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Polymorphism in Human Cytomegalovirus UL40 Impacts on Recognition of Human Leukocyte Antigen-E (HLA-E) by Natural Killer Cells*

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Background: Human cytomegalovirus (CMV) can manipulate natural killer (NK) cell function.
Results: Polymorphisms in *UL40* modulate the interaction between HLA-E and activating and inhibitory CD94-NKG2 receptors.
Conclusion: Variation in *UL40* may provide a further mechanism for CMV to control NK cell function.
Significance: CMV persistence may be enhanced by modifying NK cell function.

Natural killer (NK) cell recognition of the nonclassical human leukocyte antigen (HLA) molecule HLA-E is dependent on the presentation of a nonamer peptide derived from the leader sequence of other HLA molecules to CD94-NKG2 receptors. However, human cytomegalovirus can manipulate this central innate interaction through the provision of a "mimic" of the HLA-encoded peptide derived from the immunomodulatory glycoprotein UL40. Here, we analyzed *UL40* sequences isolated from 32 hematopoietic stem cell transplantation recipients experiencing cytomegalovirus reactivation. The UL40 protein showed a "polymorphic hot spot" within the region that encodes the HLA leader sequence mimic. Although all sequences that were identical to those encoded within HLA-I genes permitted the interaction between HLA-E and CD94-NKG2 receptors, other *UL40* polymorphisms reduced the affinity of the interaction between HLA-E and CD94-NKG2 receptors. Furthermore, functional studies using NK cell clones expressing either the inhibitory receptor CD94-NKG2A or the activating receptor CD94-NKG2C identified UL40-encoded peptides that were capable of inhibiting target cell lysis via interaction with CD94-NKG2A, yet had little capacity to activate NK cells through CD94-NKG2C. The data suggest that *UL40* polymorphisms may aid evasion of NK cell immunosurveillance by modulating the affinity of the interaction with CD94-NKG2 receptors.

Human natural killer (NK)[4] cells express a complex array of receptors, many of which are specific for major histocompatibility class I (MHC-I) proteins (1). One such group of receptors is the CD94-NKG2 family that consists of an invariant CD94 subunit covalently associated with either inhibitory (NKG2A or -B) or activating NKG2 (NKG2C, -E, and -H) molecules (2). Both inhibitory and activating CD94-NKG2 receptors recognize the MHC-I class Ib molecule human leukocyte antigen E (HLA-E) (3, 4). Unlike class Ia MHC-I molecules, HLA-E is essentially monomorphic and has an unusual peptide binding groove that is ideally suited to bind a conserved set of peptides derived from the leader sequences of other HLA-I proteins (5, 6).

The interaction of the inhibitory CD94-NKG2A receptor with HLA-E is considered to act as a checkpoint in NK cell immune surveillance. As HLA-E binds leader peptides derived from other MHC-I proteins, any viral process that impairs MHC-I synthesis could potentially lead to reduced surface expression of HLA-E. Moreover, as the loading of these peptides is dependent on the transporter associated with antigen processing (TAP), the interaction of HLA-E and CD94-NKG2A

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[4] The abbreviations used are: NK, natural killer; CMV, cytomegalovirus; HSCT, hematopoietic stem cell transplantation; SPR, surface plasmon resonance; RU, response unit; TAP, transporter associated with antigen processing.

allows NK cells to indirectly monitor both antigen processing and the expression of MHC-I (5, 7). In contrast, the biological role of the activating CD94-NKG2 receptors remains unclear, although they may have a role in antiviral immunity. Coculture of peripheral blood lymphocytes with human cytomegalovirus (CMV)-infected fibroblasts results in the expansion of CD94-NKG2C$^+$ NK cells (8). Moreover, individuals who are seropositive for CMV have increased numbers of NKG2C$^+$ cells, suggesting a degree of interplay between CMV and the expression of the CD94-NKG2 receptors.

Human CMV belongs to the β-herpesviridae family of herpes viruses and establishes a lifelong persistent infection (9). To escape recognition by CD8$^+$ T cells, CMV encodes several proteins such as US2, US3, US6, and US11 that interfere with antigen presentation and the expression of MHC-I molecules (10). Although the expression of such proteins may augment NK cell recognition of infected cells as a result of the loss of ligands for inhibitory receptors, a number of other CMV encoded proteins that may inhibit NK cell responses. For example, UL18 can act as a ligand for LILRB1, whereas other proteins such as UL16 can impair the expression of ligands for activating NK receptors (11). Human CMV also appears to specifically manipulate the interaction between HLA-E and CD94-NKG2 receptors via expression of the UL40 glycoprotein. Residues 15–23 of UL40 in the laboratory strain AD169 are identical to residues 3–11 of most HLA-C allotypes (12, 13) and, when expressed in the context of UL40, can bind HLA-E in a TAP-independent manner. Importantly, fibroblasts infected with *UL40*-deleted viruses show enhanced killing compared with wild-type viruses, indicating UL40 is active in productive viral infections (14). Thus, UL40 can provide a peptide that can bind HLA-E even when other CMV-encoded immunoevasins are active, promoting the inhibitory interaction between HLA-E and CD94-NKG2A.

Biochemical and functional studies have shown that the sequence of the peptide bound to HLA-E impacts on the interaction with CD94-NKG2 receptors (15–18). Structural and biochemical data demonstrated that the side chains of P5 and P8 directly contacted CD94-NKG2A, and changes to these residues can directly affect binding to the CD94-NKG2 receptors (17, 19). Given the central role of peptide in the interaction between HLA-E and CD94-NKG2 receptors, variation in residues 15–23 of UL40 may modulate recognition of HLA-E by CD94-NKG2 receptors and hence the capacity of NK cells to recognize CMV-infected cells (15, 18, 20). Earlier studies identified some variation in *UL40* sequences in the region encoding the HLA-E-binding peptide in laboratory strains of CMV (12, 21). Studies on clinical isolates of CMV isolated from kidney and lung transplant recipients also identified a number of distinct *UL40* sequences, but the capacity of these variant peptides to bind HLA-E or to impact on recognition of HLA-E by CD94-NKG2 receptors was not assessed (22, 23). In addition, Magri *et al.* (24) identified a UL40 isolate containing a substitution at the P2 anchor residue of the HLA-E-binding epitope, resulting in markedly reduced HLA-E surface expression. However, it is not clear whether this mutation may be the result of passage *in vitro*. Another recent study (25) identified a clinical strain of CMV in which the HLA-E epitope from UL40 is missing P1, also leading to reduced HLA-E surface expression. Therefore, it appears that variation in *UL40* can impact on HLA-E surface expression; however, its impact on recognition by CD94-NKG2A and CD94-NKG2C remains largely unknown. Here, we examined the extent of polymorphism in the *UL40* gene in a clinical cohort of hematopoietic stem cell transplant (HSCT) recipients, and we assessed the capacity of such polymorphisms to impact HLA-E binding, the affinity of the interaction between HLA-E and CD94-NKG2A and -NKG2C, and activation of NK cell clones expressing either CD94-NKG2A or -NKG2C. The data showed that the predominant *UL40* species encoded a peptide identical to that present in most HLA-C allotypes (VMAPRTLIL) that bind HLA-E and interact with both CD94-NKG2A and -NKG2C with high affinity. However, other isolates encoded peptides that bind HLA-E but failed to interact with either activating or inhibitory CD94-NKG2 receptors or alternatively bound with a lower affinity but that preferentially signaled through CD94-NKG2A. Our observations highlight the complex association between CMV and the immune system and suggest a novel mechanism by which the virus can modulate the affinity of the interaction between CD94-NKG2 receptors and HLA-E to favor its escape from immunosurveillance.

