Widespread discrepancy in Nnt genotypes and genetic backgrounds complicates granzyme A and other knockout mouse studies

Daniel J Rawle††, Thuy T Le††, Troy Dumenil†, Cameron Bishop†, Kexin Yan†, Eri Nakayama†, Cameron Bishop†, Phillip I Bird†, Andreas Suhrbier†,4*

1QIMR Berghofer Medical Research Institute, Brisbane, Australia; 2Department of Virology I, National Institute of Infectious Diseases, Tokyo, Japan; 3Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Monash University, Melbourne, Australia; 4Australian Infectious Disease Research Centre, GVN Center of Excellence, Brisbane, Australia

Abstract

Granzyme A (GZMA) is a serine protease secreted by cytotoxic lymphocytes, with Gzma-/- mouse studies having informed our understanding of GZMA's physiological function. We show herein that Gzma-/- mice have a mixed C57BL/6J and C57BL/6N genetic background and retain the full-length nicotinamide nucleotide transhydrogenase (Nnt) gene, whereas Nnt is truncated in C57BL/6J mice. Chikungunya viral arthritis was substantially ameliorated in Gzma-/- mice; however, the presence of Nnt and the C57BL/6N background, rather than loss of GZMA expression, was responsible for this phenotype. A new CRISPR active site mutant C57BL/6J GzmaS211A mouse provided the first insights into GZMA's bioactivity free of background issues, with circulating proteolytically active GZMA promoting immune-stimulating and pro-inflammatory signatures. Remarkably, k-mer mining of the Sequence Read Archive illustrated that ≈27% of Run Accessions and ≈38% of BioProjects listing C57BL/6J as the mouse strain had Nnt sequencing reads inconsistent with a C57BL/6J genetic background. Nnt and C57BL/6N background issues have clearly complicated our understanding of GZMA and may similarly have influenced studies across a broad range of fields.

Editor's evaluation

This paper is of great interest as a serendipitous discovery that, in the course of investigating the physiological role of granzyme A, has revealed the significance of the Nnt gene mutation in the inflammatory responses in mouse models. For many researchers in the fields of medicine and biology using C57BL/6 mice, the data obtained in this study will provide a useful opportunity to revisit previous findings and to gain new insights.

Introduction

Granzyme A (GZMA) is a granule trypsin-like serine protease (trypase) secreted by cytotoxic lymphocytes such as NK cells (Fehniger et al., 2007; Wu et al., 2019), NKT cells (Gordy et al., 2011), and CD8+ cytotoxic T lymphocytes (Suhrbier et al., 1991). The traditional view has been that GZMA is a cytotoxic mediator that is secreted into the immunological synapse, entering the target cell via perforin pores, whereupon certain cytoplasmic proteins are cleaved, resulting in the initiation of cell death pathway(s) (Liesche et al., 2018; Martinvalet et al., 2008; Wu et al., 2019; Zhou et al., 2020).
A key tool in the quest to understand the physiological role of GZMA has been the use of Gzma^-/- mice (Ebnet et al., 1995). For instance, control of viral infections can be compromised in Gzma^-/- mice (Loh et al., 2004; Müllbacher et al., 1996; Pereira et al., 2000; Riera et al., 2000), with cytotoxic lymphocytes from these mice reported to be less able to kill target cells (Pardo et al., 2002; Pardo et al., 2004; Shresta et al., 1999; Susanto et al., 2013). Like granzyme B (GzmB), GZMA has thus been classified as a cytotoxic granzyme (Golstein and Griffiths, 2018; Mpande et al., 2018; Muraro et al., 2017; Zhou et al., 2020), although in several studies a role for GZMA in mediating cellular cytotoxicity was not observed (Ebnet et al., 1995; Joeeckel and Bird, 2014; Regner et al., 2009; Regner et al., 2011; Smyth et al., 2003). In a range of settings, GZMA has also been associated with the promotion of inflammation, providing an additional or alternative view of its physiological role, although consensus on mechanisms has remained elusive (Metkar et al., 2008; Park et al., 2020; Santiago et al., 2020; Santiago et al., 2017; Schanoski et al., 2019; Shimizu et al., 2019; van Daalen et al., 2020; Wensink et al., 2015; Wilson et al., 2017), with a number of potential intracellular and extracellular targets for GZMA reported. These include pro-IL-1β (Hildebrand et al., 2014), SET complex proteins (Mandrup-Poulsen, 2017; Mollah et al., 2017), gasdermin B (Zhou et al., 2020), mitochondrial complex I protein NDUFS3 (Martinvalet et al., 2008), protease activated receptors (Hansen et al., 2005; Sower et al., 1996; Suidan et al., 1994; Suidan et al., 1996), TLR2/4 (van Eck et al., 2017), and TLR9 (Shimizu et al., 2019). Gzma^-/- mice have also been used to show a role for GZMA in inter alia diabetes (Mollah et al., 2017), cancer (Santiago et al., 2020), bacterial infections (García-Laorden et al., 2016; García-Laorden et al., 2017; van den Boogaard et al., 2016), and arthritis (Santiago et al., 2017). GZMA's bioactivity has generally (Plasman et al., 2014; Santiago et al., 2017), with a number of potential intracellular and extracellular targets for GZMA reported. These include pro-IL-1β (Hildebrand et al., 2014), SET complex proteins (Mandrup-Poulsen, 2017; Mollah et al., 2017), gasdermin B (Zhou et al., 2020), mitochondrial complex I protein NDUFS3 (Martinvalet et al., 2008), protease activated receptors (Hansen et al., 2005; Sower et al., 1996; Suidan et al., 1994; Suidan et al., 1996), TLR2/4 (van Eck et al., 2017), and TLR9 (Shimizu et al., 2019). Gzma^-/- mice have also been used to show a role for GZMA in inter alia diabetes (Mollah et al., 2017), cancer (Santiago et al., 2020), bacterial infections (García-Laorden et al., 2016; García-Laorden et al., 2017; van den Boogaard et al., 2016), and arthritis (Santiago et al., 2017). GZMA's bioactivity has generally (Plasman et al., 2014; Schanoski et al., 2019; Zhou et al., 2020), but not always (Shimizu et al., 2019; van Eck et al., 2017), been associated with GZMA's protease activity, with circulating GZMA in humans shown to be proteolytically active (Spaeny-Dekking et al., 1998).

After infection with the arthritogenic alphavirus, chikungunya virus (CHIKV) (Suhrbier, 2019), infected Gzma^-/- mice showed a substantially reduced overt arthritic foot swelling when compared to infected C57BL/6J (6J) mice (Wilson et al., 2017). However, we show herein that active site mutant Gzma^-S211A mice generated by CRISPR on a 6J background showed no significant differences in CHIKV arthritic foot swelling when compared with 6J mice. The apparent contradiction was resolved when it emerged that Gzma^-/- mice had a mixed 6J and C57BL/6N (6N) background. Gzma^-/- mice retained expression of the full-length nicotinamide nucleotide transhydrogenase (Nnt) gene, whereas 6J mice have a truncated Nnt with a 5 exon deletion (Fontaine and Davis, 2016). The presence of full-length Nnt and the mixed 6N/6J genetic background (rather than absence of GZMA expression) emerged to be responsible for amelioration of CHIKV arthritic foot swelling in Gzma^-/- mice. As much of our understanding of the physiological role of GZMA comes from studies in Gzma^-/- mice, we used the Gzma^-S211A mice to gain new insights into GZMA function that were not compromised by genetic background.

The enzyme, nicotinamide nucleotide transhydrogenase (NNT), also known as proton-translocating NAD(P)+ transhydrogenase (EC 7.1.1.1), is located in the inner mitochondrial membrane and catalyzes the conversion of NADH plus NADP+ to NAD+ plus NADPH, while H+ is pumped from the inter-membrane space into the mitochondrial matrix (Rydstrom, 2006). NNT thereby sustains mitochondrial antioxidant capacity through generation of NADPH (Ward et al., 2020), with the loss of active NNT in 6J mice associated with reduced ability to detoxify reactive oxygen species (ROS) via the glutathione and thioredoxin pathways (McCambique et al., 2019; Meimardou et al., 2018; Ronchi et al., 2013; Rydstrom, 2006). As redox regulation is involved in many cellular processes (Gambhir et al., 2019; Lingappan, 2018; Sun et al., 2020) and genetic backgrounds are known to affect phenotypes (Leskov et al., 2017; Morales-Hernandez et al., 2018; Rao et al., 2020; Salerno et al., 2019; Voznilek et al., 2018; Williams et al., 2021; Wolf et al., 2016), we sought to determine how other studies in GMO mice might have been affected by 6J vs. 6N background differences using the Nnt gene as a genetic marker. k-mer mining of RNA-Seq datasets deposited in the NCBI Sequence Read Archive (SRA) revealed that ≈27% of Run Accessions and ≈38% of BioProjects listing the mouse strain as ‘C57BL/6J’ had Nnt reads inconsistent with a 6J background. Although reported as underappreciated in the metabolism literature (Fontaine and Davis, 2016), potential problems associated with differences in Nnt and/or other background genes clearly extends well beyond this field and are not restricted to Gzma^-/- mice.
Results

CHIKV inflammatory arthritis in Gzma<sup>S211A</sup> mice

We reported previously that the inflammatory arthritis induced by CHIKV infection (manifesting as overt foot swelling) was significantly lower in Gzma<sup>-/-</sup> mice than in C57BL/6J (6J) mice (Wilson et al., 2017); an observation we confirm herein (Figure 1a). Injection of proteolytically active, but not proteolytically inactive, recombinant mouse GZMA induced inflammatory foot swelling, illustrating directly that GZMA's protease activity is able to drive pro-inflammatory responses (Schanoski et al., 2019). To confirm and extend these findings, a new homozygous GZMA active site mutant mouse was generated using CRISPR technology in 6J mice, with the reactive site serine changed to alanine (Gzma<sup>S211A</sup>) (Susanto et al., 2013; Figure 1—figure supplement 1a–c). Loss of enzyme activity was confirmed by BLT assays (Suhrbier et al., 1991; Figure 1—figure supplement 1d). Intracellular staining (Schanoski et al., 2019) showed that expression of the GZMA proteins in resting splenic NK cells (Fehniger et al., 2007) was comparable for Gzma<sup>S211A</sup> and 6J mice (Figure 1—figure supplement 1e).

When infected with CHIKV, viral titers in feet were not significantly different for Gzma<sup>S211A</sup> and 6J mice (Figure 1—figure supplement 1f), arguing that enzymically active GZMA has no significant antiviral activity against CHIKV. This is consistent with our previous study using Gzma<sup>-/-</sup> mice that also concluded that GZMA has no important antiviral function (Wilson et al., 2017). Surprisingly, however, foot swelling following CHIKV infection was not significantly different between the Gzma<sup>S211A</sup> and 6J mice (Figure 1a). If GZMA's pro-inflammatory bioactivity (Wilson et al., 2017) depends on its protease activity (Schanoski et al., 2019), the results from Gzma<sup>-/-</sup> and Gzma<sup>S211A</sup> mice (Figure 1a) would appear to provide contradictory results.

The effective amelioration of CHIKV arthritic foot swelling in 6J mice following treatment with Serpinb6b (an inhibitor of GZMA) also supported the view that GZMA promotes inflammation in this setting (Wilson et al., 2017). However, Serpinb6b also inhibited CHIKV foot swelling in Gzma<sup>S211A</sup> mice (Figure 1—figure supplement 1g), arguing that Serpinb6b can inhibit other unknown proteases involved in promoting arthritic inflammation. The contention is supported by the broad inhibitory activity of the human orthologue, SerpinB6 (Strik et al., 2004).

Taken together, these new data argued that the role of GZMA in promoting CHIKV arthritis and the results obtained from Gzma<sup>-/-</sup> mice (Wilson et al., 2017) required re-evaluation.

Gzma<sup>-/-</sup> mice have a mixed C57BL/6N-C57BL/6J genetic background

To reconcile the apparent contradictory data from Gzma<sup>S211A</sup> and Gzma<sup>-/-</sup> mice, and cognizant of previously described issues with knockout mice (Teoh et al., 2014), whole-genome sequencing (WGS) of
Gzma<sup>-/-</sup> mice was undertaken (NCBI SRA; PRJNA664888). This analysis unequivocally demonstrated that Gzma<sup>-/-</sup> mice have a mixed genetic background, with ≈60% of the genome showing single-nucleotide polymorphisms (SNPs) and indels present in the C57BL/6N (6N) genome (Mekada et al., 2015; Simon, 2013), with the rest reflecting a 6J background (Figure 3—figure supplement 1). The strain origin of the BL/6-III ES cells used to generate Gzma<sup>-/-</sup> mice was only reported as C57BL/6 (Ebnet et al., 1995). As ES cells from 6J mice have a low rate of germline transmission, ES from 6N mice were frequently used to generate knockout mice (Fontaine and Davis, 2016), with our studies clearly arguing that BL/6-III ES cells also have a 6N background. The reported backcrossing of Gzma<sup>-/-</sup> mice onto C57BL/6 mice (Müllbacher et al., 1999) also clearly did not involve extensive backcrossing onto 6J mice.

Importantly, a large body of literature has resulted from the use of inbred Gzma<sup>-/-</sup> mice (and Gzma<sup>-/-</sup>/Gzmb<sup>-/-</sup> double knockout mice derived from them) with 6J mice used as controls, without being aware of the presence and potential confounding influence of the 6N background (Supplementary file 1).

**Gzma<sup>-/-</sup> mice and 6N (Gzma<sup>+/+</sup>) mice both have reduced foot swelling after CHIKV infection**

An explanation for the significantly lower CHIKV-induced foot swelling seen in Gzma<sup>-/-</sup> mice is that the partial 6N background is influencing the foot swelling phenotype. To test this contention, 6J and 6N mice (both Gzma<sup>+/-</sup>) were infected with CHIKV. 6N mice showed a significant reduction in foot swelling when compared with 6J mice (Figure 1a), arguing that the 6N background, rather than loss of GZMA expression, was primarily responsible for amelioration of foot swelling in Gzma<sup>-/-</sup> mice.

