Supplemental methods

Mice and cell cultures

Female WT C57BL/6 (BL/6), OVA-specific CD8+ TCR transgenic OT-1 (C57BL/6-Tg(TcraTcrb)1100Mjb/J), BL/6 SCID (B6.CB17-Prkdc,SCID./SzJ) and BL/6 CD61 KO (B6;129S2-Itgb3tm1Hyn/J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed in the St Michael's Hospital Research Vivarium. The St. Michael's Hospital Animal Care Committee approved all animal studies.

Murine bone marrow-derived megakaryocytes were cultured as previously described [52]. Briefly, adult mice (8 to 10 weeks of age) were euthanized and their bone marrows harvested from femurs and tibia by flushing in DMEM (Gibco, Invitrogen, Burlington, Canada) with a 23-gauge needle. A single cell suspension was made and filtered through a 70 μM filter. Cells were cultured for 3 to 5 days at 37°C and 5% CO2 in DMEM medium with $5x10^{-5}$ M β2-mercaptoethanol, 2 mM L-glutamine, 100 μg/ml penicillin/streptomycin, containing 10% fetal bovine serum for mouse myeloid colony-forming cells (Stem Cell Technology, Vancouver, Canada) and 40 ng/ml of recombinant murine TPO (Cedarlane, Burlington, Canada). Cells were then loaded on a 1x g bovine serum albumin (BSA, Sigma-Aldrich, St Louis, USA) 2 step gradient (3% and 1.5%) to enrich mature megakaryocytes.

To isolate CD8+ T cells, spleen and lymph nodes were harvested from euthanized wild type (WT) C57BL/6 and OT-1 mice. Tissues were macerated with a syringe plunger in RPMI 1640 medium (Gibco) and applied through a 40 μm filter. CD8+ T cells were then isolated using the StemCell Technologies EasySep Negative selection Mouse CD8+ T cell isolation kit according

to the manufacturer's instruction. The OT-1 and BL/6 CD8+ T cells were 97.7% (±0.9) and 93.3% (±5.4) of the total cells after isolation, respectively (n=3-4, data not shown).

Chemicals and antibodies

Anti-mouse OVA 257-264 (SIINFEKL) peptide bound to H-2Kb (MHCI-OVA) antibody conjugated with Allophycocyanin (APC), anti-CD69-phycoerythrin (PE), purified anti-CD63, anti-CD41 Brilliant Violet 605 and APC, anti-CD3-PerCP/Cy5.5, anti-CD8a-APC/Cy7, Annexin V conjugated with Fluorescein (FITC), Annexin V binding buffer, anti-CD86-PE, purified anti-CD63 antibody, anti-CD19 AF647 and goat anti-mouse IgG conjugated with AF594 were from Biolegend (San Diego, USA). Purified anti-MHCI-OVA antibody, anti-CD8-FITC, anti-CD25-PE, cell stimulation cocktail plus protein inhibitors and 7-Amino-actinomycin D (7-AAD) were from eBioscience (San Diego, USA). Purified anti-CD41 and anti-I-A/I-E-FITC antibodies were purchased from BD Pharmingen. Anti-early endosome antigen 1 (EEA1) antibody came from R&D Systems (Minneapolis, USA). The Lysosomal-associated membrane protein 1 (LAMP1) antibody conjugated with DyLight 488, anti-CD62P (P-selectin) AF488 antibody and goat anti-mouse IgG DyLight 555 secondary antibody were from Novus Biologicals (Littleton, USA). Goat anti-Rat IgG AF555 secondary antibody was purchased from Abcam (Cambridge, USA). Nocodazole and Albumin from bovine serum (minimum 98% electrophoresis) were from Sigma-Aldrich. MG-132 was purchased by MedChem Express (Monmouth Junction, USA). Purified EndoFit ovalbumin was purchased from InvivoGen (San Diego, USA), anti-CD80-PE, DAPI, DQ-Ovalbumin and Ovalbumin conjugated with an AF647 from Molecular Probes (Eugene, USA). Goat anti-Mouse IgG FITC-conjugated was purchased from Invitrogen (Carlsbad, USA). Anti-MHC class I Kb was purchased from Serotec (Oxford, United Kingdom). Violet Proliferation Dye 450 was from BD Biosciences (Mississauga, Canada).

