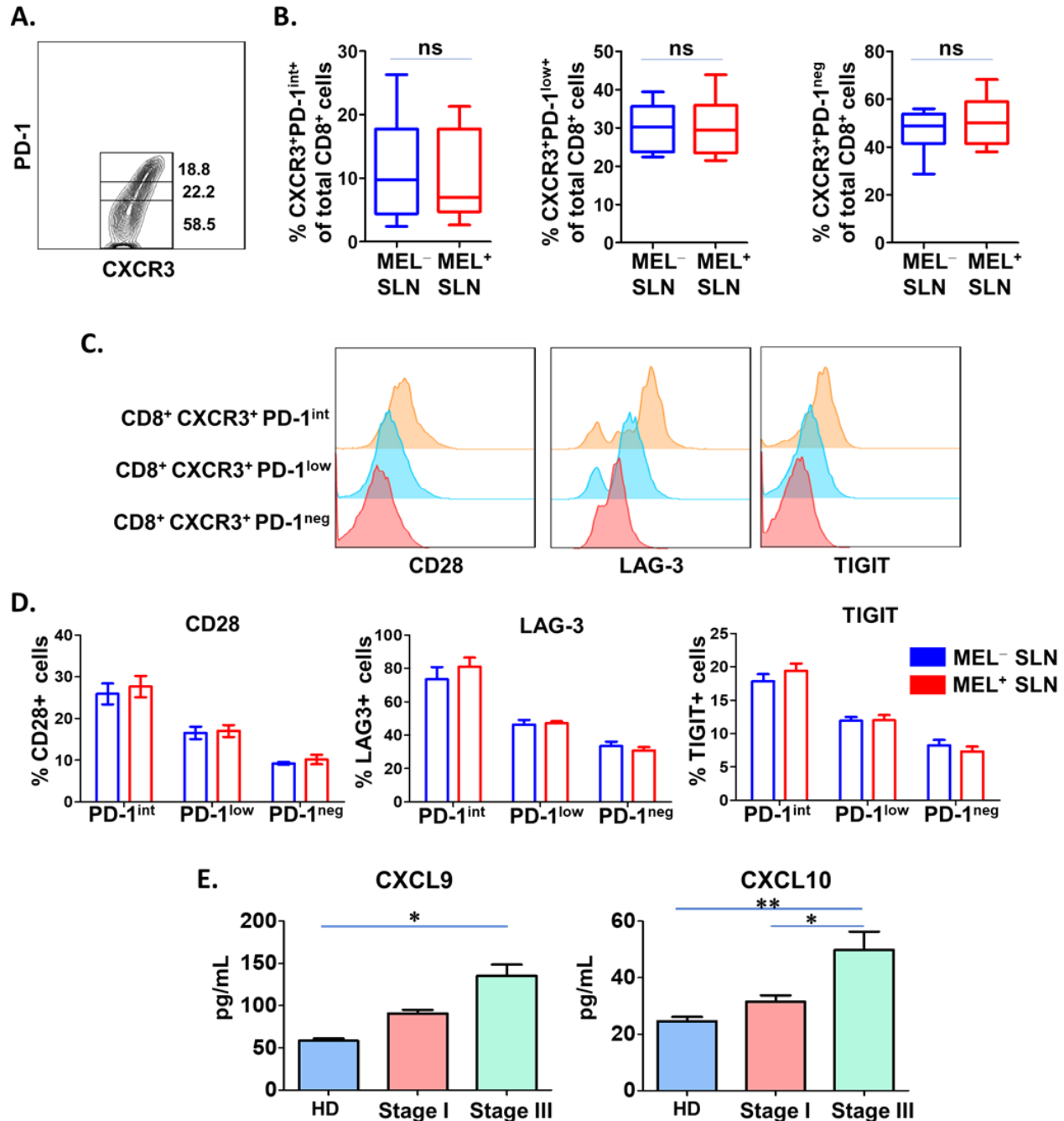


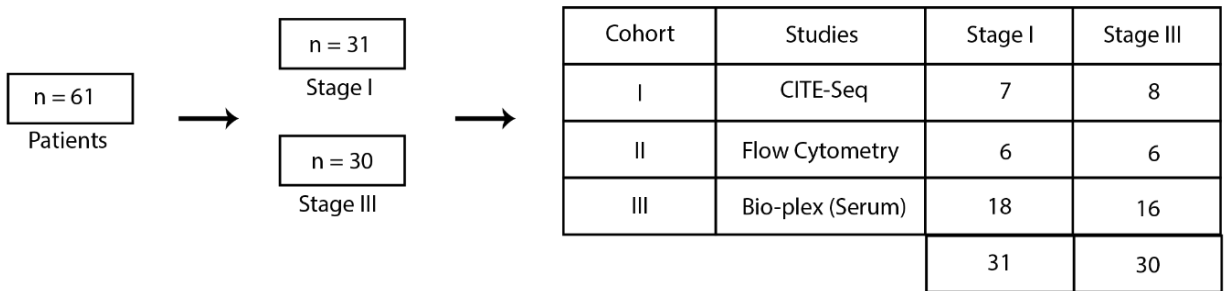
Supplemental Figure 1



Supplemental Figure 1. A–D. Identification of three subsets of CD8⁺ T cells within SLN based on the cell surface expression of CXCR3 and PD-1. A, B. SLN samples from stage I (MEL⁻ SLN; n = 6) and stage III (MEL⁺ SLN, n = 6) melanoma patients were analyzed for the expression of immune cell markers by flow cytometry. A, B. Representative contour plot (A) and bar graphs (B) show the identification of three subsets of CD8⁺ T cells within the MEL⁻ and MEL⁺ SLN tissue based on CXCR3 and PD-1 expression. C, D. Histograms (C) and bar graphs (D) showing the expression of CD28, LAG-3, and TIGIT on the three subsets of CD8⁺ T cells – CD8⁺CXCR3⁺PD-1^{int}, CD8⁺CXCR3⁺PD-1^{low}, and CD8⁺CXCR3⁺PD-1^{neg} within the SLN tissues. E. **Stage III melanoma patients have elevated circulating levels of CXCL9 and CXCL10 chemokines.** Levels of CXCL9 and CXCL10 in serum samples collected from healthy donors (n = 3), stage I melanoma patients (n = 18), and stage III melanoma patients (n = 16). One of the serum samples was excluded from the analysis due to the presence of a contaminant in the sample (see Table S9). Data, mean ± SEM, P values: *, p≤0.05; **, p≤0.005.

