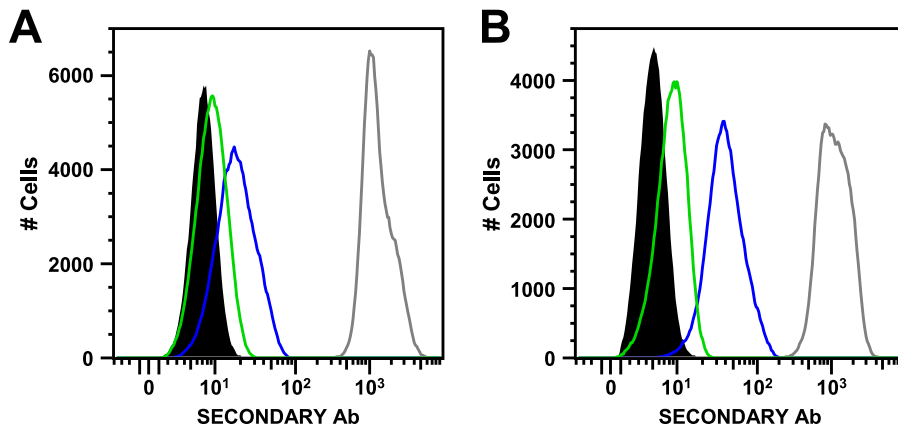
FIG. 4. Indirect staining of lymphocytes with 3D12, DT9, and W6/32 antibodies. Representative staining results are shown for CD3+ lymphocytes (gated on appropriate forward and side scatter, live cells, and CD3 expression) from two HIV-1-uninfected subjects: a −35 *TT* subject (homozygous for *HLA-Cw*07*) (A) and a −35 *CC* subject (*HLA-Cw*05/06*) (B). Data are shown for unstained cells (filled black area), 3D12 staining (green), DT9 staining (blue), and W6/32 (pan-class I) staining (gray).

HLA-C surface expression between the *CC* and *TT* subjects is greater than previously recognized. To better quantify this difference, we calculated the ratio of HLA-C (3D12 staining subtracted from DT9 staining) to total class I protein on the cell surface (measured by W6/32 staining). The proportions of actual HLA-C binding to W6/32 binding ranged from 1.0 to 4.6%, 1.0 to 3.4%, and 0.4 to 2.6% for −35 *CC*, *CT*, and *TT* subjects, respectively. There was a significant difference in the ratios of HLA-C to W6/32 binding between −35 *CC* and *TT* subjects (Fig. 5E) ($P = 0.0061$; Mann-Whitney test), but again, this difference was driven exclusively by the low

