NKG2D discriminates diverse ligands through selectively mechano-regulated ligand conformational changes

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DOI: 10.15252/embj.2021107739

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Review Timeline:

| Event                        | Date       |
|------------------------------|------------|
| Submission Date              | 14th Jan 21|
| Editorial Decision           | 30th Mar 21|
| Revision Received            | 9th Oct 21 |
| Editorial Decision           | 3rd Nov 21 |
| Revision Received            | 10th Nov 21|
| Accepted                     | 18th Nov 21|

Editor: Ieva Gailite / David del Alamo

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)
Dear Wei,

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received comments from three reviewers, which are included below for your information.

As you can see from the reports, while the reviewers appreciate the novelty of the study, they also indicate a number of concerns, in particular regarding the physiological relevance of the proposed model of mechanical regulation of NKG2D ligand binding for NK cell function and the physiological range of the values used for molecular dynamics simulations and the final model of NKG2D activation. Furthermore, they also raise technical concerns regarding protein purity and activity, as well as the used NK cell line.

Based on the interest expressed in the reviewers' comments and your willingness to engage in a major revision as expressed in the pre-decision consultation, I would like to invite you to address the comments of all reviewers in a revised version of the manuscript. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage.

We have extended our 'scooping protection policy' beyond the usual 3-month revision timeline to cover the period required for a full revision to address the essential experimental issues. This means that competing manuscripts published during revision period will not negatively impact on our assessment of the conceptual advance presented by your study. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/transparent-process

Please feel free to contact me if have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving the revised manuscript.

With best regards,

Ieva

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Referee #1:

"NKG2D sensitively discriminates diverse ligands through selectively mechano-regulated ligand conformational changes" applies single cell biophysical approaches, molecular dynamics simulations, and mathematical modeling to understand how the activated NK receptor NKG2D recognizes its cognate ligands, MICA, MICB, ULBP3, and ULBP1. Although prior biophysical solution phase studies had suggested that NKG2D binds MICs and ULBPs with similar affinity, the authors find that applied force induces the formation of catch bonds between NKG2D and MICA, and to a lesser extent between NKG2D and MICB. By contrast, ULBPs do not form NKG2D catch bonds. These differential affinities correlate with the capacity of the ligands to stimulate IFN\(\gamma\) release by NK cells (MICA > MICAB > ULBP3), implying that it is the affinity for NKG2D under force that dictates the functional activity of each ligand. Subsequently, the authors perform molecular dynamics simulations to investigate the basis for NKG2D catch bond behavior, and they identify candidate amino acid residues on MICA that could mediate interactions with NKG2D under force. They then mutate these residues and document a decrease in both affinity under force and functional responses.
This study extends the purview of immunoreceptor catch bond formation to NKG2D and demonstrates a previously unappreciated discrepancy in functional strength between different NKG2D ligands. NKG2D apparently uses catch bond formation under force to discriminate between ligands, analogous to how the TCR uses catch bonds to distinguish strong from weak antigens. What is missing, in the case of NKG2D, is a biological rationale for this discrimination. The authors make at attempt at providing one in their Discussion, but the argument is not particularly compelling. All NKG2D ligands function as indices of stress, infection, and transformation. Why would NKG2D need to distinguish them? Beyond this conceptual issue, I have a few other comments and concerns, which are listed below.

1) The core point of the paper is that the differential NKG2D affinities of each ligand under force explain their differential stimulatory capacities. The only functional readout, however, is secreted IFNγ (e.g. Fig. 2b). Including additional responses (degranulation, phosphorylation events) would strengthen the authors’ argument.

2) Low purity/folding/functionality of the ULBP3 and MICB preparations could explain, at least in part, the authors’ observations. They should include data validating the proper folding and behavior of their purified proteins.

3) The authors make a great deal of the unexpected correlation they observe in Fig. 2f. This is sort of a meaningless point. As the authors state, the in situ off rates of MICA, MICB, and ULBP3 are very similar in no-force conditions, and this similarity artificially enhances the calculated correlation with IFNγ EC50.

4) For BFP assays, it is stated that adhesion frequency was kept under 15% to ensure that most interactions involved signal receptors and single ligands. Could the authors remind me why 15% is such a critical number and why it makes them feel comfortable that they are measuring single bond kinetics?

5) Fig. 4h is unsatisfying. The authors should perform a more extensive concentration series to map out a complete dose response. Also, why does the 3A mutation decrease the maximal IFNγ response without altering the EC50? For comparison, the authors obtained a different pattern of results (change in EC50, no change in maximal response) in Fig. 2b.

6) On p7, it is stated that "Our results demonstrated that MICA (EC50=22.680.74nM) had a stronger ability to stimulate NK cell functions than MICB (EC50=44.41.84nM) and ULBP1 (EC50=92.493.02nM) . . .". The authors meant to say "ULBP3", correct?

Referee #2:

Fan and colleagues have studied mechanisms by which the NK cell receptor NKG2D can discriminate among its many ligands. They suggest that catch bonds between NKG2D and its ligands allow for mechanical forces to favor MICA as a ligand over the others to activate the receptor. The manuscript needs a little work (as discussed below) but is certainly worth publishing.

The use of micropipette and biomembrane force probe to study the adhesion frequency after contacts of various durations and extrapolating from there the on and off rates is quite standard. The experimental work here appears quite good. The display of data needs a little work - our fields have all moved away from bar charts (Fig 1) and more towards showing all the data points assessed with a box to show confidence interval of the mean. Bar charts bias the viewer by the bulk of the area of the square instead of based on the height alone. The comment applies to all the figures in this paper - show the data, not a bar chart. Statistical comparisons should be made. The number of independent experimental replicates should be given.

The use of two axes on one plot is also non-standard. Plotting one variable against the would make more sense to me, with different colors or shapes for the different ligands (Fig 1def).

The work on cytokine secretion is fine (Fig. 2). The work on force-dependent bond lifetimes (i.e., catch bonds) is well done (Fig 3), and reveals that ligands can be discriminated by the receptor based on bond lifetimes under 10 pN force. There should be some mention about the rate of pulling (retraction) - was this varied in the experiments?

The molecular dynamics work will always come with some criticism from the readers, as many in structural biology have grown weary of the extreme (!) forces applied during MD simulations to emulate force-mediated molecular dissociations in the timespans (nanoseconds) that can be simulated by MD. The tearing apart and unfolding of domains, for example, is a common consequence of these extreme forces as is visible in Fig 4a for MICA. The formation of H bonds during this extreme unfolding cannot be the cause of stabilization because these H bonds were certainly formed with waters prior and after disruption - there is no net change in energy of the system by the switching of an H bond from water to another molecular partner. There is no mention in the article about explicit waters and what their role would be in these H bonds. In the MD simulation, do these intermediate steps for MICA disappear when these selective H bonds are eliminated? What do these intermediate steps represent in the real world, for example, in the kinetic proof-reading model?

Alanine mutagenesis is a brutal way to test the hypothesis that H bonds are formed as an intermediate step. The elimination of the side chains in alanine may change many other local features required for proper shape of the fold and not just elimination of
the backbone. The 3A mutant still shows a catch bond behavior and still elicits interferon gamma.

Figure 6C is profound and important and adds an important capture of the data in the earlier part of the paper into a single figure that supports how the ligands are discriminated. There should be some mention about the relationship between signaling (in the NK cell) and the force on the receptors - is there a feedback loop? The speculative ideas in the Discussion about forces in the cirrhotic liver and soft tumors is important but should be clearly labeled as speculation.

Referee #3:

The manuscript by Fan et al. addresses the role of mechanical force of recognition by NKG2D of its different ligands. The authors use single cell mechanical assays as well as cytokine release to relate in situ molecular binding properties to the stimulation of Natural Killer cells via their NKG2D receptor.

The study is timely and based on solid experience of the authors with micropipette-based mechanical assays. It addresses the delicate problem of ligand degeneracy and discrimination by NKG2D receptors, which has no satisfactory interpretation yet, despite the importance of this issue in immunotherapy.