Supplemental Methods

Patient Cohorts and Study Design. For this study, one SLN sample was obtained from each of 27 patients (13 stage I and 14 stage III). These samples were divided into two cohorts (Cohort I and II). In addition, serum samples were collected from 34 patients (Cohort III; 18 stage I and 16 stage III) (Please see **Supplemental Scheme I** and **Table 1** (1a, 1b, and 1c; see below) for details of the Cohorts.



Supplemental Scheme 1: Details of SLN and serum sample Cohorts.

Table 1: Cohort Details

Table 1a: Cohort I (CITE-Seq)					
#	Specimen Code*	Stage	Gender	Age	Experiment
1	678	I	M	50	CITE-Seq
2	724	I	F	50	CITE-Seq
3	797	I	F	29	CITE-Seq
4	799	I	F	61	CITE-Seq
5	800	I	F	62	CITE-Seq
6	808	I	M	70	CITE-Seq
7	809	I	M	61	CITE-Seq
8	736	III	F	40	CITE-Seq
9	895	III	F	67	CITE-Seq
10	903	III	M	41	CITE-Seq
11	940	III	F	39	CITE-Seq
12	955	III	M	75	CITE-Seq
13	958	III	F	39	CITE-Seq
14	967	III	F	47	CITE-Seq
15	969	III	M	76	CITE-Seq

*Samples attributed to the missing ID numbers were not used in the study for one of the following reasons: a) not collected, b) low viability, or c) low cell number.