---

**Figure 2.** RNA-Seq of Gzma<sup>-/-</sup> + chikungunya virus (CHIKV) vs. 6J + CHIKV day 6 feet. (a) Selected Ingenuity Pathway Analysis (IPA) Diseases and Function annotations for the 469 downregulated differentially expressed genes (DEGs) for Gzma<sup>-/-</sup> + CHIKV vs. 6J + CHIKV (full set of annotations shown in Supplementary file 2c). (b) IPA cytokine upstream regulators (USRs) downregulated in CHIKV-infected feet of Gzma<sup>-/-</sup> mice (Gzma<sup>-/-</sup> + CHIKV vs. 6J + CHIKV; Supplementary file 2e) plotted by p-value and z-score. Black circles – minor USRs for Gzma<sup>-/-</sup> + CHIKV vs. 6J + CHIKV not identified for 6J + CHIKV vs. 6J mock (see also Figure 4e). (c) RNA-Seq identified 1557 DEGs upregulated in feet for 6J + CHIKV vs. 6J mock infection (Supplementary file 2g). RNA-Seq of Gzma<sup>-/-</sup> + CHIKV vs. 6J + CHIKV day 6 feet identified 469 downregulated DEGs in Gzma<sup>-/-</sup> mice associated with the reduced foot swelling (Supplementary file 2b). Only 19 of these DEGs were shared by these datasets. (d) Gene Set Enrichment Analysis (GSEA) of downregulated DEGs from Gzma<sup>-/-</sup> + CHIKV vs. 6J + CHIKV day 6 feet (Supplementary file 2b) vs. all genes (preranked by fold change) from feet 6J + CHIKV vs. 6J mock infection (Supplementary file 2f).

---

Rawle, Le, et al. eLife 2022;11:e70207. DOI: https://doi.org/10.7554/eLife.70207

4 of 31
The reduced foot swelling in 6N and Gzma−/− mice was unlikely to be due to reduced viral loads as there were no significant differences in viremia for 6N, 6J, Gzma−/−, or GzmaS211A mice (Figure 1b).

RNA-Seq of CHIKV foot swelling for Gzma−/− vs. 6J mice
To gain insights into the reduced foot swelling in Gzma−/− mice (Figure 1a), RNA-Seq was undertaken on day 6 (peak arthritis) to compare gene expression in feet from CHIKV-infected Gzma−/− mice vs. 6J mice (NCBI BioProject PRJNA664644; full gene list in Supplementary file 2a). Differentially expressed genes (DEGs) (n = 1073) were generated after application of a q < 0.01 filter (Supplementary file 2b). When the 1073 DEGs (for Gzma−/− + CHIKV vs. 6J + CHIKV; Supplementary file 2b) were analyzed for Diseases and Functions using Ingenuity Pathway Analysis (IPA), the dominant annotations were associated with decreased cell movement (often leukocyte migration) (Figure 2a, Supplementary file 2c). These findings are consistent with immunohistochemistry data showing significantly reduced T cell and NK cells in the arthritic infiltrates in feet after CHIKV infection of Gzma−/− mice when compared with infected 6J mice (Wilson et al., 2017). IPA upstream regulator (USR) analysis of the 1073 DEGs (Supplementary file 2d) also illustrated that a series of pro-inflammatory cytokine USRs were downregulated in the infected feet of Gzma−/− + CHIKV vs. 6J + CHIKV mice (Figure 2b, Supplementary file 2e), consistent with the reduced foot swelling seen in infected Gzma−/− mice when compared with infected 6J mice (Wilson et al., 2017).

We previously characterized the CHIKV arthritis signature by undertaking RNA-Seq of infected feet during peak foot swelling relative to control uninfected feet (6J + CHIKV vs. 6J mock infection) (Wilson et al., 2017). We reanalyzed the data (FastQ files NCBI BioProject PRJNA431476) using STAR aligner, RSEM EdgeR, and the more recent mouse genome build (GRCm38 Gencode vM23); all genes are shown in Supplementary file 2f, and a DEG list (with filters q < 0.01, fold change >2) is shown in Supplementary file 2g. The latter 2201 DEGs (for 6J + CHIKV vs. 6J mock infection) were analyzed by IPA, with the USRs shown in Supplementary file 2h. Of these USRs, 103 cytokine USR annotations showed significant upregulation (positive z-scores) after CHIKV infection (Supplementary file 2i). As might be expected, key pro-inflammatory cytokine USRs such as TNF, IFNG, IL6, and IL1B (Suhrbier, 2019) were upregulated during CHIKV arthritis (Supplementary file 2i) were downregulated (negative z-scores) in the ameliorated foot swelling seen in Gzma−/− mice (Figure 2b, Supplementary file 2e). Curiously, however, five minor USRs (PPBP, CTF1, WNT7A, NAMPT, TIMP1) were downregulated in Gzma−/− mice, but were not upregulated during CHIKV infection (Figure 2b, black circles; Supplementary file 2e, yellow). Furthermore, of the 469 DEGs downregulated during CHIKV arthritis in Gzma−/− mice (Supplementary file 2b), only 19 were upregulated DEGs for CHIKV arthritis (Figure 2c, Supplementary file 2g). Gene Set Enrichment Analysis (GSEA) similarly revealed that DEGs downregulated in Gzma−/− mice were not significantly enriched in the upregulated genes for CHIKV arthritis in 6J mice (Figure 2d). Thus, although feet from CHIKV-infected Gzma−/− mice showed annotations associated with reduced cellular infiltrates and pro-inflammatory cytokines, this amelioration of arthritic signatures was associated with downregulation of genes largely not associated with CHIKV arthritis in 6J mice. In summary, arthritis amelioration in Gzma−/− mice was due to the downregulation of a largely distinct set of genes (and some distinct pathways), again arguing that the 6N background plays a key role in this phenotype.

Gzma−/− mice have an intact nicotinamide nucleotide transhydrogenase gene
There are multiple genes associated with inflammation and/or arthritis that differ between Gzma−/− (mixed 6N/6J background) and 6J mice (Supplementary file 3). One gene that has been highlighted as a key difference between 6J and 6N mice is Nnt (Freeman et al., 2006; Mekada et al., 2009; Ripoll et al., 2012; Ronchi et al., 2013; Vozenilek et al., 2018; Figure 3—figure supplement 1c, red box). The function of NNT is primarily to sustain mitochondrial antioxidant capacity through the generation of NADPH, which supports the antioxidant capacity of the glutathione and thioredoxin systems (McCambridge et al., 2019; Meimardou et al., 2018; Ronchi et al., 2013; Rydstöm, 2006; Ward et al., 2020). These systems are generally viewed as having broad anti-inflammatory activities (Ghezzi, 2021; Yodoi et al., 2017).

6N mice have a full-length Nnt gene with 21 protein-coding exons, whereas 6J mice have an in-frame 5-exon deletion removing exons 7–11 (Freeman et al., 2006). Confusingly, the MM10 mouse
build numbers the Nnt exons differently and includes the noncoding exon 1 that is located before the ATG start site. According to this numbering (which is used herein), 6J mice have lost exons 8–12 of 22 exons. The Nnt gene is located only ~6.2 megabases from the Gzma gene on mouse chromosome 13, so >30 backcrosses would be required to segregate these two loci (Silver, 2008; Figure 3—figure supplement 2). The close association of Nnt and Gzma genes also means Gzma<sup>+</sup>-Gzmb<sup>−</sup> double-knockout mice (Supplementary file 1) would also very likely have full-length Nnt. Another Gzma<sup>−</sup> mouse generated using 129/SvJ ES cells (Shresta et al., 1997) would likely have the same issue as 129/SvJ mice also have a full-length Nnt gene.

Alignment of the WGS of Gzma<sup>−</sup> mice (PRJNA664888) to the standard 6J MM10 mouse genome build allowed identification of the neomycin cassette insertion site into the Gzma gene that was used to generate the Gzma<sup>−</sup> mice (Ebnert et al., 1995; Figure 3a). Curiously, this alignment shows a 12-nucleotide insertion in the 6J genome at the Nnt exon 8–12 deletion junction (Figure 3b). The 12 nucleotides are also absent in other 6N WGS data (Figure 3—figure supplement 3a), indicating this is not a unique feature of Gzma<sup>−</sup> mice. This insertion in 6J may have accompanied deletion of Nnt exons 8–12 during the generation of 6J mice (Fontaine and Davis, 2016; Figure 3—figure supplement 3b).

Although a 6N genome sequence is available, it is poorly annotated, hence the C3H/HeJ genome build was used for alignments as it also has a full-length Nnt gene (Figure 3—figure supplement 3c).

Alignment of WGS reads from Gzma<sup>−</sup> mice to the C3H/HeJ genome clearly showed that Gzma<sup>−</sup> mice had a full-length Nnt gene, whereas 6J mice had the expected ~16 kb deletion (Figure 3c). The approach (Figure 3c) was further validated using other 6N and non-6J WGS submissions (Figure 3—figure supplement 3d).

Sashimi plots of RNA-Seq reads aligned to the C3H/HeJ build clearly illustrated that Nnt mRNA from 6J mice was missing exons 8–12, whereas in Gzma<sup>−</sup> mice the full-length Nnt mRNA was expressed (Figure 3d, top). Alignment to the 6J genome and viewed by Sashimi plot showed that exons 7 and 13 are linked in the Nnt mRNA from 6J mice, consistent with expression of a truncated Gzma mRNA species. In contrast, exons 7 and 13 are not linked in the Nnt mRNA from Gzma<sup>−</sup> mice (Figure 3d, blue arrow) as the mRNA, but not the MM10 genome build, contains exons 8–12.

These results were confirmed by RT-PCR using primers located on either side of the exon 8–12 deletion (Huang et al., 2006; Figure 3e). Gzma<sup>−</sup> mice have the longer 6N Nnt PCR product as this Nnt mRNA includes exons 8–12, whereas 6J mice, Gzma<sup>521Ts</sup> (generated by CRISPR on a 6J background), and type I IFN receptor knockout (Ifnar<sup>−/−</sup>) mice (also on a 6J background Swann et al., 2007), all showed a shorter PCR product, consistent with the deletion of exons 8–12 in the Nnt mRNA (Figure 3e). In a separate RT-PCR run, Gzmb<sup>−</sup> mice (Wilson et al., 2017) were shown to be missing Nnt exons 8–12, consistent with a 6J background (Figure 3e).

**The Nnt deletion promotes CHIKV-induced foot swelling**

To determine whether the Nnt gene deletion seen in 6J mice might be responsible for promoting the arthritic foot swelling in CHIKV-infected mice, we generated 6N<sup>Nnt<sup>−</sup>8-12</sup> mice wherein exons 8–12 of Nnt were deleted from 6N mice using CRISPR (Figure 4—figure supplement 1). 6N<sup>Nnt<sup>−</sup>8-12</sup> mice thus have the same deletion of Nnt exons as 6J mice. As before, CHIKV-induced foot swelling was significantly higher in 6J mice when compared with 6N mice (Figure 4a). Importantly, foot swelling in 6N<sup>Nnt<sup>−</sup>8-12</sup> mice was significantly higher than in 6N mice, with the Nnt exon 8–12 deletion increasing foot swelling to levels comparable to those seen in 6J mice (Figure 4a). There were no significant differences in viremia between the mouse strains (Figure 4b). This data illustrates that the absence of a functional Nnt gene (6N<sup>Nnt<sup>−</sup>8-12</sup>) can by itself promote overt foot swelling after CHIKV infection. The data also argues that the presence of functional Nnt gene in Gzma<sup>−</sup> mice likely contributes to the ameliorated foot swelling seen in Gzma<sup>−</sup> mice.

Histology and H&E staining of arthritic feet illustrated that the increased foot swelling seen in 6N<sup>Nnt<sup>−</sup>8-12</sup> mice (compared with 6N mice) was due primarily to increased edema (Figure 4c and d), with no significant differences in cellular infiltrates (Figure 4—figure supplement 2a and b). Edema is a recognized feature of alphaviral arthritides and is well described in CHIKV mouse models (Gardner et al., 2010; Poo et al., 2014; Prow et al., 2019). Immunohistochemistry with anti-CD3 also showed no significant differences in T cell numbers in the inflammatory infiltrates (Figure 4—figure supplement 2c and d). To further characterize the role of Nnt in CHIKV arthritis, day 6 feet from 6N
+ CHIKV vs. 6N + CHIKV were compared using RNA-Seq (Supplementary file 4a and b). IPA USR analysis (Supplementary file 4c) provided 14 cytokine annotations with positive z-scores (Supplementary file 4d) that were associated with the increased foot swelling in 6N ∆Nnt8-12 mice (Figure 4e, brown circle). These 14 cytokine USRs were also upregulated during CHIKV arthritis (Figure 4e, yellow circle).
Figure 4. 6N<sup>ΔNnt8-12</sup> mice. (a) 6N<sup>ΔNnt8-12</sup> mice have the same Nnt exon deletion as 6J mice. Age-matched female 6N<sup>ΔNnt8-12</sup>, 6N, and 6J mice were infected with chikungunya virus (CHIKV) and foot swelling measured over time (n = 5 mice and 10 feet per group). Foot swelling was significantly higher in 6N<sup>ΔNnt8-12</sup> mice when compared with 6N mice on days 2–7 (day 2 p=0.0026, day 7 p=0.0027, t-tests, parametric data distributions; days 3–6 p=0.003, Kolmogorov–Smirnov tests, nonparametric data distributions). Foot swelling was significantly lower in 6N mice when compared with 6J mice (day 2 p=0.042, day 6 p=0.001, day 7 p=0.0005, t-tests, parametric data distributions; days 3 and 5, p=0.002, Kolmogorov–Smirnov tests, nonparametric data distributions). (b) Viremia for the same mice as in (a). (c) H&E staining of feet from 6N<sup>ΔNnt8-12</sup> and 6N mice day 6 post infection showing subcutaneous edema (*). (d) Percentage of foot section area showing overt subcutaneous edema (statistics by Kolmogorov–Smirnov test). (e) RNA-Seq data for four comparisons was analyzed by Ingenuity Pathway Analysis (IPA) and cytokine upstream regulator (USR) overlaps shown. Only cytokine USRs with positive z-scores associated with increased foot swelling are shown.

The online version of this article includes the following figure supplement(s) for figure 4: Figure 4 continued on next page.
brown overlap). The IPA cytokine classification also includes chemokines, with no chemokine USRs identified within these 14 annotations (Figure 4e, brown circle), consistent with the lack of a significant cell migration phenotype (Figure 4—figure supplement 2). In contrast, several chemokine annotations were identified for the Gzma⁻/⁻ mice (Figure 4e, blue circle, blue text) consistent with the reduced inflammatory infiltrate (Wilson et al., 2017). Furthermore, only 8/27 cytokine USRs identified for Gzma⁻/⁻ + CHIKV vs. 6J + CHIKV were also identified for the 6Nnt⁻/⁻ + CHIKV vs. 6N + CHIKV comparison (Figure 4e, blue-brown overlap), arguing that other 6N background genes apart from Nnt ameliorated inflammatory cytokine activity in the feet of CHIKV-infected Gzma⁻/⁻ mice.

