The EMBO Journal Vol.18 No.15 pp.4250–4260, 1999

Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2-A and the activating receptor CD94/NKG2-C to HLA-E

Mar Valés-Gómez, Hugh T.Reyburn[1], Robert A.Erskine, Miguel López-Botet[2] and Jack L.Strominger[3]

Department of Molecular and Cellular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138, USA, [1]Department of Pathology, Cambridge University, Tennis Court Road, Cambridge CB2 1QP, UK and [2]Servicio de Inmunología, Hospital de la Princesa, Universidad Autónoma de Madrid, Diego de León 62, Madrid 28006, Spain

[3]Corresponding author
e-mail: jlstrom@fas.harvard.edu

M.Valés-Gómez and H.T.Reyburn contributed equally to this work

The lytic function of human natural killer (NK) cells is markedly influenced by recognition of class I major histocompatibility complex (MHC) molecules, a process mediated by several types of activating and inhibitory receptors expressed on the NK cell. One of the most important of these mechanisms of regulation is the recognition of the non-classical class I MHC molecule HLA-E, in complex with nonamer peptides derived from the signal sequences of certain class I MHC molecules, by heterodimers of the C-type lectin-like proteins CD94 and NKG2. Using soluble, recombinant HLA-E molecules assembled with peptides derived from different leader sequences and soluble CD94/NKG2-A and CD94/NKG2-C proteins, the binding of these receptor–ligand pairs has been analysed. We show first that these interactions have very fast association and dissociation rate constants, secondly, that the inhibitory CD94/NKG2-A receptor has a higher binding affinity for HLA-E than the activating CD94/NKG2-C receptor and, finally, that recognition of HLA-E by both CD94/NKG2-A and CD94/NKG2-C is peptide dependent. There appears to be a strong, direct correlation between the binding affinity of the peptide–HLA-E complexes for the CD94/NKG2 receptors and the triggering of a response by the NK cell. These data may help to understand the balance of signals that control cytotoxicity by NK cells.

Keywords: HLA-E/kinetics/NK receptors/peptide dependence

Introduction

Natural killer (NK) cell cytotoxicity is regulated by the action of a large number of distinct receptors. The interaction of these receptors with their ligands results in transmission of either a positive or a negative signal, and the balance of these activating and inhibiting stimuli determines the behaviour of the NK cell (Yokoyama, 1993; Lanier *et al*., 1997; Leibson, 1997). Expression, on the target cell, of molecules of the class I major histocompatibility complex (MHC) is one of the major influences regulating the behaviour of NK cells. The basis of this phenomenon is NK cell expression of class I MHC binding receptors able to either inhibit or activate cytotoxicity and cytokine production by the NK cell (Parham, 1997).

Two major types of class I MHC protein-binding receptors are expressed on human NK cells: the immunoglobulin superfamily molecules, termed killer cell immunoglobulin-like receptors (KIRs), and a heterodimer formed from the C-type lectin-like molecules CD94 and NKG2 (reviewed in Lanier, 1998b). Both receptor families contain two subfamilies, highly homologous in their extracellular domain, but differing markedly in their transmembrane and cytoplasmic tails. Inhibitory receptors, the p58, p70 and p140 KIRs and the CD94/NKG2-A complex, have a long cytoplasmic tail containing paired immunoreceptor tyrosine-based inhibition motif (ITIM) units (Vivier and Daeron, 1997). The activating p50 KIR and the CD94/NKG2-C receptor have a short cytoplasmic tail with a charged residue in the transmembrane sequence through which they associate with the KARAP/DAP12 molecule (Olcese *et al*., 1997; Lanier *et al*., 1998b). The function of the inhibitory receptors is relatively well understood: deficient cell surface expression of class I MHC proteins results in a markedly enhanced susceptibility of the target cell to lysis by circulating NK cells; interaction of the inhibitory NK receptor with these class I MHC molecules results in blockade of the cytotoxic activity and sparing of the target cell from NK lysis. The function of the activating NK receptors is unknown, but it has been suggested that they may activate NK cytotoxicity in situations where allele-specific loss of expression of MHC proteins results in the absence of an inhibitory interaction (Colonna, 1997).

The ligands for the KIRs are the classical class I MHC molecules HLA-A, -B and -C. HLA-C proteins, the major ligands mediating dominant resistance to NK lysis (Ciccone *et al*., 1992; Colonna *et al*., 1993), interact with p58 KIR (Wagtmann *et al*., 1995; Fan *et al*., 1996; Valés-Gómez *et al*., 1998b), while HLA-A and -B molecules bind p70 and p140 KIRs (Wagtmann *et al*., 1995; Dohring *et al*., 1996; Pende *et al*., 1996). Interaction of the inhibitory KIR with its specific class I MHC ligand induces phosphorylation of tyrosine residues in the cytoplasmic tail of the receptor and recruitment of the SHP-1 protein tyrosine phosphatase which acts to interrupt NK cell activation (Leibson, 1997; Renard *et al*., 1997). Ligation of activating KIRs results in phosphorylation of the tyrosine residues in the immunoreceptor tyrosine-based activation motif (ITAM) units of the associated KARAP/DAP12 molecule and positive signalling, probably via Syk and ZAP70 protein tyrosine kinases (Brumbaugh *et al*., 1997; Lanier *et al*., 1998b).

The inhibitory CD94/NKG2-A heterodimer and the activating CD94/NKG2-C receptor use the same mechanisms as the KIRs to mediate inhibition or activation of NK cell cytotoxicity (Houchins *et al*., 1997; Carretero *et al*., 1998; Lanier *et al*., 1998a; Le Drean *et al*., 1998). However, these receptor pairs do not seem to bind classical class I MHC proteins, but instead recognize the non-classical class I MHC molecule HLA-E (Borrego *et al*., 1998; Braud *et al*., 1998; Lee *et al*., 1998b). Surface expression of HLA-E depends on its binding to nonamer peptides derived from the signal sequences of other class I MHC proteins (Braud *et al*., 1997; Lee *et al*., 1998a). Thus the interaction of CD94/NKG2-A with HLA-E can be thought of as a strategy by which NK cells monitor expression of classical class I MHC molecules indirectly, via peptide-induced stabilization, and hence surface expression of HLA-E (Lanier, 1998a). It has, however, been suggested that not all class I MHC leader sequences contain peptides able to bind HLA-E; a threonine for methionine polymorphism in the signal sequence of many HLA-B alleles has been thought to render these leaders non-permissive for HLA-E binding (Braud *et al*., 1997) and therefore apparently invisible for recognition by the CD94/NKG2-A complex (Braud *et al*., 1998; Lee *et al*., 1998b). Recently, an additional level of complexity has been identified, in that it appears that the sequence of the HLA-E-bound peptide can influence the binding to CD94/NKG2-A (Llano *et al*., 1998; Brooks *et al*., 1999). However, these experiments are based on cellular assays and it is difficult to correlate the observed behaviour with the action of a single receptor–ligand pair.

In the present study, an *in vitro* system has been established which reproduces directly with purified soluble proteins the pattern of recognition associated with CD94/NKG2-A and CD94/NKG2-C in cellular assays. This system has been used to address a number of issues. The association and dissociation rate constants of the interactions between the CD94/NKG2 receptors and HLA-E have been defined, and the binding of the inhibitory and activating CD94/NKG2 complexes to HLA-E compared. The data presented here show first that the CD94/NKG2-A–HLA-E–peptide interaction had kinetic parameters strikingly similar to those of the p58 KIR binding to HLA-C (Valés-Gómez *et al*., 1998b). Secondly, the inhibitory receptor binds to HLA-E with a higher affinity than the activating receptor. Finally, whether the peptide loaded onto the HLA-E molecule has any role in this interaction has been investigated. These data indicate that the sequence of the bound peptide can markedly influence the affinity of CD94/NKG2-A and CD94/NKG2-C binding to HLA-E and that even subtle variation in the affinity of this interaction can have significant functional consequences.

Results

Expression and refolding of soluble CD94/NKG2 heterodimers

Constructs encoding the extracellular portions of the CD94, NKG2-A and NKG2-C molecules were expressed separately in *Escherichia coli* where they formed inclusion bodies that could be purified and then solubilized in 8 M urea. In preliminary experiments analysing the refolding ability of CD94 and NKG2 proteins, separately and together, no homodimer formation was observed, i.e. a water-soluble, disulfide linked dimer of M_r 32 kDa was seen only when both CD94 and NKG2 proteins were included in the refold (data not shown). In refolding experiments in which CD94 was incubated alone, soluble species of M_r 17 kDa (consistent with a soluble monomeric CD94 protein) were produced, whereas despite repeated attempts, it has not proved possible to produce either refolded, soluble NKG2-A or NKG2-C in the absence of CD94 protein in the refolding mix. These data are consistent with the observation that CD94 can be expressed on the cell surface in the absence of NKG2 proteins, whereas in the absence of CD94, NKG2-A proteins are retained in the endoplasmic reticulum (ER) and do not reach the cell surface (Lazetic *et al*., 1996; Brooks *et al*., 1997; Carretero *et al*., 1997). The dimeric material obtained by refolding CD94 and NKG2-A or NKG2-C together could be purified to homogeneity by a combination of anion-exchange chromatography and gel filtration (Figure 1A–C). Analysis of this protein on SDS–PAGE showed it to be a disulfide-linked dimer, M_r 32 kDa, which on reduction was seen to be composed of two subunits of M_r 17 and 15 kDa (Figure 1D), the predicted M_r of the recombinant CD94 and NKG2 proteins. Soluble, monomeric CD94 protein was also obtained in these refolds and this also could be isolated as a pure species (Figure 1).

