Supplementary methods

Quantification of M protein. Blood from MM-bearing mouse was collected every two weeks after transplant and serum was isolated. The samples were analyzed using Sebia Hydrasys serum protein electrophoresis system (Hydrasys 2 Scan). Gamma and albumin fraction were quantified to calculate a gamma globulin/albumin ratio (G/A ratio), hereafter referred to as M-band. We assessed tumor growth by modeling log M-band over time using mixed-effects models with random intercepts. To compare tumor growth between groups, we performed least-squares means contrasts, and all contrasts were adjusted for multiple comparisons using Tukey's honest significant difference test. Myeloma relapse was assigned using an M-band of 0.282 as a cutoff point as described[1].

Stem cell mobilization. Recombinant human G-CSF (Amgen, Thousand Oaks, CA) was diluted with PBS and 10 μg/mouse was administered subcutaneously for four sequential days before transplant[2]. AMD3100 (Tocris, Minneapolis, MN) was diluted with PBS and given subcutaneously at 100 μg/mouse per dose one hour before mobilized grafts were harvested[3].

Cell preparation for flow cytometry. For human samples, cryopreserved BM mononuclear cells and PBSCs were thawed and incubated with Human TruStain FcX (BioLegend) prior to surface staining with antibodies. Mice were euthanized and BM, blood and spleen were harvested. Blood and spleen samples were lysed with Geys red blood cell lysis buffer prior to incubation with purified Rat anti-mouse CD16/CD32 antibody (BioLegend, clone: 2.4G2). Antibodies used for FACS analysis are described in Supplementary table 1. Surface staining with fluorescently tagged antibodies was performed for 30 minutes on ice. For intracellular staining, cells were fixed and permeabilized using Foxp3 transcription factor staining kit (eBioscience) after surface staining and were incubated with intracellular antibodies for 1 hour at room temperature. For intracellular cytokine staining, cells were stimulated for 4 hours at 37°C with phorbol myristate acetate (PMA;

5 mg/mL) and ionomycin (50 mg/mL) (Sigma-Aldrich, St. Louis, MO) and brefedlin A (BioLegend). All samples were acquired on a BD FACSymphony A3 Cell Analyzer (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR). FlowSOM analysis was performed with 3,000 cells per mouse and 10,000 cells per patient after downsampling[4].

In vitro Treg suppression assay. We performed in vitro Treg suppression assays as described previously[5]. Briefly, we injected G-CSF and AMD-3100 or control vehicle to Foxp3-GFP-DTR mice and harvested spleen as the source of Treg. We labeled CD8 T cells from B6.Ptprca (CD45.1) mice using Tag-it Violet Proliferation and Cell Tracking Dye (CTV, from BioLegend). CTV-labeled CD8 T cells were seeded at 5 x 10^4 / well in 96-well round plates with DCs from naïve C57Bl/6 mouse at 5 x 10^3 /well in the absence or presence of sort purified GFP^+ Tregs 5 x 10^4 cells / well, and supplemented with anti-CD3 (2C11, 1μg/ml). Cells were cultured for 72 hours and harvested for CTV dilution analysis of $CD45.1^+$ CD8 T cells. Data was analyzed and the division index[6] was calculated using FlowJo software.

Supplementary table 1

Mouse

Supplier	Target	Fluorochrome	Clone	Cat No.
BD	CD44	APC-Cy7	IM7	560568
BD	CD152 (CTLA-4)	APC-R700	UC10-4F10-11	565778
BD	TNF	BB700	MP6-XT22	566510
BD	CD45	BUV395	30-F11	564279
BD	CD138	BUV395	281-2	740240
BD	CD4	BUV496	GK1.5	612952
BD	Ly108	BUV661	13G3	741679
BD	PD-1	BUV737	J43	749422
BD	CD8	BUV805	53-6.7	612898
BD	TIGIT	BV421	1G9	142111
BD	CD62L	BV480	MEL-14	746726
BD	TIM-3	BV605	RMT3-23	119721
BD	DNAM-1	BV650	TX42.1	133621
BD	CD155	BV750	3F1	747250
BD	CD69	BV785	H1.2F3	104543
BD	CD25	PE	7D4	558642
BD	TCF-1/TCF7	PE	S33-966	564217
BD	CD38	PE-Cy7	90	102712
BD	CD3ε	PE-Dazzle594	145-2C11	100348
BioLegend	CD49d	AF647	R1-2	103614
BioLegend	CD62L	AF700	MEL-14	104426
BioLegend	KLRG1	APC	2F1	138412
BioLegend	IFNγ	BV421	XMG1.2	505830
BioLegend	CD73	BV605	TY/11.8	127215
BioLegend	CD90.2	BV605	53-2.1	140318
BioLegend	CD4	BV650	GK1.5	100469
BioLegend	CD3ε	BV711	145-2C11	100349
BioLegend	IFNγ	BV786	XMG1.2	505838
BioLegend	CX3CR1	FITC	SA011F11	149020
BioLegend	CD39	PE-Cy7	Duha59	143806
BioLegend	PD-1	PE-Cy7	RMPI-30	109110
BioLegend	CD304 (Nrp-1)	PE-Dazzle594	3E12	145218
eBioscience	TOX	eFluor660	TXRX10	50-6502-82
eBioscience	Foxp3	PE-Cy5	FJK-16s	15-5773-82

