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Supplemental Information

Hepcidin-Mediated Hypoferremia Disrupts Immune Responses to Vaccination and Infection

Joe N. Frost, Tiong Kit Tan, Munawar Abbas, Sarah K. Wideman, Michael Bonadonna, Nicole U. Stoffel, Katherine Wray, Barbara Kronsteiner, Gaby Smits, Dean R. Campagna, Tiago L. Duarte, José M. Lopes, Akshay Shah, Andrew E. Armitage, João Arezes, Pei Jin Lim, Alexandra E. Preston, David Ahern, Megan Teh, Caitlin Naylor, Mariolina Salio, Uzi Gileadi, Simon C. Andrews, Susanna J. Dunachie, Michael B. Zimmermann, Fiona R.M. van der Klis, Vincenzo Cerundolo, Oliver Bannard, Simon J. Draper, Alain R.M. Townsend, Bruno Galy, Mark D. Fleming, Marie C. Lewis, and Hal Drakesmith

Supplementary Information for Frost et al, Hepcidin-mediated hypoferremia disrupts immune responses to vaccination and infection

Supplementary Figures 1-7 and legends

and

Supplementary Methods, related to Flow Cytometry in STAR Methods
(Supplementary Methods figures 1-6)

Figure S1

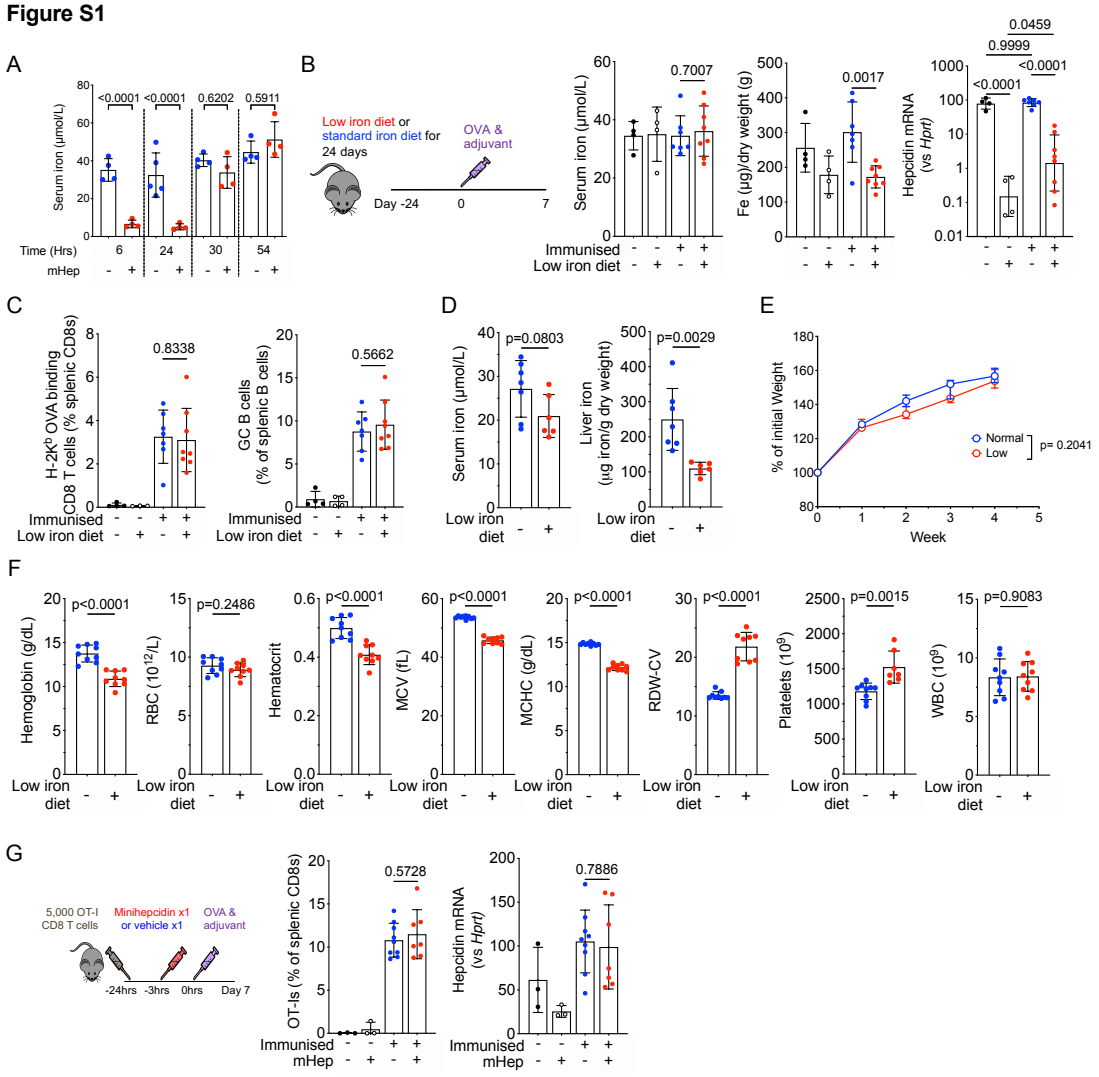


Figure S1. Depleted iron stores or a single minihepcidin injection at the time of immunisation are insufficient to impair adaptive immune responses relating to figure 1

A serum iron levels were measured after a single injection of minihepcidin. Mean ± sd. Two-way ANOVA, multiple comparisons for treatment effect at each time point.

B Experimental design to test the effect of low (2ppm) diet on adaptive immune response to immunisation. Serum iron levels, non-heme iron content of the liver, and liver hepcidin mRNA were measured. Mean ± sd. Students two-tailed T-test, unpaired.

C Frequency of OVA specific CD8 effector cells (as a % of CD8 T-cells) and GC B-cells (as a % of B cells) were measured by flow cytometry at 7 days post immunisation. Mean ± sd. Students two-tailed T-test, unpaired.

