METHODS

Antibodies and reagents

For *in vivo* experiments, azide-free anti-CD8β antibody (53-5.8; BD Biosciences, Oxford, United Kingdom [UK]), control rat IgG (Sigma, Poole, UK) or anti-CD40 (3/23; Serotec, Oxford, UK) were used. Anti–CCR3-phycoerythrin and anti–IL-12 were from R&D Systems (Abingdon, UK); anti–IL-18 was from MBL Ltd (Watertown, Mass). Biotin or phycoerythrin-labeled anti-CD44 (IM7.8.1), anti-CD25 (PC61), and Gr-1–fluorescein isothiocyanate were from Caltag-MedSystems (Towcester, UK) and antiperforin-phycoerythrin (eBioOMAK-D) from Insight Biotec (Wembley, UK). Neutralizing azide/endotoxin-free anti–IFN-γ (R4-6A2), anti-CD40L (MR1), and control antibody were from BD Biosciences. mouse plasmacytoid dendritic cell antigen 1 (mPDCA1) antibody, specific for plasmacytoid DC, was from Miltenyi Biotec (Bisley, UK). The T1/ST2 (DJ8) T_H2-specific antibody was from MD Biosciences (Zurich, Switzerland). All other antibodies were from BD Biosciences. Streptavidin-Qdot-800 (Cambridge Bioscience UK, Cambridge, UK) was used to reveal biotin antibody staining. PE-H-2K^b/SIINFEKL Pro5 Pentamer (ProImmune Ltd, Oxford, UK) staining was used to identify MHC class I–restricted ovalbumin-specific CD8 cells. CpG 1826 DNA (TLR-9 agonist) was from VHBio (Gateshead, UK), and palmitoyl-cysteinyl-seryl-lysyl-lysine lipopeptide (Pam_3CSK4, TLR1/2 agonist) was from EMC Microcollections (Tubingen, Germany). SII, ISQ, and control vesicular stomatitis virus peptide RGYVYQGL (RGY) were synthesized by Perbio Mimotopes (Tattenhall, UK). Other reagents were purchased from Sigma.

Cell separations and flow cytometry

Dendritic cells were purified from B6 splenocytes by labeling with anti–CD11c-biotin (0.5 μg/10^6 cells) followed by antibiotin microbeads (Miltenyi Biotec). CD11c$^+$ cells were selected by 2 rounds of magnetic selection (autoMACS; Miltenyi Biotec) and were >80% CD11c$^+$ MHC class II^{hi} CD14$^-$. CD4/CD8 T cells (>98% purity) were isolated from spleen/lymph node by using anti-CD4/CD8 microbeads. High purity, allophycocyanin (APC)-depleted CD8s and CD44^{hi}/CD44^{lo} CD8 separations were performed by negative selection and further magnetic separation after labeling with 0.05 μg anti–CD44-biotin per 10^8 cells. Cell fractionations and staining were performed in PBS + 1% FCS. Cell cultures were in Dulbecco modified Eagle medium (Invitrogen, Paisley, UK) + 10% FCS (Sera-Lab, Loughborough, UK) + L-glutamine (2 mmol/L), nonessential amino acids (1 mmol/L each), gentamicin (50 μg/mL), and 2-mercaptoethanol (50 μmol/L). Recombinant IL-12 (Peprotech, London, UK) and IL-18 (R&D Systems) were added at 10 ng/mL. Anti-CD3 (145-2C11) was used to coat 24-well plates (1 μg/mL in PBS, 4°C overnight), whereas anti-CD28 (37.51) was added to cultures at 1 μg/mL. Cytokine profiles were determined by restimulation of washed cells in the presence of 3 μmol/L monensin followed by intracellular cytokine staining as described.[10] Flow-cytometric analysis was using a FACScalibur (BD Biosciences), with gating on live cells determined by forward versus side scatter. IFN-γ secretion was measured in supernatants of cultured CD8 naive or memory cells (2×10^6/mL) collected at sequential time points and measured by ELISA using antibody pairs (BD Biosciences according to the manufacturer's protocol).