The authors found that NKG2D exhibits in situ catch bound behaviour with ligand MICA1, explaining the stronger potency of this ligand bound to surfaces to trigger cytokine release by NK cells. While the experimental study is overall convincing, some detailed information on the biological material is missing and several important issues should be addressed before considering the work for publication.

Additionally, the relevance of the in silico and theoretical modeling has to be better justified.

Major concerns.

1) choice of cells: why are the authors working with expanded NK cells rather than freshly isolated NK cells? How long the expanded cells are kept? The authors should provide explicit characterization of their cell production and stability (cytometry).

2) molecules production and properties: basic biochemical characterization of ligands produced in the study should be provided (SDS PAGE and cytometry). Additionally, the authors rely on ref. 11 for the in-solution measurement: where is the proof that their own molecules exhibit the same binding properties? Why are there no values for off-rate in solution for MICB, ULBP1 and ULBP3?

3) can the off-rate values at 0 force be extrapolated from the BFP data obtained at non-zero force? This would reinforce the confidence in data presented in graph 3F, on which the essential part of the study is relying. How does the fast pulling applied to BFP before reaching force clamp affects the adhesion frequency (fig. 3c) and lifetime (fig. 3f) estimates? More generally, the similarities and differences between the techniques used in fig. 1 and fig. 3 should be better explained.

4) molecular dynamics simulations: steered molecular dynamics was performed at constant pulling force (which rate is ambiguous: 7nN/s on page 9 and 7pN/s on page 25): how much is it relevant to interpret constant force-data obtained with the BFP? How much is it relevant physiologically? In absence of convincing arguments in this direction, the claim of conformational change in the title is quite speculative.

5) the kinetic proofreading model is based on several parameters which are non-experimental, as the authors put it: kp, kon-rebinding, µ, as well as the time of 300 s chosen to assess the activation. How many steps are actually taken? At the end, taking into account all these unknowns, the author should provide arguments for the values of unknowns parameters and better justify how necessary is the model for supporting their conclusions.

Minor concerns.

1) additional supporting data should be provided: determination of surface density of ligands and receptors, the calibration of the BFP spring constant

2) in-situ force free kinetics and Fig.1: the graphs showing simultaneously in-situ and in-solution data should span a similar interval on left and right axis (1D, 1E, 1F), even if expressed in different units. The authors should explicit the formula they use to fit the data in fig 1C, with free parameters kon and koff and show explicitly that the 2 parameters are no correlated.

3) the authors claim in the discussion that their model reconcile the 2 models proposed in the literature to explain ligand discrimination by NKG2D: induced fit and rigid adaptation. Those general mechanisms usually refer to molecular scale processes in solution, so the statement is not meaningful to my opinion. It is neither necessary, since the authors emphasize in the introduction the various contractions of those models with experimental evidences.

page 9: fig 3g not 2g.
Page 9: reformulate: "render NKG2D the power to discriminate ..."
page 11: reformulate: "reveal that the activation of both ..."
page 13: fig 6d not 6c
page 19: explicit pHAGE and iRES acronyms?
Page 21: 5 mim → 5 min
Point-to-Point Response

We would like to thank the reviewers for their constructive and critical evaluations. We have extensively revised our manuscript according to reviewers’ constructive comments. In the revised manuscript, all revisions are colored as red for helping reviewers to check our revision quickly. Below is our point-to-point response to the comments. The comments from the reviewers are shown in blue, and our responses are shown in black.

Reviewer 1:

"NKG2D sensitively discriminates diverse ligands through selectively mechano-regulated ligand conformational changes" applies single cell biophysical approaches, molecular dynamics simulations, and mathematical modeling to understand how the activated NK receptor NKG2D recognizes its cognate ligands, MICA, MICB, ULBP3, and ULBP1. Although prior biophysical solution phase studies had suggested that NKG2D binds MICs and ULBPs with similar affinity, the authors find that applied force induces the formation of catch bonds between NKG2D and MICA, and to a lesser extent between NKG2D and MICB. By contrast, ULBPs do not form NKG2D catch bonds. These differential affinities correlate with the capacity of the ligands to stimulate IFNg release by NK cells (MICA > MICAB > ULBP3), implying that it is the affinity for NKG2D under force that dictates the functional activity of each ligand. Subsequently, the authors perform molecular dynamics simulations to investigate the basis for NKG2D catch bond behavior, and they identify candidate amino acid residues on MICA that could mediate interactions with NKG2D under force. They then mutate these residues and document a decrease in both affinity under force and functional responses.

This study extends the purview of immunoreceptor catch bond formation to NKG2D and demonstrates a previously unappreciated discrepancy in functional strength between different NKG2D ligands. NKG2D apparently uses catch bond formation under force to discriminate between ligands, analogous to how the TCR uses catch bonds to distinguish strong from weak antigens. What is missing, in the case of NKG2D, is a biological rationale for this discrimination. The authors make at attempt at providing one in their Discussion, but the argument is not particularly compelling. All NKG2D ligands function as indices of stress, infection, and transformation. Why would NKG2D need to distinguish them? Beyond this conceptual issue, I have a few other comments and concerns, which are listed below.
We thank the reviewer for the constructive comments. The raised question is essential and is exactly what we want to address in this study. Although there is no direct evidence, it is believed that NKG2D distinguishes different ligands to accomplish various extents of functional requirements, such as maintaining immune homeostasis in different tissues under physiological conditions, and carrying out immune defenses when different pathogens invade or cancers occur (Balint et al., 2018; McFarland & Strong, 2003).

Although all the NKG2D ligands function as indices of stress, infection and transformation, evidences have shown that the functions of different NKG2D ligands were not equal. For instance, NKG2D ligands can induce different levels of ERK/Akt phosphorylation, thus activating different cytotoxic abilities of NK cells (Cosman et al., 2001; Dunn et al., 2003; Sutherland et al., 2002). Moreover, MICA can induce a higher level of IFN-γ mRNA expression than ULBP1 (Wang et al., 2020). Balint et al. have also reported that distinct NKG2D ligands are not equivalent in their abilities to reorganize IL-15 receptors at the nanoscale and to elicit distinct cortical actin remodeling and degranulation (Balint et al., 2018). ULBP1, but not other NKG2D ligands, on the target cell is able to trigger the lysis of regulatory T cells in the immune response to an intracellular pathogen (Roy et al., 2008). Moreover, MIC family is more frequently and abundantly expressed on the surface of tumor cells (Dhar & Wu, 2018), which correlates with overall survival of tumor patients better than ULBP family; thus, MIC family expression has been reported as an indicator for poor prognosis (Fang et al., 2014; Li et al., 2009; McGilvray et al., 2010) However, these distinct biological functions induced by different NKG2D ligands are all accomplished through their binding with the NKG2D receptor, strongly suggesting that NKG2D receptor needs to be able to distinguish different ligands to induce different functions.

However, the discrimination of different NKG2D ligands has long been uninvestigated. One possible reason is that the differences of ligands can only be seen at an optimized or physiological density or concentration, unfortunately, many functional assays were done by overexpression or over high concentration of these ligands which could inevitably diminish their functional differences. This is like our results that show no difference in activating NKG2D-mediated NK cell activation at the saturating condition of these ligands (Figs 2B, and 5B). Another possible reason may be due to technical limitations of soluble or biochemical binding assays which were unable to detect the NKG2D/ligand interaction parameters in situ and under mechanical force application, thereby the role of mechanical force had been ignored in the past. Therefore, it was commonly suggested that NKG2D is not capable in discriminating these ligands but rather is of degenerated recognition based on
previous similar solution-based binding kinetics of NKG2D/ligand interactions (McFarland & Strong, 2003).

