Supplemental Data

Bone Marrow Is a Major Reservoir and Site of Recruitment for Central Memory CD8$^+$ T Cells

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Supplemental Experimental Procedures

Mice

C57Bl/6 and congenic CD45.1 mice were from Jackson Labs (Bar Harbor, ME), Taconic (Germantown, NY), or Charles River (Wilmington, MA). T-GFP mice, TCR transgenic P14 mice (Pircher et al., 1989), and P14×T-GFP double transgenic mice were described previously (Manjunath et al., 2001). OT-IxRAG$^{-/-}$ mice, which produce only CD8 T cells recognizing an OVA peptide in H2K^b were provided by H. Ploegh (HMS). VCAM-1 mutant mice crossed to Tie2-Cre mice (Koni et al., 2001) and PSGL-1$^{-/-}$ mice were described previously (Xia et al., 2002). Animals were kept in a SPF/VAF facility on standard lab chow and sterile water. All procedures complied with National Institutes of Health guidelines.

Antibodies

Hybridomas for monoclonal antibodies (mAbs) 9A9 and 5H1 to mouse E- and P-selectin, respectively, were provided by B. Wolitzky (Hoffman LaRoche, Nutley, NJ). Hybridomas for mAbs Mel-14 (anti-L-selectin), TIB213 (anti-CD11a), and FIB504 (anti-β7 integrin) were a gift from E.C. Butcher (Stanford University). Anti-VCAM-1 MK 2.7 hybridoma cells were from ATCC (Manassas, VA). mAbs were purified from culture supernatant on a HPLC ion-exchange column (Bakerbond). Anti-human/mouse CXCL12 (clone 79014.111) was from R&D (Minneapolis, MN). The following flow cytometry reagents were from PharMingen (San Diego, CA): anti-CD16/32 Fc block; phycoerythrin (PE)-conjugated mAbs to α4, α5, PSGL-1, α4β7, IL-7R, CD11a, CD11b, CD25, CD43 (1B11), CD44, CD69, CD122, IFN-γ, and IL-2; biotinylated anti-CD8β; FITC-conjugated anti-CD44, anti-CD45.2; APC-conjugated anti-L-selectin; P-selectin-Ig chimera; isotype control rat IgG; and PerCP-conjugated streptavidin. CCL19-Ig chimera was used to stain CCR7 with PE- or FITC-conjugated goat-anti-human Ig (Caltag Laboratories, Burlingame, CA) as described (Manjunath et al., 2001).

mAbs from PharMingen were used to stain human BM: FITC-anti-CD3, ECD-anti-CD45RA, Cy-Chrome-anti-CD8, APC-Cy7-labeled anti-CD62L, and anti-CCR7 (clones 2H4 or 3D12). Goat-anti-mouse IgM-PE or goat-anti-rat IgG-PE (Jackson Immunoresearch Lab, West Groove, PA) was used as the secondary Abs.

Human BM Cells

Heparinized human BM from iliac crest aspiration was obtained from healthy adult volunteers after informed consent in accordance with guidelines approved by the IRBs of DFCI. BM MNL were isolated by Ficoll-Hypaque gradient centrifugation (1.077 g/ml; Pharmacia, Piscataway, NJ).

In Vitro Differentiation of T Cells

10 μg/ml gp33-41 or SIINFEKL peptide (gift from H. Plough) was added for 1 hr at 37°C to fresh splenocytes expressing the P14 or OT-I TCR, respectively. Cells were washed and cultured in complete media (CM) consisting of RPMI 1640 (BioWhittaker, Walkersville, MD) with 10% FBS and standard supplements. After 48 hr, cells were washed and incubated for 5 days in CM with 20 ng/ml human IL-15 or murine IL-2 (R&D) to generate Ag-specific T_{CM}s or T_{Eff}s, respectively. CM with fresh cytokines was added every second day. The phenotype of differentiated cells was routinely tested by measuring activation markers (CD25, CD44, and CD69), L-selectin, and GFP by FACS.