EXPERIMENTAL PROCEDURES

Patient Demographics—A total of 152 HSCT patients that had undergone stem cell transplantation for a range of hematological malignancies were recruited from the Royal Adelaide Hospital in Adelaide, the Royal Melbourne and The Alfred Hospitals in Melbourne, and Westmead Hospital in Sydney over the period 2002–2007. Of these patients, 44 were identified as having CMV viremia as assessed by PCR and had plasma samples available (Table 1). Ethics approval was gained from all participating institutions, and written informed consent to the study was obtained from all donors and recipients.

CMV UL40 Amplification, Cloning, and Sequencing—DNA was extracted from plasma samples corresponding closest to the date of reactivation using QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Primers to amplify the *UL40* gene were designed using the sequences for human herpesvirus 5 strain AD169 (GenBank™ accession number NC_001347) and wild-type strain Merlin (NC_006273), which were 5′-GGCTCTGTCTCGTCGTCATT and 3′-CGACACCGATCGATTTTCTT (Geneworks, Australia). PCR was performed with a DNA concentration typically in the range of 2–5 ng/μl using 4 units of AmpliTaq Gold polymerase Low DNA (Applied Biosystems, CA) (5 units/μl) with the following cycling parameters: one cycle of 94 °C for 12 min followed by 40 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 90 s and a final extension of 72 °C for 7 min. Multiple independent PCRs were performed for each sample, and the PCR products were cloned into pGEM®-T Easy Vector System (Promega).

A consensus sequence from each patient was derived using ChromasPro version 1.5 (Technelysium Pty. Ltd., Queensland, Australia) in most cases using multiple sequences from independent PCR products. These sequences were deposited in GenBank™ with accession numbers JQ060965 to JQ060996. They were aligned against the human herpes virus 5 AD169

strain using the ClustalW function within BioEdit software version 7.0.5 (Tom Hall, Ibis Therapeutics, Carlsbad, CA). A phylogenetic tree was constructed using the DNA-Dist function of the program to produce an unrooted neighbor joining tree using a distance matrix, with a Kimura two-parameter algorithm. This was then viewed in TreeView version 1.6.6 (26).

Production of Soluble, Recombinant Proteins—Production of recombinant HLA-E and CD94-NKG2 was performed essentially as described previously (17). The proteins were produced in *Escherichia coli* strain BL21 (DE3) pLysS, purified from inclusion bodies, and refolded by dilution. CD94 was expressed from vectors that contained a sequence allowing for an in-frame fusion with a peptide that facilitated enzymatic biotinylation. CD94-NKG2 complexes were concentrated using a stirred cell (Amicon, Beverly Hills, MA), subjected to size exclusion chromatography, and then further purified using a Mono Q column (GE Healthcare). HLA-E/peptide was refolded with human β2m and chemically synthesized peptides corresponding to the UL40 variants (Genscript). HLA-E/peptide complexes were purified by anion exchange and size exclusion chromatography. All samples were again re-purified by size exclusion within 24 h prior to analysis by surface plasmon resonance (SPR).

Surface Plasmon Resonance Analysis—SPR experiments were performed using a ProteOn XPR36 instrument (Bio-Rad) at 25 °C in a buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl (HBS), and 0.05% Tween (HBS-T). Streptavidin was diluted into 10 mM sodium acetate (pH 4.0), and 500 RU was immobilized to three flow cells of a GLC sensorchip by amine coupling. N-terminally biotinylated CD94-NKG2A and CD94-NKG2C (~300 RU) were coupled to two flow cells of a ProteOn GLC streptavidin sensorchip. A separate flow cell containing immobilized streptavidin without recombinant CD94-NKG2 protein served as the control channel. Recombinant HLA-E was again subjected to size exclusion chromatography immediately before injection over the test and control surfaces at a flow rate of 30 μl/min. Results from at least two independent experiments were analyzed. After subtraction of data from the control cells, the interactions were analyzed with ProteOn Manager software (version 2.1) and Prism (GraphPad), and K_D values were derived from the equilibrium fit option of the software packages.

Isolation and Culture of NK Cells—NK cells from healthy donors were isolated using the RosetteSep method (StemCell Technologies, Vancouver, British Columbia, Canada). Only those populations displaying >95% of $CD56^+$ $CD3^-$ NK cells were selected. Purified NK cells were then cultured on irradiated feeder cells in the presence of 2 μg/ml phytohemagglutinin (Invitrogen) and 100 units/ml recombinant interleukin-2 (proleukin; Chiron, Emeryville, CA) to obtain, after limiting dilution, clonal NK cells. Proliferating NK cell clones used for further analysis were selected on the basis of the expression of CD94-NKG2A and CD94-NKG2C receptors, as determined by flow cytometric analysis with the mAbs Z270 (27) and anti-NKG2C mAb (IgG2b, 1381, R&D Systems).

Peptide Loading and Cytolytic Activity Assays—The murine TAP2-deficient T cell lymphoma, RMA-S, cell line cotransfected with human β2 microglobulin and the HLA-E*01033 allele (kindly provided by J. E. Coligan) (28) was cultured in complete Roswell Park Memorial Institute medium. RMA-S-HLA-E transfectants were cultured overnight at 37 °C in the absence or presence of peptide after which the cells were stained with the anti-HLA class I mAb A6.136 or isotype control and analyzed by flow cytometry to determine cell surface levels of HLA-E.

NK cell clones derived from different donors were tested for cytolytic activity via ^{51}Cr-release assays as described (29) in the presence or absence of anti-CD94 mAb Y9 (IgM, 10 μg/ml). Target cells used in these experiments were RMAS-HLA-E (described above) and the human erythroleukemic cell line K562 transfected with HLA-E*0103 (HLA-E-K562, kindly provided by E. H. Weiss). Both cell lines were incubated overnight at 37 °C, either alone or in the presence of the synthetic peptides (200 μM). Peptides were kept throughout the assay to ensure higher levels of HLA-E expression.

RESULTS

Variation in CMV UL40 Sequences Isolated from HSCT Recipients—Previous studies had identified variations in CMV *UL40* sequences isolated from renal and lung transplant recipients but had not assessed the impact of these polymorphisms on peptide binding to HLA-E or NK cell activation (22, 23). Because hematopoietic cells are both critical for the control of CMV and are thought to be a major reservoir of latent virus, we assessed the extent of *UL40* polymorphism in the context of hematological malignancy and the significance of such polymorphisms for NK cell function. From a cohort of 152 patients who received HSC transplants for a variety of hematological malignancies, 99 patients were CMV-seropositive prior to transplantation. Of these, 46 patients had evidence of CMV infection or reactivation as defined by the presence of viral DNA in plasma samples. With the exception of one patient who was CMV-seronegative but who received a transplant from a seropositive donor, all patients were CMV-seropositive. Plasma was obtained from 44 of these patients, and cDNA encoding *UL40* was amplified by PCR from the plasma of 32 recipients, then cloned, and sequenced. Of these 32 patients, 16 had acute myeloid leukemia, and five had non-Hodgkin lymphoma, although there were no more than three patients with other malignancies, including chronic myeloid leukemia, acute or chronic lymphoid leukemia, and multiple myeloma. There was significant variability in pre-transplant conditioning with some patients receiving a myeloablative regimen involving total body irradiation, whereas others had reduced intensity conditioning in the absence of irradiation (Table 1). CMV prophylaxis was varied, either being delivered routinely to patients throughout the first 100 days post-transplant or alternatively only upon detection of viral DNA in the plasma as revealed in weekly testing. During the course of monitoring, eight patients had a second or third episode of CMV viremia. In all cases, sequencing of *UL40* cDNA amplified from later time points in these patients revealed identical sequences to those found in the initial reactivation.