Human

Supplier	Target	Fluorochrome	Clone	Cat No.
BD	TOX	APC	REA473	130-120-709
BD	CD39	BB515	TU66	565469
BD	TIM-3	BB700	7D3	746178
BD	CD3	BUV395	SK7	564001
BD	CD8	BUV496	RPA-T8	612942
BD	CD69	BUV563	FN50	748764
BD	CCR7	BUV661	2-L-A	749824
BD	CD28	BUV737	CD28.2	612815
BD	CD4	BUV805	SK3	612887
BD	Granzyme B	BV510	GB11	563388
BD	CD25	BV605	2A3	562660
BD	Ki67	BV650	B56	563757
BD	DNAM-1	BV711	DX11	564796
BD	CXCR5	BV750	RF8B2	747111
BD	TCF-1	PE	S33-966	564217
BD	CD127	PE-Cy5	A019D5	351324
BD	CX3CR1	R718	2A9-1	752200
BioLegend	CD45RA	APC-Cy7	HI100	304128
BioLegend	TIGIT	BV421	A15153G	372710
BioLegend	PD-1	BV786	EH12.2H7	329930
eBioscience	Eomes	PE/eFluor610	WS1928	61-4877-42
eBioscience	c-Maf	PE-Cy7	sym0F1	25-9855-82
Invitrogen	Foxp3	PE	236A/E7	12-4777-42

Supplementary table 2

Patient characteristics

#	Age	Gender	Stage (R-ISS)	Cytogenetic disease risk	Treatment prior to ASCT	Response at ASCT	Months to progressive myeloma after transplant
1	71	Male	III	High	VRD	VGPR	5
2	69	Female	III	High	VCD	sCR	6
3	70	Male	III	High	VRD, KPd, DVd, DPd, KRd	PR	13
4	67	Male	II	Standard	VCD	sCR	64
5	53	Female	N/A	High	RD, KCd	VGPR	NR at 1 month
6	57	Female	N/A	Standard	VRD, Bor maintenance	VGPR	11
7	67	Male	II	Standard	VRD	VGPR	8
8	61	Female	II	High	VRD, RD, CarD-PACE	VGPR	27
9	56	Male	N/A	Not done	VCD	VGPR	64
10	69	Male	II	High	VRd, KRd	VGPR	14
11	58	Female	II	High	VRd	CR	20
12	69	Male	N/A	High	VRD+XRT, KRD,KRD-PACE	PR	17
13	56	Male	N/A	High	VRD	sCR	NR at 11 months
14	63	Male	N/A	Standard	VRD	sCR	NR at 4 months
15	70	Female	N/A	High	RD+XRT, KD, Drd	PR	15
16	65	Male	III	High	VRD	VGPR	12
17	56	Female	II	High	VCD, VRD	VGPR	NR at 6 months
18	73	Male	II	High	IRD	VGPR	NR at 36 months

VRD / VRd: Bortezomib + Lenalidomide + Dexamethazone

VCD: Bortezomib + Cyclophosphamide + Dexamethazone

KRd / KPd / KCd: Carfizomib + Lenalidomide or Pomalidomide or Cyclophosphamide+ Dexamethazone

DVd/ DRd/ DPd: Daratumumab + Bortezomib or Lenalidomide or Pomalidomide + Dexamethazone

CarD-PACE: Carfizomib + Dexamethazone + Cisplatin + Doxorubicin + Cyclophosphamide + Etoposide

