Negative Regulation Of Nk Cell Activation By Cbl-B And Tam Receptors

Leilani Marie Chirino
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Negative Regulation Of Nk Cell Activation By Cbl-B And Tam Receptors

Abstract
Natural Killer (NK) cells are innate immune cells equipped with the ability to rapidly kill stressed cells that are neoplastic or virally-infected. NK cells are especially important in settings where these stressed cells down-regulate MHC class I molecules and evade recognition by cytotoxic T cells. However, the activity of NK cells alone is often suboptimal to fully control tumor growth or to clear viral infections. Thus, the enhancement of NK cell function is necessary to fully harness their anti-tumor or anti-viral potential. TAM receptors (Tyro3, Axl, and Mer) are receptor tyrosine kinases (RTKs) that are expressed by multiple immune cells including NK cells. Although RTKs typically enhance cellular functions, TAM receptor ligation blocks NK cell activation. The mechanisms by which RTKs block NK cell signaling downstream of activating receptors are unknown. We demonstrate that TAM receptors attenuate NK cell responses via the activity of the E3 ubiquitin ligase Cbl-b. Specifically, we show that Tyro3, Axl, and Mer phosphorylate Cbl-b, and Tyro3 ligation activates Cbl-b by phosphorylating tyrosine residues 133 and 363. Ligation of TAM receptors by their ligand Gas6 suppresses activating receptor-stimulated NK cell functions, such as IFNγ production and degranulation, in a TAM receptor kinase- and Cbl-b-dependent manner. Moreover, Gas6 ligation induces the degradation of LAT1, a transmembrane adaptor protein required for NK cell activating receptor signaling, in WT but not in Cbl-b knock-out (KO) NK cells. Together, these results suggest that TAM receptors may attenuate NK cell function by phosphorylating Cbl-b, which in turn dampens NK cell activation signaling by promoting the degradation of LAT1. Our data, therefore, support a mechanism by which RTKs attenuate, rather than stimulate, signaling pathways via the activation of ubiquitin ligases. Based on our findings, the TAM/Cbl-b pathway has key molecules that can be targeted therapeutically for the treatment of cancers and viral infections. In collaboration with Progenra, Inc, we tested several novel small molecule inhibitors that target Cbl-b. Several of the inhibitors tested enhanced NK and T cell effector functions. One inhibitor tested in vivo did not have any effect in decreasing tumor burden, likely due to the poor half-life of the in vivo formulation. More inhibitors in development will be tested in the future. These inhibitors provide a promising novel therapeutic strategy to enhance NK and T cell activation in the setting of a variety of cancers and possibly chronic viral infections.

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NEGATIVE REGULATION OF NK CELL ACTIVATION BY CBL-B AND TAM RECEPTORS

Leilani M. Chirino
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ABSTRACT

NEGATIVE REGULATION OF NK CELL ACTIVATION BY CBL-B AND TAM RECEPtorS

Leilani M. Chirino
Taku Kambayashi

Natural Killer (NK) cells are innate immune cells that rapidly kill stressed cells that are neoplastic or virally-infected. NK cells are crucial in settings where these stressed cells down-regulate MHC class I molecules and evade recognition by cytotoxic T cells. However, the activity of NK cells alone is often suboptimal to fully control tumor growth or to clear viral infections. Thus, the enhancement of NK cell function is necessary to fully harness their anti-tumor or anti-viral potential. TAM receptors (Tyro3, Axl, and Mer) are receptor tyrosine kinases (RTKs) that are expressed by multiple immune cells including NK cells. Although RTKs typically enhance cellular functions, TAM receptor ligation blocks NK cell activation. The mechanisms by which RTKs block NK cell signaling downstream of activating receptors are unknown. We demonstrate that TAM receptors attenuate NK cell responses via the activity of the E3 ubiquitin ligase Cbl-b. Specifically, we show that TAM receptors phosphorylate Cbl-b, and Tyro3 ligation activates Cbl-b by phosphorylating tyrosine residues 133 and 363. Ligation of TAM receptors by their ligand Gas6 suppresses activating receptor-stimulated NK cell functions, such as IFNγ production and degranulation, in a TAM receptor kinase- and Cbl-b-dependent manner. Moreover, Gas6 ligation induces the degradation of LAT1, a transmembrane adaptor protein required for NK cell activating receptor signaling, in WT but not in Cbl-b knock-out (KO) NK cells. Together, these results suggest that TAM...
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LIST OF ABBREVIATIONS

NK (natural killer), LAKs (lymphokine-activated killer cells), DCs (dendritic cells), Graft-versus-host disease (GVHD), Killer-cell immunoglobulin-like receptors (KIRs), Immunoreceptor tyrosine-based activation motif (ITAM), DNAX-activating protein of molecular mass 12 kD (DAP12), mammalian target of rapamycin (mTOR), phosphatase and tensin homolog (PTEN), DAG kinase (DGK), Ras guanyl nucleotide-releasing protein (RasGRP), phosphatidic acid (PA), RING (Really Interesting New Gene), linear ubiquitylation assembly complex (LUBAC), deubiquitinating enzymes (DUBs), Casitas B-lineage lymphoma proto-oncogene (Cbl), Cbl-b (Casitas B-lineage lymphoma b), ubiquitin associated (UBA), KO (knock-out), son-of-sevenless (SOS), mitogen-activated protein kinase (MAPK), Src-like adaptor proteins (SLAP), recombination-activating gene 2 (RAG2), SH2-domain-containing protein tyrosine phosphatase-1 (SHP-1), CIN85 (Cbl-interacting protein of 85 kDa), IFN-γ (interferon gamma), Tumor necrosis factor alpha (TNFα), TAM (Tyro3, Axl, Mer), RTK (receptor tyrosine kinase), LAT (linker for activation of T cells), EGFR (epidermal growth factor receptor), SOCS (suppressor of cytokine signaling), TLR (Toll-like receptor), TKB (tyrosine kinase binding), RF (ring finger), ATP (adenosine triphosphate), ELISA (enzyme-linked immunosorbent assay), i.p. (intraperitoneal), PMA (Phorbol 12-myristate 13-acetate), PI3K (Phosphoinositide 3-kinase), PLCγ1 (Phospholipase C gamma 1).
CHAPTER 1: Introduction$^1$

The Innate Immune System

The human body has two lines of defense against foreign pathogens and tumors - the innate and adaptive immune systems. The innate immune system is the first responder - both fighting the infection and helping induce the adaptive immune system. This first response relies on the recognition of foreign pathogens and tumor cells by germline-encoded receptors of innate immune cells, whereas the adaptive immune system uses variable antigen-specific receptors[1]. These receptors are a result of gene segment rearrangements. Without an innate immune response, the body becomes susceptible to infection despite having an intact adaptive immune system.

The first barrier to a pathogen is the skin; however, as soon as a pathogen infiltrates the skin, the first response by the innate immune system begins with preformed molecules[1]. These include antimicrobial enzymes and peptides that break down bacterial cell walls and membranes, respectively, in addition to the complement system - a group of plasma proteins that targets pathogens for lysis and phagocytosis[1]. After this first phase, cells of the innate immune response sense these proteins as foreign to the host. Three key players of the second phase of the innate immune response include dendritic cells (DCs), macrophages, and natural killer (NK) cells.

Dendritic cells (DCs) are antigen-presenting cells that act as messengers between the innate and adaptive immune systems. Their main function is to process antigens and present it on their cell surface to T cells - a diverse class of adaptive

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immune cells with an array of effector functions[1]. DC presentation of antigen to T cells activates naive T cell signaling. Macrophages are cells that engulf and digest cellular debris, foreign substances, microbes, cancer cells, and anything else that does not have proteins specific to healthy cells on its surface in a process called phagocytosis[1]. Phagocytic cells not only kill invaders but also recruit other immune cells with effector responses that are appropriate for the task at hand. These cells help maintain normal immune homeostasis in the face of constantly changing microbes in the environment.

The focus of this thesis is on NK cells. NK cells are another type of innate immune cell that were first described in the 1970s as large granular lymphocytes with the ability to spontaneously kill tumor cell lines[2]. This killing was different from that performed by cytotoxic T cells (CD8+ T cells), as the eradication of tumor cells by NK cells occurred without previous exposure to the tumor (priming). CD8+ T cells are a subclass of T cells that kill virus-infected and tumor cells that express MHC class I (MHC I), and they require prior exposure to the antigen before making enough T cells to fight the tumor[1].

As opposed to T cells that recognize target cells through their unique T-cell receptor created by gene rearrangement, NK cells express several germline-encoded activating and inhibitory receptors that bind to stress proteins, viral proteins, IgG Fc, and MHC I molecules[3]. It is the balance of the signals that are generated from the ligation of numerous activating and inhibitory receptors that dictates the activation of NK cells. One key aspect of NK cell function is their ability to kill cells that have down-regulated MHC I, a process known as “missing-self” recognition[4],[5]. As NK cells express inhibitory receptors that bind to MHC I molecules, cells with down-regulated MHC I disinhibit NK cells and become targets of NK cell-mediated cytotoxicity. This is an important function of NK cells as tumor cells and virally infected cells often down-
regulate MHC I molecules to evade recognition by CD8⁺ T cells. Thus, NK cells can complement the activity of CD8⁺ T cells that can no longer recognize and kill such cells.

**Strategies to enhance NK cell function for the treatment of tumors and infections**

NK cells use similar effector functions as CD8⁺ T cells. NK cells kill target cells through the release of granules, which contain proteins such as perforin and granzymes. Perforins form pores in the cell membrane of a target cell, allowing granzymes to travel into its cytoplasm and induce apoptosis by damaging key processes for cell survival[6].

Another important function of an NK cell is the production of inflammatory cytokines, such as interferon-gamma (IFNγ) and tumor necrosis factor alpha (TNFα). These cytokines recruit T cells to the site of infection or malignancy and impact the function and maturation of myeloid cells[1].

In addition to CD8⁺ T cells, harnessing the cytotoxic potential of NK cells to fight tumors and viral infections may be beneficial for many reasons. 1) NK cells are able to eliminate transformed cells and virally infected cells in an MHC I/antigen-independent manner, 2) NK cells are activated and respond to a variety of different stimuli, including stress-induced proteins, mismatched MHC I, and missing/down-regulated MHC I, 3) cancers and viral infections have developed evasion tactics to avoid T cell recognition (such as the down-regulation of MHC I), and lastly 4) NK cells potentially cause less immunopathology, such as immunopathology induced by graft-versus-host disease (GVHD), compared to T cells[7]. Thus, the enhancement of NK cell function is an attractive therapeutic strategy against tumors and viral infections.

Currently, NK cell-based therapies have failed in clinical trials for multiple reasons, mainly technical. There have been technical difficulties growing them ex vivo,
and once infused into patients, they often do not persist and expand and have suboptimal effector functions[8]. However, because they are safer to use than T cells, it is worth developing strategies to enhance NK cell function for therapies.

The enhancement of NK cell function can be accomplished in a variety of ways. Three major strategies include:

1. Targeting inhibitory signaling pathways and negative regulators of NK cell activating signaling pathways.
2. Manipulation of inhibitory/activating receptors expressed by NK cells.
3. Cytokine-mediated activation and expansion of NK cells.

NK cells are stimulated or inhibited by ligation of transmembrane receptors. These receptors assemble signaling molecules that distribute a signal, which determines the function of the cell[3]. For this thesis, we have focused on studying inhibitory signaling pathways and negative regulators of NK cell signaling, the latter which may inhibit a signal by limiting the lifetime of these multiprotein signaling complexes.

**NK cell activation**

When an NK cell engages a target cell, the NK cell receives both activating and inhibitory signals from a variety of ligands expressed by the target cell. When interacting with an unstressed healthy cell, NK cells are not activated because the net activating and inhibitory inputs the NK cell receives are balanced. However, tumor cells and a variety of infected cells often increase activating ligands (e.g., NKG2D ligands) and/or decrease inhibitory ligands (e.g. MHC I) on their cell surface. In these scenarios, the NK cell is activated by the increase in activating signals and/or a decrease in inhibitory signals it receives from the target cell. Thus, it is plausible that NK cell function could be
further enhanced by the pharmacological blockade of inhibitory signals or negative regulators.

Compared to T cells, the signal transduction pathways leading from NK cell activating receptors are more complex. In part, this complexity stems from the fact that NK cells use multiple activating receptors to detect their targets. Activating NK cell receptors such as NK1.1, CD16, and activating killer-cell immunoglobulin-like receptors (KIRs) or Ly49 receptors utilize immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor molecules, such as DNAX-activating protein of molecular mass 12 kD (DAP12), CD3ζ, and FcεRγ to initiate signal transduction[9]. Other activating receptors, such as NKG2D and 2B4, use YxxM motif-containing costimulatory adaptors and SAP, respectively, to transduce their signals[3]. Although these activating receptors initiate signal transduction through different proximal mechanisms, many of the critical signaling pathways are shared and regulated in a similar manner. For example, we previously reported that the signaling and NK cell function downstream of all three of these activating receptor families converge upon the adaptor molecule known as SLP-76[10]. Some of the key distal signaling pathways that emanate from SLP-76 include the activation of phosphoinositide 3-kinases (PI3K)s, Vav, and phospholipase C (PLC)γ - all molecules required for NK cell effector functions (Fig. 1.1).
PI3Ks are lipid kinases that regulate a wide variety of signaling pathways downstream of immunoreceptor engagement. When activated, PI3Ks are recruited to the plasma membrane where they phosphorylate the membrane-associated lipid molecule PtdIns(4,5)P2 (PIP2) to form PtdIns(3,4,5)P3 (PIP3). PIP3 molecules can then localize pleckstrin homology (PH) domain-containing proteins to the plasma membrane, allowing for protein-protein interactions to occur[3],[11]. One critical signaling molecule that is

![Diagram of NK cell signaling pathways](image-url)
activated by PIP₃ is AKT. PIP₃ allows the co-localization of AKT and its kinase PDK-1 through their PH domains, leading to AKT phosphorylation[11].

Class I PI3Ks are heterodimeric proteins comprised of a p85 (regulatory) and p110 (catalytic) subunit. Both p85 and p110 subunits have several isoforms with varying functions[11]. The p110δ isoform has been suggested to be critical for NK cell production of IFNγ, GM-CSF, MIP1α, and MIP1β[12]. Moreover, p110δ-PI3K is important for the elimination of BCR/ABL⁺ leukemic cells, and NK cells lacking PI3K had defects in Ca²⁺ mobilization following ionomycin stimulation. Thus, PI3K-p110δ may play a general role in promoting Ca²⁺-triggered exocytosis, which would impact both degranulation and cytokine secretion[13]. Furthermore, PI3K-induced mammalian target of rapamycin (mTOR) signaling is important following NK cell IL-15 priming. IL-15 priming of NK cells enhances their functional capacity including the expression of cytotoxic granules and an increased IFNγ response[14]–[16]. Pharmacological inhibition of PI3K, mTOR, and AKT inhibits IFNγ production following IL-15 priming of stimulated NK cells. PI3K signals are also crucial for the proliferation and granzyme B expression of IL-15-primed NK cells during murine cytomegalovirus infection[17].

**Negative regulation of NK cells**

NK cells have several intracellular negative regulators to prevent over-activation downstream of activating receptors and thus prevent autoimmunity. The phosphatase and tensin homolog (PTEN) and the SH2 domain-containing inositol 5'-phosphatase (SHIP) are negative regulators of the PI3K pathway. PTEN and SHIP are lipid phosphatases that dephosphorylate PtdIns(3,4,5)P₃ to PtdIns(4,5)P₂ and PtdIns(3,4)P₂, respectively, thereby decreasing the levels of PIP₃. Given the positive role that PI3K plays in NK cell cytotoxicity and cytokine production, PTEN and SHIP would be expected
to inhibit these functions. However, PTEN conditional KO[18] and SHIP1-deficient[19] NK cells did not produce more IFNγ in response to cytokine stimulation or anti-NK1.1 stimulation. These data suggest that removing a negative regulator of PI3K signaling, such as PTEN or SHIP, may result in loss and not enhancement of NK cell effector function. Another potential target is DAG kinase (DGK), which is a family of negative regulators of the DAG signaling pathway (Fig. 1.1). DAG is created when active PLCγ cleaves PIP_2 into IP_3 and DAG[3],[20]. The generation of IP_3 increases intracellular Ca^{2+} levels and subsequent activation of Ca^{2+}-dependent processes. DAG directly activates PKC family members and Ras guanyl nucleotide-releasing protein (RasGRP), leading to downstream activation of pathways such as ERK, NFκB, and AKT[21],[22]. DGK enzymes phosphorylate DAG into phosphatidic acid (PA), thereby regulating the balance of IP_3 and DAG signaling in a cell[23].

Our lab has shown that DGKζ KO mice displayed enhanced degranulation and IFNγ production compared to WT NK cells following activating receptor stimulation in vitro[24]. Moreover, DGKζ KO NK cells also displayed significantly enhanced cytotoxicity against the NK cell-sensitive tumor cell line YAC-1. Thus, therapeutic targeting of distal negative regulators of NK cell activation, such as DGKζ, may represent a strategy for improving the clinical efficacy of NK cells.

**Ubiquitin ligases as targets to enhance immune cell function**

In this thesis, we are focusing on another strategy to enhance NK cell activation by targeting a different type of negative regulator: E3 ubiquitin ligases. E3 ubiquitin ligases are a large family of proteins that function downstream of several receptor-signaling pathways during the regulation of cell proliferation and survival. They are engaged in the regulation of the turnover and activity of many proteins, making them
potential intracellular targets for enhancing NK cell function (Fig. 1.1). In addition, there is evidence to suggest that the abnormal regulation of some E3 ligases is involved in the development of various cancers[25]. Furthermore, some E3 ubiquitin ligases are frequently overexpressed in human cancers, correlating with an increased rate of chemoresistance and a poor clinical prognosis[25].

