SUPPLEMENTARY INFORMATION

SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 2. Reduced DEN-induced HCC Development in 129/SvJxC57BL/6J *Gadd45b*$^{-/-}$ Mice

(A-C) Numbers of tumours (≥0.5 mm) (A), tumour surface area (B), and maximum tumour diameter (C) in livers of *Gadd45b*$^{+/+}$ (n=20) and *Gadd45b*$^{-/-}$ (n=26) 129/SvJxC57BL/6J males 11 months after DEN injection (5 mg/kg).

(D) Gross liver morphology in representative mice from (A-C). Arrowheads indicate tumours.

(E) Images of H&E staining showing the liver histology in representative mice from (A-C). Hatched lines (top) indicate tumour areas. Solid lines (top) denote the areas magnified in the bottom panels. The arrowhead (bottom, right) indicates a typical immunoinflammatory aggregate.

(F) Number of histologically confirmed HCCs per examined liver sections from individual mice in (A-C). Each symbol represents an individual mouse. Horizontal lines denote means.

(G) IHC analysis showing the percentages of proliferating (*i.e.*, Ki-67^{+}) cells per field at 400x in *Gadd45b*$^{+/+}$ (n=25) and *Gadd45b*$^{-/-}$ (n=24) HCCs from (A-C).

(H) Images of IHC staining of representative tumour sections from (G).

(I) IHC analysis showing the percentages of apoptotic (*i.e.*, cleaved caspase 3^{+}) cells per field at 400x in *Gadd45b*$^{+/+}$ (n=24) and *Gadd45b*$^{-/-}$ (n=24) HCCs from (A-C).

(J) Images of IHC staining of representative tumour sections from (I).

(K) Western blots showing total proteins in tumour (T) and non-tumour (N) liver tissues from Figure 2. Each lane is from an individual tumour. Actin is shown as a loading control. WT, *Gadd45b*$^{+/+}$; KO, *Gadd45b*$^{-/-}$.

(A-C, G and I) Values denote means ± SEM.

(A-C, F) *, p<0.05; **, p<0.01.

(E, H and J) Scales and magnifications are shown.

Figure S2, related to Figure 2 and Figure 3. Increased TLS Formation, Macrophage Infiltration and Proinflammatory TAM Activation in HCCs from *Gadd45b*$^{-/-}$ Mice

(A and B) Percentages of HCCs (A) and combined HCCs and preneoplastic foci (B) containing TLSs in C57BL/6J *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice from Figure 2. Each symbol represents an individual mouse. Horizontal lines denote means. *, p<0.05.

(C and D) Percentages of *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice on the 129/SvJxC57BL/6J (C) or C57BL/6J (D) background from Figure S1 and Figure 2, respectively, exhibiting at least one HCC containing TLSs.

(E) Images of IHC staining of representative tumour sections showing cells expressing the indicated macrophage and proinflammatory or antiinflammatory activation markers in the sparse immunoinflammatory infiltrates of *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ HCCs from Figures 3A-3B. Scales and magnifications are shown.

Figure S3, related to Figure 3. Increased Hepatocellular Apoptosis, but Not Proliferation in DEN-Treated *Gadd45b*$^{-/-}$ Mice

(A) TUNEL assays showing the percentages of apoptotic cells in livers of 15-17 days old *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice 48 hr after treatment with DEN (100 mg/kg; +, n=6) or saline (-; *Gadd45b*$^{+/+}$, n=5; *Gadd45b*$^{-/-}$, n=4).

(B) Images of TUNEL staining of representative liver sections from (A).

(C) ALT levels in the sera of *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice from (A). IU, international units.

(D) Cell death grade, assessing both necrotic and apoptotic cells, in *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ livers from (A). Values denote medians; whiskers extent to the 75th percentile.

(E) Images of H&E staining of representative liver sections from (D). Arrowheads indicate dead hepatocytes.

(F) Western blots showing total and phosphorylated (P) proteins in livers from *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice at the indicated time points after treatment with DEN as in (A).

(G) BrdU labelling assays showing the percentages of proliferating cells in *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ livers from (A). As expected due to their young age, mice exhibited a high basal hepatocyte turnover.

(H) Images of BrdU staining of representative livers sections from (G).

(A, C and G) Values denote means ± SEM. *, $p<0.05$; **, $p<0.01$.

