Interactions between NKG2x Immunoreceptors and HLA-E Ligands Display Overlapping Affinities and Thermodynamics[1]

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The NKG2x/CD94 family of C-type lectin-like immunoreceptors (x = A, B, C, E, and H) mediates surveillance of MHC class Ia cell surface expression, often dysregulated during infection or tumorigenesis, by recognizing the MHC class Ib protein HLA-E that specifically presents peptides derived from class Ia leader sequences. In this study, we determine the affinities and interaction thermodynamics between three NKG2x/CD94 receptors (NKG2A, NKG2C, and NKG2E) and complexes of HLA-E with four representative peptides. Inhibitory NKG2A/CD94 and activating NKG2E/CD94 receptors bind HLA-E with indistinguishable affinities, but with significantly higher affinities than the activating NKG2C/CD94 receptor. Despite minor sequence differences, the peptide presented by HLA-E significantly influenced the affinities; HLA-E allelic differences had no effect. These results reveal important constraints on the integration of opposing activating and inhibitory signals driving NK cell effector functions. ***The Journal of Immunology,* 2005, 174: 2878–2884.**

Natural killer cells counter the immune-evasion strategies of viruses and tumors by eliminating cells with reduced expression of classical MHC class I proteins or the induced expression of MHC class I-like self-Ags, both common responses to pathological changes within cells (1). NK cell surface receptor (NKR)[3] families that mediate NK effector functions include the C-type lectin-like NKG2x receptors (x = A, B, C, E, and H). These closely related receptors, 81–94% identical in their C-type lectin-like ectodomains (CTLDs; Fig. 1*a*), form disulfide-linked heterodimers with CD94, another CTLD molecule, and bind the nonclassical MHC class I protein HLA-E (2), an interaction that has been coarsely modeled structurally (Fig. 1*b*) (3, 4). HLA-E is structurally homologous to classical MHC class I proteins (5, 6), also requiring association with peptide for cell surface expression. In contrast to MHC class I, however, HLA-E only binds a restricted set of nonamer peptides that are primarily derived from the leader sequences of HLA-A, -B, -C and -G molecules (6–9), though peptides derived from other cellular (10) and viral (11, 12) sources have been identified. Two nonsynonymous alleles of HLA-E are retained in the human population, maintained through strong balancing selection, distinguished by a single sequence dimorphism at position 107: glycine (E*0101; HLA-E^{G}) or arginine (E*0103; HLA-E^{R}) (13). Biophysical studies have shown that both allelic and peptide sequence differences have significant effects on HLA-E solution stability, correlating with differential cell surface expression levels (5). However, the effect of this dimorphism on receptor interactions has not previously been directly studied.

NKG2A/B and NKG2E/H are near-identical splice variants that would be predicted to behave similarly with regard to ligand interactions. NKG2A/B/CD94 is an inhibitory receptor, signaling through two ITIM sequences ($^{V}/_{I}$xYxx$^{L}/_{V}$) in the intracellular domain. The activating receptor NKG2C/CD94 associates with the ITAM-containing adapter protein DAP12. Previous studies with NKG2A and NKG2C have shown that the limited sequence variation allowed for the bound peptide is sufficient to influence the strength of the HLA-E/NKG2x/CD94 interaction by ~30-fold (9, 14, 15). Although not yet formally demonstrated, NKG2E/H/CD94 is almost certainly another activating receptor, based on the presence of a lysine residue in its transmembrane domain and the lack of an ITIM motif in its intracellular domain. We draw distinctions between the NKG2x/CD94 receptors and two outlier members associated with the family: NKG2F and NKG2D. NKG2F is missing large, otherwise-conserved sections of the CTLD, raising the question of whether it can functionally fold, consistent with its observed intracellular retention (16). The more distantly related NKG2D immunoreceptor is homodimeric and recognizes the induced expression of various MHC class I-like self Ags, such as the MHC class I-related chains A and B and the CMV UL16-binding proteins, interactions that have been extensively studied biophysically (17–19).

Because NK cells bear complex mixtures of activating and inhibitory NKRs, NK cell activation is the product of integrating diverse opposing activating and inhibitory signals received during target cell engagement—though it is not understood how these opposing signals are integrated or what parameters govern the relative contributions of the different signals in the overall equation. Studies of $\alpha\beta$ TCRs have shown that, of the possible microscopic biophysical parameters describing receptor/ligand interactions (affinity, kinetics, thermodynamics) that might affect delivered receptor signal strength, an empirical equation coupling TCR/peptide MHC (pMHC) complexation heat capacity (ΔC°_{p}) and $t_{1/2}$ best predicts output activation potential (20). Previous studies of NKG2A and NKG2C (14, 21, 22) show that the inhibitory receptor

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[3] Abbreviations used in this paper: NKR, NK cell surface receptor; CTLD, C-type lectin-like ectodomain; pMHC, peptide MHC; R_{max}, maximum analyte responses; RU, resonance units; SEC, size exclusion chromatography; SPR, surface plasmon resonance; T_m, solution thermal stability.

