METHODS

Mice

HLA-B*57:01/H2-D^d (α3) Tg and HLA-B*57:01 Tg/H2-K^{b}D^b knockout (Tg/KO) mice were generated as previously described.[E1,E2] HLA-B*57:01 Tg/H2-K^{b}D^b KO/PD-1 KO (Tg/DKO) mice were generated by backcrossing Tg/KO to Pdcd1$^{tm1.1Shr}$/J mice (The Jackson Laboratory). WT mice were animals from the Tg strain that tested negative for the transgene and expressed intact mouse MHC-I molecules. Experimental mice, female if not otherwise indicated, were older than 12 weeks. All mice were bred and housed under specific-pathogen–free conditions in the Association for Assessment and Accreditation of Laboratory Animal Care International (aka AAALAC)-accredited animal facility of the US FDA's Division of Veterinary Medicine (Silver Spring, Md) in compliance with the Institutional Animal Care and Use Committee regulations of the FDA (protocol 2017-63).

In vitro cell culture assays

In vitro T-cell responses to flucloxacillin (FLX) or abacavir (ABC) were measured in cultures of purified CD8$^+$ T lymphocytes from spleens or LNs as previously described.[E1,E2] Briefly, single cell suspensions were obtained by macerating the organs through 70 μm cell strainers, red blood cell lysis, and purification using a mouse CD8α$^+$ T Cell Isolation Kit (Miltenyi Biotec). Purified CD8$^+$ T lymphocytes (3×10^5 cells per well) were cocultured with irradiated (3000 rad) autologous feeders pulsed overnight with 10 μg/mL of ABC or 150 μg/mL of FLX (unless otherwise indicated), at a 1:2 CD8$^+$ T-cell to feeder ratio. Drug was added to the cultures at different concentrations, as specified in the figures. Cell culture media consisted of RPMI 1640 supplemented with 2 mmol L-glutamine, 1 mmol sodium pyruvate, 1× MEM Non-Essential Amino Acids, 1× MEM Vitamin Solution, 10 mmol HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Gibco). Heat-inactivated, normal mouse serum (0.5%) (Equitech-Bio, Kerrville, Tex) was supplemented in the media for the first 48 hours of culture and subsequently replaced by 10% heat-inactivated, low-endotoxin FBS (Premium Select FBS; Atlanta Biologicals, Flowery Branch, Ga)-containing media (cRPMI). Cultures prolonged up to 14 days were supplemented with 50 IU/mL of recombinant IL-2 (Proleukin [aldesleukin]; Prometheus, San Diego, Calif) every other day, starting on day 5.

Morphologic changes in cells were captured with bright-field microscope images using a model IX81 inverted microscope (Olympus, Tokyo, Japan). Secretion of IFN-γ or GZMB in cell culture supernatants was measured by ELISA using mouse IFN-γ or GZMB kits (eBioscience; Thermo Fisher Scientific). Alternatively, cytokine production was tested using IFN-γ ELISpot microplates (R&D Systems, Minneapolis, Minn). Purified splenic CD8$^+$ T cells were cultured without or with drug-pulsed irradiated splenocytes (at a 1:4 to feeder ratio) and in the absence or presence of 125 μg/mL FLX, as per the manufacturer's instructions.

In some experiments, anti-HLA antibody (clone B1.23.2, eBioscience) or anti-mouse MHC-I antibodies (H2-K^b [clone Y3, BioXcell] and H2-D^b [clone B22-249.R, Invitrogen]) were used at 10 μg/mL, 1 hour before adding FLX to the coculture.

For intracellular cytokine analysis, 0.9×10^6 splenocytes per well were cultured in 96-well plates with 0 or 250 μg/mL FLX in cRPMI for 16 hours. Brefeldin A solution (eBioscience) was subsequently added for 4 additional hours at a final dilution of 1/1000.

