MHC class I molecules co-stimulate NK1.1 signaling and enhance Ca\(^{2+}\) flux in murine NK cells

Murine NK cells express MHC class I specific inhibitory receptors of the Ly49 family [1]. MHC class I molecules act as gatekeepers for NK cell tolerance and only in the absence of MHC class I on surrounding cells, tolerance is broken and NK cell react against “missing self” [2]. MHC class I molecules are not only ligands for inhibitory NK cell receptors, but may also inhibit NK cell function themselves [3–5]. MHC class I molecules lack signaling motifs in their intracellular domains [6], and their direct inhibitory roles have been suggested to depend on MHC class I associated proteins at the NK cell surface [6].

We used a recently developed Ca\(^{2+}\) flux assay in mouse NK cells to ask if cross-linking of MHC class I would influence signaling downstream of activating receptors [7]. When the activating receptor NK1.1 and MHC class I molecules were co-cross-linked, we found an additive effect on Ca\(^{2+}\) flux, characterized by an enhanced peak and a sustained slope of the calcium release curve (Fig. 1A–C; Supporting information Fig. S1). Cross-linking MHC class I molecules alone also triggered a Ca\(^{2+}\) flux response, but with a different kinetics characterized by late onset and a slower rise (Fig. 1A–C; Supporting information Fig. S1). In the absence of extracellular Ca\(^{2+}\), the magnitude of Ca\(^{2+}\) flux was decreased but co-stimulation persisted, showing that the effect of MHC class I co-cross-linking did not depend exclusively on extracellular Ca\(^{2+}\) (Fig. 1D). The MHC class I molecule H2K\(^{b}\) bind the inhibitory Ly49C receptor in cis [8]. To allow analysis of Ly49C+ NK cells independently, we included an antibody in the panel that stained Ly49C+ NK cells but did not trigger NK cell inhibition. The effects of cross-linking H2K\(^{b}\) was similar on Ly49C+ and Ly49C- NK cells (Fig. 1E), showing that the simultaneous expression of a Ly49 receptor with the potential to bind H2K\(^{b}\) in cis was not a prerequisite for the co-stimulatory effect of H2K\(^{b}\).

Because of the unusual calcium flux profile following H2K\(^{b}\) cross-linking, we asked if this response was targeted by Ly49 receptor-mediated inhibition. To test this, we included a fluorochrome-conjugated antibody against the inhibitory receptor Ly49G2, which in this case transmits inhibitory signals following cross-linking [7]. When Ly49G2 was co-cross-linked with H2K\(^{b}\), the Ca\(^{2+}\) signal was aborted compared to Ly49G2- NK cells in the same sample (Fig. 1F). Similarly, the additive effect between NK1.1 and H2K\(^{b}\) was reduced in the presence of Ly49G2 cross-linking (Fig. 1F). This experiment revealed that the Ca\(^{2+}\) flux response downstream of H2K\(^{b}\) triggering was indeed a real signaling response, since it could be actively dampened by cross-linking of an inhibitory Ly49 receptor. To exclude that that the additive effect of the anti-H2K\(^{b}\) antibody was dependent on FcγRIIIa (CD16), we added two blocking antibodies against FcγRIIIa (clone 2.4G2 and clone 93) to our assay. This did not prevent the additive effect of anti-H2K\(^{b}\) on NK1.1-mediated Ca\(^{2+}\) flux (Fig. 1G), suggesting that the enhancing effect of anti-H2K\(^{b}\) was independent from Fc receptor binding.

Poly-I:C, a TLR 3 agonist, induced NK cell activation in vivo as shown by upregulation of the activation marker CD69 but also of NK1.1, NKp46, and H2K\(^{b}\) (Fig. 2B). Poly-I:C enhanced the response to all stimuli in our system, including both anti-H2K\(^{b}\) alone and of anti-H2K\(^{b}\) + anti-NK1.1 (Fig. 2B), suggesting that cross-linking of H2K\(^{b}\) augments NK1.1 signaling by a general enhancement of signaling capacity additive to TLR priming. These data also demonstrate that Poly I:C stimulation operates, at least partly, at the level of early Ca\(^{2+}\) signaling. Finally, to test if the augmented Ca\(^{2+}\) flux response after H2K\(^{b}\) and NK1.1 co-cross-linking affected IFN-γ secretion and degranulation, we switched to a stimulation protocol based on coating plates with antibodies, adding NK cells and allowing them to be stimulated by the antibodies over 4 h. NK1.1 stimulation resulted in both IFN-γ and degranulation, but adding an antibody to H2K\(^{b}\) to the plate did not affect these responses (Fig. 2C and D).

