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A Peptide Antagonist Disrupts NK Cell Inhibitory Synapse Formation

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Productive engagement of MHC class I by inhibitory NK cell receptors depends on the peptide bound by the MHC class I molecule. Peptide:MHC complexes that bind weakly to killer cell Ig-like receptors (KIRs) can antagonize the inhibition mediated by high-affinity peptide:MHC complexes and cause NK cell activation. We show that low-affinity peptide:MHC complexes stall inhibitory signaling at the step of Src homology protein tyrosine phosphatase 1 recruitment and do not go on to form the KIR microclusters induced by high-affinity peptide:MHC, which are associated with Vav dephosphorylation and downstream signaling. Furthermore, the low-affinity peptide:MHC complexes prevented the formation of KIR microclusters by high-affinity peptide:MHC. Thus, peptide antagonism of NK cells is an active phenomenon of inhibitory synapse disruption. The Journal of Immunology, 2013, 190: 2924–2930.

Natural killer cells are an important component of the innate immune system that provide a rapid immune response through cytokine secretion and direct lysis of stressed, infected, or transformed cells (1). Their functions are controlled by a balance of signals transduced by activating and inhibitory receptors. The inhibitory receptors include killer cell Ig-like receptors (KIRs), CD94:NKG2A, the leukocyte Ig-like receptors, and NKR-P1. The KIR and CD94:NKG2A receptors have MHC class I ligands. During infection or tumorigenesis, MHC class I may be downregulated, leading to loss of inhibitory signals (2). KIR specificity for MHC class I is determined by oligomeric motifs on MHC class I, such as the Bw4 motif for KIR3DL1, or residue 80 for the HLA-C–specific inhibitory KIR (3). In addition, these receptors are sensitive to the peptide bound by MHC class I, and so inhibition of NK cells expressing specific KIR may be mediated by only a subset of expressed peptide:MHC complexes (4–9). In particular, the inhibitory KIRs, KIR2DL2, and KIR2DL3 recognize a subset of HLA-C alleles with an asparagine at position 80, and binding of these receptors to HLA-C is modulated by residues 7 and 8 of the bound peptide. In general, large hydrophobic residues are permissive at P7 and small residues permissive at P8 (7, 10). We have recently shown that a peptide variant, which by itself does not inhibit KIR2DL2/3–positive NK cells can antagonize the inhibition owing to a peptide that strongly inhibits NK cells, as opposed to being functionally neutral (10). This finding suggests that NK cells could be sensitive to small changes in peptide repertoire, in addition to MHC class I downregulation.

Following engagement of cognate MHC class I on a target cell, the KIRs form microclusters at the inhibitory immune synapse (11). Inhibitory signaling by KIRs is subsequently determined by the presence of ITIMs (V/I/LxYxxL/V) in their cytoplasmic tails. Phosphorylation of these ITIMs leads to recruitment of Src homology protein tyrosine phosphatase 1 or 2 (SHP-1/2) (12–15). SHP-1/2 dephosphorylates Vav and leads to a block in membrane-proximal NK cell activation signals (16). This block is thought to precede actin cytoskeletal rearrangement (17, 18). Recent work has shown that the activating receptors 2B4 and CD2 can colocalize at inhibitory synapses, with inhibitory KIRs indicating that inhibitory signals do not prevent recruitment of at least some activating receptors to the immune synapse (19). However, evidence exists that they may alter the membrane organization of some receptors, such as NKG2D (17). Our previous work has shown that an antagonist peptide bound to MHC class I can recruit inhibitory KIRs to the contact area between effector and target cell but does not induce inhibitory signaling (10). Thus inhibitory signaling can be fine-tuned by the peptide:MHC complexes presented to NK cells. In this study, we set out to investigate the mechanism by which KIRs engaged by antagonist peptide:MHC complexes interfere with inhibitory signaling.

