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Mouse granzyme K has pro-inflammatory potential

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Running title: Granzyme K is a pro-inflammatory protease

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Abstract:

Granzymes (gzms) are key components of T killer (Tc) cells believed to mediate pro-apoptotic activities. Recent evidence suggests that gzms also possess non-cytotoxic activities that contribute to host defense. Here we show that Tc cells from lymphocytic choriomeningitis virus (LCMV)-infected wild type and granzyme A/B deficient mice express similar levels of gzmK protein, with both mouse strains efficiently controlling infection. GzmK, in recombinant form or secreted by *ex vivo*-derived LCMV-immune gzmAxB$^{-/-}$ Tc cells, lacks pro-apoptotic activity. Instead, gzmK induces primary mouse macrophages to process and secrete interleukin-1β, independent of the ATP receptor P2X7. Together with the finding that IL-1Ra (Anakinra) treatment inhibits virus elimination but not generation of cytotoxic Tc cells in wild type mice, the data suggest that Tc cells control LCMV via non-cytotoxic processes that involves gzmK.

Keywords and Abbreviations:

Key words: T killer cell, virus control, orphan granzymes, cell death, inflammation

Abbreviations: gzm, granzyme; Hu, human; IL-1β, Interleukin-1 beta; IL-1Ra, IL-1 receptor antagonist (Anakinra); i.p., intraperitoneal; ko; knock out; LCMV, Lymphocytic Choriomeningitis virus; MEF, mouse embryonic fibroblast; Mo, mouse; NK, Natural Killer; PEMØ, peritoneal macrophage; perf, perforin; p.i., post infection; rec., recombinant; SET, nucleosome assembly protein; SLO, streptolysin O; SN, supernatant; Tc: T killer cell; wt, wild type.

Introduction:

The cytotoxic cell granule secretory pathway is considered indispensable for eliminating tumor and virally infected cells. The underlying process is mediated by the pore-forming protein, perforin (perf), which delivers a family of serine proteases, namely the granzymes (gzms), to cells, presumably targeted for destruction. The gzms have been characterized by DNA sequencing of counterparts in mouse, rat and man and by the availability of recombinant proteins for functional and structural analyses [1] [2]. Based on their transfer into target cells and secretion extracellularly, gzms were originally anticipated to act both intracellularly and mediate extracellular proteolytic events [3]. However the discovery that isolated gzms induced apoptosis in the presence of perf [4, 5] encouraged the notion that the proteases acted primarily as death effectors.

Human (Hu)gzmA was reported to induce pro-inflammatory cytokines in human monocytes and mouse macrophages more than 10 years ago [6] which may be due to processing of interleukin (IL) -1 [7]. The significance of this observation was not appreciated until recently when human- and mouse (Mo)gzmA were observed to lack cytotoxic activity *in vitro* [8] except under very restricted conditions [8, 9]. In the absence of the apparent cytotoxicity mediated by human- and (Mo)gzmA, the possibility that the protease might act *in vivo* to induce pro-inflammatory cytokines was re-evaluated. In this regard, isolated human- and (Mo)gzmA as well as human Natural killer (NK) and mouse T killer cells secreting the protease were shown to induce human monocytic cells or pre-sensitized mouse peritoneal macrophages PEMØ, respectively, to express and secrete the pro-inflammatory cytokines IL-1β, tumor necrosis factor (TNF)-α and/or IL-6 [8]. In addition, caspase-1 inhibition was found to reduce (Hu)gzmA-induced IL-1β and TNF-α secretion by stimulated human monocytes suggesting that gzmA may be another activator of the inflammasome platform systems [10]. The physiological importance of this phenomenon was then validated by showing that gzmA knockout (ko) mice (gzmA$^{-/-}$) resist the lethal effects of LPS [8]. Together with the recent finding that (Mo)gzmM augments TLR4-driven inflammation and endotoxicosis [11], these observations set a biological precedent indicating that (Hu/Mo)gzms may have additional functions besides acting as pro-apoptotic mediators.

The highly cationic gzmK from human and mouse has tryptase-like substrate preference similar to (Hu)gzmA but the fine specificity is undoubtedly unique [12]. Similar to (Hu)gzmA, the original report indicated that isolated Rat and (Hu)gzmK are cytotoxic *in vitro* [13]. A more recent study suggests that isolated (Hu)gzmK induces biochemical events similar to (Hu)gzmA in target cells. Here (Hu)gzmK processes the nucleosome assembly protein (SET) releasing the DNAse NM23H1, which translocates to the nucleus. The apurinic endonuclease 1 (Ape-1) is also cleaved by (Hu)gzmK. On the other hand, caspase-3 activation is undetectable [14-16]. (Hu)gzmK may also cleave the tumor suppressor, p53, sensitizing tumor cells for apoptosis induction [17] and process a vasolin-containing protein contributes to endoplasmic reticulum stress and caspase-independent cytotoxicity [18]. Compared to the proposed cytotoxic role for gzmK, the protease has been found by immunoassays [19] to be elevated in the plasma of patients with sepsis [20] and in the bronchoalveolar fluid of patients with asthma and viral pneumonias [21] suggesting that gzmK may have additional biological functions.

Perf$^{-/-}$ mice demonstrate the critical role of the granule-secretory pathway in the recovery from LCMV infection [22]. However, compared to perf, the contribution of gzmA, gzmB and orphan gzms in the host defense against this pathogen is controversial [23, 24]. After intraperitoneal (i.p.) challenge with LCMV, gzmB$^{-/-}$ and gzmAxB$^{-/-}$ mice clear the virus albeit somewhat less vigorously than wild type (wt) mice. A recent study showed that (Mo)gzmK is expressed as mRNA by *ex vivo* LCMV-immune Tc cells, independent of their expression of gzmA and/or gzmB [25]. GzmK has been suggested to contribute to the clearance of influenza virus in mice [26, 27] but overall the biological function(s) of this gzm family member remains incompletely characterized. The purpose of this report is to re-examine the cytotoxic activity of (Mo)gzmK and, due to its similar substrate specificity to (Hu/Mo)gzmA, determine whether the protease has pro-inflammatory effects.

Results:

LCMV infection in mice is readily controlled in the absence of gzmA and B

Previous studies demonstrated that although perf is essential for optimal control of LCMV infection [22], gzmA and B play a marginal role [23, 24]. To verify this supposition, we compared survival and viral titers in mice lacking gzmA and B (gzmAxB$^{-/-}$) to those without perf and the two gzms (perfxgzmAxB$^{-/-}$). After challenge with 1x10^5 PFU LCMV-WE, all perfxgzmAxB$^{-/-}$ (9/9) died but only 1/9 gzmAxB$^{-/-}$ and none of the wild type (wt) B6 mice succumbed to the virus during the 30 day observation period (Figure 1A). At day eight after inoculation, hepatic virus titers were similar increased in the WT as well as the gzmAxB$^{-/-}$ and triple ko mice with somewhat higher levels in the ko mouse strains (Figure 1B). However, while the level of virus gradually declined to background levels in gzmAxB$^{-/-}$ and B6 mice, no reduction of virus load was observed in the liver of perfxgzmAxB$^{-/-}$ mice during the entire observation period (Figure 1B). The data are consistent with previous studies [23] emphasizing that the control of LCMV infection, including viral elimination, is strictly dependent on perf but that neither gzmA nor gzmB are obligatory participants.