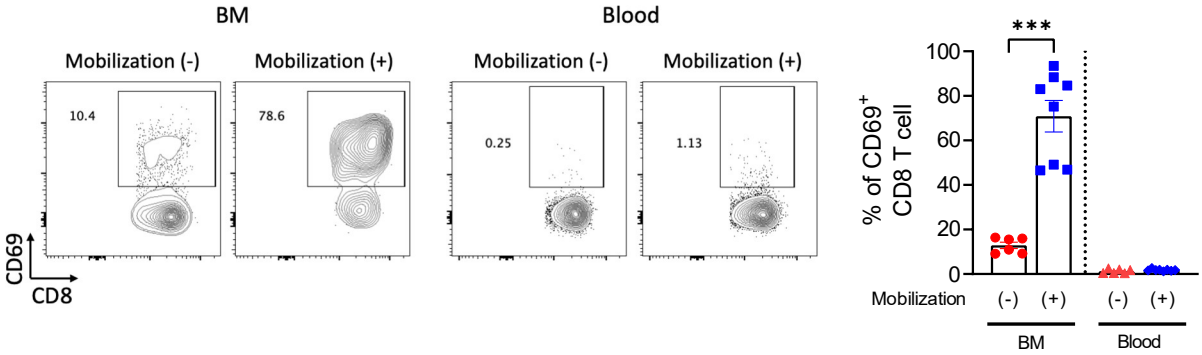
IRD: Ixazomib + Lenalidomide + Dexamethazone