Materials and Methods

Cell lines and culture

We used, as target cells, a TAP-deficient cell line 721.174 (20), which was pulsed exogenously at 26°C with VAPWNSFAL (FA), VAPWNSDAL (DA), or an equal mix of both peptides (Peptide Protein Research, Hampshire, U.K.). NKL cells, which lack KIR expression (with the exception of KIR2DL4), have been transfected with a functional KIR2DL3 (NKL-2DL3) or an ITIM-mutated KIR2DL3 (NKL-2DL3.YF), both conjugated to enhanced GFP (eGFP). In the ITIM-mutated KIR2DL3, tyrosines in positions 282 and 312 (Y282 and Y312) have been replaced by a phenylalanine. The KIR2DL3–GFP fusion constructs were generated by...
subcloning RT-PCR–amplified cDNAs encoding KIR2DL3 into plasmid pcDNA3.1 (Invitrogen, Life Technologies, Paisley, U.K.) already containing the eGFP sequence. Substitution of KIR2DL3 residues Y282 and Y312 with phenylalanine was achieved by sequential site-directed mutagenesis by PCR, using the Expand High Fidelity PCR System and dNTPack (Roche Diagnostics, Burgess Hill, U.K.). For expression in NKL cells, KIR2DL3-GFP was cloned into the retroviral vector pB2, and the Phoenix packaging cell line was used to produce retroviral particles.

The 721.174 cells were cultured in R10 medium [RPMI 1640 medium supplemented with 1% penicillin/streptomycin (Invitrogen) and 10% FBS (Globepharm, Guildford, UK)], whereas the NK cell lines—NKL, NKL-2DL3, and NKL-2DL3.2YF—were cultured in R10 medium supplemented with 100 IU recombinant IL-2 (gift from National Cancer Institute Bio-Metric Research Branch, Bethesda, MD) and the PhoPack retroviral packaging cell line (Addgene, Cambridge, MA), then coincubated at a 2:1 E:T ratio with either NKL-2DL3 or NKL-2DL3.2YF in one well of a chambered cover slide. Live cells were imaged at 37°C, 5% CO2, with R10 as the imaging medium by live cell microscopy with laser lines of 488 nm and a 63 μm oil immersion objective (TCS SP2 RS; Leica). Images were acquired (every 5 s) with Leica Application Suite Advanced Fluorescence software (Leica Microsystems, Milton Keynes, U.K.). Images were acquired (every 5 s) with Leica Application Suite Advanced Fluorescence Software and analyzed with Leica Confocal Software (both from Leica).

KIR2DL3 accumulation

The 721.174 cells were cultured with 10 μM peptide overnight at 26°C. NKL cells were stained with 1 μM DiO (Vybrant DiO; Invitrogen) in RPMI 1640 medium for 30 min at 37°C, then washed and resuspended in R10 medium. Next, 721.174 was mixed at a 2:1 E/T with DiO-stained NKL, NKL-2DL3, or NKL-2DL3.2YF in one well of a chambered cover slide. Live cells were imaged at 37°C, 5% CO2, with R10 as the imaging medium by resonance scanning confocal microscopy with laser lines of 488 nm and a 63 × 1.2 numerical aperture water immersion objective (TCS SP5 RS; Leica Microsystems, Milton Keynes, U.K.). Images were acquired (every 5 s) with Leica Application Suite Advanced Fluorescence Software and analyzed with Leica Confocal Software (both from Leica).

Statistical analysis

All statistical analyses were performed using GraphPad Prism, version 5 (GraphPad Software, La Jolla, CA).