Moreover, in the physiological condition, NKG2D ligand recognition to activate NK cells is under dynamic physical contact, and thereby mechanical ligand loading is inevitably exerted on receptor/ligand bonds. For example, to search for ligands on target cells, NK cells highly dynamically patrol in tissues and interact with target cells, which exert traction forces on the molecular complex of immune receptors -including NKG2D- binding with their ligands (Brown et al, 2012; Brown et al, 2011; Deguine et al, 2010; Le Saux et al, 2019) Other than the cell migration, membrane bending upon cell-cell contact could also generate mechanical force on receptor/ligand bond (Zhu et al, 2019). It has also been reported that the stiffness of normal tissues and tumor tissues are quite different, and these differences have a significant impact on regulating immune cells activation (Basu et al, 2016; Eagle et al, 2009; Tian et al, 2015). For example, the softness of tumor cells prevents cytotoxic immune cell killing (Liu et al, 2021), the stiffening of tumor cells could enhance the immune cell killing sensitivity (Tello-Lafoz et al, 2021). Altogether, considering NKG2D ligand discrimination happens in a very dynamical and mechanical microenvironment, neglecting mechanical force effect on NKG2D/ligand binding would very likely miss the identification of this essential biological function of NKG2D. This part has been incorporated into the ‘Introduction’ section of the revised manuscript (Page 4-7, colored as red).

In this work, we thereby applied single-molecule mechanical assay to detect the in-situ mechanical force-dependent binding kinetics of NKG2D ligand interaction to reveal the capability of NKG2D ligand recognition. We reveal very different mechanical force-dependent binding affinity of NKG2D interacting with its different ligands, which perfectly correlated with the potency of these ligands in stimulating NKG2D-mediated NK cells. Our results suggest that in addition to the amount of NKG2D ligands, the mechanical microenvironment presented on infected or tumor cells could also affect NKG2D ligand recognition and downstream NK cell response. This finding sheds some light on the potential effect of the mechanical stiffness of tissue microenvironment on the efficacy of tumor immunotherapy.

1) The core point of the paper is that the differential NKG2D affinities of each ligand under force explain their differential stimulatory capacities. The only functional readout, however, is secreted IFNg (e.g. Fig. 2b). Including additional responses (degranulation, phosphorylation events) would strengthen the authors’ argument.
Thank the reviewer for this constructive suggestion. We have performed new experiments to characterize the expression of degranulation marker CD107a and detected the phosphorylation levels of NKG2D downstream kinase ERK (pERK) in NK cells upon different ligand stimulations using flow cytometry (Figs R1, and R2). Our data showed that under same ligand concentration for stimulations, the percentages of CD107a+ cells and pERK+ cells under MICA stimulation are significantly higher than those under MICB and ULBP3 stimulations (Figs R1A and B, and R2A and B). Moreover, the levels of CD107a+ cells and pERK+ cells under different ligand stimulations highly correlate with their corresponding IFN-γ release (correlation coefficient r = 0.9969) and in-situ binding affinities (r ≈ 0.99) under mechanical force except for 0 pN (Figs R1C-H, and R2C-H), demonstrating that the functional readouts of IFN-γ release, the degranulation and phosphorylation levels in NK cells under different ligand stimulations are all well correlated with force-dependent ligand binding affinities, further supporting our conclusion that differential affinities of NKG2D ligands under force can explain their differential stimulatory capacities. We also update these data in the revised manuscript (Fig EV2).
Figure for reviewers removed
Figure for reviewers removed
2) Low purity/folding/functionality of the ULBP3 and MICB preparations could explain, at least in part, the authors' observations. They should include data validating the proper folding and behavior of their purified proteins.

Thank the reviewer for pointing this question out. To demonstrate the proper folding and behavior of our purified proteins, we first added chromatograms on Superdex 75 size exclusion column (SEC) of recombinant NKG2D ligands (Fig R3A). Their purity and proper folding were further validated and confirmed by SDS-PAGE and monoclonal antibody staining by flow cytometry (Fig R3B-D). Moreover, the receptor binding data of Biolayer interferometry characterization (Fig R10), our single-molecule data (Figs 3, and 5), and ligand stimulation data (Figs 2, and R1, and R2) that show ligand’s capability to activate NK cells also validate their proper behaviors. Altogether, these data demonstrate that our purified proteins have high purity, uniformity, specificity and proper stimulating capacity.
3) The authors make a great deal of the unexpected correlation they observe in Fig. 2f. This is sort of a meaningless point. As the authors state, the in situ off rates of MICA, MICB, and ULBP3 are very similar in no-force conditions, and this similarity artificially enhances the calculated correlation with IFNγ EC50.
Thank the reviewer for pointing this out. We agree with this point. Indeed, the \textit{in-situ} off rates of MICA, MICB, and ULBP3 are very similar in no-force conditions. Although similar, they are positively correlated with the inverse of EC50 of ligand stimulation, opposite to the reasonable correlation which should be negative. Such that, this correlation analysis in Fig. 2F can be set as a negative control for the reasonable negative correlations of mechanical force dependent binding affinities with ligand functional potencies, further supporting the important role of mechanical force in NKG2D ligand discrimination.

4) For BFP assays, it is stated that adhesion frequency was kept under 15% to ensure that most interactions involved signal receptors and single ligands. Could the authors remind me why 15% is such a critical number and why it makes them feel comfortable that they are measuring single bond kinetics?

Thank the reviewer for raising up this question. The behavior of biomolecules at the single-molecule level follows Poisson distribution. Thus, in order to ensure that the receptor/ligand binding is from single-bond events, significant amounts (>85%) of touch events should be no-binding events. To achieve this, the site densities of both the receptor and the ligand in our BFP experiments were usually controlled at low levels. Its details can be referred to previously published work (Chesla \textit{et al.}, 1998). Accordingly, the formation of receptor/ligand bond in our single-molecule binding assay is rare, thus following the Poisson process. Mathematically, to predict the probabilities of numbers of bond formation as a function of adhesion probability, we use the following equation (Formula 1) (Chesla \textit{et al.}, 1998):

\[
p_n = \frac{(1-P_{aa})^{1-\alpha}}{n!} \ln^n (1 - P_{aa})^{1-\alpha}.
\]

where \(p_n\) is the probability of having \(n\) bonds, \(P_{aa}\) is the actual adhesion probability, \(n\) is the number of the bonds that mediate the adhesion, and \(\alpha\) is the faction of miscounting false non-adhesions, which is very small (almost 0) in the BFP force clamp assay.

According to this equation, when \(P_{aa}\) is about 15%, the probability of having a single bond is about 93%. Thus, to ensure the majority of adhesion events in the BFP measurements are single-bond events, the adhesion frequency is maintained at low frequency (<15% on average) by adjusting recombinant protein coating densities on the BFP microspheres, as described in the ‘Force clamp-assay of BFP’ section of ‘Materials and Methods’ (Page 27).
5) Fig. 4h is unsatisfying. The authors should perform a more extensive concentration series to map out a complete dose response. Also, why does the 3A mutation decrease the maximal IFNg response without altering the EC50? For comparison, the authors obtained a different pattern of results (change in EC50, no change in maximal response) in Fig. 2b.

We thank the reviewer for this constructive suggestion. We thereby performed the suggested experiments by adding more ligand concentrations to complete the dose response curves (Fig R4). Indeed, both MICA 3A and MICA TAT mutation slightly increase the half effective concentrations (EC50) of MICA in IFN-γ releasing without significantly changing their maximal responses (Fig R4A and B). In contrast, both mutations of MICA significantly suppressed NKG2D-mediated IFN-γ release from NK cells in the concentrations near EC50 (Fig R4C and D). These data together suggest that these three force-induced binding sites locating at the NKG2D/MICA binding interface can partially contribute to NKG2D-mediated NK cell activation by MICA. We also update this in the revised manuscript (Fig 5).

*Figure for reviewers removed*
6) On p7, it is stated that "Our results demonstrated that MICA (EC50=22.68±0.74nM) had a stronger ability to stimulate NK cell functions than MICB (EC50=44.4±1.84nM) and ULBP1 (EC50=92.49±3.02nM) . . .". The authors meant to say "ULBP3", correct?