XRT: Radiation therapy

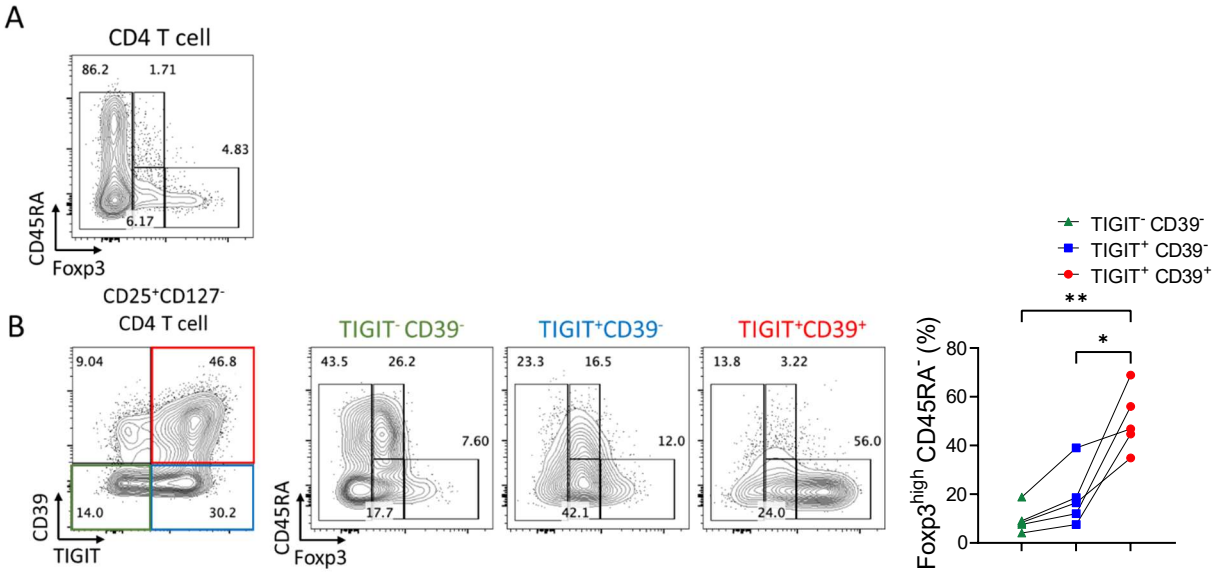
N/A: not available

NR: Not reached

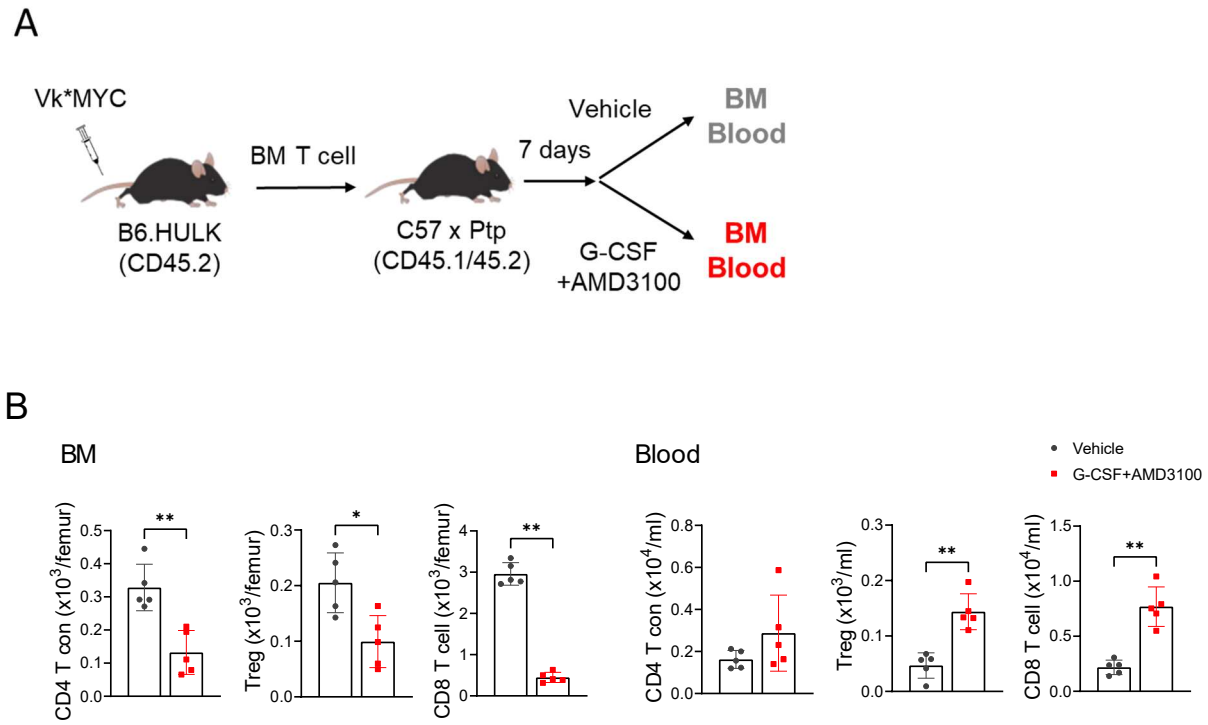
Supplementary Figures



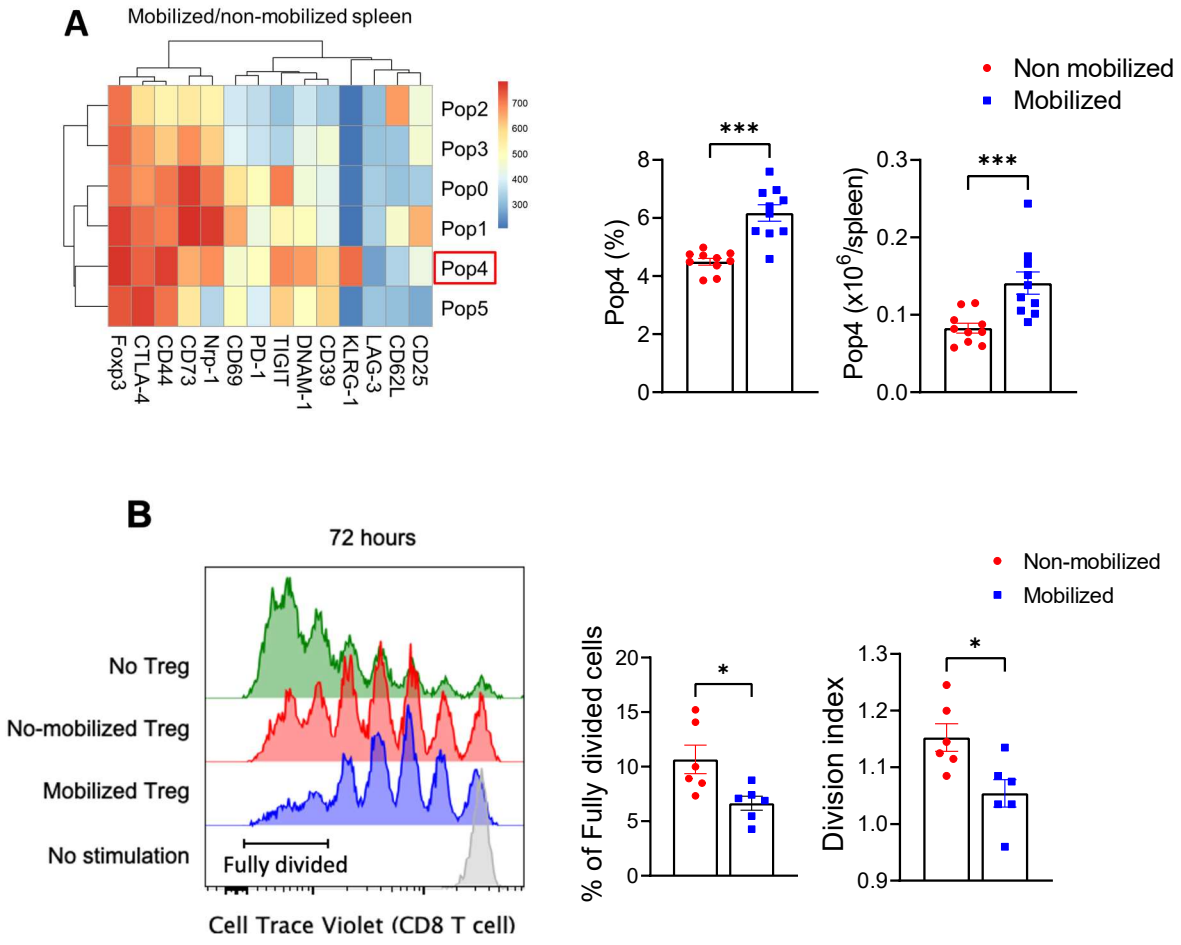
Supplementary Figure 1. CD69⁺ CD8 T cells in BM were resistant to G-CSF based mobilization. Representative flow cytometry plots of CD69⁺ CD8 T cell in blood and BM of mice treated with G-CSF or vehicle and frequency of CD69⁺ cells within CD8 T cells (n = 6-8 /group from 2 independent experiments). Data represents mean ± SEM. Mann-Whitney test was used for 2 sample comparison. ****P* <.001