Results

An antagonistic peptide disrupts inhibitory signaling

We have previously shown that a variant of the naturally processed tissue inhibitor of metalloproteinase 1–derived, HLA-Cw*0102–restricted nonamer peptide VAPWSFAL (FA) is a strong inhibitor of KIR2DL3+ primary NK cells and that a second variant VAPWNSDAL (DA) acts as a peptide antagonist for this subset (10). On KIR2DL3+ primary NK cells, the increase of membrane CD107a expression (a marker of degranulation) due to HLA-Cw*0102+–positive, TAP-deficient 721.174 B cells is lost in the presence of FA (FA 174, Fig. 1A, 1B). In contrast, DA does not modify membrane CD107a expression (DA 174) and further prevents the FA-induced decrease (DA:FA 174). To understand how these peptides affect NK cell signal transduction, we analyzed the responses of NK cells transfected with KIR2DL3 (NKL-2DL3) to 721.174 cells, presenting FA and DA peptides on HLA-Cw*0102 (20). Because NK cells constitutively express membrane CD107a, we could not use surface expression of this molecule as a readout of NK cell activity (Supplemental Fig. 1). We therefore measured the duration of intercellular contacts as a marker of productive engagement between NK and 721.174 target cells by live cell imaging, as previous work has established that contact times are shorter if a productive inhibitory interaction takes place (21). No significant difference was found in the contact times between untransfected NKL 721.174 cells and 721.174 cells in the presence or absence of peptide (Fig. 1C). In contrast, for NKL-2DL3 the strong KIR-binding peptide FA dramatically reduced the contact time between NKL-2DL3 and 721.174 cells, compared with no peptide or the antagonist peptide DA (from 279 ± 29 s to 169 ± 13 s, Fig. 1D). This decrease was unabrogated using an equimolar mix of DA and FA, consistent with our previous data showing that DA can antagonize the inhibition due to FA. Moreover these differences in contact time were dependent on inhibitory signaling, as they were unabrogated in the NKL cell line transfected with a KIR2DL3 construct in which the tyrosine residues within the two ITIMs had been mutated to phenylalanine (NKL-2DL3.2YF) (Fig. 1E).

As Vav1 is the primary substrate of SHP-1, we investigated the effects of the DA/FA mix on inhibitory signaling. Consistent with our observations on contact time, FA alone induced dephosphorylation of the SHP-1 substrate Vav1, whereas the addition of DA to FA restored Vav1 phosphorylation (Fig. 2A, 2B). Similarly, downstream signaling through Erk1 and Erk2, required for actin reorganization and NK cell degranulation (22, 23), is reduced in response to FA alone, but restored in the agonist:antagonist FA: DA peptide mix (Fig. 2C, 2D). Furthermore, titration of the peptide concentrations within the agonist:antagonist mix resulted in significantly greater Vav1 phosphorylation in the presence of DA than in its absence (Fig. 3A, 3B). Overall, we found that in the presence of small amounts of FA peptide, there was a substantial decrease in Vav1 phosphorylation, which was significantly decreased in the presence of the DA peptide (Fig. 3C). These data are similar to those observed for titrations of the same peptides in CD107a assays of primary NK cells (10). In those experiments, in the presence of the antagonist peptide, a linear decrease in Vav1 phosphorylation occurred that was not observed for the single peptide. Similarly, in these experiments linear regression analysis of the data in Fig. 3C demonstrated that a linear decrease in Vav1 phosphorylation occurred for the peptide mix experiments (r² = 0.94), but not for the single FA peptide experiments (r² = 0.67),
for which a one-phase decay curve provided a superior fit ($r^2 = 0.97$) (Supplemental Fig. 2).

An antagonistic peptide disrupts tight clustering of KIRs at the inhibitory synapse

To investigate how DA might affect the initiation of inhibitory signaling, we compared the inhibitory synapses formed in the presence of DA, FA, or the FA:DA peptide mix. We used NKL cells expressing KIR2DL3 C-terminally tagged with eGFP to determine the distribution of KIR2DL3 at the immune synapse between target and effector cells. The increase in intensity of eGFP fluorescence at the contact area, as compared with a noncontact area, was then determined. The addition of FA, DA, or the DA:FA mix induced similar levels of KIR2DL3 accumulation at the contact area between NK cells and 721.174 targets (Fig. 4A: 1.58 ± 0.04–, 1.54 ± 0.04–, and 1.55 ± 0.03–fold, respectively).