(B and E) Solid lines (middle panels) denote the areas magnified in the right panels.

(B, E and H) Scales and magnifications are shown. UT, treated with saline.

Figure S4, related to Figure 4. Host Reconstitution with BM-Derived Cells of Donor Origin

(A) Dot plots of representative FACS analyses of spleen and BM cells showing the reconstitution of CD45.2/Ly-5.2$^+$ hosts with CD45.1/Ly-5.1$^+$ haematopoietic-derived cells of donor origin in chimaeras generated using Protocol A.

(B) Images of immunofluorescence staining of representative liver sections showing successful repopulation with CD45.1/Ly-5.1$^+$, F4/80$^+$ Kupffer cells of donor origin in chimaeras generated as in (A).

(C) Images of IHC staining with anti-F4/80 antibody of representative spleen (left) and liver (middle, right) sections from *Gadd45b*$^{+/+}$ mice at the indicated times after a single intravenous injection of clodronate or PBS liposomes.

(D) Dot plots of representative FACS analyses of spleen and BM cells showing the reconstitution of CD45.2/Ly-5.2$^+$ hosts with CD45.1/Ly-5.1$^+$ haematopoietic-derived cells of donor origin in chimaeras generated using Protocol B.

(E) Images of immunofluorescence staining of representative liver sections showing successful repopulation with CD45.1/Ly-5.1$^+$, F4/80$^+$ Kupffer cells of donor origin in chimaeras generated as in (D).

(B and E) Red, F4/80; green, CD45.1/Ly-5.1 (top) or CD45.2/Ly-5.2 (bottom); yellow, merged; blue, DAPI.

(B, C, E) Scales and magnifications are shown.

Figure S5, related to Figure 5. Increased Immune Infiltration, Proinflammatory TAM Activation and Effects of CD8$^+$ T-Cell Depletion in Fibrosarcomas and/or Ovarian Tumours from *Gadd45b*$^{-/-}$ mice

(A) Images of IHC staining of representative tumour sections from Figure 5B.

(B) Volumes of subcutaneous MCA-203 fibrosarcomas from Figure 5C measured *ex vivo* at day 20.

(C) Dot plots showing the FACS analysis of representative CD8$^+$ and CD4$^+$ T-cell populations in the spleens of *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice treated with anti-CD8 or isotype control antibody, as indicated.

(D) Images of IHC staining of representative tumour sections from Figure 5E.

(A and D) Scales and magnifications are shown.

(E) Histograms showing the FACS analysis of representative TAM populations from Figure 5I-5J. The percentages of MHC-II^{high} cells (Figure 5I) and medians of the MHC-II fluorescence (vertical red and blue bars) (Figure 5J) are shown.

(F) qRT-PCR showing the relative mRNA levels of the indicated antiinflammatory activation markers in CD11b$^+$ TAMs from the *Gadd45b*$^{+/+}$ (n=22) and *Gadd45b*$^{-/-}$ (n=10 or n=12) mice shown in Figures 5K-5L.

(B and F) Values denote means ± SEM. *, p<0.05; **, p<0.01.

Figure S6, related to Figure 6. *Gadd45b* Loss Enhances Proinflammatory Macrophage Activation by Upregulating p38 Signalling

(A) qRT-PCR showing the relative mRNA levels of the indicated proinflammatory and antiinflammatory activation genes in BMDMs from *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice after a 24-hr co-culture with ID8-Luc cells.

(B and C) qRT-PCR showing the relative mRNA levels of the indicated proinflammatory activation genes in *Gadd45b*$^{-/-}$ BMDMs left untreated (-) or treated with LPS and IFNγ (+) for 12 hr in the presence (+) or absence (-) of the p38 inhibitors, Skepinone-L (10 µM) (B) or SB203580 (20 µM) (C).

(A-C) Values denote means ± SEM (A, n=4; B and C, n=3). *, p<0.05; **, p<0.01; ***, p<0.001.

Figure S7, related to Figure 7. Germline Transmission of the *Gadd45b^F* Allele

(A) PCR showing the correct integration of the proximal loxP site of the conditional *Gadd45b* knockout allele (*Gadd45b^F*) in mice crossed to the G1 generation. Oligonucleotides 1 and 2 detect the proximal loxP site as a DNA fragment of 355 bp and the wild-type allele as a DNA fragment of 300 bp.

