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Serglycin determines secretory granule repertoire and regulates NK cell and CTL cytotoxicity.

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Abbreviations: Con A; concanavalin A, CTL, cytotoxic T lymphocyte; GAG, glycosaminoglycan; Gzm A, granzyme A; Gzm B, granzyme B; MFI, median fluorescence intensity; natural killer, NK; SG, serglycin; TEM, transmission electron microscopy; 2mercaptoethanol, 2ME.

Key words: cytotoxic T lymphocyte, natural killer cell, serglycin, cytotoxic granules, perforin, granzymes

Abstract

The anionic proteoglycan serglycin is a major constituent of secretory granules in CTL/NK cells, and is proposed to promote the safe storage of the mostly cationic granule toxins, granzymes and perforin. Despite the extensive defects of mast cell function reported in serglycin gene-disrupted mice, no comprehensive study of physiologically relevant CTL/NK cell populations has ever been reported. We show that the cytotoxicity of serglycin-deficient CTL and NK cells is severely compromised, but can be partly compensated in both cell types when they become activated. Reduced intracellular granzyme B levels were noted, particularly in CD27+CD11b+ mature NK cells while serglycin-/- TCR-transgenic (OTI) CD8 T cells also had reduced perforin stores. Culture supernatants from serglycin-/- OTI T cells and IL-2-activated NK contained increased granzyme B, linking reduced storage with heightened export. By contrast, granzyme A was not significantly reduced in cells lacking serglycin, pointing to differentially regulated trafficking and/or storage

for the two granzymes. A quantitative analysis of different granule classes by transmission EM showed a selective loss of dense-core granules in serglycin-/- CD8+ CTLs, but other granule types were maintained quantitatively. Our findings show that serglycin plays a critical role in the maturation of dense-core cytotoxic granules in cytotoxic lymphocytes and the trafficking and storage of perforin and granzyme B, whereas granzyme A is unaffected. The skewed retention of cytotoxic effector molecules markedly reduces CTL/NK cell cytotoxicity, but this is partly compensated by activating the cells by physiological means.

Introduction

Central to the capacity of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells to kill virus-infected or transformed cells is their ability to store toxins including the pore-forming protein perforin and a family of granzyme serine proteases, and to release quanta of both toxins upon encountering successive target cells [1, 2]. Upon accessing the immunological synapse, membrane pore formation by perforin facilitates granzyme entry into the target cell cytosol, bringing about target cell death through apoptotic and non-apoptotic mechanisms [3]. By storing pre-formed toxins in specialised 'secretory granules', NK cells provide a constitutive 'first line of defence' against pathogens. Over the next 4-7 days, activated CD8+ CTLs develop an open, basophilic cytoplasm that contains secretory granules similar to those of NK cells, an important component of the adaptive immunity [4]. Intracellular toxin stores are critical for enabling CTLs to kill many infected target cells in succession, a process known as 'serial killing' [5, 6].

The early ultrastructural studies of CTL and NK cells identified subsets of granules with distinct morphologies. Cell fractionation later demonstrated that cytotoxic activity was localised to granules containing an electron-dense core and a variable peripheral 'rim' of vesicular/amorphous electron-lucent material [7-9]. In addition to perforin and granzymes A and B (GzmA, GzmB), the granules were rich in a chondroitin sulphate proteoglycan now known to be serglycin [1, 2, 10-12], as in mast cell granules [10, 13]. Mature mouse serglycin has 152 amino acids, including a region of 21 alternating serine/glycine residues [14, 15]. Each serine residue is a potential glycosaminoglycan (GAG) attachment site, resulting in the dense GAG clustering not seen in other proteoglycans. The presence of the GAG enhances

protease-resistance, while cell-type-specific variations in GAG content (type, number, length and extent of sulphation) can also influence serglycin's binding partners [14, 16]. Serglycin was more recently identified in many hematopoietic and some non-hematopoietic cells including endothelial and embryonic stem cells [14, 17].

In lymphocytes and mast cells, the GAG is primarily chondroitin-4-sulphate (14), whose negative charge density enables it to bind to highly basic (cationic) proteases in mast cells, confining them to the secretory granules until exocytosis [11]. It is also postulated that the active proteases and serglycin are secreted as a macromolecular complex that then dissociates to deliver active protease at the desired extracellular site [18]. Due to the strong biophysical similarities of granzymes and mast cell proteases, the same hypothesis was extrapolated to granzyme storage, release and function in killer lymphocytes [10, 11].