Perhaps consistent with the arthritis literature in general (Kato, 2020; Ma and Xu, 2013; Ogata et al., 2019; Suhrbier, 2019), pronounced foot swelling in all comparisons (including MitoTEMPO, see below) was associated with upregulation of TNF, IFNG, and IL-6 USRs (Figure 4e, red box). IL-13 was also a consistently upregulated USR, with IL-13 associated with resolution of arthritic inflammation (Schett, 2019), with peak CHIKV arthritis in 6J mice associated with a significant resolution phase signature (Prow et al., 2019).

**MitoTEMPO ameliorates CHIKV arthritis in 6J mice**

As NNT’s primary function is to sustain mitochondrial antioxidant capacity (McCambridge et al., 2019; Meimaridou et al., 2018; Ronchi et al., 2013; Rydström, 2006; Ward et al., 2020), the data argues that the reduced arthritic foot swelling in 6N mice (and to some extent in Gzma⁻/⁻ mice) is due to increased mitochondrial antioxidant capacity. MitoTEMPO has been widely used as an experimental antioxidant treatment to scavenge mitochondrial ROS in a variety of disease settings (Aoyama et al., 2012; Li et al., 2018; To et al., 2020; Vincent et al., 2020; Wu et al., 2020). Treatment of CHIKV arthritis in 6J mice with MitoTEMPO from day 3 to 8 post infection significantly ameliorated peak foot swelling (Figure 5a). Viremia was not significantly affected by MitoTEMPO treatment, even when treatment was initiated on day 0 (Figure 5b).

Histology and H&E staining revealed that MitoTEMPO treatment significantly reduced the cellular infiltrate in feet day 6 post CHIKV infection of 6J mice when compared with PBS treatment (Figure 5c and d). Edema was also significantly reduced by MitoTEMPO treatment (Figure 5e and f); additional images are shown in Figure 5—figure supplement 1. RNA-Seq analysis (Supplementary file 5a and b) and IPA USR analysis (Supplementary file 5c) illustrated that MitoTEMPO treatment was associated with downregulation of 24 cytokine USRs (Figure 4e, green circle; Supplementary file 5d), with 10 of these also downregulated in 6N vs. 6Nnt⁻/⁻ mice (Figure 4e, green and brown circle overlap). MitoTEMPO treatment in 6J mice and an intact Nnt gene in 6N mice thus provided overlapping anti-inflammatory activities. However, MitoTEMPO was also able to inhibit a series of additional pro-inflammatory arthritic responses including the chemokine CXCL8 and complement factor 5 (C5), with complement promoting arthritic infiltrates in a related alphavirus, Ross River virus (Morrison et al., 2007). Inhibition of CXCL8 and/or C5 is consistent with the reduced cellular infiltrate (Figure 5c and d).

**Reinvestigation of the physiological role of GZMA using poly(I:C)**

Our current understanding of the physiological function of GZMA comes, to a large extent, from multiple studies comparing Gzma⁻/⁻ (and Gzma⁻/⁻/Gzmb⁻/⁻) mice with 6J mice (Supplementary file 1). Given the results herein, many of the reported phenotypes are likely to have arisen, at least in part, from an intact Nnt gene and/or the 6N background, complicating any conclusions regarding the physiological role of GZMA.

To gain new insights into GZMA’s activity in vivo, we sought to find an experimental setting where high levels of GZMA are secreted. We have shown previously that humans, nonhuman primates, and mice have elevated serum GZMA levels after infection with CHIKV (Schanoski et al., 2019; Wilson et al., 2017). Infection of mice with a series of other RNA viruses also resulted in high serum GZMA levels early in infection, with NK cells identified as the likely source (Schanoski et al., 2019). Resting
NK cells constitutively contain abundant levels of GZMA protein (Fehniger et al., 2007), which is usually stored in granules as a mature protease, with the low pH of the granule preventing (premature) proteolytic activity (Stewart et al., 2012).

Polyinosinic:polycytidylic acid (poly(I:C)) can often mimic aspects of the innate responses to RNA viruses (Prow et al., 2017). We thus injected poly(I:C) i.v. into 6J mice and showed that serum GZMA levels reached mean peak serum levels of ≈20 ng/ml of serum after 2 hr, with levels dropping to...
baseline after 24 hr (Figure 6a). Although poly(I:C) treatment is known to activate NK cells (Djeu et al., 1979; Fehniger et al., 2007; Miyake et al., 2009; Ngoi et al., 2008), this rapid and prodigious poly(I:C)-induced release of GZMA into the circulation, to the best of our knowledge, has hitherto not been reported. Type I interferons (IFNs) are also rapidly induced by poly(I:C) (Dempoya et al., 2012; Santiago-Raber et al., 2003), and NK cells express the type I IFN receptor and can respond to type I IFNs (Madera et al., 2016; Mizutani et al., 2012). Injection of poly(I:C) i.v. into Ifnar\(^{-/-}\) mice resulted in a significantly blunted elevation of GZMA (Figure 6a). Type I IFNs thus appear to augment this rapid GZMA release; however, the absence of type I IFN signaling does not prevent GZMA secretion, consistent with previous data (Schanoski et al., 2019). Poly(I:C) treatment of Gzma\(^{S211A}\) mice resulted in serum GZMA levels not significantly different from those seen in 6J mice (Figure 6a), illustrating that the active site mutation does not significantly affect production, secretion, or stability. Finally, although GZMB and GZMA are often considered to be co-expressed (Supplementary file 1), in this setting no serum GZMB was detected (Figure 6—figure supplement 1). GZMB and perforin proteins are not expressed in resting NK cells and appear only after \(\approx 24\) hr of appropriate stimulation (Fehniger et al., 2007).

**RNA-Seq after poly(I:C) injection in Gzma\(^{S211A}\) vs. 6J mice**

Gzma\(^{S211A}\) and 6J mice are both on a 6J genetic background, are both missing Nnt exons 8–12, and both show similar levels of serum GZMA secretion after poly(I:C) treatment (Figure 6a). Thus, the only difference between these strains is that GZMA in Gzma\(^{S211A}\) mice is enzymically inactive (Figure 1—figure supplement 1d). Gzma\(^{S211A}\) and 6J mice were injected with poly(I:C) (as in Figure 6a), with feet and spleens removed 6 hr later and analyzed by RNA-Seq (NCBI BioProject PRJNA666748). The rationale for this time point was to capture early transcriptional events after the peak of serum GZMA. The sample preparation strategy and RNA-Seq data overview is provided in Figure 6—figure supplement 2. The full gene list for feet is provided in Supplementary file 6a, with the 199 DEGs listed in Supplementary file 6b. For spleen, the full gene list is shown in Supplementary file 6c and the four DEGs in Supplementary file 6d. This represents the first study of GMO mice targeting Gzma that is free from the potentially confounding influence of the mixed 6J/6N background.
Circulating proteolytically active GZMA would appear to have limited influence in the spleen as only four DEGs were identified in the spleen of poly(I:C)-treated Gzma<sup>S211A</sup> vs. 6J mice (Supplementary file 6d). Interestingly, the only significantly upregulated DEG in Gzma<sup>S211A</sup> spleens was Mid1, a gene involved in controlling granule exocytosis by cytotoxic lymphocytes (Boding et al., 2014; Boding et al., 2015). GSEA also illustrated that neither up- nor downregulated DEGs identified in the feet were enriched in spleen (Figure 6—figure supplement 3), arguing that in this setting the activity of GZMA in the periphery is not significantly recapitulated in spleen.

Of the 199 DEGs identified in the feet of poly(I:C)-treated Gzma<sup>S211A</sup> vs. 6J mice, 100 were downregulated, with the top annotations associated with negative regulation of protease activity and negative regulation of cell signaling after Cytoscape analysis (Figure 6b, Table 1, Supplementary file 6).

| Category               | Description                                                                 | FDR value   |
|------------------------|-----------------------------------------------------------------------------|-------------|
| GO Process             | Negative regulation of cellular protein metabolic process                   | 1.91E-06    |
| UniProt Keywords       | Protease inhibitor                                                          | 9.20E-06    |
| SMART Domains          | SERine Proteinase INhibitors                                                | 2.60E-05    |
| GO Process             | Negative regulation of catalytic activity                                   | 6.73E-05    |
| GO Process             | Negative regulation of nitrogen compound metabolic process                 | 7.86E-05    |
| GO Process             | Negative regulation of cellular metabolic process                           | 7.86E-05    |
| InterPro Domains       | Serpin superfamily                                                          | 1.10E-04    |
| GO Process             | Negative regulation of molecular function                                   | 1.30E-04    |
| GO Function            | Enzyme inhibitor activity                                                   | 1.50E-04    |
| UniProt Keywords       | Serine protease inhibitor                                                   | 1.50E-04    |
| GO Process             | Negative regulation of macromolecule metabolic process                      | 1.90E-04    |
| GO Process             | Negative regulation of protein modification process                         | 2.60E-04    |
| GO Process             | Negative regulation of hydrolase activity                                   | 2.80E-04    |
| GO Function            | Serine-type endopeptidase inhibitor activity                                | 6.60E-04    |
| GO Process             | Negative regulation of phosphate metabolic process                         | 7.40E-04    |
| GO Function            | Endopeptidase inhibitor activity                                            | 8.50E-04    |
| GO Process             | Negative regulation of endopeptidase activity                               | 0.001       |
| GO Process             | Negative regulation of intracellular signal transduction                    | 0.0014      |
| GO Process             | Negative regulation of protein phosphorylation                             | 0.0014      |
| GO Process             | Regulation of protein metabolic process                                     | 0.0015      |
| GO Process             | Negative regulation of MAPK cascade                                         | 0.0061      |
| GO Process             | Negative regulation of cellular process                                     | 0.0063      |
| GO Process             | Negative regulation of biological process                                   | 0.0071      |

### Immune/inflammation signatures stimulated by circulating proteolytically active GZMA

Circulating proteolytically active GZMA would appear to have limited influence in the spleen as only four DEGs were identified in the spleen of poly(I:C)-treated Gzma<sup>S211A</sup> vs. 6J mice (Supplementary file 6d). Interestingly, the only significantly upregulated DEG in Gzma<sup>S211A</sup> spleens was Mid1, a gene involved in controlling granule exocytosis by cytotoxic lymphocytes (Boding et al., 2014; Boding et al., 2015). GSEA also illustrated that neither up- nor downregulated DEGs identified in the feet were enriched in spleen (Figure 6—figure supplement 3), arguing that in this setting the activity of GZMA in the periphery is not significantly recapitulated in spleen.
This result supports the view that GZMA is proteolytically active in vivo (Spaeny-Dekking et al., 2000) and that GZMA’s proteolytic activity mediates cell signaling events under physiological conditions. IPA Disease and Functions analysis of the 199 DEGs from GzmaS211A + polyIC vs. 6J + polyIC; Supplementary file 6b) identified downregulation (negative z-scores) of a series of inflammation and leukocyte activation signatures (Figure 6b, Supplementary file 6f). IPA USR analysis (core analysis with direct and indirect interactions) indicated downregulation of a series of cytokine, immune receptor, and transcription factor USRs (Figure 6b, Supplementary file 6g). An IPA USR analysis using the direct-only interaction option, which largely limits the analysis to transcription factors, showed STAT6, STAT3, NFATC2, JUN, NFKB1, and STAT1 to be the dominant downregulated transcription factor signatures in GzmaS211A mice by z-score and p-values (Figure 6b, Figure 6—figure supplement 4a). These transcription factors are associated with various innate and adaptive immune responses, with NFATC2 playing a central role in the activation of T cells during the development of an immune response. STAT3 and NF-κB have previously been shown to be activated in macrophages by recombinant GZMA in vitro (Santiago et al., 2020), with monocytes/macrophages reported as targets for GZMA activity in a variety of settings (Garzón-Tituaña et al., 2021; Metkar et al., 2008; Santiago et al., 2017; Spencer et al., 2013; Uranga et al., 2016). Interrogation of the Molecular Signature Database (MSigDB) (Subramanian et al., 2005) using GSEAs also identified gene sets associated with activated monocytes (GSE19888) that were significantly enriched in the downregulated genes for GzmaS211A + polyIC vs. 6J + polyIC (Figure 6—figure supplement 4b). This observation supports the contention that monocytes/macrophages are activated by circulating GZMA (Figure 6b). IPA of the 150 core enriched genes from these GSEAs also identified STAT6, STAT3, NFATC2, JUN, NFKB1, and STAT1 to be the dominant downregulated transcription factor signatures in GzmaS211A mice by z-score and p-values (Figure 6b, Figure 6—figure supplement 4a). These transcription factors are associated with various innate and adaptive immune responses, with NFATC2 playing a central role in the activation of T cells during the development of an immune response. STAT3 and NF-κB have previously been shown to be activated in macrophages by recombinant GZMA in vitro (Santiago et al., 2020), with monocytes/macrophages reported as targets for GZMA activity in a variety of settings (Garzón-Tituaña et al., 2021; Metkar et al., 2008; Santiago et al., 2017; Spencer et al., 2013; Uranga et al., 2016). Interrogation of the Molecular Signature Database (MSigDB) (Subramanian et al., 2005) using GSEAs also identified gene sets associated with activated monocytes (GSE19888) that were significantly enriched in the downregulated genes for GzmaS211A + polyIC vs. 6J + polyIC (Figure 6—figure supplement 4b). This observation supports the contention that monocytes/macrophages are activated by circulating GZMA (Figure 6b). IPA of the 150 core enriched genes from these GSEAs also identified STAT6, STAT3, NFATC2, JUN, NFKB1,
and STAT1 as significant USRs, even though only 10 of these 150 genes were significant DEGs for \textit{Gzma}^{S211A} vs. 6J (Supplementary file 6h). TheseUSR signatures would thus appear to be a consistent feature of the RNA-Seq data for \textit{Gzma}^{S211A} + poly(I:C) vs. 6J + poly(I:C).

**Summary of CHIKV foot swelling results and GZMA’s protease bioactivity**

The CHIKV foot swelling data so far is summarized in Figure 7a and argues that the 6N background, which includes a functional Nnt gene, rather than the absence of GZMA expression, causes the ameliorated foot swelling in \textit{Gzma}^{−/−} mice (Figure 1a). The presence of an intact Nnt gene can itself reduce foot swelling, although other 6N background genes in \textit{Gzma}^{−/−} mice also contribute (Figure 4e). MitoTEMPO treatment and NNT activity share anti-inflammatory activities (Figure 4e), presumably because both are involved in mitochondrial ROS mitigation. As CHIKV-infected \textit{Gzma}^{S211A} mice (on a 6J background) show no reduction in foot swelling, the data argues that GZMA is not a major player in CHIKV arthritis.