Confocal microscopy

BSA-enriched MKs were grown on uncoated glass coverslips for 24h before being pulsed with ovalbumin fixed in 4% paraformaldehyde (PFA, EM grade, Hatfield, USA) for 20 minutes. The PFA was quenched in PBS with 10 mM glycine. Cells were then permeabilized with Triton-X100 (0.1% for 10 minutes) and incubated for 1h with a blocking solution of 3% BSA in PSB containing 4% goat serum (Abcam, Cambridge, USA). Staining was performed in 1% BSA PBS for 1h and coverslips were mounted (Mounting solution, Dako, Glostrup, Denmark). MKs were imaged using a Zeiss LSM 700 confocal fluorescent microscope (Zeiss, Oberkochen, Germany) with a 63x oil-immersion objective. Z-stack images and corresponding controls were acquired with the same settings and processed in exactly the same way using IMARIS software 8.0.4 (Bitplane, Zurich, Switzerland). Briefly, contrasts were linearly adjusted and voxel colocalizations were quantified in z-stacks by manual thresholding. Manders split colocalization coefficient was measured to quantify the colocalization between OVA and LAMP1 (15 minutes) and CD62P (60 minutes) using the FIJI Colocalization threshold plugin (n=4).

In vivo antigen-specific $CD8^+$ T cell activation

MKs were prepared and pulsed with 500 µg/mL DQ-OVA for 24h as previously described, and washed twice in PBS. OT-1 or WT BL/6 mice were infused with $8x10^5$ OVA-pulsed MKs using a 25-gauge needle via tail vein. To ensure that only intact cells were transfused, the MK integrity after injection into a saline solution was ascertained by microscopy (data not shown). After 48h, mice were anesthetized and blood was collected by cardiac puncture. Mice were sacrificed and spleens and lymph nodes were harvested. $CD8^+$ T cells were stained with anti-CD8-APC/Cy7 and anti-CD3-PerCP/Cy5.5 (Biolegend) and the level of activation of the $CD8^+$ $CD3^+$ cells was

assessed with CD69-PE (Biolegend) by flow cytometry. A least 10 000 CD8+ CD3+ cells were analyzed.

Platelet Preparation

Platelets were prepared from whole blood drawn by cardiac puncture of WT mice into PBS with citrate/phosphate, dextrose, adenine (CPDA) by centrifugation at 210xg for 15 minutes. The platelet-rich-plasma (PRP) was obtained and further centrifuged at 1000xg for 10 minutes to concentrate platelets. Platelets were subsequently washed in PBS and resuspended at final concentration of 10^9 cells/mL.

In vitro splenocyte depletions.

Splenocytes were depleted of CD19+ B lymphocytes before the transfer with an EasySep Magnetic sorting kit (StemCell Technologies, Vancouver, Canada) according to the manufacturer's guidelines. The level of depletion was verified by flow cytometry (≥98%, data not shown).

ITP adoptive transfer model

A well-characterized active murine ITP model was utilized as previously described.[33] Briefly, to generate a source of platelet/MK-specific splenocytes, CD61 KO mice were transfused weekly for 5 weeks with either 10^8 intact platelets or $8x10^5$ cultured MKs washed with phosphate-buffered saline (PBS). Serum IgG anti-CD61 antibody was then assessed by flow cytometry. The immune CD61 KO mice were sacrificed, their spleens harvested and splenocyte suspensions were prepared by macerating the spleens and applying them through a 40 µm filter in PBS. One week before ITP induction, SCID mice were prebled via the Saphenous vein and 1 day before

induction, they were injected intraperitoneally (IP) with rabbit anti-asialo GM-1 (Wako Pure Chemical Industries Lfd, Osaka, Japan) to remove natural killer cells. Six hours before induction, the mice were irradiated with 200 cGy to enhance engraftment of immune CD61 KO mouse splenocytes. ITP was induced by injecting the pretreated SCID mice IP with 7.5×10^4 splenocytes. Some SCID mice received CD19-depleted splenocytes to induce CD8+ T cell-mediated ITP.[33] Mice were bled weekly and the blood was diluted in PBS-CPDA (1:100) and platelet counts were measured with a Beckman Coulter Counter-LH750 hematology analyzer (Beckman Coulter, Brea, USA). On day 14, mice were sacrificed and bone marrow was harvested from the femurs. The CD8+ CD3+ T cells in these tissues were analyzed by flow cytometry.

Statistics

Statistical analysis was performed using GraphPad Prism 6.07 software (GraphPad Software, San Diego, CA). The test performed for each dataset comparison is specified in the legend. Gaussian distribution of the data was assessed prior to performing the indicated statistical analysis. Wilcoxon and Student t-tests were used. Multi-experimental comparisons were subjected to a one-way or two-way ANOVA, followed by Tukeys post-test for multiple comparisons. The Pearson or Spearman test assessed correlations. Results are expressed as mean ± standard deviation (SD) and p values (P) < 0.05 were considered statistically significant (****P<0.0001; ***P<0.001; **P<0.01; *P<0.05: NS = not significant). Only relevant statistical tests are represented.