D serum iron and liver non-heme iron content in mice maintained on a low or normal iron diet for 4-weeks. Mean ± sd. Students two-tailed T-test, unpaired.

E change in growth of mice maintained on a low or normal iron diet for 4-weeks starting at 4-weeks of age. Interquartile range, two-way ANOVA.

F blood hematology parameters in mice maintained on a low or normal iron diet for 4-weeks, left to right, hemoglobin, RBC (red blood cell count), hematocrit, MCV (mean corpuscular volume), MCHC (mean corpuscular hemoglobin content), RDW-CV (red cell distribution width), platelet count, WBC (white blood cell count). Mean ± sd. Students two-tailed T-test, unpaired

G Experimental design to test the effect of a single minihepcidin injection at the time of immunisation. Left, frequency of OVA specific OT-I effect cells as a % of total CD8s at 7 days post immunisation. Right, liver hepcidin mRNA. Mean ± sd. Students two-tailed T-test, unpaired.

Figure S2

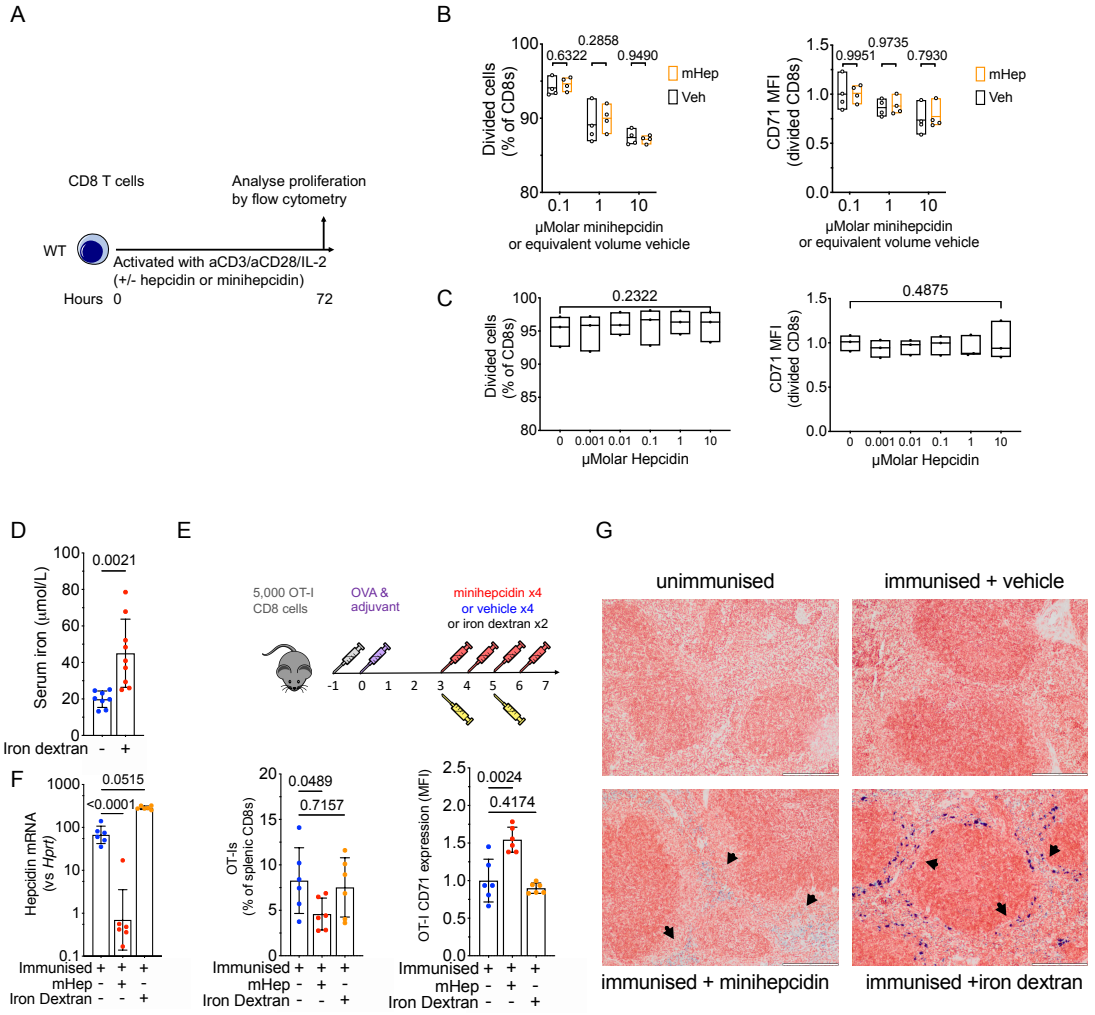


Figure S2. Hepcidin does not directly alter CD8 T cell proliferation *in vitro*. Splenic macrophage iron loading is not associated with suppressed responses to immunisation. Relating to figure 1.

A Scheme for *in vitro* CD8 T cell treatment with hepcidin or minihepcidin.

B Left, % of CD8 T cells divided (according to dilution of cell trace violet) and (right) normalised relative CD71 MFI measured by flow cytometry after treatment with mini-hepcidin or equivalent amount of vehicle control. Mean ± range. Two way ANOVA, reporting multiple comparisons p value of minihepcidin vs vehicle % of CD8 T cells divided (according to dilution of cell trace violet), paired within each biological replicate.

C Left, % of CD8 T cells divided (according to dilution of cell trace violet) and (right) normalised relative CD71 MFI measured by flow cytometry after treatment with indicated concentration of hepcidin. Mean ± range. One-way ANOVA effect of hepcidin concentration, paired within each biological replicate.

D Top, serum iron levels 24 hours after a single injection of 4mg of iron dextran. Mean ± sd. Students two-tailed T-test, unpaired.

E Experimental design to determine effect of macrophage iron loading induced by two doses of 2mg of iron dextran injection on CD8 T cell responses to immunization.