Homing of In Vitro-Generated T_{CM} and T_{Eff}

P14×T-GFP T_{CM}s and T_{Eff}s were labeled with tetramethylrhodamine (TRITC, 30 mg/ml; Molecular Probes, Eugene, OR) and centrifuged over FBS to remove dead cells. 5×10^7 T_{CM}s ($TRITC^+GFP^+$) or T_{Eff}s ($TRITC^+GFP^-$) were mixed with 5×10^7 fresh naive P14×T-GFP splenocytes ($TRITC^-GFP^+$) and transfered to syngeneic recipient mice. Some input cells were saved to assess the concentration of transferred $TRITC^+$ ($[TRITC]_{input}$) and GFP^+ cells ($[GFP]_{input}$). After 2 or 24 hr, recipients were anesthetized by i.p. injection of ketamine (5 mg/ml, Fort Dodge Animal Health) and xylazine (1 mg/ml, Phoenix Pharmaceutical), and cardiac blood was obtained. The animals were then sacrificed, and the frequency of homed cells was determined by FACS in single-cell suspensions from spleens, PLN, MLNs, liver, lungs, and BM. Results are expressed as percentage of $TRITC^+$ ($[TRITC_{organ]}$) or GFP^+ ($[GFP_{organ}]$) cells among live leukocytes. To correct for differences in the input between individual experiments, the homing index (HI) was calculated; HI = $[TRITC_{organ}] / [GFP_{organ}] : [TRITC_{input}] / [GFP_{input}]$. Results were also expressed as number of homed cells per 10^6 injected cells. For BM, the number of homed cells was calculated by multiplying by five the number of cells harvested from two hindlegs, assuming that two limbs contain ~20% of all BM cells (Boggs, 1984). The role of Gαi signaling was assessed by incubating P14 T_{CM} (5×10^6/ml) overnight in CM with IL-15 and 100 ng/ml pertussis toxin (PTX, CalBiochem, La Jolla, CA). PTX-treated cells were labeled with TRITC, whereas control cells treated with diluent (1:1000 DMSO; Sigma) were labeled green with 30 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes). Labeled cells were used for competitive homing experiments as described above; dyes were swapped between experiments.

Animal Preparation and Intravital Microscopy

Mice were anesthetized, and the hair in the neck and scalp was removed with hair removal lotion (Nair, Carter Products, NY). A PE-10 polyethylene catheter (Becton Dickinson) was inserted in the right common carotid artery for injection of cells and fluids. The animal's head was immobilized in a stereotactic holder (David Kopf Instruments, Tujunda, CA) on a Plexiglass stage, and the frontoparietal skull was exposed for IVM analysis.

An intravital microscope (IV 500, Mikron Instruments, San Diego, CA) with water immersion objectives (Zeiss, Germany) was used. Small boluses (~50 μl) of T cells (10^7/ml) labeled with 2.5 μg/ml calcein-AM (Molecular Probes) were injected into the carotid artery and visualized in the left frontoparietal skull under fluorescent epi-illumination by using a video-triggered Xenon arc stroboscope (Chadwick Helmuth, El Monte, CA), a FITC filter set, and a 10× objective (Zeiss Achroplan, NA 0.3). Scenes were recorded with a silicone-intensified target camera (VE 1000-SIT, Dage MTI, Michigan City, IN), a time base generator (For-A, Japan), and a Hi-8 VCR (Sony, Japan). In some experiments 100 μg/mouse mAb to VCAM-1, P- or E-selectin, or CXCL12 were injected i.v. 10 min before cell injection; T cells were incubated with mAbs to L-selectin or β7 (100 μg/10^7 cells, 37°C) for 10 min before injection.

Intravascular cell behavior was assessed as the rolling fraction (RFx: number of interacting cells in each venule or sinusoid per 100 passing cells), the sticking fraction (SFx: number of firmly adherent [≥30 s] cells per 100 rollers), and the sticking efficiency (SE: the number of firmly adherent cells in the total flux of cell entering a vessel).

Chemotaxis Assay

Chemotaxis experiments were performed in 24-well plates with 5 μm pore filters (Costar, Cambridge, MA). 5×10^5 T_{CM}s or T_{Eff}s were loaded in the upper chamber. The lower chamber contained media without or with various concentrations of CXCL12. After 90 min at 37°C, cells in the bottom well were counted by FACS. The chemotactic index was calculated as ratio of cells that migrated to CXCL12 versus migrated cells with media alone. Percentage of migrated cells among input cells was also determied. In some experiments, anti-CXCL12 (100 μg/ml) was added to the lower chamber or PTX treated cells were used.

