NK cell intrinsic regulation of MIP-1α by granzyme M

N Baschuk1, N Wang2, SV Watt1, H Halse1, C House1, PI Bird3, R Strugnell2, JA Trapani1,4,5, MJ Smyth1,4,5,6,7,8 and DM Andrews1,4,5,8

Granzymes are generally recognized for their capacity to induce various pathways of perforin-dependent target cell death. Within this serine protease family, Granzyme M (GrzM) is unique owing to its preferential expression in innate effectors such as natural killer (NK) cells. During Listeria monocytogenes infection, we observed markedly reduced secretion of macrophage inflammatory protein-1 alpha (MIP-1α) in livers of GrzM-deficient mice, which resulted in significantly impaired NK cell recruitment. Direct stimulation with IL-12 and IL-15 demonstrated that GrzM was required for maximal secretion of active MIP-1α. This effect was not due to reduced protein induction but resulted from heightened intracellular accumulation of MIP-1α, with reduced release. These results demonstrate that GrzM is a critical mediator of innate immunity that can regulate chemotactic networks and has an important role in the initiation of immune responses and pathogen control.

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Subject Category: Immunity

Natural killer (NK) cells are lymphocytes of the innate immune system crucial for the defence against infection and cancer.1,2 NK cells directly recognize infected cells and induce target cell death by the delivery of perforin (Pfp) and granzymes (Grzs), particularly GrzB.3 Besides this direct cytotoxic function, NK cells have an important regulatory role during inflammation.4,5 They are embedded in a reciprocal crosstalk-network with immune cells such as macrophages, neutrophils and dendritic cells. This allows them to shape the immune response via the release of cytokines and chemokines.1,6

In humans and rodents, three distinct Grz subfamilies exist. GrzA and K have trypsin-like activity; GrzB and H (Human) and B-G (rodent) exhibit chymotryptic activity, whereas GrzM processes its substrates at the carboxy side of long, hydrophobic side chain amino acids such as Met and Leu.7 Human, mouse and rat GrzMs share very similar substrate patterns and various structural characteristics.8,9 In humans and rodents, GrzM is expressed constitutively and at high levels in NK cells, in subsets of innate T cells such as γδ T cells10,11 and in CD8+ CD27− CD45RO− T effector cells.12 Recent studies have suggested that GrzM delivered by CD8 T cells can regulate Human Cytomegalovirus replication by targeting heterogeneous ribonucleotide particles.13 GrzM is able to induce Pfp-dependent target cell death in vitro, with some studies suggesting that this occurs independently of caspase,-14–16 whereas others show a requirement of caspase activity.17–19 Interestingly, GrzM−/− mice have no defect of NK cell-mediated cytotoxicity, so the physiological relevance of these cell death pathways and GrzM’s major biological role are still to be elucidated.

Our recent studies have demonstrated a critical role for GrzM in the regulation of LPS-induced inflammation and suggest that this Grz has functions other than direct cytotoxicity.20 We chose to determine the requirement of GrzM during Listeria monocytogenes infection where the pathophysiology of the inflammatory process is well characterized. L. monocytogenes is a Gram-positive bacillus causing severe, sometimes lethal food-borne, infections with a human case-fatality rate that can exceed 30%. Unless recognized and treated, Listeria infections can result in significant morbidity and mortality. Importantly, mouse models of Listeria infection have demonstrated a clear role for NK cells and APCs in the control of disease21,22 where chemokine signaling networks are key components during the crosstalk that leads to effective immunity.

As part of this network, the chemokine macrophage inflammatory protein 1 (MIP-1α) belongs to the family of C-C chemokines, important for NK cell recruitment23–25 to the liver during MCMV infection.26,27 While the major source of MIP-1α is thought to be macrophages,28,29 NK cells can secrete MIP-1α in a cytokine-dependent manner.30,31 Given our recent identification that GrzM can shape cross-talk during inflammation20 and the growing appreciation that Grzs are more than just inducers of apoptosis,20,32 we sought to determine whether GrzM could shape immunity by regulating...
chemokine networks. In the absence of GrzM, we observed enhanced survival following Listeria challenge, and this was associated with a reduction in liver-specific NK cell recruitment, augmented effector function of NK cells in the liver and reduced liver pathology. We show that GrzM intrinsically regulates the release of Mip-1x from NK cells and that this is dependent upon IL-12/IL-15 signaling. Whereas GrzM and MIP-1x co-localise to cytotoxic granules, direct proteolytic processing of MIP-1x does not occur; rather GrzM is required for the efficient release of mature chemokine. These results demonstrate that GrzM can act as an immune stimulus and suggests its potential role as an intrinsic adjuvant.