F Left to Right. Liver hepcidin mRNA expression. The frequency of antigen specific splenic OT-I CD8 T cells and normalised relative MFI of surface CD71 expression by OT-I CD8 T cells 7 days after OVA and adjuvant immunisation were detected with flow cytometry. Mean ± sd. One-way ANOVA with correction for multiple comparisons.

G Representative Perls' staining of spleen sections to resolve macrophage iron loading (blue staining, indicated by black arrowheads). Magnification 100x, scale bar 200µm.

Figure S3

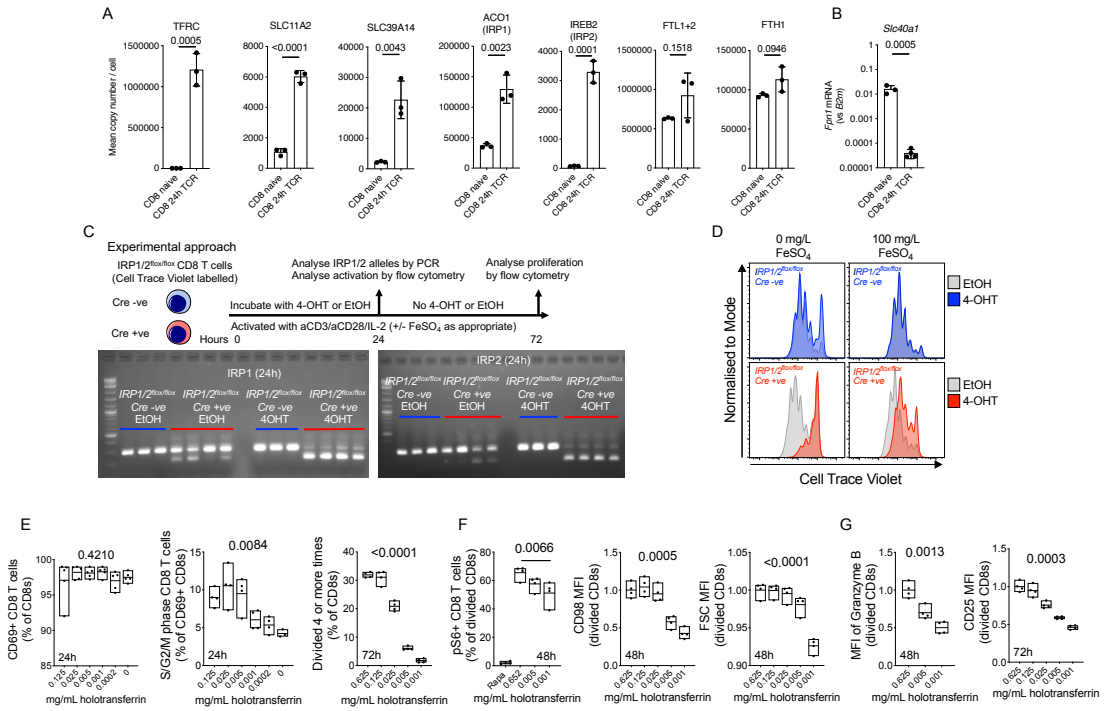


Figure S3. CD8 T cell activation and proliferation involves remodelling of iron homeostasis, requires IRP1 and IRP2, and is dependent on extracellular iron levels. Relating to figure 4.

A Mean copy number per cell of TFRC, SLC11A2, SLC39A14, ACO1, IREB2, FTL1 and 2 and FTH from CD8 T cells from P14 transgenic mice, naïve or activated *in vitro* for 24 hours. Data is derived from reference 11. Mean ± sd. Students two-tailed T-test, unpaired.

B Ferroportin mRNA in CD8 T cells magnetically isolated from the lymph nodes of C57BL/6 mice. Naïve cells were immediately snap frozen whereas 24 hour activated cells were snap frozen after 24 hours of activation with anti CD3, CD28 and IL-2. Ferroportin mRNA was measured by qRT-PCR using taqman probes against *Fpn/Slc40a1* and *B2m*. Mean ± sd. Students two-tailed T-test, unpaired.

C Experimental design used to investigate role of IRP1 and IRP2 in CD8 T cells. Genomic PCR and gel electrophoresis results showing IRP1/2$^{flox/flox}$ CreERT2 +ve and IRP1/2$^{flox/flox}$ CreERT2 -ve CD8 T cell IRP1 alleles and IRP2 alleles after 24 hours of activation and treatment with EtOH or 4-OHT. The smaller migrating bands are the recombined 'KO' alleles. A small amount of leaky recombination is observed in EtOH treated IRP1/2$^{flox/flox}$ CreERT2 +ve, but the level of recombination is much higher in 4OHT treated cells.

D Flow cytometry based measurement of CD8 T cell proliferation resolved by dilution of cell trace violet for Cre- and Cre+ IRP1/2$^{flox/flox}$ CD8 T-cells treated with EtOH or 4-OHT in the presence or absence of added 100mg/mL FeSO4 as a source of non-transferrin bound iron. Representative histograms of CD8 T cell CTV profiles at 72 hours post activation. The % of cells which had diluted CTV more than once reported as 'divided.

E Left to right: Frequency of CD69+ activated cells and cells in S/G2/M phases of the cell cycle, as indicated by their >2N DNA content resolved by increased staining with DNA content stain DAPI, detected by flow cytometry. Flow cytometric analysis of the dilution of cell trace violet labelling with division was used to assay proliferation of CD8 T cells, indicating the frequency of CD8 T cells which had divided more than 4 times. Mean ± range. One way ANOVA, effect of iron, paired within each biological replicate.

F Left to right: % of CD8 T cells positive for pS6, relative CD98 expression (MFI) and relative size (FSC-A MFI) of divided cells was measured for CD8 T cells by flow cytometry. Mean ± range. One way ANOVA, effect of iron, paired within each biological replicate.

G Left to right: Granzyme B and CD25 expression (MFI) presented for divided cells. mean ± range One way Anova, effect of iron, paired within each biological replicate. Mean ± range. One way Anova, effect of iron, paired within each biological replicate.