Consistent with a role in regulating CTL/NK killing, serglycin is secreted into the immune synapse with perforin and the granzymes following conjugate formation [11]. However, whether this mechanism contributes significantly to cytotoxicity has never been explored, either biochemically or in serglycin-null mice. Although perforin is known to perforate target cell membranes [3, 19, 20] and the molecular mechanism of GzmB-induced apoptosis is well understood [21-23], whether serglycin influences the function of either molecule or their synergy is largely unexplored. The generation of serglycin-deficient mice [17, 24] now makes it possible to address these issues. Previous studies have largely centred on mast cells, and the only study of lymphocytes examined mitogen (concanavalin A, Con A)-activated polyclonal T cell populations [24]. These cell populations were not activated in a physiologically-relevant manner, however the study suggested that the granules lacked the dense core associated with cytotoxic effector molecule storage. A loss of GzmB was observed, but both GzmA and perforin expression were considered normal and cytotoxicity was not reduced.

In the current study, we found that physiologically relevant cytotoxic populations of both NK cells and CD8+ CTLs have quite profound defects in target cell killing if they lacked serglycin, but that the defect is partly compensated by cell activation. We traced this deficiency to a failure to retain and store perforin and GzmB, with GzmB

constitutively exported from the cell. Careful quantitation of granules present in the CTLs showed that the reduction of dense-core vesicles was not compensated by an increase in other granule types, suggesting that serglycin plays an important role in granule biogenesis.

Results

CTL/NK cell cytotoxicity is severely compromised in serglycin-deficient mice.

The cytotoxic activity of serglycin-deficient CTL and NK cells was firstly assessed in standard (4h) ^{51}Cr release assays. We found a consistent and marked reduction in the cytotoxicity of both NK and CD8 T cells in the absence of serglycin. Purified naïve splenic serglycin-/- NK showed markedly reduced killing of YAC-1 mouse lymphoma targets, as 3 to 4 times as many serglycin-/- as wild-type B6 NK cells were typically required to achieve a given level of cell lysis. The reduction in cell death became less marked when the NK cells with activated with IL-2, but a consistent deficit in cell death remained (Figure 1A). For T cells, we noted robust peptide-restricted cytotoxicity for OTI T cells incubated with peptide-pulsed EL4 targets, however serglycin-/- OTI T cells had minimal cytotoxicity; indeed, the deficiency was as severe as that of OTI T cells lacking both GzmA and GzmB (Figure 2A). As with NK cells, the deficit in cytotoxicity of the CD8+ CTL was partially compensated by increasing the activation time of the killer cells *in vitro* from 4 to 7 days. Importantly, the activation status of NK and CD8 T cells was identical between wild-type and serglycin-deficient mice, as determined using a panel of antibodies to cell surface markers (see Materials and methods).

Serglycin-deficient killer cells contain reduced GzmB.

Given the markedly reduced cytotoxicity of serglycin-null CTL/NK cells, we examined the expression of granzymes and perforin by intracellular antibody staining and FACS. A clear bimodal pattern of GzmB expression was evident in B6 NK1.1+ CD3- NK cells (Figure 3A indicates FACS gating strategy). Approximately 60% of NK cells expressed high levels of GzmB (GzmB^{hi}) (Figure 3B); the remainder had considerably lower levels, but were still distinguished from syngeneic *GzmB* gene-disrupted NK cells (Figure 3B). Remarkably, no GzmB^{hi} NK cells could be identified

in serglycin-/- splenocytes, whereas the population with low expression was unaltered. Interestingly, this deficit in granzyme levels was restricted to GzmB, as the serglycin-/- NK cells showed wild type GzmA levels (Figure 3C). This selectivity indicated that serglycin differentially regulates the expression or retention of Gzms A and B in NK cells.

To further address the apparent lack of a $GzmB^{hi}$ population, the NK cells were phenotyped for CD27 and CD11b (Figure 4A), markers that distinguish immature (CD27+CD11b-), mature cytotoxic (CD27+CD11b+) and terminally differentiated (CD27-CD11b+) NK subsets [25]. All three populations were equally represented in B6 and serglycin-/- NK cells (Quadrants 1, 2 and 3, respectively in Figure 4A), indicating that serglycin plays no role in NK cell proliferation or differentiation into specific functional NK subsets. However, although GzmB expression in serglycin-null splenic NK cells was mildly reduced in the immature CD27+CD11b- NK cells (Quadrant 1), there was a marked GzmB reduction in the CD27+CD11b+ mature active NK cells (Quadrant 2, $p<0.0001$) and a lesser, though significant extent of GzmB reduction in the CD27-CD11b+ (Quadrant 3, $p<0.0001$) terminally differentiated cells (Figure 4B). This reduction was reflected in the reduced GzmB MFI in both quadrant 2 and 3 in serglycin- deficient NK (Figure 4C). As the CD27+CD11b+ subset has the greatest cytotoxic activity [25], this change was likely to account for the reduced cytotoxicity of serglycin-deficient NK cells. Consistent with previous results, GzmA expression was unchanged in each of the NK cell sub-populations (Figure 4D). Our FACS-based and functional data were supported by Western blot findings: GzmB was readily detected in B6 naïve NK cells purified by negative selection and FACS sorting, but was greatly reduced in serglycin-/- NK cells. In contrast, perforin expression levels were equivalent irrespective of serglycin status (Figure 1B, upper panel). In IL-2-activated NK cells, improved cytotoxic function of serglycin–deficient cells was reflected in near-equivalent levels of expression of both GzmB and perforin as in WT controls (Figure 1B, lower panel).