Results

GrzM-deficient mice are less susceptible to L. monocytogenes infection. To evaluate the role of GrzM during microbial infection, WT and GrzM −/− mice were infected i.v. with 10⁴ L. monocytogenes. During Listeria infection, WT mice lost an average of 12.5 and 20.0% of their initial body weight on days 2 and 3 post infection, respectively. In contrast, GrzM −/− mice were significantly less sensitive (P<0.0001) to bacterial infection and lost only 7.5% on day 2 and 8.4% on day 3 post infection (Figure 1a).

In the first 24 h, there was no difference in the recovery of viable bacteria from the livers and spleens of WT or GrzM −/− mice (Figure 1b). However, by 72 h, the difference in the weight loss was clearly reflected in the bacterial burden of the recipients. GrzM −/− mice displayed significantly fewer viable bacteria in livers (P=0.0017) and spleens (P=0.0048) compared with WT mice.

The reduced growth of viable bacteria in the organs of GrzM −/− mice coincided with significantly lower ALT levels (P=0.0069) in the serum 72 h after the infection, suggesting reduced hepatocellular damage (Figure 1c). Histological analysis of livers from infected WT and GrzM −/− mice after 24 and 72 h revealed multiple foci consisting of moderate to marked necrosis and a severe inflammatory mononuclear infiltrate in WT mice (Figure 1d). In contrast, the corresponding lesions in GrzM −/− mice were fewer and milder, with reduced necrosis and cellular infiltrate. TUNEL analysis showed massive apoptosis of hepatocytes in WT livers 72 h after infection (Figure 1e) where TUNEL + cells were widely distributed and prevalent throughout the organs. In contrast, GrzM −/− organs contained far fewer TUNEL + areas.

Overall, survival after Listeria infection was significantly enhanced (P<0.0001) for mice deficient in GrzM (Figure 1f). Collectively, these results showed a strong influence of GrzM on the immunopathology of L. monocytogenes infection and indicated a likely role for GrzM in the early inflammatory response.

Altered recruitment and function of GrzM −/− liver NK cells following L. monocytogenes infection. In order to expand our understanding of GrzM during L. monocytogenes-induced inflammation, the recruitment of NK cells to the liver and spleen was investigated. Twenty-four hours after infection, GrzM −/− mice displayed significantly reduced absolute numbers of NK cells (P=0.0459) in the livers (Figure 2a) but not in the spleens when compared with WT mice (Figure 2b). No effect on CD4⁺, CD8⁺ T cells and macrophages were observed in the spleen (Supplementary Figure 2). A more detailed investigation of liver NK cells 24 h after infection revealed comparable percentages of mature NK cells (CD27⁺/11b⁺, CD27⁺/11b⁻) in WT and GrzM −/− mice (Figure 2c). A more detailed investigation of liver NK cells during the first 72 h of infection similar absolute numbers of CD4⁺, CD8⁺ T cells and macrophages were found in the livers of WT and GrzM −/− mice (Figures 2d–f).

Thus, the absence of GrzM resulted in a specific defect in liver NK cell recruitment following Listeria infection, which seemed counterintuitive to the improved bacterial clearance observed in GrzM −/− mice. As NK cell effector functional is strongly dependent on appropriate priming and activation, we sought to determine any functional differences between NK cells in WT and GrzM −/− mice following Listeria challenge. In the livers, the frequency of NK cells positive for intracellular IFNγ was significantly increased (P=0.0115) in GrzM −/− mice at this time point (Figure 2g). Thus, the reduced infiltration of NK cells in GrzM-deficient mice correlated with increased activation of these cells, concomitant with the reduced bacterial burden.

Intrinsic capacity of GrzM −/− NK cells to secrete MIP-1x is impaired. The chemokine MIP-1x is known to induce NK cell trafficking and migration to inflammatory sites. As we had observed fewer NK cells in the livers of Listeria-challenged GrzM −/− mice (Figure 2), we next sought to determine any effect of GrzM on MIP-1x production. To reveal whether GrzM deficiency influences the production and/or release of MIP-1x in vivo, the amount of chemokine secreted in bulk leukocyte cultures from livers of GrzM −/− and WT mice 24 h after L. monocytogenes infection was determined.