Yes, thanks to the reviewer for pointing out this typo. We have corrected this mistake in our revised manuscript (Page 9, Line 21, red-colored).
Reviewer 2

Fan and colleagues have studied mechanisms by which the NK cell receptor NKG2D can discriminate among its many ligands. They suggest that catch bonds between NKG2D and its ligands allow for mechanical forces to favor MICA as a ligand over the others to activate the receptor. The manuscript needs a little work (as discussed below) but is certainly worth publishing.

We feel grateful to the reviewer for the positive evaluation of our manuscript.

The use of micropipette and biomembrane force probe to study the adhesion frequency after contacts of various durations and extrapolating from there the on and off rates is quite standard. The experimental work here appears quite good. The display of data needs a little work - our fields have all moved away from bar charts (Fig 1) and more towards showing all the data points assessed with a box to show confidence interval of the mean. Bar charts bias the viewer by the bulk of the area of the square instead of based on the height alone. The comment applies to all the figures in this paper - show the data, not a bar chart. Statistical comparisons should be made. The number of independent experimental replicates should be given.

Thank the reviewer for this helpful suggestion. We have changed all the bar charts of our results into data points (Figs 1C-E, and 3C, and 5D and E, and EV2C and F, and EV3J), except for the ratio obtained by dividing the average of two affinities (Figs 1H, and 6I), EC50s (Figs 2C, and 5C) extracted from GraphPad Prism (only have mean and error) and the mean lifetime's comparison (Fig 3E). But we showed corresponding lifetime distributions of Fig 3E in the Expanded View figure (Fig EV3H), which is also the common way to demonstrate all raw data in single-molecule studies.

The use of two axes on one plot is also non-standard. Plotting one variable against the would make more sense to me, with different colors or shapes for the different ligands (Fig 1def).

Thanks for this suggestion. We have accordingly updated Fig 1D-F with one axis as shown below (Fig R5C-E) and in our revised manuscript (Fig 1C-E).
Figure for reviewers removed
The work on cytokine secretion is fine (Fig. 2). The work on force-dependent bond lifetimes (i.e., catch bonds) is well done (Fig 3), and reveals that ligands can be discriminated by the receptor based on bond lifetimes under 10 pN force. There should be some mention about the rate of pulling (retraction) - was this varied in the experiments?

We thank the reviewer for the positive evaluation of our work. The loading rates (pulling rates) in all BFP experiments are not varied in our experiments. We analyzed the loading rates of NKG2D binding with MICA, MICB, ULBP1 and ULBP3 respectively in the BFP experiments. The loading rates of NKG2D binding with ligands are around 225-231 pN/s, and there is no significant difference between the loading rates of all four ligands (ordinary one-way ANOVA test, $P = 0.1410$) (Fig R6). Thus, these data showed that the loading rates of our BFP system are very stable. And in the revised manuscript, we have mentioned this in the main text (Page 10, Line 9, red-colored) and added the results in Fig EV3I-N.

*Figure for reviewers removed*
The molecular dynamics work will always come with some criticism from the readers, as many in structural biology have grown weary of the extreme (!) forces applied during MD simulations to emulate force-mediated molecular dissociations in the timespans (nanoseconds) that can be simulated by MD. The tearing apart and unfolding of domains, for example, is a common consequence of these extreme forces as is visible in Fig 4a for MICA. The formation of H bonds during this extreme unfolding cannot be the cause of stabilization because these H bonds were certainly formed with waters prior and after disruption - there is no net change in energy of the system by the switching of an H bond from water to another molecular partner. There is no mention in the article about explicit waters and what their role would be in these H bonds. In the MD simulation, do these intermediate steps for MICA disappear when these selective H bonds are eliminated? What do these intermediate steps represent in the real world, for example, in the kinetic proof-reading model?

We completely agree with the reviewer on the limitation of MD simulations with/without force. In our opinion, MD simulations are not able to provide definitive conclusions on a biological process. Thus, the aim of MD simulations is to provide information with which testable hypotheses can be proposed. Of course, real wet experiments must be performed to test the hypotheses. And this is indeed the strategy used in the present study. That is, we extract useful information from MD simulations and then propose a hypothesis to be validated by wet functional and single-molecule experiments.

The reviewer is right that there is no net change in the energy of system by switching of an H-bond from water to another molecular partner if water is included. But the energy will certainly be differed if only consider the interaction between the molecular partner without considering water molecule. We think that the hydrogen bonds between water molecules and amino acids could stabilize the protein conformation and smooth the whole association/dissociation process, and the switching of an H bond from water to another molecular partner could decrease the dissociation tendency between two proteins, and also increase their rebinding possibility.

MD simulations are not able to generate all possible intermediate steps of dissociation process but can provide some of the possible intermediate steps. That is to say, in the real world, if two protein molecules interact by the way like the intermediate states, whatever
from the new encounter or dissociation process, they have more chance to bind/rebind in a certain fashion, compared with those without the intermediate states.

As for the intermediate steps in the kinetic proof-reading model (Dushek et al., 2009), the output signaling is generated after a series of intermediate steps \((C_i \text{ and } U_i, i = 1, 2, 3, \ldots, s-1)\) following the initial binding \((C_1)\) of a ligand and NKG2D. These intermediate steps represent the phosphorylation on receptors, enzymatic activations and other modification steps on receptors (McKeithan, 1995).

Alanine mutagenesis is a brutal way to test the hypothesis that H bonds are formed as an intermediate step. The elimination of the side chains in alanine may change many other local features required for proper shape of the fold and not just elimination of the backbone. The 3A mutant still shows a catch bond behavior and still elicits interferon gamma.

The reviewer’s concern that the 3A mutant may change the fold of protein is reasonable, and thus we designed the new mutant (MICA TAT mutant) in the target site to test our hypothesis: D15T, S17A, and Q83T. For the similar simulation of TAT mutant, the same simulation procedure used in our manuscript was adapted, and three independent SMD simulations of force-driven MICA TAT mutant/NKG2D dissociation were performed. The simulations show that the intermediate states still exist in the force-driven dissociation process, which is similar with that in MICA-WT (Fig 4A and D and F) and MICA 3A mutant (Fig R7A and D and F). Analyzing the intermediate states, it is found that T15 mutation of MICA could form the new and unstable hydrogen bond with K186 of NKG2D (Fig R7E)., and the contact area between T15, A17, and T83 of MICA mutant and NKG2D increased slightly (Fig R7B and E). The corresponding BFP experiments also show that weaker ‘catch bond’ with smaller maximum mean bond lifetime and smaller optimal force in MICA TAT mutant binding with NKG2D, which is similar with the results for MICA 3A mutant (Fig 5A). These results on MICA 3A mutant and TAT mutant suggest that the weakening of ‘catch bond’ is resulted by the sidechains of these three residues, and not by the local conformational change induced by the mutations. The new simulation results on NKG2D dissociation with MICA 3A and TAT mutants are also presented on Page 12 (Line 8-12, red-colored) and Fig EV4E-K.

Regarding the issue of 3A mutant that still shows catch bond and still elicits IFN-\(\gamma\), this is because we only partially abolished the force-induced binding between NKG2D and MICA through 3A or TAT mutation. As force-induce bindings between NKG2D and MICA also
involved many other interaction residues and networks (D15-S17, Q83-E85 and etc.), not only sidechains of these residues but also VDW interaction, hydrogen bonds formed by the backbone atoms. Mutating them all would be too brutal to reach meaningful and reasonable interpretation, thus we decided to perform minimum intervention on MICA by only mutating three residues (D15, S17 and Q83) which only form hydrogen bonds via sidechains with NKG2D. As other force-enhanced binding sites still existed, weaker catch bond can still be formed, and the IFN-γ secretion can still be released from NK cells at a significantly lower level, further supporting our conclusion that force-dependent NKG2D binding is essential for NKG2D mediated NK cell activation.

*Figure for reviewers removed*
Figure 6C is profound and important and adds an important capture of the data in the earlier part of the paper into a single figure that supports how the ligands are discriminated. There should be some mention about the relationship between signaling (in the NK cell) and the force on the receptors - is there a feedback loop? The speculative ideas in the Discussion about forces in the cirrhotic liver and soft tumors is important but should be clearly labeled as speculation.