Figure S4

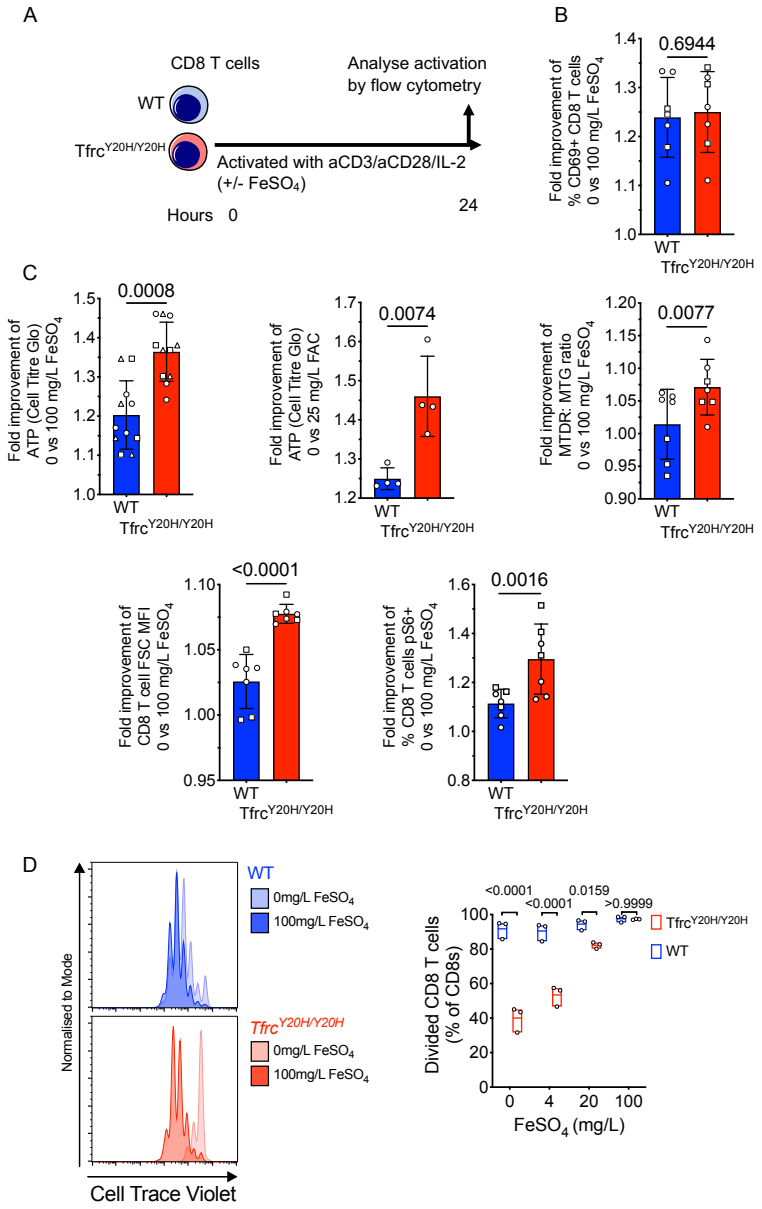


Figure S4. Iron uptake via the transferrin receptor is required for optimal activation prior to division, relating to figure 4.

A Experimental scheme for investigation of effect *Tfrc*$^{Y20H/Y20H}$ mutation of CD8 T cell activation *in vitro*. In all cases the improvement in the parameter in question in iron-supplemented cells vs non-iron-supplemented cells is reported.

B % of CD69+ cells was measured by flow cytometry. Mean ± sd. Two-way ANOVA blocking for effect of experiment replicate and genotype, reported p value is genotype effect.

C ATP production was measured using Cell Titre Glo kit. The MFIs of mitotracker deep red (MTDR) and mitotracker green (MTG) were measured by flow cytometric analysis and the ratio of MTDR to MTG was calculated. FSC MFI and the % of pS6 expressing cells was measured by flow cytometry. Presented as a combination of two or three independent experiments (indicated by different data point shapes). Mean ± sd. Two-way ANOVA blocking for effect of experiment replicate and genotype, reported p value is genotype effect.

D WT and *Tfrc*$^{Y20H/Y20H}$ activated for 72hours in the presence or absence of $FeSO_4$, proliferation was resolved by flow cytometric analysis of cell trace violet dilution and the % of cells which had diluted CTV more than once reported as 'divided'. Left: Representative cell trace violet profiles and effect of genotype and iron on % of CD8 T cells divided. Mean ± range. Right: Two-way ANOVA, multiple comparisons for treatment at each iron level.

