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Cbl ubiquitin ligases mediate the inhibition of natural killer cell activity

Omri Matalon and Mira Barda-Saad

The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel

ABSTRACT

Natural killer (NK) cells are essential for killing transformed and virally infected cells. To prevent auto-reactivity, NK cell activation is inhibited by inhibitory receptors that activate the tyrosine phosphatase SHP-1, which dephosphorylates signaling molecules crucial for NK cell activation. Initially, only a single SHP-1 substrate was identified in NK cells, the GEF VAV1. We recently demonstrated that under inhibitory conditions, LAT, PLCγ1 and PLCγ2 serve as novel SHP-1 substrates in NK cells. Furthermore, we showed that during NK cell inhibition, LAT is ubiquitylated by c-Cbl and Cbl-b, leading to its proteasomal degradation, abolishing NK cell cytotoxicity. Here, we address the mechanism through which the Cbl proteins are activated following inhibitory receptor engagement. We demonstrate that during NK cell inhibition, the expression level of the Cbl proteins significantly increases. These data suggest that inhibitory KIR receptors regulate the stability of the Cbl proteins, thereby enabling Cbl-mediated inhibition of NK cell cytotoxicity.

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Natural killer (NK) cells are key components of the immune system, crucial for killing cancerous and virally infected cells. NK cells mediate their effects through their cytotoxicity toward the target cells, and production of cytokines that activate the adaptive immune response. To prevent auto-reactivity, NK cell activation is regulated by a dynamic balance between activating and inhibitory signals, transduced upon engagement of activating and inhibitory receptors expressed on the NK cell surface.[1]

NK cell activation is dependent on the activity of the phospholipase Cγ (PLCγ) family of proteins, including PLCγ1 and PLCγ2.[2-4] PLCγ isoforms are responsible for the cleavage of membrane-associated phosphatidylinositol 4,5-bisphosphate into inositoltriphosphate and diacylglycerol, which induce intracellular Ca^{2+} mobilization and activation of signaling pathways essential for NK cell effector functions.[5-7]

To perform their activity, PLCγ1/2 must be recruited to the contact site of the NK cell with its target, known as the NK immunological synapse (NKIS). In T cells, PLCγ1 recruitment is mediated by the transmembrane adaptor protein, the linker for activation of T cells (LAT).[8] LAT contains several tyrosine residues, which upon phosphorylation, serve as docking sites for the formation and integration of signaling complexes crucial for cellular activation.[5,9,10] We recently showed that following NK cell activation, PLCγ1/2 are recruited to the NKIS via LAT tyrosine 132, where they undergo tyrosine phosphorylation that regulates their activity. We showed that the formation of these LAT:PLCγ complexes is crucial for NK cell cytotoxicity.[11]

The inhibition of NK cell activity is regulated by several mechanisms that ensure self-tolerance to the host. These mechanisms comprise the activity of inhibitory receptors specific to MHC class I molecules, including the killer-cell immunoglobulin-like receptors (KIR). Inhibitory receptors block NK cell activation by promoting dephosphorylation of signaling proteins via the recruitment and activation of the protein tyrosine phosphatases, SH2-domain-containing protein tyrosine phosphatase-1 (SHP-1) and SHP-2.[12-15] SHP-1 activity is a key inhibitory mechanism attenuating NK cell activation. However, VAV1 was the only known direct substrate of SHP-1.[16] We recently revealed that LAT, PLCγ1 and PLCγ2 serve as additional substrates of SHP-1 during NK cell inhibition. We demonstrated that SHP-1 dephosphorylates LAT tyrosine 132, thereby blocking the recruitment of PLCγ1/2 to the inhibitory NKIS, resulting in diminished cancer cell killing.[11]

Lymphocyte activity is also regulated by signaling molecule ubiquitylation, which results in their internalization or degradation. This modification is mediated by the E3

CONTACT Mira Barda-Saad Mira.Barda-Saad@biu.ac.il The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 5290002, Israel.

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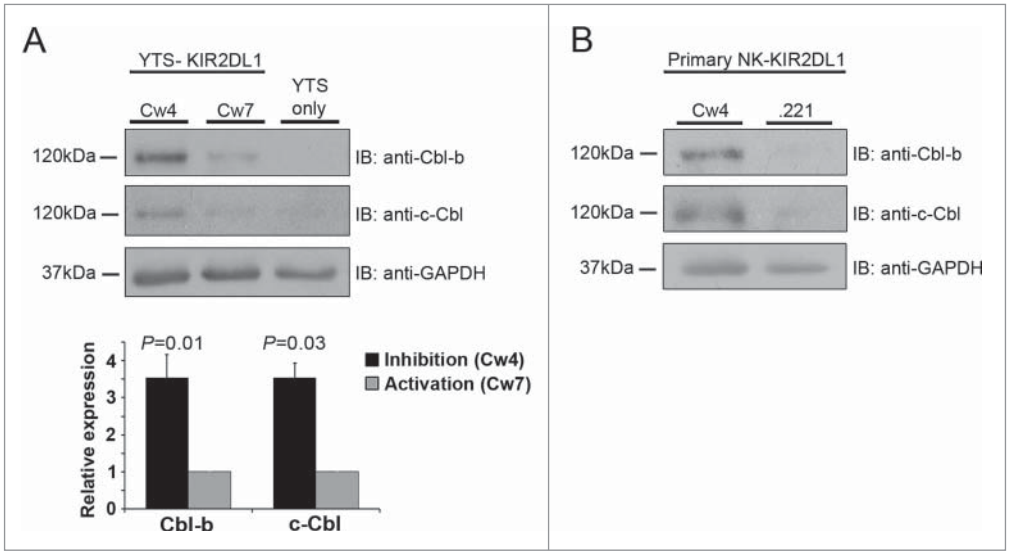