The expression of perforin and granzymes was also investigated in OTI CD8+ CTLs. Consistent with the near-absent target cell death inflicted by serglycin-/- T cells activated *in vitro* for four days (Figure 2A), minimal levels of both perforin and GzmB were detectable by Western blot (Figure 2B, left) and FACS (Figure 2C, left).

However, the reduction in expression of GzmB, and to some extent perforin in serglycin-/- T cells was less marked after 7 days of activation (Figure 2B, right, and Figure 2C, right), consistent with the increased cytotoxicity of serglycin-/- cells observed with longer activation time. We also performed intra-cellular staining and FACS analysis for both cytotoxic molecules (Figure 5). Almost 70% of OTI T cells expressed measurable perforin, compared with 20% of the day 7 serglycin -/- OT1 T cells (Figure 5B). Consistent with the Western blot findings, GzmB expression levels were also consistently reduced in the serglycin-/- OTI T, although to a lesser extent (Figure 5C). Overall, both the FACS and Western blot findings for GzmB/perforin expression were consistent with the observed reduced levels of cytotoxicity. In contrast to the activated T cells, a more minor reduction in perforin expression was observed in IL-2-activated NK cells lacking serglycin, and there was also a less obvious impact of serglycin deficiency on GzmB levels.

Consistent with these findings, the reduced GzmB expression also resulted in reduced GzmB substrate (Boc-AAD-SBzl) hydrolysis (Figure 6). In the day 4-activated serglycin-/-OTI T cells, GzmB activity was barely detectable above the background substrate hydrolysis in GzmAB-/- OTI T cell lysate (Figure 6A). After 7 days of activation, GzmB proteolytic activity was still far below that in the wild-type OTI cells ($p < 0.0001$). The small reduction in GzmB protein noted in the IL-2 activated serglycin-/- NK cells produced only a modest reduction in GzmB activity in these cells ($p = 0.0022$) (Figure 6B).

Reduced intracellular GzmB results from increased constitutive secretion.

We reasoned that if serglycin is necessary for the accurate trafficking and storage of GzmB in cytotoxic granules, then the reduced intracellular stores (and cytotoxicity) might reflect increased constitutive secretion. To address this possibility, the amount of GzmB secreted over a 24h period into the culture supernatant of activated OTI T cells and NK cells was measured using a GzmB–specific ELISA assay. Approximately 2000 pg/ml of GzmB had accumulated in the supernatants of both 5- or 7-day-activated wild-type OTI T cells. In the serglycin-/- T cells, the amount of secreted GzmB was increased by ~50% by day 5 ($p=0.0250$), and was more than doubled by day 7 ($p<0.0001$) (Figure 7A). Similarly increased secretion was found with activated NK cells, as more than twice the amount of GzmB (3586 pg/ml) was

secreted by serglycin-/- NK cells as by wild-type B6 NK (1513 pg/ml) (p=0.0222) (Figure 7B). The specificity of the assay was demonstrated in that GzmAB-/- OTI T cells activated in the same way had levels of activity no different from medium-alone controls (data not shown).

Serglycin- deficient OTI T cells lack mature cytotoxic granules.

It had been previously reported that granules of various morphology are present in immune cells that lack serglycin, however dense core granules, a hallmark of secretory lysosomes are absent. This was observed in mast cells [17] and mitogen-activated lymphocytes [24], and led to the proposal that serglycin is necessary for the maturation (but not necessarily the biogenesis) of the dense core granules. To address this issue further, wild-type OTI and serglycin-/- OTI T cells were examined by TEM to classify and enumerate granules with various morphologies and electron densities. When comparing the wild-type and the serglycin-/- OTI T cells, no difference was discerned in the nuclei, Golgi bodies, mitochondria, ribosomes or rough endoplasmic reticulum. Cytoplasmic granules were morphologically heterogeneous and could be classified into 4 groups based on ultrastructural features: (1) amorphous – granules were uniformly electron dense, (2) vesicular – granules contained many small vesicles, (3) dense core – granules resembled 'classic' mature cytotoxic granules and contained a large dense core that was often surrounded by many small vesicles, and (4) endo/lysosomal –granules within this group contained whorls of membranes and small vesicles typical of late endosomes and lysosomes; examples of each are shown (Figure 8A and B). Using cellular samples that were coded and read in a 'blinded' fashion, more than 30 CTLs containing a total of at least 80 granules were examined for each mouse genotype (Table 1). Whereas the amorphous, vesicular and endo/lysosomal granules were similar in number in the wild-type and serglycin-/-OTI T cells, the dense core granules were virtually absent from the serglycin-deficient cells (Table 1, Figure 8C). Of 81 granules examined, only one dense-core granule cold be identified in serglycin-/- OTI T cells (1.2%) whereas the corresponding number in wild-type OTI was 24/106 (22.7%). Although morphologically similar, this single granule was also smaller (Figure 8B), than all 24 'type 3' granules in wild-type OTI T cells, being 338 nm in diameter, whereas the 24 assessable granules in wild-type OTI T had a mean diameter of 590 nm (+/- 45.72;).