Figure S5

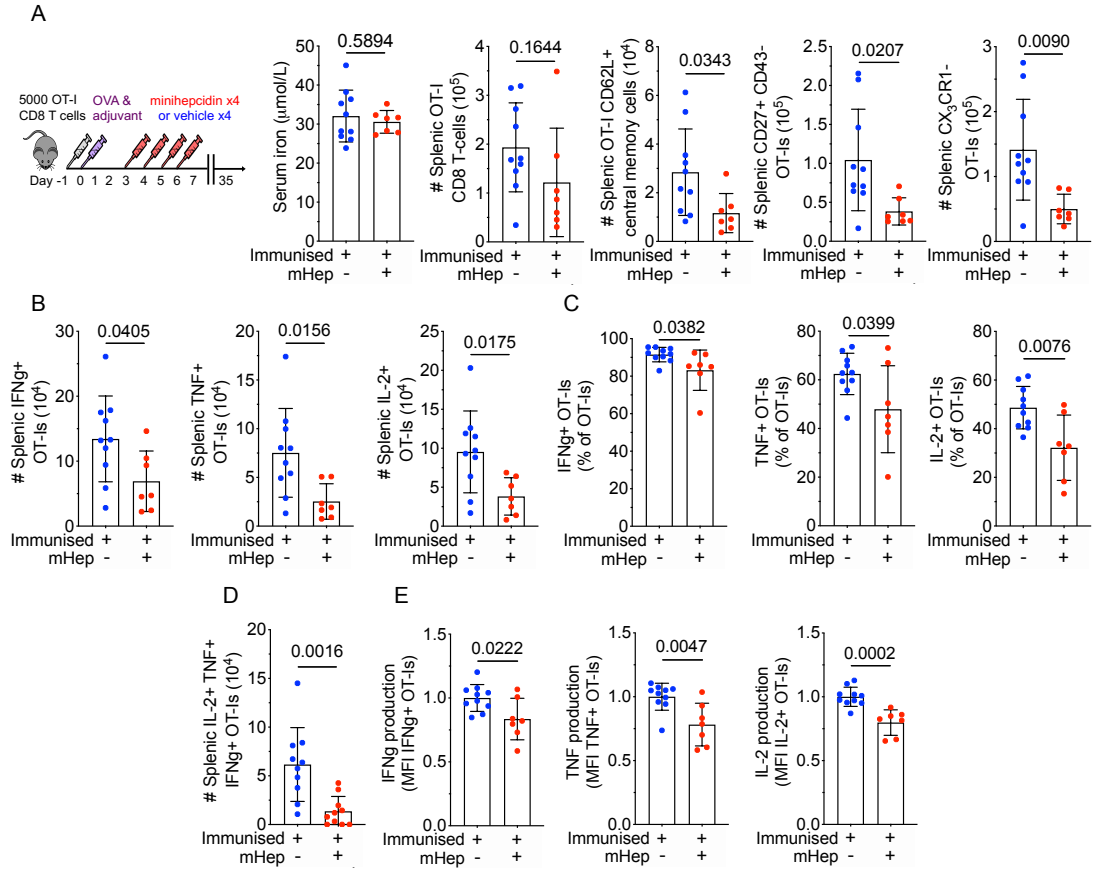


Figure S5. Transient hypoferremia during the primary CD8 T-cell response to immunisation impairs the quality and magnitude of CD8 T-cell memory, relating to figure 5.

A Experimental design for investigation of the effect of serum iron deficiency during the primary immune response on the magnitude and quality of CD8 T cell memory at 35 days post primary immunisation. Serum iron concentration at day 35 was measured. The number of total OT-I memory cells, as well as those with a CD62L+, CD27+ CD43- or CX_3CR1- surface phenotype was measured by flow cytometry in the spleen. Cytokine production by splenic OT-I memory cells on day 35 post immunisation measured by intracellular cytokine staining and flow cytometry after restimulation of total splenocytes *ex vivo* with SIINFEKL. Mean+/- SD. Students two tailed T-test.

B Total number of IFNγ+, TNFα, IL-2+ OT-I memory cells. Mean ± sd. Students two tailed T-test, unpaired.

C % of OT-I memory cells which can produce IFNγ, TNFα and IL-2.

D Number of triple positive IL-2+, TNF+, IFNg+ memory cells per spleen is also shown. Mean ± sd. Students two tailed T-test, unpaired.

E relative normalised MFI of TNFα, IFNγ and IL-2 for TNFα+, IFNγ+ and IL-2+ cells respectively. MFI normalised to average of vehicle treated group. Mean ± sd. Students two tailed T-test, unpaired.