expression of HLA-Cw7 ($P = 0.0032$ for comparing −35 *CC* with −35 *TT* subjects homozygous for *HLA-Cw*07*). As before, there was no significant difference in the ratios of HLA-C to W6/32 binding between −35 *CC* and *TT* subjects who were not homozygous for *HLA-Cw*07* ($P = 0.0896$). From this observation, we concluded that −35 *CC* subjects expressed 1.76 times more HLA-C molecules than −35 *TT* subjects considered as a whole but 5 times more HLA-C molecules than −35 *TT* subjects who were homozygous for *HLA-Cw*07*. Therefore, the protective −35 SNP genotype does not associate with increased expression of HLA-C.
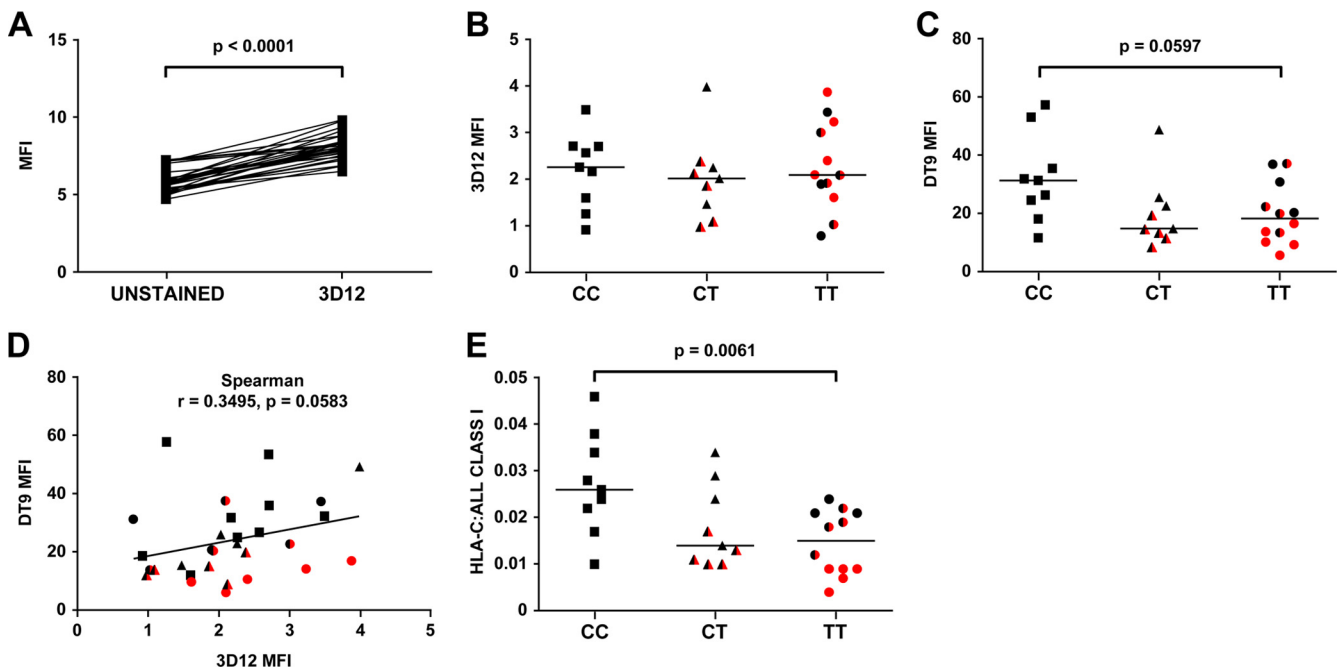

FIG. 5. Indirect staining of lymphocytes from 30 healthy volunteers, using 3D12 and DT9. (A) Paired comparisons of MFI of unstained cells and cells stained with 3D12. (B and C) Indirect staining (with 3D12 and DT9, respectively) of live CD3+ lymphocytes, corrected for background autofluorescence of unstained cells. (D) Correlation of background-corrected DT9 and 3D12 staining. (E) Ratios of HLA-C levels (3D12 staining subtracted from DT9 staining) to total class I protein levels (W6/32 staining). For panels B to E, red and half-red symbols denote individuals homozygous and heterozygous for *HLA-Cw*07*, respectively. Statistical significance was calculated using the Mann-Whitney test.

Rather, the nonprotective allele associates with the greatly reduced expression of a single HLA-C allele, the *Cw*07* allele.

DISCUSSION

Previous studies of Caucasians have indicated that a single-nucleotide polymorphism 35 kb upstream of the *HLA-C* gene associates with control of the HIV-1 set point (17, 18) as well as with a slower progression to a CD4 count below 200 and an increased time to death (52). Transcriptional data showing that this SNP associates with differences in *HLA-C* expression levels and the linkage of −35 SNP alleles with *HLA-C* alleles (17, 52) have all been taken as evidence that this SNP is a marker for the *HLA-C* locus. As a result, there has been much speculation on a role for HLA-C in controlling HIV-1 infection. HIV-1 selectively downregulates surface HLA-A and -B, but not HLA-C and -E, via the action of the Nef protein (11–13, 56). The infected cells may therefore be relatively resistant to HLA-A- and -B-restricted CD8 T-cell lysis but continue to express HLA-C. While it has been shown that recognition of infected cells by HLA-C-restricted CD8 T cells is unaffected by HIV-1 Nef expression (1), these responses are relatively weak (4, 25) and are consistently associated with high levels of viremia in HIV-1-infected subjects, even when targeting the Gag protein (26). This suggests that they contribute little to viral control *in vivo*. HLA-C molecules also act as ligands for the inhibitory KIR2DL receptors (28, 45), so infected cells expressing high levels of HLA-C may be less vulnerable to NK cell recognition (1, 12, 13). However, it is not known whether the level of HLA-C expressed by the target cell is critical, and in addition, NK cells lacking inhibitory receptors for HLA-C and HLA-E molecules would still be able to kill HIV-1-infected T cells (5).