(B) PCR showing the correct integration of the distal loxP site of the conditional *Gadd45b* knockout allele in mice from (A). Oligonucleotides 3 and 4 detect the distal loxP site as a DNA fragment of 355 bp. The wild-type *CD79b* allele was co-amplified as a control (585 bp) to verify the integrity of the DNA samples.

(A and B) 7-digit numbers denote individual mice. Control 1 and 2 are negative and positive PCR controls, respectively.

(C) Map showing the positions of oligonucleotides 1 and 5 detecting the excised *Gadd45b* allele (*Gadd45b$^{\Delta}$*), following Cre-mediated recombination.

SUPPLEMENTARY MATERIALS AND METHODS

Macrophage Isolation

BMDMs were prepared from 6 to 10 weeks old *Gadd45b$^{-/-}$* and *Gadd45b$^{+/+}$* C57BL/6J mice as previously described (21). Briefly, bone marrow (BM) cells were harvested by flushing the femurs and tibiae with ice-cold 1x PBS. Following the removal of red blood cells with RBC lysis buffer (Sigma-Aldrich), BM cells were cultured at the concentration of 1 x 10^6 or 2 x 10^6 cells/mL in DMEM high-glucose GlutaMAX™ supplemented with 1% FBS, 25 mM HEPES, antibiotics (100 U/mL penicillin/streptomycin), 2 mM L-glutamine (Gibco) and 20 ng/mL mouse recombinant M-CSF (Peprotech) for 7 days.

BMDM/Tumour Cell Co-Culture

Briefly, BMDMs were seeded at the density of 1 x 10^5 cells/mL in complete DMEM high-glucose GlutaMAX™, without M-CSF, onto Transwell cell culture inserts (BD Biosciences), the bottom of which consists of a high-density PET membrane (0.4 μm-pore size) that is permeable to fluids but not to cells. Transwells were then inserted into 6-well plates containing 8 x 10^5 tumour cells/mL, and cells were co-cultured for 24 hours in a humidified incubator in 5% CO_2 at 37°C, after which time, BMDMs were harvested from inserts using TRIzol® reagent (Invitrogen). Time-matched controls, consisting of BMDMs cultured in transwell inserts without tumour cells, were set-up in parallel for each experiment.

Generation of *Gadd45b*$^{F/F}$ mice

The gene targeting strategy was devised on the basis of *Gadd45b* NCBI transcript NM_008655.1. The targeting vector contained a puromycin resistance (PuroR) cassette, flanked by short flippase (Flp) recombinase recognition target (FRT) sites, and a thymidine kinase (TK) cassette and was constructed using a BAC clone from the C57BL/6J RPCIB-731 BAC library. Exons 3 and 4 of the *Gadd45b* gene, containing the majority of the ribosomal L7Ae domain-coding region, were flanked by Cre recombinase recognition (loxP) sites, resulting in the excision of the loxP-flanked exons, with *Gadd45b* loss of function, upon Cre-mediated recombination. A poly-adenylation signal from the human Growth Hormone-coding gene (hGHpA) was inserted at the 3'-end of the distal loxP site in order to stop transcription of the constitutive excised *Gadd45b* allele (*Gadd45b*$^{\Delta}$). The targeting vector was linearized using NotI and electroporated into Taconic Biosciences C57BL/6NTac ES cells, and recombinant ES cell clones were isolated following positive (PuroR) and negative

(TK) selection. Correct homologous recombination was verified by Southern blot and PCR analysis of resistant ES cell clones, using Taconic Biosciences in-house protocols (data not shown). Four independent homologous recombinant ES cell clones were expanded and frozen, after further validation.

Following ES-cell microinjection into BALB/c blastocysts, chimaeras (G0) were obtained from three independent validated ES cell clones. Chimerism was evaluated by the coat-colour contribution of the C57BL/6NTac ES cells to the BALB/c host. One line of highly chimeric males (B-B12) was selected for germline transmission breeding with C57BL/6 Flp Deleter females (C57BL/6-Tg(CAG-Flpe)2Arte), carrying a ubiquitously expressed *in vitro*-evolved *Flp* (*Flpe*) gene, thus resulting in the excision of the PuroR cassette with generation of C57BL/6 *Gadd45b*$^{F/+}$ mice (G1), heterozygous for the conditional loxP-flanked *Gadd45b* allele. The presence of the wild-type *Gadd45b* allele and proximal and distal loxP sites of the *Gadd45b*^{F} allele, as well as the absence of the *Flpe* transgene, were verified by PCR analysis of ear samples.