Figure 1. Increased expression of Cbl-b and c-Cbl during NK cell inhibition versus activation. (A) YTS NK cells expressing the inhibitory KIR2DL1 receptor (YTS-KIR2DL1 cells) were incubated with either inhibitory 721.221-Cw4 (Cw4), or activating 721.221-Cw7 (Cw7) target cells at 37°C for 20 min, followed by cell lysis. Cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane that was immunoblotted using the indicated antibodies. Cbl-b and c-Cbl expression levels were measured by densitometric analysis, relative to the loading control, using ImageJ. (B) Primary NK-KIR2DL1 cells were incubated with inhibitory 721.221-Cw4, or activating 721.221 (.221) target cells at 37°C for 20 min, and the cells were lysed. Cell lysates were subjected to Immunobloting (IB) with anti-Cbl-b and anti-c-Cbl antibodies. Data are representative of at least 3 independent experiments.

ubiquitin ligases c-Cbl and Cbl-b, which negatively regulate immune-cell activation.[17-23] In T cells, we extensively explored the role of the Cbls in terminating T cell activity by controlling the stability of signaling proteins, including WASp,[18] Nck[19] and LAT.[22] This ubiquitylation dependent degradation serves as a mechanism for blocking signal propagation delivered following activating receptor engagement.[17-23] However, until recently no studies described the role of ubiquitylation as a mechanism for NK cell inhibition via inhibitory receptor engagement. We revealed that at the inhibitory NKIS, LAT is ubiquitylated by c-Cbl and Cbl-b on lysines 52 and 204, leading to its proteasomal degradation, and thereby abolishing NK cell activation and cytotoxicity. Expression of the ubiquitylation resistant LAT significantly increased NK cell mediated killing of inhibitory target cells, converting the inhibitory NK cell response into an activating one.[11]

We found that LAT ubiquitylation is dependent on its phosphorylation,[11] suggesting that only LAT molecules that escape SHP-1-mediated dephosphorylation are targeted to proteasomal degradation. These data imply that NK cell inhibition relies on cooperative activity of SHP-1 and the Cbls to ensure immune tolerance to the host. However, while the mechanism through which inhibitory receptors recruit and activate SHP-1 was previously explored, the mechanisms responsible for promoting Cbl-mediated inhibition of NK cell cytotoxicity are still unknown.

To address the Cbl activation mechanisms, we investigated whether Cbl expression levels differ following NK cell inhibition versus activation. To this end, primary NK cells isolated from donor blood, and the YTS NK cell line, expressing the inhibitory KIR2DL1 receptor, were incubated with either inhibitory 721.221 target cells that express the cognate HLA- Cw4 molecule (i.e. KIR2DL1 ligand), or with activating 721.221 target cells expressing no HLA or the HLA-Cw7 molecule. Using western blot analysis we demonstrated that the expression levels of both c-Cbl and Cbl-b are upregulated in NK cells during the establishment of an inhibitory interaction, as compared to an activating one (Fig. 1). Potentially, accumulation of the Cbl proteins enables their ubiquitylation of LAT, thereby inhibiting NK cell cytotoxicity.

Several mechanisms might be responsible for the elevated expression of Cbl-b and c-Cbl following NK cell inhibition vs. activation. These include the increased expression of the Cbl protein at the transcriptional level following NK cell inhibition, or protein degradation following cellular activation. However, it is not known whether the expression of the Cbls is regulated by inhibitory or activating signals, and therefore further investigation is required to determine the precise mechanism through which inhibitory receptors promote the E3 ligase activity of Cbl-b and c-Cbl. The identification of these mechanisms is critical for the full understanding of how the Cbl proteins mediate ubiquitylation dependent degradation of key signaling molecules that regulate NK cell function.

Abbreviations

Cbl	Casitas-B-lineage lymphoma
CD3	Cluster of differentiation 3
DAG	Diacylglycerol
GEF	Guanine nucleotide exchange factor
IP3	Inositoltriphosphate
KIR	Killer-cell immunoglobulin-like receptor
LAT	Linker for activation of T cells
MHC	Major histocompatibility complex
NK	Natural killer
NKIS	Natural killer immunological synapse
PIP2	Phosphatidylinositol 4,5-bisphosphate
PLCγ	phospholipase Cγ
SHP-1	SH2-domain-containing protein tyrosine phosphatase-1
TCR	T-cell receptor

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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