At the amino acid level, 28 different sequences were identified. Strikingly, the greatest variability in these sequences was found in the region that encoded potential HLA-E-binding peptides (residues 15–23 in the laboratory strain AD169) (Fig. 1, highlighted in *black*). Notably residue 22 that corresponds to P8, a

residue critical for the interaction with CD94-NKG2A, was the most variable, having a Kabat-Wu value of 8, which, although not high compared with values for germ line-encoded complementarity determining regions (CDR) regions of the T cell receptor or Ig genes, is nevertheless comparable with that associated with polymorphisms observed in residues lining the peptide binding pockets of MHC molecules (30, 31). There was also a dimorphism at the end of the signal sequence (position 38) where the consensus sequence encoded a Cys but was a Ser in 15 isolates, both residues being permissive for recognition by signal peptidase (32).

TABLE 1

Patient demographics

The abbreviations used are as follows: TBI, total body irradiation; MMF, mycophenolate mofetil.

	Pre-transplant conditioning	
	Myeloablative ($n = 19$)	**Reduced intensity ($n = 13$)**
Mean age at transplant (range)	41 (21–55 years)	55 (43–64 years)
Recipient sex	14 female, 5 male	4 female, 9 male
Disease		
Acute lymphoid leukemia	2	
Acute myeloid leukemia	9	7
Chronic lymphoid leukemia		1
Chronic myeloid leukemia	2	1
Multiple myeloma	1	1
Non-Hodgkin lymphoma	3	2
Severe aplastic anemia	1	1
Myelofibrosis	1	
Conditioning regimen		
Busulfan plus cyclophosphamide	8	
Cyclophosphamide plus TBI	9	
Etoposide plus TBI	1	
Fludarabine plus cyclophosphamide		4
Fludarabine plus melphalan		8
Melphalan		1
V16 plus TBI	1	
Immunosuppression		
Cyclosporine A/tacromilus plus methotrexate	17	8
Cyclosporin A plus MMF		2
Cyclosporin A	2	2
Unknown		1

Phylogenetic analyses of the *UL40* sequences suggested that the cohort was split into two main clades of which the 02-R010 isolate included a single branch that resembled the *UL40* sequence of the AD169 (Fig. 2*A*). Interestingly, with the other major branch was a cluster of sequences possessing an alternative initiation codon, nine nucleotides upstream of that present in AD169. Thus, the translated protein of these isolates would encode an additional three amino acids (Fig. 2*B*).

The amino acid sequences corresponding to the potential HLA-E-binding peptide, together with that present in AD169, are shown in Table 2. A total of nine potential HLA-E-binding peptides were identified from 34 sequences, with 15 isolates having an identical sequence as that found in AD169. In addition, there were three cDNAs that encoded peptide sequences that differed from those previously identified in recent studies of the polymorphism in UL40 in renal transplant recipients (22), VMAPQSLLL, VMAPRSLIL and VMAPRTLFV. (Letters underlined within the UL40 peptide sequence indicate differences from the most common peptide sequence VMAPRTLIL.) Hence, there was a degree of sequence variation in clinical isolates of CMV, particularly in the region encoding the potential HLA-E-binding peptide.

Ability of UL40-encoded Peptide Mimics to Bind to HLA-E—As the expression of *UL40* had been shown to modulate HLA-E expression through the provision of an HLA-E-binding peptide (12), we then assessed the capacity of peptides corresponding to the putative HLA-E binding sequences isolated from HSCT recipients together with those identified previously (22) to bind to HLA-E. TAP-deficient RMA-S cells transfected with HLA-E were cultured in the presence or absence of peptide at 37 °C overnight, and the expression of HLA-E was analyzed by flow cytometry.

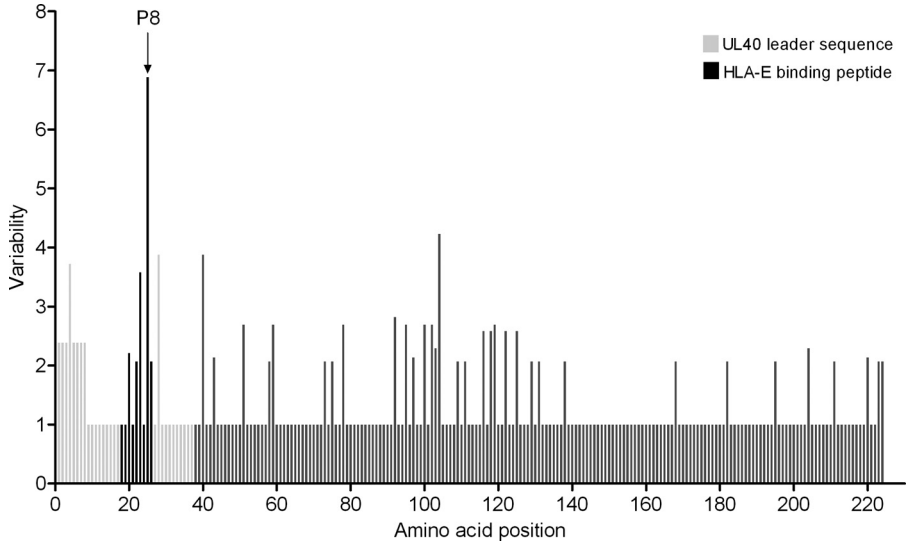
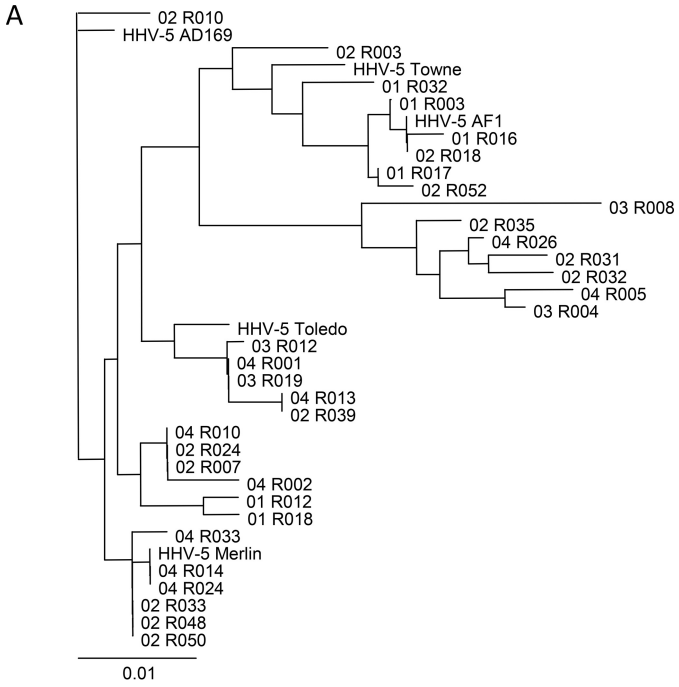


FIGURE 1. **Variability across the CMV UL40 protein sequence isolated from HSCT recipients.** A Wu-Kabat plot for variability was constructed to determine variability throughout the UL40 amino acid sequence. The plot did not make any assumptions in regard to differences in the start or stop codons of the ORF, and unresolved amino acids were counted as differences. The region encoding the predicted leader sequence is shown in *light gray*, with the potential HLA-E-binding peptide shown in *black*. P8 of the potential HLA-E-binding peptide is indicated by the *arrow*.