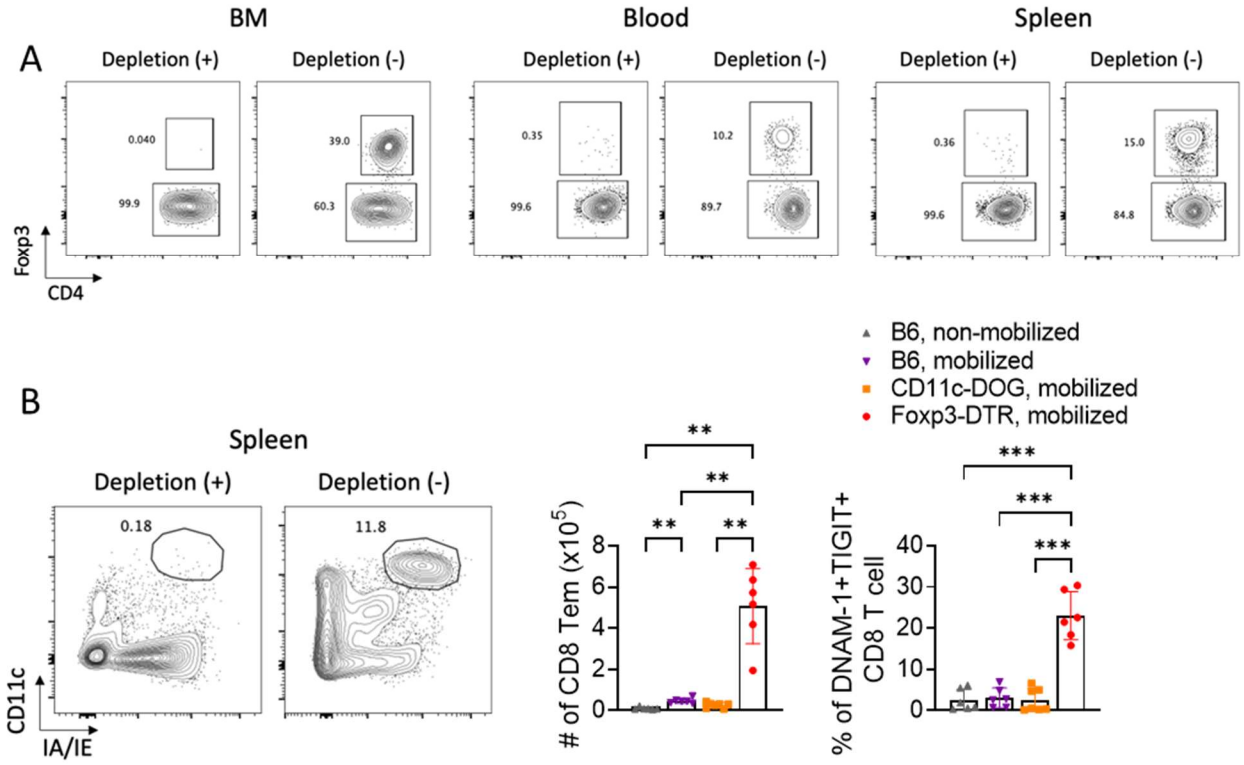
Supplementary Figure 2. Foxp3^{high} CD45RA^{-} activated Tregs are enriched in the TIGIT^{+}CD39^{+} Treg population. We performed FACS analysis on PBSC to examine Treg subsets as described in the literature[7]. (**A**) Representative flow cytometry plots demonstrating the gating strategy of Treg subsets as determined by Foxp3 and CD45RA expression. (**B**) Representative flow cytometry plots of three populations gated by TIGIT and CD39 expression and the frequency of Foxp3^{high}CD45RA^{-} activated Tregs in each population. Connecting line is in paired sample. One-way ANOVA was used for multiple comparison. *P <.05, **P <.01.