Supplemental figure legends

Figure S1: **OVA processing and presentation by MKs follow a dose-response consistent with CD8+ T cell activation.**

MKs presented a dose-response fluorescence with decreasing concentrations of DQ-OVA after a 24hr pulse (A), which matched MK MHC-OVA presentation on plasma membrane (B). OT-1 CD8+ T cell activation based on CD69 expression followed the same profile (C). (A to C. One-way ANOVA with a Tukey correction for multiple testing; n=5-11; mean with SD; ****P<0.0001; ***P<0.001; **P<0.01; *P<0.05)

Figure S2: **MKs express MHC class I and co-stimulatory proteins but not MHC class II.**

Mature MKs expressed on their surface MHC class I molecules (solid line), but not MHC class II (dotted line) (A). They were also shown to have the co-stimulatory proteins CD80 (solid line) and CD86 (dashed line) on their plasma membrane (B). In both graphs, the grey curve corresponds to the negative control (FMO).

Figure S3: **Cytometry analysis of the CD8+ T cell in co-culture with MKs, DQ-OVA level in co-cultured MKs and CD8+ T cell proliferation profiles.**

CD69 expression by CD8+ proliferation dye V450+ T cells at 24hr, 48hr and 72hr in response to OVA presentation (black histogram) was measured by flow cytometry (A). The CD8+ T cell did not show any CD69 increase in absence of OVA antigen at any time point (grey histogram, A). The level of DQ-OVA was measured in the MKs at 24, 48 and 72hr during co-culture to ensure a proper processing of the OVA antigen (B). Representative histograms of the OT-1 CD8+ T cell proliferation in response to MK OVA cross-presentation (C). (B. One-way ANOVA with a

Tukey correction for multiple testing; n≥5; mean with SD; ****P<0.0001; ***P<0.001; **P<0.01; *P<0.05)

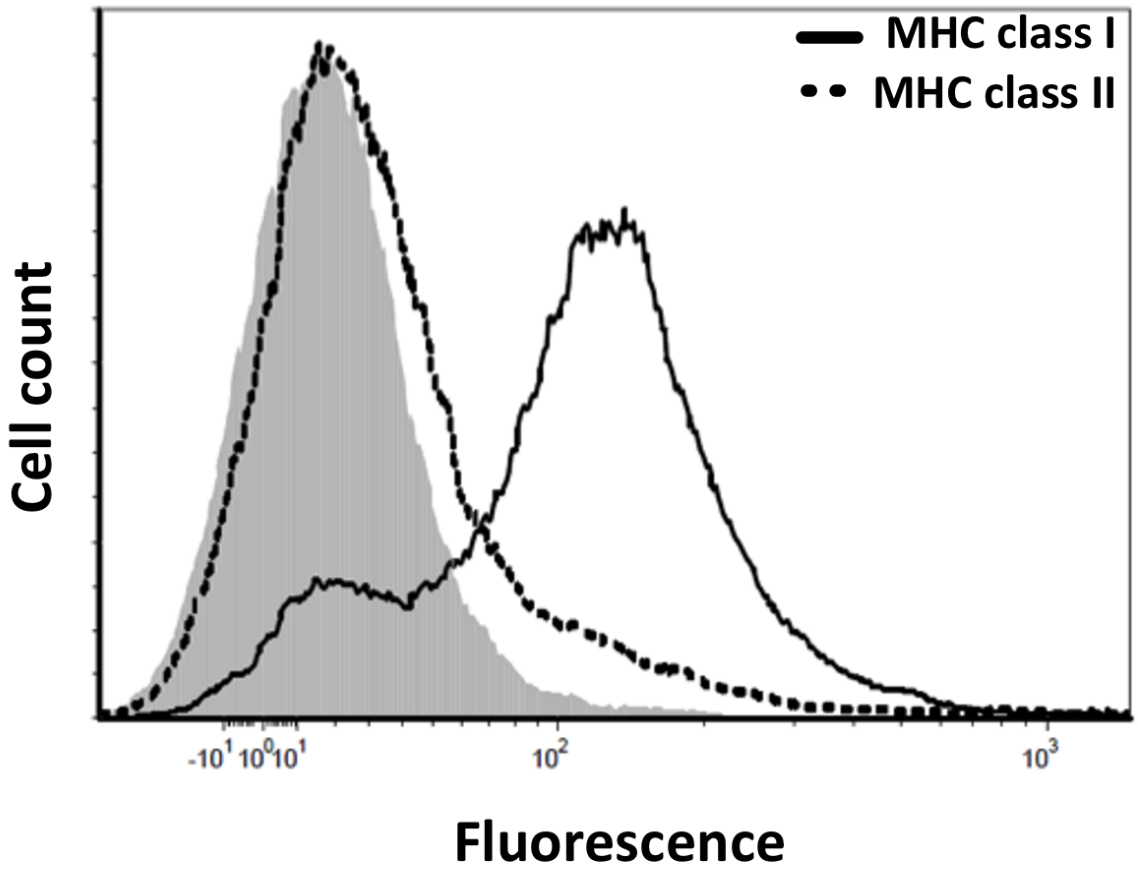
Figure S4: **Cytometry analysis of spleen CD8+ T cells response to OVA cross-presentation.**

Spleen CD 8+ CD3+ T cells were anaylsed by flow cytometry 48hr post transfusion with OVA+ MKs and the percentage of CD69+ cells was measured in OT-1 and WT BL/6 mice (A). The ratio between the presence MHCI-OVA on the plasma membrane and DQ-OVA signal was measured by flow cytometry to confirm the specific impairment of antigen cross-presentation by B2M KO MKs despite their ability to have normal OVA uptake and degradation compared to WT BL/6 MKs (C).