In vivo treatment

Mice were shaved at the abdomen area 3 days before drug administration (day −3). RA (MP Biomedicals) (100 μg in 50 μL of 100% DMSO) and/or FLX (50 mg in 100 μL of 70% of DMSO [Sigma]) was painted on the abdominal area on days 1, 2, and 3 for sensitization. All animals were treated with RA solution. FLX capsules (Apotex) were dissolved to 10 mg/mL in Water For Injection (aka WFI) for Cell Culture (Gibco) by vigorous shaking at room temperature. Drug solutions were filtered with 0.22 μm filters, formed into aliquots, and stored at −20°C. FLX (2.3 mg/250 μL of WFI) was provided by oral gavage on days 8 and 9. Anti-CD4 antibody (clone GK1.5, BioXcell) was injected intraperitoneally at 0.25 mg per dose on day −3, 1, 4, and 7. Spleen, LN, and liver samples were collected after euthanasia on day 10 unless indicated otherwise.

Blood cell phenotype analysis and expression of HLA and H2-K^b molecules

Blood was collected from the ear of the mice into MiniCollect Z Serum Separator tubes (Greiner Bio-One), with 75 μL used for testing. On red blood cell lysis (ammonium–chloride–potassium lysing buffer; Gibco) and blocking nonspecific binding with mouse Fc Block (BD Pharmingen, Franklin Lakes, NJ), samples were stained with the following antibody cocktail: anti–CD4-BV605 (clone RM 4-5, BD Biosciences), anti–CD8a-BV711 (clone 53-6.7, BD Biosciences), anti–H2K^b-FITC (clone AF6-88.5.5.3, eBioscience), anti–B220-PE/Cy7 (clone RA3-6B2, BioLegend, San Diego, Calif), anti–HLA B/C-APC (clone B1.23.2, eBioscience), anti–CD19-AF700 (clone eBio1D3 [1D3], eBioscience), and antiCD3e-APC-Cy7 (clone 17A2, BioLegend) for 30 minutes at 4°C. Alternatively, HLA and H2-K^b were stained with specific antibodies conjugated with phycoerythrin in house. Sample acquisition and data analysis were performed using LSR Fortessa X20-SORP and Diva 18 6.2/8.0.2 (BD Biosciences), respectively.

Liver enzyme levels in serum

Blood was collected at the time of euthanasia by cardiac puncture into MiniCollect tubes. Serum was separated by spinning samples at 3000 × *g* for 10 minutes. ALT (BioAssay Systems) levels were measured using commercially available assay kits.

Histology

Sections of the liver were obtained at the time of euthanasia and submerged into 10% neutral buffered formalin. Fixed liver samples were processed into paraffin blocks and sectioned at 5 μm onto positively charged slides. H&E staining was performed by Histoserv.

Gene expression analysis

Liver biopsy samples were immediately submerged in TRIzol (Invitrogen) on excision, flash-frozen, and stored at −80°C until processing. Sample processing was conducted as previously described.[E1] Briefly, tissues were mixed with 2 mm zirconia beads (BioSpec Products, Bartlesville, Okla) and homogenized using a Precellys 24 Cryolys system (Bertin Technologies, Montigny le Bretonneux, France). Total RNA was extracted from the homogenated following the TRIzol protocol as per the manufacturer's directions. One microgram of total RNA per sample was reverse transcribed using the High Capacity cDNA RT Kit (Applied Biosystems; Thermo Fisher Scientific) as per the manufacturer's instructions. Expression of individual genes was evaluated using 40 ng of the original RNA with optimized TaqMan probe and primer sets (Thermo Fisher Scientific) in 2× Universal Master Mix (Thermo Fisher Scientific) as indicated by the manufacturer. TaqMan plates were run on a Viia7 Real-Time system (Thermo Fisher Scientific). Data normalization was performed using *Gapdh* as a housekeeping gene. Fold change was calculated considering the average value of all untreated mice for each gene. Results from animals of the same treatment group were collapsed and plotted as log_2-transformed data in a heat map generated by GraphPad Prism 7.02 software (GraphPad Software, San Diego, Calif).