The \textit{Gzma}^{S211A} poly(I:C) data is summarized in Figure 7b. Poly(I:C) induces high levels of circulating GZMA, which is potentiated by type I IFNs, with NK cells the likely source of GZMA (Schanoski et al., 2019). IPA of RNA-Seq data comparing \textit{Gzma}^{S211A} + poly(I:C) vs. 6J + poly(I:C) supports the view that GZMA’s proteolytic function is required for its bioactivity (Schanoski et al., 2019; Figure 6b, Table 1). Circulating proteolytically active GZMA promotes certain immune-stimulating and pro-inflammatory activities (Schanoski et al., 2019; Shimizu et al., 2019; van Daalen et al., 2020; Wensink et al., 2015), with STAT6, STAT3, NFATC2, JUN, NFKB1, and STAT1 identified as dominant transcription factor USRs (Figures 1 and 7b; Figure 6—figure supplement 4a). Consensus regarding the molecular target(s) of extracellular GZMA’s protease activity (Figure 7b, indicated as ??) remains elusive, but may include protease-activated receptors (Hansen et al., 2005; Sower et al., 1996; Suidan et al., 1994; Suidan et al., 1996) and/or pro-IL-1 (Hildebrand et al., 2014; the latter can become extracellular when cells lyse (Afonina et al., 2015).

**Minor role for GZMA in CHIKV infection and arthritis**

If proteolytically active GZMA is present and has immune-stimulating and pro-inflammatory activities (Figure 7b), why does it have no significant role in driving the overt CHIKV arthritic foot swelling (with no ameliorated foot swelling in \textit{Gzma}^{S211A} mice, Figure 7a)? Firstly, the serum GZMA levels during CHIKV infection and arthritis (=0.2 and =0.075 ng/ml) were substantially lower than those seen after poly(I:C) treatment (=20 ng/ml) (Figure 7c), and very much lower than the ≈5 µg of recombinant GZMA-injected intraplantar to generate overt foot swelling in the absence of any other stimuli (Schanoski et al., 2019). Secondly, when the DEGs (q < 0.05) that were upregulated in feet during CHIKV peak viremia (day 2) and peak arthritis (day 7 in the Wilson et al. study) (6J + CHIKV vs. 6J mock infection) were compared with DEGs upregulated by proteolytically active GZMA (6J + poly(I:C) vs. \textit{Gzma}^{S211A} + poly(I:C); Supplementary file 6b), only small overlaps were evident, 27 genes for day 2 and 20 genes for day 7 (Figure 7c, Supplementary file 6). Thirdly, although CHIKV infection showed upregulation of many transcription factor and cytokine USRs (Supplementary file 2h), with some of these also upregulated by poly(I:C) treatment (Supplementary file 6g), the magnitude of the effects (by p-value and z-scores) was very much smaller for poly(I:C) treatment (shown for cytokine USRs, Figure 7d). So CHIKV infection (day 2) and arthritis (day 6) stimulate immune and inflammation pathways that overlap with those stimulated by GZMA, but GZMA only plays a minor role in stimulating these pathways during CHIKV viremia (day 2) and arthritis (day 6).

**6J SRA accessions with Nnt exon reads inconsistent with a 6J background**

Given the data presented herein and elsewhere (Bourdi et al., 2011; Fontaine and Davis, 2016; Leskov et al., 2017; McCambridge et al., 2019; Mekada et al., 2009; Rao et al., 2020; Ripoll et al., 2012; Rydström, 2006; Toye et al., 2005; Vozenilek et al., 2018) and given that redox regulation affects many cellular processes (Gamblhir et al., 2019; Lingappan, 2018; Sun et al., 2020), Nnt emerges as a legitimate focus of concern. In addition, the presence of Nnt exons 8–12 provides a useful genetic marker to illustrate that a mouse strain is not on a pure 6J background, with genetic backgrounds, as shown herein and elsewhere, able to have a profound influence on phenotype.
Research article

Immunology and Inflammation

Rawle, Le, et al. eLife 2022;11:e70207. DOI: https://doi.org/10.7554/eLife.70207  15 of 31

Leskov et al., 2017; Morales-Hernandez et al., 2018; Salerno et al., 2019; Vozenilek et al., 2018; Williams et al., 2021; Wolf et al., 2016). We thus undertook a k-mer mining approach to interrogate the NCBI SRA (Figure 8—figure supplement 1a), which (at the time of analysis) contained 61,443 RNA-Seq Run Accessions listing ‘C57BL/6J’ in the strain field of the metadata.

For ‘C57BL/6J’ Run Accessions, k-mer mining was used to count the number of RNA-Seq reads with sequence homology to Nnt exon 2 or exon 9, with these two exons being of similar length (203 bp for exon 2 and 192 bp for exon 9). RNA-Seq analysis of 6J tissues would ordinarily provide reads for exon 2, whereas the presence of exon 9 reads would be inconsistent with a pure 6J background. A conservative k-mer mining approach was used; (i) only an exact match for ‘C57BL/6J’ in the strain field was allowed, (ii) Run Accessions with small or large compressed file sizes (<200 Mb and >1500 Mb) were excluded, (iii) nucleotide mismatches for the 31-nucleotide k-mers were disallowed, (iv) where there were technical replicates, only one was mined, and (v) a read count of ≥10 per exon was used as a cutoff. This k-mer mining analysis revealed that 1008 Run Accessions had reads aligning to both Nnt exons 2 and 9, indicating full-length Nnt (Nnt+), which is not consistent with a 6J background (Supplementary file 7a). In contrast, 267 Run Accessions had equivocal results (Supplementary file 7c). Therefore =27% (1008 of 3744) of Run Accessions listing ‘C57BL/6J’ in the strain field had sequencing reads not consistent with a 6J background. The k-mer mining approach was validated for a selected group of Run Accessions using NCBI BLAST alignments, which illustrated excellent concordance with the k-mer mining read count data (Supplementary file 7d).

The startlingly high percentage (=27%) of SRA Accessions listing ‘C57BL/6J’ but having Nnt reads inconsistent with a 6J genetic background argues that the Nnt gene and associated mixed 6N/6J genetic backgrounds are widely underappreciated in a broad range of research areas. It should be noted that a large number of Run Accessions (n = 206,586) list ‘C57BL/6’ in the strain field and thus do not provide information on the substrain (Mekada et al., 2009) being used.

BioProjects comparing mice with truncated Nnt to mice with full-length Nnt

Based on the results of k-mer mining of ‘C57BL/6J’ Run Accessions, BioProjects (n = 373) were grouped into three categories: (i) BioProjects where all the Run Accessions with ‘C57BL/6J’ in the

---

**Figure 8.** k-mer mining of BioProjects where Nnt+ mice were compared with Nnt mice. The NCBI Sequence Read Archive (SRA) database was interrogated by k-mer mining for BioProjects where (i) some Run Accessions (listing 6J as the mouse strain) had reads compatible with a 6J background (reads for Nnt exon 2, but not exon 9) and (ii) other Run Accessions in that BioProject (listing 6J as the mouse strain) had reads not compatible with a 6J background (reads for Nnt exons 2 and 9). The methodology is described in Figure 8—figure supplement 1a, validated by BLAST alignments (Figure 8—figure supplement 1b), with raw data in Supplementary file 7d and e.

The online version of this article includes the following figure supplement(s) for figure 8:

**Figure supplement 1.** k-mer mining methodology and validation.

**Figure supplement 2.** Bruce4 ES cell line and /L28RA+ mouse Nnt genotypes.
strain field had RNA-Seq reads that were consistent with 6J (Nnt-) (62%), (ii) BioProjects where all the Run Accessions with ‘C57BL/6J’ in the strain field were not consistent with 6J (Nnt+) (23%), and (iii) BioProjects where some Run Accessions with ‘C57BL/6J’ in the strain field were Nnt- and others were Nnt+ (n = 57; 15%). Thus, 38% (15% plus 23%) of BioProjects had Run Accessions with ‘C57BL/6J’ strain listings not compatible with a 6J background.

Of the 57 aforementioned BioProjects, 43 had at least one publication associated with the study. These BioProjects were then manually interrogated to identify studies where it was clear (from the paper and the metadata) that comparisons had been made between two groups, where all the Run Accessions in one group were Nnt+, and all the Run Accessions in the other group were Nnt- (Figure 8, Figure 8—figure supplement 1b, Supplementary file 7d and e). Aside from the CHIKV BioProject described herein, several others emerged (Figure 8). For example, Mir31−/− mice showed reduced CD8 T cell dysfunction during chronic viral infection when compared to 6J mice (Moffett et al., 2017); however, Mir31+/− mice were Nnt+ (Figure 8, BioProject PRJNA385694; Supplementary file 7d). Rel−/−;Nfkb1−/−;Cd4−/−;Rela−/− mice were compared with 6J mice to implicate RIPK1 and IKK in thymocyte survival (Webb et al., 2019); however, the control 6J mice were Nnt+ (Figure 8, BioProject PRJEB30085; Supplementary file 7d). Bruce4 ES cells were reported to be on a 6J background (Ank et al., 2008) and were derived from a B6 mouse strain congenic for the Thy1.1 allele from an NZB mouse (Hughes et al., 2007; Köntgen et al., 1993), with these ES cells clearly Nnt- (Figure 8, accession SRR923434; Supplementary file 7d). The reported differences between Bruce4 and 6J genomes were thus likely more to do with the background than with genetic instability (Hughes et al., 2007). Il28ra−/− mice were generated using Bruce4 cells and were compared with 6J mice (Ank et al., 2008); however, RNA-Seq analysis of Il28ra−/− mice showed that (like Bruce4 cells) these mice had full-length Nnt (Figure 8—figure supplement 2). Myd88−/−;Tirf−/− double knockout mice were compared with 6J mice during infection with Staphylococcus aureus (Scumpia et al., 2017); however, the 6J (wild-type) mice were Nnt+ (Figure 8, BioProject PRJNA382450; Supplementary file 7e). Female Cystatin C−/− mice display significantly lower clinical signs of experimental autoimmune encephalomyelitis (EAE) when compared with 6J mice (Hoghooghi et al., 2020); however, Cystatin C−/− mice were all Nnt+ (Figure 8, BioProject PRJNA662247; Supplementary file 7d). Deletion of Nr1h3 resulted in reduced chromatin access at a large fraction of Kupffer-cell-specific enhancers (Sakai et al., 2019); however, Nr1h3−/−, but not the wild-type control, were all Nnt+ (Figure 8, BioProject PRJNA528435; Supplementary file 7d). Sesn1−/− mice were used to show that loss of Sesrin1 aggravates disuse-induced muscle atrophy when compared with 6J mice (Segalés et al., 2020); however, Sesn1−/− mice were all Nnt+ (Figure 8, BioProject PRJNA563889; Supplementary file 7d).

Of the 57 BioProjects containing Nnt+ and Nnt- Run Accessions, several contained comparisons in which one group contained a combination of Nnt+ and Nnt- Run Accessions, while the other group(s) contained either all Nnt+ or all Nnt- Run Accessions (Supplementary file 7f).

Whether the Nnt differences (or other background gene differences) would have significantly affected the interpretation of phenotypes in the aforementioned studies remains to be established. It is worth noting that herein we have only compared phenotypes of homozygotes (Figure 7a), with intermediate phenotypes potentially seen for heterozygotes (Ronchi et al., 2013). Our k-mer mining analysis also did not distinguish between Nnt+ and Nnt−, doing so would require extraction of Nnt reads, alignment to the C3H/HeJ genome, and analysis using Sashimi plots (as in Figure 3d). Nevertheless, the data argues that differences in Nnt or other background gene differences are widely underappreciated in a range of research fields and have the potential to compromise a wide range of studies.

Discussion

Despite a large body of literature, no clear consensus has emerged regarding the physiological function of GZMA (see ‘Introduction’). This lack of consensus might now, at least in part, be explained by the extensive use of Gzma−/− mice (Supplementary file 1). We show herein that this mouse strain is on a mixed 6J/6N genetic background and contains a full-length Nnt gene, with both Nnt and other 6N background genes, rather than loss of GZMA expression, responsible for the ameliorated CHIKV arthritis phenotype. Whether all the phenotypes reported for Gzma−/− mice (Supplementary file 1) are compromised by Nnt and/or the mixed background remains unclear and would require new experiments to resolve, similar to those described herein for CHIKV arthritis. However, Nnt has been
reported to affect redox regulation and activation in macrophages (Ripoll et al., 2012; Salerno et al., 2019), with an intact Nnt gene conceivably reducing cross-presentation (Dingjan et al., 2016; Nalle et al., 2020) and CD8 T cell responses (Oberkampf et al., 2018). In addition, 6N vs. 6J background differences (as herein) have shown clear phenotypes in a wide range of settings (Leskov et al., 2017; Morales-Hernandez et al., 2018; Rao et al., 2020; Salerno et al., 2019; Vozenilek et al., 2018; Williams et al., 2021; Wolf et al., 2016).

The data from Gzma<sup>S211A</sup> mice (that were generated using CRISPR on a 6J background) represents the first in vivo assessment of the physiological function of GZMA without the confounding influence of differences in Nnt or other genes associated with the mixed genetic background. The results from this analysis support the view that the physiological activity of GZMA is mediated by its protease activity (Figure 7b; Schanoski et al., 2019). This is an important point because protease-independent functions have been documented for several proteases (Calhan and Seyrantepe, 2017; McNutt et al., 2007; Pu et al., 2019; Szabo et al., 2016). One of the proposed activities for GZMA, the binding to TLR9 and potentiation of TLR9 signaling (Shimizu et al., 2019), was not reported to require GZMA’s protease activity. However, using mice defective in TLR9 signaling, we were unable to find evidence that TLR9 is required for GZMA’s pro-inflammatory activity (Supplementary file 8). Our studies also support the view that secreted extracellular GZMA has biological activity in the absence of perforin (or GZMB) (Figure 6—figure supplement 1). This contrasts with the traditional view of GZMA as a mediator of cell death, which requires perforin to deliver GZMA to the cytoplasm, where a series of target molecules are cleaved (Liesche et al., 2018; Wu et al., 2019; Zhou et al., 2020). Cleavage of SET complex proteins (Mandrup-Poulsen, 2017; Mollah et al., 2017) would similarly require delivery of GZMA to the cytoplasm. Although we cannot formally exclude translocation of circulating GZMA into the cytoplasm via some unknown mechanism, the Gzma<sup>S211A</sup> RNA-Seq experiment does ostensibly exclude perforin as NK cells only produce perforin (and GZMB) protein after ≈24 hr of appropriate stimulation (Fehninger et al., 2007). Extracellular target candidates for GZMA include protease-activated receptors (Hansen et al., 2005; Sower et al., 1996; Suidan et al., 1994; Suidan et al., 1996) and may also include pro-IL-1 (Hildebrand et al., 2014; Figure 7c) as pro-IL-1 can become extracellular when cells lyse (Afonina et al., 2015). Overall one might speculate that in such settings NK-derived GZMA synergizes with type I IFN responses to act as a systemic alarmin (Figure 7b). Gzma<sup>S211A</sup> mice will provide an invaluable tool for future studies into further refining our understanding of the role and molecular targets of GZMA.

