Supplementary Information

Equilibrium SPR Analysis

We analyzed the plots in **Fig. 2** using the Hill equation (1):

$$R = Rmin + (Rmax - Rmin)\frac{[m157]^{nH}}{K_{0.5}+[m157]^{nH}} \quad (1)$$

where *R* is the response in RU of the SPR assay; *Rmin* and *Rmax* are the minimum and maximum response values in RU, respectively; *[m157]* is the free m157 molar concentration (in SPR experiments, the bound fraction is negligible compared to the free ligand, and consequently *[m157] total ~ [m157] free*); $K_{0.5}$ is an apparent dissociation constant; and *nH* is the Hill coefficient.

Fluorescence Anisotropy Equilibrium Analysis

Using polarizers, it is possible to record the emission that is parallel ($I_{//}$) and perpendicular ($I_{\perp}$) to the excitation beam. When a fluorescent molecule is excited with a polarized beam, the fluorescence emission is also polarized. However, the extent of polarization of this emission decreases as the fluorescent molecule rotates in solution. As a consequence, large proteins yield a greater extent of polarization and the values of $I_{//}$are typically higher than $I_{\perp}$. Accordingly, when an interaction in solution of a labeled protein (Ly49-FITC) with a non-labeled protein (m157) occurs, the complex tumbles slower than Ly49H-FITC alone and consequently more polarization is registered. The degree of polarization is quantified as the anisotropy (*r*), and is equal to $r = \frac{I_{//}-GI_{\perp}}{I_{//}+2GI_{\perp}}$, where *G* is a correction factor $G = \frac{S_V}{S_H}$ that accounts for the different sensitivities of detection for vertically (S_V) and horizontally (S_H) polarized light (2).

To obtain an equation that describes the anisotropy (*r*) as a function of total m157, we first derived the partition function according to the following reaction scheme:

$$\text{Ly49} + \text{m157} \rightleftharpoons \text{Ly49m157}$$

$$Ks1 = \frac{[Ly49m157]}{[Ly49][m157]} = 2\,k = KG1 \quad (2)$$

$$\text{Ly49m157} + \text{m157} \rightleftharpoons \text{Ly49(m157)}_2$$

$$Ks2 = \frac{[Ly49(m157)_2]}{[Ly49m157][m157]} = \frac{k'}{2} \quad (3)$$

Global:

$$\text{Ly49} + 2\,\text{m157} \rightleftharpoons \text{Ly49(m157)}_2$$

$$KG2 = Ks1\,Ks2 \quad (4)$$

where *Ks1* and *Ks2* are the sequential macroscopic binding constants for the first and second site, respectively; *KG1* and *KG2* are the global macroscopic constants, such that *KG1=Ks1* and *KG2=Ks1.Ks2*; and *k* and *k´* are the microscopic binding constants for the first and second site, respectively. It should be pointed out that the union of the first m157 to one of the protomers is identical in nature to the union to the other protomer. This was assumed because Ly49s are symmetrical homodimers (3), and supports that *Ks1=2k* and *Ks2=k´/2*. We then derived the following equation system (4, 5):

1. Partition function, Ξ:

$$\Xi = 1 + KG1[m157] + KG2[m157]^2 \quad (5)$$

2. Bound ligand density, $\bar{\nu}$:

$$\bar{\nu} = \frac{[m157]}{\Xi}\left(\frac{\partial \Xi}{\partial [m157]}\right) = \frac{KG1[m157]+2KG2[m157]^2}{1+KG1[m157]+KG2[m157]^2} \quad (6)$$

3. Mass balance for m157:

$$[m157]free = [m157]total - \bar{\nu}[Ly49]total \quad (7)$$

4. Relationship between the fluorescence anisotropy signal, *r*, and $\bar{\nu}$:

$$r = rmin + (rmax - rmin)\,\bar{\nu} \quad (8)$$

where *rmin* and *rmax* are the minimum (in the absent of m157) and maximum (from extrapolation to [m157]→∞) fluorescence anisotropy signals measured, respectively. We solved this equation system to obtain an expression that links *r* with total m157 concentration. The resulting equation was fitted to the experimental points via a nonlinear least squares method, utilizing the Levenberg-Marquard algorithm (6).