The prevailing model for the effects of the protective −35 C allele is that it is associated with higher cell surface levels of HLA-C protein than those with the nonprotective T allele (17, 18, 30, 48, 52). Although DT9, the best available anti-HLA-C antibody, was used to provide support for this hypothesis, this antibody is known to cross-react with HLA-E (7). It was suggested that DT9 binding to lymphocytes may be entirely specific for HLA-C, based on the observation that the nominally HLA-E-specific antibody MEM-E/08 does not stain lymphocytes (52). However, we found that the HLA-E-specific antibody 3D12, which does not cross-react significantly with any HLA-A, -B, or -C allotype, binds to lymphocytes. Thus, HLA-E is expressed at low levels on the surfaces of lymphocytes, consistent with earlier data showing that lymphocytes express HLA-E (5, 14, 41). We found that there was no significant difference in 3D12 staining when subjects were grouped according to their −35 SNP genotype. Therefore, our results show not only that the differences in DT9 staining associated with these genotypes are due to differences in HLA-C surface expression but also that the difference between HLA-C expression levels in donors with −35 CC and TT alleles is greater than previously recognized. However, our results for DT9 staining also show an important difference from those reported previously (52). Like Thomas et al. (52), we observed consistently higher levels of DT9 staining of cells from −35 CC subjects than of those from −35 TT subjects, both for healthy

volunteers (P = 0.0459) and for individuals infected with HIV-1 (P = 0.0413); we also found considerable overlap in the ranges of DT9 staining levels between CC and TT subjects, reflecting a wide spread of HLA-C expression across the different alleles. However, in our data set, cells from TT subjects homozygous for *HLA-Cw*07* had the least DT9 staining, by far (Fig. 3). Indeed, the difference in staining levels between CC subjects and TT subjects homozygous for *HLA-Cw*07* was highly significant (P = 0.004 for healthy volunteers and P = 0.0043 for HIV-1-infected individuals). The difference in staining between TT subjects homozygous for *HLA-Cw*07* and all other TT subjects was highly significant for healthy volunteers (P = 0.0007) but less significant for HIV-1-infected individuals (P = 0.0196). Importantly, there was no statistically significant difference in staining between CC subjects and TT subjects if *HLA-Cw*07* homozygotes were excluded, both for the healthy volunteers and for the HIV-1-infected subjects. Note that a similar pattern of particularly low DT9 antibody binding to HLA-Cw7 was seen in the data on the staining of HIV-1-uninfected subjects presented by Thomas et al. (52).

Even when the contribution of the constant background of surface HLA-E expression to the level of DT9 staining for the healthy volunteer samples is taken into account, the picture does not change. Although the significance of the difference in expression levels increased between CC and TT subjects (P = 0.0061) (Fig. 5E), this difference was still driven entirely by the very low staining of cells from the *HLA-Cw*07* homozygotes (P = 0.0032 for CC versus TT *Cw*07/Cw*07* subjects and P = 0.0896 for CC versus TT non-*Cw*07/Cw*07* subjects). Overall, we estimate that levels of surface HLA-C expression were approximately 1.76-fold greater for PBMC from CC subjects than for cells from TT subjects, but this difference was almost all accounted for by the approximately 5-fold-decreased expression of HLA-C in −35 TT donors who were also homozygous for *HLA-Cw*07* compared with that in −35 CC subjects. Therefore, we concluded that the −35 SNP is actually most strongly associated with intrinsically low expression of HLA-Cw7 rather than with high expression of several −35 C-associated HLA-C allotypes. We cannot exclude, however, the possibility that other HLA-C alleles may also have different intrinsic levels of expression that could contribute to the overall levels of surface staining.