The ability to reduce CHIKV arthritis in 6J mice with MitoTEMPO might suggest such antioxidant drugs have potential utility as anti-inflammatory treatments for alphaviral arthritides (Suhrbier et al., 2012; Zaid et al., 2021). However, MitoTEMPO treatment may simply be correcting (at least in part, Figure 4e) the Nnt defect in 6J mice by scavenging excess mitochondrial ROS arising from the loss of functional NNT (Ward et al., 2020). The argument that similar antioxidant treatments would be effective in human diseases may thus not be overly compelling, given that most humans have a functional Nnt gene. Perhaps noteworthy is that >300 papers listed in PubMed use MitoTEMPO in 6J mice, with many reporting effective disease amelioration with MitoTEMPO treatments, for example (Aoyama et al., 2012; Li et al., 2018; To et al., 2020; Vincent et al., 2020; Wu et al., 2020). Unfortunately, antioxidants have not shown clear benefits in human clinical trials (Casas et al., 2020; Jiang et al., 2020; Kovacic, 2020; Steinhubl, 2008).

The Jackson Laboratory generated the 6J inbred mouse strain in the 1920s–1930s, with this mouse strain the most frequently used mouse strain in biomedical research. Although differences in the Nnt gene (or other background genes) have previously been reported as underappreciated in metabolism research (Fontaine and Davis, 2016), the data herein and elsewhere argue that this issue extends to other areas of research (Bourdi et al., 2011; Leskov et al., 2017; McCambridge et al., 2019; Morales-Hernandez et al., 2018; Rao et al., 2020; Ripoll et al., 2012; Salerno et al., 2019; Vozenilek et al., 2018; Williams et al., 2021; Wolf et al., 2016), with age effects also reported (Ghosh et al., 2014; Ubaida-Mohien et al., 2019). Of concern was that ≈27% of SRA Run Accessions and ≈38% of BioProjects listing C57BL/6J as the mouse strain had Nnt sequence data not consistent with a pure 6J background. Mouse strain listing errors or inadequate backcrossing to 6J would thus appear to be common for SRA RNA-Seq submissions. The full extent to which Nnt and/or genetic backgrounds have complicated interpretation of knockout mouse studies remains to be addressed, but may require extensive new experiments such as those described herein for GZMA.
### Materials and methods

#### Key resources table

| Reagent type (species) or resource | Designation          | Source or reference                          | Identifiers     | Additional information                      |
|-----------------------------------|----------------------|----------------------------------------------|-----------------|---------------------------------------------|
| Strain, strain background (chikungunya virus) | CHIKV                | Dr. P. Roques (CEA, Fontenay-aux-Roses, France) | KT449801.1      | Isolate LR2006-OPY1                         |
| Chemical compound, drug           | TRizol               | Sigma-Aldrich                                | Cat# 15596026   |                                             |
| Chemical compound, drug           | MitoTEMPO            | Sigma-Aldrich                                | Cat# 1334850-99-5|                                             |
| Commercial assay, kit             | TruSeq RNA Sample Prep Kit (v2) | Illumina                               | SCR_010233     |                                             |
| Commercial assay, kit             | TruSeq Stranded mRNA library preparation kit | Illumina                                  | SCR_010233     |                                             |
| Commercial assay, kit             | QIAamp DNA Micro Kit | QIAGEN                                       | Cat# 56304      |                                             |
| Commercial assay, kit             | iScript cDNA Synthesis Kit | Bio-Rad                                      | Cat# 1708890  |                                             |
| Commercial assay, kit             | Q5 Hot Start High-Fidelity DNA Polymerase | NEB                                   | Cat# MD493S     | Enzyme                                      |
| Other                             | Illumina HiSeq 2000 Sequencer | Illumina                                      | RRID:SCR_010233 | Sequencing platform                         |
| Other                             | NextSeq 550          | Illumina                                     | RRID:SCR_016381| Sequencing platform                         |
| Other                             | NovaSeq 6000         | Illumina                                     | RRID:SCR_016387| Sequencing platform                         |

| Software, algorithm               | k-mer_mining_SRA     | GitHub                                      | https://github.com/CameronBishop/k-mer_mining_SRA |

| Cell line (Cercopithecus aethiops) | Vero cells           | ATCC                                        | RRID:CVCL_0059 |
| Cell line (Aedes albopictus)       | C6/36 cells          | ATCC                                        | RRID:CVCL_2230 |
| Strain, strain background (Mus musculus) | CS7BL/6J | Animal Resources Centre (Canning Vale, WA, Australia) | IMSR_JAX:000664 |

| Strain, strain background (M. musculus) | CS7BL/6N | The Jackson Laboratory | Stock no. 005304 |
| Strain, strain background (M. musculus) | CS7BL/6-Gzma<sup>−/−</sup> | Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia | Knockout mouse |
| Strain, strain background (M. musculus) | CS7BL/6J-Gzmb<sup>−/−</sup> | Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia | Knockout mouse |
| Strain, strain background (M. musculus) | CS7BL/6J-Gzma<sup>S211A</sup> | The Australian Phenomics Network, Monash University, Melbourne, Australia (this paper) | Mutant mouse |
| Strain, strain background (M. musculus) | CS7BL/6N<sup>∆Nnt8-12</sup> | The Australian Phenomics Network, Monash University, Melbourne, Australia (this paper) | Knockout mouse |

---

*Continued on next page*
Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information
--- | --- | --- | --- | ---
Strain, strain background (M. musculus) | CS7BL/6J-Ifnar<sup>-/-</sup> | Dr P. Hertzog (Monash University, Melbourne, Australia) | Knockout mouse |
Strain, strain background (M. musculus) | CS7BL/6-Il2ra<sup>-/-</sup> | Bristol-Myers Squibb (PMID:25901316) | Knockout mouse |
Sequence-based reagent | Nnt_RTPCR_F1 | This paper | PCR primers | AACAGTGCAAGGAGGTGGAC |
Sequence-based reagent | Nnt_RTPCR_R1 | This paper | PCR primers | GTGCCAAGGTAAGCCACAAT |
Software, algorithm | FastQC | Babraham Institute | RRID:SCR_014583 |
Software, algorithm | MultiQC | PMID:27312411 | RRID:SCR_014982 |
Software, algorithm | Cutadapt | DOI: https://doi.org/10.14806/ej.17.1.200 | RRID:SCR_011841 |
Software, algorithm | STAR | PMID:23104886 | RRID:SCR_004463 |
Software, algorithm | RSEM | PMID:21816040 | RRID:SCR_013027 |
Software, algorithm | EdgeR | PMID:27280887 | RRID:SCR_012802 |
Software, algorithm | ‘Ingenuity Pathway Analysis’ (IPA) | QIAGEN | RRID:SCR_008653 |
Software, algorithm | Cytoscape | PMID:14597658 | RRID:SCR_003032 |
Software, algorithm | STRING | PMID:3047243 | RRID:SCR_005223 |
Software, algorithm | ‘Gene Set Enrichment Analysis’ (GSEA) | PMID:16199517 | RRID:SCR_003199 |
Software, algorithm | ‘Integrative Genomics Viewer’ (IGV) | PMID:21221095 | RRID:SCR_011793 |
Software, algorithm | minimap2 | PMID:29750242 | RRID:SCR_018550 |
Software, algorithm | BigQuery | Google Cloud Platform | RRID:SCR_001011 |
Software, algorithm | fasterq-dump | SRA tool kit | sra-tools v 2.9.1 |

**Cell lines and CHIKV**

Vero (ATCC#: CCL-81) and C6/36 cells (ATCC# CRL-1660) were cultured as described (Nguyen et al., 2020). Cells were checked for mycoplasma using MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland). FBS was checked for endotoxin contamination using RAW264.7-HIV-LTR-luc cells (Johnson et al., 2005) before purchase. CHIKV (isolate LR2006-OPY1; GenBank KT449801.1; DQ443544.2) was a kind gift from Dr. P. Roques (CEA, Fontenay-aux-Roses, France), was propagated in C6/36 cells, and titers determined by CCID<sub>50</sub> assays (Nguyen et al., 2020). Virus was also checked for mycoplasma (La Linn et al., 1995).

**Mice and CHIKV infections**

C57BL/6J mice were purchased from Animal Resources Centre (Canning Vale, WA, Australia). C57BL/6N mice were purchased from The Jackson Laboratory (stock no. 005304). Gzma<sup>-/-</sup> mice were generated as described (Ebnet et al., 1995) and were provided by the Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia. Gzmb<sup>-/-</sup> mice were generated as described (Müllbacher et al., 1999) and were backcrossed onto C57BL/6J mice a total of 12 times and were provided by the Peter MacCallum Cancer Centre. The Australian Phenomics Network, Monash University, Melbourne, Australia, used CRISPR to generate (i) Gzma<sub>S211A</sub> mice on a 6J background and (ii) 6N<sup>ΔNnt8-12</sup> mice on a 6N background. Ifnar<sup>-/-</sup> mice (Yan et al., 2020) were kindly provided by Dr P. Hertzog (Monash University, Melbourne, Australia). Ifnar<sup>-/-</sup> mice were used to generate a chimeric Ifnar<sup>-/-</supaversal:en
University). Il28ra−/− mice (Ank et al., 2008) were kindly provided by Bristol-Myers Squibb (Souza-Fonseca-Guimaraes et al., 2015). All GMO mice were bred in-house at QIMR Berghofer MRI.

Female mice 6–8 weeks old were infected with 10⁴ CCID₅₀ CHIKV (isolate LR2006 OPY1) s.c. into each hind foot, with foot measurements and viremia determined as described (Gardner et al., 2010; Nguyen et al., 2020; Prow et al., 2018).

RNA-Seq of feet of CHIKV-infected GMO mice
Mice were infected with CHIKV, feet collected on day 6, and RNA samples prepared as described previously (Hazlewood et al., 2021; Rawle et al., 2021; Wilson et al., 2017) with minor modifications. Briefly, library preparation and sequencing were conducted by the Australian Genome Research Facility (Melbourne, Australia) (Hazlewood et al., 2021) or were conducted in-house (Rawle et al., 2021). RNA concentration and quality was measured using TapeStation D1K TapeScreen assay (Agilent). cDNA libraries were prepared using a TruSeq RNA Sample Prep Kit (v2) (Illumina Inc, San Diego, USA), which included isolation of poly-adenylated RNA using oligo-dt beads or total RNA library preparation with rRNA depletion (NEBNext Ultra II Directional RNA Library Prep Kit for Illumina and NEBNext rRNA Depletion Kit v2). Paired end reads were generated using the Illumina HiSeq 2000 Sequencer (Illumina Inc) (100 bp) or Illumina NextSeq 550 platform (75 bp). Per-base sequence quality for >90% bases was above Q30 for all samples. Raw sequencing reads were assessed using FastQC (v0.11.8) (Simons, 2010) and MultiQC (v1.7) (Ewels et al., 2016) and trimmed using Cutadapt (v2.3) (Martin, 2011) to remove adapter sequences and low-quality bases. Trimmed reads were aligned to the GRCm38 primary assembly reference and the GENCODE M23 gene model using STAR aligner (v2.7.1a) (Dobin et al., 2013), with more than 95% of reads mapping to protein coding regions. Counts per gene were generated using RSEM (v1.3.1) (Li and Dewey, 2011), and differential expression analysis was undertaken using EdgeR in Galaxy (Varet et al., 2016) with default settings and a count sum >1 filter applied. Counts were normalized using the TMM method and modeled using the likelihood ratio test, glmLRT().

Bioinformatic analyses
Pathway analysis of DEGs in direct and indirect or direct-only interactions was investigated using IPA (QIAGEN; Shannon et al., 2003). Enrichment for biological processes, molecular functions, KEGG pathways, and other gene ontology categories in DEG lists was elucidated using the STRING database (Szklarczyk et al., 2019) in Cytoscape (v3.7.2) (Shannon et al., 2003). GSEA (Subramanian et al., 2005) was performed on a desktop application (GSEA v4.0.3) (http://www.broadinstitute.org/gsea/) to look for enrichment of DEGs in full gene sets preranked by fold change.

WGS of Gzma−/− mice
QiAamp DNA Micro Kit (QIAGEN) was used to purify genomic DNA from Gzma−/− mice spleen as per the manufacturer’s instructions. DNA was sent to the Australian Genome Research Facility (AGRF) for WGS using the Illumina NovaSeq platform with 150 bp paired end reads. The primary sequence data was generated using the Illumina bcl2fastq 2.20.0.422 pipeline. Reads were mapped to the mm10 genome assembly (GRCm38) using BWA-mem, and .bam files were provided. Mapped reads were viewed in Integrative Genomics Viewer (IGV) (Robinson et al., 2011) and 6N features identified manually based on previous publications (Mekada et al., 2015; Simon, 2013).

Alignment to mouse genomes
FastQ files were generated as described or were downloaded from the SRA using Aspera. Reads were trimmed using Cutadapt (Martin, 2011) and mapped using STAR aligner (v2.7.1a) for RNA-Seq or minimap 2 (Li, 2018) for WGS data. IGV was used to visualize reads mapping to the Nnt gene after mapping to the GRCm38 primary assembly reference for the truncated version of the gene and to the mouse C3H_HeJ_v1 reference (GCA_001632575.1) to observe full-length Nnt.

Nnt RT-PCR
RT-PCR was undertaken essentially as described using the primer set (F1 AACAGTGCAAGGGACGTTGGAC, R1 GTGCCAAGGTAAGCCACAAT) (Integrated DNA Technologies; Huang et al., 2006). RNA was extracted from testes using TRIzol (Sigma-Aldrich) according to the manufacturer’s instructions.
cDNA was generated using iScript cDNA Synthesis Kit (Bio-Rad) and Q5 Hot Start High-Fidelity DNA Polymerase (NEB) was used for PCR.