Figure S5: **MK self-presentation of CD61-derived antigens triggers a T cell-mediated thrombocytopenia in an ITP adoptive transfer model.**

The workflow depicted the experimental steps of the ITP adoptive transfer model, including the immunization of CD61 KO mice with platelets or MKs, followed by the transfer of their splenocytes into SCID recipients (A). The CD61 KO mouse immunization was confirmed by tittering platelet-specific IgG in their serum by flow cytometry. Serum was prepared and incubated with WT or CD61 KO platelets and then labeled with a fluorescent anti-mouse IgG secondary antibody. The serum titration ranged from a reciprocal of serum dilution of 1;200 to 1:25600. The inserts showed the overlay of the highest (solid line) and lowest (dashed line) dilution with a dilution of 1:100 with CD61 KO platelets, as to assess the non-specific binding (B).

A


MHC class I
MHC class II
Cell count
-10^1 10^0 10^1
10^2
10^3
Fluorescence

B

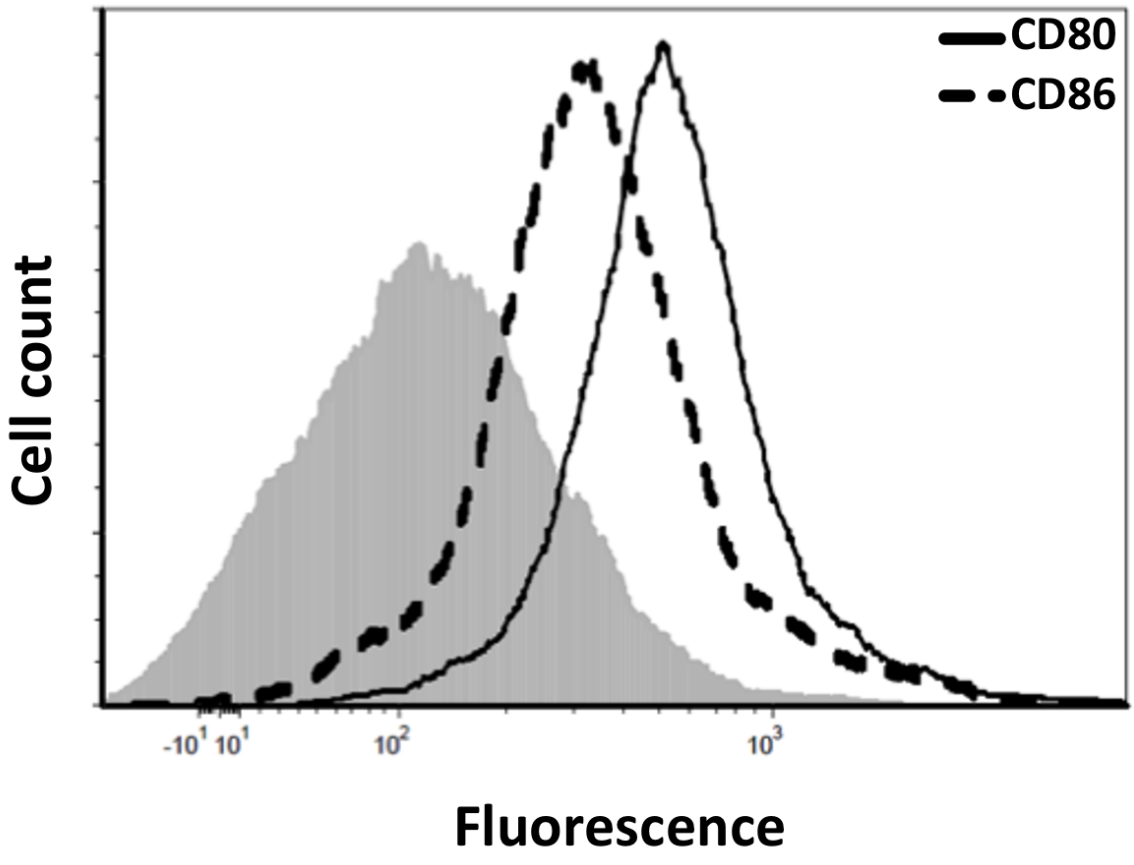

CD80
CD86
Cell count
-10^1 10^1
10^2
10^3
Fluorescence

Figure S2

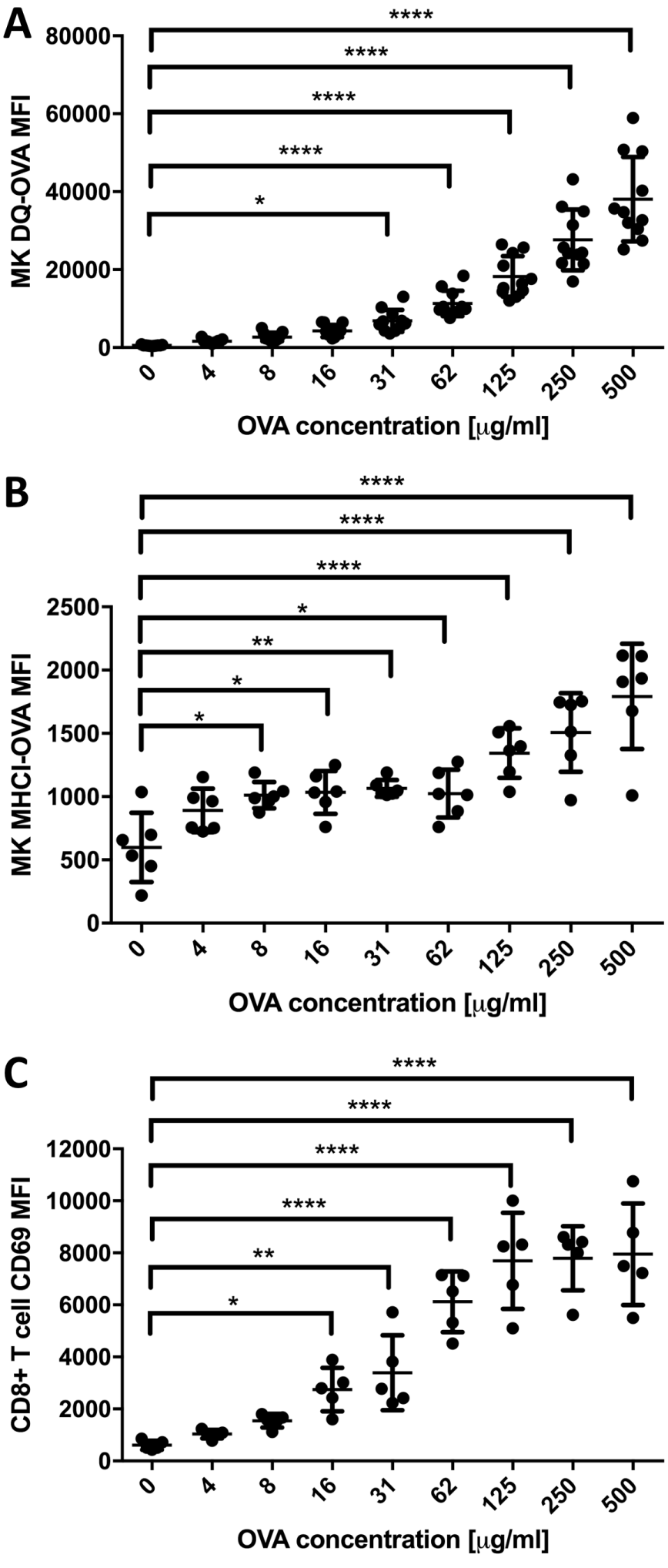
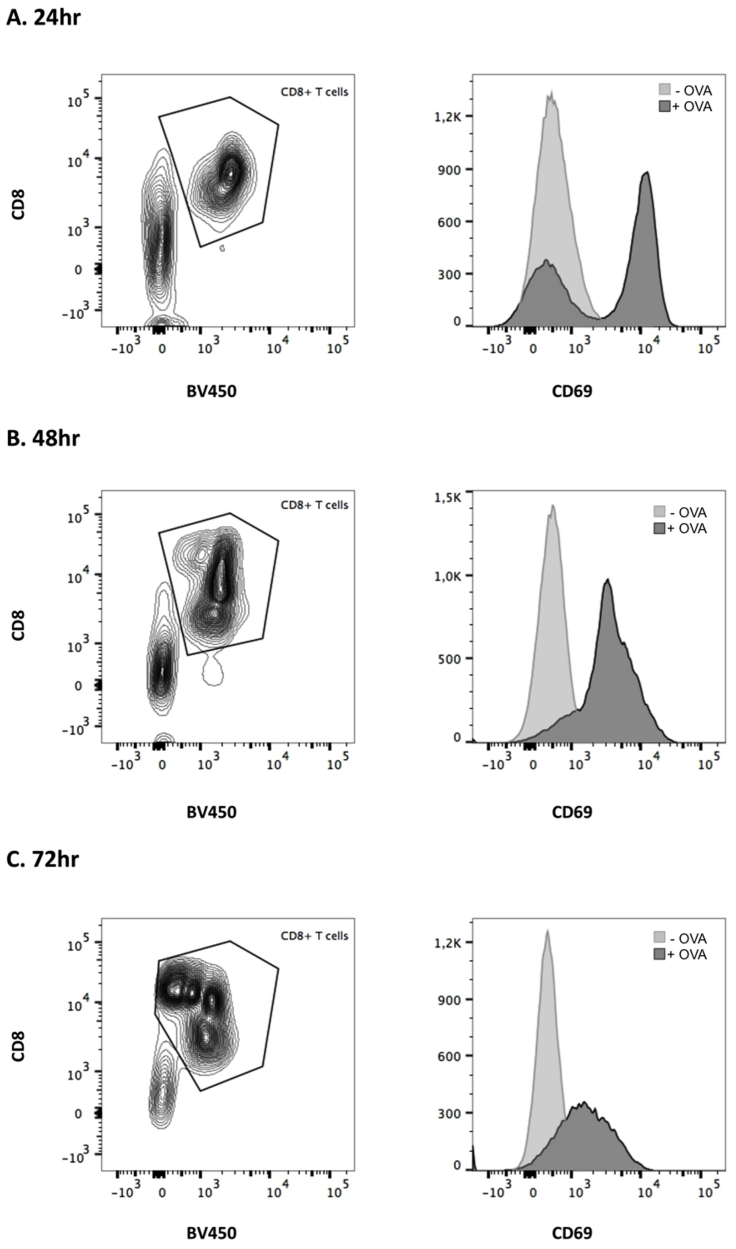
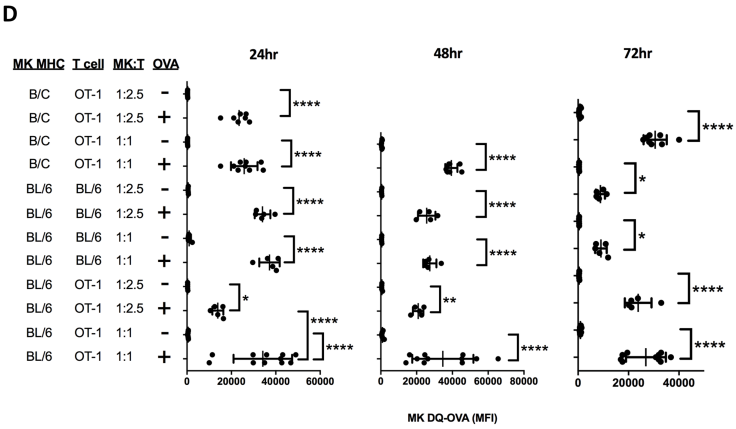