Granzyme K is expressed in LCMV-immune Tc cells from wild-type and gzmAxB-deficient mice

To test the assumption that other gzms beside gzmA and B might contribute to perf-mediated control of LCMV infection in gzmAxB$^{-/-}$ mice, virus-immune Tc cells (d 8 p.i.) were evaluated for the expression of perf and gzm-specific mRNAs and their respective intracellular proteins. As reported previously [25], virus-immune Tc cell from B6, gzmAxB$^{-/-}$ and perfxgzmAxB$^{-/-}$ mice expressed similar levels of the gzmK transcript as well as the mRNA for gzmA, gzmB and perf expected for the respective ko mice (Figure 1C). No transcripts for gzmC-G and gzmM were detectable (for gzmM see also Supp. Fig. 1C). To ensure that the various effector populations expressed gzmK protein, its presence in CD8$^+$ Tc cells of uninfected and LCMV infected mice (day 8 p.i.) was determined using a recently developed rabbit anti-rec(Mo)gzmK antibody. Although gzmK was undetectable in Tc cells from non-infected mice, Tc cells from all three infected mouse strains contained similar levels of the protease (Figure 1D). As expected, only Tc cells from LCMV immune B6 but

not gzmAxB$^{-/-}$ and perfxgzmAxB$^{-/-}$ mice expressed the gzmA and gzmB proteins (data not shown).

LCMV-immune Tc cells that express gzmK and lack gzmA and gzmB are non-cytotoxic

To determine whether LCMV-immune gzmAxB$^{-/-}$ Tc cells that expressed primarily gzmK induce apoptosis in MEF cells, we measured multiple parameters including PS externalization, PI uptake, mitochondrial depolarization ($\Delta\Psi_m$) and ROS generation. Despite the fact that LCMV-immune B6 and gzmAxB$^{-/-}$ mice both readily controlled LCMV infection, only ex vivo derived LCMV-immune B6 Tc cells induced cell death in target cells *in vitro* as defined by the abovementioned parameters (Figure 2A). LCMV-immune gzmAxB$^{-/-}$ Tc cells, on the other hand, were only marginally cytotoxic while LCMV-immune perfxgzmAxB$^{-/-}$ Tc cells were completely inactive. On the other hand, LCMV-immune gzmAxB$^{-/-}$ NK cells, which expressed perf and gzmM, but no gzmK, showed residual cytotoxic activiy which was however at least five times lower than those of gzmA$^{-/-}$ mice (Supp. Figure 1A).

Recombinant mouse gzmK and gzmA are not cytotoxic

To compare the cytotoxic potential of isolated rec(Mo)gzmK with rec(Mo)gzmA and B, EL4 cells were incubated with increasing concentrations of the proteases (150-1200nM) delivered by SLO and cell death was determined by PI uptake, annexin reactivity (PS exposure), $\Delta\psi$, and ROS generation. Although neither (Mo)gzmA nor (Mo)gzmK elicited a cytotoxic response at all concentration, (Mo)gzmB showed the predicted concentration-dependent induction of cell death (Figure 2B), including cleavage of caspase 3 (Figure 2C). To ensure that rec(Mo)gzmA and rec(Mo)gzmK manifested enzymatic activity and to verify their predicted differences in cleavage specificity[12], their amidolytic activities were compared against four different chromogenic substrates. Both gzms were proteolytically active with each exhibiting distinct preferences for the four related chromogenic substrates, especially Tos-Gly-pro-Arg-pNA (Table I). Finally, a cell survival assay was performed [28] to exclude the possibility that gzmK might induce cell death that is undetected by the parameters mentioned above. Re-growth of EL4 cells was unimpaired for targets incubated only with the rec(Mo)gzmK (1000 nM) or after the protease was delivered by SLO (Figure 2D).

Recombinant mouse granzyme K induces macrophages to release IL-1β

Although rec(Mo)gzmA and rec(Mo)gzmK have different substrate specificities, we speculated that the tryptase-like activity of (Mo)gzmK might mimic (Mo)gzmA in its capacity to induce pre-activated PEMØ to express and secrete mature IL-1β. In this regard, rec(Mo)gzmK was added to LPS-primed PEMØ in the presence or absence SLO. The delivery of gzmK by SLO induces concentration-dependent processing of pro-IL-1β, requiring doses above 30 nM (Figure 3A and Suppl. Figure 2). SLO alone was observed to also induce detectable albeit minor maturation/secretion of IL-1β (Figure 3A and Suppl. Figure 2). At higher concentrations of gzmK (600 and 1000 nM), the protease alone was sufficient to induce release of IL-1β to levels observed for cells treated with the granzyme and SLO. Finally, the inactive pro-form of rec(Mo)gzmK did not alter maturation/secretion of IL-1β (Supp. Figure 2) eliminating the possibility that the granzyme was inducing the cytokine through a non-proteolytic mechanism.

LCMV-immune Tc cells of B6 and gzmAxB$^{-/-}$ mice induce IL-1β release from wt and P2X$_7$$^{-/-}$macrophages

LCMV-immune B6 and gzmAxB$^{-/-}$ Tc cells were added to LPS-primed PEMØ in the presence or absence of LCMV-specific immunogenic peptide, gp33. In the absence of gp33, LCMV-immune B6 and gzmAxB$^{-/-}$ Tc cells induced only marginal processing and release of IL-1β in PEMØ at an E:T ratio of 5:1. In contrast, gp33 pulsed targets responded to LCMV-immune B6 as well as gzmAxB$^{-/-}$ Tc cells by secreting substantial amounts of IL-1β (p17; Figure 3B). To exclude the possibility that the Tc cell-induced IL-1β secretion was due to ATP released from stressed PEMØ [29, 30], the response of cells from mice lacking the ATP sensor (P2X7 receptor) were used under similar conditions. While treatment with ATP (5mM) did not result in secretion of active IL-1β, wt and gzmAxB$^{-/-}$ Tc cells readily induced the release of mature IL-1β from P2X7-deficient PEMØ to an extend similar to wt PEMØ. (Figure 3C).

In vivo treatment of mice with IL-1RA inhibits virus elimination

To explore the possibility that IL-β participates in the control of LCMV infection, B6 mice were treated with IL-1Ra (Anakinra; [31]) and virus titers were monitored in liver

on days 8, 12 and 19. In contrast to mock-treated and virus-infected B6 mice (Figure 1B), recipients treated with Anakinra were unable to eliminate LCMV (Figure 4A). This outcome was not due to the absence of activated Tc because the ratio of LCMV specific Tc is comparable for untreated and treated LCMV infected mice (Figure 4B). Furthermore, LCMV-immune Tc taken from the same Anakinra treated mice showed pro-apoptotic activity similar to mock-treated Tc (Figure 4C).

Discussion:

The observation that perf$^{-/-}$ mice die from LCMV infection clearly indicates that the pore forming protein perf is essential for Tc cell (NK)-mediated control of this virus [22]. However, since perf functions chiefly to deliver gzms and other granule components into target cells, an understanding of the role(s) played by these proteins in the anti-viral response and associated tissue damage is crucial. For example, LCMV is cleared in gzmA$^{-/-}$ and gzmB$^{-/-}$ mice although recovery is delayed in the latter compared to wt controls [24] and LCMV-associated hepatitis appears to require the presence of gzmA and gzmB [23]. We have observed here that the course of virus infection was similar, but not identical for gzmAxB$^{-/-}$ and B6 mice indicating that other granule proteins are possible participants [2]. Since Tc cells are essential to control LCMV infection and gzmK is expressed in virus-immune gzmAxB$^{-/-}$ Tc cells that retain this capacity, one could speculate that gzmK together with perf may contribute to the host response that results in the elimination of the virus.

