Human natural killer cells and a subset of T cells express a repertoire of killer cell immunoglobulin receptors (KIRs) that recognize major histocompatibility complex (MHC) class I molecules. KIRs and T cell receptors (TCRs) bind in a peptide-dependent manner to overlapping regions of peptide-MHC class I complexes. KIRs with two immunoglobulin domains (KIR2Ds) recognize distinct subsets of HLA-C alleles. Here we use surface plasmon resonance to study the binding of soluble forms of KIR2DL1 and KIR2DL3 to several peptide-HLA-Cw7 complexes. KIR2DL3 binds to the HLA-Cw7 allele presenting the peptide RYRPGTVAL with a 1:1 stoichiometry and an affinity (K_d ~7 μM at 25 °C) within the range of values measured for other cell-cell recognition molecules, including the TCR. Although KIR2DL1 is reported not to recognize the HLA-Cw7 allele in functional assays, it binds RYRPGTVAL/HLA-Cw7, albeit with a 10–20-fold lower affinity. TCR/peptide-MHC interactions are characterized by comparatively slow kinetics and unfavorable entropic changes (Willcox, B. E., Gao, G. F., Wyer, J. R., Ladbury, J. E., Bell, J. I., Jakobsson, B. K., and van der Merwe, P. A. (1999) Immunity 10, 357–365), suggesting that binding is accompanied by conformational adjustments. In contrast, we show that KIR2DL3 binds RYRPGTVAL/HLA-Cw7 with fast kinetics and a favorable binding entropy, consistent with rigid body association. These results indicate that KIR/peptide-MHC class I interactions have properties typical of other cell-cell recognition molecules, and they highlight the unusual nature of TCR/peptide-MHC recognition.

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Killer Cell Immunoglobulin Receptors and T Cell Receptors Bind Peptide-Major Histocompatibility Complex Class I with Distinct Thermodynamic and Kinetic Properties

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that a single KIR will bind different peptide-MHC complexes with different affinities, raising the question as to what the affinity and/or kinetic threshold is for functional recognition?

Recently Valés-Gómez et al. (23, 24) used surface plasmon resonance to measure the affinity of KIR2DL1 and KIR2DL3 binding HLA-Cw6-peptide and HLA-Cw7-peptide complexes, respectively. The dissociation rate constant ($k_{off}$) was also estimated, but the association rate constant ($k_{on}$) could not be measured directly (23, 24). We extend this study by providing more precise kinetic measurements of the KIR2DL3/peptide-HLA-Cw7 interaction, obtaining thermodynamic data, and determining the stoichiometry of the interaction. We also use affinity measurements to quantitate the effects of the peptide on the binding affinity and the degree of cross-reactivity between KIR2DL1 and HLA-Cw7. We find that KIR2DL3 binds peptide-HLA-Cw7 with a 1:1 stoichiometry and with thermodynamic and kinetic properties very similar to other cell-cell recognition molecules. These properties differ from those reported for TCR/peptide-MHC interactions, underlining the unusual nature of TCR recognition.

**EXPERIMENTAL PROCEDURES**

**Production of Soluble Forms of KIR2DL1 and KIR2DL3—DNA encoding the extracellular portions (residues 1–224) of KIR2DL1 and KIR2DL3 were amplified from cDNA, obtained as described previously (6), using 5'-GGCAATG AGA GGA GTC AAC CAC-3' as forward primer and 5'-GGG GCC CCG GGG CAT GTG TGG CTT ACC-3' as reverse primer. The resultant fragments were digested with the restriction enzymes NcoI and NotI and ligated into pGEM2 (Promega). The final construct encodes, in tandem, the Shine-Dalgarno signal peptide and an epitope and an oligohistidine tag (Fig. 1). The expression plasmid was designated pKMATHNK1 or -2.