A
MK DQ-OVA MFI
80000
60000
40000
20000
0
****
****
****
****
*
0
4
8
16
31
62
125
250
500
OVA concentration [μg/ml]
B
MK MHCI-OVA MFI
2500
2000
1500
1000
500
0
****
****
****
*
**
*
*
0
4
8
16
31
62
125
250
500
OVA concentration [μg/ml]
C
CD8+ T cell CD69 MFI
12000
10000
8000
6000
4000
2000
0
****
****
****
****
**
*
0
4
8
16
31
62
125
250
500
OVA concentration [μg/ml]

Figure S3


A. 24hr
CD8+ T cells
CD8
BV450
- OVA
+ OVA
CD69
B. 48hr
CD8+ T cells
CD8
BV450
- OVA
+ OVA
CD69
C. 72hr
CD8+ T cells
CD8
BV450
- OVA
+ OVA
CD69


D
MK MHC
T cell
MK:T
OVA
B/C OT-1 1:2.5 -
B/C OT-1 1:2.5 +
B/C OT-1 1:1 -
B/C OT-1 1:1 +
BL/6 BL/6 1:2.5 -
BL/6 BL/6 1:2.5 +
BL/6 BL/6 1:1 -
BL/6 BL/6 1:1 +
BL/6 OT-1 1:2.5 -
BL/6 OT-1 1:2.5 +
BL/6 OT-1 1:1 -
BL/6 OT-1 1:1 +
24hr
48hr
72hr
MK DQ-OVA (MFI)