Although the molecular basis for a gzmK-mediated anti-viral response is uncertain, based on past observations, the tryptase is predicted to clear LCMV by inducing infected cells to undergo apoptosis. By studying the human system, the Fan group has shown that rec(Hu)gzmK elicits multiple pro-apoptotic pathways that could participate in anti-viral immunity. Their lab has reported that the granzyme directly processes Bid which releases cytochrome c and endonuclease G, resulting in the activation of a mitochondrial-dependent cell death pathway albeit without caspase 3 activation [14]. Rec(Hu)gzmK, similar to rec(Hu)gzmA, has also been reported to cleave SET which occurs in association with single-stranded DNA nicks in target cells [15]. Furthermore, rec(Hu)gzmK has been reported to cleave p53 into three products (p40, p35, and p13) which apparently possess pro-apoptotic activities. In addition, these fragments are proposed to augment the cytotoxic activity of gzmK,

though other biological effects cannot be excluded [17]. Finally, (Hu)gzmK was shown to cleave a vasolin-containing protein resulting in accelerated endoplasmic reticulum stress and caspase-independent cytotoxicity of tumor cells [18]. In marked contrast, the present study demonstrates that ex vivo-derived gzmK^{+} but gzmAxB$^{-/-}$ LCMV-immune Tc cells expressed only marginal pro-apoptotic/ cytotoxic activities especially when measured by the sensitive markers that assess plasma membrane and mitochondrial integrity as well as by a target cell survival assay. This finding agrees with reports showing that LCMV- and ectromelia-immune gzmAxB$^{-/-}$ Tc cells have marginal pro-apoptotic activity [25, 32, 33]. More strikingly, rec(Mo)gzmK, like (Mo)gzmA [8], completely failed to induce cell death at concentrations that suffice for gzmB-induced apoptosis. The combined results suggest that MogzmK, through non-cytotoxic processes, might be one component in the complex of molecular events that contribute to elimination of LCMV. Notably, NK cells from infected gzmAxB$^{-/-}$ mice, which did not express gzmK as expected from previous studies [34] showed low but significant cytotoxic activity, which may be due to the expression of gzmM. However, the possibility that LCMV infection in these ko mice is controlled by NK cells is less likely, since NK cells have been shown before to be unable on their own to control LCMV infection, even if they fully express the exocytosis pathway.

Offering a major paradigm shift in characterizing the functionality of the granule secretory pathway, we have learned that isolated (Mo)gzmA and *ex vivo* virus-immune mouse Tc cells containing gzmA induce IL-1β release from LPS-sensitized PEMØ [8, 35]. Since gzmA may contribute to host defense by inducing pro-inflammatory cytokines in pathogen-infected cells targeted by the granule secretory process, we speculated that (Mo)gzmK, displaying a tryptase-like activity similar but not identical to (Mo)gzmA [12], might also be an inducer of pro-inflammatory cytokines. Accordingly, we show here for the first time that nanomolar concentrations of rec(Mo)gzmK and LCMV-immune Tc containing gzmK but neither gzmA nor gzmB induce the maturation and secretion of IL-1β in LPS-sensitized PEMØ. The fact that gzmK displays a highly restricted substrate specificity that only partially overlaps with (Mo)gzmA [12], suggests that (Mo)gzmK may augment (Mo)gzmA-induced pro-inflammatory processes by differentially cleaving the same or distinct specific substrates. This assumption is supported by the fact that B6 Tc cells, which express

gzmA and gzmK together with gzmB, have a greater capacity than gzmAxB$^{-/-}$ Tc cells to induce IL-1β release from LPS-sensitized PEMØ.

Pro-IL-1β is produced in response to danger or pathogen associated molecular patterns like LPS and can be processed to the mature and secreted cytokine by the NLRP3 inflammasome, a caspase-1 activating cytosolic protein complex [30]. The NLRP3 inflammasome is activated by numerous stimuli such as bacterial toxins, uric acid crystals but also by extracellular ATP released from stressed or damaged cells. This endogenous danger signal is sensed by the purinergic receptor $P2X_7$, a ligand gated ion channel [29]. Our results show that the release of IL-1β of PEMØ induced by B6 as well as gzmAxB$^{-/-}$ Tc was not affected by the absence of $P2X_7$ receptor. This result suggests that the Tc-induced IL-1β processing is not due to ATP derived from damaged target cells and therefore strengthening the concept that pro-inflammatory granzymes such as gzmK participate in inflammasome activation.

Overall, the data show that neither gzmA nor gzmB are essential to control LCMV infection in mice. This observation, which supports previous work [23, 24], is intriguing especially when combined with results here indicating *ex vivo* anti-LCMV Tc cells from gzmAxB$^{-/-}$ mice exhibit markedly reduced cytotoxicity *in vitro*. Similar to these Tc which contain primarily (Mo)gzmK, rec(Mo)gzmK delivered by SLO also failed to mount the anticipated cytotoxic response. Thus, the anti-viral effects of *in vivo* anti-LCMV Tc cells does not correlate with their *in vitro* cytolytic activity, but rather is mediated through non-cytotoxic pathways such as inflammasome activation or uncharacterized biological effects [36].

The possibility that (Mo)gzmK induces IL-1β release which aids LCMV clearance is further supported by the observation that mice treated with Anakinra are unable to control replication, even though the ratio of LCMV-immune Tc cells generated under these conditions, including their cytotoxic potential was similar to those of untreated infected B6 mice (Figure 4B, C). These findings differ from a recent study where IL-1R$^{-/-}$ mice, challenged with influenza virus, were unable to generate virus-specific Tc [37]. This discrepancy may be due to the different approaches used to reduce IL-1β in the two systems. In the IL-1R$^{-/-}$ mice, IL-1β is expected to be unable to mediate its myriad biological effects through the IL-1R including the induction of Tc. The

blockade produced by Anakinra is predicted to be incomplete allowing the induction of virus-specific Tc cells but curtailing the anti-viral activity capacity of the cytokine during the effector phase of the host response. Although (Mo)gzmK, similar to (Hu)gzmH [36], might mediate direct antiviral effects that contribute to viral clearance, together the evidence supports the existence of a (Mo)gzmK-induced pro-inflammatory pathway that controls intracellular pathogens.

In comparison to (Mo)gzmK, isolated (Hu)gzmK has been reported to be directly cytotoxic *in vitro* when delivered by SLO or adenoviral particles [14]. However, others have been unable to demonstrate the rec(Hu)gzmk is cytotoxic *in vitro* when delivered by SLO to Hela cell targets at concentrations reaching 1000 nM (personal communication, Niels Bovenschen). Using intact Tc containing primarily (Mo)gzmK, we have verified that (Mo)gzmK delivered *in vitro* is not cytotoxic. A similar approach will be necessary to reconcile whether (Hu)gzmK is cytotoxic.

Future studies that utilize gzmK-deficient mice will be necessary to verify whether LCMV infection is controlled by gzmK and/or other granule-associated components and whether the putative anti-viral activity of gzmK is linked to the induction of pro-inflammatory cytokines in macrophage subsets or to other non-cytotoxic pathways. In contrast to LCMV, other viruses such as ectromelia depend on cytolytic gzmB [38]. Thus, depending of the virus and the tissue distribution of infection, Tc cells may use individual gzms to kill infected targets and employ one or more of the gzms to disrupt their life cycle by non-cytolytic means.

Materials and Methods:

Mouse strains - The following strains were maintained at the Max-Planck-Institut für Immunobiologie, Freiburg: C57BL/6 (B6; wild type, wt); strains deficient for gzmA ($gzmA^{-/-}$), gzmA and gzmB ($gzmAxB^{-/-}$) and perf, gzmA and gzmB deficient ($perfxgzmAxB^{-/-}$), were bred on the B6 background [23]. Mice deficent for $P2X_7$ ($P2X_7^{-/-}$) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Animal studies were conducted in accordance with the ethical guidelines of the Federation of European Laboratory Animal Science Association.

Virus - LCMV (strain WE), kindly provided by O. Utermöhlen (Cologne, Germany) was expanded in L929 fibroblasts, as described [25]. The virus-specific epitope gp33 (KAVYNFATATC; Neosystem, Strasbourg, France) for $H\text{-}2D^b$ was used for target labeling in cytotoxicity assays [25].

Disease model - Mice were infected intraperitoneally (i.p.) with 10^5 PFU of LCMV-WE according to established protocols [25, 39].

IL-1Ra (Anakinra) treatment – Mice were injected 1 day before infection with 10^5 PFU LCMV-WE with 200 µg IL-1Ra (Anakinra, Kineret ® Amgen, Breda, Netherlands) in 200 µl PBS and from then on every third day with the same dose until the end of experiment [31].