Discussion

This is the first comprehensive study on the impact of serglycin on the cytotoxic activity of physiologically relevant populations of innate (NK) and adaptive (CD8+ CTL) lymphocyte populations. We demonstrate that serglycin deficiency results in significant functional and morphological defects in both types of cytotoxic lymphocyte. The congenital absence of serglycin results in a virtually complete lack of dense core secretory granules, an extensive (though not uniform) deficiency of cytotoxic effector molecules and a major compromise in cytotoxicity of NK and CTL. The ability of naïve NK and activated OTI T cells from serglycin –null mice to induce target cell death was reduced by up to 75%, based on the fact that typically, 3-4 times as many serglycin-null killer cells were required to achieve a given level of target cell lysis. Further, the reduced cytotoxicity was accompanied by a reduction of GzmB content, which was particularly marked in the mature active sub-population of CD27+ CD11b+ NK cells, leaving only NK cells with low GzmB expression. Interestingly, there was no reduction in GzmA levels in the same cells, indicating that the trafficking and storage requirements for the two granzymes differ significantly, consistent with previous findings (discussed below).

In activated T cells, a major reduction in perforin content was also observed. Combined with the reduction in GzmB this contributed to the defect in cytotoxic activity, which was particularly marked in cells activated for a short period (4 days) in vitro: target cell death in response to these T cells was compromised almost as much as when Gzm–null effector cells were used. Notably, the defective cytotoxicity of the serglycin-/- cells was partially rescued with longer in vitro stimulation (6-7 days), which coincided with the acquisition of greater granzyme stores. The profound impact of serglycin deficiency on the function of these cells was in contrast to an earlier study that used in vitro mitogen-activated T cells, where no effects on the induction of target cell death were noted [24]. However it is important to emphasize that the present study utilized classic MHC class I restricted CTLs responding to their cognate peptide, and are thus likely to be of greater relevance physiologically.

Our studies indicate that serglycin plays a crucial role in cytotoxic granule biogenesis. Despite several ultra-structural investigations reported in the 1980s and early 1990s

[7-9, 26], we still lack a detailed understanding of the biogenesis of secretory granules in mast cells and lymphocytes [27, 28]. The fact that granule morphology in these cells is pleiomorphic has complicated these studies. A comprehensive examination of the granules in human NK cells by Burkhardt et al [9] confirmed the presence of three granule types, as defined by Neighbour [8]: Type I (dense core), Type II (multivesicular) and Type III (intermediate, having a small core and vesicular cortex). However it has still not been verified whether these forms represent functionally discrete sub-populations or maturational stages of a single secretory granule type [29]. By consensus, Type I electron dense granule are thought to represent prototypic cytotoxic secretory granules containing perforin and granzymes, in association with proteoglycan (serglycin). In support of this view, we found that the absence of serglycin resulted in the specific absence of dense core granules from CD8+ CTLs, without a compensatory increase in other granule types. Previous studies of mast cells and mitogen-activated T cells from serglycin-deficient mice found no difference in the size and number of granules but noted the presence cells of 'amorphous' granules in serglycin-/- cells, which were considered likely to be secretory granules lacking the electron dense core [17, 24]. In our quantitative study, amorphous granules were noted in the serglycin-null T cells but were not over-represented compared with serglycin-sufficient cells, where they made up approximately 40% of the total number of all granules. This suggests that these granules were not secretory granules that had failed to acquire the dense core in the absence of serglycin. Overall, our observations raise the possibility that serglycin is important for the biogenesis, rather than just the maturation of secretory granules.