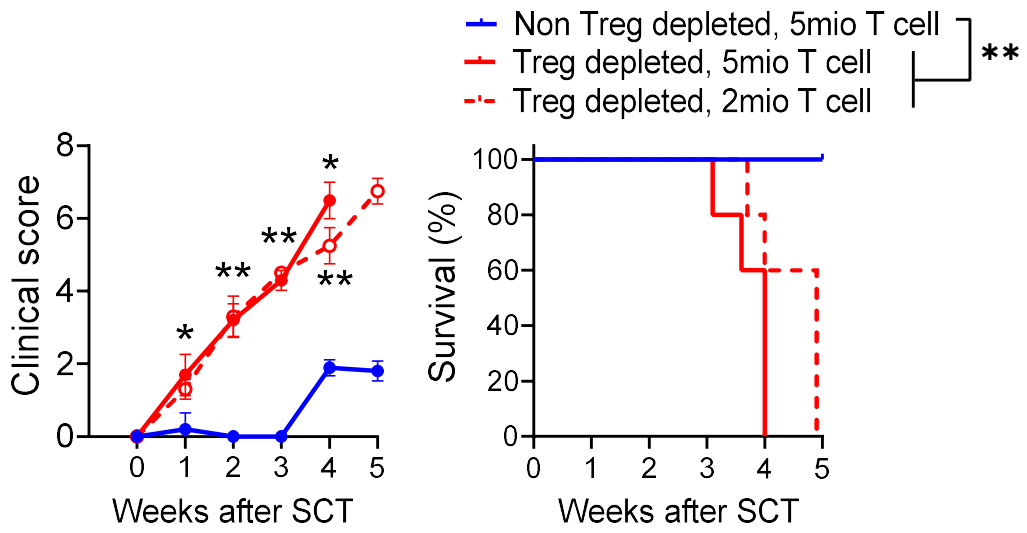
Supplementary Figure 3. BM resident Tregs are mobilized into blood during SCM. (**A**) We used B6.HULK mice (IFNγ-YFP/IL-10-GFP/FoxP3-RFP) to report Treg and performed experiments as described in Figure 2B. (**B**) Recipient C57 x Ptp (CD45.1/45.2) recipients of adoptively transferred B6.HULK T cells were treated with G-CSF+AMD3100 or control vehicle. The number of each subset of T cells in BM and blood were quantified (n = 5 / group from one experiment). Data represents mean ± SD. Mann-Whitney test for 2 sample comparison. **P* <.05, ***P* <.01.



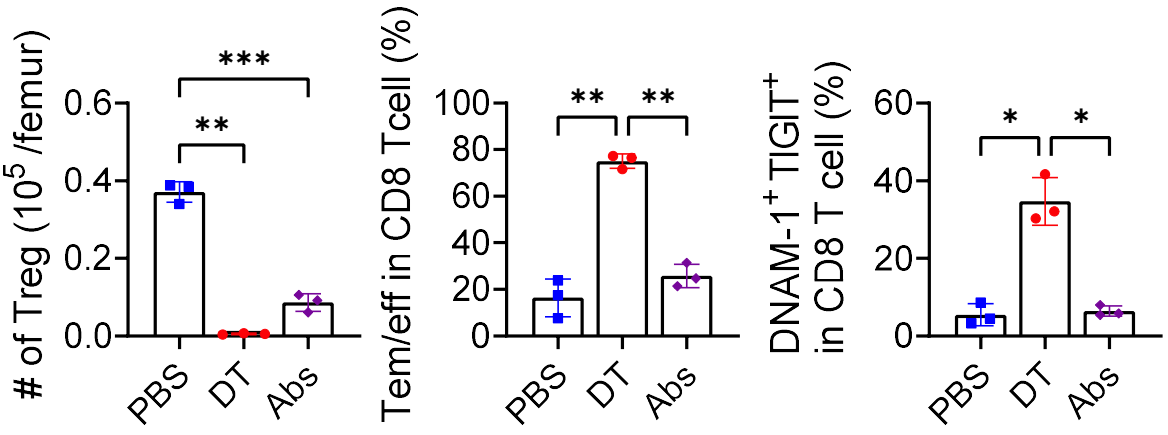
Supplementary Figure 4. Treg from mobilized grafts suppress CD8 T cell proliferation in vitro. (**A**) In order to harvest sufficient Treg for the in vitro assay, we collected Treg from mobilized spleen. We confirmed that the mobilized spleen also had increased numbers of the immunosuppressive Treg population (Pop4) that was seen in mobilized blood (Figure 3). (**B**) Sort purified Foxp3-GFP^{+} Tregs from mobilized or non-mobilized mice were cocultured with DCs from naïve C57Bl/6 mouse and CTV-labelled CD8 T cells along with anti-CD3 antibody for 72 hours. Cells were harvested for CTV dilution analysis (n = 6 / group from 2 independent experiments). Data represents mean ± SEM. Welch's t-test for two sample comparison. *P <.05, ***P <.001.