That the soluble CD94/NKG2-A and NKG2-C heterodimers were refolded correctly was tested further by assaying the refolded proteins for reactivity with the monoclonal antibody HP-3D9, which binds to a conformation-dependent epitope on CD94 (Aramburu *et al*., 1990; Phillips *et al*., 1996). Figure 2 shows that both the CD94 and the CD94/NKG2 complexes bound this antibody. Further proof of the correct refolding of the heterodimer, specific interaction of CD94/NKG2-A and NKG2-C with HLA-E, but not other soluble HLA molecules, is shown below.

HLA-E is able to bind multiple leader peptides

The extracellular portion of the HLA-E heavy chain was expressed in bacteria, and soluble HLA-E–peptide–β_2-microglobulin (β2m) complexes were obtained essentially as described (O'Callaghan *et al*., 1998a). Previously, HLA-E*0101 has been suggested to have a markedly restricted peptide-binding specificity such that it only binds nonamer peptides derived from the signal sequences of certain class I MHC proteins (Braud *et al*., 1997; Lee *et al*., 1998a). These studies suggested that HLA-E-binding peptides require the presence of a methionine residue at position 2 (P2) of the nonamer peptide, and that for this reason the leader peptides of many HLA-B alleles, which have threonine at P2, assemble HLA-E inefficiently, if at all (Braud *et al*., 1997, 1998; Lee *et al*., 1998a). However, other studies have reported that these peptides can bind to HLA-E, but that these peptide–MHC complexes do not act to inhibit or activate NK cell lysis via the CD94/NKG2 receptors (Borrego *et al*., 1998; Llano *et al*., 1998; Brooks *et al*., 1999).

In these *in vitro* refolding experiments, all the HLA leader peptides tested (see Table I for sequences) appeared competent to assemble peptide–HLA-E–β2m complexes

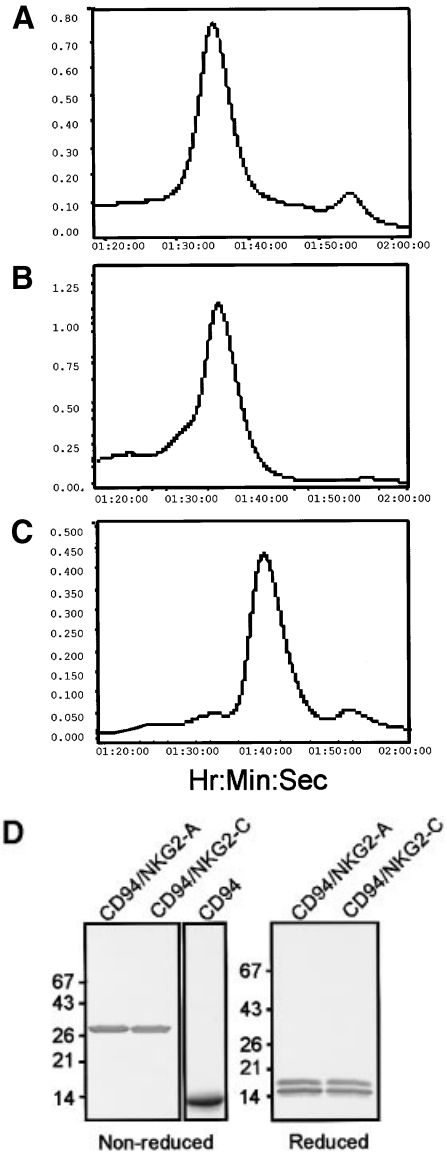

Fig. 1. Purification of CD94/NKG2-A (**A**) and CD94/NKG2-C (**B**) heterodimers and CD94 monomer (**C**) by gel filtration (see Materials and methods). (**D**) SDS–PAGE showing refolded CD94 monomer and CD94/NKG2-A and CD94/NKG2-C heterodimers. In the non-reduced gel, the heterodimer appears as an ~32 kDa band and monomeric CD94 as a 14 kDa band; in the reduced gel, the heterodimer components resolve as 17 and 15 kDa bands, respectively.

(Figure 3). Since it has been suggested that the HLA-B58 leader sequence-derived nonamer cannot bind to HLA-E, this result was surprising. To analyse this phenomenon more closely, the thermal stability of HLA-E complexed with the leader sequence-derived nonamer of HLA-B7 was compared with that of HLA-E complexed with the HLA-B58 leader sequence-derived nonamer (Figure 4). This assay has been well characterized as a direct measure of the stability of the peptide–HLA complex (Bouvier and Wiley, 1994). In these experiments, the two peptide–MHC complexes were equally stable, both having a melting temperature (T_m) of ~63°C [for comparison, the T_m of HLA-A2 assembled with the influenza matrix peptide is 65°C (Bouvier and Wiley, 1994)]. Thus, at least for peptide binding, HLA-E does not have a strict requirement for methionine at P2 of the bound peptide. In this context, it

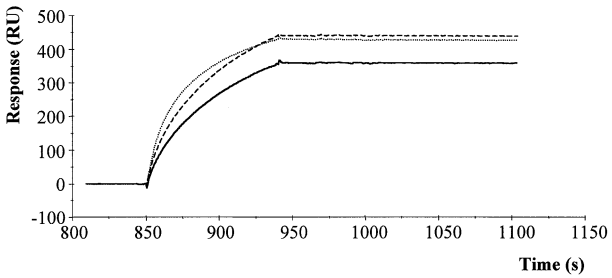

Fig. 2. Binding of conformation-sensitive monoclonal antibody (HP-3D9) to CD94, CD94/NKG2-A and CD94/NKG2-C proteins. HP-3D9 (10 μg/ml) was injected over a CM5 chip with a control surface (the control curve was subtracted from all the other values, therefore it would be a $y = 0$ line), CD94 (429 RU), CD94/NKG2-A (401 RU) and CD94/NKG2-C (334 RU). The antibody recognized monomer and heterodimers with similar dissociation rates. This experiment is representative of several others with similar results.

is worth noting that HLA-E recently has also been shown to bind some viral peptides with either leucine or glutamine at P2 of the nonamer (Ulbrecht *et al*., 1998). However, caution should be used in extrapolating these data to cell surface expression of the complex since it is also clear that HLA-E is expressed only poorly, if at all, at the surface of cells transfected with HLA-B alleles such as HLA-B51 or -B58 (Lee *et al*., 1998a; Llano *et al*., 1998). This discrepancy could be explained in a number of ways, but one plausible hypothesis might be that the HLA-B58 leader sequence nonamer is not available in the intact cell for some reason. The processing of class I MHC leader peptides which subsequently are loaded onto other MHC molecules is complex and, for Qa-1^b at least, involves shuttling of the peptide between the ER and the cytosol (Bai *et al*., 1998). Perhaps the HLA-B58 leader sequence-derived peptide is cleaved during its processing in such a way that the nonamer is generated with only a very poor efficiency. Alternatively, TAP-dependent translocation of this peptide into the ER may be inefficient.

The binding of HLA-E to CD94/NKG2-A has extremely fast kinetics and is peptide dependent

The technique of surface plasmon resonance was used to examine the interaction between soluble refolded HLA-E and CD94/NKG2-A and CD94/NKG2-C proteins. HLA-E molecules, assembled with the HLA-B7, -B58, -Cw3, -Cw4, -Cw7 and HLA-G leader sequence-derived peptide nonamers, bound specifically to the CD94/NKG2 heterodimers, but not to CD94 alone. In contrast, soluble HLA-Cw6, -Cw7 and -B8 molecules themselves bound to neither the CD94/NKG2 nor the CD94 proteins. In Figure 5, several HLA molecules, HLA-B8, HLA-Cw6 and-Cw7, were injected simultaneously over surfaces in which CD94 monomers and CD94/NKG2-A dimers had been immobilized, and compared with a control surface in which no protein was present. Binding of these proteins was not detected. The same preparations of HLA-Cw6 and -Cw7 injected over immobilized NKAT1 and NKAT2 bound to the receptors with the same specificity and kinetics as reported previously (Valés-Gómez *et al*., 1998b) (data not shown). Experiments were then performed using soluble HLA-E complexes assembled with a number of HLA leader sequence-derived peptide nonamers (Table I). Increasing concentrations of these HLA-E proteins were

Table I. Origin and sequence of the leader sequence-derived peptide nonamers used for HLA-E refolding