We observed a significant reduction (P=0.0376) in MIP-1x produced by GrzM-deficient leukocytes compared with WT (Figure 3a). In order to determine whether GrzM delivery to NK cells to macrophages could alter MIP-1x production, we co-cultured NK cells purified from LPS-stimulated WT and GrzM −/− mice with bone marrow-derived macrophages and assessed MIP-1x production using cytometric bead array (CBA). We observed a significant increase in MIP-1x (P=0.0003) produced in co-cultures of NK cells purified from GrzM −/− mice when compared with co-cultures containing WT NK cells (Figure 3b).

Whereas the requirement for GrzM in MIP-1x release from macrophages was of interest, the decrease in MIP-1x production by activated NK cells from GrzM-deficient mice suggested an intrinsic role for GrzM in MIP-1x export. We next aimed to define the pathways by which GrzM could regulate this process intrinsically. NK cells from naive WT and GzM −/− mice were stimulated ex vivo with different combinations of IL-12p70, IL-15, IL-18 and IL-2. When stimulated with IL-12 (1 ng/ml) and IL-18 (5 ng/ml) or with IL-12 (1 ng/ml) and IL-2 (1000 U/ml), similar amounts of MIP-1x or IFN-γ were detected in the supernatant after 6 and 24 h (Figures 3c and d). In contrast, stimulation of NK
cells with a combination of IL-12 (1 ng/ml) and IL-15 (10 ng/ml) led to significantly reduced amounts of secreted Mip-1α by GrzM−/− NK cells compared with WT NK cells after 3 h (P = 0.0126), 6 h (P = 0.0097) and 24 h (P = 0.0417) (Figure 3e). This effect was specific for MIP-1α as comparable amounts of IFN-γ and MIP-1β were detected in the supernatants (Figures 3f and g). MIP-1α mRNA levels of WT and GrzM−/− NK cells were similarly upregulated after IL-12/IL-15 stimulation for 4 and 24 h, indicating that the reduction in MIP-1α secretion by GrzM−/− NK cells was not due to reduced gene transcription (Figure 3h). To exclude any contribution of other Grz or Pfp, NK cells isolated from pfp−/−, GrzA−/− and GrzB−/− mice were stimulated, and the supernatants were analyzed for secreted MIP-1α. Neither NK cells from pfp−/− mice, nor from GrzA−/− or B−/− mice, showed reduced MIP-1α secretion at any time point after

Figure 1  GrzM−/− mice are less susceptible to an infection with L. monocytogenes. (a–e) Female, age-matched WT and GrzM−/− mice were infected i.v. with 10⁴ CFU of L. monocytogenes. (a) The recipients were monitored daily for weight loss and signs of illness. (b) After 12 h, on days 1 and 3 after infection mice were killed, livers and spleens were homogenized, serially diluted in PBS and plated on 5% sheep agar plates. Bacterial colonies were counted after 24 h of incubation at 37 °C. (c) Blood samples were taken 12, 24 and 72 h after infection and ALT levels were determined using the Architect plus analyser. (d) H & E staining of liver sections of WT and GrzM−/− mice 24 and 72 h after Listeria infection (N = necrotic foci, I = inflammatory infiltrations) (representative). Arrows indicate inflammatory foci. (e) TUNEL+ staining of WT and GrzM−/− mice 24 and 72 h after Listeria infection (representative). Pictures were taken with an Olympus BX-51, original magnification for images × 4 (NA 0.13, WD17 mm) or × 20 (NA 0.7, WD 0.65 mm). (f) The survival of recipients was monitored. Mice were killed when they had lost 20% of their initial body weight. Results are pooled (a) from two independent experiments with n = 10, (b and c) from six independent experiments with n = 5–15, (f) from two independent experiments, n = 12.
IL-12/IL-15 stimulation (Figure 3i). Importantly, human NK cells secreted significantly higher amounts of MIP-1α (P = 0.0137) after 24 h when stimulated with IL-12/IL-15 compared with stimulation with IL-12/IL-18 (Figure 3j).