Flow cytometry

Flow cytometry was used for immune cell subset analysis by intracellular and/or surface marker staining and conducted as described previously.[E1] Intracellular staining was performed using the BD Cytofix/Cytoperm Plus kit (BD Biosciences) as per the manufacturer's instructions. Cell viability was assessed by LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen) following the manufacturer's directions. After blocking nonspecific antibody

binding with mouse Fc Block, cells were stained for cell surface markers for 30 minutes at 4°C and subsequently acquired in a LSR Fortessa X20-SORP (BD Biosciences). Alternatively, cells were fixed/permeabilized, then stained with anti-mouse IFN-γ or GZMB antibodies for 30 minutes at 4°C before acquisition.

Antibodies used for cell surface marker staining were as follows: anti-CD3 APC/Cy7 and BV605 (clone 17A2, BioLegend), anti-CD4 BV605 and PE-Cy5 (clone RM4-5, BD Horizon, BD Biosciences), anti-CD8a BV711 and phycoerythrin (PE; clone 53-6.7, BD Horizon), anti-CD45R (B220) APC (clone RA3-6B2, eBioscience), anti-CD19 PE-Cy7 (clone eBio1D3 [1D3], eBioscience), anti–NK-1.1 APC/Cy7 (clone PK136, BioLegend), anti-CD11c efluor450 (clone N418, BioLegend), anti-CD11b BV711 (clone M1/70, BioLegend), anti-F4/80 Alexa Fluor 700 (clone BM8, BioLegend), anti-CD279 BV785 (clone 29.1A12, BioLegend), anti-Ly6C BV785 (clone HK1.4, BioLegend), anti-CD62L Alexa Fluor 700 (clone MEL-14, BioLegend), anti-CD44 PE (clone IM7, BioLegend), anti-CD25 Alexa Fluor 488 (clone eBio7D4 [7D4], eBioscience), CD69 PE-Cy5 (clone H1.2F3, eBioscience), KLRG1 PE-Cy7 (clone 2F1, Biolegend), LAG3 APC (clone eBioC9B7W, Invitrogen) anti–I-A^b ($A\beta^b$) FITC (clone 25-9-17, BioLegend), anti-CD86 BV650 (clone GL-1, BioLegend), anti-CD80 PE-Cy5 (clone 16-10A1, eBioscience), anti-CD45 BV421 (clone30-F11, BioLegend), anti-CD146 FITC (ME-9F1, BioLegend), anti-CD274 (PD-L1) PE-Cy7 (10F.9G2, BioLegend), anti–Clec4f-unconjugated (AF2784, R&D Systems), and donkey anti-goat Alexa Fluor 647 (ab150131, Abcam, Cambridge, United Kingdom). The gating strategy is shown in Fig E5.

Flow cytometry data acquisition and analysis were performed by LSR Fortessa X20-SORP and Diva 6.2/8.0.2 (BD Biosciences).

Isolation of primary hepatocyte

Mouse hepatocytes were isolated using 2 different enzyme digestion-based procedures: collagenase type I (Sigma) (method 1) and Miltenyi enzymes from the mouse liver dissociation kit (Miltenyi Biotec) (method 2). On exposure, the liver was cannulated with a 25-gauge needle placed within the portal vein. Blood flushing was conducted using a peristaltic pump, circulating 15 mL of 0.22 μm–filtered washing buffer (5.4 mmol KCl, 0.44 mmol KH_2PO_4, 140 mmol NaCl, 0.34 mmol Na_2HPO_4, 0.5 mmol EGTA, and 25 mmol Tricine in H_2O, pH 7.2, for method 1) or buffer 1 (PBS containing 30 mmol HEPES, 6.7 mmol KCl, 5 mmol glucose, and 0.2 mmol EDTA, pH 7.4, for method 2) on cutting the inferior vena cava. Subsequently, 35 mL of perfusion buffer (0.075% collagenase type I [Sigma] in calcium-free Hanks balanced salt solution with 0.002% DNAse I [Sigma] [method 1], or an enzyme cocktail of the Miltenyi liver dissociation kit in buffer 2 [PBS containing 30 mmol HEPES, 6.7 mmol KCl, 5 mmol glucose, and 1 mmol $CaCl_2$, pH 7.4] [method 2]) were used for liver digestion. The liver was excised, placed in a petri dish, and opened with forceps to let the primary hepatocyte flow into the digestion buffer (0.009% collagenase type I in calcium-free Hanks balanced salt solution with 0.002% DNAse I [method 1] or buffer 2 [method 2]). The hepatocyte suspension was applied onto a MACS Smart strain (100 μm) (Miltenyi Biotec) and centrifuged at 50 × *g* for 5 minutes. The cell pellet was resuspended into Dulbecco modified Eagle medium (DMEM) supplemented with 2 mmol L-glutamine, 100 μg/mL of penicillin/streptomycin, 1 mmol sodium pyruvate, 1× MEM Non-Essential Amino Acids (all from Gibco), and 10% heat-inactivated, low-endotoxin FBS (Atlanta Biologicals) (DMEM-10).