The myeloid-specific excision of the *Gadd45b*^{F} allele, resulting in the formation of the *Gadd45b*$^{\Delta M}$ allele, after Cre-mediated recombination, was verified by PCR. The DNA sequences of the PCR primers used were:

Oligonucleotide (Oligo) ID	DNA primer sequence
Oligonucleotide 1	5’-CTGCAGAACCCTGACTTCG-3’
Oligonucleotide 2	5’-CAGGAGAGAAGGAGTGTCAGG-3’
Oligonucleotide 3	5’-TTCGCTAGCCTCAGTTTACCAG-3’
Oligonucleotide 4	5’-CCCAAGGCACACAAAAAACC-3’
Oligonucleotide 5	5’-CTGTAGGTCTGCTTGAAGATCC-3’

Short-term DEN Treatment

For the short-term analyses of the liver response to DEN administration, 15 to 17 days old C57BL/6J *Gadd45b*$^{-/-}$ and *Gadd45b*$^{+/+}$ males were intraperitoneally injected

with a single dose of DEN (100 mg/kg; Sigma-Aldrich) and sacrificed at indicated time points. To evaluate hepatocyte proliferation, mice were intraperitoneally injected with BrdU (100 µg/kg; Sigma-Aldrich) 3 hours prior to sacrifice. Blood was collected at the indicated time points, and serum separated and flash frozen at -80° C. Serum alanine aminotransferase (ALT) levels were measured using Reflotron GPT tabs and a Reflotron Plus Chemistry Analyzer (Roche Diagnostics), according to the manufacturer's instructions. At the indicated time points, mice were sacrificed, their livers were removed, and liver tissue was fixed and paraffin-embedded for histological evaluations or stored at -80°C for molecular analyses.

Bioimaging

For bioimaging, mice were anesthetised with ketamine and xylazine (100 µg/g and 20 µg/g of body weight, respectively) and then intraperitoneally injected with 20 µg/g of body weight of D-luciferin (Invitrogen). ID8-Luc tumour bioluminescence was acquired in situ using a Hamamatsu Aequoria Darkbox Macroscopic Imaging System equipped with a Hamamatsu ORCAII-BT-1024G High Resolution BT-CCD Cooled Digital Camera (Hamamatsu Photonics), and imaging data were analysed using Wasabi™ version 1.5 software.

Histopathological Analyses

For histopathological examination, 4 µm sections of paraffin-embedded liver tissue were stained with hematoxylin-eosin (H&E) and blindly evaluated by an ECVP-board certified veterinary pathologist. The histological classification of hepatic proliferative lesions was performed according to previously published criteria (51). Foci of cellular alteration (also referred to as preneoplastic foci), hepatocellular adenomas, and

hepatocellular carcinomas were identified according to these criteria and counted. Foci of cellular alteration, adenomas and carcinomas were collectively referred to as hepatocellular proliferative lesions; adenomas and carcinomas were collectively referred to as hepatocellular neoplastic lesions or HCCs. Intratumoural inflammatory infiltrates were histopathologically evaluated and distinguished into TLS-like and non-organised infiltrates, as indicated. For the short-term analysis of the liver response to DEN, the cell death grade was scored on 400x microscopic fields according to the following scale from 0 to 3: 0, no necrotic or apoptotic cells per field; 1, <10 necrotic or apoptotic cells per field; 2, 10-30 necrotic or apoptotic cells per field; 3, >30 necrotic or apoptotic cells per field.