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**Kinetic and Thermodynamics of KIR/Peptide-MHC Interactions**

**RESULTS**

**Stoichiometry and Peptide Dependence of sKIR2DL3 Binding to HLA-Cw7—Soluble MHC class I heavy chains were expressed in E. coli and refolded in vitro with peptide and β2-microglobulin (Fig. 1). Biotinylated peptide-MHC class I complexes were produced by refolding with chemically biotinylated β2-microglobulin (28). Soluble forms of KIR2D molecules were also expressed in bacteria, either with (sKIR2DL1H and sKIR2DL3H) or without (sKIR2DL1 and sKIR2DL3) carboxyl-terminal c-myc and oligohistidine tags (see “Experimental Procedures”). sKIR2DL3H was used successfully for an x-ray crystallographic structure determination (30), indicating that it is correctly folded. Purified sKIRs migrated as monomers on size exclusion chromatography (Fig. 1B and data not shown).

Binding of KIR constructs was analyzed by surface plasmon resonance, which measures the changes in refractive index near a sensor surface (31). sKIR2DL3 was injected through 4 flow cells containing sensor surfaces to which different peptide-MHC class I complexes had been immobilized using their biotin tag (Fig. 1A, solid bar). A “background” response (measured in response units) is seen in the negative control HLA-A2 flow cell, a consequence of the high concentration, and therefore high refractive index, of injected sKIR2DL3 sample. However, a greater response is seen with injection over HLA-Cw7 complexed with the DS11 peptide, indicating binding (Fig. 1A). sKIR2DL3 bound at a much lower level to HLA-Cw7 complexed with the DS12 peptide and did not bind at all to the HLA-Cw7-DS10 complex (Fig. 1A).

In order to assess the stoichiometry of binding, HLA-Cw7-DS11 and sKIR2DL3 were mixed together, with a molar excess of sKIR2DL3 (1:1.5), and then fractionated by size exclusion chromatography (Fig. 1B). The elution position of the complex...
(~74 kDa, calculated $M_r$ 69,304) is consistent with the presence of one sKIR2DL3 molecule and one HLA-Cw7-DS11 molecule. Furthermore, whereas free sKIR2DL3 was present, no free HLA-Cw7-DS11 was detected, indicating that there was an excess of the sKIR2DL3 (Fig. 1B). Taken together, these data indicate that sKIR2DL3 binds HLA-Cw7-DS11 with a 1:1 stoichiometry.

Affinity of sKIR2DL3 and sKIR2DL1 Binding to HLA-Cw7 Peptide—The affinity of sKIR2Ds binding to peptide-MHC molecules was measured by equilibrium binding analysis on the BIAcore. A range of concentrations of sKIR2DL3 (Fig. 2A) was injected through flow cells with HLA-Cw7-DS11 or a control peptide-MHC class I complex immobilized. The binding response at each concentration was calculated by subtracting the equilibrium response measured in the control flow cell from the response in the HLA-Cw7-DS11 flow cell. Conventional (Fig. 2B) and Scatchard (Fig. 2B, inset) plots of these binding data indicate that the interaction conforms to a simple $1:1$ (Langmuir) binding model with a $K_d$ of $9 \mu M$. The results of several experiments are summarized in Table I. Other soluble recombinant forms of sKIR2DL3 bound immobilized HLA-Cw7-DS11 with a similar affinity (data not shown). These included sKIR2DL3H and a truncated version of sKIR2DL3 (comprising amino acids 1–200) which lacked the membrane-proximal stalk region. A similar affinity was measured in the reverse orientation, with sKIR2DL3H immobilized to a Ni$_2$-NTA sensor chip via its oligohistidine tag, and HLA-Cw7-DS11 in solution (Table I).

The weak interaction between sKIR2DL3 and HLA-Cw7-
DS12 peptide (Fig. 1A) was confirmed by affinity analysis (data not shown). sKIR2DL3 bound to HLA-Cw7-DS12 with an affinity ($K_D \sim 108 \mu M$) ~12-fold lower than its affinity for HLA-Cw7-DS11 (Fig. 2B). This very low affinity was confirmed in the reverse orientation, with sKIR2DL3H immobilized and HLA-Cw7-DS12 in solution.

sKIR2DL1 also bound to HLA-Cw7-DS11, albeit with a 10–15-fold lower affinity than sKIR2DL3 (Fig. 2B and Table I). This lower affinity was largely a consequence of a faster $k_{off}$ (data not shown), indicating that it is not a consequence of low sKIR2DL1 activity. No binding was detected when high concentrations (up to 3 mM) of sKIR2DL1 and sKIR2DL3 were injected over several other classical (HLA-A2 and -B35) and non-classical (HLA-E and -G1) MHC class I molecules (Table I).