Isolation of nonparenchymal cells and liver leukocytes

NPC isolation was performed upon washing the liver with 5-10 mL of 1× PBS using a peristaltic pump. The liver was removed from the abdominal cavity and placed in 5 mL of DMEM-10. Single cell suspensions were obtained by pressing the tissue through a 70 μm strainer. Cells were spun down at 50 × *g* for 5 minutes so we could discard the hepatocytes. Supernatant was collected and further spun at 500 rpm for 10 minutes. The cell pellet was resuspended for NPC and counted. For liver leukocyte preparation, NPC suspensions were subsequently run in a continuous 37.5% Percoll gradient and centrifuged at 800 × *g* for 30 minutes. Cell pellets were resuspended in DMEM-10. Alternatively, NPCs were isolated from the excised liver using the mouse liver dissociation kit (Miltenyi Biotec) in an Octo Dissociator with Heaters (Miltenyi Biotec) as per the manufacturer's instructions. Liver leukocytes were obtained by further processing the NPC solutions in a continuous 37.5% Percoll (Cytiva) gradient as indicated above.

Cytotoxicity assay

Primary hepatocytes were seeded on collagen-coated (8 $\mu g/cm^2$) E-plates (Agilent) in DMEM-10 media at a density of 20,000 cells per well. After 18 hours' incubation, liver leukocytes were added into the plates at different *E:T* ratios (see Fig 7, *A*, *B,* and *E*). FLX was also added at different concentrations in selected wells. The cocultures were maintained in an xCELLigence real-time cell analysis platform (Agilent) for up to 96 hours. Data-point readouts were taken every 15 minutes, and the data were normalized at the time point when effector cells were added. The percentage of cytolysis was calculated with the following formula: $\% \text{ Cytolysis} = [(\text{Normalized Cell Index}_{\text{no effector}} - \text{Normalized Cell Index}_{\text{effector}})/(\text{Normalized Cell Index}_{\text{no effector}})] \times 100$.

REFERENCES

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E2. Puig M, Ananthula S, Venna R, Kumar Polumuri S, Mattson E, Walker LM, et al. Alterations in the HLA-B*57:01 immunopeptidome by flucloxacillin and immunogenicity of drug-haptenated peptides. Front Immunol 2020;11:629399.

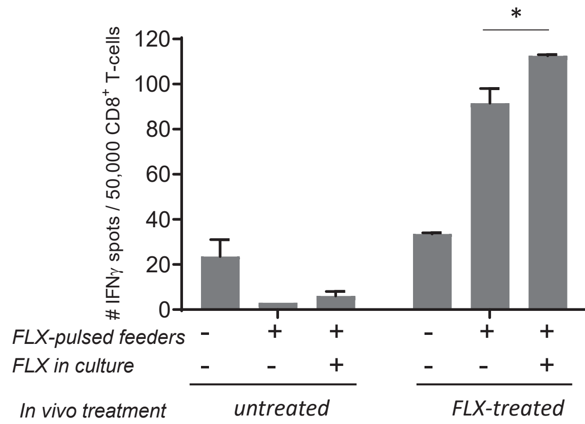

FIG E1. CD8$^+$ T cells were isolated from spleen of FLX-untreated or -treated Tg mice and cultured with drug-pulsed, irradiated feeder cells at a 1:2 ratio of CD8$^+$ T cells to feeders in the absence or presence of 125 μg/mL FLX in culture. IFN-γ yields were measured after 4 days of *in vitro* culture by ELISpot assay. Results are from 1 experiment of 2. $^*P \leq .05$.