T-cell proliferation

In vitro proliferation was measured in triplicate cultures of 0.2 mL CD8 cells (2.5×10^6/mL) in 96-well plates. After 72 hours of culture, 0.5 μCi ^{3}H-thymidine (Amersham Ltd, Amersham, UK) was added to each well overnight, cells were harvested onto glass fiber filters, and scintillation counting was performed with the Topcount system (Perkin Elmer, Beaconsfield, UK). For tracking cell division by CFSE dilution, cells were washed twice in PBS, labeled with 2.5 μmol/L CFSE (Invitrogen) for 10 minutes at 37°C, washed, and cultured for 3 days before flow cytometry. For the *in vivo* proliferation experiment, B6 CD8 cells were labeled with CFSE as described and resuspended in PBS for intravenous transfer into tail veins of B6 or OT-1 recipient mice (5×10^6 cells in 100 μL). Mice then received 25 μg SII peptide in PBS or PBS alone intraperitoneally. Three days later, axillary, inguinal, and cervical lymph nodes were stained for CD44, Vα2, and Vβ5 and analyzed by flow cytometry.

T-cell:DC cocultures

For IL-12 induction, CD8 populations as described from OT-1 mice were cultured in U-shaped microtiter wells at 10^6/mL with addition of DCs at 5×10^5/mL, peptides at 2 μg/mL, ovalbumin at 500 μg/mL, CpG at 1 μg/mL, and antibodies at 10 μg/mL. Supernatants were collected after 24 hours for IL-12 measurements. For proliferation assays, CD8 fractions from B6 mice were cultured at 2.5×10^6/mL ± DCs at 2.5×10^5/mL, with CpG, Pam_3CSK4, or poly I:C at 1 μg/mL. For bystander activation experiments, CFSE-labeled B6 CD44^{hi} CD8s, OT-1 CD44^{hi} CD8s, and DCs were prepared and cocultured at 2×10^6/mL, 1×10^7/mL, and 1×10^6/mL, respectively. SII peptide (2 μg/mL) and antibodies to IL-12 and IL-18 (both 10 μg/mL) were added as indicated. After 15 hours, 3 μmol/L monensin was added, and intracellular staining was performed after a further 4 hours.

Analysis of lung and liver infiltrates

For intrahepatic lymphocyte isolation, livers were perfused with 5 mL cold PBS/FCS via the hepatic portal vein *in situ*, then dissociated by using a nylon mesh and washed before digestion in RPMI-1640 + 10% FCS + 0.7 mg/mL collagenase type IV for 1 hour at 37°C. Cells were released by pipetting and washed in PBS/FCS. Cells were then suspended in 24% metrizamide in PBS and overlaid with culture medium before density gradient centrifugation at 1500*g*. Lymphocytes were harvested from the interface. For lung cell isolation, lungs were cut into fragments, digested as described, and used without density gradient purification.

CD8 T-cell depletion and adoptive transfer

Mice were depleted of CD8 T cells by injection of 75 μg anti-CD8β antibody intraperitoneally, or rat IgG control. Depletion was confirmed by flow-cytometric analysis of peripheral blood 24 hours later by using CD8α, CD3, CD11c, and mPDCA1 markers. This showed that there was >99% depletion of CD8 T cells with no effect on myeloid or plasmacytoid DC numbers (Fig E3). Effective CD8 T-cell depletion was also observed in lymph node, spleen, and lung cells and lasted for >3 weeks (not shown).