Immunohistochemistry, Immunofluorescence and TUNEL Assays

For immunohistochemical analysis, 4-μm sections of paraffin-embedded liver tissue were stained as previously described (52). The primary antibodies used were: anti-CD3ε (sc-1127), anti-IDO (sc-53978) (Santa Cruz Biotechnology); anti-CD45R/B220 (557390), anti-Ly6G (551459), anti-BrdU (551321) (BD Pharmingen); anti-CD8a (14-0808-82) (eBioscience); anti-F4/80 (MCA497), anti-HO-1 (4915-1050), anti-CD206 (MCA2235) (AbD Serotec); anti-IBA-1 (019-19741) (Wako Chemicals); anti-MHC-II (ab25333), anti-iNOS (ab15323), anti-F4/80 (Ab6640) (Abcam); anti-COX-2 (160106) (Cayman Laboratories); anti-YM-1/Chi3l3 (01404) (StemCell Technologies); anti-Ki-67 (RM-9106-S) (Thermo Scientific); anti-cleaved caspase 3 (9661) (Cell Signalling). The immunohistochemical evaluation of MCA-203 fibrosarcoma allografts was performed on 8-μm frozen tumour sections using the following same primary antibodies: anti-CD3ε, anti-CD45R/B220, anti-F4/80, anti-Ly6G, anti-iNOS, anti-COX-2, anti-YM-1/Chi3l3, anti-HO-1, anti-CD206. Immunoreactions were visualised using the Peroxidase DAB Substrate Kit (Vector Laboratories), and sections were counterstained using Mayer's haematoxylin. TUNEL assays were performed on 4-

µm paraffin-embedded liver sections using the DeadEnd™ colorimetric TUNEL system (Promega), according to the manufacturer's instructions. Histological images were acquired using a Nikon Eclipse E200 Microscope equipped with a Nikon Digital Sight DS-MS Camera or a Leica DM2500 Microscope equipped with a Leica DFC310 FX Digital Camera. In each case, the percentage of positive area or number of positive cells was quantified in 3 non-overlapping hot-spot fields at the 400x magnification, using the ImageJ analysis tool (http://rsb.info.nih.gov/ij/).

For immunofluorescence staining, 5-µm sections of OCT-embedded frozen liver tissue were mounted onto poly-L-lysine coated slides (Thermo Scientific) and fixed with ice-cold methanol-acetone (1:1; Sigma-Aldrich). Slides were then washed with 1x PBS-Tween (0.1%; Sigma-Aldrich), blocked with Normal Rabbit Serum Blocking Reagent (Monosan) and stained with anti-F4/80 primary antibody (ab6640; Abcam), followed by staining with Texas Red®-X-conjugated secondary antibody (T-6392; Invitrogen). Following this, slides were washed with 1x PBS-Tween (0.1%; Sigma-Aldrich), and sections were stained with either anti-CD45.1-FITC (11-0453-82; eBioscience) or anti-CD45.2-FITC (11-0454-82; eBioscience). Slides were finally mounted using VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories), and images were acquired on a Zeiss Axiovert S100 TV Inverted Fluorescence Microscope equipped with an ORCA-03G Digital CCD Camera (Hamamatsu) and analysed using MetaMorph™ software.

For double immunofluorescence staining of 4-µm sections of paraffin-embedded liver tissue with anti-IBA-1 (019-19741) (Wako Chemicals) and anti-MHC-II (ab25333), the following secondary antibodies were used: donkey anti-rabbit IgG (H+L) Alexa Fluor 555 (A-31572) (for IBA-1), and donkey anti-rabbit IgG (H+L) Alexa Fluor 488 (A-21208) (for MHC-II) (Invitrogen). Images were acquired with an Olympus BX63 upright automated fuorescence microscope equipped with an ORCA-AG CCD camera (Hamamatsu) by using MetaMorph™ software. The number of

single and double positive cells was quantified in hot-spot fields (*i.e.*, centered on intratumoural inflammatory infiltrates) at the 400x magnification, using the ImageJ analysis tool (http://rsb.info.nih.gov/ij/).

Western Blots

Western blots were performed using ECL (Amersham) as previously described (32). Proteins were extracted from bone marrow derived macrophages (BMDMs) using RIPA buffer (Sigma-Aldrich). Liver tissue was homogenised using Tissue Lyser (Qiagen), and proteins were extracted with NP-40 lysis buffer (32). The antibodies used were: anti-β-actin (sc-1616), anti-Actin (sc-1615), anti-PCNA (sc-56), anti-Cdc2/p34 (sc-954), anti-Cyclin A (sc-751), anti-CDK2 (sc-163), anti-CDK4 (sc-260), anti-c-Myc (sc-40), anti-JNK (sc-474), anti-Stat3 (sc-8019), goat anti-rabbit IgG-HRP (sc-2030), goat anti-mouse IgG-HRP (sc-2031), donkey anti-goat IgG-HRP (sc-2033) (Santa Cruz Biotechnology); anti-P-Stat1 (7649), anti-Stat1 (9172), anti-P-JNK (9251), anti-JNK (9252), anti-P-p38 (9215; 9211), anti-p38 (9212), anti-P-ERK1/2 (4377; 9101), anti-ERK1/2 (9102), anti-Cyclin B1 (4138), anti-Cyclin E1 (4129), anti-P-Stat3 (9131), anti-c-Jun (9165), anti-IκBα (4792) (Cell Signalling Technology); anti-Gadd45β (SAB2108614) (Sigma-Aldrich); donkey anti-rabbit IgG-HRP (NA934; GE Healthcare).