HLA allotype	Leader peptide sequence	k_{off} (/s)	K_D (μM)[a](CD94/NKG2-A)	Inhibition of NK lysis[b]	k_{off} (/s)	K_D (μM)[a](CD94/NKG2-C)	Activation of NK lysis[b]
-G	VMAPRTLFL	0.42	2.33	yes	1.80	10.77	yes
-Cw*0302	VMAPRTLIL	1.22	7.35	yes	ND	⩾35.4	no
-B*0702	VMAPRTVLL	1.27	11.23	yes	ND	⩾56.2	no
-B*5801	VTAPRTVLL	1.33	17.06	no	ND	⩾238	no
-Cw*0702	VMAPRALLL	ND	⩾34.05	no	ND	ND	no
-Cw*0402[c]	VMEPRTLIL	ND	⩾75.30	no	ND	ND	no

Kinetic data, corresponding to the interaction between HLA-E loaded with each peptide and CD94/NKG2-A, are related to the data obtained in cytotoxicity assays. Residues that differ from the HLA-B7-derived peptide, for which the crystal structure is known, are underlined. (Data are the averages of 2–5 experiments performed using several batches of refolded protein assayed on sensor surfaces prepared at different times.)
[a]Steady-state values obtained using the general fit option of BIAevaluation 3.0 (see Materials and methods).
[b]Inhibition or activation by HLA-E assembled with the indicated leader sequence-derived peptide through binding to the D94/NKG2-A or -C complexes (Llano *et al*., 1998).
[c]The sequence of HLA-Cw*0402 has been partially corrected and the existence of this allotype is in question (Bunce *et al*., 1997).
ND = no kinetic parameters could be obtained for these pairs of proteins: in the column corresponding to k_{off}, because of the poor binding; in the case of K_D, because the curve representing R_{eq} versus concentration did not reach saturation at the concentrations used in the experiment. This reflects either a much lower affinity or non-specific binding.

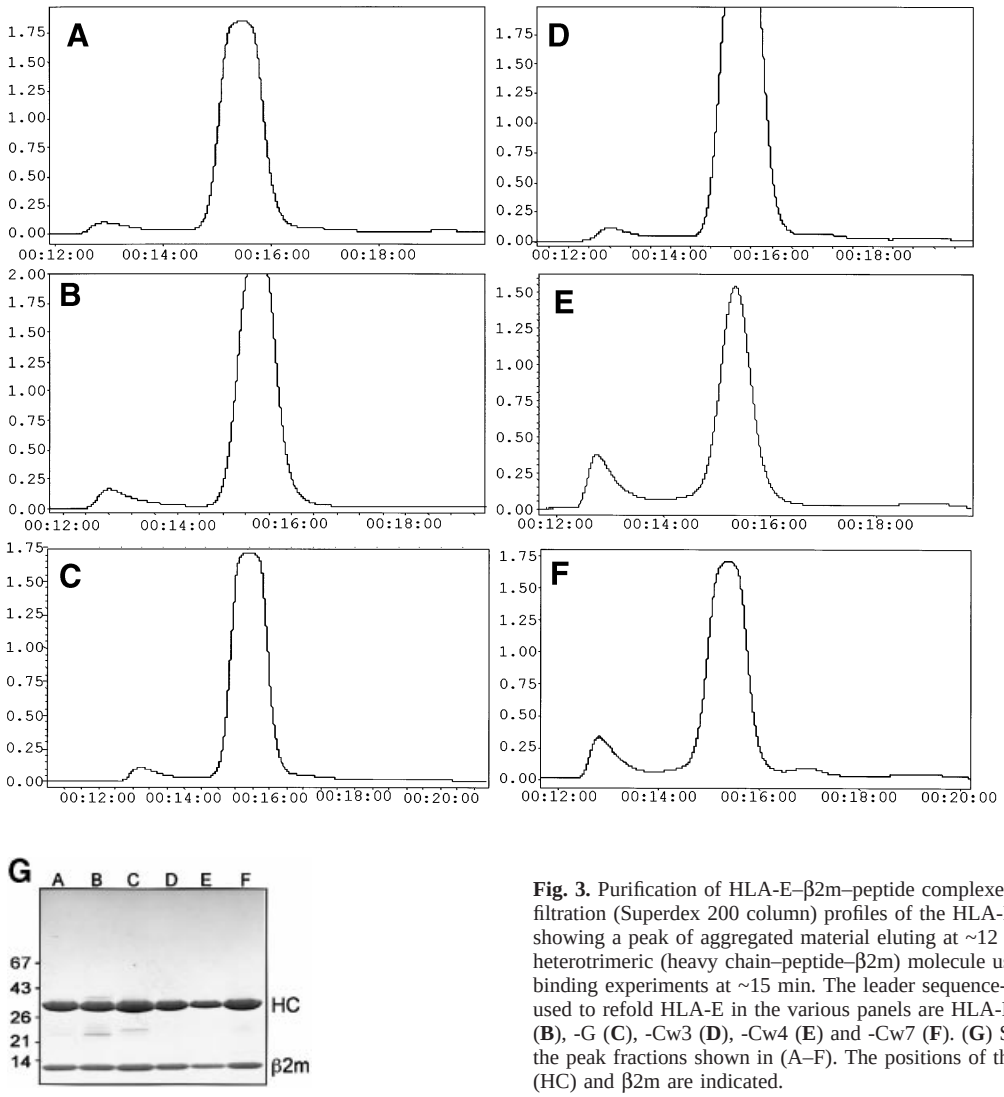

Fig. 3. Purification of HLA-E–β2m–peptide complexes. (A–F) Gel filtration (Superdex 200 column) profiles of the HLA-E complexes showing a peak of aggregated material eluting at ~12 min and the heterotrimeric (heavy chain–peptide–β2m) molecule used in the binding experiments at ~15 min. The leader sequence-derived peptides used to refold HLA-E in the various panels are HLA-B58 (**A**), -B7 (**B**), -G (**C**), -Cw3 (**D**), -Cw4 (**E**) and -Cw7 (**F**). (**G**) SDS–PAGE of the peak fractions shown in (A–F). The positions of the heavy chain (HC) and β2m are indicated.

injected over the control surface, CD94, CD94/NKG2-A and CD94/NKG2-C. The relevant curves showing increasing concentrations of HLA-E loaded with different peptides binding to either CD94/NKG2-A (Figure 6A) or CD94/NKG2-C (Figure 7A) are depicted after the control curves (no protein immobilized on the flow cell) had been subtracted. The curve corresponding to the flow cell containing immobilized CD94 alone was comparable to the control (mock immobilization) surface. This is consistent with previous data showing that CD94 monomer

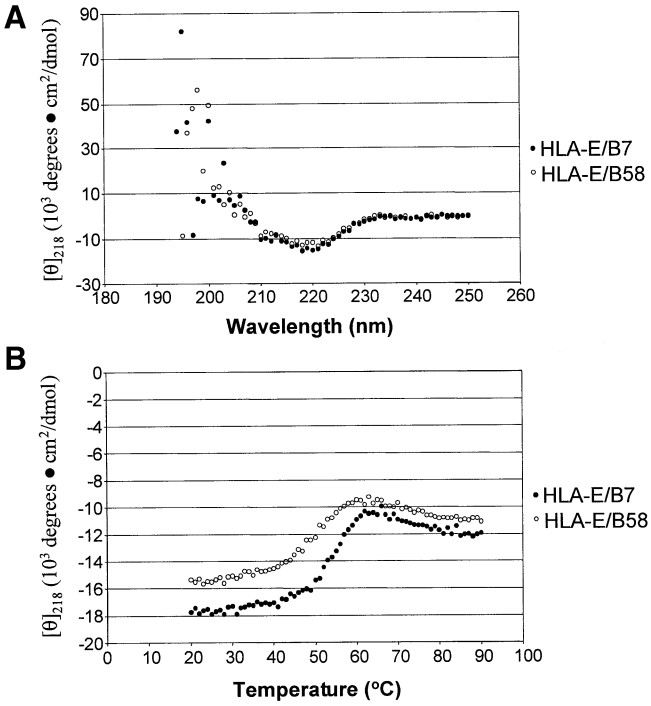

Fig. 4. (**A**) CD spectra (250–194 nm) of HLA-E in complex with the HLA-B7 and -B58 leader sequence-derived nonamer peptides. (**B**) A comparison of the thermal stability of HLA-E complexed with either the HLA-B7 or -B58 leader sequence-derived peptides. Curves were obtained by monitoring the change in CD signal at 218 nm while heating the sample from 20 to 90°C (scan rate of 0.7°C/min).