An antagonistic peptide disrupts tight clustering of KIRs at the inhibitory synapse

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**FIGURE 1.** The DA peptide antagonizes the inhibition of NK cells owing to the FA peptide. (A and B) CD107a assay gated on CD3-CD56+ NK cells using 721.174 either unloaded (No Pep) or loaded with the indicated peptides. A dot plot from one representative experiment is shown in (A) and the mean value ± SEM of three experiments in (B). (C-E) Unpulsed 721.174 cells (No Pep) or cells loaded with FA, DA, or both peptides at an equimolar ratio (DA/FA) were used as target cells for NKL (C), NKL-2DL3 (D) or NKL-2DL3.2YF (E) cells in live-cell imaging experiments. Movies were recorded for 40 min in a 37 °C chamber by confocal microscopy. The contact times of 36–63 conjugates were determined and plotted for each peptide condition. The mean contact time in seconds ± SEM is shown (gray bars). Data were analyzed using one-way ANOVA and a Newman–Keuls posttest for multiple comparisons. For all panels, significant p values are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 2.** A peptide antagonist abrogates inhibitory signaling. Analysis of cell lysates for Vav1 and Erk signaling by Western blotting. The 721.174 cells were incubated without exogenous peptide (No Pep, lane 2), or 721.174 cells loaded with DA (DA, lane 3), FA (FA, lane 4), or both peptides (DA/FA, lane 5) were incubated with NKL-2DL3 for 5 min at 37 °C. Cell lysates were analyzed by Western blot for pVav1 (A, B) and pErk1/2 (C, D) phosphorylation as well as for total Vav1 (A, bottom blot) and β-actin (C, bottom blot). Lysates of NKL-2DL3 (lane 1) and 721.174 (lane 6) alone were used to determine the basal level of phosphorylation in the two cell lines. Blots from one representative experiment are shown for Vav1 (A) and Erk1/2 (C). Data from three independent experiments (Vav1, B) or eight independent experiments (Erk1/2, D) were used to calculate the ratio of phosphorylation (p-Vav1/Vav1, p-Erk1/β-actin, or p-Erk2/β-actin) using ImageJ software and plotted as mean value ± SEM. The ratio of NKL-L3 (lane 1) and 721.174 (lane 6) is shown to demonstrate the basal ratios of phosphorylation in the cell lines (B, D). Data were analyzed using one-way ANOVA and a Newman–Keuls posttest for multiple comparisons. For all panels, significant p values are as follows: *p < 0.05.
above random fluctuations of fluorescence within the membrane. Differences in the percentage of conjugates demonstrating a ≥1.5-fold increase in KIRs at the immune synapse did not significantly vary between FA, DA, and the DA:FA mix (Fig. 4B). Therefore, the amount of KIR recruited to the immune synapse was similar under inhibitory or antagonistic conditions.

**FIGURE 3.** Comparison of inhibition of Vav1 phosphorylation in the presence or absence of antagonist peptide. Analysis of cell lysates for Vav1 by Western blotting. The 721.174 cells were incubated without exogenous peptide, or in the presence of various concentrations of the inhibitory peptide (FA) in the presence (A) or absence (B) of the antagonist peptide DA, and the lysates were assayed for Vav1 phosphorylation. The peptide concentrations for each condition in micromolar units are indicated. The mean and SEMs of three independent experiments, normalized to Vav1 phosphorylation in the absence of peptide, are shown in (C). For the FA+DA columns, the DA peptide was added to make the total peptide concentration 20 μM, as indicated in (A) lanes 2–7; and for the FA-alone columns, peptide concentrations are as in (B) lanes 2–7. Data at each concentration were compared using one-way ANOVA and a Newman–Keuls posttest for multiple comparisons. Significantly different levels of Vav1 phosphorylation between FA alone and FA+DA at the same concentrations of the FA peptides are indicated with **p < 0.01, ***p < 0.001. Other comparisons were nonsignificant.