Supplemental References

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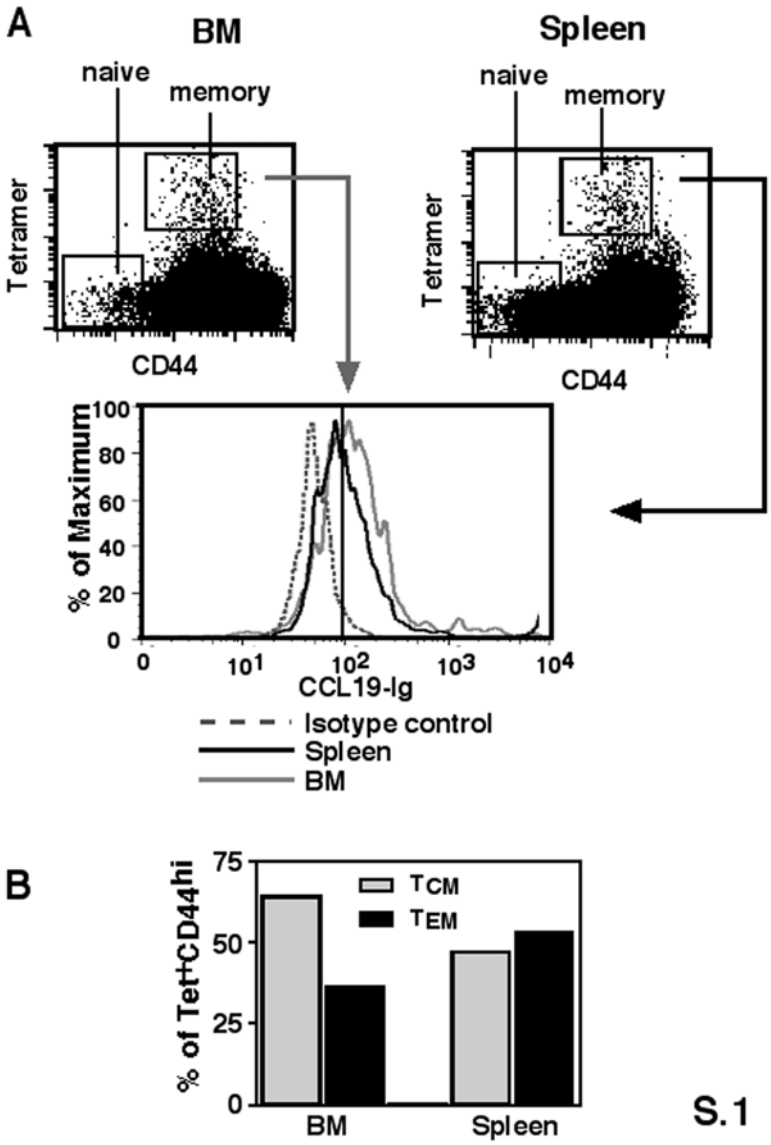

Figure S1. Distribution of $CD8^+$ T Cell Memory Subsets in BM and Spleen of Mice after In Vivo Ag Stimulation

(A) Naive CD8 cells from P14×$TCRa^{-/-}$ mice injected 8 weeks before aquired a memory phenotype ($tetramer^+CD44^{hi}$) after in vivo Ag priming and challenge. Ag-specific $Tetramer^+CD44^{hi}$ $CD8^+$ T_{CM}s and T_{EM}s were measured in BM and spleen based on CCR7 expression.

(B) Frequencies of endogenous T_{CM}s and T_{EM}s within the Ag-specific memory CD8 population.

Table S1. Subset Frequency among CD8β$^+$ T Cells in Normal Murine BM and Spleen

$CD8^+$ T Cell Subset	Frequency (% of all MNL)		Frequency (% of CD8 T cells)		Number of Cells per Organ ($\times 10^6$)		L-sel^{hi} (% in subset)	Psel-Ig$^+$ (% in subset)[a]
	BM	Spleen	BM	Spleen	BM	Spleen	BM	BM
Naïve ($CD44^{lo}CCR7^+$)	0.3 ± 0.02***	7.8 ± 0.5	31.3 ± 1.9***	67.9±1.3	0.5 ± 0.07**	4.4 ± 0.3	95.8 ± 0.9	0.7 ± 0.4
T_{CM} ($CD44^{hi}CCR7^+$)	0.51 ± 0.03**	1.7±0.2	48.0 ± 2.8***	15.4±2.7	0.8 ± 0.1	1.0 ± 0.2	84.4 ± 0.4	4.4 ± 1.5
T_{EM} ($CD44^{hi}CCR7^-$)	0.12 ± 0.01*	0.8 ± 0.2	11.3 ± 0.5*	6.7 ± 1.5	0.2 ± 0.01*	0.5 ± 0.1	26.3 ± 6.2	38.0 ± 6.7

Total cell number per organ: BM, 151.5±13.0 and spleen, 55.9±2.2. Data are presented as mean ± SEM. The frequencies of $CD8^+$ cells were determined by anti-CD8β staining. To assess P-selectin binding, L-selectin expression, rather than CCR7, was used to identify subsets, i.e., $CD44^{low/-}$L-selectin^{high} for naive cells, $CD44^{high}$L-selectin^{high} for T_{CM}s and $CD44^{high}$L-selectin$^{Low/-}$ for T_{Eff}s. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (paired Student's t test). Data are presented from five mice.