B

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                1         10         20         30
           ...|.....|.....|.....|.....|..
HHV-5 AD169 ~~~MNKFSNTRIGFTCAVMAPRTLILTVGLLCMRI
02R031      MAAAGIRFNTRIGFTCAVMTPRTLVLTLGLLCMRI
02R032      MAAAGIRFNTRIGFTCAVMTPRTLVLTLGLLCMRI
02R035      MAAAGIRFNTRIGFTCAVMAPRTLILTVGLLCMRI
03R004      MAAAGIRFNTRIGFTCAVMAPRTLILTLGLLCMRI
03R008      MAAAGIRFNTRIGFTCAVMAPRTLFVTVGLLCMRI
04R005      MAAAGIRFNTRIGFTCAVMAPRTLILTLGLLCMRI
04R026      MAAAGIRFNTRIGFTCAVMAPRTLVLTLGLLCMRI
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FIGURE 2. **Variation in UL40 protein sequences isolated from plasma of HSCT recipients.** *A,* phylogenetic representation of CMV UL40 protein sequences isolated from HSCT recipients. The genetic distance of the CMV UL40 sequences obtained from HSCT patients compared with laboratory CMV strains AD169, Towne, AF1, Merlin, and Toledo are shown using a phylogenetic tree. An unrooted neighbor joining tree using a distance matrix was used to show genetic distance that allows for varying rates of evolution. Each hospital is coded by the *first two numbers*. The scale is 0.1 nucleotide substitutions per site. *B,* eight UL40 protein sequences isolated from HSCT recipients possessed an alternative translation start site as compared with HHV AD169. Residues are numbered according to AD169 sequence.

TABLE 2

UL40-encoded HLA-E peptide mimic obtained from HSCT recipients

CMV strain	P1	P2	P3	P4	P5	P6	P7	P8	P9	No. of isolates
AD169	Val	Met	Ala	Pro	Arg	Thr	Leu	Ile	Leu	15
Toledo	–[a]	–	–	–	–	–	–	Val	–	2
Towne	–	–	–	–	–	–	–	Leu	–	5
109B	–	–	Thr	–	–	–	–	Val	–	4
	–	–	–	–	–	Ile	–	–	–	1
	–	–	–	–	–	Ser	–	–	–	1
AF1	–	–	–	–	–	Ser	–	Leu	–	2
	–	–	–	–	Gln	Ser	–	Leu	–	1
	–	–	–	–	–	–	–	Phe	Val	1

[a] – indicates amino acid identical to AD169.

In the absence of peptide, there was little evidence of HLA-E surface expression (Fig. 3). Similar results were obtained when the cells were pulsed with an irrelevant peptide, MAGE-1 (EADPTGHSY, data not shown). Incubation of RMA-S/HLA-E cells with the VMAPRTLIL sequence identical to that present in most HLA-C alleles (*e.g.* HLA-Cw*0304 and -*0401) resulted

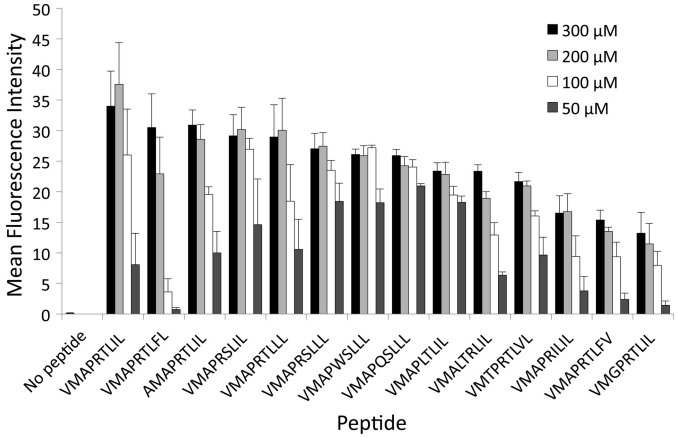

FIGURE 3. **Variant CMV UL40 peptides stabilize HLA-E surface expression.** The RMA-S cell line, cotransfected with human β2 microglobulin and the HLA-E*01033 allele, was cultured in the presence or absence of the indicated peptides at 37 °C overnight prior to staining with the mAb A6.136 (anti-HLA class I) and analyzed by flow cytometry. Plots show the difference in mean fluorescence intensity between A6.136 and isotype control and represent the mean ± S.E. of at least two experiments.

in the highest levels of HLA-E expression. In contrast, culture with the peptide VMGPRTLIL led to a modest increase in HLA-E surface expression relative to cells cultured without exogenous peptide. Coculture with the VMTPRTLVL peptide, which like VMGPRTLIL also contains a P3 substitution, also resulted in lower surface levels of HLA-E expression compared with VMAPRTLIL, suggesting that the substitution of Ala to either Gly or Thr impacted the affinity of the peptide for HLA-E. The VMAPRTLFV peptide also stabilized cell surface expression of HLA-E, although not to the extent of the VMAPRTLIL peptide. Structural studies have shown that the side chain at P8 is exposed and that Phe at P8, as seen in the leader sequence peptide derived from HLA-G (VMAPRTLFL), is well tolerated (4, 5, 33). Consequently, the key change in this sequence with respect to peptide binding to HLA-E is likely to be the Leu to Val substitution at P9, particularly as P9 has been reported to function as a dominant anchor residue (6). A number of sequences possessed a Ser at P6 that differs from both the canonical UL40 sequence and the amino acids found at residue 8 of HLA-I leader sequences. Again, each of these peptides had the capacity to stabilize cell surface expression of HLA-E. However, the substitution of Ile at P6 was not able to stabilize HLA-E surface expression to the same extent as P6 Ser. Together, the data demonstrated that all of the UL40-derived peptides were able to stabilize surface expression of HLA-E, albeit to varying degrees.

Impact of UL40 Variation on Recognition of HLA-E by CD94-NKG2 Receptors—HLA-E bound to the VMAPRTLIL peptide present in the AD169 UL40 sequence and most HLA-C alleles have been shown to interact with CD94-NKG2A (17, 34). However, the impact of *UL40* polymorphisms on recognition of HLA-E by both inhibitory and activating CD94-NKG2 receptors is unknown. Consequently, we next determined whether the sequence variability in this region of UL40 would impact the affinity of the interaction between HLA-E and the inhibitory CD94-NKG2A receptor or its activating counterpart CD94-NKG2C via SPR.