**FIGURE 4.** An antagonistic peptide disrupts tight clustering of KIR2DL3 at the inhibitory synapse. (A–E) Unpulsed 721.221 cells (No Pep) or cells loaded with control peptide (GILG), or with FA, DA, or both FA and DA (DA/FA) peptides, were used as target cells for NKL-2DL3. After a 10-min coinoculation at 37˚C, the conjugates were fixed and imaged by confocal microscopy. (A) The GFP intensity of 86–116 conjugates from three independent experiments was quantified for each peptide condition. KIR2DL3 accumulation was determined by comparing the intensity at the interface between the effector and target cells with that of the NKL-2DL3 plasma membrane at a noncontact area, and the data were plotted as a fold increase in intensity. The mean fold increase in intensity ± SEM is shown by red bars. Data were analyzed using one-way ANOVA and a Newman–Keuls posttest to compare individual groups. No significant differences were noted between the DA, FA, and DA/FA conditions. (B) With use of the same experiments as for (A), the percentage of conjugates showing a KIR2DL3 accumulation with a fold increase ≥ 1.5 was determined. Data were analyzed by a Student t test between GILG and other conditions. (C) Confocal microscopy image of 721.174 and NKL-2DL3 conjugates. The 721.174 cells were loaded with FA, DA, or both peptides at an equimolar ratio (DA/FA). The three-dimensional projection of the interface between NKL-2DL3 and 721.174 is labeled with a pseudocolor scale based on the eGFP intensity, and the arrows show the plane used for the plot profile. On the plot profile, the gray region shows the area with a KIR2DL3 accumulation >1.5 times background. (D and E) Analysis of microclusters at the inhibitory synapse. The experiments from (A) were reanalyzed to determine the density of KIR2DL3 accumulation within the area of ≥1.5 times KIR2DL3 accumulation [gray area in (C)]. The area of intensity of KIR2DL3 accumulation within this region was compared with that of NKL-2DL3 plasma membrane at a noncontact area (D). The means ± SEM are shown in red, and statistical analysis was performed as for (A). Significant values for FA versus other conditions are shown. No significant difference was observed between the DA and DA/FA conditions. (E) The percentage of conjugates showing a density of KIR2DL3 accumulation with a fold increase ≥ 2.5 plotted as mean value ± SEM. FA was compared with the other conditions using a Student t test. For all panels, significant p values are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.
However, three-dimensional reconstruction of the contact areas showed that FA and DA induced the formation of differently structured synapses. FA induced the formation of very tight and bright clusters, whereas DA induced a more diffuse accumulation lacking large areas of intense clustering (Fig. 4C). Moreover, the synapses formed when the FA:DA peptide mix was presented demonstrated a diffuse clustering of KIRs, similar to the synaptic patterning induced by DA alone. To determine the density of KIR2DL3 accumulation at the inhibitory synapse, we measured the fluorescence intensity within the area of detectable KIR2DL3 clustering (gray area in the profile plots), as opposed to the total contact area between NKL and 721.174 cells (Fig. 4C). The density of KIR2DL3 was higher in the presence of FA (Fig. 4D) and showed that only FA induced tight clustering of KIRs, as defined by a fold increase $\geq 2.5$ between the fluorescence intensity within the synapse compared with the noncontact area (Fig. 4E). These tight clusters are similar in dimension to the KIR-based inhibitory signaling microclusters described by Treanor et al. (11). Microcluster formation was present in 35 ± 7% of FA conjugates, compared with 7 ± 4% of DA conjugates and 7 ± 6% in the FA:DA mix. Thus, DA prevents the formation of microclusters containing tightly clustered KIR2DL3, which are associated with productive inhibitory signaling. Peptide antagonism therefore influences inhibitory synapse organization.