Ubiquitination is one of many posttranslational modifications that impact signaling thresholds in immune cells in a variety of ways, e.g. by targeting proteins for degradation or recruiting ubiquitin-binding proteins[26]. Ubiquitination of a substrate requires the sequential action of 3 classes of enzymes: E1 or ubiquitin activating enzyme, E2 or ubiquitin conjugating enzyme, and an E3 ubiquitin ligase. In the ubiquitination pathway, E1 activates ubiquitin by the formation of a thioester with the carboxyl group of glycine 76 on ubiquitin[27]. The ubiquitin is then transferred to a catalytic cysteine on an E2, which then associates with an E3 that is in a complex with a substrate protein. The E3 may serve as a scaffold to facilitate the transfer of ubiquitin from the E2 to the substrate, as is the case for the RING (Really Interesting New Gene) type of E3 ubiquitin ligases[28],[29]. E3 ligases can thus identify the substrate and also dictate the formation of ubiquitin linkages, driving the mono, multi-mono, or polyubiquitination of the substrate.

Ubiquitin has seven accessible lysines on its surface (K6, K11, K27, K29, K33, K48, or K63), each of which can be a point of attachment for ubiquitin chains[30]. The amino terminal methionine of ubiquitin (M1) can also serve as a point of attachment for linear chains in a reaction catalyzed by an E3 ubiquitin ligase complex called the linear ubiquitylation assembly complex (LUBAC)[31]. These ubiquitin chains can subsequently determine the fate of a substrate by changing its location in the cell, promoting or inhibiting its interactions with other proteins, or driving degradation by the lysosome or
proteasome. Ubiquitination is a dynamic and reversible event because ubiquitin chains can be cleaved by a family of ubiquitin-specific proteases called deubiquitinating enzymes (DUBs)[25].

The Cbl (Casitas B-lineage lymphoma proto-oncogene) family of proteins is comprised of E3 ubiquitin ligases that negatively regulate several tyrosine kinase-dependent pathways and are, therefore, targets to enhance immune cell functions. This family includes the homologs c-Cbl and Cbl-b, which are expressed in a variety of immune cells. c-Cbl and Cbl-b share a highly conserved N-terminal tyrosine kinase binding (TKB) domain, a RING finger (RF) domain, and a linker domain that connects the TKB and RF domains (Fig.1.2). The modified SH2 domain within the TKB recognizes phosphorylated tyrosines on substrate proteins[32]. The linker domain contains tyrosine residues that are crucial to the regulation of Cbl’s E3 function, and the RF domain is the catalytic domain that binds to ubiquitin-conjugating enzyme E2[33],[34]. This RF domain mediates the transfer of ubiquitin directly from the associated E2 to one or more lysines of the specific target protein, which provides specificity to the ubiquitination process. c-Cbl and Cbl-b differ in their C-termini, which mainly determines ubiquitin-independent functions of Cbl proteins. The C-termini include proline-rich domains, tyrosines, and ubiquitin associated (UBA) domains; the latter of which mediate other protein interactions with SH2 and SH3 domain-containing proteins, enabling diversity in signaling[35]. These carboxy-terminal interactions are involved in the control of cell-specific functions of Cbl, including the uptake of glucose, activation of osteoclasts, remodeling of bone, and cell migration[36]–[38].
The many roles of Cbl proteins

Cbl proteins can function as inhibitors, adaptors, or E3 ligases. c-Cbl binds to Grb2, competing with and preventing binding of the guanine-nucleotide exchange factor son-of-sevenless (SOS), blocking signaling through the Ras-mitogen-activated protein kinase (MAPK) pathway and inhibiting proliferation. Independent of their ubiquitin ligase activity, Cbl proteins can also act as adaptor proteins, specifically via their proline-rich C-termini where molecules with SH2 and SH3 domains can bind[39]. As an adaptor, c-Cbl recruits other adaptor molecules, such as Cbl-interacting protein of 85 kDa (CIN85) and CD2AP, which trigger signaling cascades that lead to endocytosis by dynamin-dependent, clathrin-mediated receptor endocytosis[40]. They can also recruit molecules to activated receptor tyrosine kinases (RTKs). In T cells, c-Cbl has been shown to recruit active p85 to the plasma membrane, which subsequently activates the PI3K/AKT pathway[41],[42]. Cbl-b was also found to bind p85 via its proline-rich sequence,
independent of the TKB[43] (Fig.1.2). Interestingly, p85 was still ubiquitinated, showing that Cbl-b’s role as an adaptor and E3 ubiquitin ligase are not mutually exclusive[44]. Whether Cbl proteins act as adaptors or ligases is likely cell- and context-dependent.

Cbl’s E3 ligase activity is essential for the negative regulation of signaling molecules. The type and length of ubiquitination determines the outcome of the substrate. Substrates can either be tagged with single (monoubiquitination) or multiple (>4) ubiquitins, which leads to polyubiquitination. Monoubiquitination typically disrupts a signaling complex formation by preventing binding of the ubiquitinated protein. However, monoubiquitylation is also thought to be sufficient as an internalization signal, whereas the addition of four or more ubiquitin molecules is required for recognition by the proteasome[44]. The polyubiquitin chains are generally linked by lysine residues at position 48 or 63, and this polyubiquitination induces their degradation by the 26S proteasome, but polyubiquitin chains may also modify protein function, e.g. by increased cell-surface receptor turnover or a change in location. Monoubiquitination, on the other hand, targets cell membrane-receptor associated proteins to the lysosome, attenuating cell surface mediated signals by a desensitization process. Thus, monoubiquitination and K63-linked polyubiquitination regulate protein trafficking and cell to cell interactions, whereas K48-linked polyubiquitination targets substrates to the proteasome for degradation[45]. The activation of Cbl and the subsequent ubiquitination of key signaling molecules can acutely lower the number of these molecules (via degradation) or make them inactive/unusable (via altered localization or binding), thereby functioning as a feedback mechanism to attenuate further signaling[40].

c-Cbl and Cbl-b seem to differ in the outcome of their E3 ubiquitin ligase activity. In c-Cbl, ubiquitination often results in degradation, whereas Cbl-b ubiquitination tends to affect protein localization and associations[40]. For example, Cbl-b is known to poly-
ubiquitinate the p85 subunit of PI3K, preventing its binding to CD28 and decreasing T-cell anti-tumor activity[43]. However, this generalization does not always hold true, as Cbl-b has been shown to ubiquitinate and degrade the epidermal growth factor receptor signaling complex[46].

**Regulation of Cbl proteins**

Typically, Cbl proteins are found in the cytosol in an autoinhibited state where the N-terminal TKB domain masks the RF domain, making them catalytically inactive[47]. Phosphorylation of Y36 on Cbl-b, located in the linker region between TKB and RF domains, regulates the E3 activity of Cbl-b by 2 mechanisms: one by physically separating the RF domain from the TKB domain, and the other by forming a surface to enhance binding affinity to E2s[47] (Fig.1.2). Consistent with these findings, the equivalent tyrosine in c-Cbl, i.e., Y371, has been shown to regulate its E3 ubiquitin ligase activity in a similar fashion[48],[49]. Both Cbl-b and c-Cbl’s tyrosine residues are phosphorylated by Syk and some members of the Src-like family of kinases[44],[50]. PKCθ activity has also been shown to modulate the strength of T cell signaling by phosphorylating Cbl-b, targeting it for degradation[51]. There are likely many other kinases that phosphorylate and regulate Cbl proteins that have yet to be identified.

There are examples of phosphorylation that does not lead to degradation. GSK-3 has been shown to phosphorylate Cbl-b on serine residues 476 and 480, which leads to stabilization of Cbl-b levels and constraint of T cell signaling[52]. This suggests that the fate of Cbl proteins after being phosphorylated is likely residue- and location-dependent.

Too much negative regulation of immune cells can lead to their lack of activation in the face of a cancerous or virally-infected cell. Therefore, there are mechanisms in
place to negatively regulate E3 ubiquitin ligases like Cbl. Cbl has been reported to be degraded along with its substrates EGFR and KIT[46],[53],[54]. In both cases, the degradation of the receptor and Cbl protein was dependent on the RING domain of the E3, suggesting that Cbl proteins regulate themselves via auto-ubiquitination and degradation. Not only may these mechanisms serve to circumvent autoimmunity but also may serve to avoid undesired targeting of other substrates by the activated Cbl. This ensures that specificity of the signaling pathway is maintained[55].

Cbl proteins are also regulated by other E3 ubiquitin ligases. NEDD4 directly ubiquitinates Cbl-b in vitro, and both Nedd4 and Itch stimulate the degradation of Cbl-b and c-Cbl[56]. Cbl-b degradation by Nedd4 appears to be induced by T-cell activation where co-stimulation of resting T cells by CD3 and CD28 results in a rapid degradation of Cbl-b, which is dependent on Nedd4[57].

**Cbls as regulators of T cell effector functions**

There is more known about the role of Cbl proteins in T cells than in NK cells. The importance of c-Cbl and Cbl-b in immunity and immune receptor signaling pathways was initially demonstrated by the phenotypes of the mice lacking each respective gene. c-Cbl and Cbl-b single KO mice are viable, whereas double KO are embryonically lethal[58], suggesting they have overlapping functions. Consistent with their roles as negative regulators, both c-Cbl KO and Cbl-b KO mice display hyperactive signaling downstream of the TCR. A characteristic feature of these mice is that loss of either Cbl protein lowers the activation threshold for signaling through the TCR. This leads to hypersensitivity to low affinity and avidity engagement of the TCR, as well as activates
downstream signaling pathways without the usual requirement for co-receptor stimulation[59].

Despite the similar T cell effector outcomes, the phenotypes of c-Cbl KO and Cbl-b KO mice are different, likely due to their tissue distribution. c-Cbl is expressed more in the thymus compared to Cbl-b, which is expressed more in peripheral T cells[36]. As such, c-Cbl KO T cells display an increased number of thymocytes and enhanced TCR signaling, likely due to CD4+ and CD8+ double positive thymocytes having increased levels of CD3, TCR-β, TCR-δ, Lck, and FynT[36]. Cbl-b KO mice develop autoimmunity from increased peripheral T cell proliferation and enhanced cytolytic activity of CD8+ T cells without requiring CD28 co-stimulation[36],[59]. This suggests that Cbl-b plays a critical role in T cell tolerance induction. As such, Cbl-b negatively regulates the costimulatory pathway of CD8+ T cells, functioning as a central gatekeeper of T-cell activation.

Mice lacking Cbl-b were found to be less susceptible to tumor formation in induced as well as spontaneous mouse models of cancer, and this effect was found to be mediated by CD8+ T cells[60]. The loss of Cbl-b was found to enhance antitumor reactivity of CD8+ T cells even in the absence of CD4+ T cells. Lack of Cbl-b led to a significant delay in tumor growth in both a mouse model of lung cancer where TC-1 tumors were subcutaneous implanted as well as in a model of spontaneous tumors by UV irradiation. Tumor rejection correlated with increased infiltration of CD3*CD8+ T cells into the tumor[60]. Increased tumor infiltration of CD8+ T cells also led to a significant increase in IFNγ production within the tumor microenvironment, reflecting an enhanced immune response in vivo.
Based on these findings, the Cbl family was determined from early on to be regulators of immunoreceptor families, in particular, T cell and B cell antigen receptors. T and B lymphocytes recognize antigens through their TCR and BCR, respectively. Following initial triggering of the antigen receptor, the Src family kinases Lck and Fyn are recruited and phosphorylate Zap-70, which initiates various downstream signaling pathways. ZAP-70 phosphorylates SLP-76, mediating the formation of a multi-subunit protein complex including key signaling molecules, such as PI3K, PLC, and Vav1[61]. Activation of these signaling components results in activation of PLCγ-regulated calcium influx, cytoskeletal rearrangements via Vav1, Rac, and WASP, and activation of PKCθ[62]. The activation step of PKCθ is essential for an appropriate NFκB stimulation to induce a productive T cell response in vivo[63]. Upon TCR or BCR stimulation, c-Cbl is recruited to the plasma membrane by Src-like adaptor proteins (SLAP) where it can be phosphorylated by Src-like and Syk-like kinases[64]. c-Cbl can then ubiquitinate key signaling molecules, including Src, Lck, ZAP-70, LAT, and p85[65]–[69]. Not only do Cbl family proteins ubiquitinate these downstream signaling molecules, but both c-Cbl and Cbl-b have also been shown to silence TCR signaling by internalization of the receptor complex[58].

Despite all these studies, how Cbl-b was regulating immune cell effector functions via these proteins was unknown. There were several clues that Cbl-b’s physiological functions is mediated by its E3 ligase activity. For example, the RING finger domain of Cbl proteins is evolutionarily conserved[70], and this domain is directly responsible for protein ubiquitylation[71],[72]. Therefore, not surprisingly, Cbl-b E3 ligase-defective (C373A<sup>Ki/Ki</sup>) mice phenocopy the T cell responses observed in the Cbl-b KO mouse described above, resulting in T cell hyperactivation, spontaneous autoimmunity, and impaired induction of T cell anergy in vivo[73]. Moreover, mice
carrying a Cbl-b E3 ligase-defective mutation spontaneously reject tumor cells that express human papilloma virus antigens[73].

The role of c-Cbl and Cbl-b in NK cells

Although less well-studied, NK cells are also regulated by Cbl proteins. The role of c-Cbl in NK cell cytotoxicity and cytokine production has been explored in a human NK cell line (NKL cells). Upon NKG2D and 2B4 co-ligation, NKL cells with reduced levels of c-Cbl (by siRNA knock-down) displayed enhanced cytotoxic responses and IFNγ production[74]. In addition, whereas NKG2D ligation alone does not normally induce IFNγ production by NKL cells, NKG2D stimulation alone was sufficient to elicit IFNγ production in NKL cells with reduced c-Cbl expression[74]. The enhancement in NKL cell function by c-Cbl knockdown was associated with decreased Vav ubiquitination in these cells, suggesting that Vav ubiquitination may represent the mechanism for c-Cbl-mediated inhibition. However, how exactly c-Cbl regulates Vav-mediated signaling is still unclear, since the knockdown of c-Cbl did not appreciably alter the level of total Vav or phosphorylated Vav proteins[74].

Although originally considered to be a regulator of TCR signaling, Cbl-b KO mice spontaneously rejected tumors even on a recombination-activating gene 2 (RAG2) KO background[75]. This effect was lost when NK cells were depleted or when NKG2D was blocked, suggesting that Cbl-b KO NK cells also display enhanced anti-tumor activity. In addition, metastatic tumor burden was significantly reduced when NK cells from Cbl-b KO and Cbl-b ligase mutant (C373A KI/KI) mice were adoptively transferred to a NeuT metastatic breast cancer model[75]. Together, these data suggest that Cbl-b negatively regulates NK cell function through its ubiquitin ligase domain.
Cbl-b KO NK cells display enhanced proliferation, degranulation, and IFNγ secretion \textit{in vitro}, but how Cbl-b negatively regulates NK cell function is not entirely clear. Which substrates Cbl-b targets to inhibit NK cell function likely depends on which receptors are stimulated on the NK cell surface. KIR activation recruits SH2-domain-containing protein tyrosine phosphatase-1 (SHP-1) and SHP-2, which dephosphorylates Vav1, LAT, PLCγ1, and PLCγ2 - all important for NK cell cytotoxicity\cite{3}. Specifically, SHP-1 dephosphorylates LAT tyrosine 132, blocking recruitment and activation of PLCγ1/2\cite{68}. This data suggests that inhibition of NK cell effector functions depends on the cooperation between SHP-1 and the Cbl family. LAT ubiquitination was found to be dependent on its phosphorylation, which suggests that only LAT molecules that escape SHP-1 are targeted to be degraded\cite{68}. In addition, both c-Cbl and Cbl-b protein levels were increased in human primary NK cells and in the YTS NK cell line during inhibition\cite{76}. Barda-Saad’s group hypothesized that the increase in Cbl proteins allows for more ubiquitylation of LAT, which inhibits NK cell cytotoxicity. It is unknown, however, whether the elevated expression of the Cbl proteins is due to an increase in transcription following NK cell inhibition or due to decreased protein degradation following NK cell activation.

**Cbl-b and TAM receptors**

More recently, Cbl-b was also found to be associated with TAM receptors in an \textit{in vitro} proteomics assay\cite{75}. The TAM receptor family is a group of RTKs that includes Tyro3, Axl, and Mer\cite{77}. Receptor tyrosine kinases are a large group of transmembrane proteins that translate extracellular stimuli into intracellular signals for cell proliferation, differentiation, survival and migration\cite{78}–\cite{80} (Fig. 1.2). All RTKs have a highly conserved cytoplasmic kinase domain that is activated upon growth factor binding to
receptor monomers. Activation of RTKs leads to their autophosphorylation and the phosphorylation of tyrosine residues of multiple downstream intracellular signaling molecules[81]. These are then able to initiate a variety of signal transduction cascades. Penninger’s group speculated that Cbl-b negatively regulates NK cells by ubiquitinating TAMs, which leads to their endocytosis[75]. They proposed that this endocytosed complex can then negatively regulate NK cell signaling. This speculation is not unfounded as there is evidence for Cbl proteins ubiquitinating RTKs, leading to their endocytosis[33].

The carboxyl terminus of Cbl has been implicated in the control of endocytosis of receptor tyrosine kinases (RTKs). This has been best studied in the regulation of the EGF receptor. The primary mechanism of Cbl recruitment to activated EGF receptors involves the binding of Cbl’s SH2 domain to the auto-phosphorylated tyrosine 1045 of EGFR[33]. Alternatively, an EGFR Y1045F mutant could still bind Cbl, via adaptor protein Grb2[82]. This informs us that are multiple ways for Cbl to bind RTKs. Additionally, CIN85 was also shown to bind to the carboxyl terminus of Cbl, regulating EGF and c-MET receptor endocytosis[83],[84]. Similarly, Cbl-b mediates down-regulation of activated EGFR and PDGFR via binding of CIN85 to the carboxyl terminus of Cbl-b[85].