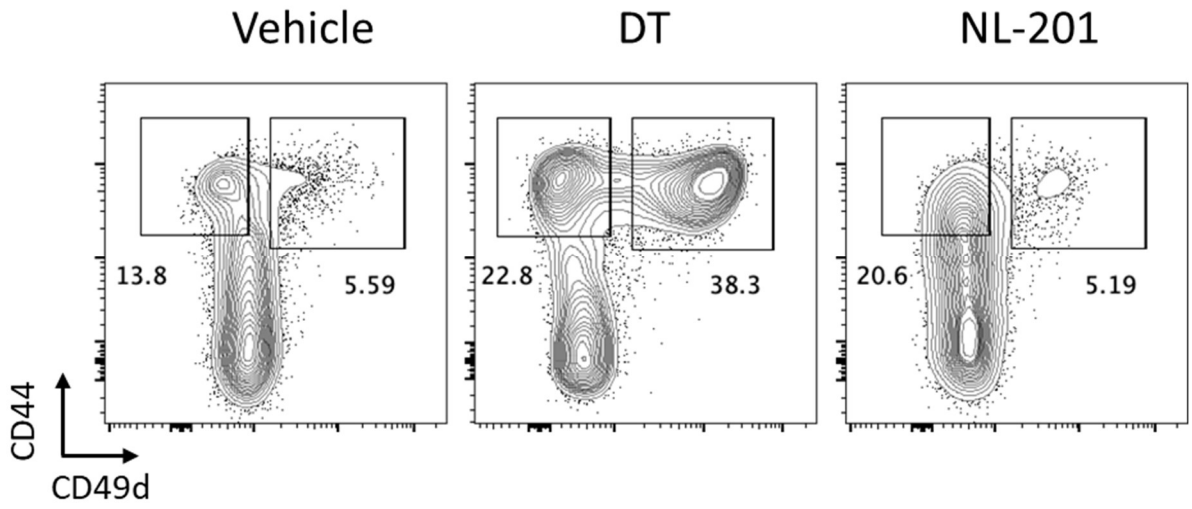
Supplementary Figure 5. DC depletion by DT administration does not generate activated effector CD8 T cells. Foxp3-GFP-DTR mice and CD11c-DTR mice were treated with DT injection. The mice were euthanized to assess the depletion efficacy of target cells. (**A**) Representative flow cytometry plots of Foxp3-GFP^{+} CD4 T cells in BM, blood, and spleen. (**B**) Representative flow cytometry plots show the depletion of the MHCII^{+}CD11c^{+} cells in spleen and the phenotype of blood CD8 T cells from DT treated CD11c-DTR mice (n = 6 – 7/group from 2 independent experiments). Data represents mean ± SEM. One-way ANOVA for multiple sample comparison. $^{**}P$ <.01, $^{***}P$ <.001.