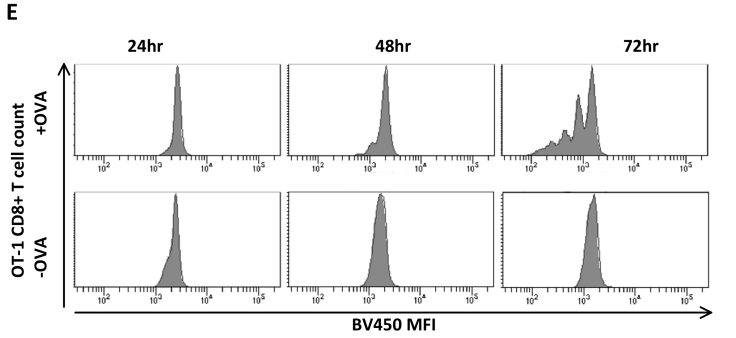
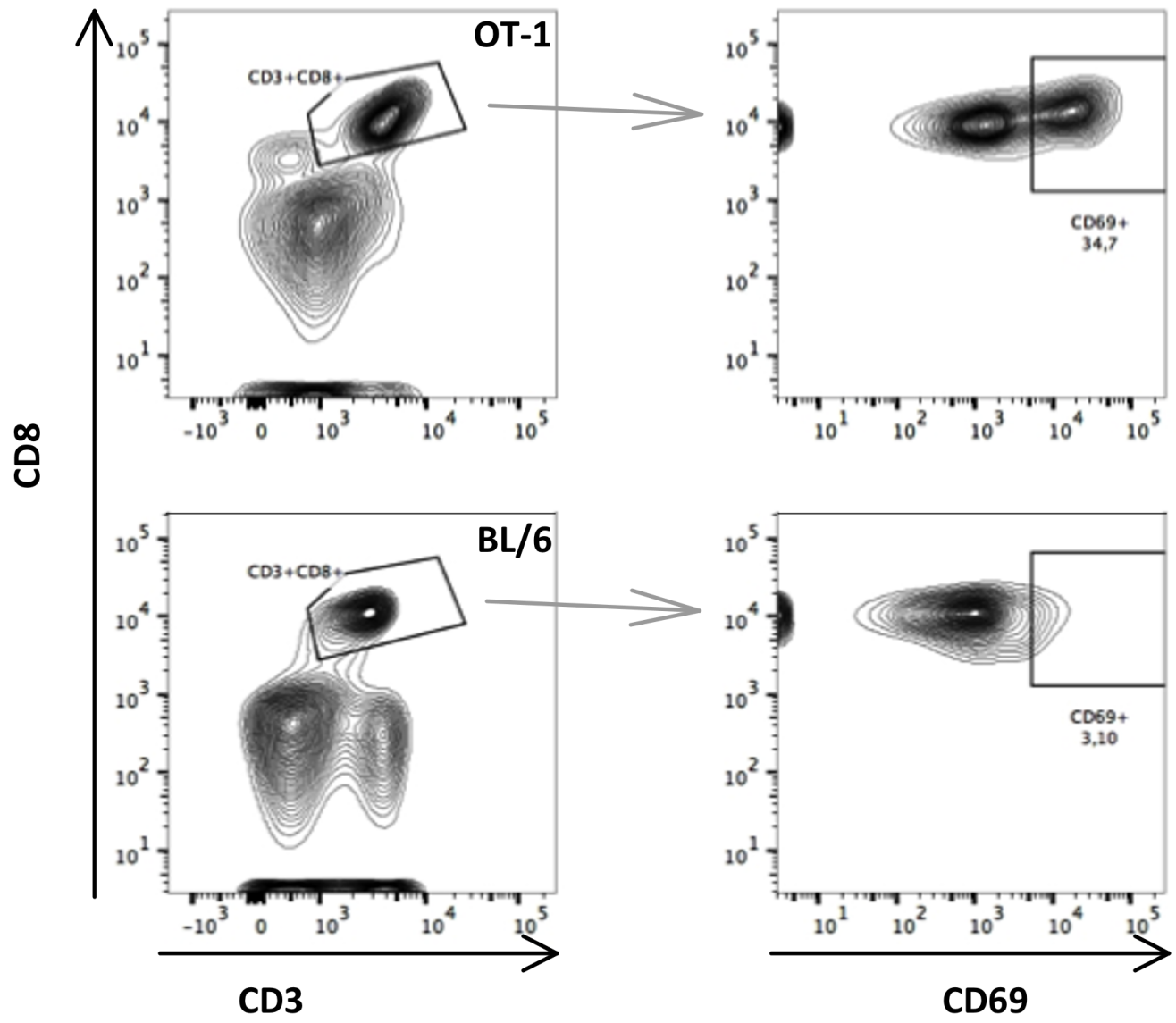