Quantitative Real-Time Polymerase-Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted with TRIzol® reagent (Invitrogen) and purified using the PureLink™ RNA Mini Kit (Invitrogen) by performing on-column DNase digestion with PureLink™ DNase (Invitrogen), according to the manufacturer's instructions. cDNA was synthesised from 1 µg of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Multiplex qRT-PCR reactions were carried

out using either an 7900HT or 7500 Fast Real-Time PCR System (Applied Biosystems), the TaqMan® Universal PCR Master Mix (Applied Biosystems) and the following predesigned TaqMan® Gene Expression Assays:

Gene	Species	Assay ID	Reporter Dye
Arg1	Mouse	Mm00475988_m1	FAM
B7-h3	Mouse	Mm00506020_m1	FAM
Cd206	Mouse	Mm00485148_m1	FAM
Chi3l3	Mouse	Mm00657889_m1	FAM
Cxcl9	Mouse	Mm00434946_m1	FAM
Cxcl10	Mouse	Mm00445235_m1	FAM
Cxcl11	Mouse	Mm00444662_m1	FAM
Gadd45b	Mouse	Mm00435123_m1	FAM
Hmox1	Mouse	Mm00516005_m1	FAM
Ido	Mouse	Mm00492590_m1	FAM
Il-6	Mouse	Mm00446190_m1	FAM
Il-12b	Mouse	Mm00434174_m1	FAM
Il10	Mouse	Mm00439614_m1	FAM
Nos2	Mouse	Mm00440502_m1	FAM
Pd-l1	Mouse	Mm00452054_m1	FAM
Pdcd1lg2	Mouse	Mm00451734_m1	FAM
Ctla-4	Mouse	Mm00486849_m1	FAM
Tnfa	Mouse	Mm00443258_m1	FAM
Nfkbia	Mouse	Mm00477798_m1	FAM
Tnfaip3	Mouse	Mm00437121_m1	FAM
18s	Human	Hs99999901_s1	VIC
18s	Mouse	Mm03928990_g1	FAM
Actin1	Mouse	Mm01304398_m1	FAM

The transcript levels of each gene were normalised to 18s mRNA levels, and $^{\Delta\Delta}$CT values were calculated relatively to a reference sample, according to previously described methods (53).

FACS Analysis

FACS analysis was performed essentially as previously described (54). Briefly, single cell suspensions were prepared from spleen and BM tissue of BM chimaeras

or peritoneal fluid from ID8-Luc tumour-bearing mice, as indicated, using a 70-µm cell strainer (BD Biosciences), and red blood cells were removed using RBC lysis buffer (Sigma-Aldrich). Cells were then washed, re-suspended in either 1x PBS or Hank's Balanced Salt Solution (Sigma-Aldrich), containing 1-2% heat-inactivated FBS and NaN_3, treated with Mouse BD Fc Block™ (BD Pharmingen), and stained with fluorochrome-conjugated antibodies or appropriate isotype controls. The antibodies used were: anti-CD45.1-FITC (11-0453-82), anti-CD45.2-APC (17-0454-82) (eBioscience); anti-CD45.2-FITC (130-102-458), anti-CD3ε-PerCP-Vio700 (130-109-243), anti-CD3-FITC (130-108-836), anti-CD4-APC (130-109-415), anti-CD8b-PE-Vio770 (130-106-316), anti-Cd8b-APC (130-106-315), anti-CD45R/B220-APC (130-102-259), anti-Ly-6G-PE (130-102-392), anti-CD11b-PerCP-Vio700 (130-097-585), anti-F4/80-PE-Vio770 (130-102-193), anti-MHC-II-APC (130-107-942) (Miltenyi Biotec). Stained cells were acquired on a DAKO Cyan or BD FACSCanto II Flow Cytometer, and data were analysed using FlowJo™ (Tree Star) or DIVA (BD Bioscience) software.

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