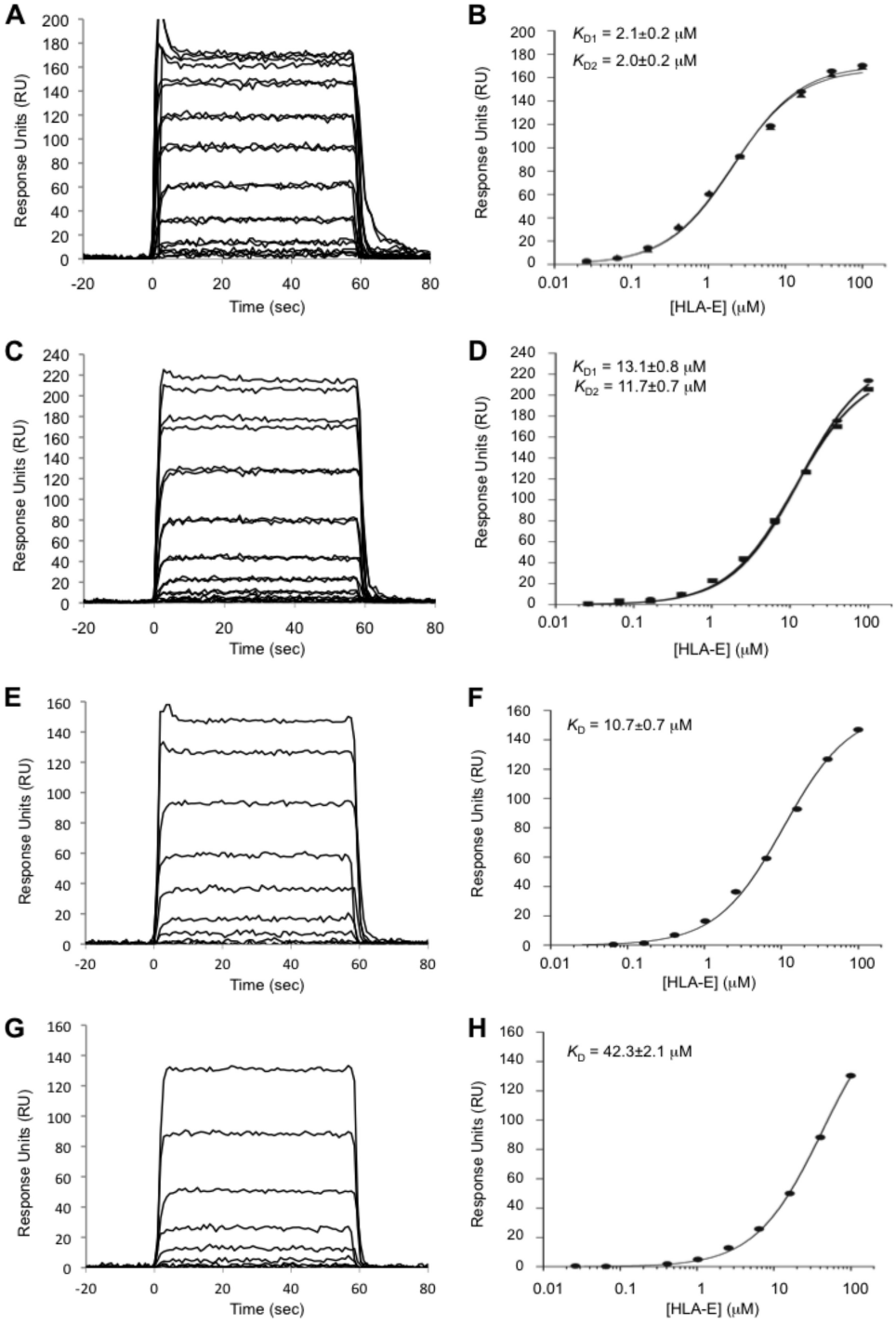

FIGURE 4. **Surface plasmon resonance analysis of the interaction between HLA-E and CD94-NKG2A or CD94-NKG2C.** CD94-NKG2A (*A, B, E,* and *F*) and CD94/NKG2C (*C, D, G,* and *H*) were biotinylated and captured on the surface of streptavidin coupled to a Proteon Bio-Rad GLC sensor chip (~300 RU), and HLA-E·VMAPRTLIL (*A–D*) or HLA-E·VMTPRTLVL (*E–H*) complexes were passed over the surface. Sensorgrams of duplicate runs (*A* and *C*) or representative experiments (*E* and *G*) show binding (RU) of increasing concentrations (0.3–100 μM) of HLA-E following subtraction from a control flow cell. Saturation plots (*B, D, F,* and *H*) show the response at equilibrium *versus* concentration of HLA-E, which was used to determine K_D values.

Injection of soluble HLA-E over immobilized CD94-NKG2A or -NKG2C indicated that the association and dissociation rates observed were extremely fast, with equilibrium reached almost immediately after the start of the injection, consistent with previous studies (15, 17, 18). As such, all subsequent analyses were based on data obtained from the equilibrium phase of the interaction. These analyses revealed that HLA-E/VMAPRTLIL bound to CD94-NKG2A with an affinity of ~2 μM (Fig. 4, *A* and *B*), similar to previously reported values (17), whereas the affinity for CD94-NKG2C was ~6-fold lower (≈12 μM) (Fig. 4, *C* and *D*).

A number of HLA-E/UL40 complexes bound to CD94-NKG2A and CD94-NKG2C with affinities similar to that observed for HLA-E complexed with the canonical sequence

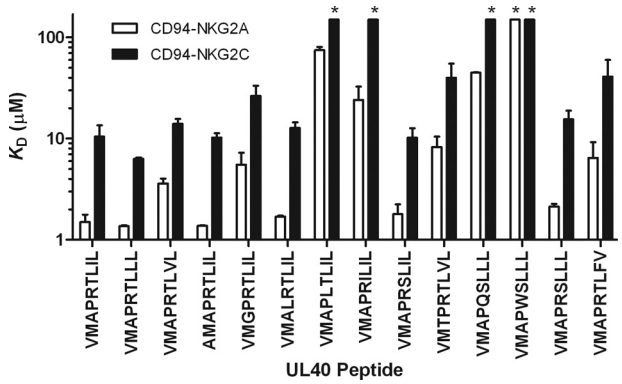

FIGURE 5. **Binding affinity of HLA-E/UL40 peptide complexes for CD94-NKG2A and CD94-NKG2C as determined by surface plasmon resonance.** CD94-NKG2A (*white bars*) and CD94-NKG2C (*black bars*) were biotinylated and captured on the surface of streptavidin coupled to a Proteon Bio-Rad GLC sensor chip, and HLA-E·UL40 peptide complexes were passed over the surface. Binding affinities (K_D, mean ± S.E., shown on a log scale) were determined by equilibrium analysis. * indicates K_D >150 μM.

VMAPRTLIL. These included HLA-E bound to VMAPRTLLL, VMAPRTLVL, AMAPRTLIL, VMALRTLIL, VMAPRSLIL, and VMAPRSLLL, all of which interacted with CD94-NKG2A and CD94-NKG2C with affinities of 1.4–3.6 and 6.3–15.5 μM, respectively (Fig. 5).