GrzM and MIP-1α co-localize within the cytotoxic granules of primary human NK cells. We next sought to assess whether GrzM has a role in the secretion of MIP-1α. Wagner et al.37 showed that HIV-specific cytotoxic CD8+ T cells co-localize GrzA and MIP-1α within the cytotoxic granules, and GrzA and MIP-1α are secreted together upon antigen-specific stimulation. To analyze how and where GrzM and MIP-1α interact within NK cells, confocal microscopy of naive human NK cells was performed. Our analysis revealed that in naive NK cells GrzM (stained green) and MIP-1α (stained red) are co-localized in the cytotoxic granules (Figure 4a). In naive NK cells, GrzM/MIP-1α-positive granules were distributed across the cytoplasm in small vesicles of relatively uniform size. Complementing these analyses, intracellular staining of MIP-1α in IL-12/IL-15-activated mouse NK cells revealed higher frequencies of MIP-1α+ GrzM+/−/− NK cells compared with MIP-1α+ WT NK cells.
cells at 4 and 24 h after stimulation (Figure 4b). A significant increase in the percentage of MIP-1α+ NK cells ($P = 0.0043$) was also observed in the livers of GrzM−/− mice 24 h after the infection with *Listeria* (Figure 4c). Thus, GrzM and MIP-1α are in close contact within the cytotoxic granules of NK cells. Furthermore, our data suggest that IL-15 is a key cytokine regulating GrzM-specific release of MIP-1α from NK cells.
Impaired migration of NK cells towards MIP-1α secreted by GrzM−/− NK cells. We have shown that GrzM−/− NK cells secrete less MIP-1α after stimulation with IL-12/-15 or during Listeria infection, and that fewer NK cells are recruited into the livers of Listeria-infected GrzM-deficient mice. To evaluate whether these two observations are causally linked, chemotaxis assays were performed. Culture supernatants from WT and GrzM−/− NK cells stimulated for 4 h with IL-12 and IL-15 were added to the bottom chambers of transwell plates. Naive WT or GrzM−/− NK cells were incubated in the top chambers, and NK cell migration was assessed 4 h later. When we used supernatant from GrzM−/− NK cells, the migration of both GrzM−/− and WT NK (P = 0.0138) cells was strongly impaired (Figure 5a). In contrast, WT and GrzM−/− NK cells showed a similar migratory capacity towards WT supernatant, indicating that the impaired migration of GrzM−/− NK cells is not a result of a defect in NK cell chemotaxis. When MIP-1α was neutralised, the migration towards WT supernatant of WT (P = 0.0265) and GrzM−/− NK cells was reduced to background levels (Figure 5b). These data demonstrate that GrzM is required for optimal NK cell recruitment, and that, in turn, this recruitment is MIP-1α-dependent.

GrzM does not process/activate MIP-1α. The ectodomain of plasma membrane-bound CD26, an aminopeptidase, truncates MIP-1α by processing the N-terminus of the LDβ form after Proline. Owing to the acidic environment of cytotoxic granules (pH 5.5), GrzM (a neutral serine protease) has suboptimal catalytic activity while being stored in that compartment, making it more likely that GrzM might process MIP-1α following delivery to the APC, as macrophages do not synthesise GrzM. As MIP-1α contains a potential site for cleavage by GrzM (Leu-Ala-Val) within the signal peptide at position 15-16-17 (Figure 6a), it was necessary to formally exclude a direct effect of GrzM processing of MIP-1α. In order
Figure 5 Migration of NK cells towards MIP-1α secreted by GrzM−/− NK cells is impaired. (a and b) Migration of naïve WT and GrzM−/− NK cells towards supernatant of WT or GrzM−/− NK cells stimulated for (a) 4 h with IL-12/IL-15 or media or (b) supernatant treated with a neutralizing antibody against MIP-1α. Results are pooled from three experiments (n = 6).