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FIGURE 1. Sequence comparisons of CTLD NKR ectodomains and the modeled NKG2x/CD94/HLA-E complex. *a*, A CLUSTALW (34) alignment of the ectodomains of NKG2A through NKG2H, NKG2D, and CD94 is shown, with sequence substitutions between NKG2x isoforms colored green or purple, the latter for the region distinguishing the NKG2E/H splice variants. Residues are numbered, with positions 197 and 225 highlighted. Blue hexagons show positions of potential N-linked oligosaccharide sites below the NKG2x sequences. Symbols above the NKG2D and CD94 sequences (for which three-dimensional structures are available) indicate paired cysteines forming disulfide linkages. Secondary structure (β-strands as gray arrows, α-helices as slashed bars, with forward slashes indicating 4_{13} and back slashes indicating 3_{10} helices), homodimerization interface residues (=) and ligand contacting residues (◇) are indicated below the NKG2D and CD94 sequences. *b*, The structure of the modeled NKG2x/CD94/HLA-E complex (3, 4) is shown in combined ribbon (NKG2x/CD94) and space-filling (HLA-E+peptide) styles, colored by chain (NKG2x: gray, yellow, orange, red, and purple; CD94: green; HLA-E α_1 domain: light blue; HLA-E α_2 domain: blue; and the bound peptide: alternately black or white, residue-by-residue). NKG2x loops predicted to provide contacts to the ligand are colored yellow, orange, and red, matching the colored bars overlaying the corresponding sequence elements in *a*. The NKG2x region corresponding to the differential NKG2E/H splice variation is colored purple. The approximate positions of residues 197 and 225, and the side chain of the P5 residue in the peptide, are indicated with red arrows. The figure was generated with PyMOL. The PyMOL Molecular Graphics System (2002); ⟨www.pymol.org⟩).

(NKG2A) has a uniformly higher affinity for ligand, which, in turn, is proposed to correlate with the presumed dominance of inhibitory signals over activating signals (23).

To sort out the complex network of signals impinging on a NK cell during interrogation of potential targets, and to fully determine the effects different NKG2x/CD94 receptors, different HLA-E alleles, and different presented peptides may have on the process, we have performed a comprehensive analysis of 23 specific interactions across varying NKG2x isoforms, HLA-E alleles, and bound peptides. The results show that activating and inhibitory NKG2x/CD94 receptors and HLA-E alleles can have completely overlapping affinities and that subtle peptide differences significantly affect the interactions, likely mediated by CD94 and previously unrecognized elements of the NKG2x moiety and consistent with previous modeling of the complexes. Qualitatively similar dissociation rate constants and negligible complexation heat capacities show that K_D values are the most distinguishing feature of different NKG2x/CD94/HLA-E interactions. These results suggest that the integration process, and the recognition mechanism, used by NKG2x NKRs is likely much more complicated than previously appreciated.

Materials and Methods

Protein expression and purification

The ectodomains of NKG2A (residues 99–233), NKG2C (residues 95–231) and NKG2E (residues 109–226), all with N-terminal 6x histidine purification tags added, and untagged CD94 (residues 31–179), were expressed in bacteria and purified as inclusion bodies, solubilized in urea, and refolded by stepwise dialysis to remove the denaturant as previously described (4). These constructs encompass the CTLD and most of the N-terminal arm linking the CTLDs to the transmembrane domains. All the constructs included exogenous, N-terminal initiator methionine residues. Refolding mixtures consisted of 2:1 molar ratios of NKG2x:CD94. Properly refolded complexes were purified over Ni-NTA affinity chromatography (Qiagen) followed by size exclusion chromatography (SEC) to yield protein >99% pure. All purified receptors showed the appropriate disulfide bonding state by comparative reducing/nonreducing SDS-PAGE and were monomeric and monodisperse by analytical SEC. NKG2A/CD94 and NKG2C/CD94 refold with ~1% efficiencies; wild-type, intact NKG2E/CD94 refolds very poorly, but a 14-residue, C-terminal deletion, based on NKG2x sequence comparisons (Fig. 1*a*), improves NKG2E/CD94 refolding efficiency to ~0.1%. The truncated NKG2E heterodimer displays identical affinities to the full-length NKG2E receptor, based on preliminary surface plasmon resonance (SPR) measurements with HLA-E+HLA-G peptide ligands (data not shown). HLA-E was purified as previously described (5) with four different peptides: VMAPRTLFL, from HLA-G;

VTAPRTLLL, from HLA-B27; VMAPRTVLL, from HLA-B7; and VMAPRALLL, from HLA-Cw7. Mutations in NKG2A and NKG2C were made using the QuikChange procedure (Stratagene). All proteins used for SPR were repurified by SEC in HBS-EA buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.02% NaN_3; Biacore AB) within 48 h of use. Protein concentrations were determined by bicinchoninic acid protein assay (Pierce), with associated errors ≤2%.