Virus titers - Aliquots of liver tissues were homogenized and used for determination of virus titers as described elsewhere [39].

Generation of ex vivo $CD8^+$ cells - On day eight post infection (p.i.), $CD8^+$ cells were positively selected from spleen using α-CD8-MicroBeads (Miltenyi Biotec Bergisch Gladbach, Germany) with an autoMACS (Milteny Biotec, Bergisch Gladbach, Germany) and resuspended in MEM/5%FCS prior to use in cytotoxic assays as described [25]. Detection of LCMV-immune CTL was conducted by double staining with CD8 antibody (BD Pharmingen, San Diego, CA, USA ; clone 53-67) and gp33- labeled pentamers (ProImmune, Oxford, UK) as described [32].

Cell lines, cell culture and reagents - The mouse cell lines 1.3E6 (Tc cell line) and EL4.F15 (thymoma) and mouse embryonic fibroblast (MEF) cell lines were cultured as described [25, 40]. Recombinant (rec.) (Mo)gzmK was produced in *E.coli* B834 (DE3) using the pET-21a vector and purified as described [41]. Rabbit immune serum (IS) specific for (Mo)gzmK was generated as described for (Mo)gzmB [28].

Analysis of pro-apoptotic processes - Cell death induced by *ex vivo* CD8-enriched Tc cells or by isolated gzms in the presence or absence of Streptolysin O (SLO), was analyzed as described [32]. Briefly, target cells were pre-treated with LCMV-immunodominant peptide gp33 for 2 h prior to incubation with *ex vivo*-derived LCMV-immune CTL at 10:1 E:T cell ratio for 4h at 37°C, 7% CO_2. Alternatively, target cells were incubated with the indicated concentration of rec.gzms +/- SLO for 4h. Subsequently, different apoptotic parameters were tested in the target population ($CD8^-$) by FACS with a FACSCalibur (BD Pharmingen, San Diego, CA, USA) and FLOWJO® (Ashland, OR, USA) software as described [8, 25]. In short:

Cell membrane and mitochondrial membrane perturbations: Phosphatidylserine (PS) exposure and Propidium Iodide PI uptake were analysed by FACS using the annexin-V-FITC kit from BD Pharmingen (San Diego, CA USA). The mitochondrial membrane potential was measured with the fluorescent probe 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$; (Molecular Probes Eugene, OR, USA) and Reactive Oxygen Species (ROS) generation with 2-hydroxyethidium (2-HE; Molecular Probes Eugene, OR, USA)

Intracellular staining - Cell populations were analyzed for cell surface markers and intracellular gzm expression as described elsewhere [40], analyzed by FACS with a FACSCalibur (BD Pharmingen San Diego, CA, USA) and FLOWJO® (Ashland, OR, USA) software.

Survival assay – Survival of target cells following their incubation with rec(Mo)gzmK in presence and absence of SLO was analyzed as described [42]. Briefly, target cells were incubated with 1000 nM rec(Mo)gzmK in the presence or absence of SLO for 4h. Afterwards ^{3}H-thymidine was added and cells were incubated for 10h at 37°C, 5%CO_2, without removing rec(Mo)gzmK and SLO. Subsequently cells were harvested and target cell survival was quantified by ^{3}H-thymidine incorporation as

described [42]. This assay gives similar result to clonogenic survival assays in agar plates [25].

Enzymatic Assays - Amidolytic activity of gzms using the following chromogenic substrates (Bachem, Weil am Rhein, Germany) was performed as described previously [43]: Bz-Arg-pNA; H-D-Pro-Phe-Arg-NA; Ac-Ile-Glu-Pro-Asp-pNA; Tos-Gly-Pro-Arg-pNA.

RT-PCR - Total RNA was extracted from up to 5 x 10^6 *ex vivo* $CD8^+$ cells, using the QIAshredder spin columns, the RNAeasy Mini Kit and the RNAse-free DNAse Kit (all from Qiagen, Hilden, Germany) according to the manufacturer's instructions, and specific transcripts were amplified. The sense/ antisense primers for *gzmA, gzmC, gzmD, gzmE, gzmF, gzmG* and *Hprt1* are described in [40] and [44], respectively. Sense/antisense primers for *gzmB*, *gzmK* and *gzmM* are described in [25]. Primers for perf are described in [23].

IL-1β release assay - B6 mice were injected i.p. with 2 ml of 4% thioglycollate (Sigma, Steinheim, Germany) in LPS-free water, three days prior to removal of peritoneal macrophages (PEMØ). On d3 post injection, mice were sacrificed and their peritoneum was washed with 10ml of PBS to collect PEMØ. The cell-suspension was washed and 8 x 10^5 cells/well were plated in a 12-well plate (500µl volume/MEM without FCS) and incubated for 1h at 37°C 5% CO_2. Subsequently, PEMØ were sensitized with 100ng/ml LPS (S. Minnesota R595) and incubated for 2h at 37°C 5% CO_2. Afterwards, supernatant (SN) was discarded and PEMØ were challenged either with LCMV-immune *ex-vivo* Tc cells or recombinant (rec.) gzms (300 µl volume/MEM without FCS) in the presence or absence of SLO, for 4-6h at 37°C 5% CO_2.

Western Blot analysis -The inactive and active form of caspase-3 as well as actin and IL-1β was determined by Western blotting under reducing conditions. Rabbit polyclonal anti-human caspase 3 IgG (active and inactive) was from Cell Signaling Technology (Danvers, MA USA), mouse anti-actin IgG was from MP Biomedicals (Aurora, OH, USA) and goat anti-IL-1β from R&D Systems (Minneapolis, MN, USA). Blots were then stained with horseradish peroxidase conjugated goat anti-rabbit IgG

or goat anti-rabbit IgG or goat anti mouse IgG all Jackson ImmunoResearch Laboratories Inc. (Suffolk, UK) followed by enhanced chemiluminescence (ECL Western Blotting Analysis System, GE Healthcare, (München, Germany).

Conflict of interest:

The authors declare no conflict of interest

Supplementary information:

Supplementary information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)

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Titles and legends to figures:

Figure 1. A Survival of wild-type, gzmAxB$^{-/-}$ and perfxgzmAxB$^{-/-}$ mice infected with LCMV-WE. Groups (9 mice each) of B6 (dashed line) or gzmAxB$^{-/-}$(dotted line) or perfgzmAxB$^{-/-}$ (line) mice were infected with 1x10^5 PFU LCMV-WE i.p. and survival was monitored for 30 days.

B LCMV-WE replication in liver of WT, gzmAxB$^{-/-}$ and perfxgzmAxB$^{-/-}$ mice. Groups of B6, gzmAxB$^{-/-}$ and perfxgzmAxB$^{-/-}$ (18 mice per strain) recipients were infected with 1x10^5 PFU LCMV-WE i.p.. 6 mice from each group were sacrificed at 8, 12 and 19 days p. i.. Virus titers of individual mice were determined by plaque titer assay as described in Materials and Methods.

C Expression of mRNA in *ex vivo* LCMV-immune CD8-enriched Tc cells from WT, gzmAxB$^{-/-}$ and perfxgzmAxB$^{-/-}$ mice. Total mRNA was isolated from *ex vivo* LCMV-immune CD8-enriched Tc cells (d8 p.i.) from wild-type, gzmAxB$^{-/-}$ and perfxgzmAxB$^{-/-}$ mice, previously infected with 1x10^5 PFU LCMV-WE i.p. Samples were analyzed by RT-PCR for their expression of transcripts using specific primers for *gzmA-G, gzmK, gzmM, Prf1* and *Hprt1*.

D Intracellular expression of gzm and perf protein in *ex vivo* LCMV-immune CD8-enriched Tc cells (d8 p.i.). *Ex vivo* LCMV-immune CD8-enriched Tc cells (d8 p.i.) of wt, gzmAxB$^{-/-}$ and perfxgzmAxB$^{-/-}$ mice, infected with 1x10^5 PFU LCMV-WE i.p. were analyzed by FACS for intracellular expression of gzmK (blue line) using the respective rabbit anti-K immune serum and the control pre-immune serum (red line).