Many thanks to the reviewer for this positive evaluation of our work and for raising up this interesting and inspiring question to help us think about the potential feedback loop between the signaling and the force on the receptors. Indeed, this is very likely the case although we still lack enough direct and convincing data. But we speculate that mechanical force exerted on the receptor-ligand binding can trigger receptor’s instar-cellular signaling and further physically regulate molecular conformations of signaling molecules and their biological functions, thereby converting mechanical signals to biochemical signals (including cytoskeleton reorganization, kinase activation, Ca$^{2+}$ flux, and further downstream gene transcription, cytokine release, etc.). These biological activities in a feedback way regulate the sub-cellular machinery to further change the mechanical force production and thereby receptor-ligand binding.

This feedback model can occur at both the molecular level and the cellular/tissue level. The important role of force in tissue development in both physiological and pathological conditions has been reviewed and discussed before extensively (Hannezo & Heisenberg, 2019; Petridou et al, 2017). Although there is still not much direct evidence for immune cell recognition activation, some have reported similar feedback may also exist and play an
important role in immune cell receptor-ligand recognition and immune cell activation. For example, previous studies have shown that mechanical forces can potentiate the activation and killing of immune cells potentially through regulating mechanical force on immune cells in a feedback manner (Basu et al., 2016; Liu et al., 2014). The activation of NK cells through NKG2D-ligand interaction has also been shown to be accompanied by cytoskeleton remodeling, mediated mainly by F-actin (Brown et al., 2012; Brown et al., 2011). Dynamic cytoskeletal elements are the main force generators, which can load force onto cell surface receptors (Zhu et al., 2019) when they bind to their ligands expressed on the target cell.

Therefore, we propose a feedback model (Fig R8) here, in which force can strengthen the NKG2D-ligand binding in a feedback manner. We have also labeled this model and the discussion about forces in the cirrhotic liver and soft tumors as speculation in the revised text in ‘Discussion’ section. (Page 18 and 19, red-colored).

Figure for reviewers removed
Reviewer 3:

The manuscript by Fan et al. addresses the role of mechanical force of recognition by NKG2D of its different ligands. The authors use single cell mechanical assays as well as cytokine release to relate in situ molecular binding properties to the stimulation of Natural Killer cells via their NKG2D receptor.

The study is timely and based on solid experience of the authors with micropipette-based mechanical assays. It addresses the delicate problem of ligand degeneracy and discrimination by NKG2D receptors, which has no satisfactory interpretation yet, despite the importance of this issue in immunotherapy.

The authors found that NKG2D exhibits in situ catch bound behaviour with ligand MICA1, explaining the stronger potency of this ligand bound to surfaces to trigger cytokine release by NK cells. While the experimental study is overall convincing, some detailed information on the biological material is missing and several important issues should be addressed before considering the work for publication.

Additionally, the relevance of the in silico and theoretical modeling has to be better justified.

We thank the reviewer for the positive evaluation of our manuscript.

Major concerns.

1) choice of cells: why are the authors working with expanded NK cells rather than freshly isolated NK cells? How long the expanded cells are kept? The authors should provide explicit characterization of their cell production and stability (cytometry).

We thank the reviewer for this important question. We used expanded NK cells mainly for the following three reasons:

  1. Expanded NK cells last long enough for us to complete multiple binding and functional experiments, including BFP kinetic measurement, site density characterization by flow cytometry and cytokine release assay, thus diminishing variation from different batches of freshly isolated NK cells from different donors.
2. Higher expression levels of NKG2D receptors on the expanded NK cells than those on primary NK cells can enhance the experimental efficiency of BFP single-molecule detection (Fig R9B and C). To detect single-molecule binding requires a suitable receptor and ligand concentration (ensure that most adhesion events are single bond events while reducing non-specific adhesion events, see ‘Force clamp-assay of BFP’ in section of ‘Materials and Methods’ (Page 27)). If the receptor concentration is too low, the ligand concentration must be increased to a very high level to ensure the appropriate adhesion frequency. But if the ligand concentration is too high, the streptavidin coated on the glass bead would be over-occupied by the ligand, thereby significantly reducing the biotin on the red blood cell binding to the streptavidinylated probe bead and impairing the BFP assembly and single-molecule experiment.

3. Functional experiments require a large number of NK cells from the same source to reduce batch effects. Usually, the proportion of NK cells in human peripheral blood is low, so it is not very feasible to perform multi-ligand and multi-concentration stimulation experiments. For example, one experiment can contain 3 different ligands with 7 distinct concentrations plus one control group, each of which needs three replications and each replication needs at least $3 \times 10^5$ cells. It may take 20 million cells in total, that is, without considering the loss in each step, at least 200mL (about $10^5$ NK/mL whole blood) of blood from the same person is required. It even requires more for multiple repeats. So, it is not quite feasible to use fresh primary NK cells for our study.

We have measured the lifetime distribution of both expanded NK and freshly isolated NK cells, and found their average lifetime was not significantly different (Fig R9A).

We expanded NK cells for 13-15 days and the expanded cells were used before Day 18. For NK cell characterization, we have performed flow cytometry analysis to verify their phenotypes. Compared with freshly isolated NK, the expanded NK cells are pretty much similar. The characteristic staining for checking the purity of expanded NK cells is shown in Fig R9D and E.
2) molecules production and properties: basic biochemical characterization of ligands produced in the study should be provided (SDS PAGE and cytometry). Additionally, the authors rely on ref. 11 for the in-solution measurement: where is the proof that their own molecules exhibit the same binding properties? Why are there no values for off-rate in solution for MICB, ULBP1 and ULBP3?

We thank the reviewer for raising up this concern. Regarding basic biochemical characterization of ligands produced in this study, please refer to Fig R3. Moreover, the receptor binding data of Biolayer interferometry characterization (Fig R10), our single-molecule data (Figs 3, and 5), and ligand stimulation data (Figs 2, and R1, and R2) that show ligand’s capability to activate NK cells also validate their proper behaviors. To explore the binding properties of our purified proteins, we did Bio-Layer Interferometry (BLI) experiments to show their binding properties with NKG2D receptor. According to the results, the binding response curves of our purified proteins with NKG2D increased in a
concentration-dependent manner (Fig R10). The values of binding kinetics of four ligands with NKG2D are consistent with our in-situ kinetics at 0 pN, with MICA (5.99×10⁻⁸ M) showing the highest affinity with NKG2D among all tested ligands (MICB, 1.09×10⁻⁷ M; ULBP1, 1.73×10⁻⁷ M; and ULBP3, 2.64×10⁻⁷ M). We also added these data in the revised manuscript (Fig EV1I-O).

As for the off-rate in solution for MICB, ULBP1 and ULBP3 in reference 11 (McFarland & Strong, 2003), they are too fast to be measured by SPR according to the author’s explanation.

Figure for reviewers removed

3) can the off-rate values at 0 force be extrapolated from the BFP data obtained at non-zero force? This would reinforce the confidence in data presented in graph 3F, on which the
essential part of the study is relying. How does the fast pulling applied to BFP before reaching force clamp affects the adhesion frequency (fig. 3c) and lifetime (fig. 3f) estimates? More generally, the similarities and differences between the techniques used in fig. 1 and fig. 3 should be better explained.

Thank Reviewer 3 for raising up these questions about BFP. We have extrapolated the off-rate values at 0 force from the BFP data (Fig 3F) as shown in Fig R11A. We constructed fitting curves using exponential function based on the experimental data points. The bond lifetimes at 0 pN for MICA, MICB, ULBP1, ULBP3 are respectively extrapolated as 0.061s, 0.047s, 0.085s, and 0.012s. These data indeed reinforce the conclusion that force is essential to regulate the off-rate of NKG2D with ligands.