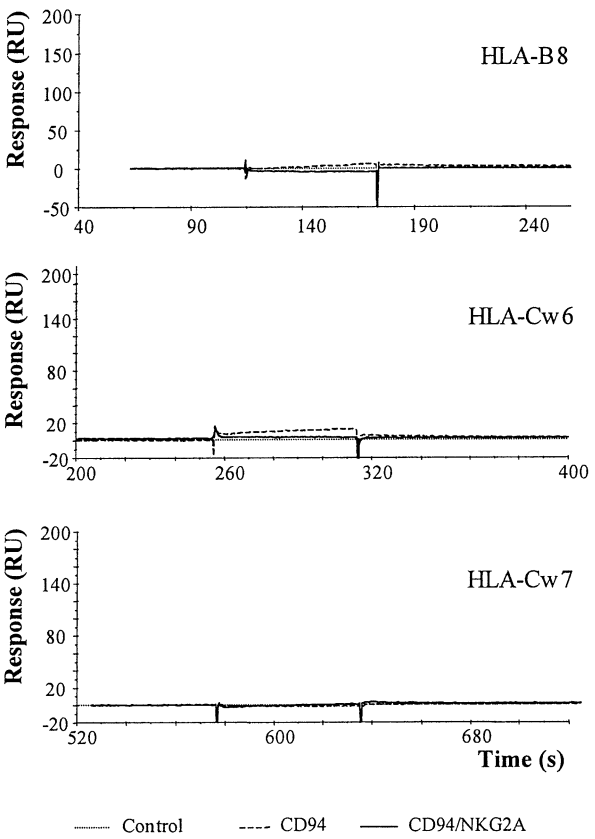

Fig. 5. HLA-B8, -Cw6 and -Cw7 do not bind CD94 or CD94/NKG2-A. HLA-B8, -Cw6 and -Cw7 (all at 45 μM) were injected over a chip containing a control surface, CD94 (544 RU) and CD94/NKG2-A (597 RU).

does not interact with HLA class I (Braud *et al.*, 1998). HLA-E molecules loaded with the HLA-B7, -B58, -Cw3, -Cw4, -Cw7 and HLA-G leader sequence-derived peptide nonamers all bound both CD94/NKG2-A and CD94/NKG2-C, but with widely differing affinities. The association and dissociation rate constants of the CD94/NKG2–HLA-E interactions appeared to be very fast, as judged by the shapes of the curves, similar to those observed for the binding of the Ig superfamily KIRs to HLA-C (Valés-Gómez *et al.*, 1998b). Kinetic data were analysed using both the general fit option and the simultaneous fit option provided by BIAevaluation 3.0 (see Materials and methods), and, where possible, the rate constants of the interaction between CD94/NKG2-A, CD94/NKG2-C and HLA-E were determined in experiments involving the injection of increasing concentrations of peptide–HLA-E complex. The averages of the measured dissociation rates (k_{off}) of CD94/NKG2-A binding to HLA-E loaded with different leader sequence-derived peptide are depicted in Table I. From the dissociation constants, we can calculate the half-life of these complexes ($t_{1/2}$): HLA-E (G) 1.6 s, HLA-E (Cw3) 0.57 s, HLA-E (B7) 0.54 s, and HLA-E (B58) 0.52 s. Notably, HLA-G binds with a much slower dissociation rate which probably contributes to the increased affinity of the interaction. For CD94/NKG2-C, the only k_{off} that could be calculated with some confidence was that corresponding to HLA-E (G) with a value of 1.8/s ($t_{1/2}$ = 0.39 s). These dissociation rate constants should be taken as minimal estimates as they are subject to sources of error such as rebinding (Valés-Gómez *et al.*, 1998b). The saturation plots represented in Figures 6B and 7B correspond to steady-state analysis of representative data performed using the global fit option of BIAevaluation 3.0 in the same experiment as shown in Figures 6A and 7A. In Table I, the average of the values for K_D obtained in multiple different experiments are given for comparison. These data revealed that (i) HLA-E, assembled with any of the six peptides used, bound both the CD94/NKG2-A and CD94/NKG2-C receptors with very rapid association and dissociation rate constants, as seen from the dissociation constants and from the shapes of the curves shown in Figures 6A and 7A; and (ii) that the affinity of this interaction varied with the peptide bound to the HLA-E molecule and with the use of either CD94/NKG2-A or CD94/NKG2-C (Figures 6 and 7; Table I).

Discussion

NK cells can express a large repertoire of receptors able to bind to class I MHC molecules (Moretta *et al.*, 1996). Thus, in order to achieve a precise understanding of the biology of the individual receptor–ligand systems, it is necessary to isolate any given receptor–ligand interaction from the multitude of class I-binding receptors expressed on an NK cell. With this purpose, the interaction of the CD94/NKG2-A and CD94/NKG2-C receptors with HLA-E has been reconstituted *in vitro* using purified, soluble proteins.

The CD94, NKG2-A and NKG2-C proteins were expressed in *E.coli*, refolded together and the hetero-

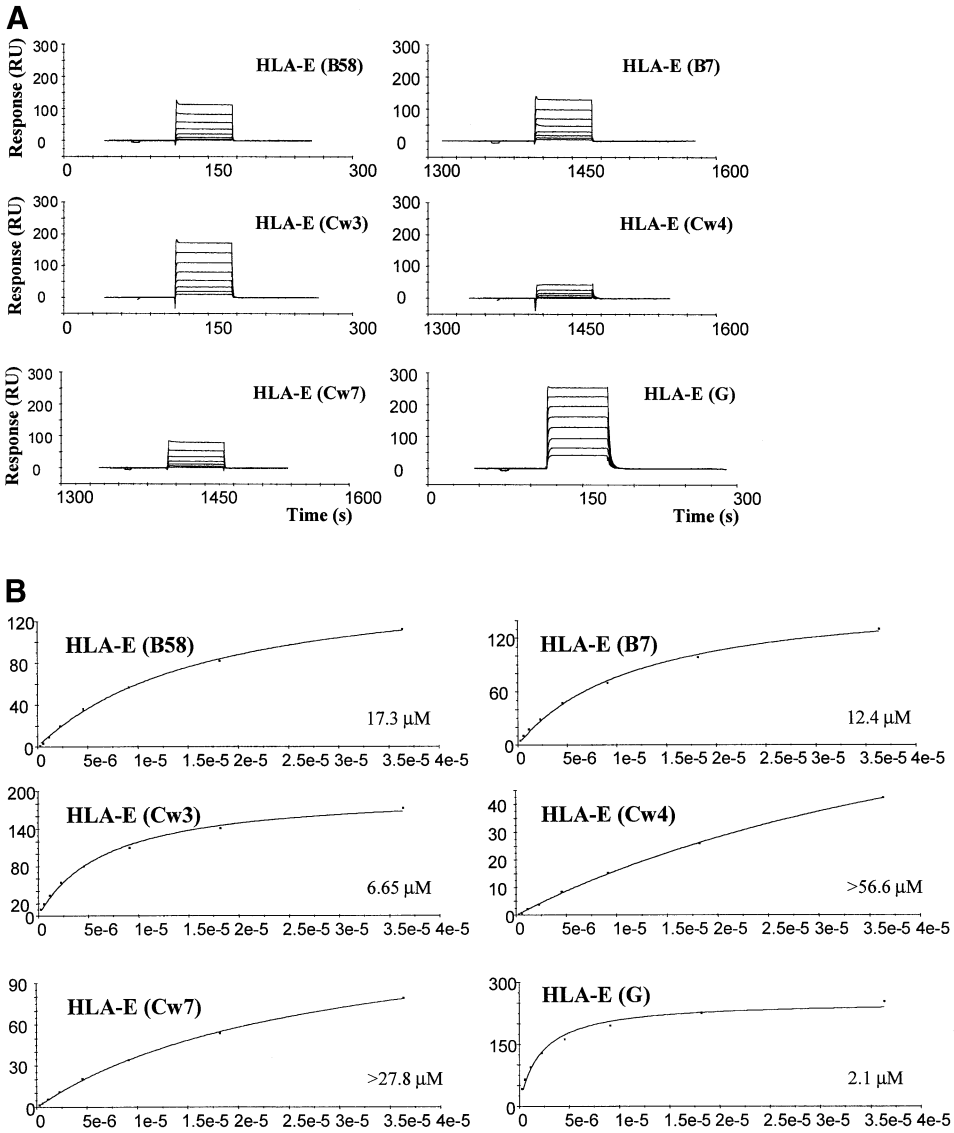

Fig. 6. Peptide specificity in the recognition of HLA-E by CD94/NKG2-A. (**A**) Increasing concentrations (0.28, 0.57, 1.14, 2.27, 4.55, 9.1, 18.18 and 36.36 μM) of HLA-E, refolded using HLA-B58, -B7, -G, -Cw3, -Cw4 and -Cw7 leader sequence-derived peptide nonamers, were injected over the same surface used in the experiment shown in Figure 2. The curves represent the binding of HLA-E to CD94/NKG2-A after the control flow cell had been subtracted. (**B**) Steady-state saturation plots. The amount of RU at equilibrium was plotted against the concentration for each receptor–ligand pair. This experiment is representative of several, using different surface chips and various batches of refolded protein, yielding similar results in all cases.

dimeric complexes purified to homogeneity. Several pieces of evidence indicate that the refolded CD94/NKG2 molecules are refolded correctly: first, a water-soluble, disulfide-linked heterodimer is formed only if both CD94 and NKG2 proteins are included in the refold (Figure 1). Secondly, the heterodimers are recognized by conformation-sensitive monoclonal antibodies including HP-3D9 (Figure 2) and HP-3B1 (data not shown). Thirdly, consistent with the data obtained in cellular assays (Borrego *et al*., 1998; Braud *et al*., 1998; Lee *et al*., 1998b), the recombinant CD94/NKG2-A and CD94/NKG2-C complexes bind specifically to HLA-E (Figures 5–7).