Figure 6 GrzM does not process MIP-1α. (a) Human recombinant full-length GST-tagged MIP-1α (sequence start mature MIP-1α: bold letters) was generated. The protein contains a GrzM consensus cleavage site in the polylinker region between GST and the start of full-length MIP-1α, as well as a potential cleavage site for GrzM in the full-length MIP-1α sequence. (b) This product was then incubated with low concentrations of human recombinant GrzM or a Serine/Alanine (S/A) mutant. The resulting products were used for immunoblotting (c) N-terminal sequencing and (d) mass spectrometry.
to do this, we utilised a GST fusion protein that incorporated full-length MIP-1α coupled to GST through a polylinker region. We incorporated a consensus cleavage site for human GrzM (Valine-Leucine-Phenylalanine) as a positive control (Figure 6a). Exposure of the fusion protein to low levels of active GrzM generated a protein product of ~10 kDa, which was not produced by a catalytically inactive (Serine/Alanine-mutated) GrzB (Figure 6b). Protein purification and mass spectrometry established that the C-terminal cleavage product had a MW of 11687 daltons (Figure 6d), which was well in excess of a product processed at the physiological site but corresponded to the mass predicted for full-length MIP-1α attached to a portion of the adjacent polylinker. N-terminal sequencing proved that processing of the GST fusion protein had taken place at the artificial site in the linker, but not within the MIP-1α polypeptide sequence, and specifically not at the potential GrzM consensus sequence (Figure 6c). This result clearly indicated that direct processing by GrzM is not responsible for MIP-1α activation or release.

Discussion
GrzM is not recognised as a principal mediator of apoptosis and is not essential for tumor control. Conversely, GrzM contributes to inflammatory processes observed during MCMV infection and LPS-induced endotoxicosis; however, the mechanism is unclear. Our data now provide a molecular target for GrzM of biological relevance to inflammation. These studies demonstrate that GrzM is required for the optimal release of MIP-1α from NK cells. Importantly, MIP-1α release from GrzA+ and GrzB− NK cells was not affected, GrzmC deficiency did not alter MIP-1α release, and the pathway was strictly dependent upon the activity of IL-12 and IL-15, indicating the strict specificity of this interaction. We have also demonstrated co-localisation of GrzM and MIP-1α in the cytotoxic granules of human NK cells. As MIP-1α is an important regulator of NK cell recruitment during inflammation, our data suggest that GrzM-dependent MIP-1α production may auto-regulate the migration of NK cells, acting as an ‘internal-adjuvant’ for the amplification of inflammation.

Twenty-four hours after L. monocytogenes infection, no difference was observed in the bacterial load from GrzM−/− and WT mice. This result indicated that GrzM had no influence on the initial infection of, or on the initial replication of the bacterium in, resident macrophages. However by 72 h, markedly fewer bacteria were recovered from the organs of GrzM−/− mice. This was a surprising outcome since GrzA and B deficiency reportedly resulted in increased pathogen burden. At later time points, GrzB−/− mice were less susceptible to Listeriosis when compared with their WT counterparts, exhibiting reduced liver damage, weight loss and considerably lower concentrations of pro-inflammatory serum cytokines (Supplementary Figure 1), all hallmarks of a ‘cytokine storm’-induced sepsis. These data suggested that GrzM might contribute to inflammation by amplifying the immune response, resulting in the high levels of cytokine production observed in WT mice. Indeed, co-incidence with the reduced Listeria burden we observed a reduction in both serum cytokine levels and NK cell recruitment to the livers of GrzM-deficient mice.

As the function of NK cells is strongly dependent on appropriate priming, this observation suggested that the effects we observed in GrzM-deficient mice may arise from a more targeted immune response. After infection, NK cells are activated by cytokines and chemokines as well as by direct cell–cell contact. These interactions result in activation of NK cells and production of pro-inflammatory cytokines. Conversely, NK cells can influence the behaviour of accessory cells in return, either by the secretion of cytokines, such as IFNγ, or by cytotoxicity sculpting the subsequent immune response by altering the ability of the APC to continually stimulate inflammatory leukocytes. Hence, the reduced recruitment of NK cells to the liver in GrzM−/− mice could heavily influence crosstalk at the site of infection, resulting in a reduced inflammatory cytokine milieu. Our data suggest that, within the livers of GrzM−/− mice, the cellular immune-milieu and the resulting crosstalk is more beneficial for the host compared with the WT situation. This altered milieu results in the bacterial infection being contained more efficiently within the liver, preventing the development of aggressive systemic bacteremia.