It is evident from studies primarily in mast cells that although the serglycin core is common to a wide range of cells containing secretory granules, the nature of glycosaminoglycan side chains varied, both with regard to carbohydrate backbone (heparin or chondroitin sulphate) and to the degree of sulphation [14]. This may have important consequences for the type of molecules that serglycin could interact with and hence a profound effect on the ultimate function of the cell. For example, in connective tissue type mast cells the glycosaminoglycan side chains of serglycin are heparin whilst mucosal mast cells have chondroitin sulphate, and it is possible that the different protease repertoire of connective tissue type versus mucosal type mast cells could, at least partly, be a consequence of the differential glycosaminoglycan type

expressed by the two mast cell subclasses [27]. However it has been shown that not all of the proteases contained within the granules are dependent on serglycin for their storage [17, 27]. One possibility would be that these non-serglycin-dependent proteases are stored in complex with proteoglycans other than serglycin. However, the presence of non-serglycin proteoglycans in mast cell granules has not been established so it remains to determine how the mast cell serglycin- independent proteases of mast cells are safely stored and delivered to their site of action [30]. Our findings in lymphocytes follow a similar pattern: in mitogen activated T cells only GzmB, but not GzmA or perforin was decreased by the lack of serglycin [24]. Examining the net charge of these molecules revealed that predicted charge interactions did not account for binding. Although GzmA and GzmB differ in their dependency for serglycin but both have an equivalent high charge (39). In order to explain these differences, a close structural examination of both proteases (23) revealed the presence of a highly charged "patch" on the GzmB surface, which was absent from GzmA. By contrast it should be noted that mMCP4 and mMCP6 have an overall charge of +16.7 and -2.2 respectively but both are dependent on serglycin (39). Similarly, we revealed that only GzmB is decreased in serglycin-null naïve NK cells, but in contrast to the study by Grujic et al [24] the reduction in GzmB did detrimentally affect target cell death. In contrast to NK cells, perforin expression was also significantly compromised in serglycin-/- antigen-specific T cells, again consistent with the marked loss in cytotoxicity of these cells.

We show that in T cells and activated NK cells, the absence of serglycin results in increased constitutive secretion of GzmB. This may imply either that GzmB is directly mis-sorted from the trans Golgi network to a secretory pathway, or that GzmB is in fact correctly sorted via the mannose 6 phosphate (M6P) receptor pathway to granules, but that serglycin-deficient granules are not able efficiently store the protease. Consistent with such a mechanism, inefficient storage and increased secretion of the granzymes is also found in patients with I cell disease, due to a mutation of the phosphotransferase responsible for the mannose modification necessary for binding to the M6P receptor [31]. In contrast to the findings reported here, in mast cells lacking serglycin, direct sorting of proteases to degradative lysosomes rather than increased secretion was thought to account for reduced protease

stores [32]. Thus, serglycin is thought to have a principal role in retention of proteases within the granules rather than mediating their sorting [32].

An earlier report suggested that a macromolecular complex consisting of perforin, GzmB and serglycin, which resembled a virus particle, was exocytosed and entry into the target cell was facilitated via an endosomal mechanism [33, 34]. Thus, in this model GzmB delivery was entirely dependent on the uptake of a large serglycin/granzymes complex. This suggestion relies on very tight binding of granzymes to serglycin, as one would expect rapid dissociation to take place with the release of the complexes upon degranulation. More recent kinetic and biochemical data has suggested a simpler mechanism, whereby uncomplexed GzmB enters the cell via perforin pores, consistent with some of the earliest hypotheses on cytotoxic lymphocyte-mediated cell death [3, 23, 35]. Our findings that day 7 activated OTI T cells and IL-2 activated NK cells in particular are capable of quite significant levels of target cell death in the absence of serglycin, support the notion that alternate storage and exocytic mechanisms exist. Expansion of the TEM studies with immuno-EM of activated T cells alone and conjugated to target cells may give us insight into these possibilities.

Materials and methods

Mice. C57BL/6 (B6) mice were purchased from the Walter and Eliza Hall Institute, Melbourne. The previously described serglycin B6 knockout (serglycin-/-) mice [17] were crossed with B6.OTI (ovalbumin-specific, H-2^b-restricted T-cell receptor transgenic) (OTI) mice [36] to create serglycin-/- OTI. The perforin or granzyme-deficient strains B6.GzmB-/-, B6.GzmAB -/-, GzmAB-/-.OTI and Pfp-/-.OTI have been described previously [37]. All experiments were approved by the Animal Ethics and Experimentation Committee (E486) at the Peter MacCallum Cancer Centre and carried out there.

Primary cells. CTL cultures were generated from the various OTI mice by activation with the OVA_{257}- specific peptide SIINFEKL [38]. Spleen single cell suspensions were cultured in RPMI supplemented with 2mM L-glutamine, 50U/ml penicillin and

50ug/ml streptomycin, 50uM 2mercaptoethanol (2ME), 100uM non-essential amino acids, 1mM sodium pyruvate, 10% (v/v) FCS (RPMI plus), 100U/ml (0.67pM) human rIL-2 (Peprotech, NJ, USA) and 1□M SIINFEKL peptide. At day 4, activated CTL were purified by Ficoll separation and re-cultured in fresh medium (at 5 x 10^5 cell/ml) for a further 3 days, without peptide. Splenic NK cells were isolated by negative selection using AutoMacs kits (MACS, Miltenyi Biotec, according to the manufacturer's instructions) [23], then cultured in the same medium (7x 10^5 cells/ml) containing 1000U/ml human rIL-2 for 3-6 days, with the medium replenished every 3 days. Naïve NK cells were purified by negative selection from spleens of B6 and serglycin-/- mice using a mouse NK cell enrichment kit (EasySep) (according to the manufacturer's instructions), and positively sorted (BD. AriaII, CA, USA) for NK1.1 positive/CD3 negative cells [39].