SPR analysis

SPR measurements were conducted in HBS-EP buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.05% P-20); Biacore AB) using a Biacore 3000 system. NKG2x/CD94 receptors were covalently coupled to a CM5 research-grade chip using standard amine-coupling techniques. Reference flow cells contained either amine-coupled NKG2D or were left blank; results were identical in either case. Reverse measurements, using NKG2x/CD94 as the analyte and HLA-E as the ligand, failed as amine-coupling of HLA-E abrogated receptor binding (modeling studies suggest that three HLA-E lysine residues, 146, 170, and 174, are close enough to the interface that coupling through any of these residues could sterically block binding; only lysine 230 in NKG2A and NKG2C lies in the interface, but is possibly not accessible to coupling). Coupling biotinylated HLA-E, through an engineered C-terminal BirA biotinylation site (24), to streptavidin-coated sensor chips does not yield stable surfaces, also precluding accurate reverse affinity determinations.

Affinity analyses were typically conducted using ligand densities yielding maximum analyte responses (R_{max}) of ~500–2000 resonance units (RU), although similar K_Ds were obtained at densities with R_{max} values of 25 RU. HLA-E was injected over the surface at a flow rate of 20 μl/min. Raw sensorgrams were corrected using the double-subtraction protocol of Myszka and coworkers (25) by subtracting both the reference flow cell response and the average of eight buffer injections. Average equilibrium responses were measured for five to six concentrations of HLA-E bracketing the K_D, except in the case of NKG2C with HLA-E^R+Cw7 where concentrations of HLA-E were below the determined K_D (Fig. 2). Reported K_Ds are the average of at least three independent experiments and were fit with a simple 1:1 steady-state Langmuir binding model (Response = K_A*[HLA-E]*R_{max}/(K_A*[HLA-E]+1) using BIAevaluation 3.0 software (Biacore AB). Error estimates were propagated from the SE of the K_A. Interaction thermodynamic parameters were determined by measuring the equilibrium K_Ds at a series of temperatures ranging from 283 to 310 K and analyzing the data as previously described (18, 26).

Results

Affinities of NKG2x/CD94/HLA-E interactions

Previous studies have reported a total of 10 NKG2x/CD94/HLA-E affinities, restricted to analyses of two receptor isoforms (NKG2A and NKG2C) and one HLA-E allele (HLA-E^R). To fully explore the interaction space of NKG2x/CD94 immunoreceptors, we have determined a total of 23 NKG2x/CD94/HLA-E affinities by SPR, varying three parameters: NKG2x identity (spanning the unique receptor specificities encoded by NKG2A, -C, and -E); both HLA-E alleles (HLA-E^R and -E^G); and four different peptides (Figs. 2 and 3 and Table I). The peptides studied were derived from the nonclassical MHC class I protein HLA-G and three classical MHC class I proteins, HLA-B7, -Cw7, and -B27. Six of these measurements replicate previous results and were conducted both to confirm the newer analyses and insure that completely self-consistent data were generated across the near-complete set of 23 interactions. Only one target interaction, NKG2C/CD94/HLA-E^G+Cw7, was so weak as to preclude accurate quantitation. It is presumed that the splice variants, NKG2B and NKG2H, would behave identically to NKG2A and NKG2E, respectively, as the only sequence differences between variants occur well outside of the ligand-binding CTLD. Protein for these experiments was refolded in vitro from bacterial inclusion bodies and purified using established methodology (4).

Examination of a tabulation of these affinity results show that K_D values range from 0.7 μM to >0.1 mM (Table I). These affinities span a significant portion of the range of affinities previously reported for NKRs. Peptide/HLA-E^R and HLA-E^G complexes bound with essentially identical K_Ds to all three NKG2x/

