Supplementary Information

Distinct cell death pathways induced by granzymes collectively protect against intestinal *Salmonella* infection

Amanpreet Singh Chawla, Maud Vandereyken, Maykel Arias, Llipsy Santiago, Dina Dikovskaya, Chi Nguyen, Neema Skariah, Nicolas Wenner, Natasha B. Golovchenko, Sarah J. Thomson, Edna Ondari, Marcela Garzón-Tituaña, Christopher J. Anderson, Megan Bergkessel, Jay C. D. Hinton, Karen L. Edelblum, Julian Pardo, Mahima Swamy

Graphical abstract
Supplementary Figure 1. A. Representative immunofluorescent micrographs to confirm the specificity of the GzmA and GzmB antibodies. Images show cells expressing GzmA (red) and GzmB (green) in the epithelial layer of a jejunal villus in WT, GzmA KO and GzmB KO. Sections were counterstained with phalloidin to show actin (white) and DAPI to show nuclei (blue on left panel). Scale bar=5µm. B. Gating strategy of GzmA/B+ cells in the small intestine epithelium. Total CD45+ were gated as live, single, GzmAB+ cells. These GzmA/B+ cells can be further divided into TCRβ+, TCRγδ+ and TCR- subsets. C. Gating strategy of IEL in the small intestine - Total IEL were gated as live, single cell, CD45+CD103+ cells. CD45+CD103+ cells can be further subdivided into TCRβ+ (CD8αα+ and CD8αβ+),
TCRγδ+ (CD8αα- and CD8αβ-) and TCR− subsets. D. Absolute numbers of CD103+ IEL in WT littermate controls, GzmA KO, GzmB KO and GzmA/B dKO mice, with gating strategy shown in Suppl. Fig 1C E. Comparison of the percentage of positive WT and GzmA/B dKO IEL, from co-housed mice, for CD103, CD69, CD160, Lag-3, LILRB4, TIGIT and CD96 surface markers (n=3 each), with gating strategy shown in Suppl. Fig 1C. All data are presented as mean ± SEM. P values were calculated for (C-D) by ordinary one-way ANOVA with Sidak’s multiple comparisons. Where no p-values are shown, no significance was found.
Supplementary Figure 2

**A.** Bar graph showing total bacterial (SL1344) counts in ileum, caecum, and colon of WT mice 4 days post infection (n=6).

**B.** Comparison of the expression of CCR9 (left panel) and α4β7 (right panel) on ex vivo IEL and IEL cultured with retinoic acid and 100 ng/ml IL-15/Ra.

**C.** Bar graph showing the number of CD45+ cells collected in the SI epithelium after ex-vivo (n=3) or cultured (n=2) IEL transfer into Rag2−/− mice. Bar graphs show the ratio of GzmA positive cells in the TCRβ and TCRγδ populations, in the 1:1 mix before the transfer (n=2) and 4 weeks after the transfer, isolated from the adoptively transferred Rag2−/− mice (n=4). All data are presented as mean ± SEM.

**D.** Competitive transfer of cultured WT and GzmA/B dKO IEL (from cohoused mice) into Rag2−/− mice. WT and GzmA/B dKO IEL were mixed at a ratio of 1:1 before the transfer. Bar graphs show the ratio of GzmA positive cells in the TCRβ and TCRγδ populations, in the 1:1 mix before the transfer (n=2) and 4 weeks after the transfer, isolated from the adoptively transferred Rag2−/− mice (n=4). All data are presented as mean ± SEM.

**E.** Percentages of GzmA/B+ cells out of CD45+ cells isolated from the small intestinal epithelia of Rag2−/− mice, and from Rag2−/− mice that received IEL from either WT or GzmA/B dKO mice 4 weeks earlier, and that were then infected with SL1344. These data are from the mice shown in Fig. 2G-I. P values were calculated for (A) using ordinary one-way ANOVA with Sidak’s multiple comparisons. ***p<0.001.
Supplementary Figure 3. A. Bar graphs comparing intestinal permeability in cohoused naïve WT and GzmA/B dKO mice (n=5/group). The concentration of FITC dextran was measured in the serum of the mice 4h after the gavage. B. Transcript fold change in the IL-6, IL-18 and TNF in small intestinal tissue of cohoused WT and GzmA/B dKO mice 3 days post *Salmonella* infection (n=3/group). C. Schematic showing the set-up of invitro MODE-K killing assay by IEL. MODE-K was infected with SL1344-lux for 0.5 h and then extracellular bacteria was killed using gentamycin. IEL were added to infected MODE-K and bioluminescence was acquired at different time points. D. Graph showing the correlation between bacterial OD and bacterial bioluminescence. E. IEL do not kill uninfected MODE-K. Bar graph showing the survival, measured using crystal violet method, of uninfected MODE-K in the presence or absence of WT IEL (n=3/group). All data are presented as mean ± SEM. P values were calculated for (A and E) using unpaired t-test and for (B) ordinary one-way ANOVA with Sidak’s multiple comparisons. ns, not significant.

Supplementary Figure 4. Cohoused WT (n=10) and Prf1 KO (n=14) mice were orally infected with SL1344-GFP and culled 5dpi. Weight loss (A) and CFU/mg in MLN, spleen and liver at the time of sacrifice (B) are shown. Data were pooled from 2 independent experiments. All data are presented as mean ± SEM. P values were calculated for bacterial counts using the Mann-Whitney U-test and for all other comparisons, two-way ANOVA was used, with multiple comparisons using Sidak’s multiple comparisons tests. ns: not significant.
Supplementary Figure 5

A. Mean fluorescent intensity (MFI) of GzmA (left panel) and GzmB (right panel) in naïve GzmA KO (n=3), GzmB KO (n=2) compared to their WT littermate controls (n=3) mice.

B. Comparison of the percentage of GzmA⁺ (left panel) or GzmB⁺ (right panel) IEL in GzmA KO, GzmB KO, and WT littermate controls (n>5/strain).

C-D. Transcript fold change in Gzma and Gzmb in GzmA KO (C) and GzmB KO (D) as compared to WT littermate controls 5 days post Salmonella infection from mice in figure 4.

E. Dot plots comparing intestinal permeability in naïve GzmB KO and WT littermate control mice (n=5/group). The concentration of the FITC dextran was measured in the serum of the mice 4h after the gavage. Data were pooled from 2 independent experiments. All data are represented as mean ± SEM, except A where it is represented as mean ± SD. P-values were calculated for (B) by ordinary one-way ANOVA with Sidak’s multiple comparisons, (C) by unpaired t-test. ns: not significant. Where no p-values are shown, no significance was found.
**Supplementary Figure 6.** Chemokine and cytokine levels in plasma of naïve and orally infected (A) GzmA sKO (n=12) and (B) GzmB sKO (n=12), compared to their WT littermate controls (n>9) mice from figure 4, 5 days post oral *Salmonella* infection. All data are represented as mean ± SEM. P-values were calculated for (A-B) by ordinary one-way ANOVA with Sidak’s multiple comparisons, (C) by unpaired t-test. Standard annotations were used to denote significance: ns, not significant, ** p<0.01, *** p<0.001.

**Supplementary videos 2 and 3.** Representative time-lapse confocal microscopy videos of either (2) co-housed WT or (3) GzmA/B dKO IEL (in green) co-cultured with WT enteroids. Frames were acquired every 2.5 minutes for 90 min.