In order to study the effect of fast pulling on adhesion frequency (Fig R11B) and bond lifetime (Fig 11C), we analyzed the loading rate (pulling rate) of NKG2D binding with MICA. We divided the loading rate into two groups: the fast group (faster than 230 pN/s) and the slow group (slower than 230 pN/s). According to our results, there was no significant difference in the adhesion frequency (t-test, $P = 0.6594$, Fig R11B) and bond lifetime (t-test, $P = 0.0967$, Fig R11C) between the fast-loading-rate and the slow-loading-rate groups. These data demonstrate that the fast pulling before reaching force-clamping phase has no significant effects on adhesion frequency and lifetime compared with those measured with slow pulling in our BFP experiment.

Figure for reviewers removed
We have added an explanation (Fig R12) about the similarities and differences between Micropipette adhesion frequency assay used in Fig 1 and Force clamp assay of BFP in Fig 3. Briefly, the former measures the binding frequency as a function of contact duration to extract the force-free 2D kinetics by nonlinearly fitting the data with a probabilistic model (Chesla et al., 1998). The latter detects bond formation and dissociation by monitoring the edge of BFP in real time (Chen et al., 2008; Liu et al., 2014). Both assays are mechanically based using ultrasensitive force techniques to detect in-situ binding kinetics at the level of mostly single molecular interaction (Chen et al., 2008). We also illustrated the setups and workflows (Fig R12) and listed the similarities and differences (Tables R1 and 2) of these two assays for better understanding these two assays. Detailed methods and setups are described in the ‘Micropipette adhesion frequency assay’ (Page 26) and ‘Force clamp assay of BFP’ section of ‘Materials and Methods’ (Page 27) in our revised manuscript.

Figure for reviewers removed
Table R1. Similarities of micropipette adhesion frequency assay and force clamp assay of BFP.

| Similarities                                      |
|--------------------------------------------------|
| 1. Mechanical based setup                        |
| 2. Use RBC as ultra sensitive force sensor       |
| 3. Measure in-situ binding kinetics that can be used on live cell |

Table R2. Differences of micropipette adhesion frequency assay and force clamp assay of BFP.

| Differences                                      |
|--------------------------------------------------|
| Micropipette adhesion frequency assay            |
| Force clamp assay of BFP                         |
| What we measure                                  |
| Binding frequencies under various contact durations at certain surface densities of receptor and ligand. |
| Bond lifetimes under various preset force at certain surface densities of receptor and ligand. |
| How and what we get                               |
| Fit binding frequency vs. contact duration with theoretical model to get \( A_c K_a \), then multiply them to obtain \( A_c k_{on} \). |
| Bond lifetimes under certain force, from which we get \( k_{off} \), under certain force by calculate its reciprocal. |
| Way to acquire data                              |
| Kinetics are extracted from fitting data with theoretical model |
| Kinetics are obtained through actual measurement |
| Theoretical model                                |
| \[ P_a = 1 - \exp[-m_r m_l A_c K_a \left(1 - \exp(-k_{off} t_c)\right)] \] |
| \[ k_{off}(F) = K^0 \exp\left(\frac{F}{F_0}\right) \] |
| Obtained kinetics under force or not             |
| Obtain kinetics under no force                   |
| Obtain kinetics under a certain force            |
| Role of force sensor                             |
| Determine whether adhesion occurs                |
| Calculate the value of the force                 |
| Single molecule level or not                     |
| Single or several molecules-level                |
| Single molecule level                            |

4) molecular dynamics simulations: steered molecular dynamics was performed at constant pulling force (which rate is ambiguous: 7nN/s on page 9 and 7pN/s on page 25): how much is it relevant to interpret constant force-data obtained with the BFP? How much is it relevant physiologically? In absence of convincing arguments in this direction, the claim of conformational change in the title is quite speculative.
We thank the reviewer for pointing this out. The loading rate of applied force in steered molecular dynamics should be 7 pN/ns and 14 pN/ns, as described in ‘Molecular dynamics simulations’ of section ‘Materials and Methods’ (Page 28). The words “7nN/s and 14nN/s” on original Page 9 are typos, which have been corrected in the revised manuscript (Page 11, Line 13, red-colored). In fact, the force-driven dissociation processes obtained with MD simulations are much faster than those used by BFP experiments, and the loading rate of force in MD simulations is ~105 higher than that in BFP experiments, which results from the balance between current computational power, similar loading speed is also used in most MD studies on force-driven protein dissociation. This is also a well-known limitation of MD simulations, which has also been pointed out by reviewer 1. Nevertheless, the MD simulation is used to provide information and formulate testable hypotheses, the conclusion of the study is ultimately reached through the functional and single-molecule experimental results.

We think that MD simulation could give us important clues about the dissociation process at the atomic level, in which intermediate states could be identified, though they could not accurately reproduce the physiological dissociation. The key residues in the intermediate state could be observed visually, leading to a testable hypothesis. Accordingly, BFP experiments employed with the mutagenesis were carried out to validate our hypothesis and the roles of these selective residues. By experimental confirmation, we believe that the general conformation changes in the dissociation process should be reasonable and represent the physiological cases approximately.

5) the kinetic proofreading model is based on several parameters which are non-experimental, as the authors put it: kp, kon-rebinding, μ, as well as the time of 300 s chosen to assess the activation. How many steps are actually taken? At the end, taking into account all these unknowns, the author should provide arguments for the values of unknowns parameters and better justify how necessary is the model for supporting their conclusions.

We thank the reviewer for this question. Considering the receptor clustering upon activation in NK cells, we introduced a fast ligand-receptor rebinding step (kon-rebinding) into account, as proposed in kinetic proofreading model for T cell responses to antigen binding (Dushek et al., 2009). Once NKG2D binds with ligands, it may go through a series of forward and/or backward steps (e.g., phosphorylation and dephosphorylation by signaling molecules, fast reassociating or disassociating from the ligand, etc.). As these intermediate steps are very complicated and have not been clearly understood, we assumed a simplified model
with multiple intermediate steps (the same forward rate constant $k_p$ and the same fully
disassociation rate constant $\mu$ for all intermediate states). According to the experimental
measurements, we have fixed some of the parameters such as the simulation time which was
set as 300 s to ensure the full phosphorylation of NKG2D (Sutherland et al., 2002), and the
values of $k_{\text{on-initial}}$ and $k_{\text{off}}$ that directly came from our BFP measurements of corresponding
ligands binding with NKG2D on cells (Figs 1D, and 3F). Based on these settings, we then
set up a series of simulations to mimic the active signal respectively induced by three ligands
MICA, MICB and ULBP3. Comparing the simulated signals with the observed activation
signals for the three ligands under different forces, we could iteratively identify the possible
numerical ranges of unknown parameters for the current model. For instance, Fig R13A
shows the simulation results for three ligands with different values of the rate constant ($k_p$
varies from 1 to 5 s\(^{-1}\)) and the number of steps ($\text{steps}$ vary from 4 to 20) of forward steps
under optimal force 10 pN. Fig R13B shows the simulation results for three ligands under
different forces, as $k_{\text{on-rebinding}}$ varies from 0.1 to 100 s\(^{-1}\). After iteratively comparing the
simulated signals with the observed activation signals for the three ligands, we set $k_p$ as 2 s\(^{-1}\),
$k_{\text{on-rebinding}}$ as 10 s\(^{-1}\) (>> $k_{\text{on-initial}}$), and $\text{steps}$ as 10 as currently appropriate parameter values to
ensure simulation results consistent with experimental observations.
Figure for reviewers removed
Minor concerns.