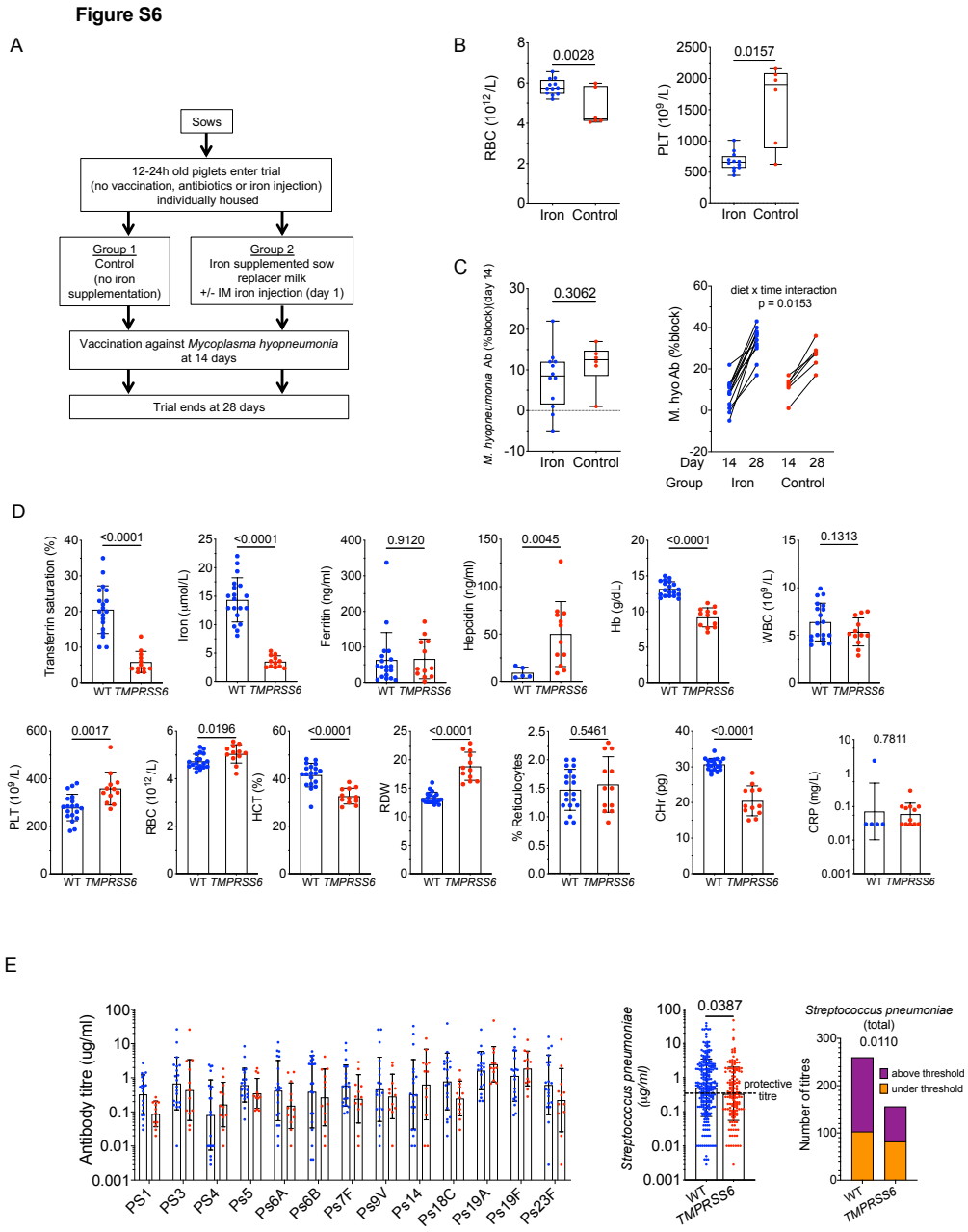


Figure S6. Piglet iron supplementation study, and IRIDA patient phenotype and response to *Streptococcus pneumoniae* vaccination, relating to figure 6.

A Experimental design for investigation of effect of iron supplementation of piglet physiology and vaccine responses.

B RBC and platelet counts were measured at 28 days of age in piglets. Mean +/- range.

C Anti-M. hyopneumoniae antibody presented as % block on day 14 (baseline, day of immunisation), left. Mean ± sd Students two tailed T-test, unpaired. Change in % block with time: % block on day 14 and day 28, with lines connecting paired data for each piglet, right. Two-way ANOVA reporting significant interaction of effect of diet and time.

D Transferrin saturation, serum iron, ferritin, hepcidin, Hb, haematological parameters and CRP of IRIDA patients expressing mutant *TMPRSS6* and non-anaemic wild-type *TMPRSS6* controls. Mean ± sd, geometric mean ± geometric sd for logged CRP data. Students two tailed T-test, unpaired.

E Ab concentrations against all *Streptococcus pneumoniae* serotypes analysed individually, and in combination. Mean ± SD. Students two tailed T-test, unpaired. Rightmost panel: Analysis of the frequency of measurements for both genotypes with Ab concentrations against each *Streptococcus pneumoniae* serotype that exceeds the protective threshold of 0.35ug/mL. Fisher exact test.

Figure S7

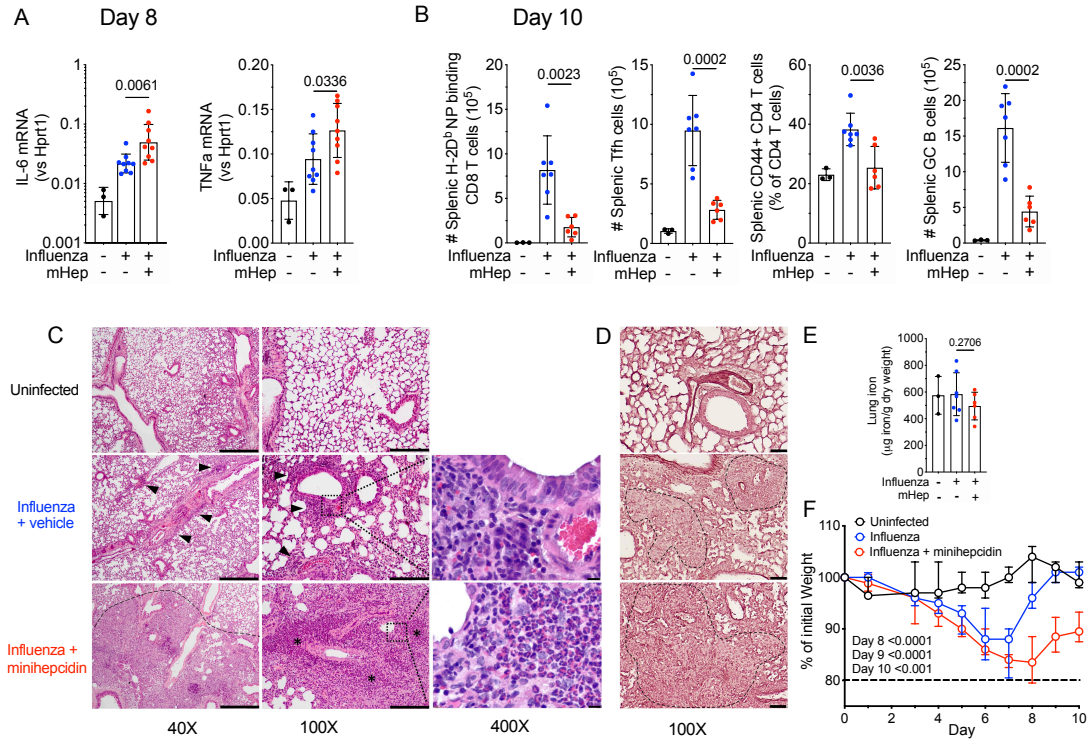


Figure S7. Serum iron deficiency increases lung inflammation but suppresses adaptive immune responses to influenza infection, relating to figure 7

A Expression in whole lung lobe of *Il6* and *Tnfa* mRNA on day 8 post-infection. Mean ± sd. Students two tailed T-test, unpaired.