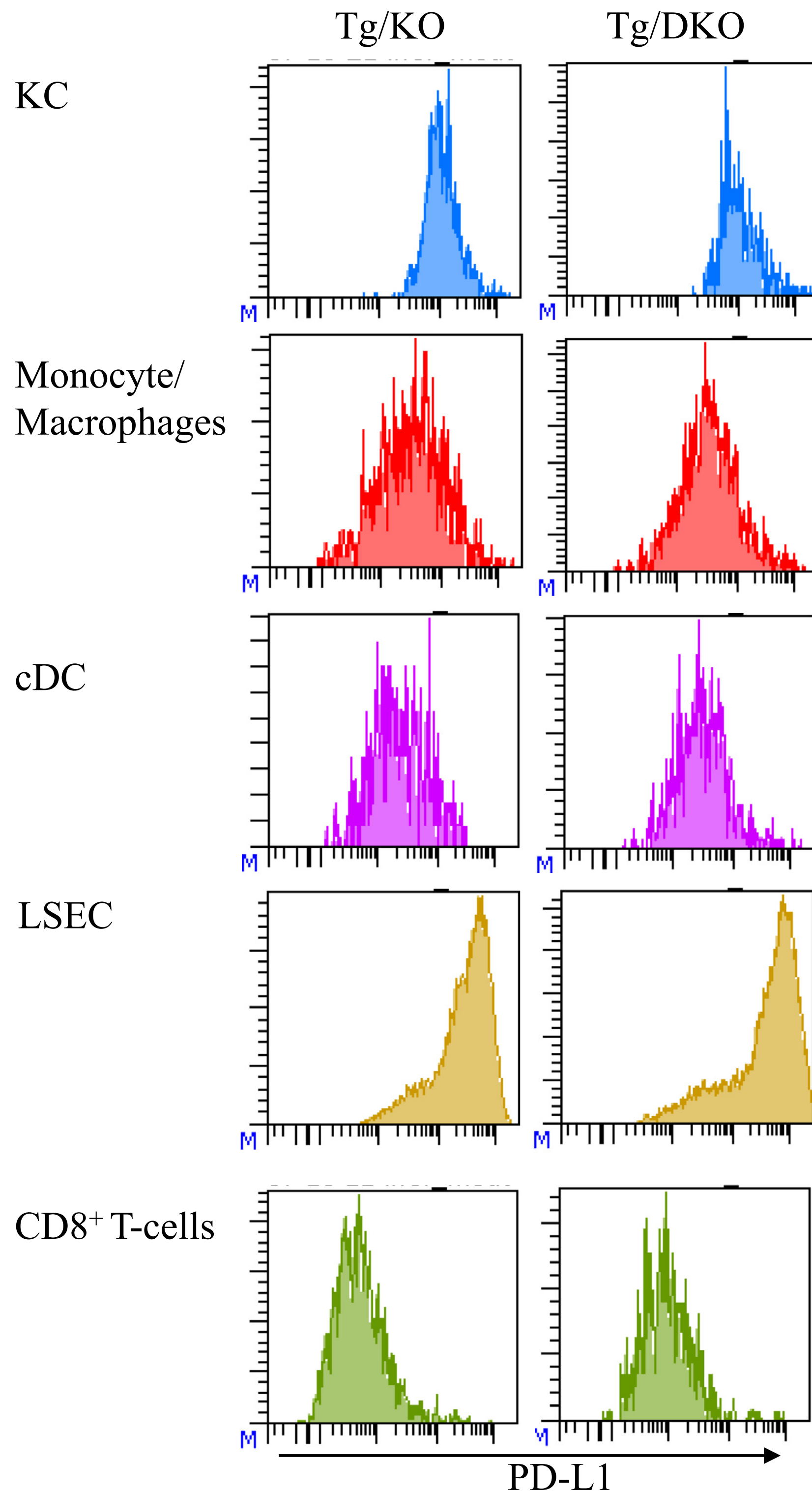

FIG E2. PD-L1 expression on different liver NPC subsets in Tg/KO and Tg/DKO animals. $CD45^+$ cells include KCs gated on $CD11b^+Ly6C^-F4/80^+Clec4f^+$, and monocyte/macrophages gated on $CD11b^+Ly6C^+$. cDC gated on $CD11c^+CD11b^+$ and $CD8^+CD3^+$ T cells. LSECs are $CD45^-CD146^+$ cells. *cDC,* Conventional DC.