MIP-1α is involved in the recruitment of NK cells to the liver; however, it has been assumed that the source of this chemokine is tissue-resident APC activated via TLR signals. Similar to other groups, we have found that NK cells can be a source of MIP-1α; however, its release is specifically dependent upon the activity of GrzM. Our data demonstrate that GrzM and MIP-1α co-localise in the cytotoxic granules, suggesting that GrzM may be a promising candidate to process or to truncate MIP-1α. The highly related murine and human MIP-1α are synthesised as 92 aa precursor proteins, which are matured by peptidases that cleave the hydrophobic signal sequence. As GrzM is a protease, our primary focus for the mechanism by which it increases MIP-1α output was to test for processing of MIP-1α by GrzM. Despite GrzM clearly being capable of cleaving at an accessible but artificial GrzM consensus sequence, no physiologically relevant interaction between GrzM and MIP-1α could be detected. Mouse and human GrzM exhibit very similar, although not identical, substrate specificities, as is the case for all Grzs. For example, both mouse and human GrzB cleave after selected aspartate residues but have different extended substrate preferences that reflect the different evolutionary pressures faced by the different species over many millions of years.

Despite this, both are strongly pro-apoptotic, albeit they activate subtly different apoptotic pathways. All of the known functions of the granule exocytosis pathway are conserved across species, and as both mouse and human granules containing GrzM and MIP-1α, it is likely that a similar mechanism underpins MIP-1α release in both species.

A combination of cytokines is the key to inducing NK cell activation, as single agents do not stimulate NK to effector function. This complex requirement is very relevant to both the clinical application of NK cells and to studies of NK cell regulation of adaptive immunity, as optimal NK cell activation is required in order to elicit the appropriate immune response. For example, overstimulation of NK cells results in a reduced CD8 T-cell response limiting the generation of functional memory CD8 T cells. It has been shown that the combination of IL-18 and IL-12 induce IFN-γ production, whereas IL-15/IL-12 induces IL-10, MIP-1α and TNF-α.
Interestingly, optimal IFN-γ production requires priming via IL-18 and subsequent secondary stimulation (4 h after priming) by IL-12. A similar mechanism has been demonstrated for Pfp and GrzB upon IL-15 priming and subsequent IL-12 stimulation. The observation that GrzM-dependent MIP-1α production occurs only after IL-15 and IL-12 stimulation improves our understanding of GrzM biology and potentially the adoptive transfer of NK cells. Signal transduction following IL-15 stimulation has been extensively mapped, facilitating future studies that will identify the interaction between IL-15 and Granzyme M (GrzM). Using the appropriate NK cell stimulation to activate chemokine production and to amplify immune responses may prove useful for cellular therapies aimed at enhancing CD8 T-cell memory. Significantly, NK cell therapy is gaining traction in many clinical settings but the field remains young and the key components regulating the immune response are not well defined.

Collectively, our study shows for the first time that GrzM can specifically regulate the release of the β-chemokine MIP-1α. Furthermore, we have demonstrated that the interaction between GrzM and MIP-1α is sensitive to cytokine stimulation combinations. These results demonstrate that GrzM can influence the recruitment of immune cells to inflammatory sites and that this interaction may be amenable to therapeutic targeting through specific cytokine stimulation of adoptively transferred NK cells.

Materials and Methods

Mice and bacterial infections. All experimental procedures and tissue/sample collections were approved by the Animal Ethics Committee at the Peter MacCallum Cancer Centre and the Department of Microbiology and Immunology of the University of Melbourne. B6.GrzM-deficient (GrzM<sup>C2/C2</sup>), B6.Ppf-deficient (ppf<sup>C2/C2</sup>) and GrzB-deficient (GrzB<sup>C2/C2</sup>) mice were generated<sup>23</sup> and maintained at the Peter MacCallum Cancer Centre under SPF conditions. Age-response are not well defined. GrzB<sup>C2/C2</sup> mice were infected with <i>L. monocytogenes</i> after infection. Liver and spleen tissues were homogenized and the number of viable cells was quantified by two-way ANOVA test. To study bacterial clearance, recipients were killed on pre-determined days after infection. Liver and spleen tissues were homogenized and the number of viable cells was quantified by two-way ANOVA test. Mice were considered to have succumbed to disease and were killed when they had lost 20% of their starting body weight. ALT levels were measured using the Architect plus analyser (ci 4100, Abbott Park, IL, USA) to facilitate counting of migrated cells. A neutralizing antibody against MIP-1α was used to prevent MIP-1α from binding to receptors on the target cell surface. Control slides were incubated with only secondary antibodies or the mismatching antibodies. After staining, specimens were analysed with the Olympus BX-51 microscope at × 4 (NA 0.13, WD 17 mm) and × 20 (NA 0.7, WD 0.65) magnifications.