This binding had kinetic characteristics remarkably similar to those of Ig superfamily KIRs binding to HLA-C, both interactions being characterized by extremely rapid association and dissociation rate constants. Presumably this reflects functional constraints on the kinetics of the interaction which are required for effective NK cell immunosurveillance (Valés-Gómez *et al*., 1998b), i.e. the association rate constant is sufficiently fast that the rate-limiting factor for the interaction will be the diffusion of the molecules in the membrane (Bell, 1978). CD94/NKG2 complexes are expressed at relatively high levels on NK cells; thus the availability of the HLA-E–peptide–β2m complex will be a major factor in deciding the outcome of the interaction between the NK cell and the target cell.

Another striking parallel between the binding of the two Ig domain KIRs to HLA-C and the binding of CD94/NKG2 to HLA-E is that the activating receptor binds to the peptide–MHC complex with a much lower affinity than the inhibitory receptor in both cases (Figures 6 and 7; Table I). As has been discussed previously (Valés-Gómez *et al*., 1998a), these data suggest that if an activating and an inhibitory receptor compete for binding to a given HLA ligand then the inhibitory receptor is more likely to bind. It seems plausible to suggest that this phenomenon

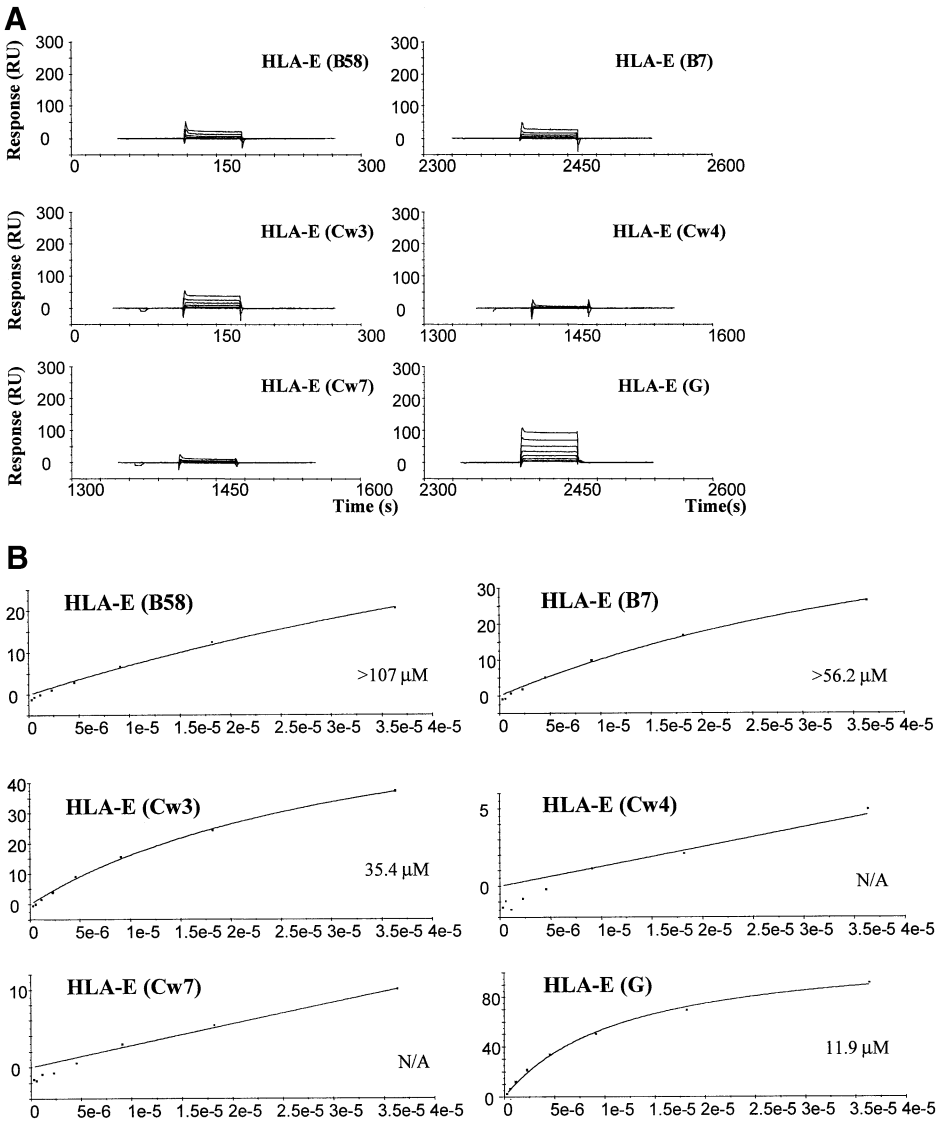

Fig. 7. Peptide specificity in the recognition of HLA-E by CD94/NKG2-C. Increasing concentrations (0.28, 0.57, 1.14, 2.27, 4.55, 9.1, 18.18 and 36.36 μM) of HLA-E, refolded using HLA-B58, -B7, -G, -Cw3, -Cw4 and -Cw7 leader sequence-derived peptide nonamers, were injected over the same surface used in the experiment shown in Figure 2. The curves represent the binding of HLA-E to CD94/NKG2-C after the control flow cell had been subtracted. (**B**) Steady-state saturation plots. The amount of RU at equilibrium was plotted against the concentration for each receptor–ligand pair. This experiment was also repeated several times yielding similar results in all cases.

may contribute to the observed dominance of inhibition over activation (Moretta *et al*., 1995). However, the molecular basis of the weaker binding to HLA-E of the CD94/NKG2-C receptor is unclear. These proteins are both type II membrane glycoproteins, and comparison of the NKG2-A and NKG2-C sequences reveals that the bulk of the sequence variation between these proteins is found in the membrane-proximal N-terminus of the molecule. The only striking sequence variation occurring in the C-terminal, membrane-distal portion of the molecule occurs at residue 197. The crystal structure of the CD94 protein recently has been solved and a model for the CD94/NKG2-A heterodimer proposed (Boyington *et al*., 1999). In this model, residue 197 was suggested to be a component of the HLA-E-binding site and thus perhaps this lysine for glutamic acid change between the NKG2-A and NKG2-C proteins is important for the altered binding affinity.

These experiments also show that another major factor controlling the outcome of the interaction between HLA-E and the CD94/NKG2-A or CD94/NKG2-C receptors is the nature of the bound peptide. Recent experiments, published while this manuscript was in preparation, have provided evidence that the primary structure of the peptide bound to HLA-E can affect CD94/NKG2 recognition (Llano *et al*., 1998; Brooks *et al*., 1999). The experiments presented here have examined the effect of the peptide closely and indicate that recognition of the peptide–HLA-E complex at the cell surface by the CD94/NKG2 receptors is a critical checkpoint for surveillance of class I MHC protein expression. As predicted from cellular assays, HLA-E molecules assembled with the HLA-B7, HLA-Cw3 or the HLA-G leader sequence-derived peptide nonamer bound to the CD94/NKG2-A receptor (Figure 6). The difference among the affinity constants of the interactions between these three HLA-E heterotrimers and

CD94/NKG2-A are notable, the HLA-E molecule loaded with the HLA-G leader sequence-derived peptide binding to CD94/NKG2-A with the highest affinity (Table I). Surprisingly, HLA-E molecules loaded with a leader sequence-derived nonamer peptide from either HLA-B58, HLA-Cw*0702 or HLA-Cw*0402 also bound to CD94/NKG2-A. These observations were unexpected since a variety of experiments have suggested that expression of these HLA alleles does not confer protection from NK lysis via CD94/NKG2-A signalling (Borrego *et al*., 1998; Braud *et al*., 1998; Lee *et al*., 1998b; Llano *et al*., 1998). Strikingly, however, the affinity of binding of these complexes is weaker than that of the HLA-E/B7, -G or -Cw3 leader sequence-derived peptide complexes that are able to trigger inhibitory signalling in NK cells. These data, therefore, strongly suggest that there is a marked threshold effect of receptor–ligand binding leading to NK cell response in this system. In this context, it is interesting to compare the two sets of cytotoxicity data published (Llano *et al*., 1998; Brooks *et al*., 1999). The former group, using 100 μM B58 peptide, could not demonstrate any protective effect of this peptide in cytotoxicity assays, whereas the latter group using 300 μM peptide, present in the medium during the assay, could demonstrate protection. Thus, increasing the number of HLA-B58-derived peptide–HLA-E complexes, i.e. increasing the valency of the HLA-E–CD94 interaction, appears to compensate for the lower binding affinity.

A similar pattern is apparent for the interaction between CD94/NKG2-C and HLA-E (Figure 7). It has been noted previously, in cytotoxicity assays, that activation of lysis of 721.221 cells loaded with HLA-E-binding peptides is very poor (Llano *et al*., 1998), the exception to this being 721.221 cells loaded with the HLA-E-binding G1 leader sequence-derived peptide. Inspection of the affinity constants of the various peptide–HLA-E–CD94/NKG2-C interactions reveals that only the HLA-E–G leader sequence-derived peptide binds CD94/NKG2-C with an affinity (~10 μM) consistent with triggering of a response by the NK cell (Figure 7).