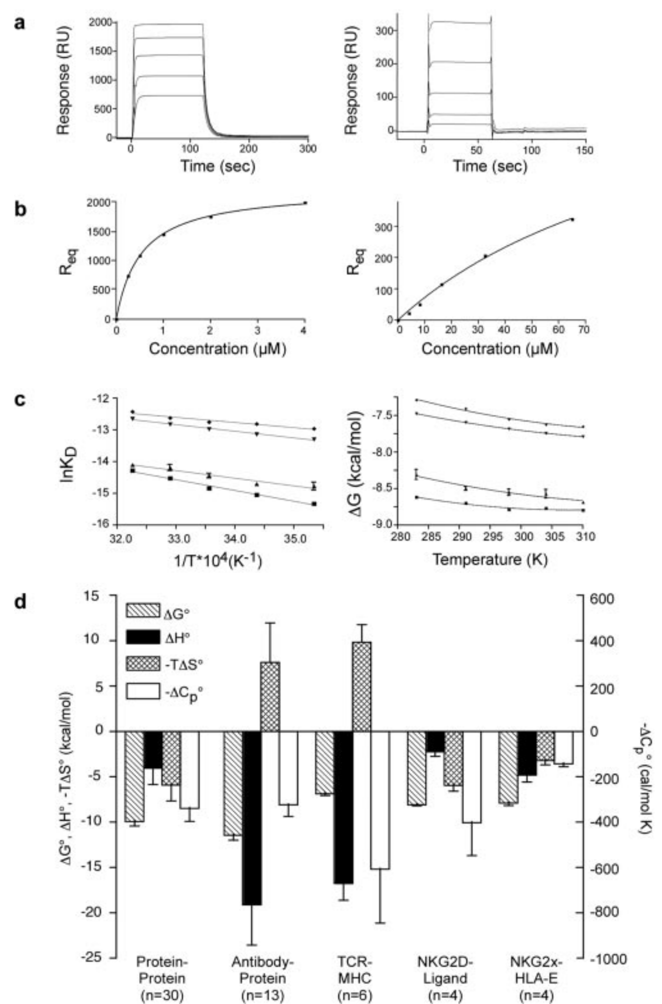

FIGURE 2. Determination of affinities and thermodynamic parameters for NKG2x/CD94/HLA-E interactions by SPR. *a*, Sensorgrams of the highest (NKG2A/CD94/HLA-E^G+G, *left panel*) and lowest (NKG2C/CD94/HLA-E^G+Cw7, *right panel*) affinity interactions. HLA-E complexes were injected at 20 μl/min over NKG2x-coupled dextran in serial dilutions ranging from 0.25 to 4.0 μM (*left*) or from 3.9 to 62.5 μM (*right*) at 298 K. The data were double-referenced by subtracting an inline NKG2D control surface and the average of eight buffer injections. *b*, Plots of SPR equilibrium responses (R_{eq}) as a function of the concentration of HLA-E (micromolar). K_Ds were determined by fitting the data with a 1:1 Langmuir binding model. *c*, van't Hoff (*left panel*) or three-parameter (*right panel*) fits to the same data (with K_D converted to ΔG°) for four representative interactions: NKG2A/CD94/HLA-E^G+G (■); NKG2E/CD94/HLA-E^G+G (▲); NKG2C/CD94/HLA-E^G+G (▼); and NKG2A/CD94/HLA-E^G+B7 (●). Affinities were determined over temperatures ranging from 283 to 310 K. *d*, Average ΔG°, ΔH°, −TΔS°, and $ΔC_p$° values for NKG2x/CD94/HLA-E from Table II are graphically compared with several classes of binding interactions. Error bars represent SEs of measurement.

CD94 receptors tested, highlighting the reproducibility of these measurements and strikingly demonstrating that this allelic dimorphism does not affect NKR affinity. The relative K_D values are completely consistent across these interactions when broken down by either receptor or peptide identity, demonstrating that the effects of alternate receptor or peptide use on the interactions are effectively independent. The inhibitory receptor NKG2A/CD94 and the stimulatory receptor NKG2E/CD94 display essentially identical affinities for peptide/HLA-E complexes, with K_Ds ranging from 0.7 μM to ~20 μM. These interactions are uniformly 6-fold stronger than NKG2C/CD94/HLA-E interactions, which have K_Ds