Serum alanine aminotransferase (ALT). Blood was taken either from the retro-orbital sinus or by cardiac puncture at the indicated times, and serum ALT levels were measured using the Architect plus analyser (cl 4100, Abbott Park, Chicago, IL, USA), as an indicator of hepatic cell damage.

Flow cytometry. The following antibodies from e-Bioscience (San Diego, CA, USA) were used: CD8-PE-CY7 (clone 53-6.7), CD4-APC-Cy7 (clone RM4-5), NK.1.1-PE (PK 136), CD3-Pacific Blue (17A2), CD69-FITC (H1.2F3), CD11b-APC-Cy7 (M170), Ly6G-PE (1A8), F4/80-APC (BM8), IFN-γ-FITC (XMG1.2), Mic-1x-APC (FFM3s), CD27-PE-Cy7 (LG.7F9). Single-cell suspensions of livers and spleens from WT and GrzM<sup>C2/C2</sup> mice were processed at indicated times after Listeria infection and stained for immune-cell subsets. For intracellular staining, the Cytotox/Cytoperm kit (554715; BD Biosciences Pharmingen, San Diego, CA, USA) from BD was used according to the manufacturer’s instructions. For intracellular MIP-1α staining at 24 h, Golgi stop was added 6 h before cells were harvested.

Ex vivo stimulation of NK cells. Single-cell suspensions of spleen from naive WT, GrzM<sup>C2/C2</sup>, ppf<sup>C2/C2</sup> or GrzB<sup>C2/C2</sup> mice were enriched for NK cells (NK1.1<sup>+</sup>CD3<sup>−</sup>) by negative selection with a mouse NK cell isolation kit II (Miltienyi Biotech, Bergisch Gladbach, Germany) and sorted to purity (BDCA, San Jose, CA, USA). Human NK cells were enriched from Buffy-coats (EasySep negative enrichment kit, Stemcell Technologies, Vancouver, BC, Canada; 19055). Mouse or human NK cells were stimulated with 1 ng IL-12/10 ml and 10 ng IL-15/10 ml, 1 ng IL-12/10 ml and 5 ng IL-18/10 ml, or 1000 U/ml IL-2 and 1 ng IL-12/10 ml for 3, 6 and 24 h or for 4 and 24 h. Cells were used for surface marker staining, intracellular staining or to extract RNA for RT-PCR. Supernatants were harvested for CBA assays or were analysed by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

Cytometric bead array. The CBA Flex Systems Kit (BD Biosciences) was used to measure IFN-γ, MIP-1α and MIP-1β according to the manufacturer's instructions.

Semi-quantitative real-time PCR analysis for Mip-1α. NK cells enriched from single-cell suspensions of spleen cells from naive WT and GrzM<sup>C2/C2</sup> mice were stimulated with cytokines as described above. Total RNA was extracted by using the RNeasy mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Quantitative real-time PCR was performed on the ABI4800 light cycler, using cytochrome with high ROX for internal calibration (Agilent Technologies, Santa Clara, CA, USA). The following conditions were used for real-time PCR: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s. A dissociation curve was determined to analyse primer specificity that was 95 °C for 15 s, 95 °C for 15 s and 95 °C for 15 s. All data were collected after the annealing step for each cycle and for the dissociation curve, after the annealing step and the final 95 °C denaturation step. Data from each sample were quantified relative to the housekeeping gene L32. All samples were run in triplicate and data reflect the normalised ΔΔC<sub>T</sub> (10000/[2ΔΔC<sub>T</sub>]) of the triplicate wells. Primers were as follows: mouse Mip-1α: forward: 5'-GCCATATGGAGCTGACAC-3'; mouse Mip-1α reverse: 5'-TGCTCCCAAGACGCTCAG-3'.

Co-culture experiments. NK cells enriched from single-cell suspensions of spleen cells from naive or LPS-injected WT and GrzM<sup>C2/C2</sup> mice (2 × 10<sup>6</sup>/ml) were co-cultured with naive bone marrow-derived macrophages (1 × 10<sup>6</sup>/ml) at a ratio of 1:5. Cells were incubated 4 h at 37 °C in air containing 5% CO<sub>2</sub>. Culture supernatants were then analysed by CBA as described above.