**TAM receptors**

The TAM receptors are key regulators of homeostasis with a relevant role in tissue repair, vascular remodeling, and removal of apoptotic bodies[86]. Aside from a role in maintaining homeostasis, they have also been implicated in tumorigenesis, metastasis, and chemoresistance, making TAM receptors targets for therapy. In immune
homeostasis, they play two key roles: 1) inhibiting pro-inflammatory pathways and 2) promoting tissue repair after adaptive immune responses.

Tyro3 and Axl are the most similar in genomic structures, sharing the same number and size of exons[87]. However, Axl and Mer have the most similar tyrosine kinase domain sequence[88],[89]. The human TAM receptors share 31-36% identical (52-57% similar) protein sequences in the extracellular regions, whereas they share 54-59% sequence identity (72-75% similarity) in their tyrosine kinase domain[88]. The differences in post-translational modifications, such as glycosylation, phosphorylation, and ubiquitination may account for the variability in TAM expression observed across tissues and cell types[90],[91].

Tyro3, Axl, Mer all share a common structure with extracellular regions composed of two immunoglobulin-related domains[90],[92] linked to two fibronectin type-III repeats, and cytoplasmic regions that contain an intrinsic tyrosine kinase domain[93],[94],[95]. These ectodomains are hypothesized to be important in cell to cell contact, adhesion, and migration[77]. The kinase domain has a conserved sequence, including an ITIM-like sequence KW(I/L)A(I/L)ES[77]. The typical ITIM motif is S/I/V/LxYxxI/V/L. This motif typically becomes phosphorylated by enzymes of the Src kinase family, allowing them to recruit other enzymes such as the phosphotyrosine phosphatases SHP-1 and SHP-2 or the inositol-phosphatase SHIP[3]. These phosphatases decrease the activation of molecules involved in cell signaling. This may be one of many ways that TAM receptors can negatively regulate inflammation.

**Ligands of TAM receptors**

The ligands to TAM receptors include Gas6 and ProteinS[96]–[99], which bind to receptors with a 2:2 stoichiometry[77]. Both are glutamic acid-containing proteins that
are carboxylated by reduced Vitamin K. Gas6 is a growth potentiating factor,[100],[101] which contains multiple N-terminal gamma-carboxyglutamic acid residues, four epidermal growth factor-like repeats and a C-terminal sex hormone-binding globular-like region that is sufficient for receptor activation[98]. ProteinS shares the same domain structure with the exception of thrombin cleavage sites, allowing it to act as an anticoagulant[102]. The gamma-carboxylation makes Gas6 and ProteinS biologically active[103] and increases their ability to bind to calcium, which is important for their binding to phosphatidylserine that is exposed on the surface of apoptotic cells[104]. Gas6 and ProteinS act as a bridge, attaching to phosphatidylserine and the TAM receptors, allowing for stronger signaling downstream of TAM receptors[105], as well as phagocytosis of apoptotic cells[77].

Gas6 binds to all 3 TAM receptors, with a preference for Axl and Tyro3 over Mer[96]–[99],[106],[107], and ProteinS has preference for binding to Tyro3, followed by Mer but not Axl[97],[106]–[110]. Typically, Gas6 binds to a TAM receptor and activates the receptor via dimerization, which then elicits PI3K-AKT, MAPK, STAT and NFκB pathways[111]. Not only can TAMs be activated by these ligands, but they can also be activated via ligand-independent dimerization, ligand-dependent dimerization, heteromeric dimerization of two different TAM receptors, heteromeric dimerization with a non-TAM receptor, and trans-cellular binding of extracellular domains[77],[109].

**TAM receptors regulate innate immune responses**

In hematopoietic cells, TAM receptors are primarily expressed by cells of the innate immune system. How TAM receptors regulate immune responses is a topic of immense interest, as therapeutic targets are being developed to target each individual receptor. The frequently observed co-expression of the receptors and their ligands,
together with the presence of conserved ITIM-like domains of all 3 receptors, suggests that TAMs negatively regulate inflammation[112].

TAM receptors negatively regulate innate immune responses upon activation with ligands Gas6 or ProteinS. It remains to be determined where the Gas6 and ProteinS are derived from. However, in several cell types, signaling appears to be autocrine or paracrine in nature[108],[113],[114]. Macrophages co-express ligands and receptors[115], but in NK cell activation, the data suggests that both Gas6 and ProteinS are present in stromal cells, and these likely stimulate the TAM receptors on NK cells[116]. In addition, an important source of ProteinS for TAM-expressing macrophages and dendritic cells may be activated T cells[117]. The latter is an elegant feedback mechanism, where ProteinS is only expressed in activated but not resting T cells, and selective deletion of T-cell derived ProteinS enhanced APC activation and cytokine production in an antigen-specific, TAM-RTK-dependent manner[118]. More recently, ProteinS was found to be produced by B6F10 melanoma cells, leading to reduced polarization of anti-tumor macrophages by binding to Mer and Tyro3 on the surface of macrophages[119].

Regardless of the form of activation, signaling pathways downstream of TAMs not only control the magnitude of the immune response by dampening inflammation but also likely integrate the phasing out of the innate immune response. The latter is accomplished by active processes that favor the resolution of inflammation and recovery of tissue function via the clearance of apoptotic cells and the restoration of vascular integrity[104]. These data suggest that TAM signaling functions at the interface of innate and adaptive immunity to tame the activity of innate cells and prevent unnecessary consequences of inflammation[120].
There is clear evidence for the role of TAM receptors in dampening inflammation in mice lacking Tyro3, Axl, and Mer. Mice lacking all 3 TAM receptors appear normal at birth but develop a severe lymphoproliferative disorder accompanied by autoimmunity, resulting from hyperactivation of antigen-presenting cells that typically express all 3 receptors[112]. The peripheral lymphoid organs of these mice were enlarged by hyperproliferation of B and T cells, which were also constitutively activated. All of these mice also developed autoimmunity, had elevated levels of autoantibodies, and were similar histologically to rheumatoid arthritis and lupus. It has been hypothesized that mechanistically, these phenotypes may be due to excessive activation of innate immune cells resulting in aberrant activation of self-reactive lymphocytes[112]. These features indicate that TAM receptors normally serve as a self-extinguishing regulatory mechanism that limits the severity and time course of inflammatory immune responses.

TAM signaling pathways have been shown to function as negative feedback regulators of DC inflammation, where TAMs are only engaged after the inflammatory receptors, such as Toll-like (TLR) receptors, have been activated. Once the TLR is engaged, the first wave of pro-inflammatory cytokines is released, propagating inflammation by activating cytokine receptors such as type I interferon receptors (IFNAR)[114]. The latter then induces Axl receptor signaling. Gas6 or ProteinS-induced activation of Axl in APCs inhibits NFκB activation and cytokine production downstream of TLR3, 4, and 9[114] (Fig. 1.3).

Not surprisingly, TAM receptors were found to be required to clear apoptotic bodies. The phosphatidyl serine residues on the surface membrane of apoptotic bodies bind to Gas6, serving as a means for recognition and subsequent phagocytosis by TAM receptor-expressing macrophages[121]. Thus, Tyro3, Axl, and Mer triple KO mice display a defect in clearing apoptotic bodies, which causes a lupus-like spontaneous
autoimmune disease[112]. This phenotype likely results from the accumulation of apoptotic cells and subsequent tissue necrosis in addition to the constitutive activation of the immune system[112]. Mechanistically, TAM receptors were found to mediate Vav1/RhoA/Rac1-dependent actin cytoskeleton rearrangements that are required for phagocytosis[122],[123]. Also, TAM receptors mediate the immunosuppressive effects of apoptosis where Mer has been shown to be essential in generating tolerogenic dendritic cells upon cellular apoptosis[124]. Lack of Mer generates dendritic cells that enhance T cell stimulation, which is associated with increased susceptibility to autoimmunity. In addition, TAM receptor ligation blocks pro-inflammatory cytokine release by macrophages[112],[114],[125]. The TAM KO mice were found to have abnormal regulation of cytokine release, including elevated levels of IL-12 produced by hyperactivated macrophages[112]. MerKD mice were also found to have elevated TNFα and NFκB following LPS treatment[126], suggesting that Mer is involved in attenuating a proinflammatory cytokine response in macrophages (Fig. 1.3).

**FIGURE 1.3 TAM receptors can both positively and negatively regulate cells.** In tumor cells, TAM receptor activation leads to phosphorylation of ERK and AKT, enhancing cell proliferation and survival. In dendritic cells, TAM receptor activation leads to phosphorylation of STAT1, which increases transcription of E3 ubiquitin ligases SOCS1 and 3, inhibiting TLR and cytokine signaling. In macrophages, TAM receptor activation by
Other roles for TAM receptors

Not only are TAM receptors responsible for mediating the immunosuppressive effects of apoptosis, they are also responsible for repairing damaged tissues. Gas6 and Mer contribute to tissue repair by activating the GTPase RhoA, which transcriptionally upregulates the hepatocyte growth factor - a key player in tissue reparation[127]. Thus, TAM receptors are responsible for creating an environment - both anti-inflammatory and reparative- to restore tissue homeostasis.

In addition, TAM receptors control immune responses at the interface of the innate and adaptive immune systems[75]. Once adaptive immune cells are activated, they need to inform innate immune cells to prevent uncontrolled and chronic activation. As mentioned previously, activated T cells produce ProteinS, which binds to TAM receptors on dendritic cells, inhibiting DC activation, and therefore, controlling antigen-specific immune activation[118]. TAM signaling functions bidirectionally as TAM receptors also directly influence T cells to control the magnitude of the adaptive immune response. As such, neutralization of Mer in DCs resulted in enhanced T cell cytokine production[128].

TAM receptors in NK cells

As previously described, TAM receptors are also expressed by NK cells[116]. Ligands Gas6 and ProteinS are expressed in bone marrow stromal cells and activate TAM receptors on immature NK cells. Interestingly, the expression of TAM receptors is necessary for proper NK cell development and functional maturation[116]. In the absence of TAM receptor signaling, immature NK cells were unable to acquire
expression of inhibitory and activating receptors, which are required for late
differentiation and effector function[120]. As described above, as part of an elegant
feedback mechanism, T cells also secrete ProteinS when activated and can therefore
engage TAM ligand/receptor and subsequently dampen the innate immune response. In
mature NK cells, TAM receptors attenuate NK cell function. Accordingly, the inhibition of
TAM receptor signaling prevents metastasis in a mouse tumor model in an NK cell-
dependent manner[75]. The mechanism by which TAMs inhibit NK cell function is not
known.

**Investigating how Cbl-b and TAM receptors work together to negatively regulate
NK cells.**

Despite the fact that Cbl-b and TAM receptors were found to interact in an *in vitro*
proteomics assay[75], the mechanism by which they interact to inhibit NK cell responses
is unknown. Penninger’s group speculates that Cbl-b negatively regulates NK cells by
ubiquitinating TAM receptors, which leads to their endocytosis[75]. They further
speculate that this endocytosed complex is what negatively regulates NK cells. This
model leaves several unanswered questions. How is Cbl-b activated to ubiquitinate TAM
receptors? How can an endocytosed complex negatively regulate signaling?

Given that both Cbl-b and the TAM receptor family are targets for cellular
immunotherapy, it is critical to understand their biology to better develop targeted
therapeutics and improve clinical trial outcomes. Therefore, for my thesis work, I wanted
to determine how TAMs, which as RTKS that are typically associated with enhancement
of signaling, can inhibit NK cell effector functions. In addition, I was interested in
understanding what role Cbl-b plays in this negative regulation of NK cell signaling. We
used recombinant protein and primary NK cells to answer these questions. Lastly, I used
Cbl-b KO mice to test several Cbl-b inhibitors in collaboration with Progenra, Inc, to assess their efficacy as anti-tumor immunotherapies.
CHAPTER 2: TAM receptors attenuate murine NK cell responses via E3 ubiquitin ligase Cbl-b

Abstract

TAM receptors (Tyro3, Axl, and Mer) are receptor tyrosine kinases (RTKs) that are expressed by multiple immune cells including natural killer (NK) cells. Although RTKs typically enhance cellular functions, TAM receptor ligation blocks NK cell activation. The mechanisms by which RTKs block NK cell signaling downstream of activating receptors are unknown. In this report, we demonstrate that TAM receptors attenuate NK cell responses via the activity of E3 ubiquitin ligase Cbl-b. Specifically, we show that Tyro3, Axl, and Mer phosphorylate Cbl-b, and Tyro3 ligation activates Cbl-b by phosphorylating tyrosine residues 133 and 363. Ligation of TAM receptors by their ligand Gas6 suppresses activating receptor-stimulated NK cell functions such as IFNγ production and degranulation, in a TAM receptor kinase- and Cbl-b-dependent manner. Moreover, Gas6 ligation induces the degradation of LAT1, a transmembrane adaptor protein required for NK cell activating receptor signaling, in WT but not in Cbl-b knock-out NK cells. Together, these results suggest that TAM receptors may attenuate NK cell function by phosphorylating Cbl-b, which in turn dampens NK cell activation signaling by promoting the degradation of LAT1. Our data therefore support a mechanism by which RTKs attenuate, rather than stimulate, signaling pathways via the activation of ubiquitin ligases.

Introduction

The TAM receptor family is a group of receptor tyrosine kinases (RTKs) that includes Tyro3, Axl, and Mer[77]. TAM receptors have a highly conserved cytoplasmic
kinase domain that is activated upon binding with their ligands Gas6 or ProteinS[101],[129]. TAM activation leads to their auto-phosphorylation and the phosphorylation of tyrosine residues of multiple downstream intracellular signaling molecules[77]. In hematopoietic cells, TAM receptors are primarily expressed by cells of the innate immune system.

One important function of TAM receptors is to clear apoptotic bodies. The phosphatidyl serine residues of the surface membrane of apoptotic bodies bind to Gas6, serving as a means for recognition and subsequent phagocytosis by TAM receptor-expressing macrophages[121]. Thus, Tyro3, Axl, and Mer triple KO mice display a defect in clearing apoptotic bodies, which causes a lupus-like spontaneous autoimmune disease[112]. In addition, TAM receptor ligation blocks pro-inflammatory cytokine release by macrophages and dendritic cells (DCs)[112],[114],[125].

In addition to macrophages and DCs, TAM receptors are also expressed by NK cells[116]. NK cells are innate lymphocytes that play an important role in killing cancerous and virally infected cells by direct cytotoxicity and through the production of inflammatory cytokines such as IFNγ[3]. Interestingly, the expression of TAM receptors is necessary for proper NK cell development and functional maturation[116]. In mature NK cells, TAM receptors attenuate NK cell function. Accordingly, the inhibition of TAM receptor signaling prevents metastasis in a mouse tumor model in an NK cell-dependent manner[75].

RTKs are typically associated with stimulation, rather than inhibition, of activating receptor-mediated signaling pathways. For example, ligand binding to epidermal growth factor receptor (EGFR) stimulates the ERK, MAPK and AKT pathways, which leads to
cell growth and proliferation[130]. However, TAM receptors are unique within the RTK family in that they are able to both promote and inhibit signal transduction pathways.

The mechanism by which TAM receptors inhibit innate immune cells has only been well described in DCs, where the TAM receptor Axl is induced upon Toll-like receptor (TLR) activation by foreign pathogens. Axl associates with the type I interferon receptor, which activates the JAK/STAT pathway and drives the transcription of the suppressor of cytokine signaling (SOCS1 and 3) genes[114],[131], which are ubiquitin ligases that inhibit cytokine receptor and TLR-mediated signaling.

Cbl-b, another E3 ubiquitin ligase, was also found to be associated with TAM receptors in an *in vitro* proteomics assay[75]. Cbl-b ubiquitinates TAM receptors, causing decreased surface expression, likely through lysosomal degradation[75]. Although this interaction appeared to be important for the TAM receptor function in NK cells, the mechanism by which TAM receptors attenuate NK cell function is still unclear.

In this report, we find that while TAM receptors are targets for Cbl-b-mediated ubiquitination, Cbl-b is also a phosphorylation target for TAM receptors. Our data suggest that TAM receptors may attenuate NK cell function by phosphorylating Cbl-b, promoting the degradation of LAT1, a key protein necessary for activating receptor-mediated signaling in NK cells[68].

**Results**

**Gas6 inhibits IFNγ production and degranulation by WT NK cells stimulated by ligation of activating receptors**

We first tested several concentrations of the TAM receptor ligand Gas6 to determine which concentration was most effective in inhibiting NK cell activation. A dose
range experiment of the effect of Gas6 on the inhibition of NK cell IFNγ production has been previously published[75]. In this report, they tested a narrow range of Gas6 concentrations (0, 200, 400, and 800 ng/ml). We, therefore, tested a wider range of Gas6 concentrations in stimulated IL-2-expanded NK cells (Lymphokine Activated Killer cells, LAKs) by ligating the activating receptors NK1.1 and NKG2D in the presence or absence of Gas6 at either 0, 100, 500, or 2,500 ng/ml, and measured the production of IFNγ and cell-surface CD107a expression (a surrogate for degranulation) (Figure 2.1).

We found that while the concentration of 500 ng/ml led to Gas6-mediated inhibition of NK cell responses, the effect was minimal at 100 ng/ml and was even enhanced in some instances at a concentration of 2,500 ng/ml. Based on this data and the data that was previously published[75], we consistently used a Gas6 concentration of 450 ng/ml for all experiments.