Figure 2 A Induction of pro-apoptotic markers by *ex vivo* LCMV-immune Tc cells from WT, gzmAxB$^{-/-}$ and perfxgzmAxB$^{-/-}$ mice. Gp-33-pulsed or untreated MEF target cells were incubated with *ex vivo* LCMV-immune CD8-enriched Tc cells of the indicated strain (4h, 10:1 effector/target ratio). Subsequently, PS exposure on plasma membrane (annexin-V-FITC) and PI uptake and in parallel $\Delta\Psi_m$ loss ($DiOC_6$) and ROS generation (2-HE) were analyzed by three-color flow cytometry in the cell population negative for CD8 expression as described in Materials Methods. Data in the panels are represented as the mean±S.E.M. of three independent experiments.

B rec(Mo)gzmA and rec(Mo)gzmK are not cytotoxic: analyses of apoptotic parameters during gzmA, gzmB and gzmK-induced cell death in EL4 cells. EL4 cells were incubated with the indicated amounts of gzms in the presence of a sublytic

dose of SLO for 4 hr and the highest amount of gzm was also probed without SLO indicated by 1200 nM - SLO. Subsequently, PS exposure on plasma membrane (annexin-V-FITC) and PI uptake and in parallel $\Delta\Psi_m$ loss ($DiOC_6$) and ROS generation (2-HE) were analyzed by three-color flow cytometry. **C** EL4 cells were incubated or not with indicated amounts of rec.(Mo)gzmA, rec.(Mo)gzmB and rec.(Mo)gzmK in presence and absence of SLO for 1h at 37°C. Subsequently, lysates were prepared and Caspase-3 and cleaved Caspase-3 was analyzed by WB, with Actin serving as control. **D** Survival assay of EL4 cells treated with rec.(Mo)gzmK in presence and absence of SLO as described in Materials and Methods.

Figure 3

A Induction of IL-1β release by rec(Mo)gzmK. Adherent B6 PEMØ, previously sensitized with thioglycollate *in vivo* and challenged with LPS *in vitro* were incubated with the indicated amounts of gzmK +/- 0,25µg/ml SLO, or ATP as positive control, for 3h and SN were subjected to WB analysis using anti-IL-1β specific Ab. Aliquots of the following preparations were used as standards for WB analysis: SN of ATP sensitized pre-activated B6 PEMØ; SN of thioglycollate pre-sensitized and LPS challenged B6 PEMØ.

B Induction of IL-1β release by ex vivo CD8 cells expressing gzmK. Thioglycollate pre-sensitized and LPS challenged adherent B6 PEMØ were incubated with *ex vivo*-derived LCMV-immune Tc cells from B6 or gzmAxB ko mice at an E:T ratio of 5:1 in presence or absence of the LCMV-specific peptide gp33. SN was taken after 6h and subjected to WB analysis using anti-IL-1β specific Ab. As control, SN from thioglycollate pre-sensitized and LPS challenged B6 PEMØ, and subsequently stimulated with 5mM ATP, was analysed accordingly. Aliquots of the following preparations were used as standards for WB analysis: rec.IL-1β, p17; SN of ATP sensitized pre-activated wt B6 PEMØ; SN of wt thioglycollate pre-sensitized and LPS activated B6 PEMØ.

C Induction of IL-1β release by ex vivo CD8 cells expressing gzmK by targerts lacking the P2X7 receptor. Experimental procedure as in figure 3B but with macrophages from $P2X_7^{-/-}$ mice. Controls were prepared individually for this experiment with $P2X_7^{-/-}$ macrophages (SN of ATP sensitized pre-activated PEMØ; SN of wt thioglycollate pre-sensitized and LPS activated PEMØ).

Figure 4 A Anakinra inhibits recovery from LCMV infection**.** B6 mice received Anakinra before infection ($1x10^5$ PFU LCMV-WE i.p) and every third day thereafter. Three mice from each group were sacrificed at 8, 12 and 19 days and hepatic viral replication determined.

B Anakinra treated mice infected with LCMV express levels of LCMV specific Tc cells similar to untreated controls. Mice were first treated eith Anakinra (200 µg) one day before infection and then every third day. After sacrifice (day 8), splenic CD8 cells were isolaed and virus specific Tc were determined by pentamer staining. Livers from these mice were used to generate data reported in figure 4A.

C Anakinra treated and control Tc mediate similar apoptotic effects against EL4 cells. Gp-33-pulsed or untreated EL4 target cells were incubated with *ex vivo* LCMV-immune CD8-enriched Tc cells (4h, 10:1). Subsequently, PS exposure on plasma membrane (annexin-V-FITC) and PI uptake were analysed via flow-cytometry.

Tables:

Table 1: Amidolytic activity of gzmA and gzmK on chromogenic substrates

Substrates	gzmA	gzmK
	specific activity units/µg protein	
Pro-Phe-Arg-pNA	107	115
Tos-Gly-Pro-Arg-pNA	9	120
H-D-Ile-Pro-Arg-pNA	25	31
Bz-Arg-pNA	0	3

Titles and legends to supplementary figures:

Supplementary Figure 1. Cell death induced by *in vivo* LCMV-activated NK cells is reduced in the absence of granzymes A and B. NK cells from gzmA-/- or gzmAxB-/- mice previously inoculated with PBS, polyIC (0,1 mg; 24h) or LCMV (10^5 pfu; 72h) were enriched by MACS selection. **A** NK cells were stained with Cell Tracker Green and incubated with Yac-1 cells at different e:t ratio for 4h. Subsequently, 7AAD incorporation in target cells was analysed by FACS. Numbers correspond to the % of cells as indicated by vertical bars. **B** Expression of mRNA in *ex vivo* LCMV-immune NK cells from gzmA$^{-/-}$ and gzmAxB$^{-/-}$ mice. Samples were analyzed by RT-PCR for their expression of transcripts using specific primers for *gzmA-G, gzmK, gzmM, Prf1* and *Hprt1*. **C** Expression of mRNA in *ex vivo* LCMV-immune CD8-enriched Tc cells from WT, gzmAxB$^{-/-}$ and perfxgzmAxB$^{-/-}$ mice. Total mRNA was isolated from *ex vivo* LCMV-immune CD8-enriched Tc cells (d8 p.i.) from wild-type, gzmAxB$^{-/-}$ and perfxgzmAxB$^{-/-}$ mice, previously infected with $1x10^5$ PFU LCMV-WE i.p. Samples were analyzed by RT-PCR for their expression of gzmM transcripts. As positive control served NK cells from gzmA$^{-/-}$ mice.

Supplementary Figure 2. Induction of IL-1β release by rec(Mo)gzmK. Adherent B6 PEMØ, previously stimulated with thioglycollate *in vivo* and stimulated with LPS *in vitro,* were incubated for 2 hr with the indicated concentrations of gzmK in the presence and absence of SLO (0.25 µg/ml), or with ATP which acted as positive control. SN were subjected to WB analysis using anti-IL-1β specific Ab. The following were used as controls: Positive controls are 100 pg of rec. IL-1β (p17) and LPS-sensitized PEMØ treated with 5 mM ATP resulting in p17 processing. Unstimulated (no band detectable) or LPS only stimulated PEMØs (pro-form p32 detectable) served as negative controls.