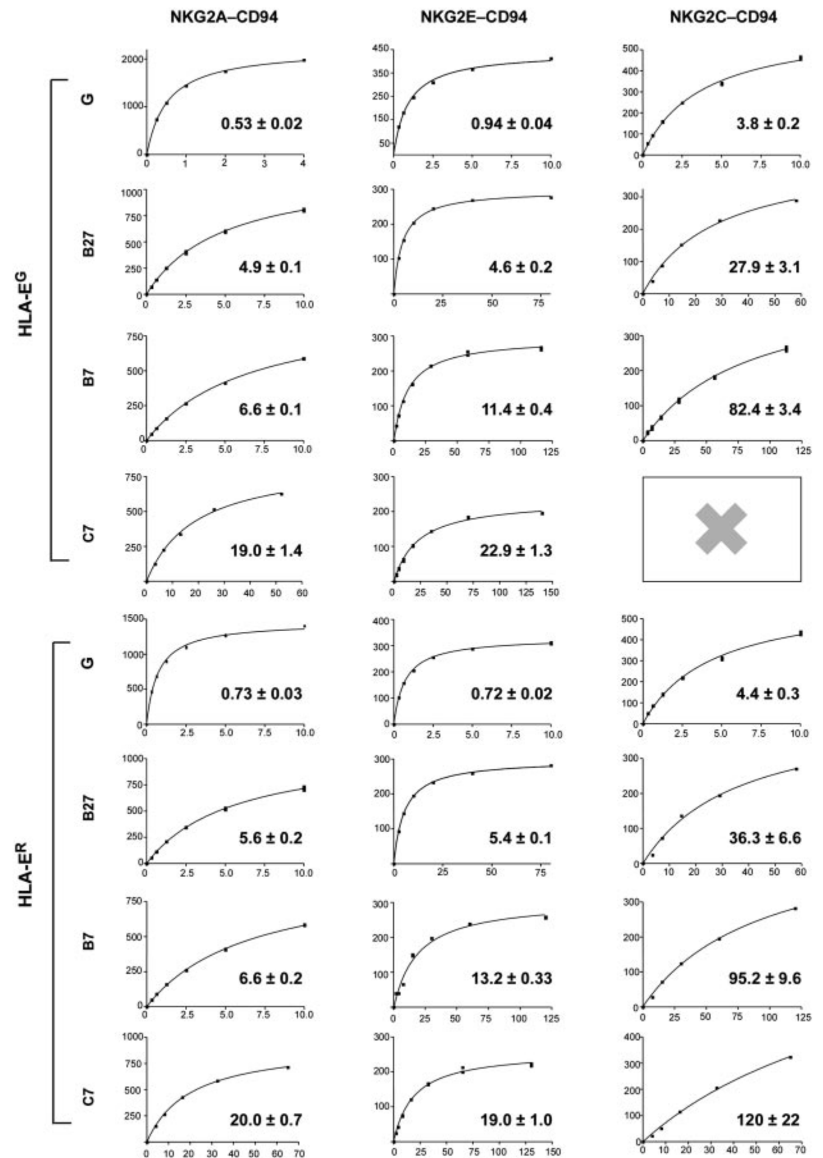

FIGURE 3. Determination of 23 NKG2x/CD94/HLA-E affinities. For each indicated interaction, HLA-E concentration (on the abscissa, in micromolar concentration) is plotted vs R_{eq} (on the ordinate, in RU) and fit using a 1:1 Langmuir binding model. The resulting K_D estimates (in micromolar concentration) and propagated error are also shown on each plot.

ranging from ~4 μM to >0.1 mM. Differential peptide presentation by HLA-E has the most dramatic effect on receptor/ligand affinity, with the HLA-G-derived peptide showing the strongest interactions, the B27-derived peptide uniformly showing ~8-fold weaker affinities, the B7-derived peptide uniformly showing ~17-fold weaker affinities than the HLA-G peptide and the Cw7-derived peptide showing the weakest affinities, uniformly ~29-fold weaker than the HLA-G peptide. The significance of the dominance of the HLA-G-derived peptide is not known, but the expression of HLA-G is limited to placental trophoblast cells, indicating a possible role in gestational immunity (27).

Peptide/HLA-E complexes bind to NKG2x/CD94 receptors with very fast association and dissociation kinetics (Fig. 2*a* and additional data not shown), consistent with what are relatively weak affinities when considered in absolute terms. We attempted to measure the associated kinetic parameters (k_{on} and k_{off}), but were unable to achieve completely satisfactory fits to the data, even when collected at lower temperatures. The advertised limits of the Biacore 3000 instrumentation used in these studies are $\leq 10^5$ $M^{-1}s^{-1}$ (k_{on}) and $\leq 10^{-1}s^{-1}$ (k_{off}). Vales-Gomez et al. (23) have previously reported k_{off} values ranging from of 0.4 s^{-1}, for the NKG2A-CD94/HLA-E^R+G peptide complex, to 1.8 s^{-1} for the NKG2C/CD94/HLA-E^R+G peptide complex. This range of k_{off} values corresponds to complex $t_{1/2}$ values of ~2-~0.4 s, values that are all considerably shorter than physiologically relevant αβ TCR/pMHC complex half lives, which typically range from a few seconds to almost a minute (20). Our analysis yields qualitatively consistent estimates. However, qualitatively, we do not observe what we believe are significant differences in the dissociation rates of any of the complexes, particularly in comparison with the TCR/pMHC range.

Thermodynamics of selected NKG2x/CD94/HLA-E interactions

NKG2D binds to a number of structurally distinct MHC class I homologues, a well-characterized example of highly degenerate recognition (17). An analysis of the interaction thermodynamics showed that NKG2D achieves this degeneracy through a recognition mechanism termed "rigid adaptation" (18), distinct from the "induced fit" mechanism typical of cross-reactive αβ TCRs. However, in contrast to NKG2D, NKG2x/CD94/HLA-E interactions are not demonstrably degenerate, reflected in the significant effects on affinity of even relatively minor peptide sequence substitutions (Table I). Therefore, NKG2x/CD94 NKRs would not, a priori, need to use anything other than conventional, rigid protein-rigid

Table I. *Steady-state K_D (μM) for NKG2x/CD94/HLA-E interactions*[a]