**Histology**

Histology and H&E staining were undertaken as described (Rawle et al., 2021). Sections were scanned using Aperio AT Turbo (Aperio, Vista, CA) and analyzed using Aperio Image-Scope software (Leica Biosystems, Mt Waverley, Australia) (v10). Quantitation using Positive Pixel Count v9 was used to generate blue/red pixel ratios as a measure of leukocyte infiltrates, as described (Poo et al., 2014).

**MitoTEMPO treatment**

Mice were injected i.v. daily, on the indicated day post CHIKV infection, with 62.5 µg of MitoTEMPO (Sigma-Aldrich) in 150 µl of PBS.

**RNA-Seq of poly(I:C) injection for Gzma<sup>S211A</sup> vs. 6J mice**

Age-matched female Gzma<sup>S211A</sup> and 6J mice were injected i.v. with 250 µg of poly(I:C) in 150 µl of PBS. After 6 hr, mice were euthanized, spleen and whole feet were harvested, and RNA isolated as described previously (Prow et al., 2019; Wilson et al., 2017). Three RNA pools were generated for each mouse strain, whereby each pool contained equal amounts of RNA from four feet from four mice, or two spleens from two mice. RNA-Seq of polyadenylated RNA was then undertaken in-house at QIMR Berghofer MRI. RNA integrity was assessed using the TapeStation system (Agilent Technologies), and libraries were prepared using the TruSeq Stranded mRNA library preparation kit (Illumina). Sequencing was performed on the Illumina NextSeq 550 platform with 75 bp paired end reads. Per-base sequence quality for >92% bases was above Q30 for all samples. Raw sequencing reads were then processed as above.

**k-mer mining**

An exact-match (31 mer) k-mer mining approach was used to identify RNA-Seq read files (Accessions) with C57BL/6J mice listed as the mouse strain, but where Nnt reads were incompatible with 6J background. Metadata associated with the National Center for Biotechnology Information's SRA was screened using the Google Cloud Platform's BigQuery service with the Structured Query Language (SQL) command: SELECT m.bioproject, m.acc, m.sample_name, m.platform, m.mbytes, m.mbases FROM nih-sra-datastore.sra.metadata as m, UNNEST (m.attributes) as a WHERE m.organism = 'Mus musculus' and m.assay_type = 'RNA-Seq' and a.v = 'C57BL/6J.' Technical replicates for the same sample were collapsed by taking only the first accession for each Biosample. Accessions were then filtered on the basis of their compressed size so that only those between 200 Mb and 1500 Mb were retained; we found that read files of this size provided adequate sequencing depth to detect Nnt exon reads. Accessions were sorted according to BioProject and used as input for a bioinformatics pipeline executed on the Google Cloud Platform, which allowed access to the ‘SRA in the cloud’ database. A copy of our Bash script to automate the pipeline is available at https://github.com/CameronBishop/k-mer(mining)_SRA (Bishop, 2021). Accession read files were converted to FastQ format using fasterq-dump (SRA tool kit). BBduk version 38.87 (Bushnell, 2020) was used with default parameters to screen each read for sequence homology to exons 2 and 9 of the Nnt gene. Reads sharing at least one 31-mer with either exon were counted as a ‘match’ for that exon. FastQ files with at least 10 matches to exon 2 and 0 matches to exon 9 were classed as consistent with a 6J genotype (truncated Nnt), while FastQ files with at least 10 matches to each exon were classed as not consistent with a 6J genotype. Results were curated using BigQuery to confirm that for each Accession’s metadata entry the ‘strain_sam’ field (or equivalent) of the metadata ‘Attributes’ table was listed as C57BL/6J.

BioProjects were identified where some read files contained exon 2 reads and no exon 9 reads, whereas others contained both exon 2 and exon 9 reads. The literature and Gene Expression Omnibus submissions associated with these BioProjects were then consulted to identify BioProjects where mice with full-length Nnt had been compared with mice with truncated Nnt.
Determination of GZMA levels in mouse serum samples

Mouse serum was collected in Microvette 500 Z gel tubes (Sarstedt) with GZMA levels determined using a GZMA ELISA kit (MyBioSource, San Diego, CA, MBS704766) according to the manufacturer’s instructions.

Statistics

Statistical analysis of experimental data was performed using IBM SPSS Statistics for Windows, version 19.0. The t-test was performed when the difference in variances was <4, skewness was >-2, and kurtosis was <2, otherwise the Kolmogorov–Smirnov test was used.

Data, code, and GMO mouse availability

All data are provided in the article and accompanying supplementary files. Raw sequencing data generated for this publication has been deposited in the NCBI SRA. RNA-Seq NCBI BioProjects: (i) 6J + CHIKV vs. 6J mock infection, day 2 and day 7 feet (PRJNA431476); Gzma<sup>−/−</sup> + CHIKV vs. 6J + CHIKV, day 6 feet (PRJNA664644); (ii) 6N<sup>∆Nnt8-12</sup> + CHIKV vs. 6N + CHIKV, day 6 feet (PRJNA779556); (iii) 6J + MitoTEMPO + CHIKV vs. 6J + PBS + CHIKV (PRJNA779556); and (iv) 6J Gzma<sup>S211A</sup> + poly(l:C) vs. 6J + poly(l:C) feet and spleen 6 hr (PRJNA666748). WGS of Gzma<sup>−/−</sup> mice NCBI BioProject PRJNA664888.

A copy of our code to automate the k-mer mining pipeline is available at https://github.com/CameronBishop/k-mer_mining_SRA (Bishop, 2021; copy archived at swt:1:rev:372d29b02972d96e8eff7b-6c431883ea88dfb5c50). CRISPR GMO mouse lines 6N<sup>∆Nnt8-12</sup> and Gzma<sup>S211A</sup> are available on request.

Acknowledgements

We thank the following staff at QIMR Berghofer MRI for their assistance; animal house staff, Dr I Anraku (BSL3 facility manager), Dr R Johnston (Bioinformatics), and Dr Viviana Lutzky (for proof reading). We thank Dr Dion Kaiserman (Monash University, Australia) for supplying recombinant GZMA. We also thank Dr Mark Heise (University of North Carolina) for valuable discussions.

Additional information

### Funding

| Funder                              | Grant reference number | Author          |
|-------------------------------------|------------------------|-----------------|
| National Health and Medical Research Council | APP1141421             | Andreas Suhrbier |
| National Health and Medical Research Council | APP1173880             | Andreas Suhrbier |

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

### Author contributions

Daniel J Rawle, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – review and editing; Thuy T Le, Kexin Yan, Investigation; Troy Dumenil, Cameron Bishop, Data curation, Formal analysis, Visualization, Writing – review and editing; Eri Nakayama, Investigation, Conceptualization; Phillip I Bird, Conceptualization, Funding acquisition, Methodology, Resources, Writing – review and editing; Andreas Suhrbier, Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review and editing

### Author ORCIDs

- Troy Dumenil [http://orcid.org/0000-0002-3032-8360](http://orcid.org/0000-0002-3032-8360)
- Cameron Bishop [http://orcid.org/0000-0002-5710-9942](http://orcid.org/0000-0002-5710-9942)
- Andreas Suhrbier [http://orcid.org/0000-0001-8986-9025](http://orcid.org/0000-0001-8986-9025)
Ethics

All mouse work was conducted in accordance with the "Australian code for the care and use of animals for scientific purposes" as defined by the National Health and Medical Research Council of Australia. Mouse work was approved by the QIMR Berghofer Medical Research Institute animal ethics committee (P2235 A1606-618M), with infectious CHIKV work conducted in a biosafety level-3 (PC3) facility at the QIMR Berghofer MRI (Australian Department of Agriculture, Water and the Environment certification Q2326 and Office of the Gene Technology Regulator certification 3445).

Decision letter and Author response

Decision letter https://doi.org/10.7554/eLife.70207.sa1
Author response https://doi.org/10.7554/eLife.70207.sa2

Additional files

Supplementary files

• Supplementary file 1. Summary of studies using Gzma−/− mice. Compilation of studies employing Gzma−/− or Gzma−/− Gzmb−/− double KO mice that reveal a phenotype or do not show a phenotype.

• Supplementary file 2. RNA-Seq of Gzma−/− chikungunya virus (CHIKV) vs. 6J + CHIKV and 6J + CHIKV vs. 6J mock infection. Datasets for RNA-Seq and Ingenuity Pathway Analysis (IPA) of mice feet day 6 post infection from CHIKV-infected Gzma−/− mice vs. CHIKV-infected 6J mice and of mice feet day 7 post infection from 6J + CHIKV vs. 6J mock infection.

• Supplementary file 3. Genetic differences between Gzma−/− and C57BL/6J. Genetic differences potentially involved in inflammation or arthritis are indicated. Nnt not included. Differences in introns not included.

• Supplementary file 4. RNA-Seq for 6N NntΔ8-12+ chikungunya virus (CHIKV) vs. 6N + CHIKV. Datasets for RNA-Seq and Ingenuity Pathway Analysis (IPA) of mice feet day 6 post infection; 6N NntΔ8-12+ CHIKV vs. 6N + CHIKV.

• Supplementary file 5. RNA-Seq of chikungunya virus (CHIKV)-infected mice treated with MitoTEMPO. Datasets for RNA-Seq and Ingenuity Pathway Analysis (IPA) of mice feet day 6 post infection; 6J + MitoTEMPO + CHIKV vs. 6J + PBS + CHIKV.

• Supplementary file 6. RNA-Seq of Gzma5211A vs. 6J mice injected with polyinosinic:polycytidylic acid (poly(I:C)). Datasets for RNA-Seq, Ingenuity Pathway Analysis (IPA), and Cytoscape analyses of mice feet and spleen taken 6 hr after injection with poly(I:C); Gzma5211A vs. 6J.

• Supplementary file 7. k-mer mining of the NCBI Sequence Read Archive (SRA). Datasets for exact-match (31 mer) k-mer mining approach to identify full-length Nnt reads (6N background) in accessions listing C57BL/6J mice as the mouse strain.

• Supplementary file 8. No evidence for TLR9 involvement. (a) Tlr9−/− and 6J mice were injected intraplantar into the feet with 5 µg recombinant mouse granzyme A (GZMA) in 20 µl and foot swelling measured over time as described (Schanoski et al., 2019) (n = 4 mice and four feet per group; statistics by Kolmogorov–Smirnov tests). Tlr9−/− mice were derived from 129/Ola × C57BL/6F1 progeny (http://www.myv.ne.jp/obs/index.files/tlr_eng.htm). (b) As for (a) using Tlr9MTBI/Mmjx and 6J mice. Tlr9MTBI/Mmjx mice have a Tlr9 missense point mutation and do not respond to oligonucleotides containing CpG motifs (https://www.jax.org/strain/014534). (c) Tlr9−/− mice (like 6J) do not encode the full Nnt gene. (d) Female 8–10-week-old Tlr9−/− and 6J mice (n = 6 mice and 12 feet per group) were infected with chikungunya virus (CHIKV) and feet measured over time. Statistics by Kolmogorov–Smirnov tests. (e) Female C57BL/6-J-Tlr9MTBI/Mmjx mice (n = 6 mice and 12 feet per group) were infected as for (d). (f) Viremia for the mice in (d). After GZMA injection, Tlr9−/− mice showed increased foot swelling (a), whereas C57BL/6-J-Tlr9MTBI/Mmjx mice showed no significant difference (b). Tlr9−/− mice also have the Nnt deletion (c); however, they are on a mixed 129/Ola and C57BL/6 background (Hemmi et al. 2000), with 129/SvJ mice showing increased inflammatory infiltrates in certain settings (Hoover-Plow et al., 2008). After CHIKV infection, foot swelling was again increased in Tlr9−/− (d), but not Tlr9MTBI/Mmjx mice (e). Tlr9−/− mice did not show an increased viremia (f). These data do not support a contention that TLR9 is required for GZMA’s bioactivity.

• Transparent reporting form

• Source data 1. Source data for DNA gel images. Source data for DNA gel images in Figure 3e, Figure 1—figure supplement 1b,c, and Figure 4—figure supplement 1e.
Data availability

Five supplementary files have been provided which constitute source data for all the results cited in the manuscript. Raw sequencing data was uploaded to SRA: BioProject accessions: PRJNA666748, PRJNA664888, PRJNA664644, PRJNA779556.

The following datasets were generated:

| Author(s)       | Year | Dataset title                                                                 | Dataset URL                                                                 | Database and Identifier |
|-----------------|------|-------------------------------------------------------------------------------|----------------------------------------------------------------------------|-------------------------|
| Suhrbier A      | 2020 | RNA-Seq of Granzyme A proteolytic site mutant mice injected with Poly (I:C)    | https://www.ncbi.nlm.nih.gov/bioproject/PRJNA666748                       | NCBI BioProject, PRJNA666748 |
| Suhrbier A      | 2020 | Whole genome sequencing of a Granzyme A knock out mouse                       | https://www.ncbi.nlm.nih.gov/bioproject/PRJNA664888                       | NCBI BioProject, PRJNA664888 |
| Suhrbier A      | 2020 | RNA-Seq of Granzyme A knockout mice infected with chikungunya Virus          | https://www.ncbi.nlm.nih.gov/bioproject/PRJNA664644                       | NCBI BioProject, PRJNA664644 |
| Suhrbier A      | 2021 | RNA_Seq of C57BL/6N Nnt knockout mice and MitoTEMPO treatment of C57BL/6J mice infected with CHIKV | https://www.ncbi.nlm.nih.gov/bioproject/PRJNA779556                       | NCBI BioProject, PRJNA779556 |

References

Afonina IS, Müller C, Martin SJ, Beyaert R. 2015. Proteolytic Processing of Interleukin-1 Family Cytokines: Variations on a Common Theme. *Immunity* 42:991–1004. DOI: https://doi.org/10.1016/j.immuni.2015.06.003, PMID: 26084020

Ank N, Iversen MB, Bartholdy C, Staeheli P, Hartmann R, Jensen UB, Dagnaes-Hansen F, Thomsen AR, Chen Z, Haugen H, Klucher K, Paludan SR. 2008. An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. *Journal of Immunology* 180:2474–2485. DOI: https://doi.org/10.4049/jimmunol.180.4.2474, PMID: 18250457

Aoyama T, Paik Y-H, Watanabe S, Laleu B, Gaggini F, Fioraso-Cartier L, Molango S, Heitz F, Merlot C, Szyndralewiez C, Page P, Brenner DA. 2012. Nicotinamide adenine dinucleotide phosphate oxidase in experimental liver fibrosis: GKT137831 as a novel potential therapeutic agent. *Hepatology* 56:2316–2327. DOI: https://doi.org/10.1002/hep.25938, PMID: 22806357