Figure 1

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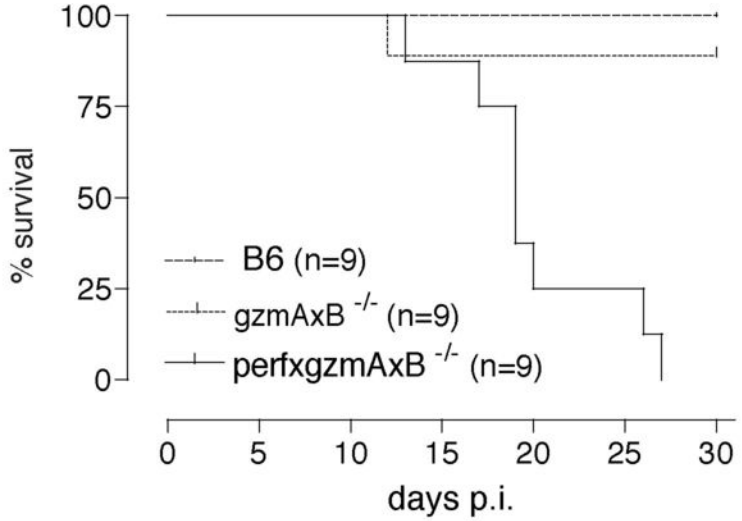

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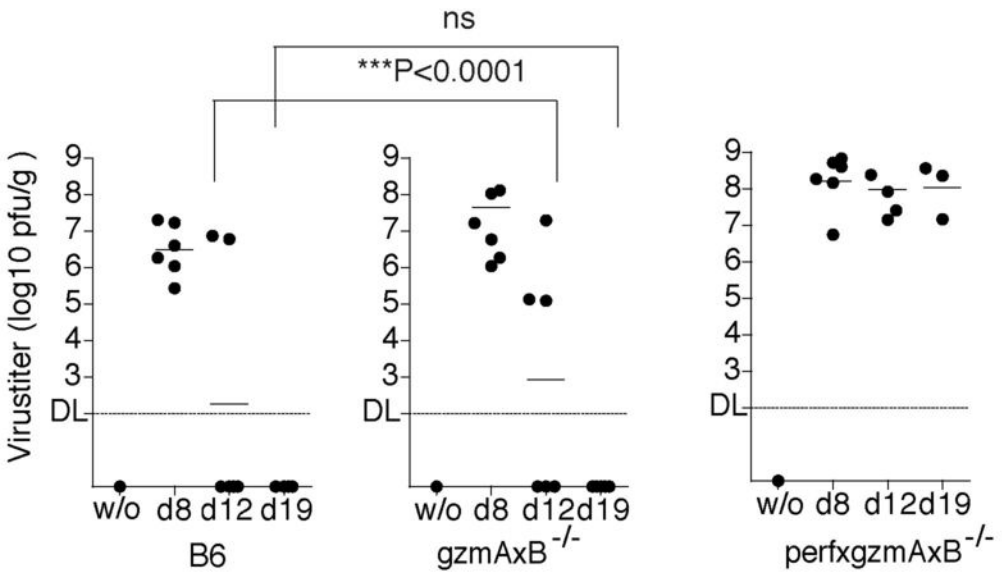

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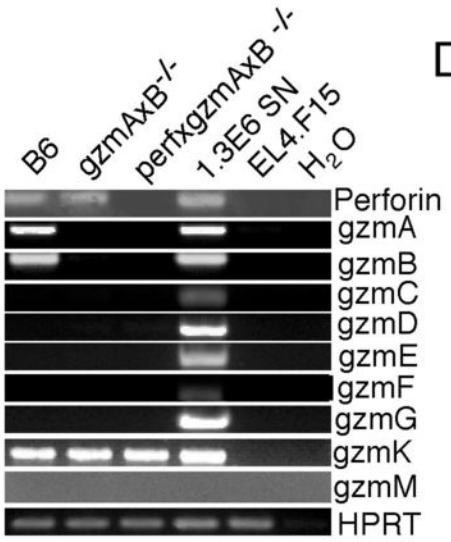

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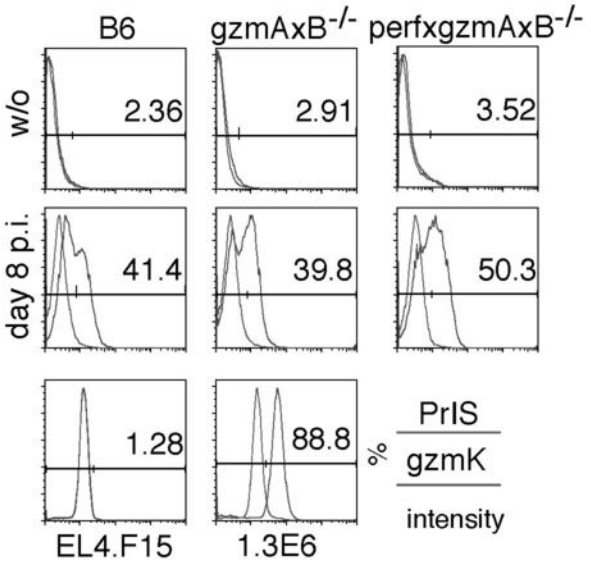
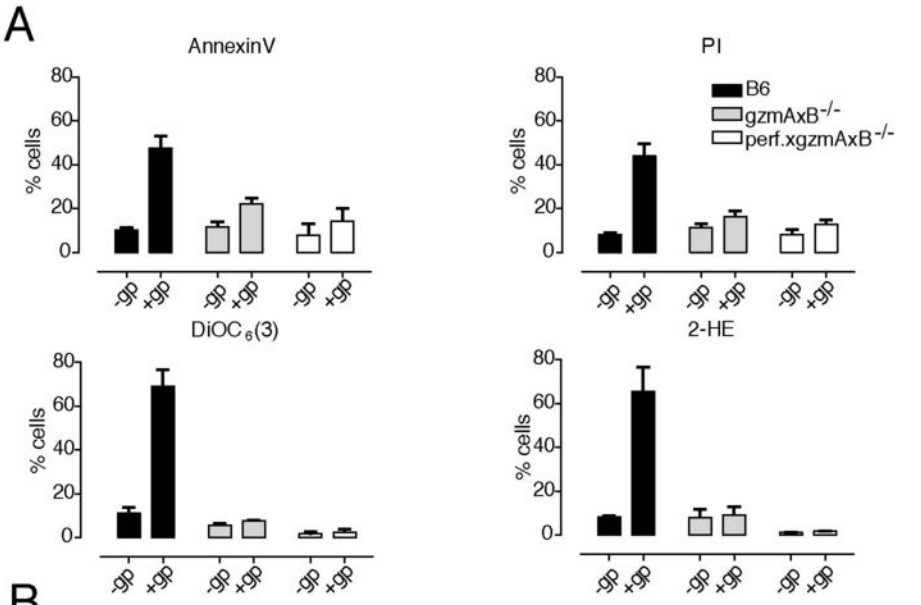