HLA-E complexed with UL40 peptide variants that contained amino acids other than Arg at P5 such as VMAPLTLIL, VMAPQSLLL, and VMAPWSLLL interacted weakly with both CD94-NKG2A and CD94-NKG2C consistent with structural studies showing the importance of P5-Arg (19). Interestingly, although the presence of Ser rather than Thr at P6 had little impact on the affinity of HLA-E for CD94-NKG2A or -NKG2C, the presence of an Ile at this position (VMAPRILIL) reduced the affinity of HLA-E for both CD94-NKG2A and -NKG2C to 24 and >150 μM, respectively. There were also three peptides, VMGPRTLIL, VMTPRTLVL, and VMAPRTLFV, which when refolded with HLA-E clearly interacted with both CD94-NKG2A and -NKG2C but with lower affinities than the consensus HLA-E/VMAPRTLIL peptide, resulting in a 2–5-fold increase in K_D values (Figs. 4, *E–H*, and 5). In conclusion, we found that variation in UL40 sequences significantly impacted the ability of HLA-E to bind to CD94-NKG2A and CD94-NKG2C, with changes in peptide residues P3, P5, P6, and P9 in particular, decreasing the affinity for both receptors.

UL40 Sequence Variation Impacts NK Cell Cytolytic Activity—Data obtained from SPR experiments demonstrated that sequence variation in UL40-derived peptides bound to HLA-E impacted the affinity of its interaction with CD94-NKG2A and CD94-NKG2C. However, it was unclear whether these differences in affinity translated into different functional outcomes. Therefore, we tested the ability of UL40 peptides to modulate target cell recognition by NK cell clones expressing either CD94-NKG2A or CD94-NKG2C.

As expected, in the absence of exogenous peptides, NK cell clones efficiently lysed K562 cells transfected with HLA-E (HLA-E-K562, Fig. 6). Incubation of target cells with the peptide VMAPRTLFL derived from the HLA-G leader sequence, which in complex with HLA-E has a high affinity for CD94-NKG2A, inhibited lysis by NK cell clones that expressed NKG2A. Consistent with this effect being dependent on recognition by CD94-NKG2A, the addition of a blocking mAb specific for CD94 reversed the inhibition. Coculture of K562-HLA-E cells with the peptide VMAPRTLLL, which corresponds to sequences present in certain HLA-A and -C leader sequences and which we also found in 16% of our UL40 isolates, was capable of protecting target cells from lysis by NK cell clones such as 25.3 and 50.3, which expressed high levels of NKG2A. In contrast, coculture of targets with VMAPRTLLL induced only modest levels of protection from NK cell clones expressing low levels of NKG2A such as 2.1.2 (Fig. 6).

Similarly, stabilization of HLA-E with the UL40-derived peptides VMAPRILIL, VMGPRTLIL, and VMTPRTLLL did not confer protection from 2.1.2 consistent with both the low expression of NKG2A on this clone and the low affinity of the interaction between CD94-NKG2A and HLA-E when bound to these peptides. However, coculture with either the VMGPRTLIL or VMTPRTLVL peptides reduced lysis by NK cell clones that expressed high levels of NKG2A. Similarly, the peptide VMAPRILIL was also capable of inhibiting target cell lysis via a CD94-dependent mechanism, although the degree of inhibition varied between NK cell clones. In contrast, the peptides VMAPQSLLL and VMAPWSLLL that lacked Arg at P5 had no capacity to confer protection consistent with both the SPR data (Figs. 5 and 6*C*) and structural studies showing that the Arg makes critical contacts with NKG2A (19).

The capacity of UL40 peptides bound to HLA-E to be recognized by NK cells expressing the activating receptor CD94-NKG2C was also assessed. In the absence of exogenous peptide only low levels of lysis of RMA-S-HLA-E cells were observed following culture with NK cell clones that lacked NKG2A but expressed NKG2C (Fig. 7). Coculture of these cells with peptides corresponding to either HLA-I leader sequences or UL40 sequences had little capacity to modulate target cell lysis by NK cell clones such as 54.10 that expressed low levels of NKG2C. However, stabilization of HLA-E by culture with the VMAPRTLFL peptide sensitized them for lysis by clones that expressed high levels of NKG2C. Similarly, coculture of RMAS-HLA-E target cells with the peptides VMAPRTLLL and to a lesser extent VMGPRTLIL also enhanced lysis via a CD94-dependent mechanism by NKG2C^{hi} clones. In contrast, culture of RMA-S-HLA-E cells with the peptides VMAPRILIL and VMTPRTLVL did not substantially augment target cell lysis (Fig. 7). As expected, culture with the peptides VMAPQSLLL and VMAPWSLLL had no impact on target cell recognition by NKG2C$^+$ NK cell clones (data not shown). Together, the functional data show that *UL40* polymorphisms that impacted the affinity of the interaction between CD94-NKG2 receptors and HLA-E also regulated the extent of signaling through either CD94-NKG2A or -NKG2C. Furthermore some of the UL40-encoded variants may have a "tuned" affinity that can sustain functional interactions with CD94-NKG2A but not CD94-NKG2C.

DISCUSSION

Phylogenetic analyses of *UL40* sequences obtained from HSCT recipients undergoing CMV reactivation identified two distinct lineages. One of these lineages resembled the UL40 sequence from the AD169 strain of CMV, although it