	HLA-G Leader VMAPRTLFL	HLA-B27 Leader VTAPRTLLL	HLA-B7 Leader VMAPRTVLL	HLA-Cw7 Leader VMAPRALLL	Normalized, Relative K_D by Receptor
NKG2A/CD94 (inhibitory)	HLA-E[R]: **0.7 ± 0.08**	HLA-E[R]: 5.6 ± 0.2	HLA-E[R]: **11.9 ± 0.6**	HLA-E[R]: **20.0 ± 0.7**	1
	HLA-E[G]: 0.7 ± 0.04	HLA-E[G]: 5.3 ± 0.4	HLA-E[G]: 12.3 ± 0.4	HLA-E[G]: 19.5 ± 1.4	1
NKG2E/CD94 (activating)	HLA-E[R]: 0.7 ± 0.1	HLA-E[R]: 6.2 ± 0.7	HLA-E[R]: 13.2 ± 0.3	HLA-E[R]: 20.3 ± 1.5	1
	HLA-E[G]: 0.7 ± 0.05	HLA-E[G]: 4.9 ± 0.3	HLA-E[G]: 11.1 ± 0.4	HLA-E[G]: 22.8 ± 1.3	1
NKG2C/CD94 (activating)	HLA-E[R]: **4.4 ± 0.3**	HLA-E[R]: 28 ± 3.1	HLA-E[R]: **94 ± 9.6**	HLA-E[R]: **120 ± 22**	~6
	HLA-E[G]: 4.3 ± 0.6	HLA-E[G]: 36 ± 6.6	HLA-E[G]: 82 ± 3.4	HLA-E[G]: ND	~6
Normalized, relative K_D by peptide	1	~8	~17	~29	

[a] Affinities that replicate previously reported estimates (14) are in bold. For comparison, dissociation constants have been normalized either by receptor, with the tightest association set to unity (*right column*), or by HLA-E/peptide complex, with the tightest association again set to unity (*bottom row*).

protein binding mechanisms. To confirm this supposition, we performed a thermodynamic analysis of four representative NKG2x/CD94/HLA-E[G] interactions, spanning three receptors (NKG2A, -C, and -E), two peptides, and covering a range of affinities (Table II). Thermodynamic parameters were estimated using either conventional linear van't Hoff or multiparameter fitting (Fig. 2*c*) (18, 26). Both fitting methods give essentially the same results (Table II), consistent with the near linearity of the data and the low $\Delta C°_p$ values calculated from the multiparameter fits (Fig. 2*c*). Thermodynamic parameters calculated by these methods are consistent with NKG2x/CD94 immunoreceptors interacting with HLA-E ligands as rigid species (Fig. 2*d*), though complexities unexplored in this simplistic analysis, and the absence of component and complex structures, can also affect this interpretation.

Mutagenesis of putative key NKG2x/CD94/HLA-E interface residues

Examination of a multiple sequence alignment of the ectodomains of NKG2A, -C, and -E reveals that at only two positions do NKG2A and -E contain the same residue where NKG2C does not: position 197 (Glu in NKG2A and -E and a Lys in NKG2C); and position 225 (Ile in NKG2A and -E and a Met in NKG2C). Indeed, residue 197 has been previously proposed to be a primary determinant of the 6-fold affinity differential between NKG2A/E and NKG2C for HLA-E (2, 14). However, this position is predicted to be too far away in the model of the complex to constitute part of the direct interface, whereas position 225 is located on a loop that could mediate contacts between the NKG2x/CD94 receptors and HLA-E (Fig. 1*b*). To test whether either of these positions are critical for the higher affinity of NKG2A/CD94 compared with NKG2C/CD94, we swapped the residues at these positions between NKG2A and C by mutagenesis and tested binding to HLA-E[G]+G peptide. We found that none of these mutations reversed the binding behavior of NKG2A and NKG2C, producing effects of 20% or less on affinity: the NKG2A(E197K)/CD94/HLA-E[G] K_D is 0.8 ± 0.1 μM and the NKG2C(K197E)/CD94/HLA-E[G] K_D is 3.4 ± 0.2 μM; the NKG2A(I225M)/CD94/HLA-E[G] K_D is 0.7 ± 0.02 μM and the NKG2C(M225I)/CD94/HLA-E[G] K_D is 3.5 ± 0.1 μM (compare with the wild-type K_Ds in Table I: NKG2A, 0.7 μM; NKG2C, 3.8 μM). The mutagenesis at position 197 mutation also confirms that the lower measured NKG2C affinities are not due to artifacts of amine-coupling ligands to sensor chips, which could result in cross-linking through the ε-amino group on lysine 197, which is adjacent to the putative interface, as the NKG2C(K197E) mutant affinity is unchanged relative to wild type.