**Binding Kinetics**—Although binding and dissociation were very fast, it was possible to analyze both the association and dissociation phases of binding (Fig. 3). Global fitting with mono-exponential rate equations derived from the simple 1:1 Langmuir binding model produced reasonable fits, yielding a $k_{on}$ of $1.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ and a $k_{off}$ of $1.2 \text{ s}^{-1}$ (Fig. 3A). The rate constants did not change significantly when the level of immobilized HLA-Cw7-DS11 varied 2-fold (Table II), indicating that binding was not substantially affected by mass transport or rebinding artifacts. The excellent agreement between calculated $K_D$ (Table II) and the $K_D$ determined by equilibrium binding (Table I) further supports the notion that these kinetic constants are correct.

Because recent studies have shown that the kinetics of TCR binding to peptide-MHC are strongly temperature-dependent (32), we analyzed the temperature dependence of the KIR/peptide-MHC interaction (Fig. 3B). The $k_{off}$ of a TCR/peptide-MHC interaction increased ~40-fold as the temperature was raised from 5 to 25 °C (32). In contrast, the $k_{off}$ of the sKIR2DL3/HLA-Cw7-DS11 interaction increased a modest ~4-fold over the same temperature range (Fig. 3B). Arrhenius plots yield an activation energy of $13 \text{ kcal mol}^{-1}$ for sKIR2DL3/HLA-Cw7-DS11 dissociation (Fig. 3B, inset), far lower than the ~30 kcal mol measured for TCR/peptide-MHC dissociation (32).

**Thermodynamic Analysis**—The enthalpy change ($\Delta H$) that accompanies KIR binding to peptide-MHC was estimated by van’t Hoff analysis, which involves measuring the dependence of affinity on temperature (Fig. 4A). Because the enthalpy and entropy vary with temperature, the non-linear form of the van’t Hoff equation was used (see “Experimental Procedures”). At 25 °C favorable enthalpic ($\Delta H_m \sim -4.1 \text{ kcal mol}^{-1}$) and entropic ($-\Delta S_m \sim -3.1 \text{ kcal mol}^{-1}$) changes contribute in approximately equal measure to the binding energy ($\Delta G_m \sim -7.2 \text{ kcal mol}^{-1}$). The heat capacity derived from this fit ($\Delta C_p \sim -100 \text{ cal mol}^{-1}$), which is a measure of the dependence of the binding enthalpy and entropy change on temperature, is well within the range determined for other protein/protein interactions (33). Similar values for $\Delta H_m$ and $-\Delta S_m$ were obtained when using the linear form of van’t Hoff equation (data not shown), which assumes that the enthalpy is temperature-independent.

**DISCUSSION**

**Peptide Dependence**—Although several studies have demonstrated that NK cell recognition is dependent on the peptide as well as the MHC on target cells, no studies have measured directly the effect of peptide on the affinity of a KIR for a peptide-MHC class I complex. Consistent with functional (21) and binding (18) assays, we found that the affinity of sKIR2DL3 binding to HLA-Cw7-peptide was dramatically affected by the nature of the peptide (Fig. 1A and Table I). sKIR2DL3 bound to HLA-Cw7-DS11, -DS12, and -DS10 with a $K_D \sim 7 \mu M$, $\sim 115 \mu M$, and >3 mM, respectively (Table I). In agreement with this, Vázquez-Gómez et al. (18) found that sKIR2DL3 bound to HLA-Cw7-DS11 but not to HLA-Cw7-DS10. Interestingly, Mandelboim et al. (21) showed that killing by several NK clones specific for group 2 HLA-C alleles was inhibited when target cells expressed HLA-Cw7 loaded with the DS12 peptide. This suggests that affinities as low as $K_D$