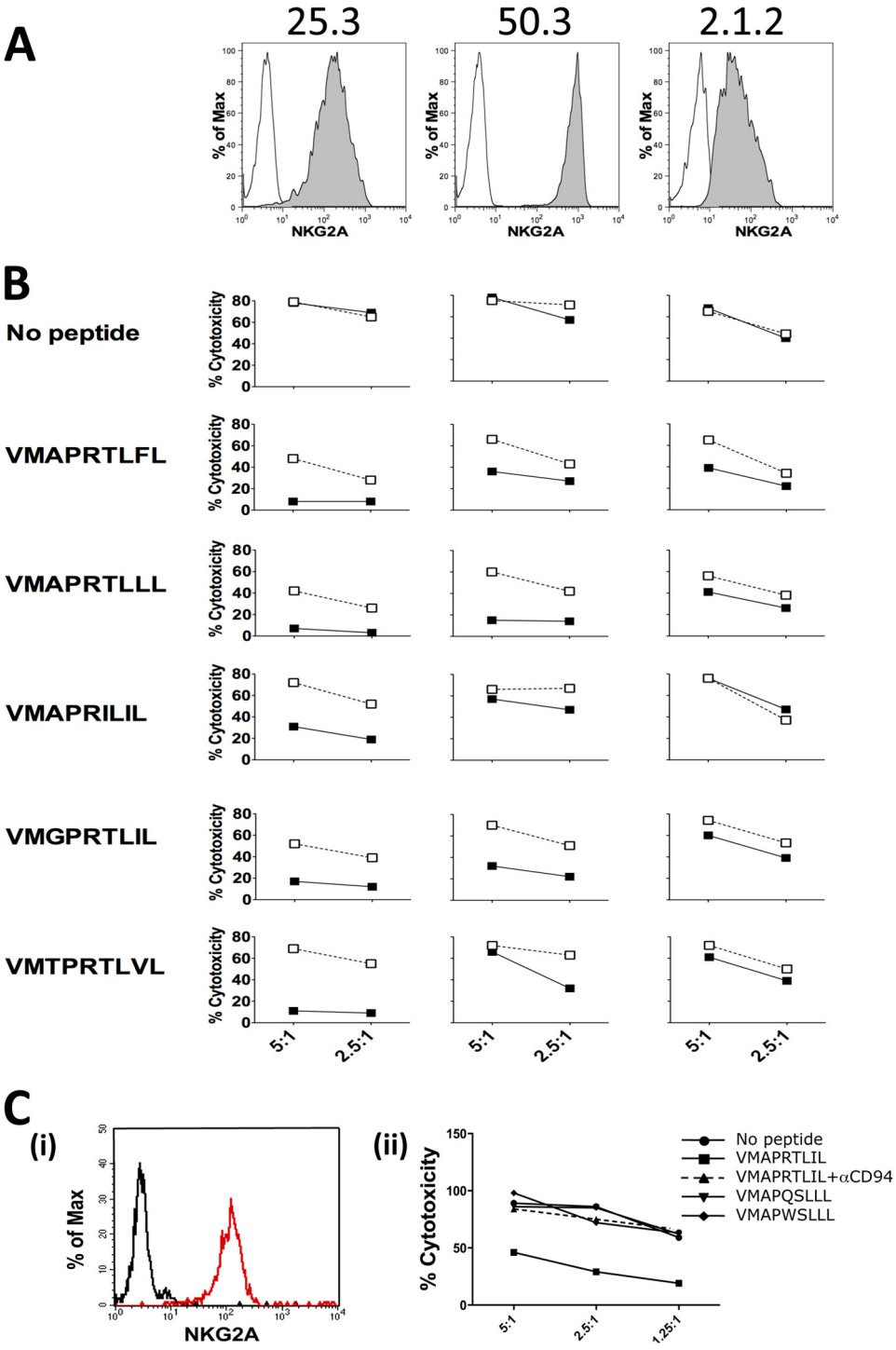

FIGURE 6. **Ability of HLA-E/UL40 peptide complexes to inhibit NK cell-mediated lysis.** *A,* flow cytometric histograms show NKG2A expression (*filled histograms*) compared with isotype control (*open histograms*) on NK cell clones. *B,* NK cell clones (25.3, 50.3, and 2.1.2) were tested for cytolytic activity against K562/HLA-E in a 4-h ^{51}Cr-release assay at the indicated effector to target cell ratios in the absence (*solid lines, filled squares*) or presence (*dotted lines, open squares*) of an anti-CD94 mAb Y9 (IgM, 10 μg/ml)). The cell lines were incubated overnight at 37 °C, either alone (no peptide) or in the presence of the indicated synthetic peptides (200 μM). *C,* peptides that had substitutions at P5 failed to protect target cells from lysis by NKG2A expressing NK cells. *Panel i,* histogram showing NKG2A expression (*red line*) compared with isotype control (*black*); *panel ii*, cytolytic activity of an NKG2A$^+$ NK cell clone (20.3) against K562/HLA-E loaded with the indicated peptides (200 μM).

was composed of only a single sequence. The remaining sequences could be divided into a number of groups, each consisting of five or more members, the most distinctive of which possessed an alternative initiation codon resulting in an additional three N-terminal amino acids. Surprisingly, polymorphism in *UL40* was largely clustered around the region encoding the leader sequence that had been proposed to mimic residues 3–11 of HLA class I molecules, which suggest that it may impact the interaction of HLA-E with CD94-NKG2A and CD94-NKG2C.

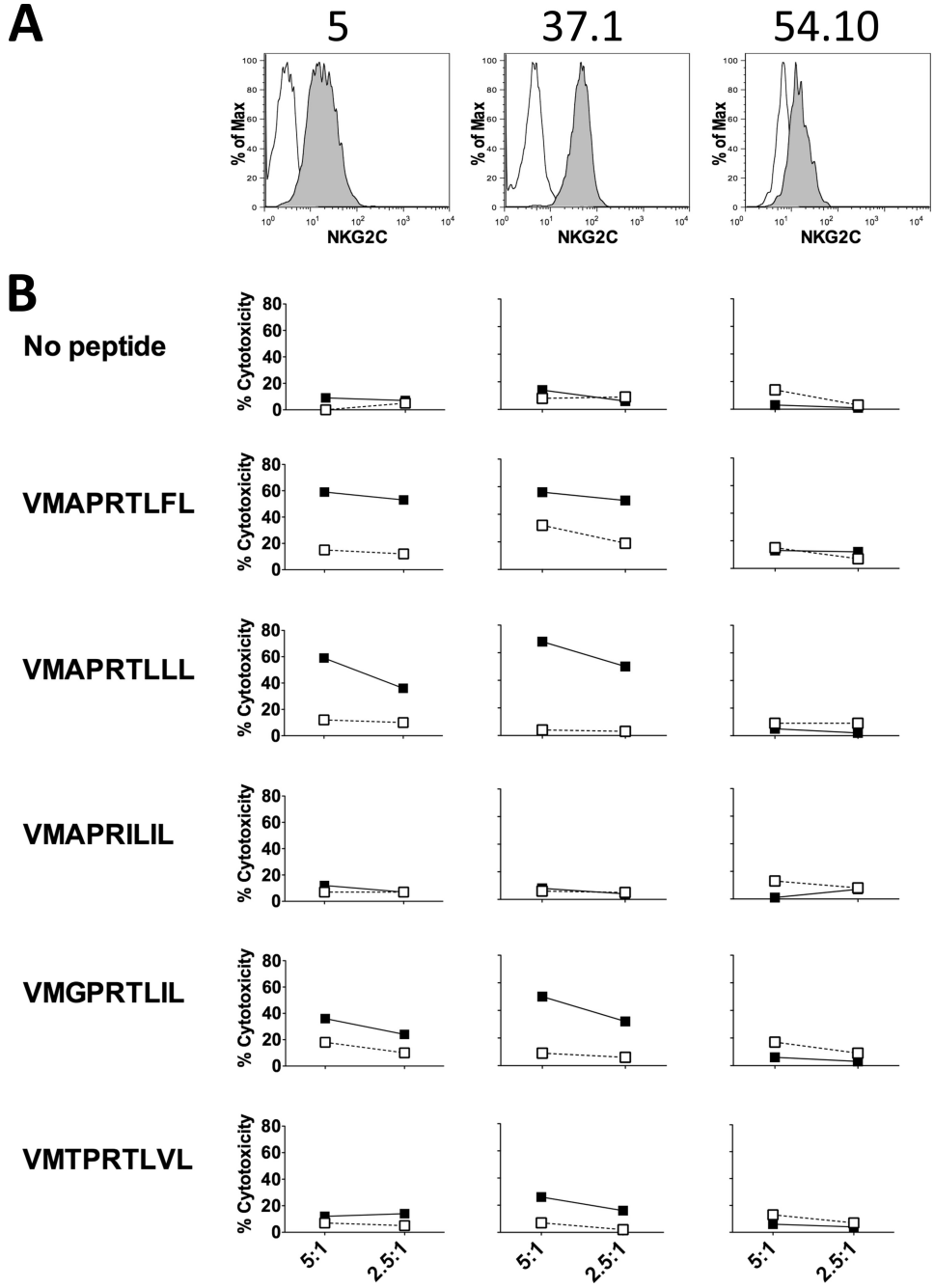

FIGURE 7. **Ability of HLA-E/UL40 peptide complexes to induce NK cell-mediated lysis.** *A,* flow cytometric histograms show NKG2C expression (*filled histograms*) compared with isotype control (*open histograms*) on NK cell clones (clone 5, 37.1, and 54.10). *B,* NK cell clones were tested for cytolytic activity in a 4-h ^{51}Cr-release assay against RMA-S/HLA-E at the indicated E:T ratios in the absence (*solid lines, filled squares*) or presence (*dotted lines, open squares*) of an anti-CD94 mAb Y9 (IgM, 10 μg/ml). The cell lines were incubated overnight at 37 °C, either alone (no peptide) or in the presence of the indicated synthetic peptides (200 μM).