Figure 2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DiOC6(3)
2-HE
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B6
gzmAxB-/-
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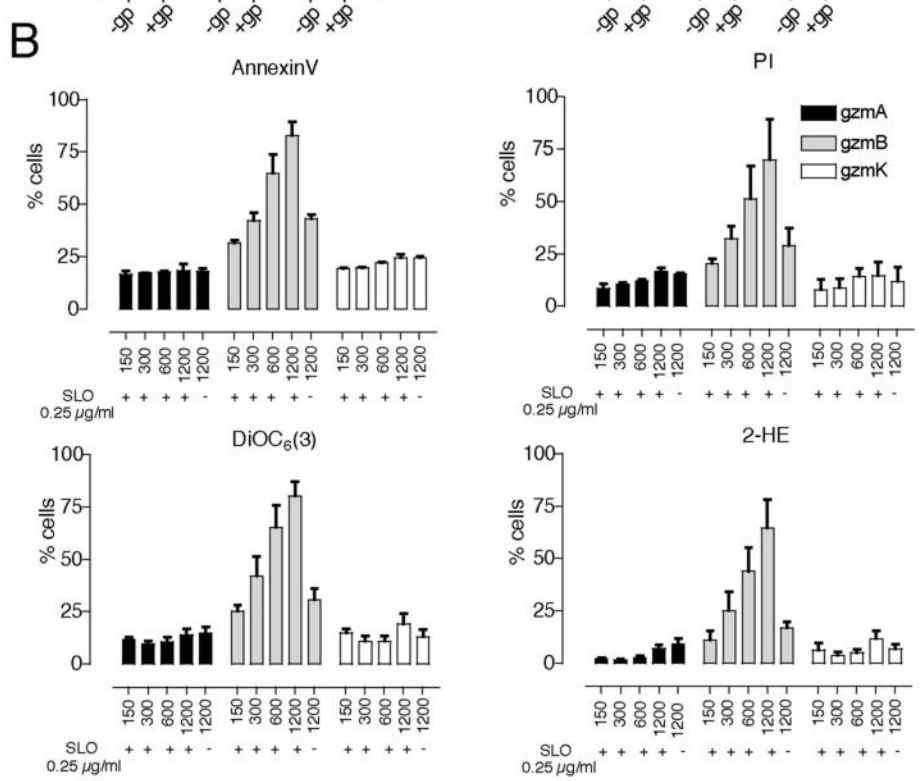
+gp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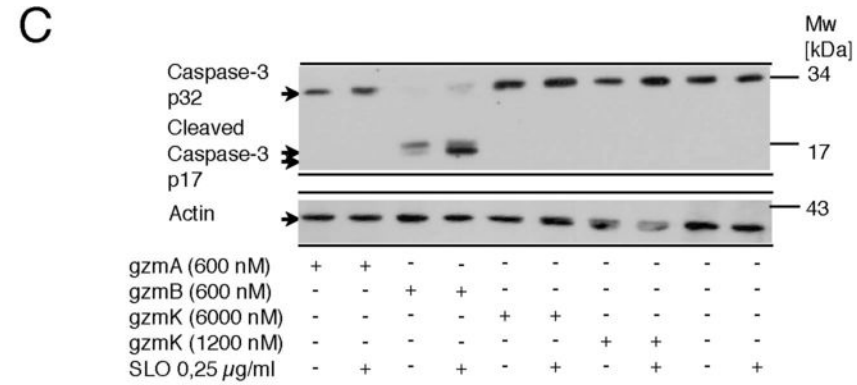
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Cleaved Caspase-3 p17
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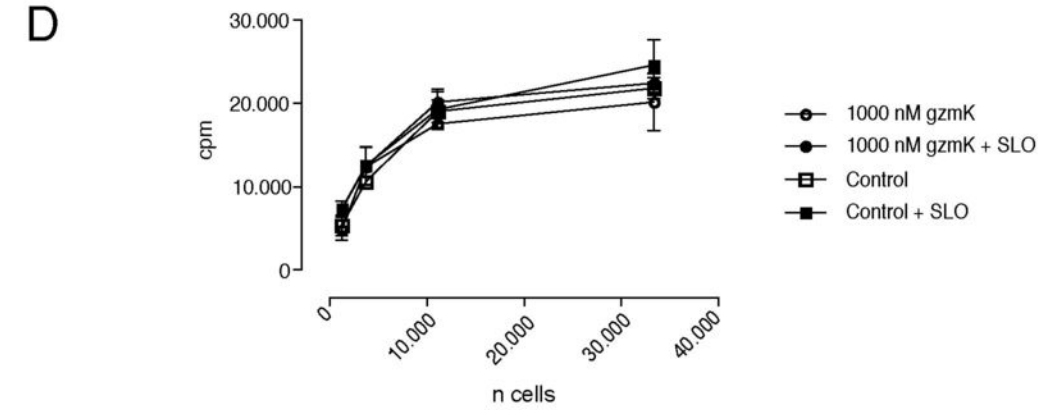
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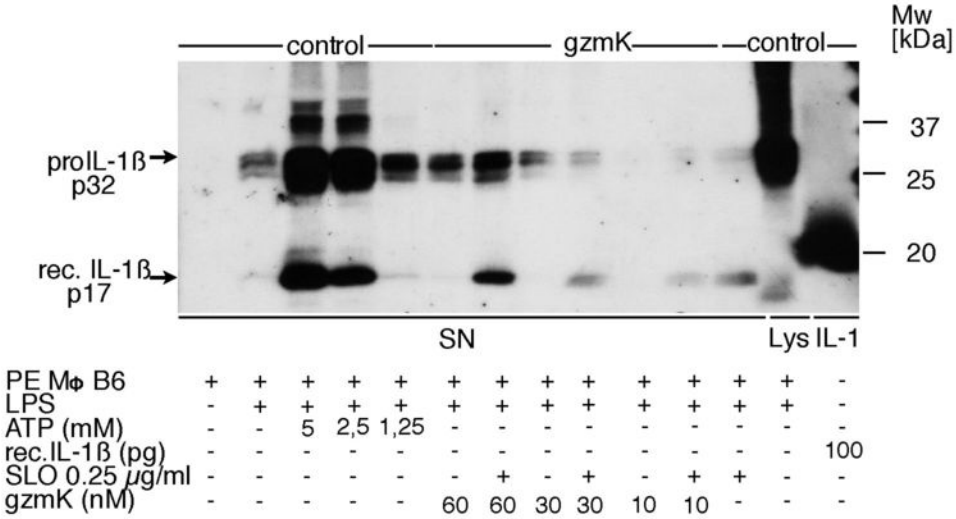
1000 nM gzmK + SLO
Control
Control + SLO

Figure 3

A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LPS - + + + + + + + + + + + + -
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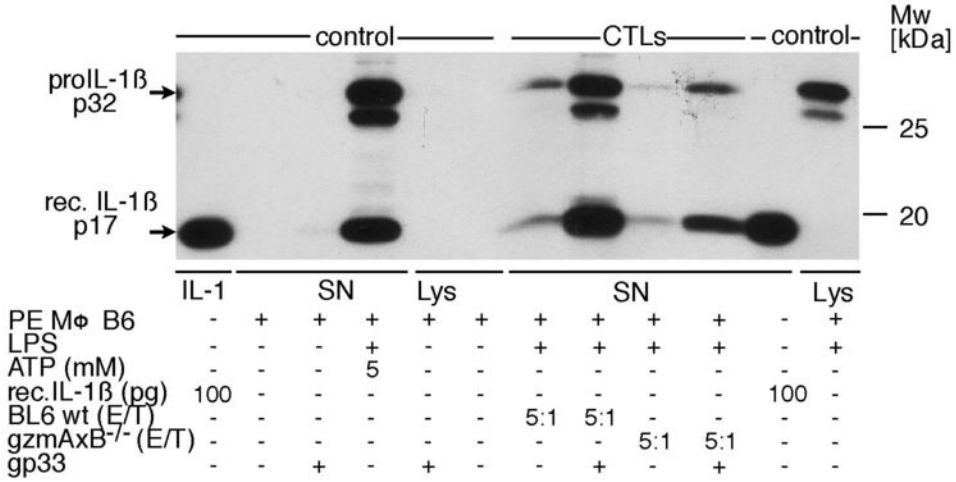
gzmK (nM) - - - - - 60 60 30 30 10 10 - - -

B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control
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proIL-1ß p32
rec. IL-1ß p17
IL-1
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PE Mφ B6 - + + + + + + + + + - +
LPS - - - + - - + + + + - +
ATP (mM) - - - 5 - - - - - - - -
rec.IL-1ß (pg) 100 - - - - - - - - - 100 -
BL6 wt (E/T) - - - - - - 5:1 5:1 - - - -
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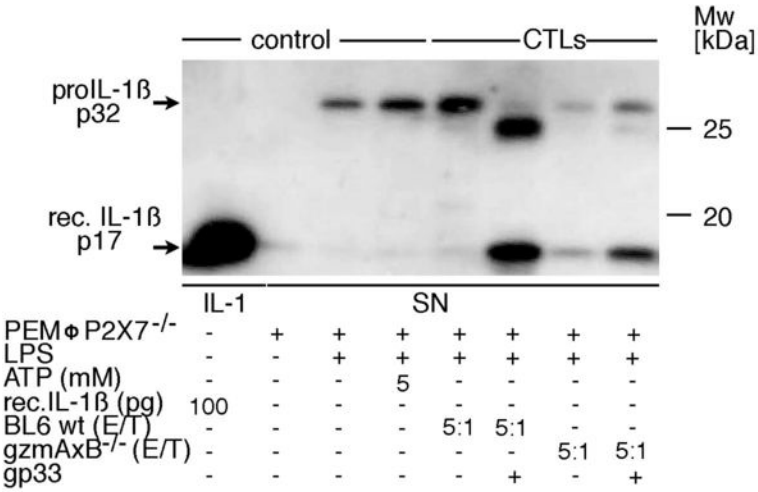
gp33 - - + - + - - + - + - -