**FIGURE 2.1.** NK cells are optimally inhibited by Gas6 at 500ng/ml. LAKs were serum and IL-2-starved for 2 hours, pre-treated with either 0, 100, 500, or 2,500 ng/ml of Gas6 for 2 hours, and stimulated with anti-NK1.1 or anti-NKG2D for 5 hours and analyzed by flow cytometry. Cumulative data are represented as % of control ± SEM of n=3 mice for anti-NK1.1 and anti-NKG2D. *p< 0.05 by paired Student t test comparing Gas6 conditions 0 to 500 ng/mL.
To examine the effect of TAM receptor-mediated signaling on NK cell activation, we stimulated LAKs by ligating the activating receptors NK1.1 and NKG2D in the presence or absence of Gas6 and measured the production of IFNγ and cell-surface CD107a expression (a surrogate for degranulation). The addition of Gas6 significantly decreased the proportion of IFNγ⁺ and CD107a⁺ anti-NK1.1- or anti-NKG2D-stimulated LAKs (Fig. 2.2A-C). To test whether a similar effect was observed in NK cells removed freshly ex vivo, NK cells from WT mice pre-treated with the viral mimetic PolyI:C were stimulated in the presence or absence of Gas6. Similar to LAKs, a decreased fraction of IFNγ⁺ and CD107a⁺ NK cells was detected in freshly isolated NK cells treated with Gas6 and stimulated with anti-NK1.1 (Fig. 2.3A-C). These data demonstrate that TAM receptor ligation negatively regulates NK cell activation.

**FIGURE 2.2.** Gas6 inhibits IFNγ production and degranulation by WT LAKs stimulated through activating receptors. LAKs were serum/IL-2-starved for 2 hours, pre-treated with or without Gas6 for 2 hours, and left unstimulated (Unstim) or stimulated with PMA/Iono, anti-NK1.1, or anti-NKG2D for 5 hours and analyzed by flow cytometry. (A) Representative flow cytometric plots of IFNγ⁺ and CD107a⁺ LAK cells are shown. (B) The fraction of IFNγ-expressing and (C) CD107a-expressing LAKs from 4 independent experiments are shown (n = 4 mice for anti-NK1.1 and n=5 mice for anti-NKG2D). *p<0.05 and **p<0.01 by paired Student t test.
TAM receptor Tyro3 phosphorylates tyrosine residues 133 and 363 of Cbl-b

It has been previously shown that TAM receptors are direct targets of the ubiquitin ligase Cbl-b and that these interactions are important for TAM receptors to attenuate NK cell function[75]. To further examine the interaction between TAM receptors and Cbl-b, we generated recombinant phosphorylated Tyro3 and a truncated Cbl-b composed of the tyrosine kinase binding and RING finger domains (TKB+RF). As previously reported[75], the addition of Cbl-b to purified Tyro3 induced the ubiquitination of Tyro3 (Fig. 2.4A).

While Cbl-b typically needs to be pre-activated by Lck-mediated phosphorylation to carry out its ubiquitinating function[132], we unexpectedly found that Cbl-b was able to ubiquitinate Tyro3 in the absence of Lck. Given that Tyro3 is a kinase, this raised the possibility that Tyro3 might be phosphorylating and activating Cbl-b. Indeed,
phosphorylated Cbl-b was detected when Cbl-b was mixed with both Tyro3 and ATP (Fig. 2.4B). These data suggest that not only was Tyro3 a ubiquitination target of Cbl-b, but also that Cbl-b was a phosphorylation target of Tyro3. Furthermore, recombinant Axl and Mer also phosphorylate Cbl-b (Fig. 2.4B).

![Image](image_url)

**FIGURE 2.4.** Cbl-b ubiquitinates Tyro3, and Tyro3, Axl, and Mer phosphorylate Cbl-b *in vitro*. (A) HA-tagged Tyro3 was incubated with or without truncated Cbl-b (TKB+RF), E1, E2, ubiquitin, and ATP in vitro for 1 hour and immunoblotted with anti-HA antibody. (B) Truncated Cbl-b (TKB+RF) was incubated with or without recombinant Tyro3, Axl, or Mer and with or without ATP *in vitro* and immunoblotted with anti-phosphotyrosine antibody.

To test whether Cbl-b is phosphorylated upon TAM receptor ligation in NK cells, we next examined Cbl-b phosphorylation in LAKs treated with Gas6. Consistent with our purified protein assay results, the treatment of LAKs with Gas6 induced the phosphorylation of Cbl-b (Fig. 2.5A). To ensure that the phosphorylation was TAM kinase-dependent, we pre-treated LAKs with the pan-TAM kinase inhibitor BMS777607 for 30 minutes prior to stimulation with Gas6; this resulted in the reduction of Cbl-b phosphorylation to baseline levels (Fig. 2.5B), suggesting that the induction of Cbl-b phosphorylation by Gas6 was TAM kinase-dependent. The ligation of activating receptors such as Ly49D and CD16 can also lead to phosphorylation of the Cbl-b homologue c-Cbl[133], likely as a feedback mechanism to attenuate NK cell overactivation. To test whether Gas6 has a cumulative effect in enhancing Cbl-b
phosphorylation with an activating receptor stimulus, we stimulated LAKs with anti-NK1.1 with or without Gas6. Stimulation of LAKs with anti-NK1.1 antibody PK136 led to minimal phosphorylation of Cbl-b, which was synergistically enhanced by Gas6 (Fig. 2.5B). These data collectively suggest that TAM receptors directly phosphorylate Cbl-b.

Tyrosines 106, 133, and 363 have been previously shown to be required for the ligase activity of Cbl-b[48],[49],[132]. Phosphorylation of Tyr363 opens Cbl-b from its auto-inhibitory confirmation, allowing E2 and substrates to bind to Cbl-b[48]. To determine which of the tyrosines on Cbl-b could potentially be phosphorylated by Tyro3, we examined Tyro3-dependent phosphorylation of Cbl-b mutants harboring single Y to F mutations at residues Tyr106, Tyr133, and Tyr363, and a triple mutation of all tyrosines (Y106/133/363F). Following co-incubation of Y133F, Y363F, and Y106/133/363F mutants with Tyro3, we found a decrease in phospho-Cbl-b in comparison to WT Cbl-b (Fig. 2.6A). These data suggest that Tyr133 and Tyr363 are the primary tyrosine residues that are phosphorylated by Tyro3.
To test whether phosphorylation of Cbl-b at Tyr133 and/or Tyr363 by Tyro3 is important for its ubiquitin ligase function, we examined the ubiquitination of the Cbl-b mutants and Tyro3. Tyr106, Tyr133, and Tyr363 have been previously shown to be required for the auto-ubiquitination of Cbl-b[49]. Ubiquitinated Tyro3 and Cbl-b bands were detected when Tyro3 was mixed with WT Cbl-b (Fig. 2.6B). These ubiquitination bands were slightly decreased in the Y133F mutant and almost undetectable in the Y363F and triple mutants. These results show that Tyro3 regulates the function of Cbl-b by phosphorylating Tyr363 of Cbl-b.

**FIGURE 2.6.** TAM receptor Tyro3 phosphorylates tyrosine residues 133 and 363 of Cbl-b. (A) 10XHis-tagged Y→F Cbl-b point mutants were incubated with Tyro3 and ATP, followed by immunoblotting with anti-phosphotyrosine antibody. Y3F refers to Y106/133/363F triple mutant. (B) 10XHis-tagged Y→F Cbl-b point mutants were incubated with Tyro3, E1, E2, ubiquitin, and ATP for 1 hour and immunoblotted with anti-ubiquitin antibody. One representative of three independent experiments is shown.

**TAM receptors attenuate NK cell function by phosphorylating Cbl-b, which promotes LAT1 degradation**

Cbl-b negatively regulates NK cell function by ubiquitinating key signaling molecules and targeting them for re-localization or degradation[68]. Thus, we
hypothesized that TAM receptors might inhibit NK cell function by activating Cbl-b through its phosphorylation. To test this possibility, we first examined whether the kinase activity of TAM receptors was necessary for the suppression of NK cell activation. Cells were pretreated with the pan-TAM kinase inhibitor BMS777607 for 2 hours prior to stimulation with plate-bound anti-NK1.1 or anti-NKG2D in the presence of Gas6. The suppressive effect of Gas6 on LAK IFNγ production and degranulation was partially reversed by BMS777607, suggesting that Gas6 may regulate NK cells at least in part by the kinase activity of TAM receptors (Fig. 2.7A-B).

Next, we determined whether Cbl-b was functionally required for TAMs to inhibit NK cells. Cbl-b KO and WT LAKs were stimulated with plate-bound anti-NK1.1 or anti-NKG2D antibodies in the presence or absence of Gas6, and the supernatants were
assayed for IFNγ by ELISA at 24 hours. Consistent with our flow cytometric data, the addition of Gas6 to WT LAKs significantly inhibited IFNγ release. However, Gas6 treatment displayed no inhibitory effect towards anti-NK1.1 or anti-NKG2D-stimulated Cbl-b KO LAKs (Fig.2.8A-B). In fact, Cbl-b KO LAKs have enhanced IFNγ release compared to WT LAKs (Fig.2.8A), consistent with previously published studies[75]. Together, these data suggest that the phosphorylation of Cbl-b is required for the Gas6/TAM pathway to inhibit pro-inflammatory cytokine production by NK cells.

FIGURE 2.8. Cbl-b is required for TAM receptors to attenuate NK cell function. WT and Cbl-b KO LAKs were serum/IL-2-starved for 2 hours, followed by Gas6 treatment for 2 hours, and stimulated with anti-NK1.1 or anti-NKG2D antibodies for 24 hours. IFNγ content in the supernatants was measured by ELISA. (A) Representative data for 1 experiment. (B) Cumulative data are represented as % of control ± SEM of n=4 mice for anti-NK1.1 and n=3 mice for anti-NKG2D. *p< 0.05 and ***p <0.001 by paired Student t test. ns, not significant.

To gain insight into how the TAM/Cbl-b pathway may be regulating NK cell signaling, we assessed degradation of PLCγ1, the p85 subunit of PI3K, and LAT1- key signaling molecules downstream of NK cell activating receptors and known targets for ubiquitination and degradation by Cbl-b[68]. Upon Gas6 ligation, only LAT1 levels were
decreased over time in WT LAKs (Fig. 2.9A), whereas Cbl-b KO LAKs were unaffected (Fig. 2.9B).

Lastly, we asked whether TAM/Cbl-b activation is followed by a negative feedback response, which would allow the NK cell to return to baseline and respond to a cancerous or virally-infected cell. We hypothesized that in order for this to occur, Cbl-b would need to be degraded. To test whether Cbl-b is degraded after TAM receptor ligation in NK cells, we next examined total levels of Cbl-b in LAKs treated with Gas6. The treatment of LAKs with Gas6 led to a decrease in total Cbl-b (Fig. 2.10A). To ensure that Cbl-b was being proteolytically degraded, we pre-treated LAKs with MG132 (proteasome inhibitor) and chloroquine (lysosome inhibitor); however, these pre-treatments were toxic to the cells (Fig. 2.10B).

FIGURE 2.9. TAM receptors attenuate NK cell function by phosphorylating Cbl-b, which promotes LAT1 degradation. (A) LAKs were serum and IL-2-starved for 4 hours followed by Gas6 stimulation for various time points. Cell lysates were immunoblotted with anti-PLCy1, anti-p85, anti-LAT1, and β-actin antibodies. (B) LAKs from WT and Cbl-b KO mice were serum and IL-2 starved for 4 hours followed by Gas6 stimulation for various time points. Cell lysates were immunoblotted with anti-LAT1 and β-actin antibodies. The relative ratio of LAT1 to β-actin for each time point, normalized to the 0 time-point for each genotype, is shown below the blots. One representative of three independent experiments is shown.
Together, these data support a novel mechanism whereby TAM receptors negatively regulate NK cell function by phosphorylating Tyr363 of Cbl-b, which in turn dampens NK cell activation signaling by promoting the degradation of positive signaling molecules such as LAT1. This attenuation in signaling may then negatively signal back to Cbl-b, which in turn is degraded, allowing NK cells to reset (Fig. 2.11).

FIGURE 2.10 Cbl-b may be degraded after Gas6/TAM stimulation. (A) LAKs were serum and IL-2-starved for 4 hours followed by Gas6 stimulation for various time points. Cell lysates were immunoblotted with anti-Cbl-b and β-actin antibodies. (B) LAKs were serum and IL-2-starved for 4 hours and left either untreated or treated with MG132 and chloroquine followed by Gas6 stimulation for various time points. The relative ratio of Cbl-b to β-actin for each time point is shown below the blots. One representative of three independent experiments is shown.
Discussion

Previously published data showed that Gas6 inhibited NK cell responses but the range of Gas6 concentration was narrow (200, 400, and 600 ng/mL)[75]. We found that while the concentration of 500 ng/ml led to Gas6-mediated inhibition of NK cell responses, the effect was minimal at 100 ng/ml and was even enhanced in some instances at a concentration of 2,500 ng/ml. The enhancement of NK cell responses may be due to enhanced survival of NK cells treated with high concentrations of Gas6, as Gas6 has an effect on cell survival and proliferation. Because this experiment is done under serum-starved conditions, NK cells treated with higher concentrations of Gas6 may survive better than cells treated with a lower concentration of Gas6.

FIGURE 2.11 Gas6/TAM signaling inhibits NK cell activation through Cbl-b. Gas6 binds and activates TAM receptors, which directly phosphorylates Cbl-b. Phosphorylated Cbl-b, in turn, ubiquitinates and targets LAT1 for degradation. NK1.1/NKG2D signaling is attenuated by decreased LAT1 protein, which is required for signal transduction downstream of NK cell activating receptors. Lastly, this signaling attenuation may lead to the degradation of Cbl-b as a form of negative feedback regulation.
In addition, the percent of Gas6-mediated inhibition does not appear to be substantial with an average of about 30% reduction of IFNy and CD107a. However, in comparison to the previously published work where reduction was about 15%. This 15% reduction was enough to produce a biological response. Therefore, although the *in vitro* experiment data does not seem substantial, based on the previously published work, this is enough to create a biological response. In order to address the seemingly inadequate Gas6-mediated reduction in IFNy and CD107a, we used alternative approaches, such as ProteinS instead of Gas6. ProteinS, however, did not reduce IFNy and CD107a. It is well documented that the best inhibition of TAM receptors occurs when Gas6 is bound to phosphatidylserine[105]. Therefore, we prepared liposomes consisting of phosphatidylserine with Gas6 or Protein. Our control of PS liposome alone led to reduction in IFNy and CD107a. Human NK cells express an inhibitory receptor, CD300, which binds to PS[134-136]. Therefore, the effect we saw with PS alone may be due to direct inhibition through a similar receptor in murine NK cells.

The *in vitro* biochemical data shown in Figure 2.4B was variable. Tyro3 phosphorylated large quantities of Cbl-b. Although Axl and Mer also phosphorylated Cbl-b, the amount of phosphorylation is not equivalent to that phosphorylated by Tyro3. The recombinant proteins were derived from different sources, and thus, are likely of varying quality. In addition, we did not test for whole amounts of recombinant protein being added into the assay. Therefore, each experiment may have different amounts of kinase, and thus, lead to differing amounts of phosphorylated Cbl-b.

We showed that ligation of TAM receptors leads to phosphorylation of Cbl-b. The ligation of activating receptors such as Ly49D and CD16 can also lead to phosphorylation of Cbl-b homologue c-Cbl[133],[137], likely as a feedback mechanism to
attenuate NK cell overactivation. Our data showed that Gas6 has a cumulative effect in enhancing Cbl-b phosphorylation with co-incubation of an activating receptor stimulus NK1.1 and Gas6. TAM ligation with Gas6 and anti-NK1.1 stimulus may separately lead to the phosphorylation of Cbl-b and appear to be synergistic. Although the phosphorylation of Cbl-b via the ligation of NK1.1 and TAM receptors may be synergistic, they are likely independently leading to the phosphorylation of Cbl-b. In addition, it is unknown which tyrosine(s) is/are phosphorylated by each agent, and, therefore, the mechanism and sites of phosphorylation can be different.

TAM receptors are RTKs that inhibit the pro-inflammatory function of a variety of cell types. While it has been shown that TAM receptors attenuate DC function through the transcriptional induction of SOCS proteins[114], the mechanism by which TAM receptors attenuate activating receptor-stimulated NK cell function is unknown. In this report, we provide data that supports a mechanism by which TAM receptors attenuate pro-inflammatory responses via post-translational modification of the E3 ubiquitin ligase Cbl-b. By activating Cbl-b through its phosphorylation, TAM receptor ligation can inhibit NK cell function potentially by inducing the degradation of key molecules necessary for activating receptor signaling, such as LAT1.

RTKs are typically associated with stimulation rather than inhibition of activating receptor-mediated signaling pathways[130]. Accordingly, the tyrosine kinase activity of TAM receptors activates ERK, AKT, and NFkB – key signaling pathways required for NK cell cytokine production[77]. Thus, it is puzzling that TAM receptors attenuate, rather than augment, NK cell function. Our data reveal a mechanism by which RTKs inhibit activating receptor stimulation in NK cells. Namely, by phosphorylating and activating
Cbl-b, TAM receptors can selectively suppress signaling through activating receptors by targeting LAT1 for degradation.

Cbl-b has been shown to negatively regulate T cells, macrophages, and more recently NK cells[138]. In T cells, Cbl-b has been shown to ubiquitinate not only the T cell receptor, but also downstream signaling molecules such as p85, PLCγ, and PLCθ – which are all required for T cell effector function[139]. In NK cells, Cbl-b has also been shown to ubiquitinate key signaling molecules including the transmembrane adaptor protein LAT1[68]; LAT1 is part of the multi-molecular signaling complex that forms downstream of activating receptors and is required for signaling and cytolytic activity[140],[141].