The structural basis of the observed variation in binding affinity for CD94/NKG2-A between HLA-E loaded with different peptides is unclear. Inspection of the sequences (Table I) indicates that substitution at residues P2, P3, P6, P7 and P8, compared with the HLA-B7-derived peptide, for which the three-dimensional structure is known, had marked effects on the interaction of the peptide–HLA-E complex with CD94/NKG2-A and CD94/NKG2-C. The crystal structure of HLA-E in complex with the nonamer peptide from the signal sequence of HLA-B7 (O'Callaghan *et al*., 1998b) shows that P2, methionine in all of the peptides studied except for threonine in the HLA-B58 sequence, is buried deep in the B pocket. This T for M substitution, however, reduces the affinity of the HLA-E–receptor interaction sufficiently to prevent both the inhibition and activation of lysis by CD94/NKG2-A and CD94/NKG2-C, respectively. The P3 side chain occupies the shallow D pocket and projects towards the α2 helix. It could therefore be speculated that the marked decrease in binding to CD94/NKG2 observed for the Cw*0402 peptide, glutamic acid for alanine at P3, might result from protrusion of the glutamic acid side chain out of the groove and neutralization of the charge on the P5 arginine. Residue 6, threonine in most sequences and positioned laterally with its -OH pointing up and possibly involved in a hydrogen bond, is arginine in the -Cw7-derived peptide. This substitution also has a drastic effect on the binding of the peptide–HLA-E complex to CD94/NKG2-A or -C. Residue 7, valine or leucine, is buried in the E pocket pointing down, and this alternation of two similar hydrophobic residues seems to have only minor effects on the affinity of the interaction. P8, phenylalanine in the HLA-G-derived peptide but leucine or isoleucine in all of the others, results in a strikingly higher affinity of its HLA-E complex for both CD94/NKG2-A and -C and seems clearly to be an important contact residue. It interacts with the rim of the peptide-binding groove, but is solvent exposed (O'Callaghan *et al*., 1998b). Thus, changes in the peptide sequence at residues P6 and P8, at least, may directly influence binding to CD94/NKG2, whereas substitutions at P2, P3 and P7 are more likely to affect receptor binding indirectly by influencing peptide binding.

Whatever the molecular basis of these phenomena, these data emphasize that the action of the Ig superfamily KIRs and the lectin-like CD94/NKG2 receptors appears to be complementary. Expression on the target cell surface of HLA-E molecules complexed with peptides derived from the leader peptide of HLA-B allotypes bearing the Bw4 public epitope (e.g. HLA-B58) and several very common HLA-C alleles fails to mediate either inhibition or activation of CD94/NKG2-expressing NK clones (Llano *et al*., 1998). However, these are exactly the HLA molecules with well-defined reactivity with Ig superfamily KIRs (reviewed in Moretta *et al*., 1996; Lanier, 1998b). Reciprocally, Ig superfamily KIRs reactive with HLA-B molecules bearing the Bw6 epitope and the majority of HLA-A alleles examined have not been defined, although there is evidence that some may exist (Vyas *et al*., 1998). However, these HLA molecules have leader peptides able to assemble HLA-E–peptide–β2m complexes competent to signal productively via CD94/NKG2 receptors.

Finally, if a critical threshold of signalling, a product of affinity and valency, exists for CD94/NKG2 function, then protection from NK cell lysis may require larger or smaller numbers of cell surface HLA-E molecules, depending on the binding affinity of the HLA-E–peptide complex for CD94/NKG2-A. This model is consistent with the idea that expression of class I MHC molecules such as HLA-B58 or -Cw7 does not signal protection via CD94/NKG2-A, because an insufficient number of cell surface HLA-E leader sequence derived-peptide complexes form to compensate for the lower binding affinities of these interactions. Similarly, HLA-E molecules assembled with the HLA-B7-derived peptide and the HLA-G-derived peptide bound CD94/NKG2-A with a 5-fold different affinity. Thus, the appearance of susceptibility to NK lysis would be predicted to require a more profound down-regulation of the class I MHC protein for HLA-G-expressing cells than for HLA-B7-expressing cells.

Here it is interesting to consider CD94/NKG2-A recognition of HLA-C-derived peptides in complex with HLA-E. HLA-Cw7, which at least in Caucasian populations is markedly the most common HLA-C allele (gene frequency of 33%, phenotype frequency of 62%) (Bunce *et al*.,

1997), does not mediate inhibition via HLA-E (Llano *et al*., 1998), presumably because the HLA-E–Cw7 leader sequence-derived peptide complex binds CD94/NKG2-A with only a low affinity (Figure 6). In transfection systems, overexpression of other HLA-C alleles can result in assembly of sufficient HLA-E to interact productively with CD94/NKG2-A (Phillips *et al*., 1996; Sivori *et al*., 1996; Llano *et al*., 1998), but it is possible that this level of expression is non-physiological. Normally, HLA-C proteins are expressed on the cell surface at ~10% of the level of HLA-A and -B molecules. The molecular basis of this phenomenon is a matter for debate (Neefjes and Ploegh, 1988; McCutcheon *et al*., 1995; Neisig *et al*., 1998), but this observation does provoke the speculation that a lower level of HLA-C leader sequence-derived peptide might be available for binding to HLA-E. If this is so and given that the nonamer peptide from HLA-Cw3 binds CD94/NKG2 with an affinity comparable with that of HLA-B7, the numbers of HLA-E–HLA-C leader sequence-derived peptide complexes formed might be insufficient to protect from NK cell attack via interaction with CD94/NKG2-A. Thus, in a cell with a selective down-regulation of HLA-A and -B proteins, the remaining low level of expression of HLA-C alleles might result in inadequate levels of cell surface HLA-E protein to mediate protection from NK attack via CD94/NKG2-A inhibitory signalling. For example, the US2 and US11 proteins encoded by human cytomegalovirus promote rapid proteolytic degradation of HLA-A and -B proteins, but not of HLA-C (Schust *et al*., 1998; Cohen *et al*., 1999). Similarly, expression of the HIV-1 nef protein results in enhanced internalization and degradation of HLA-A and -B molecules, but not HLA-C (Le Gall *et al*., 1998). These attempts by viruses to evade immunosurveillance by HLA-A- and -B-restricted CTL, while avoiding attack by NK cells, could be frustrated by a failure of recognition of HLA-E complexed with HLA-C leader sequence-derived peptides. Thus, deficient recognition of these complexes, mediated either by a failure of the complex to bind CD94/NKG2-A or by low level expression of HLA-C, may represent a selective advantage for the host.

Materials and methods

Peptides and monoclonal antibodies

Peptides were synthesized by the Biopolymers Laboratory at Harvard Medical School. Monoclonal antibody HP-3D9, specific for the CD94 protein (Aramburu *et al*., 1990), was purchased from Pharmingen (San Diego, CA).

Cloning, expression, refolding and purification of recombinant proteins

Soluble extracellular portions of the CD94, NKG2-A and NKG2-C genes were generated by PCR, using as template cDNA prepared from an NK cell line and the primer pairs 5′-GGAATTCCATATGAAAAATTCTTTT-ACTAAACTGAGTA-3′ and 5′-CGGGATCCTTAAATGAGCTGTTG-CTTACAGAT-3′ for CD94. The 5′ primers 5′-GGAATTCCATAT-GCAGCGTCACAACAATTCTTCCCTGAATAC-3′ (NKG2-A) and 5′-GGAATTCCATATGCCTTTCCTGGAGCAGAA-3′ (NKG2-C), and the 3′ primer 5′-CGGGATCCTAAAGCTTATGCTTACAATGATA-3′ were used to prepare the soluble NKG2 constructs. These PCR products were cloned into the pGMT7 vector and sequenced. For expression, plasmids were transformed into *E.coli* strain BL21 (DE3) (Studier *et al*., 1990). The transformed bacteria were grown and expression of recombinant protein was induced as described previously (Valés-Gómez *et al*., 1998b). The CD94, NKG2-A and NKG2-C proteins accumulated as insoluble aggregates and these inclusion bodies were purified as previously described (Valés-Gómez *et al*., 1998b). The yield of protein recovered as inclusion bodies varied from 100 to 200 mg/l of bacterial culture for CD94, NKG2-A and NKG2-C. The purified, denatured CD94 and NKG2 proteins were refolded together by dialysis to form the CD94/NKG2-A and CD94/NKG2-C heterodimers. Briefly, 50 mg of CD94 protein and 100 mg of NKG2 protein were diluted in buffer [8 M urea, 100 mM Tris pH 8.5, 50 mM glycine, 5 mM reduced glutathione (GSH) and 1 mM oxidized glutathione (GSSG)] and incubated at 10°C for 24 h. This solution was then dialysed successively against refolding buffers (0.4 M L-arginine pH 8, 100 mM Tris pH 8.5, pepstatin A and leupeptin, both at 1 μg/ml) supplemented with 4, 2 or 1 M urea, respectively in the successive changes. After dialysis against refolding buffer without urea, the mixture finally was dialysed against two changes of 10 mM sodium phosphate pH 8. The refolding mix was then centrifuged and filtered to remove any precipitated material. Soluble CD94 and CD94/NKG2 heterodimers were purified by anion-exchange and size exclusion chromatography. Refolded CD94/NKG2, in 10 mM phosphate pH 8, was circulated overnight over a DEAE–Sepharose column (Amersham Pharmacia), the column was then washed extensively with phosphate buffer and proteins eluted with a linear gradient of 0–0.5 M NaCl in 10 mM phosphate pH 8. Under these conditions, the CD94/NKG2-A and CD94/NKG2-C heterodimers eluted early in the gradient (~184 mM NaCl for CD94/NKG2-A and 189 mM NaCl for CD94/NKG2-C) separate from a later peak of aggregated material and refolded but non-NKG2 associated CD94 protein. These two peaks were collected separately, concentrated and then size-fractionated on a Hiload 16/60 Superdex 200 column to obtain pure CD94/NKG2 heterodimers and CD94 monomers (Figure 1D). The yields of purified, refolded protein were of the order of 3–5% for the heterodimer and 15–20% for the CD94 monomer.