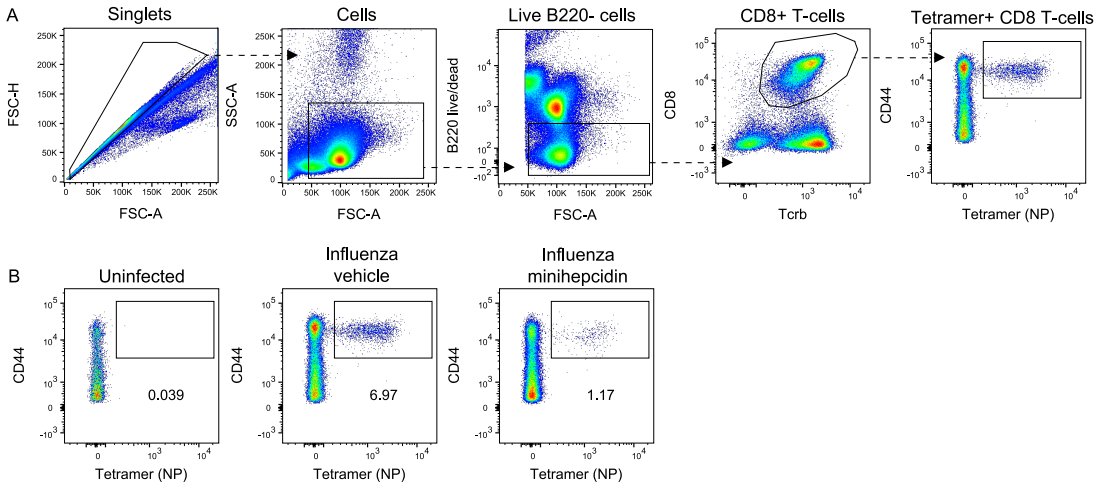
B Splenic influenza NP specific CD8 effector cell number, CD4 T follicular helper cell number, frequency of CD44+ effector CD4s as a % CD4 T cells and GC B cell number on day 10 post influenza virus infection. Mean ± sd. Students two tailed T-test, unpaired.

C H&E staining of representative lung sections from mice on day 10 post influenza infection from each group. 40x, scale bar 500µm, arrows/area delineated by dotted line indicate inflammatory infiltrate. 100x, scale bar 200µm, arrowheads point to areas of lymphocytic infiltration in the perivascular/peribronchiolar compartment, asterisks highlight multiple foci of polymorphonuclear cell aggregation. 400x, scale bar 10µM, frequent polymorphonuclear cells indicated by intense pink eosin staining cytoplasm.

D Representative lung sections stained with Orcein stain, to resolve elastic fibres, from day 10 post infection. 100x, scale bar 50µm. Inflamed areas with breakdown of lung structure delineated with dotted line.

E Lung non-haem iron content 10 days post influenza virus infection. Mean ± sd. Students two tailed T-test, unpaired.

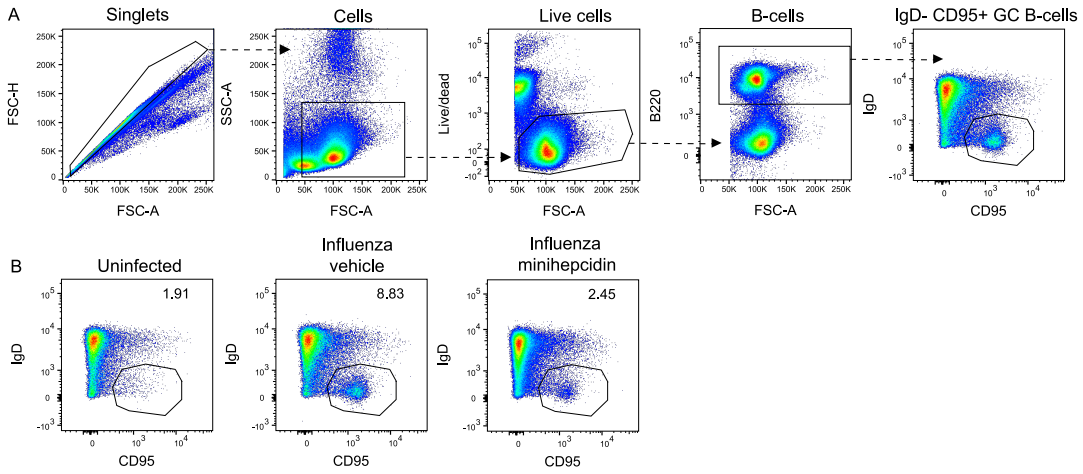
F Percentage of initial starting weight was measured throughout the experiment. Mean ± range. Two-way ANOVA, with multiple comparisons for effect of minihepcidin treatment at each time point.



Supplementary Methods Figure 1.

A Example gating scheme for Tetramer+ CD8 effector cells.

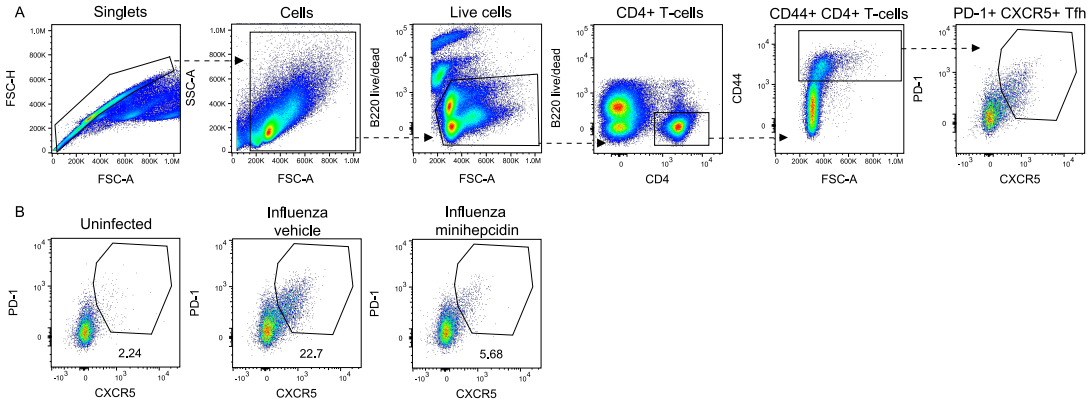
B Representative effect of minihepcidin on Tetramer+ CD8 effector cells.



Supplementary Methods Figure 2.

A Example gating scheme for germinal centre B cells.