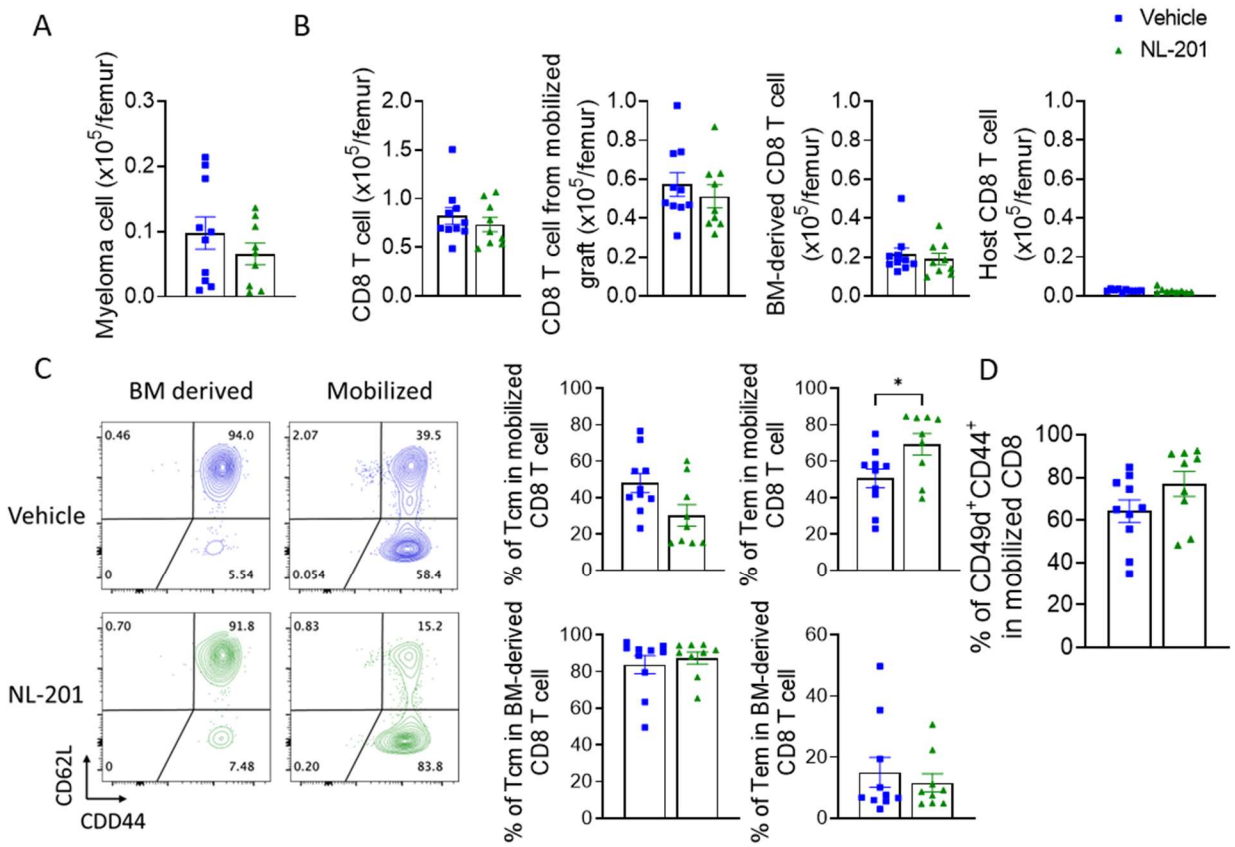
Supplementary Figure 6. Complete depletion of Treg peri-transplant results in lethal toxicity. Foxp3-GFP-DTR mice were used as recipients and donors. Myeloma-bearing Foxp3-GFP-DTR mice were transplanted with $10x10^6$ BM and 5 or 2 $x10^6$ T cells. Recipient mice were treated with DT (160ng twice weekly for 5 weeks) to deplete Treg or control vehicle for non-Treg depleted control groups after transplant. Clinical score and survival were shown (n = 5/group from one experiment). No mice died of myeloma in this experiment. Data represents mean ± SEM. One-way ANOVA for multiple sample comparison and Log-rank test for survival data. **P* <.05, ***P* <.01.



Supplementary Figure 7. Antibody-mediated Treg depletion approach failed to generate activated effector CD8 T cells. Foxp3-GFP-DTR mice were treated with DT, antibodies (combination of anti-CTLA4, anti-GITR, and anti-CD25 antibodies), or vehicle controls for 1 week. BM was harvested to enumerate Foxp3-GFP$^+$ CD4 T cells and the frequency of effector/effector memory and DNAM-1$^+$TIGIT$^+$ CD8 T cells in each group. Data represents mean ± SEM. One-way ANOVA for multiple sample comparison. *P <.05, **P <.01, ***P <.001.



Supplementary Figure 8. NL-201 increases the frequency of CD49d^{neg} memory CD8 T cells in mobilized grafts. MM-bearing Foxp3-GFP-DTR or C57Bl/6 donor mice were treated with DT, NL-201, or vehicle during mobilization with G-CSF and AMD3100. Representative flow cytometry plot of CD44 and CD49d expression on CD8 T cells in mobilized graft.



Supplementary Figure 9. NL-201 increased effector memory T cell differentiation. MM-bearing B6. HULK donor mice (IFNγ-YFP x IL-10-GFP x FoxP3-RFP reporter) were treated with NL-201 or control vehicle during mobilization with G-CSF and AMD3100. Recipient mice (CD45.1/CD45.2) were lethally irradiated and transplanted with mobilized CD8 T cells from MM-bearing B6.HULK donors (CD45.2) and BM and CD4 T cells from PTPRCA donors (CD45.1). Recipient mice were euthanized and BM was harvested 2 weeks after transplant. (**A**) The total number of myeloma cells, (**B**) total number of all CD8 T cells and the number of CD8 T cells derived specifically from mobilized spleen, engrafted BM, and the recipient compartment were measured in BM. (**C**) Representative flow cytometry plot and total number of memory CD8 T cells derived from mobilized spleen and engrafted BM. (**D**) The frequency of antigen-experienced memory CD8 T cells derived from mobilized spleen. (n = 9-10 /group from 2 independent experiments). Mann-Whitney test for 2 sample comparison. *P <.05.

References

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