### Table II

**Summary of kinetic data**

| Immobilized     | Soluble      | Immobilization level | $k_{on}$ | $k_{off}$ | $K_{dissoci}$ |
|-----------------|--------------|----------------------|----------|-----------|--------------|
| HLA-Cw7-DS11    | sKIR2DL3     | ~1500                | 210,000 ± 23,000 | 1.1 ± 0.13 | 5.2          |
|                 | sKIR2DL3     | ~750                 | 190,000 ± 36,000 | 1.2 ± 0.15 | 6.4          |

*Values are means ± S.D. of three determinations.
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~115 μm are sufficient to mediate inhibition. Paradoxically, however, HLA-Cw7-loaded with DS10 peptide (KYFDEHYEY), which we and others (18) show does not bind to sKIR3DL3, was a better inhibitor of most of these NK clones than HLA-Cw7-DS12 (21). In only one of the clones studied (dp10.7) did the functional inhibition data (21) correlate with the direct binding data. A likely explanation for these results is that these NK clones express multiple KIRs, highlighting the importance of using purified KIRs to analyze the binding specificity.

Stoichiometry—Based on the structural similarity between KIR2DL1 and hematopoietic receptors (e.g. growth hormone receptor, which binds growth hormone with a 2:1 stoichiometry), Fan et al. (34) proposed that a single peptide-MHC class I complex might bind to two KIR molecules, providing a possible mechanism for signaling. However, our results suggest that sKIR2DL3 binds to soluble HLA-Cw7-DS11 in a 1:1 complex. First, free sKIR2DL3 but no free HLA-Cw7-DS11 is detected when sKIR2DL3 and HLA-Cw7-DS11 are mixed in a ratio 1.5:1. Second, the sKIR2DL3/HLA-Cw7-DS11 complex migrated on gel filtration at the position expected for a 1:1 complex. Third, SDS-polyacrylamide gel electrophoresis of the peak complex indicated that there were equimolar amounts of sKIR2DL3 and HLA-Cw7 heavy chain (data not shown). Finally, the standard Langmuir 1:1 binding model fits very well to the equilibrium binding and kinetic data. We cannot exclude a second sKIR2DL3 site on HLA-Cw7 with a much lower affinity (e.g. Kd > 200 μm). However, such a second site would need to achieve a much higher “physiological” affinity at the cell/cell interface in order to contribute to KIR2DL3 binding (35).

The 1:1 binding stoichiometry suggests that, despite some structural similarities between KIRs and hematopoietic receptors, they do not bind their ligands in the equivalent manner. Indeed, comparison of the recently determined KIR2DL3 crystal structure with Ig superfamily and fibronectin type III domains indicates that KIRs bear a closer resemblance to Ig domains than to the fibronectin type III domains of hematopoietic receptors (30).

Affinity, Kinetics, and Thermodynamics—The affinity measured here between soluble forms of KIR2DL3 and the HLA-Cw7-DS11 peptide-MHC complex agrees well with the affinity measured independently by Valés-Gómez et al. (24) (Kd ~ 9 μm) for sKIR2DL3 binding HLA-Cw7-DS11. Valés-Gómez et al. (24) measured a similar affinity (Kd ~ 10 μm) between soluble forms of sKIR2DL1 and HLA-Cw6-peptide. This consistency between completely independent studies suggests that these affinity measurements are likely to be correct.

These affinities are well within the range of affinities measured for many other cell-cell recognition molecules, including TCR/peptide-MHC interactions (Table III). However TCR/peptide-MHC interactions differ from other cell-cell molecule interactions in that low affinity of TCR binding is a consequence of a relatively slow koff, rather than a fast kon. Unlike Vales-Gómez et al. (23, 24), we were able to obtain precise estimates of the binding kinetics of the sKIR2DL3/HLA-Cw7-DS11 interaction. This revealed that, in contrast to TCR/peptide-MHC interactions, the low affinity is a consequence of a faster koff, whereas the kon is unremarkable, being typical of other cell surface protein/protein interactions (Table III). Furthermore, the KIR binding kinetics did not show the strong temperature dependence observed with TCR binding.