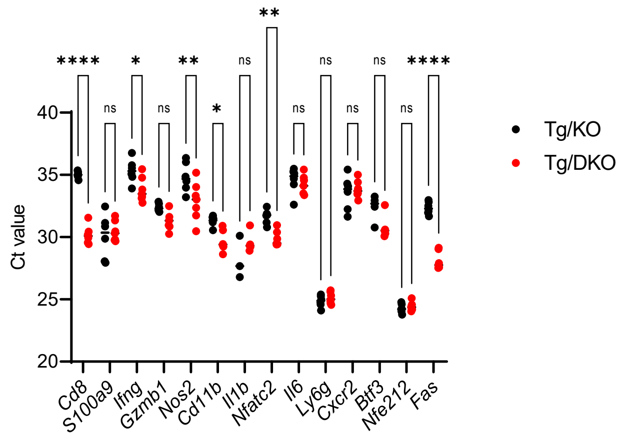

FIG E3. Real-time PCR values *(C_t)* of mRNA of different genes in perfused livers from drug-naive Tg/KO and Tg/DKO animals (n = 3-6 per group).

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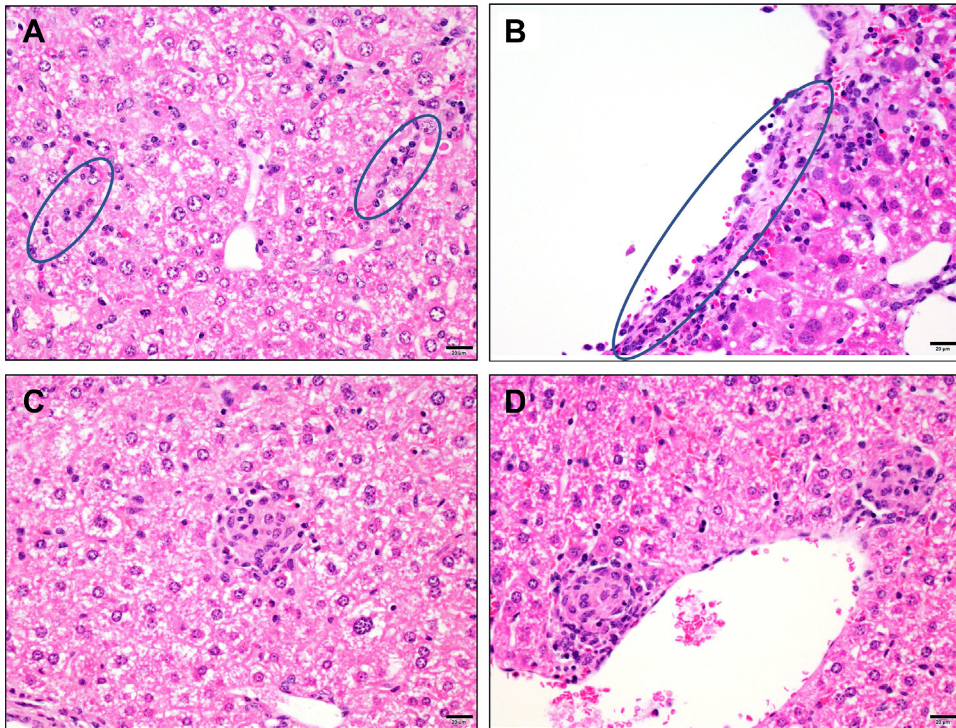

FIG E4. Histopathologic alterations in liver sections of Tg/DKO treated with aCD4Ab + FLX. H&E staining of fixed liver sections (scale bar = 20 μm) depicting representative images of sinusoidal lymphocytosis **(A)**, endotheliitis **(B)**, and granulomas (**C** and **D**) from 2 representative animals.