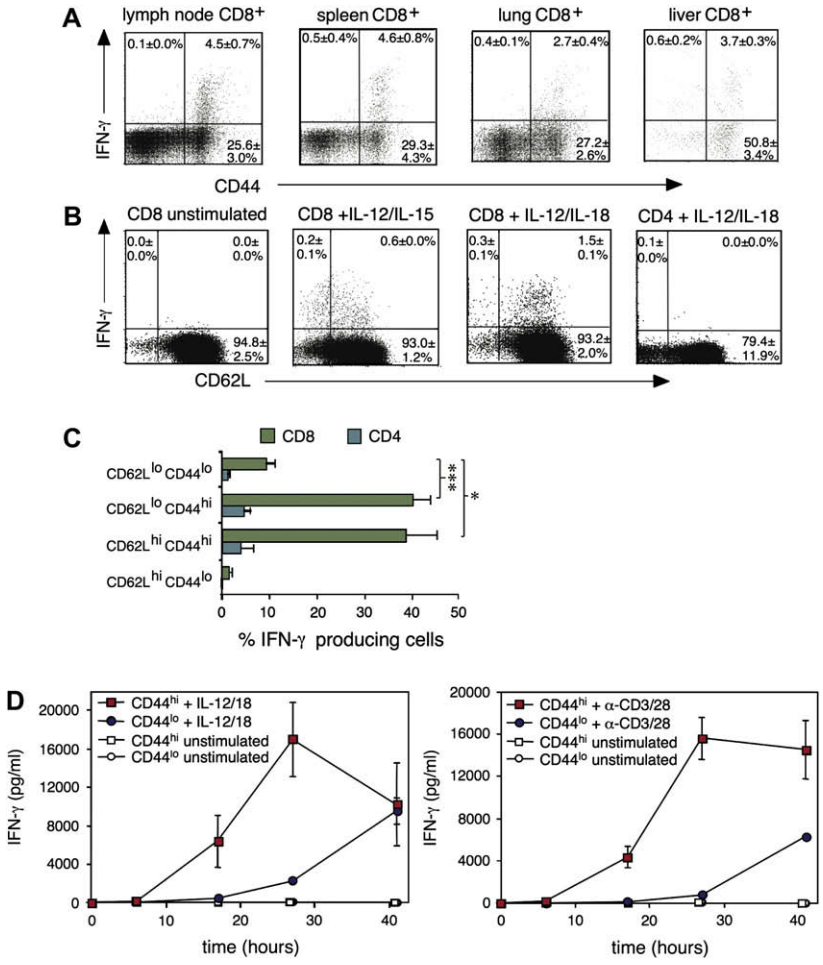

FIG E1. Early IFN-γ synthesis in central and effector memory CD8 Tms. **A,** CD8 Tms ($CD44^{hi}$) are the major source of TCR-triggered early IFN-γ. CD8 T cells purified from tissues of untreated mice were stimulated with anti-CD3/CD28 for 5 hours and stained for CD44 and intracellular IFN-γ. Gated $CD8^+$ events are shown. Data refer to percent IFN-γ^+ cells in total CD8 population. **B,** CD8 but not CD4 T cells from unprimed mice produce IFN-γ after primary stimulation with cytokines alone. Lymph node CD4 or CD8 T cells were stained for CD44 (memory marker) and CD62L (lymph node homing receptor) and then stimulated with indicated cytokines for 5 hours before intracellular IFN-γ analysis. **C,** Both central memory ($CD44^{hi}$ $CD62L^{hi}$) and effector memory ($CD44^{hi}$ $CD62L^{lo}$) CD8 cells produce early IFN-γ after IL-12 + IL-18 stimulation. Splenic CD4 or CD8 cells were stained for CD62L and CD44 before IL-12/IL-18 stimulation. Spleen contained both central and effector memory cells. Percent IFN-γ^+ cells within each subpopulation are shown. **D,** Time course of IFN-γ secretion from cultures of fractionated $CD44^{hi}$ and $CD44^{lo}$ CD8 cells stimulated with IL-12/IL-18 or anti-CD3/28. Data in *A* and *B* are representative of 3 experiments; data in *C* and *D* are means ± SEMs from 3 independent experiments. $*P < .05$; $***P < .005$.