Bishop C. 2021. k-mer_mining_SRA. swh:1:rev:372d29b02972d96e8ef7b6c431883ea8dfb5c50. Software Heritage. https://archive.softwareheritage.org/swh:1:dir:439009609bd1ec2052fb1f2cbceab60dc48f5622; origin=https://github.com/CameronBishop/k-mer_mining_SRA;visit=swh:1:snp:0cb753db1380263f4e875b7e076de0778a176d5;anchor=swh:1:rev:372d29b02972d96e8ef7b6c431883ea8dfb5c50

Boding L, Hansen AK, Meroni G, Johansen BB, Braunstein TH, Bonefeld CM, Kongsbak M, Jensen BAH, Woetmann A, Thomsen AR, Odum N, von Essen MR, Geisler C. 2014. Midline 1 directs lytic granule exocytosis and cytotoxicity of mouse killer T cells. *European Journal of Immunology* 44:3109–3118. DOI: https://doi.org/10.1002/eji.201344388, PMID: 25043946

Boding L, Hansen AK, Meroni G, Levring TB, Woetmann A, Ødum N, von Essen MR, Geisler C. 2015. MID2 can substitute for MID1 and control exocytosis of lytic granules in cytotoxic T cells. *APMIS* 123:682–687. DOI: https://doi.org/10.1111/apm.12402, PMID: 25924778

Bourdi M, Davies JS, Pohl LR. 2011. Mispairing C57BL/6 substrains of genetically engineered mice and wild-type controls can lead to confounding results as it did in studies of JNK2 in acetaminophen and concanavalin A liver injury. *Chemical Research in Toxicology* 24:794–796. DOI: https://doi.org/10.1021/tx100143x, PMID: 21557537

Bushnell B. 2020. BBMap. Sourceforge. https://jgi.doe.gov

Calhan OY, Seyrantepe V. 2017. Mice with Catalytically Inactive Cathepsin A Display Neurobehavioral Alterations. *Behavioural Neurology* 2017:4261873. DOI: https://doi.org/10.1155/2017/4261873, PMID: 28133419

Casas AI, Nogales C, Mucke HM, Petraina A, Cuadrado A, Rojo AI, Ghezzi P, Jaquet V, Augsburger F, Dufresne F, Soubhye J, Deswalt S, Di Sante M, Kaludercic N, Di Lisa F, Schmidt HHHW. 2020. On the Clinical Pharmacology of Reactive Oxygen Species. *Pharmacological Reviews* 72:801–828. DOI: https://doi.org/10.1124/pr.120.019422, PMID: 32859763

Dempoya J, Matsumiya T, Imaiizumi T, Hayakari R, Xing F, Yoshida H, Okumura K, Satoh K. 2012. Double-stranded RNA induces biphasic STAT1 phosphorylation by both type I interferon (IFN)-dependent and type I IFN-independent pathways. *Journal of Virology* 86:12760–12769. DOI: https://doi.org/10.1128/JVI.01881-12, PMID: 22973045
Research article

Dingjan I, Verboogen DR, Paardekooper LM, Revelo NH, Sittig SP, Visser LJ, Mollard GF, Henriët SS, Figdor CG, Ter Beest M, van den Bogaart G. 2016. Lipid peroxidation causes endosomal antigen release for cross-presentation. *Scientific Reports* 6:22064. DOI: https://doi.org/10.1038/srep22064, PMID: 26907999

Djeu JY, Heinbaugh JA, Holden HT, Herberman RB. 1979. Augmentation of mouse natural killer cell activity by interferon and interferon inducers. *Journal of Immunology* 122:175–181 PMID: 310826.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson L, Gingeras TR. 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29:15–21. DOI: https://doi.org/10.1093/bioinformatics/bts635, PMID: 23104888.

Ebnet K, Hausmann M, Lehmann-Grube F, Müllbacher A, Kopf M, Lamers M, Simon MM. 1995. Granzyme A-deficient mice retain potent cell-mediated cytotoxicity, *The EMBO Journal* 14:4230–4239. DOI: https://doi.org/10.1092/j.1460-2075.1995.tb00097.x, PMID: 7556064.

Ewels P, Magnusson M, Lundin S, Käller M. 2016. MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics* 32:3047–3048. DOI: https://doi.org/10.1093/bioinformatics/btw354, PMID: 27312411.

Fehniger TA, Cai SF, Cao X, Bredemeyer AJ, Presti RM, Cellars L, Andrade-Gordon P, Pan Z, Baruch A, Wallace JL, Hollenberg MD, Levault KR, Brewer GJ. 2014. Relative importance of redox buffers GSH and NAD(P)H in age-related neurodegeneration and Alzheimer disease-like mouse neurons. *Aging Cell* 13:789–811. DOI: https://doi.org/10.1111/acel.12216, PMID: 24655393.

Fontaine DA, Davis DB. 2016. Attention to Background Strain Is Essential for Metabolic Research: C57BL/6 and P280834. DOI: https://doi.org/10.1016/j.immuni.2007.04.010, PMID: 17540585.

Garzón-Tituaña M, Gordy LE, Bezbradica JS, Flyak AI, Spencer CT, Dunkle A, Sun J, Stanic AK, Boothby MR, He Y-W, Zhao Z, Gardner J, Anraku I, Le TT, Larcher T, Major L, Roques P, Schroder WA, Higgs S, Suhrbier A. 2010. Chikungunya virus arthritis in adult wild-type mice. *Journal of Virology* 84:8021–8032. DOI: https://doi.org/10.1128/JVI.02603-09, PMID: 20519386.

García-Laorden MI, Stroo I, Blok DC, Florquin S, Medema JP, de Vos AF, van der Poll T. 2016. Granzymes A and B Regulate the Local Inflammatory Response during Klebsiella pneumoniae Pneumonia. *Journal of Innate Immunity* 8:258–268. DOI: https://doi.org/10.1159/000443401, PMID: 26894590.

García-Laorden MI, Stroo I, Terpstra S, Florquin S, Medema JP, van T Veer C, de Vos AF, van der Poll T. 2017. Expression and Function of Granzymes A and B in Escherichia coli Peritonitis and Sepsis. *Mediators of Inflammation* 2017:4137563. DOI: https://doi.org/10.1155/2017/4137563, PMID: 28694562.

Gardner J, Anraku I, Le TT, Larcher T, Major L, Roques P, Schroder WA, Higgs S, Suhrbier A. 2010. Chikungunya virus arthritis in adult wild-type mice. *Journal of Virology* 84:8021–8032. DOI: https://doi.org/10.1128/JVI.02603-09, PMID: 20519386.

Görlach K, Abbas AA, Sultana S, Tareen F, Sari M, Bergmann MC, Schmitt M, Müller J, Lode H, von Zglinicki T. 2012. Effects of oxidative stress on the expression of cytoprotective genes. *Aging Cell* 11:3781–3795. DOI: https://doi.org/10.1111/j.1474-9726.2012.00863.x, PMID: 33664861.

Ghezzi P. 2021. Redox regulation of immunity and the role of small molecular weight thiolis. *Redox Biology* 44:102001. DOI: https://doi.org/10.1016/j.redox.2021.102001, PMID: 33994345.

Ghos O, Lefebvre KR, Brews CJ. 2014. Relative importance of redox buffers GSH and NAD(P)H in age-related neurodegeneration and Alzheimer disease-like mouse neurons. *Aging Cell* 13:631–640. DOI: https://doi.org/10.1111/acel.12216, PMID: 24655393.

Gotlieb N, Griffiths GM. 2018. An early history of T cell-mediated cytotoxicity. *Nature Reviews. Immunology* 18:527–535. DOI: https://doi.org/10.1038/s41577-018-0009-3, PMID: 29662120.

Gordon LE, Bezbradica JS, Flyak AI, Spencer CT, Dunkle A, Sun J, Stanic AK, Boothe MB, He Y-W, Zhao Z, Van Kaer L, Joyce S. 2011. IL-15 regulates homeostasis and terminal maturation of NK cells. *Journal of Immunology* 187:6335–6345. DOI: https://doi.org/10.4049/jimmunol.1003965, PMID: 22084435.

Hansen KK, Sherman PM, Cellars L, Andrade-Gordon P, Pan Z, Baruch A, Wallace JL, Hollenberg MD, Vergnolle N. 2005. A major role for proteolytic activity and proteinase-activated receptor-2 in the pathogenesis of infectious colitis. *PNAS* 102:8363–8368. DOI: https://doi.org/10.1073/pnas.0409535102, PMID: 15919826.

Hazelwood JE, Dumenil T, Le TT, Slonchak A, Kazakoff SH, Patch AM, Gray LA, Howley PM, Liu L, Hayball JD, Yan K, Rawle DJ, Prow NA, Suhrbier A, McFadden G. 2021. Injection site vaccinology of a recombinant vaccinia-based vector reveals diverse innate immune signatures. *PLoS Pathogens* 17:e1009215. DOI: https://doi.org/10.1371/journal.ppat.1009215.

Hildebrand D, Bode KA, Rieß D, Cerny D, Walhuber A, Römmler F, Strehl A, Scharnhorst J, Orth JHC, Miethke T, Heeg K, KabatkyzKF. 2014. Granzyme A produces bioactive IL-1β through a nonapoptotic inflammasome-independent pathway. *Cell Reports* 9:910–917. DOI: https://doi.org/10.1016/j.celrep.2014.10.003, PMID: 25437548.

Hoghooghi V, Palmer AL, Frederick A, Jiang Y, Merkens JE, Balakrishnan A, Finlay TM, Grubb A, Levy E, Gordon F, Jirik FR, Nguyen MD, Schuurmans C, Visser F, Dunn SE, Ousman SS. 2020. Cystatin C Plays a Sex-Dependent Detrimental Role in Experimental Autoimmune Encephalomyelitis. *Cell Reports* 33:108236. DOI: https://doi.org/10.1016/j.celrep.2020.108236, PMID: 33027652.
Immunology 32:1980–1985. DOI: https://doi.org/10.1002/1521-4141(200207)32:7<1980::AID-IJIMM1980-3.0.CO;2-Z, PMID: 12115618

Pardo J, Bosque A, Brehm R, Wallich R, Naval J, Müllbacher A, Anel A, Simon MM. 2004. Apoptotic pathways are selectively activated by granzyme A and/or granzyme B in CTL-mediated target cell lysis. The Journal of Cell Biology 167:457–468. DOI: https://doi.org/10.1083/jcb.200406115, PMID: 15534000

Park S, Griesenauer B, Jiang H, Adom D, Mehrpouya-Bahrami P, Chakravorty S, Kazemian M, Imam T, Srivastava R, Hayes TA, Pardo J, Janga SC, Paczesny S, Kaplan MH, Olson MR. 2020. Granzyme A-producing T helper cells are critical for acute graft-versus-host disease. JCI Insight 5:124465. DOI: https://doi.org/10.1172/jci.insight.124465, PMID: 32809971

Pereira RA, Simon MM, Simmons A. 2000. Granzyme A, a noncytolytic component of CD8(+) cell granules, restricts the spread of herpes simplex virus in the peripheral nervous systems of experimentally infected mice. Journal of Virology 74:1029–1032. DOI: https://doi.org/10.1128/jvi.74.2.1029-1032.2000, PMID: 10623769

Plasman K, Demol H, Bird PI, Gevaert K, Van Damme P. 2014. Substrate specificities of the granzyme tryptases A and K. Journal of Proteome Research 13:6067–6077. DOI: https://doi.org/10.1021/pr500968d, PMID: 25383893

Poo YS, Rudd PA, Gardner J, Wilson JAC, Larcher T, Colle M-A, Le TT, Nakaya HI, Warrilow D, Allcock R, Bielefeldt-Ohmann H, Schroder WA, Khromyk AA, Lopez JA, Suhbier A. 2014. Multiple immune factors are involved in controlling acute and chronic chikungunya virus infection. PLOS Neglected Tropical Diseases 8:e3354. DOI: https://doi.org/10.1371/journal.pntd.0003354, PMID: 25474568

Prow NA, Tang B, Gardner J, Le TT, Taylor A, Poo YS, Nakayama E, Hirata TDC, Nakaya HI, Slonchak A, Mukhopadhyay P, Mahalingam S, Schroder WA, Klimstra W, Suhbier A. 2017. Lower temperatures reduce type I interferon activity and promote alphavirus arthritis. PLOS Pathogens 13:e1006785. DOI: https://doi.org/10.1371/journal.ppat.1006785, PMID: 29281739

Prow NA, Liu L, Nakayama E, Cooper TH, Yan K, Eldi P, Hazlewood JE, Tang B, Le TT, Suhbier A. 2018. A vaccinia-based single vector construct multi-pathogen vaccine protects against both Zika and chikungunya viruses. Nature Communications 9:1230. DOI: https://doi.org/10.1038/s41467-018-01366-0, PMID: 29581442

Prow NA, Hirata TDC, Tang B, Larcher T, Mukhopadhyay P, Alves TL, Le TT, Gardner J, Poo YS, Nakayama E, Lutzky VP, Nakaya HI, Suhbier A. 2019. Exacerbation of Chikungunya Virus Rheumatic Immunopathology by a High Fiber Diet and Butyrate. Frontiers in Immunology 10:2736. DOI: https://doi.org/10.3389/fimmu.2019.02736, PMID: 31849947

Pu H, Shi Y, Zhang L, Lu Z, Ye Q, Leak RK, Xu F, Ma S, Mu H, Wei Z, Xu N, Xia Y, Hu X, Hitchens TK, Bennett MVL, Chen J. 2019. Protease-independent action of tissue plasminogen activator in brain plasticity and neurological recovery after ischemic stroke. PNAS 116:9115–9124. DOI: https://doi.org/10.1073/pnas.1821979116, PMID: 30996120

Rao KNS, Shen X, Pardue S, Krzywanski DM. 2020. Nicotinamide nucleotide transhydrogenase (NNT) regulates mitochondrial ROS and endothelial dysfunction in response to angiotensin II. Redox Biology 36:101650. DOI: https://doi.org/10.1016/j.redox.2020.101650, PMID: 32763515

Rawle DJ, Le TT, Dumenil T, Yan K, Tang B, Nguyen W, Watterson D, Modhiran N, Hobson-Peters J, Diener KR, Howley PM, Haywell PM, Day JD, Suhbier A. 2021. ACE2-lentiviral transduction enables mouse SARS-CoV-2 infection and mapping of receptor interactions. PLOS Pathogens 17:e1009723. DOI: https://doi.org/10.1371/journal.ppat.1009723, PMID: 34214142