A construct encoding a soluble form of the HLA-E heavy chain (HLA-E*0101) in the pGMT7 vector was obtained by PCR using the primer pair 5′-CATGCCATGGGTTCTCATTCTTTAAAATATTTTCA-TACTTCTGTATCTCGTCCCGGCCG-3′ and 5′-CCCAAGCTTACGG-CTTCCATCTCAGGGTGACGGGCTC-3′ and template cDNA prepared from the 721.221 cell line. The constructs encoding soluble forms of the HLA-Cw6, -Cw7 and -B8 heavy chains have been described (Reid *et al*., 1996; Valés-Gómez *et al*., 1998b). Soluble HLA–peptide–β2m complexes were produced, refolded and purified essentially as described previously (Garboczi *et al*., 1992; Fan *et al*., 1996; O'Callaghan *et al*., 1998a; Valés-Gómez *et al*., 1998b).

Circular dichroism

Circular dichroism experiments were done as described previously (Bouvier and Wiley, 1994). Briefly, HLA-E complexes were diluted to 0.18 mg/ml in 10 mM MOPS pH 7.5 and CD spectra (250–198 nm) for each complex collected. Both complexes had minimum values at 218 nm and so for the thermal denaturation experiments, curves were obtained by monitoring the change in CD signal at 218 nm while heating the sample from 20 to 90°C. The experiments were carried out using an Aviv 62DS spectropolarimeter equipped with a thermo-electric temperature controller.

Surface plasmon resonance

The interaction between soluble HLA-E and the soluble CD94 or CD94/NKG2 proteins was analysed using a BIAcore 2000 instrument (BIAcore, Piscataway, NJ) as described previously (Valés-Gómez *et al*., 1998b). CD94/NKG2-A, CD94/NKG2-C and CD94 proteins were immobilized to the dextran surface by amine coupling. Around 40–50% of the protein immobilized was in a native conformation, as calculated from binding of the conformation-sensitive monoclonal antibody HP-3D9 and HLA-E protein. Control flow cells were prepared by applying the same treatment as for immobilization of a protein, but without the injection of any protein. Unless otherwise stated, analytes were injected simultaneously over the test and control surfaces, using the multichannel flow option, at a flow rate of 30 μl/min. The data collection rate was set as high as possible.

Analysis of the kinetics of the interaction between CD94/NKG2 complexes and HLA-E–β2m–peptide heterotrimers was carried out using BIAevaluation software version 3.0 (BIAcore). First, because all the curves reflecting CD94/NKG2 binding reached equilibrium very soon after injection of the HLA-E complex, steady-state affinity data were analysed using the general fit option. K_D values were derived by curve fitting analyses supplied in the BIAevaluation 3 package. Averages of the affinity constants obtained in this way are depicted in Table I. Then the kinetic parameters of each set of curves were analysed using the simultaneous fit option. The numbers obtained in both ways were

comparable. Where applicable, Scatchard plots were inspected and gave comparable values for the affinity constants.

Acknowledgements

We would like to thank Dr Chris O'Callaghan (Molecular Immunology Group, University of Oxford) for helpful discussions, Dr W.Weissenhorn (Laboratory of Molecular Medicine, HHMI, The Children's Hospital, Boston, MA) for help and assistance with the circular dichroism experiments, Dr Scott Reid (Axys Pharmaceuticals Inc., CA) for the gift of the HLA-B8 construct, and Dr Dave Garboczi (NIH) for the gift of the plasmid for expression of β2m. H.T.R. acknowledges support from the Wellcome Trust. This work was supported by National Institutes of Health grant CA-47554 to J.L.S. M.L.-B. received support from the grant SAF98-0006 (Plan Nacional I+D).

References

Aramburu,J., Balboa,M.A., Ramirez,A., Silva,A., Acevedo,A., Sanchez-Madrid,F., De Landazuri,M.O. and Lopez-Botet,M. (1990) A novel functional cell surface dimer (Kp43) expressed by natural killer cells and T cell receptor-γ/δ+ T lymphocytes. I. Inhibition of the IL-2 dependent proliferation by anti-Kp43 monoclonal antibody. *J. Immunol.*, **144**, 3238 – 3247.

Bai,A., Broen,J. and Forman,J. (1998) The pathway for processing leader-derived peptides that regulate the maturation and expression of Qa-1b. *Immunity*, **9**, 413–421.

Bell,G.I. (1978) Models for the specific adhesion of cells to cells. *Science*, **200**, 618–627.

Borrego,F., Ulbrecht,M., Weiss,E.H., Coligan,J.E. and Brooks,A.G. (1998) Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J. Exp. Med*., **187**, 813–818.

Bouvier,M. and Wiley,D.C. (1994) Importance of peptide amino and carboxyl termini to the stability of MHC class I molecules. *Science*, **265**, 398–402.

Boyington,J.C., Riaz,A.N., Patamawenu,A., Coligan,J.E., Brooks,A.G. and Sun,P.D. (1999) Structure of CD94 reveals a novel C-type lectin fold: implications for the NK cell-associated CD94/NKG2 receptors. *Immunity*, **10**, 75–82.

Braud,V., Jones,E.Y. and McMichael,A. (1997) The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. *Eur. J. Immunol.*, **27**, 1164–1169.

Braud,V.M. *et al*. (1998) HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature*, **391**, 795–799.

Brooks,A.G., Posch,P.E., Scorzelli,C.J., Borrego,F. and Coligan,J.E. (1997) NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. *J. Exp. Med*., **185**, 795–800.

Brooks,A.G., Borrego,F., Posch,P.E., Patamawenu,A., Scorzelli,C.J., Ulbrecht,M., Weiss,E.H. and Coligan,J.E. (1999) Specific recognition of HLA-E, but not classical, HLA class I molecules by soluble CD94/NKG2A and NK cells. *J. Immunol.*, **162**, 305–313.

Brumbaugh,K.M., Binstadt,B.A., Billadeau,D.D., Schoon,R.A., Dick,C.J., Ten,R.M. and Leibson,P.J. (1997) Functional role for Syk tyrosine kinase in natural killer cell-mediated cytotoxicity. *J. Exp. Med*., **186**, 1965–1974.

Bunce,M., Barnardo,M.C., Procter,J., Marsh,S.G., Vilches,C. and Welsh,K.I. (1997) High resolution HLA-C typing by PCR–SSP: identification of allelic frequencies and linkage disequilibria in 604 unrelated random UK Caucasoids and a comparison with serology [corrected and republished article originally printed in *Tissue Antigens* 1996 Dec; **48** (6): 680–91]. *Tissue Antigens*, **50**, 100–111.

Carretero,M. *et al*. (1997) The CD94 and NKG2-A C-type lectins covalently assemble to form a natural killer cell inhibitory receptor for HLA class I molecules. *Eur. J. Immunol.*, **27**, 563–567.

Carretero,M., Palmieri,G., Llano,M., Tullio,V., Santoni,A., Geraghty,D.E. and Lopez-Botet,M. (1998) Specific engagement of the CD94/NKG2-A killer inhibitory receptor by the HLA-E class Ib molecule induces SHP-1 phosphatase recruitment to tyrosine-phosphorylated NKG2-A: evidence for receptor function in heterologous transfectants. *Eur. J. Immunol.*, **28**, 1280–1291.

Ciccone,E. *et al*. (1992) Involvement of HLA class I alleles in natural killer (NK) cell-specific functions: expression of HLA-Cw3 confers selective protection from lysis by alloreactive NK clones displaying a defined specificity (specificity 2). *J. Exp. Med*., **176**, 963–971.

Cohen,G.B., Gandhi,R.T., Davis,D.M., Mandelboim,O., Chen,B.K., Strominger,J.L. and Baltimore,D. (1999) The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity*, **10**, 661–671.

Colonna,M. (1997) Specificity and function of immunoglobulin superfamily NK cell inhibitory and stimulatory receptors. *Immunol. Rev.*, **155**, 127–133.

Colonna,M., Borsellino,G., Falco,M., Ferrara,G.B. and Strominger,J.L. (1993) HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc. Natl Acad. Sci. USA*, **90**, 12000–12004.

Dohring,C., Scheidegger,D., Samaridis,J., Cella,M. and Colonna,M. (1996) A human killer inhibitory receptor specific for HLA-A. *J. Immunol.*, **156**, 3098–3101.