Further differences between KIR/peptide-MHC and TCR/peptide-MHC interactions were evident in thermodynamic studies. Unlike TCR binding, which is characterized by large, unfavorable entropic changes compensated for by even larger

![Fig. 4. A, thermodynamic analysis of sKIR2DL3 binding to HLA-Cw7-DS11. Measurement of enthalpy by van’t Hoff analysis (ΔH_m) of affinity constants for the sKIR2DL3/HLA-Cw7-DS11 interaction were measured at several temperatures (5–30 °C) and converted into the standard free energy of binding (ΔG°). Values for the enthalpic (ΔH_m) and standard entropic (−TΔS°) changes at 25 °C and the specific heat capacity (C_p) were derived by fitting the non-linear form of the van’t Hoff equation to these data (see “Experimental Procedures”). B, comparison of thermodynamic properties (at 25 °C) of several macromolecular interactions. The values for protein/protein interactions (excluding antibody/protein interactions) are the mean and S.E. of 30 calorimetric determinations taken from Ref. 33. Data for TCR/peptide-MHC interactions are the mean and range of two determinations, one of which was by calorimetry (32).

| Interaction          | kon  | koff | Kd  | Ref. |
|----------------------|------|------|-----|------|
| TCR/peptide-MHC      |      |      |     |      |
| KIR2DL3/HLA-Cw7-DS11 | ~200 | ~1.2 | ~90 | 32, 36 |
| KIR2DL1/HLA-Cw6      | >200 | >2   | ~7  | 24    |
| CD8a/MHC class I     | >100 | >18  | ~200| 28    |
| CD2/CD58             | >400 | >4   | ~10 | 38    |
| Mouse CD48/CD2       | ~120 | >11  | ~90 | 39    |
| Mouse CD48/2B4       | >200 | <3   | ~15 | 40    |
| CD28/CD80            | >660 | >1.6 | ~4  | 41    |
| CTLA-4/CD80          | >940 | >0.4 | 0.46| 41    |

![Graph showing thermodynamic analysis of sKIR2DL3 binding to HLA-Cw7-DS11.](image-url)
favorable enthalpic changes, KIR binding is driven by favorable entropic and enthalpic changes at 25 °C (Fig. 4B). The latter thermodynamic characteristics are typical of protein/protein interactions (Fig. 4B) including low affinity interactions between cell-cell recognition molecules, such as the CD2/CD48 interaction.2

A key finding in this study is that TCRs and KIRs, although recognizing overlapping portions of peptide-MHC class I molecules, bind with very different kinetic and thermodynamic properties. The KIR/peptide-MHC interaction has binding properties consistent with rigid body association, whereas TCR binding has been shown to require conformational adjustments (37) and is likely to be accompanied by a reduction in conformational flexibility (32). The difference in the binding properties of KIRs and TCRs for very similar ligands supports the suggestion the TCR and not the peptide-MHC are the primary source of conformational flexibility. We have proposed that these binding properties arise from the structure of TCR antigen-binding sites, and that KIR binding is driven by favorable enthalpic changes, KIR binding is driven by favorable enthalpic changes.

Cross-reactivity of KIR2DL1 with HLA-Cw7—Although KIR2DL molecules are grouped according to whether they bind to group 1 (KIR2DL1) or group 2 (KIR2DL2 and -L3) HLA-C alleles, recent data suggest that there is some cross-reactivity. KIR2DL2 and -L3 have been shown to bind to, and reactivity. KIR2DL2 and -L3 have been shown to bind to, and cannot be inherited, they are denied the opportunity, available to other ligand-binding proteins, to acquire a more stable tertiary structure during the course of evolution.

Conclusion—We show here that a KIR binds peptide-MHC with a 1:1 stoichiometry and that the thermodynamic and kinetic features of this interaction are typical of cell/cell recognition molecules and consistent with rigid body association. This contrasts with TCR/peptide-MHC interactions, which cannot be inherited, they are denied the opportunity, available to other ligand-binding proteins, to acquire a more stable tertiary structure during the course of evolution.

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2 J. Ladbury, P. A. van der Merwe, and S. J. Davies, unpublished data.