In contrast to the dampening effect of MHC class I cross-linking seen in other systems [3–5], we found that stimulation of MHC class I molecules in primary mouse NK cells triggered a Ca\(^{2+}\) flux response in itself as well as provided an additive effect on immunoreceptor tyrosine-based activating (ITAM) motif mediated Ca\(^{2+}\) flux. The slow onset response using

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MHC class I triggering alone was distinct from the Ca$^{2+}$ flux response reported after MHC I cross-linking on T cells and B cells, in which a rapid, sharp onset of Ca$^{2+}$ flux was followed by a slow decline [9]. The response in our system bears more similarities to triggering of some NK cell receptors in human, for example, 2B4 [10], which is interesting in light of a previous study in humans showing that MHC class I molecules associate with 2B4 at the NK cell surface and that MHC class I cross-linking dampens 2B4-mediated killing responses [5]. It remains to be tested if MHC class I molecules bind 2B4 also in mouse NK cells, and if so, to which extent our results would be dependent on 2B4 signaling.
Figure 2. (A, B) Poly-I:C treatment enhances Ca^{2+} flux after receptor cross-linking and maintains the costimulatory effect of H2Kb cross-linking. (A) Cell surface staining of NK1.1, NKp46, CD69, and H2Kb. (B) Ca^{2+} flux response in Poly-I:C treated and untreated B6 mice after receptor cross-linking. Data from one mouse of two analyzed in separate experiments. (C, D) H2Kb co-cross-linking does not augment NK1.1-mediated cytokine secretion and degranulation. (C) Co-staining of IFN-γ and CD107 after cross-linking of the indicated antibodies. (D) Data from six mice in six different experiments with one mouse in each. Statistical analysis using a mixed-effect analysis (due to missing data in some groups) with Tukey’s multiple comparison test. ns = not significant.

Intriguingly, the enhanced Ca^{2+} flux did not translate into increased IFN-γ secretion or degranulation, suggesting that the enhanced Ca^{2+} flux induced by NK1.1+H2Kb may be qualitatively different compared to the response triggered by ITAM signaling alone. Ca^{2+} flux is crucial for effector lymphocyte function but signaling via activating receptors is complex and may not necessarily imply effector cell function. Strong immune cell activation might induce an exhaustion phenotype.
rather than a powerful effector cell response. Perhaps the costimulatory role of H2Kb in our model is geared towards enhanced proliferation or secretion of factors that we did not measure. Another more trivial, yet possible explanation, could be that Ca2+ flux was measured in solution using two antibodies, while IFN-γ and CD107a were measured after 4 h of stimulation using plate-bound primary antibodies alone. Two parameters differ in these settings: the type and time of stimulation, both of which might affect the experimental result and determine downstream effects resulting from the additive Ca2+ flux.

Further work needs to be performed to identify the signaling pathways involved in MHC class I signaling and which cis-binding NK cell surface proteins that might be responsible for transmitting this signaling response. The physiological relevance of MHC class I cross-linking also needs to be identified, but it is not unlikely that any protein with signaling capacity that bind to MHC class I, in trans or in cis, could be involved. Identifying the functional consequences of MHC class I induced Ca2+ flux is an important next step, requiring novel experimental models in which MHC class I triggering can also be induced by more physiological ligands in cellular systems.

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References

1 Hoglund, P. and Brodin P, Nat. Rev. Immunol. 2010. 10: 724–734.
2 Karre, K. et al., Nature 1986. 319: 675–678.
3 Petersson, M. G. et al., Scand. J. Immunol. 1995. 42: 34–38.
4 Rubio, G. et al., J. Leukoc. Biol. 2004. 76: 116–124.
5 Betser-Cohen, G. et al., J. Immunol. 2010. 184: 2761–2768.
6 Gur, H. et al., J. Exp. Med. 1990. 172: 1267–1270.
7 Ganesan, S. et al., Front. Immunol. 2018. 9: 3173.
8 Held, W. et al, Cell. Mol. Life Sci. 2011. 68: 3469–3478.
9 Pedersen, A. E. et al., Exp. Cell Res. 1998. 240: 144–150.
10 Bryceson, Y. T. et al., Blood 2006. 107: 159–166.

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