**Peptide antagonism requires recruitment of SHP-1**

To determine the mechanism that leads to the formation of tight microclusters of KIR2DL3, we investigated if the KIR2DL3-associated inhibitory signal transduction machinery was involved with this process. Images of conjugates formed between NKL-2DL3.2YF and 721.174 demonstrated that in the absence of functional ITIMs, KIR2DL3 accumulated diffusely at the interface between the two cells and did not form tight clusters, indicating that KIR association with ITIM-mediated signaling components is critical for tight cluster formation. However, immunoprecipitation of KIR2DL3 demonstrated that each of the stimulating conditions (DA, FA, or the FA:DA mix) leads to similar levels of SHP-1 recruitment to KIR2DL3 (Fig. 5A). Thus, although the association of SHP-1 with KIR2DL3 is necessary for the formation of tight KIR clusters, simply recruiting this phosphatase to KIR2DL3 does not define the critical step within the process of tight cluster formation.

In the absence of functional ITIMs, most conjugates showed a diffuse accumulation of KIR2DL3 (Fig. 5B, 5C). However, the levels of KIR2DL3 accumulation at the contact area between NK cells and 721.174 targets and the frequency of clustering (number of conjugates showing fold increase $> 1.5$) observed with both FA (1.7 ± 0.06-fold and 69.5 ± 0.5% of conjugates)
and also the FA:DA mix (1.7 ± 0.05-fold and 62.0 ± 6.5% of conjugates) were higher than with DA alone (1.4 ± 0.05-fold and 41.7 ± 6.9% of conjugates) (Fig. 5D). Furthermore, although the number of conjugates with tight clustering of KIR2DL3 at the synapse (fold increase > 2.5) was low for FA (12.0 ± 3.0% of conjugates), and also for FA:DA (6.8 ± 2.4% of conjugates), these were both higher than for DA alone (0.0 ± 0.0% of conjugates) (Fig. 5E). Thus, in the absence of ITIMs, the FA:DA peptide mix behaviors more like DA alone, whereas in their presence, the mix behaves more like the DA peptide alone. Thus, peptide:MHC complexes with low affinity for KIRs do not disrupt the passive association of high-affinity complexes with KIRs, indicating that peptide antagonism is an active process of signal disruption.

Discussion

Peptide antagonism is a mechanism by which NK cells can be released from inhibition in the absence of MHC class I down-regulation. The peptides VAPWNSFAL (FA) and VAPWNSDAL (DA) both bind HLA-Cw*0102 with similar affinities, but whereas FA is strongly inhibitory, DA does not inhibit NK cells expressing KIR2DL2 or KIR2DL3. In binding assays using KIR-Fc fusion constructs, DA induces no discernible binding of KIR-Fc fusion constructs to HLA-Cw*0102 (10). Despite this apparent lack of function, the DA peptide can induce clustering of KIR2DL3 to the inhibitory synapse. This ability is most likely because the avidity of the KIR:peptide:MHC interaction is greater at the synapse between two cells than it is as measured in solution. This avidity has recently been demonstrated for TCR:peptide:MHC class I interaction, in which the $K_D$ of the interaction between the TCR and the peptide:MHC class I complex was up to eight times lower, as measured by total internal reflection fluorescence microscopy, at an in vitro “synapse” compared with a solution measurement (24). Nevertheless, although the DA peptide can recruit KIRs and SHP-1 to the immune synapse, only the high-affinity peptide FA induced inhibition of signaling, as determined by a reduction in NK:target cell contact time and also in terms of Vav1 dephosphorylation. In this system, inhibitory signaling correlated directly with the ability to form tight inhibitory microclusters (11), and only the FA peptide induced the formation of these structures. The 721.174 cells express low levels of Cw*0102 on the cell surface in the absence of exogenous peptide (20). In the absence of TAP, it most likely binds low-affinity peptides, as has been found for the other alleles expressed by this cell line, HLA-A*0201 and HLA-B*5101 (25). These HLA-C:peptide complexes do not recruit KIRs to the immune synapse, nor induce SHP-1 recruitment to KIRs, presumably as they are unstable. However, HLA-C:DA complexes, which have a low avidity for KIRs, are sufficient to perform both of these functions, and thus this peptide uncouples SHP-1 recruitment from inhibitory signaling.