Along with LAT1, we also assessed the stability of other key signaling molecules such as PLCγ1 and the p85 subunit of PI3K after Gas6 stimulation. Although only LAT1 was degraded after 60 minutes of Gas6 stimulation, lack of degradation of these proteins does not preclude them from being involved in TAM/Cbl-b-mediated inhibition of NK cell activation. Ubiquitination can also affect localization of the protein within the cell, block posttranslational modifications, or can even alter binding to other proteins[40],[43]. Therefore, although these proteins are not degraded upon Gas6 stimulation, Cbl-b may still be regulating NK cell responses by changing their localization. In addition, although our results show that Gas6-mediated LAT1 degradation is mainly controlled by Cbl-b, other additional ubiquitin ligases can be involved LAT1 degradation.

Pathways, whether activating or inhibitory, require a regulatory mechanism to allow cells to return to baseline signaling and respond to acute changes in their environment. Cbl-b is known to self-regulate via auto-ubiquitination and degradation[46].
We, therefore, assessed the stability of Cbl-b with longer stimulation by Gas6. Total levels of Cbl-b were in fact reduced by 120 minutes. However, we were unable to verify that it was proteolytically degraded because pre-treatment with MG132 and chloroquine were toxic for the cells as shown by decreased levels of β-actin.

Our investigation was initially prompted by a previous report showing that TAM receptors are ubiquitinated by Cbl-b[75]. In this study, Gas6 treatment of NK cells led to a decrease in surface expression of TAM receptors in a Cbl-b-dependent manner. This ubiquitin-dependent endocytosis of TAM receptors was proposed to be necessary for the induction of its inhibitory signal transduction pathway. Consistent with these data, we also found that Tyro3 is ubiquitinated by Cbl-b. However, while it is possible that Cbl-b-mediated endocytosis of TAM receptors is critical for the inhibitory function of TAM receptors, it remained unclear as to how this endocytosis could subsequently inhibit NK cell functions. Instead, our data favor a model in which the inhibitory function of TAM receptors is carried out by Cbl-b-mediated ubiquitination of activating receptor-associated signaling molecules. In this model, the downregulation of TAM receptors by Cbl-b-mediated ubiquitination could play a role in feedback attenuation of TAM receptor signaling. In support of this type of negative feedback, another RTK, epidermal growth factor receptor (EGFR), has been shown to be ubiquitinated by a homolog of Cbl-b, c-Cbl[142].

Despite differences in the molecules involved, there are similar mechanisms by which TAM receptors inhibit cytokine production in DCs; in these cells, Axl ligation up-regulates the transcription of the ubiquitin ligases SOCS1 and SOCS3, which block TLR-mediated (MyD88 and TRIF) and cytokine-mediated (JAK/STAT) signaling[114]. While the increased expression of SOCS1 and SOCS3 does not affect activating receptor-
mediated activation in NK cells, TAM receptors post-transcriptionally activate Cbl-b, which downregulates components of activating receptor-mediated but not TLR- or cytokine-mediated signaling. Thus, TAM receptors may (through two independent mechanisms) induce ubiquitin ligase-mediated attenuation of signaling through TLRs, cytokines, and activating receptors, to provide broad control of signaling pathways in innate cells of the immune system.
CHAPTER 3: Enhancing NK cell responses by pharmacological inhibition of Cbl-b

Introduction

NK cells represent a promising target for treatment of tumors and chronic infections. Although the effector function of NK cells overlaps with CD8⁺ T cells, they respond to different stimuli and complement the activity of CD8⁺ T cells, especially in settings where CD8⁺ T cell responses are no longer effective. However, NK cell responses alone are often suboptimal to control tumor growth or viral infections. In addition, NK cells rapidly adjust to their environment and can display signs of exhaustion after chronic activation, making it difficult to sustain their effector function. Thus, strategies that involve the enhancement of NK cell activity are necessary to fully harness their therapeutic potential. Manipulation of signaling pathways is an attractive approach, but the research thus far is limited to mouse models and in vitro human NK cell activation assays. Most of the strategies currently being tested at the bedside involve the manipulation of cell surface receptors and cytokines to enhance the activity of NK cells in neoplastic settings. Although none of the strategies are yet fully optimized and effective, the development of novel inhibitors of signaling pathways (e.g. Cbl) and the clever combination of cytokines and receptor/ligand pairs will likely improve the effectiveness of NK cell-based immunotherapies.

Cbl-b has also been shown to be a promising immunotherapy target in various disease models. The enhanced T cell effector functions in the absence of Cbl-b prompted scientists to determine whether Cbl-b regulates anti-tumor responses. Indeed, genetic deletion of Cbl-b conferred spontaneous in vivo rejection of tumor cells
expressing human papilloma virus antigens[60]. In addition, ablation of Cbl-b in the tumor-prone ataxia telangiectasia mutated (ATM)-deficient mice reduced the incidence of spontaneous thymic lymphomas[139]. Mice lacking Cbl-b also develop significantly fewer ultraviolet B (UVB)-induced skin malignancies and reject UVB-induced skin tumors[60]. These mice also reject transplanted E.G7 and EL4 lymphomas[139]. This rejection in the absence of Cbl-b was found to be due to activated CD8+ T cells, which are resistant to regulation by regulatory T cells or TGF- β[60,139]. In fact, the adoptive transfer of CD8+T cells lacking Cbl-b improved survival of murine models of leukemia[140].

Mice harboring a Cbl-b E3 ligase-defective mutation (C373A) are resistant to the development of tumors in vivo and rejected tumors in both experimental and spontaneous tumor models[60,73,139]. This suggested that Cbl-b was negatively regulating immune cell function through its ubiquitin ligase domain[73].

Cbl-b was originally considered a regulator of TCR-induced T cell activation[36], but in another study, Cbl-b KO mice were found to spontaneously reject tumors even on a recombination-activating gene 2 (RAG2) KO background[75]. This effect was lost when NK cells were depleted, suggesting that Cbl-b KO NK cells also display enhanced anti-tumor activity[75]. In addition, metastatic tumor burden was significantly reduced when NK cells from Cbl-b KO and Cbl-b ligase mutant (C373A<sup>AK</sup>/K<sup>AK</sup>) mice were adoptively transferred to a NeuT metastatic breast cancer model. Together, these data suggest that Cbl-b negatively regulates NK cell function through the ubiquitin ligase domain[75]. Therapies to inhibit Cbl-b have been evaluated in murine models of disseminated candidiasis[141] where Cbl-b is targeted using gene silencing via siRNA or a small inhibitory peptide[142], both in a macrophage-dependent manner. Peptide-based
inhibition of Cbl-b increased cellular responses to fungal stimulation in vitro and protected mice from sepsis after systemic infection with C. albicans. To date, there has been one small phase 1 clinical trial in 3 patients to assess the inhibition of Cbl-b in humans[143]. APN401 is an autologous peripheral blood mononuclear cells (PBMCs) suspension that is transfected with an siRNA that knocks down Cbl-b. This suspension was tested in human patients suffering from metastatic solid tumors. CD56 cells were most efficiently transfected (55%), followed by CD3 (46%), CD19 (45%), and CD14 (23%) cells. Cbl-b-siRNA-transfected PBMCs produced more IFNγ and IL-2 in response to stimulation with anti-CD3 and anti-CD28 antibodies in vitro. In patients, the APN401 infusions were well tolerated with no resulting autoimmune adverse effects. These results showed that Cbl-b can be safely targeted in humans.

Although the therapeutic efficacy of inhibiting Cbl-b in humans has yet to be shown to be dependent on T cells and NK cells, murine Cbl-b KO T cells and NK cells have enhanced effector functions, including cytokine production and direct tumor killing—making them potential targets to enhance antitumor and antiviral responses.

Cbl-b makes for an excellent immune checkpoint target because of its dual role in T cells and NK cells. Even though APN401 shows promise in a phase 1 clinical trial, establishing a therapeutic paradigm consisting of removing a patient’s PBMCs, followed by transfection with Cbl-b siRNA, and re-infusion back into a patient is costly, time-consuming, and inefficient as electroporation does not guarantee siRNA is being introduced into each cell. More importantly, there are further risks of developing other cancers with this therapy as viral transduction can introduce new mutations into cells. Instead, small molecule inhibition of Cbl-b provides a safer alternative. In addition, small molecule inhibitors would make the therapy transient and reversible: two qualities sought
after for immune checkpoint blockade to prevent autoimmunity. Therefore, we set out to test several small molecule inhibitors of Cbl-b developed by Progenra, Inc.

**Results**

**Small molecule #52642 inhibits Cbl-b mediated Tyro3 ubiquitylation in a dose-dependent manner**

A small molecule is a low molecular weight organic compound of approximately 1 nm in size that may regulate a biological process[144]. Small molecules can be designed to inhibit certain domains or groups of domains in a protein, preventing its enzymatic function or its binding to other proteins. These compounds are usually ≤500Da size and are often administered orally. Their small size also allows them to translocate through the plasma membrane and interact with the cytoplasmic domain of cell-surface receptors and intracellular signaling molecules. In principle, small molecule compounds can be developed to target any portion of a molecule, regardless of the target protein’s location inside the cell[144].

A small molecule inhibitor of Cbl-b can be designed to inhibit Cbl-b in many ways. Ideally, since Cbl-b is an E3 ubiquitin ligase, a small molecule should be designed to inhibit Cbl-b’s ligase activity. This can be accomplished in several ways. A small molecule can be designed to bind to the TKB domain, preventing substrates from binding to Cbl-b. It can also be designed to bind the linker region, covering essential tyrosines and preventing phosphorylation and thus the activation of Cbl-b. In collaboration with Progenra, Inc., we tested dozens of small molecule inhibitors they developed using a high throughput screening approach. Progenra, Inc. developed the UbiPro™ Drug Discovery Platform (https://progenra.com/therapeutic-focus/rd-platform/)
to screen a diverse collection of >600,000 drug-like small molecules where they then develop selected hits into therapeutic candidate molecules. The platform is divided into 3 categories: functional, biophysical, and cellular validation. The functional screen includes TR-FRET (Time Resolved Fluorescence Resonance Energy Transfer) assay to demonstrate ubiquitination in real time and an AlphaScreen™ ubiquitination assay to achieve higher sensitivity by measuring the proximity of biotinylated ubiquitin (b-Ub) and GST-tagged substrate proteins[145]. This step is followed by biophysical assays, such as surface plasmon resonance (SPR), which uses light refraction of unlabeled substrate bound to glass, to ensure binding of the molecule to a target substrate. Differential scanning fluorimetry, which uses thermal shifts, is then used to identify compounds that are thermally stabilized. These functional and biophysical assays are then followed by cellular assays for validation.

Jurkat T cells, an immortalized human T cell line, were used for the cellular screen of compounds. Jurkat cells were treated with the compounds, their supernatant was collected, and IL-2 production was measured as a readout for T cell activation. A screen revealed a family of compounds labeled #P0052 that inhibited IL-2 secretion in Jurkat cells (data not shown). Next, these compounds were tested biochemically for their ability to specifically block Cbl-b’s E3 ubiquitin ligase activity. In a proteomics screen, TAM receptor Tyro3 was shown to be a target substrate of Cbl-b[75]. This data was validated in HEK 293T cells transfected with V5-Tyro3, FLAG-WT Cbl-b or FLAG-ligase mutant Cbl-b, and HA-ubiquitin. WT Cbl-b ubiquitinated Tyro3 in 293T cells while ligase mutant Cbl-b had a decreased ability to ubiquitinate Tyro3, validating that Cbl-b targets Tyro3 for ubiquitination (Fig. 3.1A-B).
Each compound that blocked Jurkat IL-2 production was then incubated in a cell-free ubiquitination reaction with Cbl-b and Tyro3. Several compounds of family #P0052 were shown to inhibit ubiquitination of Tyro3 when compared to control DMSO (Fig. 3.2A). To show that this compound specifically inhibits E3 and not E1 or E2 proteins, the compounds were incubated at varying concentrations with E1 and E2 in a ubiquitination reaction. No inhibition in the interaction between E1 or E2 and ubiquitin was detected with incubation with compound #52642, similar to reactions in DMSO (Fig. 3.2B). The IC50, or the concentration of an inhibitor where the response is reduced by half, was determined using ubiquitination of Tyro3 as a surrogate for Cbl-b activity. Two varying lengths of Cbl-b recombinant protein were used: full length and a truncated version with only the TKB and RF domains. Ubiquitination of Tyro3 was reduced by 50% with 4.6 μM of inhibitor #52642 (Fig. 3.2C). The IC50s using either full length or truncated Cbl-b
were similar. These results suggest that the #52642 family of compounds are specific inhibitors of E3 ligase activity.

**FIGURE 3.2** Small molecule #52642 inhibits Cbl-b mediated Tyro3 ubiquitylation in a dose-dependent manner. (A) Compounds generated by Progenra’s UbiPro™ Drug Discovery Platform were incubated with E1, E2, HA-Tyro3, and GST-Cbl-b, followed by immunoblotting with anti-HA. All compounds tested inhibited ubiquitination of Tyro3 compared to control. (B) E1 and E2 were incubated with different concentrations of inhibitor #52642 followed by immunoblotting with anti-ubiquitin. The ubiquitination status of E1 and E2 incubated with #52642 were comparable to E1 and E2 treated with DMSO. (C) IC50 was determined by incubating full length Cbl-b, truncated Cbl-b (TKB+RING domains), or DMSO with Tyro3, E1, and E2. Ubiquitination was detected by streptavidin-HRP luminescence. Small molecule #52642 inhibited the E3 ligase function of both full length and truncated forms of Cbl-b, and ubiquitination of half of the Tyro3 molecules (IC50) was inhibited at a concentration of 4.6μM.

**WT and Cbl-b KO NK cells are appropriate cellular models to test for inhibitor efficacy and specificity**

We set out to establish a model where we could test the Cbl-b inhibitors to assess their toxicity, ability to enhance T and NK cell effector functions, and specificity to Cbl-b. LAKs prepared from WT and Cbl-b KO mice were stimulated with antibodies that target several different activating receptors: NK1.1, NKG2D, 2B4, or Ly49D. NK1.1 and
Ly49D use ITAM-mediated adaptor molecules for signal transduction, whereas NKG2D and 2B4 use YxxM motif-containing costimulatory adaptors and SAP, respectively, to transduce their signals. The cells were gated on NK cells and assessed for IFNγ and CD107a (surrogate for degranulation). LAKs deficient in Cbl-b had significantly increased IFNγ+ and CD107a+ production under stimulation with anti-NK1.1 or anti-NKG2D (Fig. 3.3A-C). These data are consistent with previous findings that NK cells deficient in Cbl-b are hyperactivated[75].
To test whether a similar effect was observed in NK cells removed freshly *ex vivo*, NK cells from WT and KO Cbl-b mice were stimulated with antibodies targeting activating receptors. Similar to LAKs, an increased fraction of IFNγ* and CD107a* NK cells was detected in freshly isolated splenocytes stimulated with anti-NK1.1, anti-NKG2D, and anti-2B4 (*Fig 3.4A-C*).
FIGURE 3.4 Cbl-b KO NK cells have enhanced IFNγ production and degranulation. Splenocytes from WT and Cbl-b KO mice were left unstimulated (Unstim) or stimulated with PMA/Iono, anti-NKG2D, anti-NK1.1, anti-2B4, or anti-Ly49D for 6 hours. (A) Representative flow cytometric plots of NK cell IFNγ and CD107a expression are shown (gated on CD3−DX5+NKp46+). The fraction of (B) IFNγ-expressing and (C) CD107a−expressing splenocytes is represented as mean ± SEM of 3 independent experiments (n =3 mice per genotype per condition). *p<0.05 and **p<0.01 by paired Student t test, respectively; ns, not significant.
To test if Cbl-b is negatively regulating NK cells in a physiological setting, NK cells from WT and KO Cbl-b mice pre-treated with the viral mimetic PolyI:C were stimulated by antibodies targeting activating receptors. Similar to LAKs and fresh NK cells, an increased fraction of IFNγ⁺ and CD107a⁺ NK cells was detected in PolyI:C treated splenocytes stimulated with anti-NK1.1, anti-NKG2D, and anti-2B4 (Fig 3.5A-C).
Cbl-b KO NK cells are phenotypically normal

To ensure that there were no phenotypic differences between WT and Cbl-b KO NK cells, fresh NK cells were isolated from the splenocytes of WT or Cbl-b KO mice and analyzed for cell number, cell surface receptor repertoire, and maturation state by flow cytometry. Cell number was analyzed as percent splenic NK cells and no difference in cell number was observed between WT and Cbl-b KO NK cells (Fig. 3.6A).
The cell surface receptors analyzed are part of the Ly49 family of receptors on murine NK cells, which play a key role in NK cell function. Ly49 receptors Ly49A, Ly49G, Ly49C, and Ly49I are inhibitory receptors, whereas Ly49D and Ly49H are activating receptors[10]. This receptor family is used to phenotype NK cells because Ly49 receptors are acquired in a sequential and variegated manner during development, which yields a diverse repertoire of NK cells with various Ly49 receptor expression patterns[10]. Since each Ly49 receptor recognizes a subset of MHC I alleles, the Ly49 receptor expression pattern on an individual NK cell determines its target cell specificity. Ly49 receptor genes are activated in a specific order, and each receptor possesses a developmental time frame for the initiation of expression, which is maintained for the lifetime of the NK cell. Splenocytes from WT, Cbl-b heterozygous, or Cbl-b knockout mice were phenotyped for these Ly49 receptors by flow cytometry and there were no differences found in Ly49 receptor expression (Fig. 3.6B).