Cell lines. Mouse EL4 (B6 thymoma) and YAC-1 (A/Sn lymphoma) were maintained in DME medium, supplemented with glutamine (2mM), 50U/ml penicillin and 50ug/ml streptomycin, 50uM 2ME and 10% FCS.

Cytotoxicity assays. Death of target cells (EL4 and YAC-1) induced by CTL or NK was quantitated using ^{51}Cr release assays as previously described [40]. EL-4 were incubated simultaneously with SIINFEKL peptide and ^{51}Cr at 37^{o}C, and washed 3 times prior to incubation with CTL.

Flow cytometry. The purity and activation state of CTL/NK populations were assessed by surface staining with anti-CD8-e450, CD44 FITC, CD62L-PE, CD69-PECy7 for CTL as previously shown [37], and NK1.1-PE and PE-Cy7, CD69-PECy7, CD27-APC, CD11b-FITC, and/or NKG2D-PE (eBioscience) for NK [25]. (Data not shown). Cells were fixed and permeabilised to detect intracellular GzmB and perforin with AL647 antibody (GB11, ebioscience) or P1-8, respectively, followed by anti-rat PE secondary antibody [41]. Isotype controls comprised rat IgG2a-PE for PI-8 and mouse IgGI-APC. Activated NK and OTI T cells were gated based on morphology and single cell status prior to analysis. Naïve NK were identified in splenocytes by gating on CD3- negative (CD3-e450) and NK1.1-positive (NK1.1-AL488) cells (Figure 3A). To identify NK subsets, cells were further gated using CD27-PE and

CD11b-eFluor780 (Figure 3A) [42]. Cells were fixed and permeabilised prior to staining with anti-GzmB (as above) or anti-GzmA-APC (3G8.5, ebioscience).

Proteolysis assays. Cell lysates were prepared from CTL and NK cultures, and normalized for protein content. Hydrolysis of synthetic peptide thiobenzylester (Boc-Ala-Ala-Asp (AAD)-SBzl substrate (SM Biochemicals, CA, USA) was used to determine GzmB enzyme activity, as previously described [43, 44].

Western blot. Proteins in whole cell (from CTL and NK cultures, or from naïve NK) lysates (5-10ug) were separated on Nu PAGE 4-12% Bis Tris gels (Life technologies, USA), transferred and probed for GzmB (rat anti-mouse GzmB, clone 16G6, eBioscience, CA, USA), and perforin (P1-8) as previously described [45]. Blots were re-probed with an anti-mouse β-actin antibody (Sigma-Aldrich, USA) to confirm equal protein loading. Image J analysis was performed on all the Western blots to quantitate the comparative levels of GzmB and perforin for each of the cell types examined. This quantitation is indicated on the representative blots and the pooled quantitation is summarized in Table 2.

ELISA. Secretion of GzmB in tissue culture supernatant was quantitated using a commercial GzmB ELISA kit (eBioscience, CA, USA). Culture medium from CTL or NK cells seeded at $5x10^5$cells/ml for 24hours was harvested, centrifuged to remove cellular debris and assayed according to the manufacturer's instructions.

Transmission electron microscopy (TEM). Cells were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1M Cacodylate buffer pH7.4 followed by post-fixation in 1% osmium tetroxide, 1.5% potassium ferrocyanide in distilled water. Cells were dehydrated through a graded series of alcohol, passed through two changes of acetone and embedded in Spurrs low viscosity embedding medium [46]. Ultrathin sections were cut with a Leica EM UC7 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) and contrasted with lead citrate and aqueous uranyl acetate. Sections were examined in a JEOL 1011 Transmission Electron Microscope (JEOL, Tokyo Japan) and images captured with a MegaView III CCD cooled camera, using iTEM AnalySIS software (Olympus, Münster, Germany).