E
24hr
48hr
72hr
OT-1 CD8+ T cell count
+OVA
-OVA
BV450 MFI

Figure S4


A
OT-1
CD3+CD8+
CD69+
34,7
BL/6
CD3+CD8+
CD69+
3,10
CD8
CD3
CD69

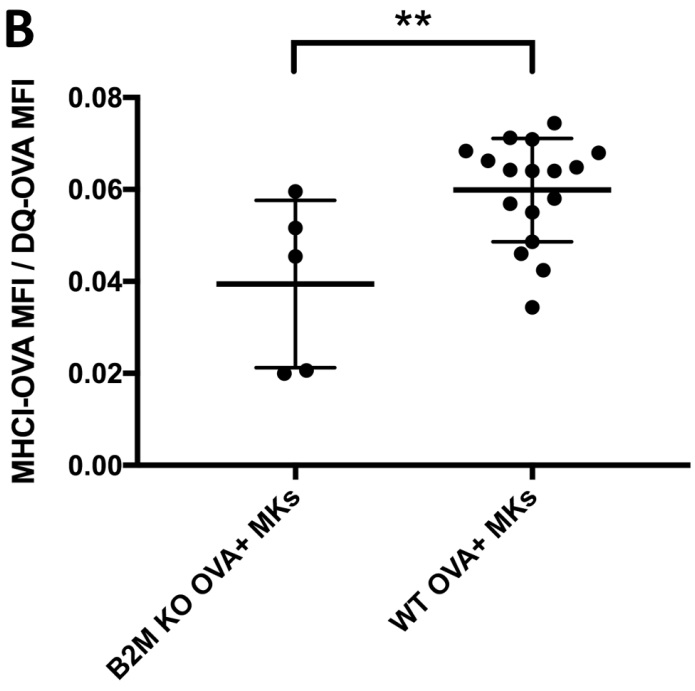
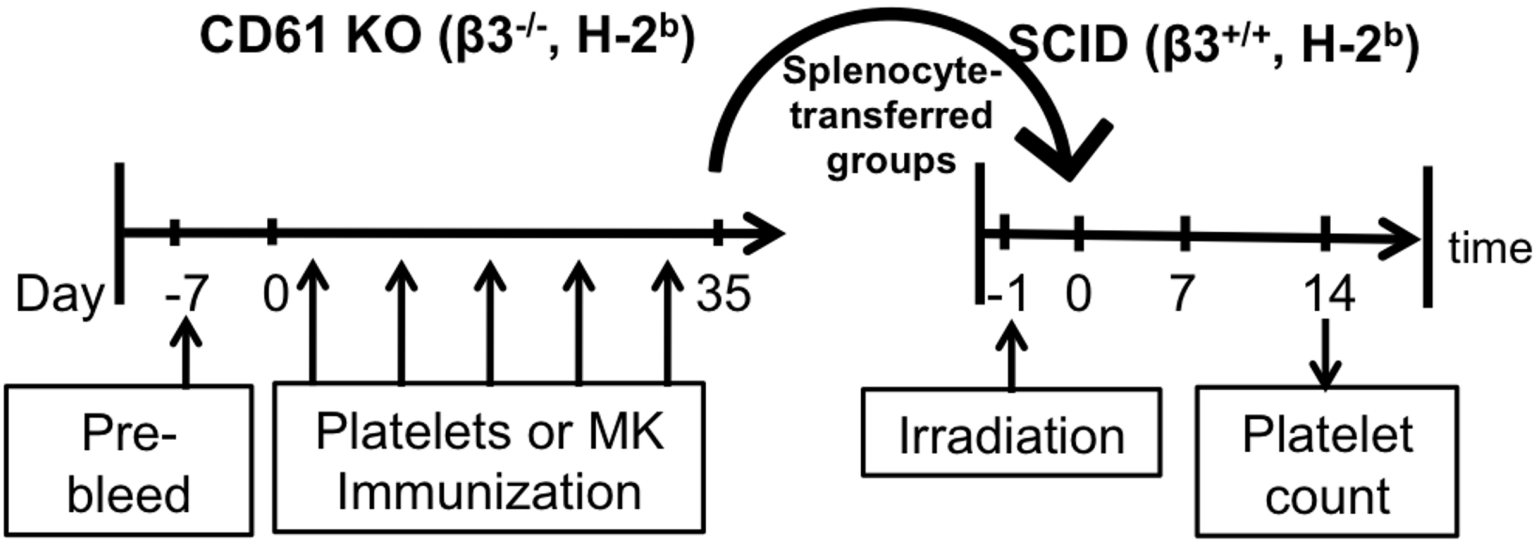

B
**
MHCI-OVA MFI / DQ-OVA MFI
0.08
0.06
0.04
0.02
0.00
B2M KO OVA+ MKs
WT OVA+ MKs

Figure S5

A



Splenocyte-transferred groups:

1. **Naive BL/6 spleen cells**
2. **Plt-immune ND spleen cells (Ab+T)**
3. **MK-immune ND spleen cells (Ab+T)**
4. **Plt-immune CD19 dep spleen cells (T)**
5. **MK-immune CD19 dep spleen cells (T)**

B