The maturation of NK cells is a 4-step developmental program, which is associated with a progressive acquisition of receptors and NK-cell effector functions[146]. Surface density of CD27 and CD11b subdivides murine NK cells into 4 subsets: CD11b(low)CD27(low), CD11b(low)CD27(high), CD11b(high)CD27(high), and CD11b(high)CD27(low). CD11b(low)CD27(low) NK cells are the most immature, whereas CD11b(high)CD27(low) NK cells are fully matured with effector functions that include cytokine production and degranulation[146]. Splenocytes from WT and Cbl-b KO mice were stained for CD11b and CD27 and gated on NK cells. There were no significant differences in maturation state between WT and Cbl-b KO NK cells (Fig. 3.6C). These studies informed us that the Cbl-b KO mouse model was an adequate model in which to study the safety and efficacy of Cbl-b inhibitors.
Cbl-b inhibitors were first tested on WT NK cells to assess for their ability to enhance cytokine production and degranulation. WT NK cells were pre-treated with either inhibitor or DMSO followed by stimulation with anti-NK1.1 or anti-NKG2D. Splenocytes were then analyzed by flow cytometry, gated on live NK cells and analyzed for IFNγ and CD107a. Although there was no enhancement of IFNγ when stimulated with anti-NKG2D, there was an enhancement of IFNγ with anti-NK1.1 stimulation and an
increase in CD107a expression with activation of both receptors when treated with inhibitor #52642 when compared to DMSO (Fig. 3.7).
FIGURE 3.7 NK cells treated with compound #52642 have enhanced degranulation and IFNγ production. Splenocytes from WT mice were pre-treated with DMSO or putative Cbl-b inhibitors (10μm), left unstimulated (Unstim) or stimulated with PMA/ Iono, anti-NKG2D, anti-NK1.1, or anti-2B4 for 6 hours. Representative flow cytometric plots of NK cell (A) IFNγ and (B) CD107a expression are shown (gated on CD3- DX5+NKp46+). (C-D) Cumulative data is shown as mean % enhancement \(((\text{DMSO - 52642-treated)/DMSO}*100)\) ± SEM of 4 independent experiments \((n = 4-5)\). *p< 0.05 and ***p <0.001 by paired Student t test, respectively.
To test whether the Cbl-b inhibitor was specific for Cbl-b or whether it may also target c-Cbl, Cbl-b KO NK cells were pre-treated with either inhibitor or DMSO followed by stimulation with antibodies targeting activating receptors NK1.1 or NKG2D. Splenocytes were then analyzed by flow cytometry, gated on NK cells, and analyzed for IFNγ and CD107. Treatment of Cbl-b KO NK cells with inhibitor #52642 led to their enhanced degranulation and IFNγ when compared to WT NK cells, suggesting that inhibitor #52642 was not specific for Cbl-b (Fig. 3.8).

**FIGURE 3.8 Putative Cbl-b inhibitor #52642 also enhances Cbl-b KO NK cells.** Splenocytes from Cbl-b KO mice were pretreated with DMSO or compound #52642 (10 μM) and left unstimulated (Unstim) or stimulated with PMA/Iono, anti-NKG2D, or anti-NK1.1 for 6 hours. Representative flow cytometric plots of NK cell IFNγ and CD107a expression are shown (gated on CD3 DX5 NKp46+). Putative Cbl-b inhibitor 52642 enhanced both IFNγ and CD107a compared to DMSO controls. One representative of two independent experiments is shown.
Cbl-b is also expressed in T cells and has been shown to be a key regulator of threshold activation. CD8\(^+\) T cells lacking Cbl-b have been shown to have enhanced anti-tumor activity, and thus is another cellular target cell for Cbl-b inhibition. We therefore sought out to assess the effect of the inhibitors on T cells. CD8\(^+\) T cells isolated from WT mice were grown in culture, labeled with CFSE, treated with DMSO or Cbl-b inhibitor #52642, and assessed for proliferation after 24 hours. Putative Cbl-b inhibitor #52642 enhanced proliferation of CD8\(^+\) T cells compared to DMSO control (Fig. 3.9A). To assess for effector functions, WT CD4\(^+\) and CD8\(^+\) T cells were pre-treated with the inhibitor #52642 and another set of compounds (#87678, 87793, and 87798), stimulated with anti-CD3, and their supernatant assayed for IL-2 production. Both CD4\(^+\) and CD8\(^+\) WT T cells had enhanced IL-2 production when treated with inhibitor #52642 (Fig. 3.9B).

![FIGURE 3.9 Putative Cbl-b inhibitor #52642 enhances proliferation and IL-2 production in CD4+ and CD8+ T cells.](image)

(A) CD8\(^+\) T cells isolated from mice were grown in culture, labeled with CFSE, pre-treated with DMSO or putative Cbl-b inhibitor #52642, and assessed for proliferation after 24 hours. (B) WT CD4\(^+\) and CD8\(^+\) T cells were pre-treated with putative inhibitors for 2 hours, stimulated with plate-bound anti-CD3, and their supernatant assayed for IL-2 production by ELISA.
87675 enhances NK and T cell effector functions and may be more specific for Cbl-b

CD4<sup>+</sup> and CD8<sup>+</sup> T cells isolated from WT mice were grown in culture, treated with a Cbl-b inhibitor, stimulated with either anti-CD3 or both anti-CD3 and anti-CD28, and assessed for IL-2 production after 24 hours. Putative Cbl-b inhibitors #87675 and #119012 increased IL-2 production by TCR-stimulated CD4<sup>+</sup> and CD8<sup>+</sup> T cells. (Fig.3.10A-B).

FIGURE 3.10 Putative Cbl-b inhibitors #87675 and #119012 increased IL-2 production by TCR-stimulated CD4<sup>+</sup> and CD8<sup>+</sup> T cells. (A) CD4<sup>+</sup> and (B) CD8<sup>+</sup> T cells were purified and pre-treated with DMSO or putative Cbl-b inhibitors (10 μM) for 2 hours and stimulated in plates coated with PBS, anti-CD3, or anti-CD3/CD28. IL-2 was measured in the supernatant 24 hours later by ELISA.

To test whether the Cbl-b inhibitors were specific for Cbl-b, Cbl-b KO CD4<sup>+</sup> and CD8<sup>+</sup> T cells were pre-treated with either DMSO or inhibitor followed by stimulation with...
anti-CD3. Both CD4\(^+\) and CD8\(^+\) WT and Cbl-b KO T cells had enhanced IL-2 production when treated with inhibitor #87675 (Fig. 3.11A-B). As with NK cells, the Cbl-b inhibitors enhanced T cell effector functions but were not specific to Cbl-b in T cells; further providing evidence that the inhibitor is not specific to Cbl-b. However, when the data was graphed as percent of control DMSO, Cbl-b KO CD4\(^+\) and CD8\(^+\) T cells had less IL-2 production compared to WT CD4\(^+\) and CD8\(^+\) T cells. The latter suggests that the inhibitor is targeting Cbl-b, as well as other proteins (Fig. 3.11C).

**FIGURE 3.11.** Putative Cbl-b inhibitors #87675 enhanced IL-2 production in Cbl-b WT T cells more so than in Cbl-b KO T cells. (A) CD4\(^+\) and (B) CD8\(^+\) T cells from WT and Cbl-b KO mice were purified and pre-treated with DMSO or putative Cbl-b inhibitors (10 μM) for 2 hours and stimulated with plates coated with PBS or anti-CD3. IL-2 was measured in the supernatant 24 hours later by ELISA. (C) Data are represented as % of control.
Cbl-b inhibitor #52642 does not reduce tumor burden in a mouse model of lung adenocarcinoma

Inhibitor #52642 was not specific for Cbl-b. Cbl-b is homologous to c-cbl; therefore, it is highly possible that the compound is also inhibiting c-Cbl. However, because both NK and T cell effector functions were enhanced and c-Cbl is another target for therapeutic inhibition, we decided to test the inhibitor *in vivo* as a potential immunotherapy. We selected the TC-1 murine lung adenocarcinoma cell line, as it has been established as an adequate subcutaneous tumor model and is sensitive to tumor killing by CD8⁺ T cells and NK cells deficient in Cbl-b[60,75]. TC-1 cells were grown in culture and injected subcutaneously into the flank of mice. After tumors were of appreciable size 1 week after injection, mice were treated intraperitoneally with either vehicle or inhibitor #52642 twice daily and tumor volume assessed by calipers. After an approximately 2-week pre-clinical trial, the mice treated with inhibitor #52642 had no reduction in tumor volumes compared to control. Mice were euthanized after losing 20% of their initial body weight (Fig. 3.12). Initially, weights were being monitored as we predicted that if the inhibitor were working, mice would initially lose weight. However, the reduction in weight correlated with tumor growth and not therapeutic efficacy.

![Graph A](image1.png)  ![Graph B](image2.png)

**FIGURE 3.12** Putative Cbl-b inhibitor #52642 is not toxic but also ineffective at reducing TC-1 tumor burden. 3 mice were treated with putative Cbl-b inhibitor #52642 and 2 mice were given vehicle (DMSO) control. After tumor engraftment, mice began treatment with #52642 twice daily. (A) Tumor volume and (B) body weight were measured daily. After 10 days, mice were euthanized because of greater than 20% weight loss in the treatment group.
Our data support the hypothesis that Cbl-b is a therapeutic target, as both NK cells and T cells that are either Cbl-b-deficient or are inhibited by an E3 ligase inhibitor have enhanced effector functions. It is left to be determined if these inhibitors also target c-Cbl. Despite the lack of specificity to Cbl-b, having an inhibitor that also targets c-Cbl may prove beneficial. c-Cbl and Cbl-b are homologues that have overlapping roles, and both are expressed in T cells and NK cells. An inhibitor that targets Cbl-b and c-Cbl may have an additive effect in enhancing T and NK cell effector functions. In addition, an immense obstacle in moving forward is the currently short half-life of the compound tested \textit{in vivo}. Once the half-life is prolonged, we can more accurately test \textit{in vivo} the safety and efficacy of these inhibitors.

**Discussion**

Ubiquitination is a post-translational modification that regulates intracellular protein levels and localization. Drug discovery in the ubiquitination field began to thrive with FDA approval of 26S proteasome inhibitors Bortezomib (approved in 2003, marketed as Velcade®) and more recently Carfilzomib (approved in 2012, marketed as Kyprolis®) for treatment of multiple myeloma. Although proteasome inhibitors demonstrate selectivity toward tumor cells and lead to apoptosis, they also suppress proteasome-mediated degradation of all intracellular proteins resulting in undesirable off-target side effects[147]. In addition, many patients developed resistance towards Bortezomib[148]. Strategies to overcome the resistance include designing inhibitors with alternative mechanisms of action that differs from current targeting methods. For example, Carfilzomib covalently binds the proteasome, causing irreversible inhibition[149].
As part of the ubiquitination process, targeting E3 ubiquitin ligases represent another strategy for drug discovery. They are the largest family in the ubiquitin system - with 700 members predicted to have ligase activity. In addition, they use distinct catalytic mechanisms, making them targets that can yield enhanced specificity and less toxicity. Currently, there are few therapies targeting E3 ligases, and their development remains a challenge. Development of E3 ligase modulators is complicated by diverse protein–protein interactions, lack of a classical enzymatic/catalytic active site, and specificity problems due to the variety of potential substrates.

Despite these challenges, there are examples of successes. RING-type E3 ligases were successfully targeted using small molecule inhibitors of protein–protein interactions, i.e., binding at substrate/receptor HIF1α/VHL[150], p53/MDM2 and p53/MDM4 interfaces[151], adaptor/receptor interface Skp1/Skp2[152] and several others[153]. However, no inhibitor has successfully been designed to specifically block E3 ligase activity.

To successfully address this problem one must take into consideration the multi-subunit nature of many E3 ligases that implies multiple druggable pockets and protein–protein interfaces. To successfully inhibit an E3 ubiquitin ligase, an inhibitor should be designed to block the linker region containing the tyrosines that need to be phosphorylated to activate Cbl’s ligase function, the RF domain to prevent E2 binding, or block the TKB binding domain to prevent substrate binding. One potential caveat to inhibiting Cbl-b is that it plays many different roles, both in positively and negatively regulating cells. Cbl proteins have 3 roles: inhibitors, adaptors, and E3 ligases, making targeting them a difficult task. We currently do not know enough of Cbl-b biology in NK cells and T cells to predict potential negative outcomes of Cbl-b inhibition.
Why did E3 ligase putative Cbl-b inhibitor #52642 enhance Cbl-b KO T and NK cells?

Small molecule #52642 enhanced NK cell and T cell effector functions. However, it also enhanced effector functions in Cbl-b KO NK and T cells, indicating that the compound was not specific for Cbl-b. One likely explanation is that it is also targeting c-Cbl, which is also expressed in both T cells and NK cells. The amino-terminal domains are highly conserved across all members of the Cbl family-making this explanation highly likely. If an inhibitor is designed to target the RING finger domain, it is likely that it would inhibit both c-Cbl and Cbl-b as their C-termini are very similar. Cbl-b and c-Cbl are highly homologous proteins; therefore, it is possible that a small molecule inhibitor designed to bind to Cbl-b may also bind to c-Cbl. One potential way to test this is developing siRNA to target Cbl-b and c-Cbl, transduce Jurkat T cells, and then measure IL-2 production levels by ELISA. As mentioned before, having an inhibitor that targets both c-Cbl and Cbl-b may be beneficial, as both play a role in negatively regulating lymphocytes.

Why did E3 ligase inhibitor #52642 not work in vivo?

Despite not being specific to Cbl-b, we predicted that the compound may still have an anti-tumor therapeutic effect in vivo given that it enhanced NK and T cell effector functions. We conducted a small pre-clinical study where we treated TC-1 lung adenocarcinoma bearing mice with vehicle or compound #52642. Although there was no toxicity associated with the compound, there was no therapeutic benefit compared to treatment with vehicle. There are several potential explanations for this.
The half-life of the compound was so short that the drug likely failed to enter enough cells and inhibit Cbl proteins despite intraperitoneal injections twice daily for the course of the study. The half-life of #52642 in vivo formulation was only 1.24 hrs, and the maximum concentration was shown to drop precipitously. In addition, the compound was not administered intravenously but via intraperitoneal injection. One consideration is that due to its short half-life, an intravenous injection may have worked better than an intraperitoneal injection. Putative Cbl-b inhibitor #87675 enhanced WT T cells more so than Cbl-b KO T cells and will, therefore, be a better option to test in vivo.

**How may the development of a Cbl-b inhibitor be accomplished?**

More than 20 small molecule inhibitors have been approved for clinical use and are being successfully used as therapies for cancer. Still, there are certain limitations that are to be considered and overcome while designing more active drugs so as to reduce the failure rates of the drugs at the clinical level. Certain small molecule inhibitors bind to multiple molecular targets including cell surface receptors and other intracellular proteins thus increasing the risk of toxicity[154]. Most of the small molecule drugs have a short life span, thus requiring daily dosing which is not the case with therapeutic monoclonal antibodies that have a longer life span[154]. Alternatively, coupling these drugs to other compounds, such as nano-carriers, so as to increase the specificity and bio-availability should be considered.
The human body needs internal rheostats to monitor responses to pathogens - those that are foreign, such as viruses, and even those that are derived from our own cells, such as tumors. With an internal rheostat, we can limit a pathogen and pathogen-induced damage while at the same time avoiding autoimmunity. This requires sensors to monitor thresholds of activation on immune cells and to modulate the magnitude of their response. In addition, a physiological immune response requires a negative regulatory mechanism that provides a variety of safeguards and exit strategies. Several receptor families are required for homeostasis of the immune response. One such family is that of TAM receptors (Tyro3, Axl, Mer), which are receptor tyrosine kinases expressed in a variety of human and murine cell types.

How receptor tyrosine kinases can act as sensors and at the same time promote signaling and cellular functions is a topic of immense interest, as RTKs are not typically associated with inhibiting signaling and cellular responses. According to the canonical view of RTKs as promoting signaling, the tyrosine kinase activity of TAM receptors activates ERK, AKT, and NFκB – key signaling pathways required for NK cell cytokine production[77]. Thus, it is puzzling that TAM receptors attenuate, rather than augment, NK cell function.

TAM receptors are not the only puzzling RTKs with dual roles. Ron is an RTK that has been shown to negatively regulate macrophages. Ron induces transcription of E3 ubiquitin ligase SOCS1, which reduces the production and response to IFNγ,
resulting in enhanced susceptibility to endotoxin challenge[155]. TAM receptors have also been shown to negatively regulate DCs by increasing the transcriptional expression of E3 ubiquitin ligase SOCS1 and SOCS3[114]. Similarly, our data reveal a mechanism by which TAMs can inhibit activating receptor stimulation in NK cells. Namely, by phosphorylating and activating Cbl-b, TAM receptors can selectively suppress signaling through activating receptors by targeting LAT1 for degradation. Our data parallels the other established mechanisms by which RTKs (TAMs and Ron) can inhibit innate immune cell activation - via E3 ubiquitin ligases. Our data takes it a step further and identifies a target substrate of Gas6/TAM-activated Cbl-b, LAT1, which is required for the cytotoxic activity of NK cells.

**The role of RTKs in negatively regulating innate immunity: Unresolved questions and future directions**

**Is the interaction between TAM and Cbl-b direct or indirect?**

We showed using recombinant proteins that Tyro3, Axl, and Mer can directly phosphorylate Cbl-b, specifically on tyrosines 133 and 363. In NK cells, we were able to show that incubation with Gas6 leads to phosphorylation of Cbl-b, and that phosphorylation was abolished with treatment with TAM inhibitor BMS777607. Nevertheless, this still does not prove that the phosphorylation is a result of a direct interaction. Even an immunoprecipitation of individual TAM receptors and immunoblotting for Cbl-b will not answer this question. Fluorescence resonance energy transfer (FRET) may provide a more definitive answer to this question. Yet, it is likely that given the supramolecular complexes that form at the immunological synapse, this story may be more complicated with other kinases being recruited to the phosphorylated
TAM receptor, thus making it difficult to answer the question of which kinase or kinases directly phosphorylate Cbl-b.