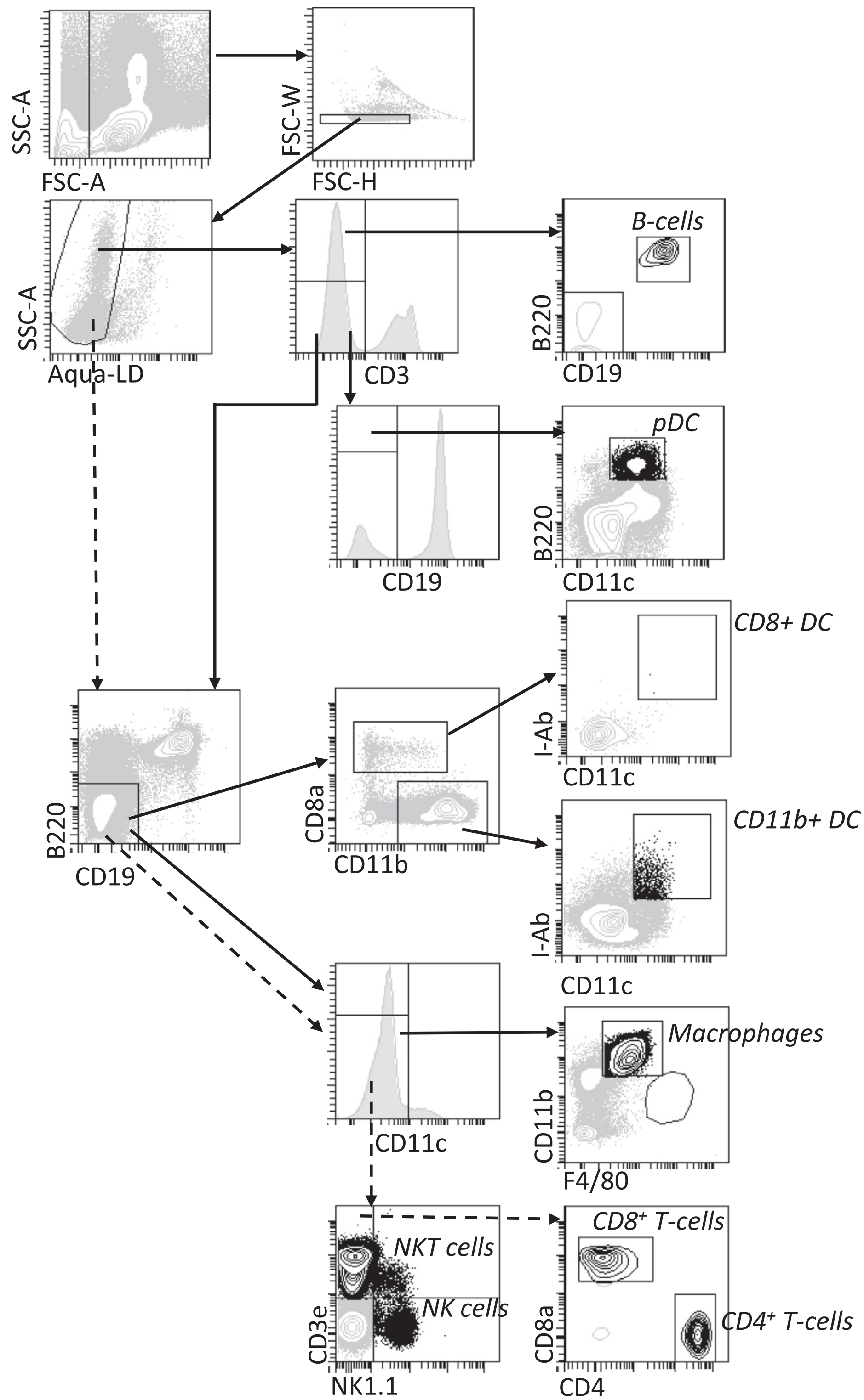

FIG E5. Gating strategy for fluorescence-activated cell sorting analysis of leukocyte subsets.