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Author Contribution Statement

Planned experiments: VRS, AJB, SE, IV, RWJ, DMA, JAT
Performed experiments: VRS, AJB, SE, JD, KT, MRJ, DMA
Analyzed Data: VRS, AJB, SE, JD, MRJ, IV, DMA, JAT
Contributed essential reagents (gene knock-out mice): GP
Wrote paper: VRS, IV, DMA, GP, JAT

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Granule type	B6 (n=106)	SG-/- (n=81)	Pa
Amorphous	45	43	0.18 (ns)
Vesicular	13	9	0.1 (ns)

Dense core	24	1	<0.0001
"other"	24	28	0.1 (ns)

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Table 1: Classification of granules in day 7 OTI T cells
Day 7- activated OTI T cells were examined by transmission electron microscopy. The granules in a total of thirty cells from each strain were classified and quantitated. [a]Fisher's exact test (2x2 contingency table).

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Cell type and effector protein	Mean +/- SEM: relative band intensity	Significance	# Lysates examined	#Western blots	#Mice
Naïve NK GzmB	0.4813+/0.1832	* p=0.0473	n=3	3	14
Naïve NK Pfp	1.461+/-0.3109	ns p=0.2120	n=3	3	14
Activated NK GzmB	0.7308+/-0.08924	* p=0.0130	n=6	5	5
Activated NK Pfp	0.8660+/-0.06051	ns p=0.0512	n=6	5	5
Day 4 T GzmB	0.1877+/-0.06354	****p<0.0001	n=6	3	6
Day 4 T Pfp	0.2503+/-0.09455	***p=0.0002	n=4	3	6
Day 7 T GzmB	0.5617+/-0.06352	****p<0.0001	n=10	5	10

Table 2: Quantitation of GzmB and perforin relative band intensity in serglycin-deficient NK and OTI T cells. Image J analysis was used to compare band intensity relative to signals detected in B6. Significance of differences was determined by unpaired t-test.

Day 7 T Pfp	0.4647+/-0.09139	****p<0.0001	n=10	5	10

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640 **Figure Legends**
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642 Figure 1: **Serglycin deficiency results in diminished NK activity and altered**
643 **effector enzyme expression.** (A) Cytotoxic activity of purified (negative selection
644 and FACS sorted) naïve splenic NK and IL-2 activated NK (day 6 and day 7)
645 against ^{51}Cr –labeled YAC-1 cells, in a 4h assay. Two independent experiments were
646 done using pools of naïve NK from 3 mice each (error bars indicate SEM, n=2) or
647 individually activated cells from 7 mice were each tested on day 6 and day 7 (error
648 bars indicate SEM, n=7). In total 8 independent experiments were performed. (B)
649 Perforin and GzmB expression was detected in whole cell lysates (5-10ug/lane),
650 separated on 4-12% gels. The purified naïve NK blot was probed sequentially for
651 perforin, GzmB and then actin, whereas individual blots were probed for perforin and
652 GzmB followed by actin for the day 7 IL-2 activated NK. NB. An empty lane was
653 excised from the day 7 IL-2 NK blot probed with GzmB and actin. Data is
654 representative of three experiments performed using 3 individual lysates prepared
655 from pools of purified naïve NK cells from a total of 14 mice. Activated NK lysates
656 were individually prepared from 5 mice and were each analysed in 5 independent
657 experiments.
658
659 Figure 2: **Serglycin deficiency results in diminished T cell cytotoxic activity and**
660 **altered effector enzyme expression.** (A) Cytotoxic activity of Day 4 and day 7
661 activated OTI T cells tested on SIINFEKL-pulsed ^{51}Cr –labeled EL-4 cells; wild-type
662 OTI (OTI) (n=3 mice), GzmAB.OTI (n=3 mice), serglycin-/- OTI (SG.OTI) (n=6
663 mice). Pooled data from 3 independent experiments. OTI (filled circles), SG.OTI
664 (filled squares), GzmAB.OTI (filled triangles). Error bars indicate SEM, n=3.
665 (B) Perforin and GzmB expression was detected in whole cell lysates (5-10ug/lane),
666 separated on 4-12% gels. Individual blots were probed for perforin and GzmB
667 followed by actin for the day 4 and day 7 activated OTI T cells. Lysates from OTI T
668 cell cultures from 2 individual serglycin-/- OTI (SG-1, SG-2) mice were compared.

Representative data of 3 experiments using lysates from day 4 T cells from 3 B6.OTI and 6 SG.OTI mice, and of 5 experiments using lysates from day 7 T cells from 7 B6.OTI and 10 SG.OTI mice. (C) Representative intracellular staining for GzmB in day 4 and day 7 activated B6, serglycin-/- (SG-/-) and GzmAB-/- OTI T cells. MFI: day 4 B6: 178 (n=7), SG-/- 49 (n=7); day 7 B6: 564 (n=7), SG-/- 327 (n=11).

Figure 3. **FACS gating strategy and intracellular FACS analysis of GzmA and GzmB in naïve NK.**

NK1.1 positive, CD3 negative naïve splenic NK cells (A) were examined by intracellular staining for GzmB (B, left) and GzmA (C, right) expression. A total of 14 mice were examined for GzmB and 4 mice for GzmA expression. Differences in median fluorescence intensity (MFI) for GzmB from 3 independent experiments were compared using unpaired t test; B6: 502, SG-/-: 235, p=0.0002, n=14).