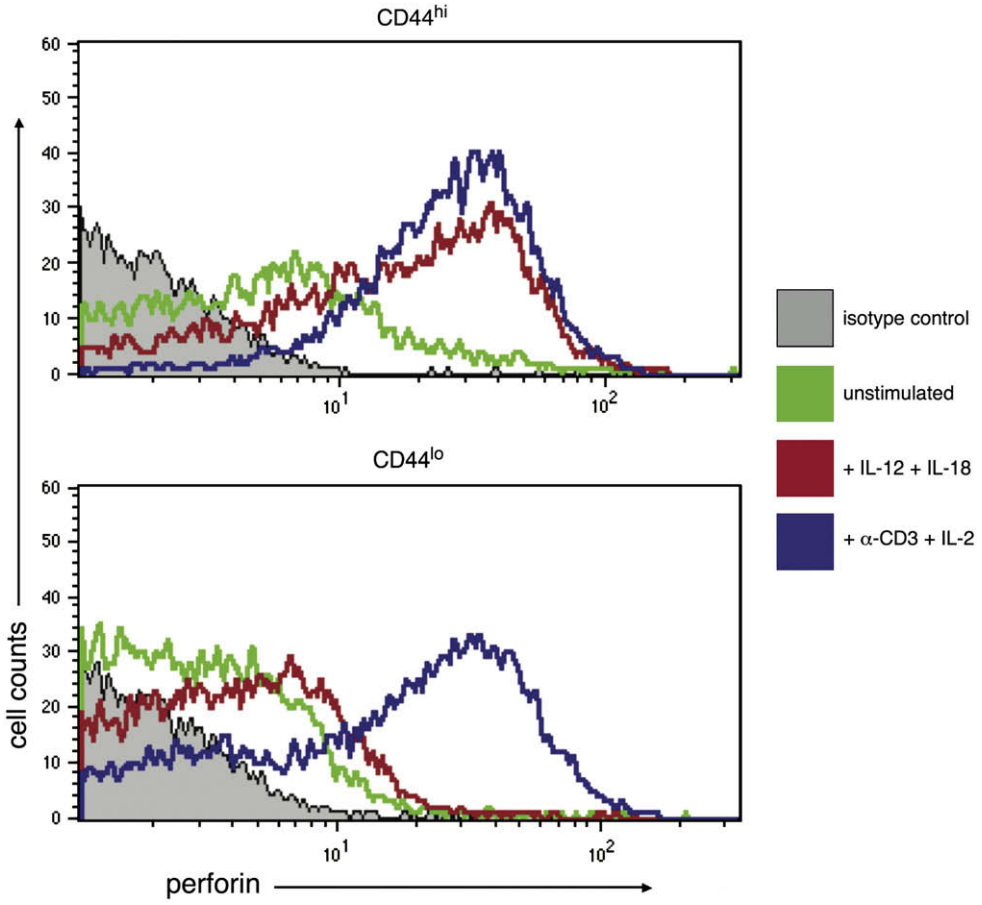

FIG E2. IL -12+ IL-18 induces perforin expression in CD8 Tms. Splenic CD8 T cells were labeled with anti–CD44-APC, washed, and cultured with IL-12 + IL-18 (10 ng/mL each) or platebound anti-CD3 + IL-2 or were unstimulated for 3 days. Cells were then fixed, permeabilized, with 0.1% Triton X100 (Sigma, Poole, UK), and stained with antiperforin-phycoerythrin or isotype control antibody before flow cytometry. Intracellular perforin staining in gated $CD44^{hi}$ (memory CD8, *upper graph*) or naive CD8 ($CD44^{lo}$, *lower graph*) is shown. Data are representative of 3 experiments.

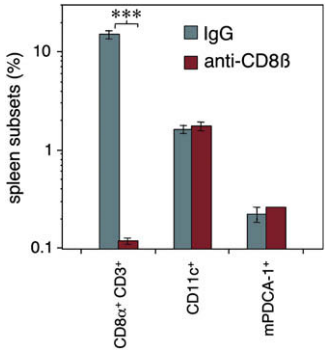

FIG E3. Proportions of CD8 T cells, total DC ($CD11C^+$) and plasmacytoid DC ($mPDCA\text{-}1^+$) in spleen 1 day after intraperitoneal injection of C57BL/6 mice with 75 μg anti-CD8β or control antibody.