**What other signaling molecules downstream of the TAM/Cbl-b pathway aside from LAT1 are targeted by Cbl-b?**

It is debated whether LAT1 alone is enough to promote NK cell cytotoxicity, and there are reports that LAT2/NTAL is also required. We attempted to address this question but the antibodies available for LAT2/NTAL western blot provided inconclusive results (data not shown). In addition, it would also be important to test functionally whether LAT1 is indeed ubiquitinated upon Gas6/TAM stimulation and whether a LAT mutant (K52R or K204R) abrogates its ubiquitination and degradation. We attempted to perform a ubiquitination immunoprecipitation followed by LAT1 western blot but were unsuccessful. Several protocol details need to be optimized to improve the ubiquitination immunoprecipitation. The reverse experiment could not be done because the available LAT1 antibodies are not suited for immunoprecipitation. Similarly, we attempted to block the proteasome and lysosome to assess whether this would stabilize LAT1 levels upon Gas6 stimulation. However, this also requires optimization as the proteasome and lysosome inhibitors are toxic to NK cells, which are already in a stressed state upon serum and IL-2 starvation.

Another way to test functionally whether LAT1 is required downstream of the Gas6/TAM pathway is by stimulating NK cells with a LAT1-independent method of stimulating NK cells, such as using IL-12 and IL-18. If the Gas6/TAM pathway requires LAT1, Gas6 should not inhibit cytokine production when stimulated by IL-12 and IL-18.
Aside from LAT1 and 2, there are likely other signaling molecules downstream of the TAM/Cbl-b pathway that may be targeted. Thus, along with LAT1, we also assessed the stability of other key signaling molecules that are required for NK cell activation, such as PLCγ1 and the p85 subunit of PI3K, after Gas6 stimulation. None of these proteins were degraded after 60 minutes of Gas6 stimulation. However, the lack of degradation of these proteins does not preclude them from being involved in the TAM/Cbl-b-mediated inhibition of NK cell activation. Ubiquitination can also lead to re-localization within the cell, can prevent proteins from binding to each other, and can also prevent the addition of post-translational modifications. Therefore, although PLCγ1 and p85 are not degraded upon Gas6 stimulation, Cbl-b may still be regulating NK cell responses by proteolysis-independent mechanisms. An unbiased approach using tandem ubiquitin-binding entities (TUBES) or TUBES in combination with mass spectrometry may yield other targets of the TAM/Cbl-b pathway that may be required for NK cell cytotoxicity[156].

**How does the cellular environment affect TAM expression?**

To maintain homeostasis and prevent autoimmunity, it is imperative that the expression of immune modulating receptors be tightly regulated. In NK cells, TAM receptor expression may be minimal until required as would be the case in a pro-inflammatory setting. We see examples of this regulation in macrophages and dendritic cells where TAM receptors are lowly expressed at baseline and expression is induced only when there are pro-inflammatory stimuli. In DCs, it is type 1 interferon receptor (IFNAR) signaling that upregulates Axl expression via the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway[114]. In bone-marrow derived macrophages, surface expression of Axl is enhanced in a pro-inflammatory environment, such as in the presence of IFNs, IL-4, and Poly I:C[157]. The intracellular
mechanism that connects pro-inflammatory stimuli to enhanced expression of Axl is not yet known.

It remains to be determined if TAM expression on NK cells requires induction by a pro-inflammatory environment. We and others have shown that NK cells minimally express TAM receptors[75] (data not shown). This provides some initial evidence that like macrophages and DCs, NK cells may need to be primed to express TAM receptors. Both DX5+ MACS purified NK cells and LAKs cultured in IL-2 express large amounts of phosphorylated Cbl-b protein even after resting from IL-2 for 4 hours. This became evident with the immunoprecipitation of Cbl-b, where there were high levels of phosphorylated Cbl-b protein being expressed at baseline (data not shown). This made it impossible to determine enhancement in phosphorylation with Gas6 treatment. There is evidence that NK cell stimulation can also lead to increases in total Cbl-b protein[76]. Therefore, one possibility is that this can be due to IL-2 driven pro-inflammatory intracellular changes, which can lead to more Cbl-b being produced and/or being phosphorylated, either dependent or independent on TAM receptor expression.

To test if TAM receptors are induced in a pro-inflammatory environment, better antibodies need to be developed as the current antibodies for murine TAM receptors are not adequate. If and when they are developed, a simple experiment would be to IL-2 starve LAKs for 18 hours, serum starve them for 4 hours, and stain for TAM receptors after 0, 30, 60, and 120 minutes of pro-inflammatory cytokine stimulation, such as with IFNs or Poly I:C.
Is receptor mediated endocytosis of the Gas6/TAM complex required for TAM signaling?

It is clear that NK cells that are deficient in Cbl proteins exhibit enhanced cytotoxicity, degranulation, anti-tumor effects, and cytokine production. The current hypothesis in the field is that upon Gas6/TAM ligation, Cbl-b gets recruited to ubiquitinate TAM receptors, and that receptor-mediated endocytosis of the Gas6/TAM complex is how Cbl-b negatively regulates NK cell activation[75]. Evidence was provided that TAM surface expression decreased over time with Gas6 ligation. However, no evidence was provided to show that the internalized Gas6/TAM complex is what drives the TAM-mediated anti-inflammatory program. This hypothesis was not unfounded as there are examples in the literature of Cbl proteins ubiquitinating and down-regulating cell-surface receptors. For example, c-Cbl can promote ligand-induced EGFR internalization in a ubiquitin-dependent manner, and either c-Cbl or Cbl-b can downregulate the TCR[58].

In light of what we have learned, it is possible that Cbl-b ubiquitinates TAM receptors after the cell has had sufficient TAM/Cbl-b-mediated inhibition. This fits well with our model, where we show that Gas6-mediated TAM activation phosphorylates and activates Cbl-b, ubiquitinating LAT1, which is required for NK cell cytotoxicity. To ensure that a cell can mount a proper anti-inflammatory response when required, after sensing enough anti-inflammatory signals, Cbl-b ubiquitinates TAM receptors, leading to their endocytosis. This stops the anti-inflammatory program, which is required if the cell were to then encounter a virus or tumor and need to mount a proper inflammatory response. There is presently little evidence of TAM receptor-mediated endocytosis as a negative-feedback mechanism. Another possible mechanism to halt signaling after a substantial
anti-inflammatory response is receptor shedding. Receptor shedding serves two purposes: 1) to remove the receptor and halt signaling and 2) to become a ligand sink, further preventing activation of receptors still present on the cell surface. There is evidence in macrophages that upon TAM stimulation, TAM receptors are shed[157]. Therefore, it is possible that negative feedback regulation of TAM signaling may be Cbl-b-independent. More works needs to be done to further elucidate how TAM signaling is regulated in NK cells.

We attempted to address the question of whether TAM receptor-mediated endocytosis was required for inhibition of NK cell effector functions. Receptor-mediated endocytosis can be clathrin-dependent or independent. The GTPase Dynamin has a role in both types of receptor-mediated endocytosis. Therefore, to address the question of whether Gas/TAM internalization is required for TAM’s inhibitory effect on NK cells, we used two dynamin inhibitors: Dyngo and Dynasaur. Unfortunately, because the compounds were too toxic to NK cells in serum-free conditions, the experiments did not yield any results as the cells died during incubation (data not shown). There is one published work using the human NK cell line NKL which used a dynamin inhibitor successfully, however, in serum sufficient media conditions[158]. It is likely that serum is required for cellular uptake of these compounds, in which case these experiments could not be performed as serum contains TAM ligands Gas6 and ProteinS.

In addition to the potential toxic effects of using these compounds, dynamin inhibitors Dyngo and Dynasaur have also been shown to block the release of cytotoxic granules. Dynamin 2 was shown to regulate granule exocytosis during NK cell cytotoxicity[159]. Therefore, we would not have been able to use CD107a as a surrogate for cytotoxicity, nor could we assess perforin release. IFNγ, once it is produced, is also
transported via vesicles to the cell surface where it is released. Cytokine secretion is a distinct process from secretion of cytotoxic granules[160]; however, in human NK cells, clathrin is also required for IFNγ release after viral stimulation[161]. Both the issues of dynamin-inhibitor toxicity and the likely dependence on dynamin for both IFNγ and CD107a release make it difficult to answer the question of whether receptor-mediated endocytosis of the Gas6-TAM complex is a requirement for TAM’s inhibition of NK cell effector functions.

Based on our model, receptor-mediated endocytosis may be part of the negative feedback mechanism to attenuate TAM signaling. There are several examples of ligand exposure leading to the downregulation of the ligand’s receptor as a means to stop signaling via that particular receptor. After an adequate anti-inflammatory response, Cbl-b may ubiquitinate TAM receptors, allowing for their degradation and a return to cellular homeostasis.

**Does Cbl-b play a role in TAM-mediated phagocytosis by macrophages?**

Although there is insufficient evidence to validate Penninger’s group’s speculation that Cbl-b ubiquitinates TAM receptors and this internalized complex leads to negative regulation of signaling in NK cells, this interaction may occur in macrophages. TAM receptors and Cbl-b are both expressed in macrophages. Mer was shown to be the key TAM receptor required for phagocyte-dependent clearance of apoptotic cells[162], but since then all TAM receptors have been shown to play a role in phagocytosis[163,157]. Macrophages use TAM receptors to sense the PS exposed on the surface of apoptotic cells, but what occurs downstream of the TAM receptors is unknown. Given our data and that of others showing the importance of Cbl-b in TAM signaling, we hypothesized that Cbl-b is required for macrophages to phagocytose
apoptotic bodies via TAM receptors. To test this, we performed an initial experiment where CFSE-labeled irradiated splenocytes were injected IV into WT and Cbl-b KO mice. If Cbl-b is required for macrophages to clear these apoptotic cells, we would have observed less CFSE+ cells in Cbl-b KO mice compared to WT. However, we observed no difference in CFSE+ cells in Cbl-KO and WT mice. Not surprisingly, this can be due c-Cbl functioning in the absence of Cbl-b. It has been shown before that c-Cbl and Cbl-b have redundant functions and either can ubiquitinate and down-regulate TCR[58]. This experiment, therefore, would need to be performed in Cbl-b and c-Cbl DKO's to answer this question.

It would also be interesting to further elucidate the possible interaction between TAM receptors and Cbl-b. Based on in vitro data we and others have shown, Cbl-b ubquitinates TAM receptors[75]. To further validate this data, it should be determined which lysines on Tyro3 can be possibly ubiquitinated by Cbl-b. Tyro3 has 12 lysine residues in the intracellular portion of the receptor. Truncation mutations of Tyro3 can help delineate which residue(s) are required for it to be ubiquitinated. If a ubiquitinated resistant Tyro3 can then be transduced into a cell line lacking all TAM receptors but expressing Cbl-b, then we could ask whether TAM receptors directly interact with Cbl-b and if Cbl-b provides a negative feedback mechanism to attenuate TAM signaling.

Do TAMs play a role in NK cell sensing of apoptotic bodies?

TAM receptors have many roles in maintaining homeostasis, not only in regulating intracellular signaling and innate immune effector functions, but also in the clearing of apoptotic bodies. When cells undergo apoptosis, apoptotic bodies express the “eat me” signal phosphatidylserine (PS) on their surfaces, which bind to serum Gas6.
Therefore, we speculate that this PS bound to Gas6 can bind to TAM receptors on the NK cell surface, inhibiting NK cell function.

It is known that interaction between NK cells and macrophages is contact-dependent[164,165], making the PS-Gas6-TAM receptor interaction likely. One possible rationale for this interaction is to prevent NK cells from killing something that is already dead. One possible experiment to test this would be to co-culture NK cells with a tumor line that expresses an NK receptor ligand, such as Rae1-RMA - a murine lymphoma cell line. Rae1-expressing RMA cells are known to activate NK cells by binding to and stimulating the NKG2D activating receptor[166]. If Rae1-RMA cells are irradiated to induce apoptosis and co-cultured with NK cells, the question would be whether Gas6 present in the serum will bind to PS on the apoptotic cell and inhibit NKG2D-mediated NK cell activation. One caveat to this experiment is that NK cells likely express a receptor that directly binds to PS[167], as we discovered when attempting to make PS-Gas6 liposomes and our PS only control inhibited NK cell activity (data not shown)[168].

**Targeting NK cells for therapies - unresolved questions and future directions**

NK cells represent a promising target for treatment of tumors and chronic infections. Although NK cell responses alone are often suboptimal to control tumor growth or viral infections, NK cells complement the activity of CD8+ T cells. This is especially true in settings where CD8+ T cells are no longer effective. Thus, strategies that enhance NK cell activity are necessary to fully harness their therapeutic potential. In this thesis, we have discussed how targeting negative regulators can be employed to enhance NK cell function. Manipulation of inhibitory signaling pathways is an attractive approach, but the research thus far is limited to mouse models and *in vitro* human NK cell activation. Most of the strategies currently being tested at the bedside involve the
manipulation of cell surface receptors and cytokines to enhance the activity of NK cells in neoplastic settings. None of the strategies are yet fully optimized nor are they effective; therefore, the development of novel inhibitors of signaling pathways (e.g., DGK and Cbl) and the targeting of inhibitory receptor/ligand pairs will likely improve the effectiveness of NK cell-based immunotherapy.

Both Cbl-b and TAM receptors have dual roles in cancer: they individually drive tumorigenesis and act on immune cells to suppress an anti-tumor response, making them exciting targets for targeted anti-tumor immunotherapy. Using these therapies to specifically enhance an immune response can be even more beneficial because these therapies can in theory be used to treat a variety of different tumor types. In addition, because they’re enhancing immune responses, not only can cancers be treated, but also difficult-to-treat viral infections where enveloped viruses mimic apoptotic cells, expressing PtdSer on their surface[169]. In this setting, Axl has been shown to be involved in T cell priming and antiviral immunity[170]. It is unknown whether NK cells are also involved, thus it would be interesting to study the role of TAM receptors in NK cell antiviral immunity.

As discussed previously in the introduction, RTKs are known to enhance signaling. As such, TAM receptors promote proliferation and survival via the ERK and AKT pathways. Not surprisingly, TAM receptors promote tumor growth, chemoresistance, metastasis, migration and invasion. There are no known mutations that make TAMs oncogenic like other RTKs. Their oncogenic potential is related to aberrant regulation of the same signaling pathways and cellular processes in which these receptors normally play a role[77]. This aberrant regulation leads to overexpression of TAMs.
It has been difficult to study immune cell TAM signaling in the context of cancer because both tumors and innate immune cells express TAM receptors. Gas6 is produced in the tumor microenvironment of melanoma and breast cancer tumors[171]. Gas6 promotes tumor survival and proliferation and inhibits the inflammatory innate immune response required for activation of anti-tumor cytotoxicity.

The first study showing that TAM signaling affects anti-tumor immunity came from Gas6 KO mice, which had impaired primary tumor and metastatic growth[172]. In this setting, tumors produced IL-10 and macrophage colony-stimulating factor (M-CSF), which instructed macrophages in the microenvironment to overexpress and secrete Gas6. This Gas6 then acts on TAM receptors present on tumor cells. Stromal cells have also been shown to produce Gas6 in the presence of multiple myeloma and metastatic prostate cancer[173]. Therapeutically targeting Gas6/Axl using an Axl inhibitor has been shown to improve the efficacy of chemotherapies[174], likely due to enhancement of immune cell responses in addition to targeting tumors that may have developed resistance to standard chemotherapies.

There is a plethora of TAM receptor inhibitors currently in development and in clinical trials[77]. Regulators of TAM receptors may also be another option for targeted therapies, such as the E3 ubiquitin ligase Cbl-b[75].

**TAMs and Cbl-b as therapeutic targets to enhance an immune response**

The activation of costimulatory T cell pathways is already used clinically for the treatment of cancers. Cbl-b would be another target for immunotherapy, as mice deficient in Cbl-b are able to spontaneously reject tumors of solid and hematopoietic types, including rejection of TC-1, EL-4, and E.G7 tumorigenic cell lines. Studies have
shown that this can be ascribed to both CD8+ and NK cells deficient in Cbl-b[32]. RNAi mediated knockdown of Cbl-b in adoptively transferred CD8+ T cells has been shown to be effective treatment for leukemia[140]. The development of small molecule inhibitors would be even more effective because it they can potentially enhance the anti-tumor activity of both T cells and NK cells.

**What are the different ways Cbl-b can be targeted?**

Cbl-b was discovered as an oncogene, where v-Cbl, a truncated splice variant containing only the TKB domain, caused leukemia in mice[175]. This truncated mutant prevented RTK ubiquitination and downregulation, mostly likely acting as a dominant-negative protein preventing the recruitment of endogenous Cbl proteins to the RTK. Other mutants that have been identified have in-frame deletions of part or all of the linker and RF domains, losing E3 ligase activity; the latter resulting from point mutations which lead to mis-splicing of the Cbl mRNA[47].

Interestingly, mice that lack Cbl proteins do not develop leukemia, yet mice that have a knock-in mutation of an RF-mutant Cbl develop myeloid leukemia[176]. The latter is likely due to a dominant negative effect, where mutant Cbl binds to activated RTKs, preventing recruitment of endogenous WT Cbl proteins. In fact, mice deficient in both c-Cbl and Cbl-b in the hematopoietic stem cell compartment develop myeloid leukemia[177]. In addition to these mutations, Cbl proteins maintain their positive adaptive function - contributing to the transforming capacity of the Cbl mutants. Therefore, a novel approach would be to also target the adaptor function of Cbl protein.