Determination of the Ly49I–m157 Stoichiometry

The normalized signal Q was calculated using the following expression:

$$Q = \frac{r - rmin}{rmin} \quad (9)$$

where *r* is the anisotropy at each m157 concentration evaluated and *rmin* is the anisotropy of free Ly49. Then, a plot of Q versus total *[m157]* was done (data not shown), including both titrations at different Ly49 concentrations. The values of $\bar{\nu}$ were calculated with expression 10, considering that at every parallel line to the x-axis traced (implying constant Q), the free m157 concentration is the same for each curve at a different Ly49 concentrations:

$$\bar{\nu} = \frac{[m157]total_1 - [m157]total_2}{[Ly49]total_1 - [Ly49]total_2} \quad (10)$$

where *[m157]total*$_1$ and *[m157]total*$_2$ are the total molar concentrations of m157 that give the same *Q* response for each fluorescence anisotropy assay done with *[Ly49]total*$_1$=1.0 µM and *[Ly49]total*$_2$=2.6 µM, respectively.

Kinetic SPR Analysis

Fig. 6A and **6B** show the association and dissociation SPR data, respectively, for Ly49H–m157 and Ly49I–m157 at 25 °C fitted with two exponential functions of the form:

$$R = R_0 + A_1\left(1 - e^{-k_{obs1}t}\right) + A_2\left(1 - e^{-k_{obs2}t}\right) \quad (11)$$

for the association phase, and:

$$R = R_0 + A_1\left(e^{-k_{obs1}t}\right) + A_2\left(e^{-k_{obs2}t}\right) \quad (12)$$

for the dissociation phase, where *R* is the SPR response in RU, A_1 and A_2 are the amplitudes of the exponential functions, k_{obs1} and k_{obs2} are the observed apparent kinetic rate constants, and R_0 is the response at *t*=0.

In **Fig. 6C**, we plotted the first derivative of the response as a function of the response for the Ly49H–m157 and Ly49I–m157 association data, according to the expression (7):

$$\frac{dR}{dt} = k_{on}[m157]Rmax - \left(k_{on}[m157] + k_{off}\right)R \quad (13)$$

where *R* is the response of the SPR signal in RU; *Rmax* is the maximum response obtained; k_{on} and k_{off} are the kinetic parameters of association and dissociation, respectively; *t* is the time in seconds; and *[m157]* is the total m157 molar concentration. This function should give a straight line if the interaction is a simple 1:1 binding, where the slope is $-(k_{on}[m157]+k_{off})$ and intercepts the y axis at $k_{on}[m157]Rmax$. This is not the

case for any of the pairs dealt with in this paper, and the plots describe a biphasic behavior. Thus, the parameters k_{on} and k_{off} do not hold a defined physical sense in these cases and should be taken as phenomenological descriptors.

In **Fig. 6D**, the natural logarithm of the response versus time for the dissociation data is shown for the two couples analyzed (7):

$$\ln\left(\frac{R_0}{R}\right) = k_{off}t \qquad (14)$$

where R is the response of the SPR signal in RU, R_0 is the response in RU when the dissociation process begins triggered by the injection of buffer alone (*[m157]=0*), k_{off} is the kinetic parameter of dissociation, and t is the time in seconds. Again, this function should yield a straight line with slope k_{off} if the interaction is a simple 1:1 binding but, instead, it renders a biphasic curve.

The equation that describes the conformational selection model follows the form (8, 9):

$$k_{obs1} = k_1 + \frac{k_{-1}}{1+K2[m157]} \qquad (15)$$

where the different constants are indicated over the different steps of the following scheme:

$$\text{Ly49} \overset{\text{K2}}{\rightleftharpoons} \text{Ly49*+m157} \underset{\text{k}_{-1}}{\overset{\text{k}_1}{\rightleftharpoons}} \text{Ly49*m157}$$

One-to-one interaction of the Ly49*m157 complex with a second m157 ligand follows the function (8, 9):

$$k_{obs2} = k_2[m157] + k_{-2} \qquad (16)$$

where the different constants are indicated over the different steps of the following scheme:

$$\text{Ly49*m157+m157} \underset{\text{k}_{-2}}{\overset{\text{k}_2}{\rightleftharpoons}} \text{Ly49*(m157)}_2$$

Selection criteria to determine binding mechanisms

To decide which of the models displayed in **Figure 7** is the most appropriate for the experimental data obtained, we performed several statistic calculations, including chi square (χ^2/n), modified Akaike criterion (MSC), Bayesian selection criterion (BIC), and Hannan-Quinn information criterion (HQIC). The equations for these statistics are as follows:

MSC. Modified Akaike Criterion [(10, 11) Scientist Handbook 1995, Akaike, 1976]:

$$MSC = \ln\left({}^{\Phi}/_{\chi^2}\right) + 2\frac{m}{n}$$

BIC. Bayesian Selection Criterion [(12, 13, 14) Buckwitz1990, Schwarz 1978, Davidian 1993]:

$$BIC = (\chi^2 + m\ln n)/n$$

HQIC.Hannan-Quinn Information Criterion [(14, 15)Davidian1993, Hannan 1987]:

$$HQIC = (\chi^2 + 2m\ln(\ln n))/n$$

where:

$$\chi^2 = \sum_{i=1}^{n}(y_i^e - y_i^t)^2/\sigma_i^2$$

$$\Phi = \sum_{i=1}^{n} (y_i^e - \bar{y})^2$$

$$\bar{y} = \sum_{i=1}^{n} \frac{y_i^e}{n}$$

σ_i = standard deviation of the experimental point *i*
n = number of experimental points
y_i^e = experimental point *i*
m = number of free parameters in the model
y_i^t = value for the best fit to a point *i*, predicted by the model
A maximum value for MSC; and minima for χ^2/n, BIC and HQIC indicate the most appropriate model.

To elucidate whether there are equally valid models to describe the interaction, we applied the Zwanzig selection criterion between two different models *u* and *v*. The equations are as follows:

T_{uv}.ZwanZig Selection Criterion between models *u* and *v* [(12, 16)Zwanzig 1980, Buckwitz 1990]:

$$T_{uv} = \sqrt{\frac{n}{4}} \frac{Q_u - Q_v}{\sqrt{\frac{1}{n}\sum_{I=1}^{n} \omega_i^2 \sigma_i^2 (u_i - v_i)^2}}$$

where:

$$Q_u = \frac{1}{n}\sum_{I=1}^{n} \omega_i \, (y_i^e - u_i)^2$$

$$Q_v = \frac{1}{n}\sum_{I=1}^{n} \omega_i \, (y_i^e - v_i)^2$$

ω_i = weight factor for point *i* (ω_i = 1)
σ_i = standard deviation of experimental point *i*
n = number of experimental points
y_i^e = experimental point *i*
u_i; v_i= value for the best fit to point *i*, predicted by the model
Tuv > 1.96 implies that model *v* is more appropriate than model *u* (significance level: α=0.05)
|*Tuv*| ≤ 1.96 means that there is no significant difference between the two models under consideration
Tuv <- 1.96 means that model *u* is more appropriate than model *v* to explain the data (significance level: α=0.05)

Employing the latter, we compared all the models with model D. If *Tuv* > 1.96, model *v* is more appropriate than model *u;* if |*Tuv*| ≤ 1.96, there are no significant differences between the two models; if *Tuv* < -1.96, model *u* is more appropriate than model *v* to describe the binding.

Thermodynamic Analysis of Ly49–m157 Interactions

Eyring equation (Eq. 17) was fitted to the experimental points at the reference temperature of 25 °C, to render the activation parameters $\Delta H^0‡$ (activation enthalpy), $\Delta S^0‡$ (activation entropy) and $\Delta Cp^0‡$ (activation heat capacity) (**Table S3**), according to classical transition state theory of absolute reaction rates (17):

$$ln\left(\frac{kh}{k_B T}\right) = \frac{\Delta S^0‡}{R} - \frac{\Delta H^0‡}{RT} + \frac{\Delta Cp^0‡}{R}\left[ln\left(\frac{T}{T_R}\right) + \frac{T_R}{T} - 1\right] \quad (17)$$

where *h* is the Planck´s constant (6.63 x 10^{-34} J.s); k_B is Boltzmann´s constant (1.38 x 10^{-23} J.K^{-1}); *R* is the gas constant (1.98 cal $mol^{-1}K^{-1}$); *T* is the absolute temperature in Kelvin degrees; *k* is the kinetic rate constant analyzed in each case; T_R is the reference temperature (25 °C); and the parameters $\Delta H^0‡$, $\Delta S^0‡$ and $\Delta Cp^0‡$

are the activation enthalpy, entropy and heat capacity, respectively. The activation free energy $\Delta G^{0}\ddagger$ was calculated according to equation 18, from the kinetic rate constants (17):

$$\Delta G^{0} \ddagger = -RTlnk \qquad (18)$$

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Table S1. Ly49H-m157 statistical selection criteria parameters for the different models proposed.