1) additional supporting data should be provided: determination of surface density of ligands and receptors, the calibration of the BFP spring constant

We thank the reviewer for this suggestion. The method we used is described in ‘Determination of molecular densities on the surfaces of NK cells and RBCs’ of section ‘Materials and Methods’ (Page 24-25) and our supporting data were shown in Fig R14A-G. We also updated the data in our revised manuscript Fig EV1B-H.
We have carried out BFP spring constant calibration using thermal fluctuation analysis as previously described (Chen et al., 2008; Ju & Zhu, 2017) or see and the results are shown in Fig R15. According to our results, the data points display a linear trend with a slope of 4.60, which is in agreement with the $k_B T$ value (around 4.12 when $t = 25\, ^\circ C$) and supporting the validity of Evans’ model we used in BFP spring constant calculation. We have added the results in Fig EV3A-D and updated our method by adding the details of BFP calibration in ‘BFP spring constant calibration’ of section ‘Materials and Methods’ (Page 23, red-colored).
2) in-situ force free kinetics and Fig.1: the graphs showing simultaneously in-situ and in-solution data should span a similar interval on left and right axis (1D, 1E, 1F), even if expressed in different units. The authors should explicit the formula they use to fit the data in fig 1C, with free parameters kon and koff and show explicitely that the 2 parameters are no correlated.

Thank Reviewer 3 for raising up these minor concerns. As for Fig 1d-f, Reviewer 1 has also pointed out that the use of two axes on one plot is non-stand, so we have changed these plots accordingly (Fig 1C-E).

The formula (Formula 2) we use in extracting force-free kinetics in Fig 1C is as below:

\[ P_a = 1 - \exp\left(-m_r m_l A_c K_a [1 - \exp(-k_{off} t_c)]\right) \]  

(2)

where \( m_r \) and \( m_l \) are the densities of the receptor and ligand respectively, \( A_c K_a \) is the effective in-situ affinity under no force, \( k_{off} \) is the in-situ off-rate under no force. \( A_c K_a \) and \( k_{off} \) can be obtained independently by fitting the experimental data with the formula. The effective in-situ on-rate \( A_c k_{on} \) under no force was obtained from corresponding \( A_c K_a \) and \( k_{off} \) according to the following formula (Formula 3):

\[ A_c k_{on} = A_c K_a \times k_{off} \]  

(3)

So the 2 parameters \( (A_c k_{on} \) and \( k_{off} \)) under no force are actually correlated with each other and dependents on the effective in-situ affinity \( A_c K_a \) under no force. We have revised
our manuscript as the reviewer suggested in ‘Micropipette adhesion frequency assay’ of section ‘Materials and Methods’ (Page 26, red-colored).

3) the authors claim in the discussion that their model reconcile the 2 models proposed in the literature to explain ligand discrimination by NKG2D: induced fit and rigid adaptation. Those general mechanisms usually refer to molecular scale processes in solution, so the statement is not meaningful to my opinion. It is neither necessary, since the authors emphasize in the introduction the various contractions of those models with experimental evidences.

We thank the reviewer for pointing out this question. We have revised our statement in the updated manuscript according to this suggestion (Page 16, Line 7-10, red-colored).

Revise the text.

page 9: fig 3g not 2g.
Page 9: reformulate: "render NKG2D the power to discriminate ...
page 11: reformulate: "reveal that the activation of both ..."
page 13: fig 6d not 6c
page 19: explicit pHAGE and iRES acronyms?
Page 21: 5 mim → 5 min

We are really sorry for our careless mistakes. Many thanks to Reviewer 3 for this kind and careful reminding. We have corrected these mistakes in the revised text and proofread the manuscript carefully.

(Page 10, Line 27, red-colored);
(Page 10, Line 22-23, red-colored);
(Page 13, Line 9-11, red-colored);
(Page 15, Line 1, red-colored);
(Page 21, Line 27-29, red-colored; and Page 22, Line 2-3, red-colored),
(Page 24, Line 22, red-colored).
References:

Balint S, Lopes FB, Davis DM (2018) A nanoscale reorganization of the IL-15 receptor is triggered by NKG2D in a ligand-dependent manner. Sci Signal 11

Basu R, Whitlock BM, Husson J, Le Floc’h A, Jin W, Oyler-Yaniv A, Dotiwala F, Giannone G, Hivroz C, Bias N et al (2016) Cytotoxic T Cells Use Mechanical Force to Potentiate Target Cell Killing. Cell 165: 100-110

Brown AC, Dobbie IM, Alakoskela JM, Davis I, Davis DM (2012) Super-resolution imaging of remodeled synaptic actin reveals different synergies between NK cell receptors and integrins. Blood 120: 3729-3740

Brown AC, Oddos S, Dobbie IM, Alakoskela JM, Parton RM, Eissmann P, Neil MA, Dunsby C, French PM, Davis I et al (2011) Remodelling of cortical actin where lytic granules dock at natural killer cell immune synapses revealed by super-resolution microscopy. PLoS Biol 9: e1001152

Chen W, Zarnitsyna VI, Sarangapani KK, Huang J, Zhu C (2008) Measuring Receptor-Ligand Binding Kinetics on Cell Surfaces: From Adhesion Frequency to Thermal Fluctuation Methods. Cell Mol Bioeng 1: 276-288

Chesla SE, Selvaraj P, Zhu C (1998) Measuring two-dimensional receptor-ligand binding kinetics by micropipette. Biophys J 75: 1553-1572

Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, Kubin M, Chalupny NJ (2001) ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity 14: 123-133

Deguine J, Breart B, Lemaitre F, Di Santo JP, Bousso P (2010) Intravital imaging reveals distinct dynamics for natural killer and CD8(+) T cells during tumor regression. Immunity 33: 632-644

Dhar P, Wu JD (2018) NKG2D and its ligands in cancer. Curr Opin Immunol 51: 55-61

Dunn C, Chalupny NJ, Sutherland CL, Dosch S, Sivakumar PV, Johnson DC, Cosman D (2003) Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity. J Exp Med 197: 1427-1439

Dushek O, Das R, Coombs D (2009) A role for rebinding in rapid and reliable T cell responses to antigen. Plos Comput Biol 5: e1000578

Eagle RA, Jafferji I, Barrow AD (2009) Beyond Stressed Self: Evidence for NKG2D Ligand Expression on Healthy Cells. Curr Immunol Rev 5: 22-34

Fang L, Gong J, Wang Y, Liu R, Li Z, Wang Z, Zhang Y, Zhang C, Song C, Yang A et al (2014) MICA/B expression is inhibited by unfolded protein response and associated with poor prognosis in human hepatocellular carcinoma. J Exp Clin Cancer Res 33: 76

Hannezo E, Heisenberg CP (2019) Mechanochemical Feedback Loops in Development and Disease. Cell 178: 12-25

Huang J, Zarnitsyna VI, Liu B, Edwards LJ, Jiang N, Evavold BD, Zhu C (2010) The kinetics of two-dimensional TCR and pMHC interactions determine T-cell responsiveness. Nature 464: 932-936

Ju L, Zhu C (2017) Benchmarks of Biomembrane Force Probe Spring Constant Models. Biophys J 113: 2842-2845

Le Saux G, Bar-Hanin N, Edri A, Hadad U, Porgador A, Schwartzman M (2019) Nanoscale Mechanosensing of Natural Killer Cells is Revealed by Antigen-Functionalized Nanowires. Adv Mater 31: e1805954