Despite the evidence that HLA-C-restricted CD8 T cells contribute little to the control of HIV-1 at the population level, it is still possible that significantly reduced expression of HLA-Cw7 could have significant effects on an individual level. Low expression of HLA-Cw7 could result in less effective presentation of peptides to CD8 T cells and in less control of viral replication. Low expression could also mean positive selection of fewer HLA-Cw7-restricted T cells in the thymus. In addition, by analogy with the suggestion that much of the benefit of protective HLA-B alleles arises from the presentation of particularly effective peptides (27), it is possible that HLA-Cw7 might bind to less-favorable peptides in HIV-1. Consistent with these possibilities, Fellay et al. showed that of all the HLA-C alleles, *HLA-Cw*07* is significantly associated with the highest mean viral load set point (17). However, the exclusion of subjects homozygous for *HLA-Cw*07* does not abolish the association of the −35 SNP with control of HIV-1 infection. This suggests that lower expression of HLA-Cw7 (a particularly

common allele in people of European descent [26.0 to 66.2%] [39]) contributes only a part of the protective effect marked by the −35 SNP. The International HIV Controllers Study recently implicated seven HLA class I alleles with control of HIV-1 and showed that *HLA-Cw*07* is a risk allele associated with high viremia and more rapid disease progression (48).

Indeed, it is becoming increasingly clear that genomewide association studies of human diseases can be hampered by the complexity of linkage disequilibrium patterns in the MHC region. These studies capture the majority of common variants in the genome that explain only a small proportion of heritability, while low-frequency polymorphisms, each conferring an intermediate increase in risk, can explain a significant proportion of the genetic susceptibility to common diseases (19, 20). Recently, Dickson et al. argued that rare variants can create synthetic association signals in genomewide association studies by occurring more often in association with one of the alleles of an SNP, which would therefore synthetically confer an increased risk for disease (16). We suggest, therefore, that the −35 SNP effect is an example of just such a synthetic association, arising as a compound effect of linkage disequilibrium. Specifically, *HLA-C* alleles associated with rapid progression tend to be in linkage disequilibrium with the −35 *T* allele, while protective alleles such as *HLA-B*57* and *-B*27* are in linkage disequilibrium with the protective −35 *C* allele. Studies on immune responses in HIV-1 have shown that HLA-B allotypes most frequently induce detectable T-cell responses and that these are generally of greater magnitude than responses restricted by either HLA-A or HLA-C. Therefore, HLA-B-restricted CD8 T-cell responses play a greater role in the immune response against HIV-1 infection (4, 25, 26). It is very well established that pairs of *HLA-B* and *HLA-C* alleles are in linkage disequilibrium and that particular haplotypes can be either protective (*B*14-Cw*0802* in Caucasians and *B*3910-Cw*1203* and *B*8101-Cw*0401* in Africans) or harmful (*B*35-Cw*0401* in Caucasians) in the context of HIV-1 disease progression (8, 17, 30, 48). *HLA-Cw*07* is in strong linkage disequilibrium with *HLA-B*07* and *-B*08*, and the latter is known to have an unfavorable impact on HIV-1 disease progression (9, 24, 35, 42, 48, 54). Although particular HLA alleles, particularly those of the *HLA-B* locus, can have a strong individual impact, overall HIV-1 control is likely to be influenced by the combination of all HLA alleles present (30).

In conclusion, it is extremely unlikely that the association of the −35 SNP with control of HIV-1 infection is a direct result of the SNP itself, and the role of increased expression of HLA-C allotypes associated with the −35 *C* allele (18, 52) is uncertain. Our data imply that it is the particularly low expression of the −35 *T*-associated HLA-*Cw*07* allele that contributes substantially to the low HLA-C expression associated with the −35 *T* allele, and possibly to the relatively high risk of disease progression.

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