Model	χ^2/n	MSC	BIC	HQIC	T_{xE2}
A	,38024552	,8767885	38178685	,3809481	59,8182106
B	,38024552	,8767885	38178685	,3809481	59,8182106
C	,61270707	68891822	,6142484	61340965	0,19286815
D	,61261307	68907165	,6141544	61331565	nc
E	,78341326	44314483	78495459	78411584	37,2134178
F	,91040019	29292091	91194152	91110277	39,5579087
G	1,1790796	03431576	18062093	17978218	60,520219
H	,93828896	26274721	93983029	93899154	41,9460656
I	,62633954	66691255	62788087	62704212	26,5286033

Table S2. Ly49I-m157 statistical selection criteria parameters for the different models proposed.

Model	χ^2/n	MSC	BIC	HQIC	T_{xE2}
A	0,91018138	3,96488671	0,91172271	0,91088396	68,4475527
B	0,91018138	3,96488671	0,91172271	0,91088396	68,4475527
C	0,38759648	4,8185658	0,38913781	0,38829906	-6,2477651
D	0,39344721	4,80358371	0,39498854	0,39414979	nc
E	0,4506049	4,66793971	0,45214623	0,45130748	23,1156578
F	0,5865461	4,40427934	0,58808743	0,58724868	43,4491859
G	0,56519712	4,44135605	0,56673845	0,5658997	39,1966106
H	0,48895381	4,58626257	0,49049515	0,4896564	28,7590971
I	0,49241134	4,57921619	0,49395267	0,49311392	33,1945277

Table S3. Ly49H-m157 and Ly49I-m157 activation free energy ($\Delta G^0‡$), enthalpy ($\Delta H^0‡$), entropy at 25 °C ($-T\Delta S^0‡$) and heat capacity ($\Delta Cp^0‡$) for each step of the model D, estimated using Eyring equation.

	Ly49H-m157	Ly49I-m157
	ka1	
$\Delta G^0‡$ (kcal/mol)	7±2	4±1
$\Delta H^0‡$ (kcal/mol)	30±20	23±8
$-T\Delta S^0‡$ (kcal/mol)	-30±20	-20±10
$\Delta Cp^0‡$ (cal/mol)	4000±3000	2000±1000
	kd1	
$\Delta G^0‡$ (kcal/mol)	2.3±0.2	6.23±0.08
$\Delta H^0‡$ (kcal/mol)	-10±8	-29±9
$-T\Delta S^0‡$ (kcal/mol)	16±9	40±10
$\Delta Cp^0‡$ (cal/mol)	-2000±1000	-2000±1000
	ka2	
$\Delta G^0‡$ (kcal/mol)	-4.83±0.03	-5.75±0.03
$\Delta H^0‡$ (kcal/mol)	-19±5	-11±1
$-T\Delta S^0‡$ (kcal/mol)	20±5	4.8±0.7
$\Delta Cp^0‡$ (cal/mol)	-2100±500	-1100±200
	kd2	
$\Delta G^0‡$ (kcal/mol)	-0.270±0.003	0.548±0.003
$\Delta H^0‡$ (kcal/mol)	-19±8	-31±7
$-T\Delta S^0‡$ (kcal/mol)	23±8	31±7
$\Delta Cp^0‡$ (cal/mol)	-2000±1000	-3000±900
	ka3	
$\Delta G^0‡$ (kcal/mol)	-8.4±0.1	-7.38±0.05
$\Delta H^0‡$ (kcal/mol)	-5±4	-25±9
$-T\Delta S^0‡$ (kcal/mol)	7±4	17±7
$\Delta Cp^0‡$ (cal/mol)	-1300±500	-3000±1000
	kd3	
$\Delta G^0‡$ (kcal/mol)	3.1±0.2	3.67±0.03
$\Delta H^0‡$ (kcal/mol)	-20±10	-21±4
$-T\Delta S^0‡$ (kcal/mol)	20±10	24±5
$\Delta Cp^0‡$ (cal/mol)	-2000±1000	-2400±500