Analyses of our cohort revealed that 15 patients had CMV isolates that encoded the VMAPRTLIL sequence identical to that in the AD169 strain and also with that found in most HLA-C alleles. The remaining 17 HSCT recipients had sequences with a variety of subtle differences, typically differing by only one or two amino acids in this region, all of which had the ability to bind to HLA-E. However, the differences in the underlying malignancies and transplant conditioning within our cohort preclude any firm conclusions connecting individual *UL40* sequences with clinical outcome.

As found with natural polymorphisms in residues 3–11 of MHC class I genes or targeted substitutions in these peptides, the ability of the CD94-NKG2 receptors to recognize HLA-E in complex with individual UL40 peptides was dependent on the sequence of the peptide bound to HLA-E. Recognition of HLA-E by both CD94-NKG2A and CD94-NKG2C was largely tolerant of UL40 polymorphisms that introduced conservative substitutions at P1, P4, P6 (Ser), and P8. In contrast, substitutions at P3 and P5 as well as the introduction of an Ile at P6 had a more marked impact on recognition of HLA-E.

Although P5 is typically an Arg in both the UL40 sequence and at residue 7 of HLA class I molecules (*i.e.* P5 of the HLA-E binding sequence), substitutions at this position to Leu, Trp, and Gln were observed in this cohort and other clinical isolates (22). P5-Arg plays a pivotal role in CD94-NKG2 recognition of HLA-E via two distinct mechanisms. First, it contributes to the overall stability of the complex forming a salt bridge to Glu-152 of the HLA-E heavy chain (6). Consistent with this finding, we observed a modest reduction in the capacity of the peptides VMAPLTLIL, VMAPQSLLL, and VMAPWSLLL relative to VMAPRTLIL to stabilize HLA-E expression. More strikingly however, these substitutions essentially abrogated the interaction between HLA-E and both CD94-NKG2A and -NKG2C, consistent with previous data showing that P5-Arg was critical for binding CD94-NKG2A and structural data indicating that the side chain of P5-Arg protrudes into a cleft between the CD94 and NKG2A subunits (19).

Earlier studies have suggested that a variety of amino acids at P8 can facilitate an interaction between HLA-E and both CD94-NKG2A and -2C (17, 18). In the AD169 UL40 sequence, residue 22 (P8) is Ile. However, in the UL40 sequences isolated from both our cohort and other CMV isolates (21–23), additional hydrophobic residues such as Val and Leu were also observed at this position resulting in HLA-E-binding peptides identical to that present in a number of additional HLA-I-allotypes (*e.g.* VMAPRTLVL, HLA-A2). In the absence of any other substitutions, the substitution of Ile to either Val or Leu had no significant impact on binding of HLA-E by CD94-NKG2A or -2C. Previous studies had shown that the peptide VMAPRTLFL derived from the leader sequence of HLA-G supported higher affinity interactions between HLA-E and CD94-NKG2A and -NKG2C as a result of the Phe at P8 (19). Although no *UL40* isolates encoded this sequence, a similar peptide containing a Phe at P8 but with Val at P9 (VMAPRTLFV) was identified. Interestingly, when complexed with this variant, HLA-E bound both CD94-NKG2A and -2C with lower affinity than HLA-E/VMAPRTLIL suggesting that substitution at P9 impacted the conformation of HLA-E or the peptide, effectively abrogating the enhanced CD94-NKG2 receptor binding associated with the presence of the Phe at P8.

The functional data were broadly consistent with the data obtained from surface plasmon resonance experiments. Peptides such as VMAPRTLIL, which in complex with HLA-E binds with high affinity to CD94-NKG2A, inhibited target cell lysis by NK cell clones expressing this receptor. Similarly, peptides that had substitutions at P5, consistent with structural models, abrogated binding to CD94-NKG2A and failed to protect target cells from lysis.

Perhaps most interestingly, a number of *UL40* isolates encoded peptides such as VMTPRTLVL and VMAPRILIL that, in functional studies, had little capacity to induce NK cell activation through CD94-NKG2C but retained the ability to inhibit target cell lysis by CD94-NKG2A$^+$ NK cells. The magnitude of these peptide-induced effects varied between NK cell clones but largely corresponded with the level of expression of either CD94-NKG2A or -NKG2C. Our observations extend the work by Llano *et al.* (35), who showed that in the HLA-I-derived peptides only the VMAPRTLFL peptide was capable of augmenting lysis via CD94-NKG2C, whereas the requirements for signaling via CD94-NKG2A were not as stringent. Our binding data showed that although the affinity of the interaction between HLA-E and CD94-NKG2 receptors was acutely dependent on peptide sequence, there was a consistent 5–6-fold difference in the K_D value for the interaction between the UL40/HLA-E complexes and CD94-NKG2A and -NKG2C irrespective of the peptide sequence. These data are inconsistent with the hypothesis that specific peptides facilitate preferential binding to CD94-NKG2A over CD94-NKG2C. Rather, the data show that the overall affinity of the interaction between HLA-E and CD94-NKG2 receptors can be modified by peptide in such a way as to favor inhibition over activation, likely stemming from differing thresholds for signaling via CD94-NKG2A and CD94-NKG2C. Thus, this preferential triggering of an inhibitory signal is suggestive of the capacity of a CMV to further refine evasion of NK cell-mediated surveillance via the CD94-NKG2 receptor family.

Surprisingly, all of the *UL40*-encoded peptides were capable of interacting with HLA-E to some extent. However, not all isolates encoded peptides that facilitate the interaction with CD94-NKG2 receptors, notably those with substitutions at P5. In this respect, these isolates are similar to the 3157 strain of CMV recently reported by Prod'Homme *et al.* (25), which has a truncated UL40 leader sequence. This truncation maintained signal peptide function but abrogated the capacity to up-regulate HLA-E resulting in a virus with a defect in NK cell evasion function. Taken together, the data suggest that the manipulation of NK cell responses via HLA-E expression may not be crucial for viral replication *in vivo*. However, much larger patient cohorts will be required to determine whether these *UL40* polymorphisms impact the establishment of chronic infection, the level of viral replication, transmission of CMV, or ultimately patient outcome.

All of the *UL40* sequences identified in this study, like those in the work of Garrigue *et al.* (22, 23) and Faure-Della Corte *et al.* (36), have been obtained from individuals with diminished cellular immunity. In immunocompetent individuals, UL40-specific T cell responses targeting the canonical VMAPRTLIL and VMAPRTLVL epitopes have been observed. These responses appear limited to individuals who lack HLA-I alleles that encode these sequences, presumably reflecting tolerance to the HLA-I-encoded peptides. However, the capacity to generate robust HLA-E restricted T cell responses may provide strong selection against the evolution of novel *UL40* sequences that differ from those encoded within the leader sequences of HLA genes. Thus, the range of *UL40* sequences observed in this cohort, as well as those observed in other situations where CD8 T cell responses are compromised, may be substantially greater than that observed within healthy donor populations.

Finally, because the variation in HLA-E-binding peptides described herein stems from polymorphisms in a putative immunoevasin, the presence of sequences such as VMTPRTLVL and VMAPRILIL in UL40 may reflect a novel viral adaptation to NK cell-mediated immune surveillance. This suggests that CMV has the capacity to fine tune the magnitude of signals transmitted through both activating and inhibitory receptors to facilitate viral persistence and/or transmission.

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