Cell migration assay. NK cells enriched from single-cell suspensions of spleen cells from naive WT and GrzM<sup>C2/C2</sup> mice were stimulated for 6 h with cytokines as described above, harvested and stored at −20 °C. On the following day, NK cells were enriched from as described above from naive GrzM<sup>C2/C2</sup> and WT mice and adjusted to 2 × 10<sup>6</sup>/ml. One hundred microlitres of the enriched naive WT and GrzM<sup>C2/C2</sup> NK cells (2 × 10<sup>5</sup>) were added into the top chamber of transwell membranes (Corning, 5 μm, CLS3421-48EA) over 600 μl of the defrosted, cell-free supernatant from the IL-12/IL-15-stimulated NK cells and incubated for 4 h. The transwells were then removed, and the plates fixed (MBF), permeabilized (0.1% Triton) and stained with DAPI (Invitrogen, Carlsbad, CA, USA) to facilitate counting of migrated cells. A neutralizing antibody against MIP-1α (Abcam, Cambridge, UK; ab 10383) was used as a specificity control for the response to MIP-1α.

Immunofluorescence. Human NK cells were enriched from Buffy-coats (EasySep negative enrichment kit, Stemcell Technologies, 19055). A total of 2 × 10<sup>6</sup> cells/ml were cytospun onto slides, air-dried, fixed in Bouins for 10 min, permealized with Triton for 5 min and blocked with 1% BSA in PBS for 30 min at 4 °C. Slides were incubated with goat anti-human MIP-1α (R&D Systems, AF-270-NA; 1:50) and mouse anti-human GrzM (1:500) for 45 min. After two washes with PBS slides were incubated with Alexa 488-conjugated anti-mouse IgG1 and Alexa 649 donkey anti-goat IgG for further 45 min (both diluted 1:1000; Molecular Probes, Grand Island, NY, USA). Control slides were incubated with only secondary antibodies or the mismatching antibodies. After staining, specimens
were washed in PBS and a drop of Dapi anti-fade (Invitrogen) was used for nucleic staining, as well as to reduce photo-bleaching. Slides were examined by confocal imaging on a Nikon CS 2, original magnification × 40 (WD 0.24 mm oil) using the NIS software (Nikon Instruments Inc., Melville, NY, USA).

Western blot analysis. Recombinant full-length human GST-tagged MIP-1α, into which a processing consensus site for human GrMZ (Valine Leucine Phenylalanine) was encoded in the polylinker region (Abnova, P01), was incubated with human recombinant GrzM or a S/A mutant (inactive) GrzM for the indicated times at 37 °C, or left on ice as a control. Products were separated on SDS-polyacrylamide gel and immunoblotting was performed as described previously using goat anti-human MIP-1α (R&D Systems, AF-270-NA) Blots were developed with ECL using the AP substrate CDP-Star (Applied Biosystems, Carlsbad, CA, USA).

N-terminal sequencing. GrzM-digested GST-MIP-1α was resolved by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane, then stained with Coomassie brilliant blue G250. The band at ~11 kDa was excised and subjected to N-terminal sequence analysis by Edman degradation (Australian Proteome Analysis Facility, Macquarie University, Sydney, Australia).

Mass spectrometry. Intact or GrzM-cleaved GST-MIP-1α were precipitated using methanol and chloroform to remove salts, according to the method of Wessel and Fluegge and dissolved in 2% formic acid for direct injection into an ESI-TOF mass spectrometer (Agilent Technologies 6220) with a constant flow of 0.25 mmol/min 50% acetonitrile in 0.1% formic acid. Data were collected in positive ion mode and voltages used were capillary voltage 3.5 kV, tube lens 100–2000 m/z and the internal reference ions were 121.050873 and 122.09798. Acquisition was performed using the Agilent Mass Hunter Acquisition software version B.02.01 (B2116.30), and analysis was performed using Mass Hunter version B.03.01.

Statistical analysis. For statistical analysis, the data were subjected to the two-tailed Student’s t-test, two-way ANOVA (weight loss experiments) or Mantel–Cox test (survival). *P-values: *P < 0.05, **P < 0.005, ***P < 0.0005.

Conflict of Interest

The authors declare no conflict of interest.

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