Li K, Mandai M, Hamanishi J, Matsumura N, Suzuki A, Yagi H, Yamaguchi K, Baba T, Fujii S,
Konishi I (2009) Clinical significance of the NKG2D ligands, MICA/B and ULBP2 in ovarian cancer: high expression of ULBP2 is an indicator of poor prognosis. Cancer Immunol Immunother 58: 641-652
Liu B, Chen W, Evavold BD, Zhu C (2014) Accumulation of dynamic catch bonds between TCR and agonist peptide-MHC triggers T cell signaling. Cell 157: 357-368
Liu Y, Zhang T, Zhang H, Li J, Zhou N, Fiskesund R, Chen J, Lv J, Ma J, Zhang H et al (2021) Cell Softness Prevents Cytolytic T-cell Killing of Tumor-Repopulating Cells. Cancer Res 81: 476-488
McFarland BJ, Strong RK (2003) Thermodynamic analysis of degenerate recognition by the NKG2D immunoreceptor: Not induced fit but rigid adaptation. Immunity 19: 803-812
McGilvray RW, Eagle RA, Rolland P, Jafferji I, Trowsdale J, Durrant LG (2010) ULBP2 and RAET1E NKG2D ligands are independent predictors of poor prognosis in ovarian cancer patients. Int J Cancer 127: 1412-1420
McKeithan TW (1995) Kinetic proofreading in T-cell receptor signal transduction. Proceedings of the national academy of sciences 92: 5042-5046
Petridou NI, Spiro Z, Heisenberg CP (2017) Multiscale force sensing in development. Nat Cell Biol 19: 581-588
Roy S, Barnes PF, Garg A, Wu S, Cosman D, Vankayalapati R (2008) NK cells lyse T regulatory cells that expand in response to an intracellular pathogen. J Immunol 180: 1729-1736
Sutherland CL, Chalupny NJ, Schooley K, VandenBos T, Kubin M, Cosman D (2002) UL16- binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells. J Immunol 168: 671-679
Tello-Lafoz M, Srpan K, Sanchez EE, Hu J, Remsik J, Romin Y, Calo A, Hoen D, Bhanot U, Morris L et al (2021) Cytotoxic lymphocytes target characteristic biophysical vulnerabilities in cancer. Immunity 54: 1037-1054 e1037
Tian M, Li Y, Liu W, Jin L, Jiang X, Wang X, Ding Z, Peng Y, Zhou J, Fan J et al (2015) The nanomechanical signature of liver cancer tissues and its molecular origin. Nanoscale 7: 12998-13010
Wang Z, Guan D, Wang S, Chai LYA, Xu S, Lam KP (2020) Glycolysis and Oxidative Phosphorylation Play Critical Roles in Natural Killer Cell Receptor-Mediated Natural Killer Cell Functions. Front Immunol 11: 202
Zhu C, Chen W, Lou J, Rittase W, Li K (2019) Mechanosensing through immunoreceptors. Nat Immunol 20: 1269-1278
Dear Prof Chen,

Thank you for the submission of your revised manuscript to The EMBO Journal. As you will see below, your article has been seen by two of the original referees, who now consider that you have properly dealt with all of their major concerns. Referee #1, however, still raises a couple of issues that will need to be discussed in the final version of your paper (see below).

Additionally, there are a few editorial issues that need your attention:

- Please, provide 3 to 5 keywords describing the topic of your paper.
- The manuscript needs a Data Availability Section where you describe the databases in which datasets have been made available. For transparency, this section is required even if just to mention that there are no public datasets associated to the results in the paper.
- Movies must be provided in ZIP folders including a text document with their legend (currently included in the manuscript). Each movie and its corresponding legend in a separate ZIP folder. Correct nomenclature is "Movie EV1". Make sure callouts in the manuscript are correct (Movies EV3 and EV4 are not called out). Movie EV3 does not play in QuickTime but plays in VLC player. Please make the movie as compatible as possible for visibility.
- Please provide the paper's synopsis composed of:
  1) a short 'blurb' text summarizing in two sentences the study (max. 250 characters). Add as well three to four 'bullet points' highlighting the main findings. Bullet points and standfirst text should be submitted as a separate manuscript file in LaTeX, RTF or MS Word format.
  2) A "synopsis image", which can be used as a "visual title" for the synopsis section of your paper. The image should be PNG or JPG format with pixel dimensions of 550 x 300-600 (width x height).

Please let me know if you have any further questions regarding any of these points. Thank you again for giving us the chance to consider your manuscript for The EMBO Journal and congratulations!

I look forward to receiving the final version of your manuscript with these minor changes included.

Yours sincerely,

David del Alamo
Editor
The EMBO Journal

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Please click on the link below to submit the revision online:

Link Not Available

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Referee #1:

The new data and discussion have substantially improved the manuscript. I have just a couple remaining questions, which the authors should be able to address textually.

1) The discriminatory capacity of MKG2D is explored at great length in Fig. 6-7, but the results don't entirely make sense. Indeed, the simulation studies (Fig 7) and the affinity measurements under force (Fig 6) seem, if anything, to dramatically overestimate the level of biological discrimination that is actually observed. Fig 2 indicates that MICA provides less than 10-fold stronger NKG2D stimulation than does ULBP3. However, Fig 6 seems to suggest a 150-fold difference in stimulatory capacity, while Fig 7 seems to suggest that, under force, ULBP3-induced signal induction actually approaches 0. Do the authors really mean to suggest that ULBP3 is incapable of eliciting NKG2D activation? I'm probably misunderstanding them, but I'm likely not the only reader who will be confused by this apparent conflict between data and interpretation. The authors should address this issue in their revised Discussion.

2) On p5, it is written, "Moreover, MIC family is more frequently and abundantly expressed on the surface of tumor cells (Dhar &
Wu, 2018), which correlates with overall survival of tumor patients better than ULBP family; thus, MIC family expression has been reported as an indicator for poor prognosis (Fang et al, 2014; Li et al, 2009; McGilvray et al, 2010). I don't understand what the authors are saying here. If MIC proteins correlate with overall survival, then how can they be indicators of poor prognosis?

Referee #2:

The authors have adequately addressed my concerns.
The authors performed the requested editorial changes.
Konishi I (2009) Clinical significance of the NKG2D ligands, MICA/B and ULBP2 in ovarian cancer: high expression of ULBP2 is an indicator of poor prognosis. *Cancer Immunol Immunother* 58: 641-652
Liu B, Chen W, Evavold BD, Zhu C (2014) Accumulation of dynamic catch bonds between TCR and agonist peptide-MHC triggers T cell signaling. *Cell* 157: 357-368
Liu Y, Zhang T, Zhang H, Li J, Zhou N, Fiskesund R, Chen J, Lv J, Ma J, Zhang H et al (2021) Cell Softness Prevents Cytolytic T-cell Killing of Tumor-Repopulating Cells. *Cancer Res* 81: 476-488
McFarland BJ, Strong RK (2003) Thermodynamic analysis of degenerate recognition by the NKG2D immunoreceptor: Not induced fit but rigid adaptation. *Immunity* 19: 803-812
McGilvray RW, Eagle RA, Rolland P, Jafferji I, Trowsdale J, Durrant LG (2010) ULBP2 and RAET1E NKG2D ligands are independent predictors of poor prognosis in ovarian cancer patients. *Int J Cancer* 127: 1412-1420
McKeithan TW (1995) Kinetic proofreading in T-cell receptor signal transduction. *Proceedings of the national academy of sciences* 92: 5042-5046
Petridou NI, Spiro Z, Heisenberg CP (2017) Multiscale force sensing in development. *Nat Cell Biol* 19: 581-588
Roy S, Barnes PF, Garg A, Wu S, Cosman D, Vankayalapati R (2008) NK cells lyse T regulatory cells that expand in response to an intracellular pathogen. *J Immunol* 180: 1729-1736
Sutherland CL, Chalupny NJ, Schooley K, VandenBos T, Kubin M, Cosman D (2002) UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells. *J Immunol* 168: 671-679
Tello-Lafoz M, Srpan K, Sanchez EE, Hu J, Remsik J, Romin Y, Calo A, Hoen D, Bhanot U, Morris L et al (2021) Cytotoxic lymphocytes target characteristic biophysical vulnerabilities in cancer. *Immunity* 54: 1037-1054 e1037
Tian M, Li Y, Liu W, Jin L, Jiang X, Wang X, Ding Z, Peng Y, Zhou J, Fan J et al (2015) The nanomechanical signature of liver cancer tissues and its molecular origin. *Nanoscale* 7: 12998-13010
Wang Z, Guan D, Wang S, Chai LYA, Xu S, Lam KP (2020) Glycolysis and Oxidative Phosphorylation Play Critical Roles in Natural Killer Cell Receptor-Mediated Natural Killer Cell Functions. *Front Immunol* 11: 202
Zhu C, Chen W, Lou J, Rittase W, Li K (2019) Mechanosensing through immunoreceptors. *Nat Immunol* 20: 1269-1278
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   - The analysis and methods used to carry out the reported observations and measurements.
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   - An explicit mention of the biological and chemical entity(ies) that are being measured.
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     - Definitions of error bars as s.d. or s.e.m.
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