The use of the two peptides FA and DA separate early synaptic events (recruitment of KIRs to the interface and SHP-1 recruitment) from later events (microcluster formation and inhibitory signaling). The DA peptide induced only early synaptic events and prevented the formation of microclusters related to FA. Thus, peptide antagonism operates at the early stage of synapse formation. In the absence of ITIMs, and hence SHP-1 recruitment, the peptide mix behaved more like the FA peptide. However, in the presence of intact ITIMs, the mix behaves more like the DA peptide. In the absence of ITIMs, the patterning of KIRs at the immune synapse is most likely due to the passive recruitment by peptide:MHC complexes and hence is determined by the affinity of the peptide:MHC complex for KIRs. Therefore, the DA peptide: MHC complexes did not alter recruitment of KIR-2DL3.2YF by the FA complexes to the synapse.

KIRs were originally shown to be recruited to the interface between effector and target cells in the absence of actin polarization, and adhesion molecules, implying that formation of the inhibitory synapse is a passive process (17, 26). However, recent work has demonstrated that formation of inhibitory synapses is more complex. In addition to recruiting SHP-1 and SHP-2, KIRs can also bind to β-arrestin 2 and the F-actin binding protein supervillin (27, 28). These molecules are reported to facilitate SHP-1 recruitment to KIRs and may assist in the recruitment of KIRs to signaling scaffolds. In addition, KIR ligation leads to the phosphorylation of crk, indicating that KIRs can have a positive signaling effect (29). Moreover, the activating receptor 2B4 has been found to colocalize with KIRs at the immune synapse. Taken together, these data show that inhibitory signaling is a multistep process. Recruitment of SHP-1 to KIRs may stabilize the low-affinity interaction between KIR2DL3 and DA:MHC, but not to the extent that it permits triggering of tight cluster formation. In our experiments, mutation of the KIR ITIMs prevented microcluster formation, implying that there is a requirement for SHP association and that microcluster formation is an active process. It may be related to a coassociation of KIRs with activating signaling complexes, which has been suggested as one explanation for the association of KIRs with phosphotyrosine moieties in microclusters and is consistent with the colocalization of KIRs and 2B4 at the immune synapse (19).

Our data help to dissect out this multistep process in the following ways: passive recruitment of KIRs to the immune synapse can occur without SHP-1 recruitment; DA can recruit SHP-1, but not induce inhibitory microcluster formation; and productive inhibitory signaling requires both high-affinity peptide:MHC:KIR interactions and ITIM signaling. This information is consistent with a threshold for inhibition as described by Almeida et al. (30) and hence also for microcluster formation. In this model, a threshold level of high-affinity peptide:MHC complexes is needed to engage KIRs and bind SHP-1, and this threshold may not be reached if SHP-1 is also sequestered by low-affinity peptide:MHC class I complexes.

Peptide antagonism is a novel mechanism for releasing NK cells from dominant inhibitory signals and is a feature of peptide: MHC class I complexes with a low affinity for KIRs. This effect, and its in vivo significance, will require testing in more physiological systems of viral infection and tumors. Our observation of altered KIR synapse patterning in the presence of an agonist:antagonist peptide pairing differs from the observation of TCR antagonists. In this system, MHC class I recruitment to the central supramolecular activation complex is decreased, but formation of this complex is not abrogated (31). Thus, the nature of synaptic patterning is not shared between these two agonist:antagonist systems, consistent with the presence of distinct mechanisms of peptide antagonism in immunocytes. Our data suggest that KIRs provide NK cells with the ability to scan the peptide repertoire of a target cell, and if the critical threshold of high-affinity peptide: MHC complexes is not reached, then the cell will be released from inhibition. Peptide antagonists can thus fine-tune the inhibition of NK cells.

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Disclosures

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