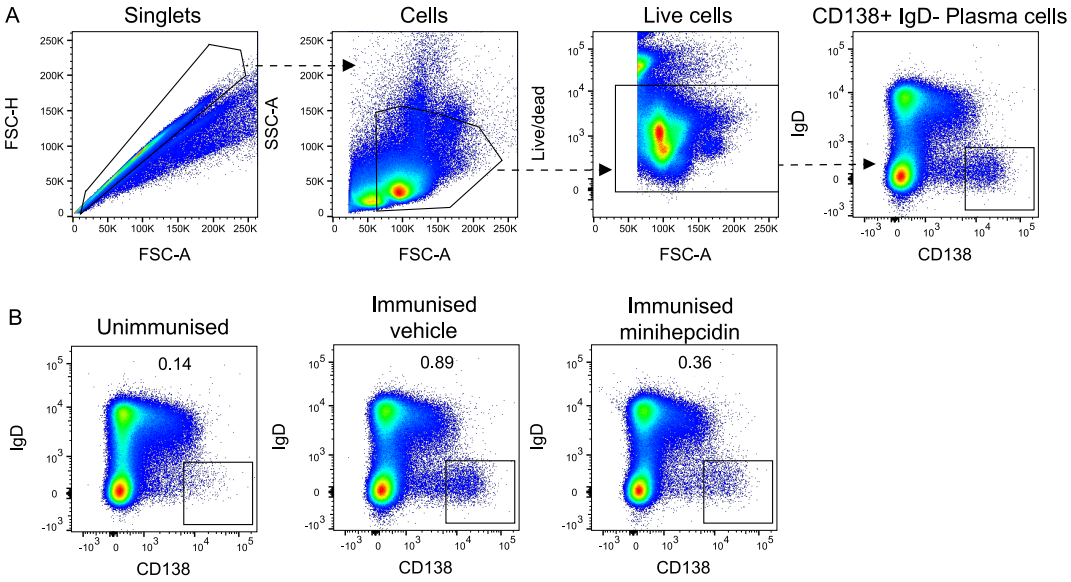
B Representative effect of minihepcidin on germinal centre B cells.



Supplementary Methods Figure 3.

A Example gating scheme for T follicular helper cells.

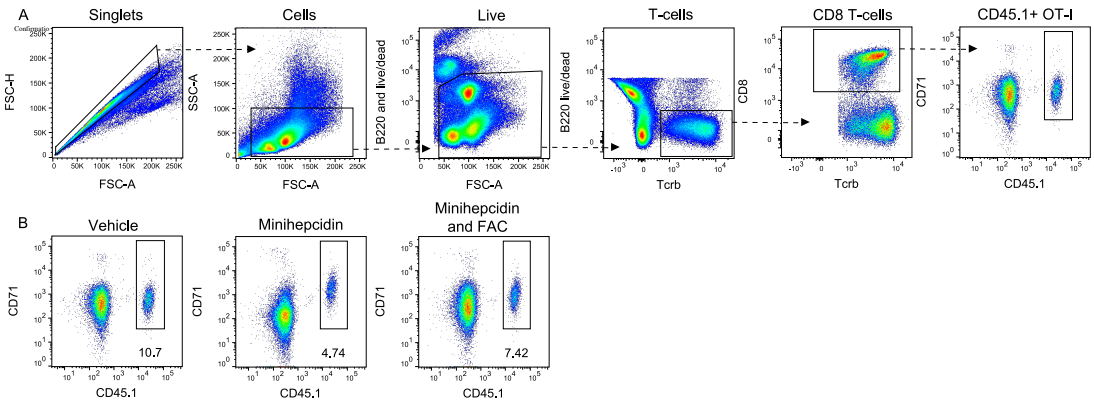
B Representative effect of minihepcidin on T follicular helper cells.



Supplementary Methods Figure 4.

A Example gating scheme for plasma cells.

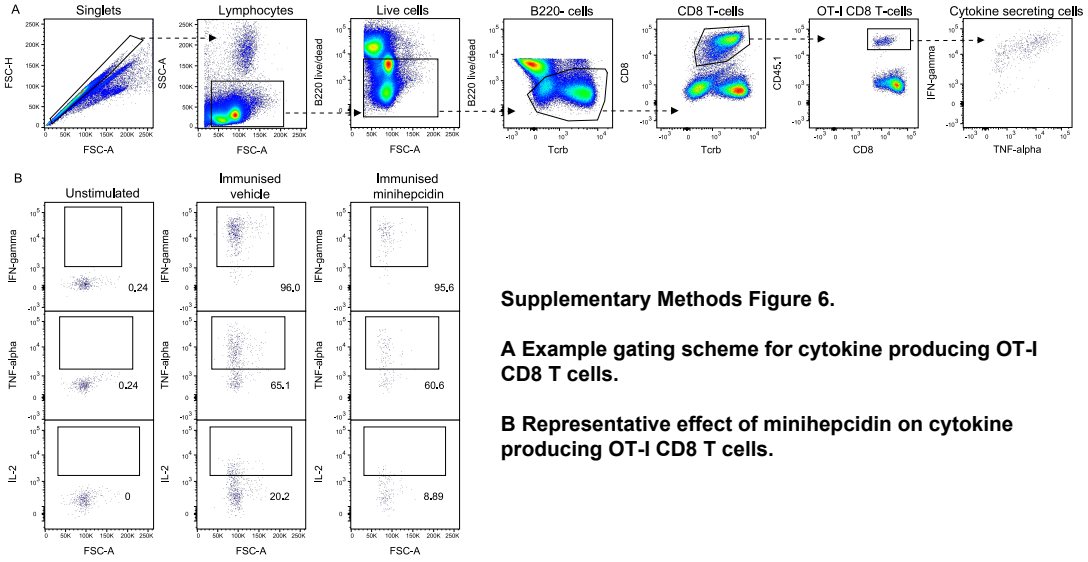
B Representative effect of minihepcidin on plasma cells.



Supplementary Methods Figure 5.

A Example gating scheme for OT-I CD8 T cells.

B Representative effect of minihepcidin on OT-I CD8 T cells.



Supplementary Methods Figure 6.

A Example gating scheme for cytokine producing OT-I CD8 T cells.

B Representative effect of minihepcidin on cytokine producing OT-I CD8 T cells.