C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SN
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LPS - - + + + + + +
ATP (mM) - - - 5 - - - -
rec.IL-1ß (pg) 100 - - - - - - -
BL6 wt (E/T) - - - - 5:1 5:1 - -
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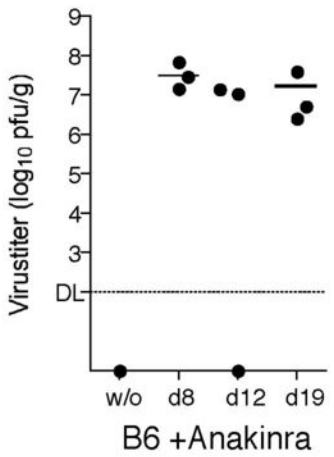
gp33 - - - - - + - +

Figure 4

A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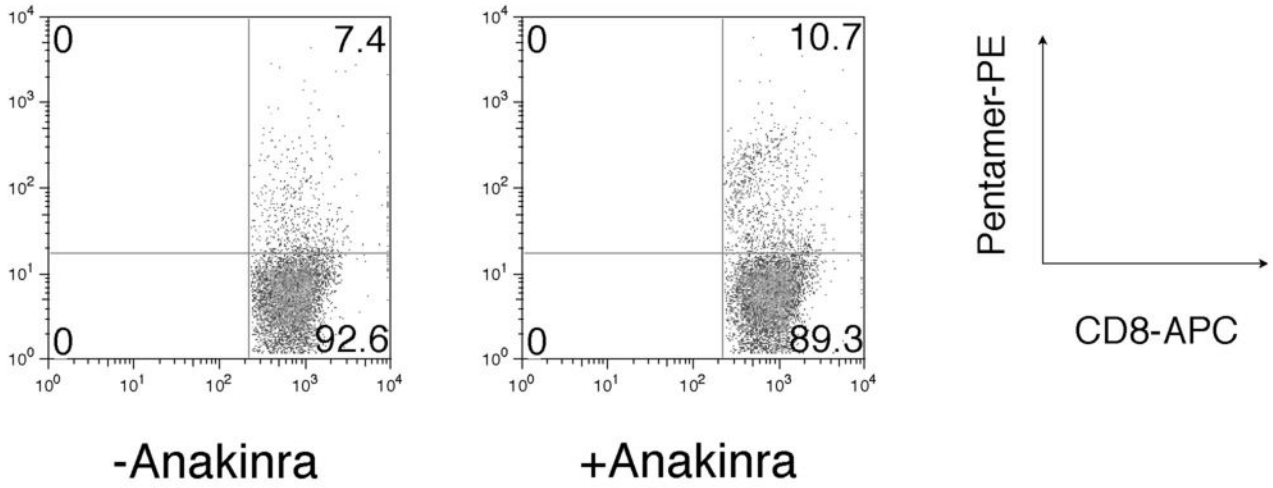
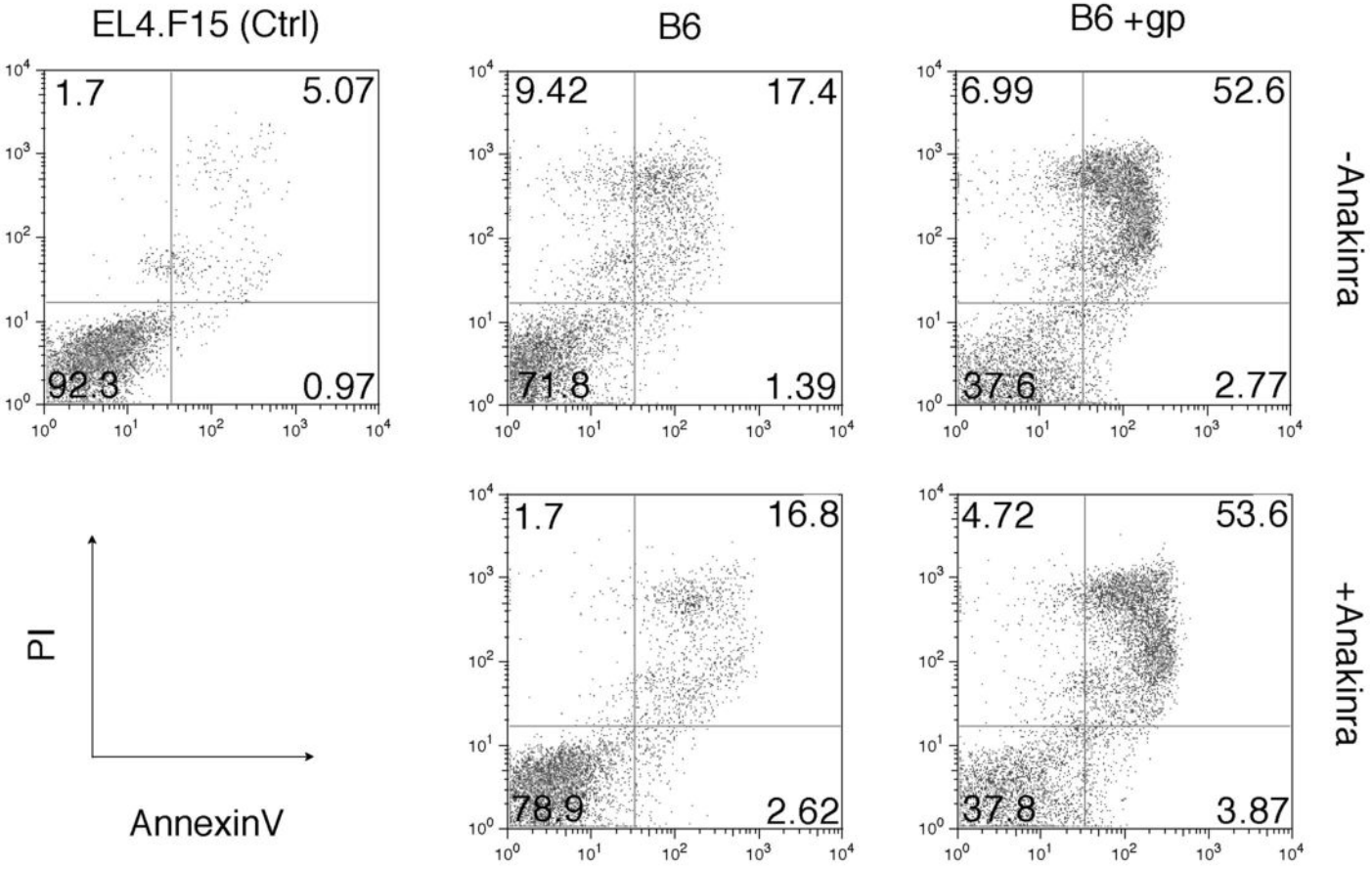
9
8
7
6
5
4
3
DL
w/o
d8
d12
d19
B6 +Anakinra

B


0
7.4
0
92.6
0
10.7
0
89.3
Pentamer-PE
CD8-APC
-Anakinra
+Anakinra

C


EL4.F15 (Ctrl)
B6
B6 +gp
1.7
5.07
92.3
0.97
9.42
17.4
71.8
1.39
6.99
52.6
37.6
2.77
-Anakinra
1.7
16.8
78.9
2.62
4.72
53.6
37.8
3.87
+Anakinra
PI
AnnexinV