Discussion

NKG2x NKR/ligand binding parameters and signal integration

The process NK cells use to integrate the opposing signals received through the diverse NKRs engaged upon interacting with a given cell, driving effector functions when abnormal MHC class Ia, Ib, and homologue combinations are encountered, yet restraining such activities when the host-specified complement is detected, remains enigmatic. A number of factors potentially contribute to the final integrated signal output, including use of different signaling pathways, differential interactions within convergent signaling pathways, differential expression levels of receptors or ligands, and the biophysical parameters of different receptor/ligand interactions. Previous studies of limited sets of NKG2x NKR receptor/ligand interactions have suggested that inhibitory NKRs may uniformly have tighter affinities than stimulatory NKRs, presuming that output signal strength correlates with affinity. Another aspect of this process is the equally enigmatic process by which the host-specific NK cell repertoire is acquired, and what combinations of stimulatory or inhibitory receptors are allowed in context of a particular MHC haplotype (28). A single-cell RT-PCR analysis of NKG2x family members from NK cells isolated from healthy donors found that, in general, cells tend to express higher levels of the inhibitory receptor NKG2A than the activating receptors NKG2C or NKG2E (29). A separate study using gene expression microarrays found that uterine decidual NK cells express NKG2C and NKG2E mRNAs at levels 3- to 5-fold higher than peripheral NK cell populations (30).

Table II. *Thermodynamic parameters of selected NKG2x/CD94/HLA-E[G] interactions*

Receptor/Peptide	$\Delta G°^{a}$ (kcal/mol)	van't Hoff Analysis: $\Delta H°$ (kcal/mol)	van't Hoff Analysis: $T\Delta S°$ (kcal/mol)	Multiparameter Fitting: $\Delta H°$ (kcal/mol)	Multiparameter Fitting: $T\Delta S°$ (kcal/mol)	Multiparameter Fitting: $\Delta C°_{p\ obs}$ (cal/mol K)
NKG2A/HLA-G	−8.4 ± 6%	−6.8 ± 7%	1.6 ± 4%	−7.1 ± 4%	1.7 ± 19%	−170 ± 44%
NKG2E/HLA-G	−8.3 ± 7%	−4.8 ± 15%	3.5 ± 3%	−5.1 ± 14%	3.5 ± 20%	−140 ± 120%
NKG2C/HLA-G	−7.3 ± 14%	−4.2 ± 7%	3.1 ± 32%	−4.4 ± 2%	3.3 ± 1%	−110 ± 4%
NKG2A/HLA-B7	−6.7 ± 3%	−3.2 ± 16%	3.5 ± 13%	−3.6 ± 9%	4.0 ± 10%	−160 ± 56%

[a] $\Delta G° = -RT\ln K_D$; K_D values are taken from Table I.

Given the many uncertain aspects of this process, we have reported in this study an exhaustive analysis of the interaction parameters of NKG2x NKRs, focusing on tractable facets of this potentially complex process for immediate study. In contrast to previous studies, our results show that inhibitory (NKG2A/CD94) and activating (NKG2E/CD94) receptors can have essentially identical affinities for the same ligand complexes, showing that the NK signal integration mechanism cannot simply depend upon a rank affinity superiority of an inhibitory receptor to prevent inappropriate autoreactivity. The span of NKG2x/CD94/ligand affinities, 2.2 logs, is quite broad and allows for considerable potential discrimination between different receptor/ligand pairs in different contexts, but this discrimination is apparently limited solely to the interaction parameter of affinity. However, the span of this range is considerably less, 1.4 logs, if HLA-G peptide complexes are excluded, as is likely the case for most cells in the body. Although there is no direct evidence that distinct NKR/ligand pairs differentially modulate NK responses to MHC class I dysregulation in different contexts, the available range of NKG2x/ligand affinities is broad enough to enable such a system. Unlike TCR-mediated signaling, where kinetics and thermodynamics apparently play crucial roles in determining output signal strength (20), NKG2x-mediated signals cannot discriminate on the basis of these parameters as the various receptor/ligand pairs do not generate discernibly different values.

Studies of HLA-E allelic polymorphism argue that balancing selection may be acting to maintain two major alleles in most populations (13), suggesting that a functional difference exists between the alleles. As our current results definitively show that these two alleles behave identically in terms of microscopic aspects of NKG2x/CD94 interactions, this difference may be solely limited to altered cell surface expression levels due to a small, but significant, difference in solution thermal stability (T_m). These results do not rule out the possibility that the dimorphism is functionally affecting interactions with other as yet unrecognized HLA-E–specific receptors.