Fan,Q.O.R., Garboczi,D.N., Winter,C.C., Wagtmann,N., Long,E.O. and Wiley,D.C. (1996) Direct binding of a soluble natural killer cell inhibitory receptor to a soluble human leukocyte antigen-Cw4 class I major histocompatibility complex molecule. *Proc. Natl Acad. Sci. USA*, **93**, 7178–7183.

Garboczi,D.N., Hung,D.T. and Wiley,D.C. (1992) HLA-A2–peptide complexes: refolding and crystallization of molecules expressed in *Escherichia coli* and complexed with single antigenic peptides. *Proc. Natl Acad. Sci. USA*, **89**, 3429–3433.

Houchins,J.P., Lanier,L.L., Niemi,E.C., Phillips,J.H. and Ryan,J.C. (1997) Natural killer cell cytolytic activity is inhibited by NKG2-A and activated by NKG2-C. *J. Immunol.*, **158**, 3603–3609.

Lanier,L.L. (1998a) Follow the leader: NK cell receptors for classical and nonclassical MHC class I. *Cell*, **92**, 705–707.

Lanier,L.L. (1998b) NK cell receptors. *Annu. Rev. Immunol*., **16**, 359–393.

Lanier,L.L., Corliss,B. and Phillips,J.H. (1997) Arousal and inhibition of human NK cells. *Immunol. Rev.*, **155**, 145–154.

Lanier,L.L., Corliss,B., Wu,J. and Phillips,J.H. (1998a) Association of DAP12 with activating CD94/NKG2C NK cell receptors. *Immunity*, **8**, 693–701.

Lanier,L.L., Corliss,B.C., Wu,J., Leong,C. and Phillips,J.H. (1998b) Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature*, **391**, 703–707.

Lazetic,S., Chang,C., Houchins,J.P., Lanier,L.L. and Phillips,J.H. (1996) Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. *J. Immunol.*, **157**, 4741–4745.

Le Drean,E. *et al*. (1998) Inhibition of antigen-induced T cell response and antibody-induced NK cell cytotoxicity by NKG2A: association of NKG2A with SHP-1 and SHP-2 protein-tyrosine phosphatases. *Eur. J. Immunol.*, **28**, 264–276.

Lee,N., Goodlett,D.R., Ishitani,A., Marquardt,H. and Geraghty,D.E. (1998a) HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J. Immunol.*, **160**, 4951–4960.

Lee,N., Llano,M., Carretero,M., Ishitani,A., Navarro,F., Lopez-Botet,M. and Geraghty,D.E. (1998b) HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc. Natl Acad. Sci. USA*, **95**, 5199–5204.

Le Gall,S., Erdtmann,L., Benichou,S., Berlioz-Torrent,C., Liu,L., Benarous,R., Heard,J.M. and Schwartz,O. (1998) Nef interacts with the mu subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules. *Immunity*, **8**, 483–495.

Leibson,P.J. (1997) Signal transduction during natural killer cell activation: inside the mind of a killer. *Immunity*, **6**, 655–661.

Llano,M., Lee,N., Navarro,F., Garcia,P., Albar,J.P., Geraghty,D.E. and Lopez-Botet,M. (1998) HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: preferential response to an HLA-G-derived nonamer. *Eur. J. Immunol.*, **28**, 2854–2863.

McCutcheon,J.A., Gumperz,J., Smith,K.D., Lutz,C.T. and Parham,P. (1995) Low HLA-C expression at cell surfaces correlates with increased turnover of heavy chain mRNA. *J. Exp. Med*., **181**, 2085–2095.

Moretta,A., Sivori,S., Vitale,M., Pende,D., Morelli,L., Augugliaro,R., Bottino,C. and Moretta,L. (1995) Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. *J. Exp. Med.*, **182**, 875–884.

Moretta,A., Bottino,C., Vitale,M., Pende,D., Biassoni,R., Mingari,M.C. and Moretta,L. (1996) Receptors for HLA class-I molecules in human natural killer cells. *Annu. Rev. Immunol*., **14**, 619–648.

Neefjes,J.J. and Ploegh,H.L. (1988) Allele and locus-specific differences in cell surface expression and the association of HLA class I heavy chain with beta 2-microglobulin: differential effects of inhibition of glycosylation on class I subunit association. *Eur. J. Immunol.*, **18**, 801–810.

Neisig,A., Melief,C.J. and Neefjes,J. (1998) Reduced cell surface expression of HLA-C molecules correlates with restricted peptide binding and stable TAP interaction. *J. Immunol.*, **160**, 171–179.

O'Callaghan,C.A., Tormo,J., Willcox,B.E., Blundell,C.D., Jakobsen, B.K., Stuart,D.I., McMichael,A.J., Bell,J.I. and Jones,E.Y. (1998a) Production, crystallization and preliminary X-ray analysis of the human MHC class Ib molecule HLA-E. *Protein Sci.*, **7**, 1264–1266.

O'Callaghan,C.A., Tormo,J., Willcox,B.E., Braud,V.M., Jakobsen,B.K., Stuart,D.I., McMichael,A.J., Bell,J.I. and Jones,E.Y. (1998b) Structural features impose tight peptide binding specificity in the nonclassical MHC molecule HLA-E. *Mol. Cell*, **1**, 531–541.

Olcese,L., Cambiaggi,A., Semenzato,G., Bottino,C., Moretta,A. and Vivier,E. (1997) Human killer cell activatory receptors for MHC class I molecules are included in a multimeric complex expressed by natural killer cells. *J. Immunol.*, **158**, 5083–5086.

Parham,P. (1997) *NK cells, MHC Class I Antigens and Missing Self*. Immunological Reviews. Vol. 155, Munksgaard, Copenhagen.

Pende,D. *et al*. (1996) The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/p70 family of inhibitory receptors that is characterised by three immunoglobulin like domains and is expressed as a 140-kd disulphide linked dimer. *J. Exp. Med*., **184**, 505–518.

Phillips,J.H., Chang,C., Mattson,J., Gumperz,J.E., Parham,P. and Lanier,L.L. (1996) CD94 and a novel associated protein (94AP) form a NK cell receptor involved in the recognition of HLA-A, HLA-B and HLA-C allotypes. *Immunity*, **5**, 163–172.

Reid,S.W. *et al*. (1996) Production and crystallization of MHC class I B allele single peptide complexes. *FEBS Lett*., **383**, 119–123.

Renard,V., Cambiaggi,A., Vely,F., Blery,M., Olcese,L., Olivero,S., Bouchet,M. and Vivier,E. (1997) Transduction of cytotoxic signals in natural killer cells: a general model of fine tuning between activatory and inhibitory pathways in lymphocytes. *Immunol. Rev.*, **155**, 205–221.

Schust,D.J., Tortorella,D., Seebach,J., Phan,C. and Ploegh,H.L. (1998) Trophoblast class I major histocompatibility complex (MHC) products are resistant to rapid degradation imposed by the human cytomegalovirus (HCMV) gene products US2 and US11. *J. Exp. Med*., **188**, 497–503.

Sivori,S., Vitale,M., Bottino,C., Marcenaro,E., Sanseverino,L., Parolini,S., Moretta,L. and Moretta,A. (1996) CD94 functions as a natural killer cell inhibitory receptor for different HLA class I alleles: identification of the inhibitory form of CD94 by the use of novel monoclonal antibodies. *Eur. J. Immunol.*, **26**, 2487–2492.

Studier,F.W., Rosenburg,A.H., Dunn,J.J. and Dubendorff,J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.*, **185**, 60–89.

Ulbrecht,M., Modrow,S., Srivastava,R., Peterson,P.A. and Weiss,E.H. (1998) Interaction of HLA-E with peptides and the peptide transporter *in vitro*: implications for its function in antigen presentation. *J. Immunol.*, **160**, 4375–4385.

Valés-Gómez,M., Reyburn,H.T., Erskine,R.A. and Strominger,J.L. (1998a) Differential binding to HLA-C of p50-activating and p58-inhibitory natural killer cell receptors. *Proc. Natl Acad. Sci. USA*, **95**, 14326–14331.

Valés-Gómez,M., Reyburn,H.T., Mandelboim,M. and Strominger,J.L. (1998b) Kinetics of interaction of HLA-C ligands with natural killer cell inhibitory receptors [published erratum appears in *Immunity*, **9**, 892]. *Immunity*, **9**, 337–344.

Vivier,E. and Daeron,M. (1997) Immunoreceptor tyrosine-based inhibition motifs. *Immunol. Today*, **18**, 286–291.

Vyas,Y., Selvakumar,A., Steffens,U. and Dupont,B. (1998) Multiple transcripts of the killer cell immunoglobulin-like receptor family, KIR3DL1 (NKB1), are expressed by natural killer cells of a single individual. *Tissue Antigens*, **52**, 510–519.

Wagtmann,N., Rajagopalan,S., Winter,C.C., Peruzzi,M. and Long,E.O. (1995) Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and functional transfer. *Immunity*, **3**, 801–809.

Yokoyama,W. (1993) Recognition structures on natural killer cells. *Curr. Opin. Immunol.*, **5**, 67–73.

Received April 16, 1999; revised June 14, 1999; accepted June 15, 1999