Figure 4. **GzmB expression is reduced in mature cytotoxic NK cells**

(A) Representative CD27, CD11b staining of NK1.1 positive, CD3 negative, single cells from B6 (from n=5 mice) and serglycin-/- (SG-/-) (from n=5 mice). (B) GzmB expression on NK populations (gated by CD27 and CD11□ status) from B6, SG-/- and GzmB-/- spleen. Representative of n=5 mice. Differences in %GzmB+ cells from B6 and SG-/- in each quadrant were compared using unpaired t test; Q1 p=0.0003, Q2 p<0.0001, Q3 p<0.0001, n=5. (C) Representative GzmB expression in quadrant 2 (CD27+, CD11b+) (MFI: B6 320, SG-/-145, GzmB-/- 82) and quadrant 3 (CD27-, CD11b+)(MFI: B6 520, SG-/- 249, GzmB-/- 88) NK populations. Differences in MFI from B6 and SG-/- were compared using unpaired t test; Q2 p<0.0001, Q3 p<0.0001, n=5. Two additional independent experiments were performed and the statistical significance for the differences in % GzmB+ cells and the MFI were equivalent. (D). GzmA expression in CD27 low and CD27 high NK subpopulations from B6 (n=4 mice) and serglycin-/- (n=4 mice).

Figure 5. **Intracellular FACS analysis of GzmB and perforin in OTI T cells.**

(A) Day 7 activated OTI T cells from pfp.OTI (pfp-/-), OTI (B6) and serglycin.OTI (SG-/-) were assessed for perforin and GzmB expression. Isotype- alone staining is shown in the left hand panels. Histograms of the perforin (B) and GzmB (C) staining

are compared for pfp-/- (B only), SG-/- and B6. The specific isotype- alone controls on B6 and SG-/- (dotted lines) are presented in (C). Perforin MFI; B6: 170, SG-/-: 64, pfp-/-: 29 and GzmB MFI; B6: 2251, SG-/-: 1244, and for isotype controls B6: 85, SG-/-: 73. Representative data from 2 experiments.

Figure 6. **GzmB activity is reduced in OTI T cells and activated NK cells.** Granzyme enzyme activity in CTL (A) and NK (B) whole cell lysates was quantitated using specific peptide substrates for GzmB (Boc-AAD-S-Bzl) and the maximum rate (mOD/min) of peptide substrate hydrolysis was determined. Statistical significance was demonstrated in GzmB activity in OTI T cells (A) at day 4 (3 individual experiments) and day 7 (6 individual experiments) (****p< 0.0001) and in NK (B) for GzmB activity (5 individual experiments) (*p=0.0022) using unpaired t test. Individual day 4 OTI T cell lysates were prepared from OTI (B6) (n=7 mice), serglycin-/-OTI (SG-/-) (n=6 mice) and GzmAB-/-.OTI GzmB-/-) (n= 3 mice) and day 7 OTI T cell lysates were from OTI (n=9 mice), serglycin-/- OTI (n=11 mice) and GzmAB-/-.OTI (n= 5 mice). Individual NK lysates were prepared from B6 (n=4 mice), serglycin -/- (SG-/-) (n=8 mice) and B6.GzmB-/- (GzmB-/-) (n= 3 mice). The data was pooled from each of the individual experiments.

Figure 7. **Constitutive secretion of GzmB is increased in serglycin-deficient OTI T and NK cells.** GzmB secreted into culture supernatant of day 5 and day 7 OTI T cells (A) and day 6 NK (B) was quantitated by ELISA. Statistical significance was determined using Mann Whitney: Day 5 T cells (*p=0.0250), day 7 T cells (****p<0.0001), and unpaired T test: NK (*p=0.0222). OTI T cell cultures were derived from OTI (B6) (n=9) and serglycin-/- OTI (SG-/-) (n=11 mice) and NK from B6 (n=2) and serglycin -/- (SG-/-) (n=3) mice. Each individual supernatant was tested once in triplicate in a total of 3 independent ELISA assays and the data pooled.

Figure 8. **Mature cytotoxic granules are absent from serglycin -/- OTI T cells.** Classification of granule types in (A) OTI and (B) serglycin-/- OTI T cells identified by transmission electron microscopy. (C) Representative transmission electron micrographs of a day 7 activated OTI T cell (top panel) and a serglycin-/- OTI T cell (bottom panel). The arrows in the micrographs on the left hand side indicate the

granules that are shown at a higher magnification on the right hand side. N: nucleus. Thirty cells of each type were examined and the granules classified and quantitated.