Structural basis of recognition

The results we present in this study may be most informative in terms of delimiting the structural details of the NKG2x/CD94/HLA-E interaction. Coarse models for this complex have been proposed on the basis of the crystal structures of CD94 (3) and the NKG2D/MHC class I-related chains A complex (Fig. 1*b*) (4). In these models, the NKG2x/CD94 heterodimer straddles the HLA-E/peptide complex much as a saddle does on horseback, with CD94 predominately contacting the α_1 domain of HLA-E, the NKG2x moiety predominately contacting the α_2 domain and both receptor domains potentially contacting the presented peptide. Systematic alanine scanning mutagenesis of the HLA-E α_1 and α_2 domain helices identified numerous residues required for binding to both NKG2A and -C (31), consistent with the overall arrangement of domains in the models. The bonds contributing to complexation would be predicted to include significant contributions from polar, hydrophobic, and electrostatic terms. The caveats associated with these models are manifest: absolute sequence identities are low enough, and the variation in C-terminal NKG2x sequences high enough, that the structure of the NKG2x molecules is likely to be significantly different from any available related structure. However, this model does predict that three loops of the NKG2x moiety contribute direct ligand-contacting residues: 170–173, 200–206, and 210–225 (Fig. 1*a*). Previous analysis of the sequences and affinities of NKG2A vs NKG2C had proposed that the very nonconservative substitution of a lysine for a glutamate at position 197, which does not lie in one of these loops, accounted for the observed affinity difference. However, consistent with the model of the complex, direct mutagenesis of this residue in both receptor backgrounds reported in this study refutes this contention. Many of the sequence differences do, indeed, fall in regions outside of these three putative contact loops and would, therefore, be predicted not to have direct effects on differential NKG2x affinities.

That NKG2A/CD94 and NKG2E/CD94 receptors have nearly identical affinities for HLA-E/peptide complexes is somewhat surprising as there are many nonconservative substitutions between these two molecules in these loops, particularly the putative ligand-contacting 210–225 loop (Fig. 1*a*). This conundrum either highlights limitations of these coarse structural models or suggests that NKG2x receptors use recognition machinery that tolerates such substitutions, as does the rigid adaptation mechanism of NKG2D. The significantly different affinities of NKG2A/NKG2E and NKG2C for HLA-E/peptide complexes is equally surprising, as there are few positions where NKG2C differs at positions where NKG2A and NKG2E are identical. One such position is 197 that has already been excluded from consideration as discussed above. The only other candidate position is 225, methionine in NKG2C but isoleucine in NKG2A and NKG2E. The residue at this position is predicted to directly contact HLA-E and may; however, again, reciprocal mutations show that this substitution has no effect. It is also possible that NKG2A and NKG2E achieve indistinguishable HLA-E/peptide affinities through different binding mechanisms. That the HLA-E allelic difference does not affect receptor affinity is completely consistent with the models as the dimorphic position lies well outside of the predicted interface as previously noted (3, 4).

Our reported tabulation of affinities reveals that the sequence of the peptide, despite relatively conservative substitutions, is the predominant determinant of receptor/ligand affinity. The peptide-binding groove of HLA-E contains deep anchor pockets at P2, P7, and P9 and shallower pockets at P3 and P6, based on both crystal structures (5, 6) and peptide binding studies (32, 33). Only the side chains of the P4, P5, and P8 residues are fully exposed to receptors, with the P1 and P6 side chains much less exposed. Previous mutational analysis of the peptide implicates the invariant arginine at P5 and the conserved hydrophobic residue at P8 as primary contacts for NKG2A/CD94 (32), consistent with models of the complex, with even conservative substitutions at the P8 position significantly affecting affinity. Our current results extend these conclusions, showing that the naturally occurring substitutions in the C-terminal four residues of the HLA-E ligand peptides have consistent 8- to 29-fold effects on receptor affinity across all three NKG2x NKRs tested, with the largest effect on affinity attributable to P8 substitutions, e.g., the 8-fold difference in the G and B27 peptide affinities.

Other positions within the nonamers derived from HLA leaders are infrequently substituted, including P2 (threonine in B27 and B58 vs methionine in most other class I-derived peptides) and P6 (alanine in Cw7 vs threonine in most other peptides). The P2 side chain is completely buried and does not indirectly alter any accessible element of the HLA-E structure (5, 6); the naturally observed P2 substitution is also unlikely to indirectly affect receptor affinities due to altered HLA-E T_ms, because the resultant HLA-E T_m difference between peptides is comparable to the T_m difference between HLA-E alleles (5), which clearly has no appreciable affect on affinity. Therefore, we would predict that the 8-fold difference in the affinities of the G and B27 peptides, which also differ at P2, is due solely to direct effects of the phenylalanine/leucine substitution at P8. The P6 substitution does have measurable effects on affinity; again assuming that we can discount the P2 substitution, compare the almost 4-fold affinity difference between the B27 and

Cw7 peptides (Table I). The conservative substitution at P7 (Leu/Val) has a lesser, though also measurable, effect; compare the over 2-fold affinity difference between the B27 and B7 peptides (Table I).

These analyses, along with the structural models, also explain the observation that peptide substitutions have equivalent effects on the affinities of all three unique NKG2x receptor specificities: NKG2A, NKG2C, and NKG2E. The models suggest that CD94, an invariant component of heterodimeric NKG2x NKRs, is providing the majority, if not all, of the contacts to the C-terminal half of the presented peptide. Therefore, peptide substitutions are likely having the same effect on the different receptors because contacts to CD94 are equivalent across the family, supporting the receptor orientation proposed in the modeling studies.

Disclosures

The authors have no financial conflict of interest.

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