Regen R, Pavlinovic L, Pavlinovic L, Krzywanski DM, Cooper TH. 2021. Nicotinamide nucleotide transhydrogenase (NNT) acts as a novel modulator of macrophage inflammatory responses. FASEB Journal 35:3550–3562. DOI: https://doi.org/10.1096/fj.11-2593545

Riera L, Gariglio M, Valente G, Müllbacher A, Museteanu C, Landolfo S, Simon MM. 2000. Murine cytomegalovirus replication in salivary glands is controlled by both perforin and granzymes during acute infection. European Journal of Immunology 30:1350–1355. DOI: https://doi.org/10.1002/1521-4141(20000530:<1350::AID-IJIMM350-3.0.CO;2-J>, PMID: 10820381

Ripple VM, Meadows NA, Bangert M, Lee AW, Kadioglu A, Cox RD. 2012. Nicotinamide nucleotide transhydrogenase (NNT) acts as a novel modulator of macrophage inflammatory responses. FASEB Journal 26:3550–3562. DOI: https://doi.org/10.1096/fj.11-199935, PMID: 22593545

Robinson JT, Thorvaldsdóttir H, Winckler W, Gutmann M, Lander ES, Getz G, Mesirov JP. 2011. Integrative genomics viewer. Nature Biotechnology 29:24–26. DOI: https://doi.org/10.1038/nbt.1754, PMID: 21221095

Ronchi JA, Figueira TR, Ravagnani FG, Oliveira HCF, Vercesi AE, Castillo RF. 2013. A spontaneous mutation in the nicotinamide nucleotide transhydrogenase gene of C57BL/6J mice results in mitochondrial redox abnormalities. Free Radical Biology and Medicine 63:446–456. DOI: https://doi.org/10.1016/j.freeradbiomed.2013.05.049, PMID: 23747984

Rydström M. 2006. Mitochondrial NADPH, transhydrogenase and disease. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1757:721–726. DOI: https://doi.org/10.1016/j.bbabio.2006.03.010, PMID: 16730324

Sakai M, Troutman TD, Seidman JS, Ouyang Z, Spann NJ, Abe Y, Ego KM, Bruni CM, Deng Z, Schlachetzki JCM, Nott A, Bennett H, Chang J, Vu BT, Patlals MP, Link VM, Texari I, Heinz S, Thompson BM, McDonald JG, et al. 2019. Liver-Derived Signals Sequentially Reprogram Myeloid Enhancers to Initiate and Maintain Kupffer Cell Identity. Immunity 51:655-670. DOI: https://doi.org/10.1016/j.immuni.2019.09.002, PMID: 31587991
Salerno AG, Rentz T, Dorighello GG, Marques AC, Lorza-Gil E, Wanshel ACBA, de Moraes A, Vercesi AE, Oliveira HCF. 2019. Lack of mitochondrial NADP(H)-transhydrogenase expression in macrophages exacerbates atherosclerosis in hypercholesterolemic mice. *Biochemical Journal* **476**:3769–3789. DOI: https://doi.org/10.1042/BCJ20190543, PMID: 31803904

Santiago L, Menaa C, Arias M, Martin P, Jaime-Sánchez P, Metkar S, Comas L, Erill N, Gonzalez-Rumayor V, Esser E, Galvez EM, Raja S, Simon MM, Sprague SM, Gabay C, Martínez-Lostao L, Pardo J, Froelich CJ. 2017. Granzyme A Contributes to Inflammatory Arthritis in Mice Through Stimulation of Osteoclastogenesis. *Arthritis & Rheumatology* **69**:320–334. DOI: https://doi.org/10.1002/art.39837, PMID: 27598995

Segalés J, Castro M, Sanz-Pampolina R, Garzón M, Ramirez-Labrada A, Tapia E, Moreno V, Layunta E, Gil-Gómez G, Garrido M, Peña R, Lanuza PM, Comas L, Jaime-Sanchez P, Uranga-Murillo I, Del Campo R, Pelegrín P, Camerer E, Martínez-Lostao L, Muñoz G, et al. 2020. Extracellular Granzyme A Promotes Colorectal Cancer Development by Enhancing Gut Cell Survival. *Cell Reports* **32**:107847. DOI: https://doi.org/10.1016/j.celrep.2020.107847, PMID: 32640217

Santiago-Raber M-L, Baccala R, Haraldsson KM, Choube D, Stewart TA, Kono DH, Theofilopoulos AN. 2003. Type-I Interferon Receptor Deficiency Reduces Lupus-Like Disease in NZB Mice. *Journal of Experimental Medicine* **197**:777–788. DOI: https://doi.org/10.1084/jem.20021996, PMID: 12642605

Schett G, Schanoski AS, Le TT, Kaiserman D, Rowe C, Prow NA, Barboza DD, Santos CA, Zanotto PMA, Magalhães KG, Baccala R, Haraldsson KM, Choubey D, Stewart TA, Kono DH, Theofilopoulos AN. 2003. Resolution of inflammation in arthritis. *Seminars in Immunopathology* **41**:1371/journal.ppat.1006496, PMID: 28704551

Shresta S, Graubert TA, Thomas DA, Raptis SZ, Ley TJ. 1999. Granzyme A initiates an alternative pathway for granzyme K expression in granzyme A- deficient cytotoxic lymphocytes. *The Journal of Biological Chemistry* **274**:19777–19785. DOI: https://doi.org/10.1074/jbc.272.32.20236, PMID: 10367905

Silver LM. 2008. In Mouse Genetics Concepts and Applications. The Jackson Laboratory. http://www.informatics.jax.org/silver/index.shtml

Simon MS. 2008. In Mouse Genetics Concepts and Applications. The Jackson Laboratory. http://www.informatics.jax.org/silver/index.shtml

Simon MS. 2013. A comparative phenotypic and genomic analysis of C57BL/6J and C57BL/6N mouse strains. *Genome Biology* **14**:R82. DOI: https://doi.org/10.1186/gb-2013-14-7-r82

Simons A. 2010. FastQC: A quality control tool for high throughput sequence data. FastQC.

Smyth MJ, Street SEA, Trapani JA. 2003. Cutting Edge: Granzymes A and B Are Not Essential for Perforin-Induced Tumor Rejection. *The Journal of Immunology** **171**:515–518. DOI: https://doi.org/10.4049/jimmunol.171.2.515, PMID: 12847210

Souza-Fonseca-Guimarães F, Young A, Mittal D, Martinet L, Bruedigam C, Takeda K, Andoniou CE, Souza-Fonseca-Guimaraes F, Street SEA, Trapani JA. 2003. Cutting Edge: Granzymes A and B Are Not Essential for Perforin-Induced Tumor Rejection. *The Journal of Immunology** **171**:515–518. DOI: https://doi.org/10.4049/jimmunol.171.2.515, PMID: 12847210

Spaeny-Dekking EH, Hanna WL, Wolbink AM, Wever PC, Kummer JA, Kummer AJ, Swaak AJ, Middeldorp JM, Huisman HG, Froelich CJ, Hack CE. 1998. Extracellular granzymes A and B in humans: detection of native species during CTL responses in vitro and in vivo. *Journal of Immunology** **160**:3610–3616. PMID: 9531325.

Spaeny-Dekking EH, Kamp AM, Froelich CJ, Hack CE. 2000. Extracellular granzyme A, complexed to proteoglycans, is protected against inactivation by protease inhibitors. *Blood* **95**:1465–1472 PMID: 10666226.

Spence CT, Abate G, Sakala IG, Xia M, Truscott SM, Eickhoff CS, Linn R, Blazevic A, Metkar SS, Peng G, Froelich CJ, Hoft DF. 2013. Granzyme A produced by y(9)s(2) T cells induces human macrophages to inhibit growth of an intracellular pathogen. *PLOS Pathogens* **9**:e1003119. DOI: https://doi.org/10.1371/journal.ppat.1003119, PMID: 23326234

Rawle, Le, et al. eLife 2022;11:e70207. DOI: https://doi.org/10.7554/eLife.70207

9 of 31
Steinhubl SR. 2008. Why have antioxidants failed in clinical trials? The American Journal of Cardiology 101:14D–19D. DOI: https://doi.org/10.1016/j.amjcard.2008.02.003, PMID: 18474268

Stewart SE, D’Angelo ME, Bird PI. 2012. Intercellular communication via the endo-lyosomal system: translocation of granzymes through membrane barriers. Biochimica et Biophysica Acta 1824:59–67. DOI: https://doi.org/10.1016/j.bbapap.2011.05.020, PMID: 21683168

Strik MCM, Wolbink A, Wouters D, Bladergroen BA, Verlaan AR, van Houdt IS, Hijlkema S, Hack CE, Kummer JA. 2004. Intracellular serpin SERPINB6 (P16) is abundantly expressed by human mast cells and forms complexes with beta-tryptase monomers. Blood 103:2710–2717. DOI: https://doi.org/10.1182/blood-2003-08-2981, PMID: 14670919

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. PNAS 102:15545–15550. DOI: https://doi.org/10.1073/pnas.0506580102, PMID: 16199517

Suhrbier A, Fernan A, Burrows SR, Saul A, Moss DJ. 1991. BLT esterase activity as an alternative to chromium release in cytotoxic T cell assays. Journal of Immunological Methods 145:43–53. DOI: https://doi.org/10.1016/0022-1759(91)90309-4, PMID: 1722495

Suhrbier A, Jaffar-Bandjee M-C, Gasque P. 2012. Arthritogenic alphaviruses—an overview. Nature Reviews. Rheumatology 8:420–429. DOI: https://doi.org/10.1038/nrrheum.2012.64, PMID: 22565316

Suhrbier A. 2019. Rheumatic manifestations of chikungunya: emerging concepts and interventions. Nature Reviews. Rheumatology 15:597–611. DOI: https://doi.org/10.1038/s41584-019-0276-9, PMID: 31481759

Suidan HS, Bouvier J, Schaerer E, Stone SR, Monard D, Tschopp J. 1994. Granzyme A released upon stimulation of cytotoxic T lymphocytes activates the thrombin receptor on neuronal cells and astrocytes. PNAS 91:8112–8116. DOI: https://doi.org/10.1073/pnas.91.17.8112, PMID: 8058766

Suidan HS, Clemetson KJ, Brown-Luedi M, Niclou SP, Clemetson JM, Tschopp J, Monard D. 1996. The serine protease granzyme A does not induce platelet aggregation but inhibits responses triggered by thrombin. The Biochemical Journal 315 (Pt 3):939–945. DOI: https://doi.org/10.1042/bj3150939, PMID: 8645180

Sun L, Wang X, Saredy J, Yuan Z, Yang X, Wang H. 2020. Innate-adaptive immunity interplay and redox regulation in immune response. Redox Biology 37:101759. DOI: https://doi.org/10.1016/j.redox.2020.101759, PMID: 33086106

Susanto O, Stewart SE, Voskoboinik I, Brasacchio D, Hagn M, Ellis S, Asquith S, Sedelies KA, Bird PI, Waterhouse NJ, Trapani JA. 2013. Mouse granzyme A induces a novel death with writhing morphology that is mechanistically distinct from granzyme B-induced apoptosis. Cell Death and Differentiation 20:1183–1193. DOI: https://doi.org/10.1038/cdd.2013.59, PMID: 23744295

Swann JB, Hayakawa Y, Zerafa N, Sheehan KCF, Scott B, Schreiber RD, Hertzog P, Smyth MJ. 2007. Type I IFN contributes to NK cell homeostasis, activation, and antitumor function. Journal of Immunology 178:7540–7549. DOI: https://doi.org/10.4049/jimmunol.178.12.7540, PMID: 17548858

Szabo R, Lantsman T, Peters DE, Bugge TH. 2016. Delineation of proteolytic and non-proteolytic functions of the membrane-anchored serine protease prostatin. Development 143:2818–2828. DOI: https://doi.org/10.1242/dev.137968, PMID: 27385010

Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P, Jensen LJ, Mering C von. 2019. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Research 47:D607–D613. DOI: https://doi.org/10.1093/nar/gky1131, PMID: 30476243

Teoh SSY, Vieuxsieux J, Prakash M, Berkowitz S, Luu J, Bird CH, Lai YS, Rosado C, Price JT, Whistock JC, Bird PI. 2014. Maspin is not required for embryonic development or tumour suppression. Nature Communications 5:3164. DOI: https://doi.org/10.1038/ncomms4164, PMID: 24445777

To EE, Erlich JR, Liang F, Li L, Liang S, Esag F, Oseghale O, Anthony D, McQuilter J, Bozinovski S, Vlahos R, O’Leary JJ, Brooks DA, Selemidis S. 2020. Mitochondrial Reactive Oxygen Species Contribute to Pathological Inflammation During Influenza A Virus Infection in Mice. Antioxidants & Redox Signaling 32:929–942. DOI: https://doi.org/10.1089/ars.2019.7727, PMID: 31190565

Toye AA, Lippati JD, Proks P, Shimomura K, Bentley L, Hugill A, Mijat V, Goldsworthy M, Moir L, Haynes A, Quarterman J, Freeman HC, Ashcroft FM, Cox RD. 2005. A genetic and physiological study of impaired glucose homeostasis control in C57BL/6J mice. Diabetesologia 48:675–686. DOI: https://doi.org/10.1007/s00125-005-1680-z, PMID: 15729571

Tumiatiram, M. Hemmes A, Uusivirta S, Koopal S, Kankainen M, Lehtonen E, Kuznetsov SG. 2015. Loss of Rad51c accelerates tumourigenesis in seaceous glands of Trp53-mutant mice. The Journal of Pathology 235:136–146. DOI: https://doi.org/10.1002/path.4455, PMID: 25270124

Ubaida-Mohien C, Lyashkov A, Gonzalez-Freire M, Tharakang R, Shardell M, Moaddel R, Semba RD, Chia CW, Gorospe M, Sen R, Ferrucci L. 2019. Discovery proteomics in aging human skeletal muscle finds change in spliceosome, immunity, proteostasis and mitochondria. eLife 8:e49874. DOI: https://doi.org/10.7554/eLife.49874, PMID: 31642809

Uraga S, Marinova D, Martin C, Pardo J, Aguilo N. 2016. Granzyme A Is Expressed in Mouse Lungs during Mycobacterium tuberculosis Infection but Does Not Contribute to Protection In Vivo. PLOS ONE 11:e0153028. DOI: https://doi.org/10.1371/journal.pone.0153028, PMID: 27055232

van Daalen KR, Reinevere JF, Bovenschen N. 2020. Modulation of Inflammation by Extracellular Granzyme A. Frontiers in Immunology 11:931. DOI: https://doi.org/10.3389/fimmu.2020.00931, PMID: 32508827