Progenra, Inc. has developed numerous small molecule peptides to bind Cbl-b and inhibit its ability to function as an E3 ubiquitin ligase. Whether these small molecules...
bind the TKB domain and block interaction with tyrosine kinases has yet to be determined. In collaboration with Progenra, Inc., we tested dozens of these compounds. There is one promising compound (#87675), which although it is efficacious in vitro, has yet to be tested in vivo. Currently, an in vivo formulation is being made to test in the near future.

Concluding thoughts

NK cells represent a promising target for treatment of tumors and chronic infections. They are safer than T cell therapies and can function when T cells are no longer adequately killing tumors. Thus, strategies that involve the enhancement of NK cell activity are necessary to fully harness their therapeutic potential.

Over the last decade, small molecule cancer drugs have been slowly replacing conventional chemotherapy. RTKs have been the main focus of molecularly targeted therapy using small molecule inhibitors, as they are usually deregulated in cancer and promote cancer cell survival and proliferation. Identifying regulators of RTKs and understanding the mechanism(s) underlying the negative regulation of innate immune responses will help in the design of novel strategies to further improve the efficacy of current drugs and possibly identify other novel targets. The advantages of using a combination of different agents that inhibit several pathways or use of small molecule inhibitors in combination with radiation therapy can also be explored. In this regard, combination therapies using small molecule drugs and monoclonal antibodies have been exploited and are emerging to be a promising anti-cancer strategy[178,179].

We have explored how an RTK (TAM receptors) and a regulator of RTKs (Cbl-b), can work together to inhibit NK cell signaling, and we tested therapeutic inhibition of Cbl-
b. Here, we provided a molecular mechanism which A) discusses the interaction between two clinical targets and B) provides a detailed mechanism that allows for further manipulation of inhibitory pathways to both target cancer cells and enhance antitumor immunity.

More work needs to be done to further understand what other signaling molecules play a role in the TAM/Cbl-b pathway. This information can help determine what other effects in addition to providing other therapeutic targets. Potential therapies targeting either TAM receptors or Cbl-b will affect other players in the pathway. In addition, this pathway may play a role in other cell types, such as macrophages.
CHAPTER 5: Materials and Methods

Mice

Cbl-b KO mice were generated and generously provided by Richard Hodes (National Cancer Institute, Bethesda, MD). SJL/Thy1.1 were created by breeding B6.SJL and B6.PL1 mice. B6.SJL, B6.PL1, and C57BL/6 mice were purchased from The Jackson Laboratory. Mice were sacrificed and analyzed between 8 and 12 weeks of age. Mice were housed in pathogen-free conditions and treated in strict compliance with the Institutional Animal Care and Use Committee regulations at the University of Pennsylvania.

Reagents, Flow cytometry, antibodies, and data analysis

Recombinant murine Gas6 was purchased from R&D systems (Minneapolis, MN).
Recombinant Axl and Mer were purchased from SignalChem (Richmond, BC, Canada).
TAM inhibitor BMS777607 was purchased from Santa Cruz Biotechnology (Dallas, TX).
Antibodies for NK cell stimulation and phenotyping were purchased from BD Pharmingen (San Diego, CA), BioLegend (San Diego, CA), eBioscience (San Diego, CA), Bio X Cell (West Lebanon, NH), and Molecular Probes, Invitrogen (Carlsbad, CA).
Western blot antibodies were purchased from Cell Signaling Technology (Danvers, MA), Santa Cruz Biotechnology (Dallas, TX), or Sigma Aldrich (St. Louis, MO). All chemicals are from Sigma Aldrich (St. Louis, MO), unless otherwise specified.

For flow cytometric analysis, single-cell suspensions were stained with fluorochrome-labeled antibodies in PBS for 30 minutes at 4°C. Fc receptors were blocked with anti-CD16/32 antibodies (BD Pharmingen). For staining of LAKs, fluorochrome-labeled antibodies against CD4, CD8, NK1.1, and NKG2D (BD Pharmingen) were used, including Live/Dead (Invitrogen). Data were acquired using a multicolor flow cytometer.
FACS Canto equipped with FACSDiva software (BD Biosciences). Data analysis was performed using FlowJo (BD Biosciences).

**Statistical Analysis**

Data are shown as mean values ± SEM. Illustrations and statistical analyses were generated using GraphPad Prism 4 (GraphPad, San Diego, CA). A p value <0.05 was considered to indicate statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001).

**Recombinant protein-specific protocols:**

**Production and purification of proteins**

Human Cbl-b TKB+RF (residues 38-429) and Tyro3 kinase catalytic domain (residues 495-810) were cloned into pET-24d(+) -based vectors for bacterial expression. Full length versions of these constructs do not grow adequately in bacteria. Tyro3 isolated from *E. coli* was found to be in a phosphorylated state, possibly due to autophosphorylation. Kinase-dead Tyro3 is not phosphorylated in bacteria (data not shown). Cbl-b point mutations (Y106F, Y133F, and Y363F), in addition to a compound mutant (Y106/133/363F) were generated from plasmid pE-10xHis-HA-Cbl-b TKB+RF by site-directed mutagenesis. WT and mutant Cbl-b TKB+RF proteins were purified from IPTG-induced bacteria transformed with the various constructs. The cells were lysed and sonicated for 30 seconds x 3 cycles, loaded onto HIS-select spin columns (Sigma), and purified according to the manufacturer’s protocol. To remove the imidazole contained in the elution buffer prior to assays, buffer exchange was performed using NAP-5 Sephadex G-25 columns (GE Healthcare) using 20 mM Tris HCl pH 8.0. Proteins were stored in 150 mM NaCl, 10% glycerol, and 10mM DTT for stability. Protein concentration was measured using Pierce 600 nm kit (Thermo Scientific).
Phosphorylation and ubiquitination assays

For *in vitro* kinase assays, ATP (0.2 mM), WT or mutant Cbl-b recombinant protein (500 nM), and p-Tyro3 (50 nM), p-Axl (100nM), or p-Mer (100nM) were added to a final volume of 50 mL in kinase assay buffer (50 mM Bicine pH8.0, 5 mM MgCl2, 0.05% Chaps, and 1mM beta-mercaptoethanol) and incubated at 25°C for 1 hour.

For ubiquitination assays, E1 (5 nM), E2 (100 nM), ATP (0.2 mM), ubiquitin (600 nM), p-Tyro3 (50 nM), and Cbl-b (50 nM) were incubated in kinase assay buffer for 1 hour at room temperature in a final volume of 20 mL. The E2 enzyme used in the assays was UBE2D3/UbcH5C (LifeSensors). The reactions were stopped with 4X sample buffer (NuPage) with 100 mM DTT and immunoblotted with anti-phosphotyrosine or with anti-ubiquitin.

To assess whether the Cbl-b inhibitors could block E3 ubiquitin ligase activity, 200 nM recombinant purified cytoplasmic domain of Tyro3 was bound to clear polypropylene 96-well plates overnight. Plates were washed 3X in PBST then blocked with 3% BSA in PBS for 2 hours. After blocking, plates were washed 3X in PBST and a mixture containing E1, E2, ATP, MgCl2 and a 1:5 ubiquitin:biotinylated-ubiquitin mix was added to all wells of the plate. Cbl-b was pre-incubated with indicated concentrations of compound (DMSO volume adjusted accordingly) for 30 minutes and then added to the reaction plate. Plates were incubated at 25°C for 45 minutes and then washed 3X with PBST. Ubiquitylation was detected with streptavidin-HRP luminescence.

To test whether the small molecules generated by Progenra, Inc. could inhibit Cbl-b mediated Tyro3 ubiquitylation, ubiquitylation reactions were performed using E1, E2, ATP, MgCl2, GST-Cbl-b, and HA-Tyro3 in the presence of indicated compounds or
vehicle control. Reaction was stopped at 1h with SDS sample buffer. Reactions were run on a 10% SDS-acrylamide gel and transferred for Western blot analysis. Membranes were then blotted with anti-HA primary, HRP secondary and signal was detected by chemiluminescence.

To verify that Cbl-b inhibitors inhibit E3 and not E1 or E2 proteins, an in vitro inhibition assay was prepared using E1, E2, ATP, MgCl$_2$, and a 1:4 mixture of WT ubiquitin and biotinylated ubiquitin. The mixture was incubated for 3 hours at 25°C in the presence or absence of indicated concentrations of compound. After 3 hours, the reaction was stopped using 2X SDS containing sample buffer, ran on a 10% SDS-acrylamide gel and transferred for Western blot analysis. Membranes were then blotted with streptavidin-conjugated HRP and signal was detected by chemiluminescence.

**NK cell-specific protocols:**

**NK cell isolation and LAK preparation**

Splenocytes from 10-12 week old WT or Cbl-b KO mice were removed and crushed with the back end of a 1ml syringe in 1mL of ammonium-chloride- potassium (ACK) lysis buffer made in-house, resuspended in complete LAK media, and filtered using 70 micron filter paper. Complete LAK media consists of MEMα, 10 mM HEPES, 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, and 1 x 10$^{-5}$M beta-mercaptoethanol. Splenocytes were then stained with 5uL of anti-DX5-biotin antibody in 500mL of complete LAK media for 20 minutes in 4°C, washed in LAK media, and enriched using 70uL of anti-biotin MACS beads (Miltenyi Biotec) in 430 mL of MACS buffer (PBS, 0.5% BSA, 0.5 mM EDTA) for 30 minutes in 4°C prior to magnetic separation. The DX5+ cells were then expanded in LAK media with 1000 U/ml human IL-2 (PeproTech) for at least 5
days to create LAKs. After 5-6 days of expansion, ~80–90% of the LAKs in culture were NK cells (CD4⁻CD8⁻NK1.1⁺).

**NK cell stimulation assays**

For NK cell activation, freshly isolated splenocytes (untreated or from Poly I:C-injected mice, 200 μg per mouse, injected i.p. 16 hours before harvesting) or LAKs were rested (deprived of IL-2 and serum) for 2 hours with 450 ng/mL Gas6 and then cultured with PE-labeled anti-CD107a (0.4 mg/ml), IL-2 (1000 U/ml), and brefeldin A (10 mg/ml) for 5 hours in 96-well tissue culture plates that were pre-coated with antibodies against NK cell–activating receptors NK1.1 (PK136) or NKG2D (HMG2A) plated at 30 μg/ml overnight at 4 °C. The plates were washed twice in serum free LAK media prior to adding the cells. Unstimulated and PMA/Ionomycin controls shown in the figures did not contain exogenous hIL-2. The cells were then stained with cell surface antibodies, fixed and permeabilized (BD Biosciences), intracellularly stained with anti-IFNγ antibody, and analyzed by flow cytometry. LAKS were gated by FSC/SSC, followed by singlet selection (FSC-W/FSC-H and SSC-W/SSC-H), exclusion of dead cells, and exclusion of CD4⁺ and CD8⁺ T cells. Splenocytes from Poly I:C-treated mice were gated by FSC/SSC, followed by singlet selection (FSC-W/FSC-H and SSC-W/SSC-H), exclusion of dead cells, and exclusion of CD3⁺ T cells, and gating on NK cells (DX5⁺/NKp46⁺)

In experiments involving the TAM inhibitor BMS777607, splenocytes were preincubated with 100 nM of the inhibitor for 2 hours before stimulation, and the inhibitor was maintained throughout the assay. All experiments were performed in serum-deprived conditions during the stimulation to avoid contamination of our cultures with Gas6, which is present in FBS. When using LAK cells, the cells were starved of IL-2 for 2-18 hours
prior to stimulation, since long term culture in IL-2 led to high baseline levels of Cbl-b phosphorylation (data not shown).

**Western Blot and Immunoprecipitation**

For immunoprecipitation of phosphotyrosine, LAKs were serum-starved for 4 hours and IL-2-starved for 18 hours, followed by incubation with the proteasome inhibitor MG132 (5 μM, SelleckChem) for 30 minutes and stimulation with 450 ng/mL recombinant murine Gas6 for various time points at 37°C. In some experiments, cells were co-incubated with MG132 and the pan-TAM inhibitor BMS777607 (300 nM), 30 minutes prior to Gas6 stimulation or incubated with anti-NK1.1 antibody (30 μg/ml) with or without 450ng/mL Gas6 for 60 minutes. Cells were washed in PBS and protein was extracted with protein lysis buffer (Pierce Crosslink Magnetic IP/Co-IP Kit; Thermo Scientific) containing a cocktail of protease and phosphatase inhibitors (dichloroisocoumarin, benzamidine hydrochloride, sodium orthovanadate, sodium pyrophosphate, and sodium fluoride).

Lysis was performed on ice for 5 minutes while vortexing followed by the addition of 10 μg deubiquitinating enzyme Usp2core (Life Sensors) for 1 hour at room temperature. 5 μg of PY20 antibody was prepared for immunoprecipitation using Pierce Crosslink Magnetic IP/Co-IP Kit. Crosslinked antibodies were incubated with lysates overnight in a rotator at 4°C. Samples were run on SDS-PAGE gels, transferred to nitrocellulose membranes, and blotted for Cbl-b.

For western blot lysates, LAKs were IL-2-starved for 18 hours followed by serum-starvation for 4 hours prior to stimulation with 450 ng/mL recombinant murine Gas6 for various time points at 37°C. Cells were then washed with PBS. Protein was extracted using 1% NP-40 lysis buffer containing a protease inhibitor cocktail (Roche) and phosphatase inhibitors for 25 minutes on a rotator at 4°C. Samples were run on SDS-
PAGE gels, transferred to nitrocellulose membranes, and blotted for the indicated protein. The band intensity of LAT1 and the loading control β-actin were measured by ImageJ and the ratio was normalized to unstimulated conditions.

**T cell-specific protocols:**

**T cell isolation**

Splenocytes from 10-12-week-old WT or Cbl-b KO mice were removed and crushed with the back end of a 1ml syringe in 1mL of ammonium-chloride- potassium (ACK) lysis buffer made in-house, resuspended in complete LAK media, and filtered using 70 micron filter paper. Complete LAK media consists of MEMα, 10 mM HEPES, 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, and 1 x 10^{-5}M beta-mercaptoethanol. Splenocytes were enriched using 70μL of anti-biotin MACS beads (Miltenyi Biotec) in 430 mL of MACS buffer (PBS, 0.5% BSA, 0.5 mM EDTA) for 30 minutes in 4°C prior to magnetic separation.

**T cell stimulation**

A 96 well flat bottom plate was coated with anti-CD3 (1μg/mL) or anti-CD3 (1μg/mL) and anti-CD28 (1μg/mL) in PBS in 4°C. T cells were pre-incubated with DMSO or 10μM of Cbl-b inhibitor for 2 hours at 37°C. 100μL of 1 million cells/mL were added per well and incubated overnight, transferred to a V-bottom plate, and the supernatant harvested 24 hours later for ELISA.

**ELISA**

**NK cell work**: LAKs made from either WT or Cbl-b KO splenocytes were deprived of serum and IL-2 for 2 hours and simultaneously pre-treated with 450 ng/mL murine Gas6
at 37°C. Cell free supernatant was collected 24 hours later after stimulation with anti-NK1.1 and anti-NKG2D, and IFNγ content was measured by ELISA (BioLegend).

**T cell work:** CD4+ and CD8+ T cells from either WT or Cbl-b KO splenocytes were pre-treated with 10μM of inhibitor for 2 hours and stimulated with either anti-CD3 or anti-CD3 and anti-CD28 for 24 hours at 37°C. Cell free supernatant was collected 24 hours later, and IL-2 content was measured by ELISA (eBioscience).

**Cell line work:**

**Transfection**

Human cell line HEK 293T (293Ts) cells were grown in culture. 18 hours prior to transfection, 293Ts were plated at 1x10^5 cells/well in a 12-well plate. Cells were checked visually to see if they were approximately 70% confluent prior to transduction. 293Ts were transfected using liposomes in serum-free Opti-MEM media (Gibco) with 1μg of V5-Tyro3, HA-Ubiquitin, and either FLAG-tagged WT Cbl-b or C373A ligase mutant Cbl-b. Protocol was followed according to the manufacturer's instructions (Lipofectamine).

**Western blotting**

Immunoprecipitation with tag V5 (V5-Tyro3) was performed, followed by immunoblotting for HA (HA-ubiquitin) and V5 (V5-Tyro3) and FLAG (FLAG-Cbl-b) and V5 (V5-Tyro3). Cells were then washed with PBS. Protein was extracted using 1% NP-40 lysis buffer containing a protease inhibitor cocktail (Roche) and phosphatase inhibitors for 25 minutes on a rotator at 4°C. Samples were run on SDS-PAGE gels, transferred to nitrocellulose membranes, and blotted for the indicated protein. The band intensity of V5-Tyro3 ubiquitination was measured by ImageJ.
Mouse model of TC-1 lung adenocarcinoma

TC1 cells are primary C57BL/6 mouse lung fibroblasts co-transformed with an activated c-H-ras oncogene and the HPV-16 E6 and E7 oncoproteins[180]. Culture conditions and TC1 inoculation protocols have been described elsewhere[60]. $2.5 \times 10^5$ TC1 tumor cells were subcutaneously injected into the shaved left flank of young (8–12 wk old) mice using a 1-cc syringe and a 27-gauge needle. Tumor growth was monitored daily with a caliper. Because TC1 cells are derived from female mice, only female animals were used for the described experiments.

TC1 tumors were allowed to grow for 1 week. We waited 24 hours after visible growth to inject intraperitoneally with the first dose of #52642. Compound #52642 was prepared in DMSO and Tween80, the mice weighed, volume of compound calculated and immediately injected intraperitoneally (0.1mL injected per 10g of body weight). Subsequently, #52642 was administered twice daily, once in the morning and once in the evening every day. Tumor volume was measured using the calculation: Volume ($\text{mm}^3$) = (width)$^2$ x length/2. Mice were euthanized in accordance with animal license protocols either when tumor volume reached 1 cm$^3$ or weight loss exceeded 20% of body weight.
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