Table 1b: Cohort II (Flow Cytometry)					
#	Specimen Code*	Stage	Gender	Age	Experiment
1	634	I	M	58	Flow Cytometry
2	635	I	F	52	Flow Cytometry
3	636	I	M	55	Flow Cytometry
4	704	I	M	90	Flow Cytometry
5	705	I	F	52	Flow Cytometry
6	706	I/II	M	53	Flow Cytometry
7	537	III	M	42	Flow Cytometry

8	549	III	F	31	Flow Cytometry
9	655	III	F	64	Flow Cytometry
10	721	III	F	88	Flow Cytometry
11	729	III	F	30	Flow Cytometry
12	734	III	M	63	Flow Cytometry

Table 1c: Cohort III (Serum-Bio-plex assay)						
#	Specimen Code*	Stage	Gender	Age	Sample	Experiment
1	596	I	M	84	Serum	Bio-plex
2	598	I	F	53	Serum	Bio-plex
3	603	I	F	67	Serum	Bio-plex
4	605	I	M	39	Serum	Bio-plex
5	608	I	M	57	Serum	Bio-plex
6	611	I	M	76	Serum	Bio-plex
7	612	I	M	44	Serum	Bio-plex
8	620	I	F	38	Serum	Bio-plex
9	627	I	F	72	Serum	Bio-plex
10	628	I	F	28	Serum	Bio-plex
11	636	I	M	55	Serum	Bio-plex
12	641	I	M	86	Serum	Bio-plex
13	644	I	M	68	Serum	Bio-plex
14	650	I	M	53	Serum	Bio-plex
15	676	I	F	49	Serum	Bio-plex
16	677	I	M	49	Serum	Bio-plex
17	680	I	F	43	Serum	Bio-plex
18	687	I	M	67	Serum	Bio-plex
19	601	III	M	52	Serum	Bio-plex
20	617	III	M	70	Serum	Bio-plex
21	618	III	M	62	Serum	Bio-plex
22	633	III	F	77	Serum	Bio-plex
23	639	III	F	66	Serum	Bio-plex
24	643	III	F	79	Serum	Bio-plex
25	651	III	M	81	Serum	Bio-plex
26	657	III	M	36	Serum	Bio-plex
27	660	III	M	39	Serum	Bio-plex
28	683	III	M	47	Serum	Bio-plex
29	684	III	M	49	Serum	Bio-plex
30	694	III	M	62	Serum	Bio-plex
31	695	III	F	39	Serum	Bio-plex
32	700	III	M	55	Serum	Bio-plex
33	708	III	M	67	Serum	Bio-plex
34	721	III	F	88	Serum	Bio-plex

SLN Digestion – A portion of the SLN was enzymatically digested in RPMI 1640 medium (Corning Life Sciences, Corning, NY) containing 5% FBS (Thermo Fisher Scientific, Waltham, MA), 1% L-glutamine (Corning Life Sciences), 1% penicillin/streptomycin (Corning Life Sciences), 0.1% DNase (Thermo Fisher) and collagenase type IV (1 mg/mL; Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37 °C. The single cell suspensions were then filtered through a 70 µm cell strainer, washed, lysed in RBC buffer (BioLegend, San Diego, CA), re-filtered through a 70 µm

cell strainer and cryopreserved. For cryopreservation, SLN cells were resuspended in cooled (4 °C) freezing media (90% FBS and 10% DMSO) and transferred into cryovials. Once aliquoted, cryovials were transferred into a freezing container (Nalgene, Rochester, NY), and stored at –80 °C for 24 hours. Cryovials were then transferred to –150 °C for storage.

Flow Cytometry – For fluorescence-activated cell sorting (FACS) analysis of the frequency and phenotype of SLN tissues, cryopreserved patient SLN samples were thawed. Cells were then washed twice and resuspended in FACS buffer [2% FBS + PBS] and stained with multi-color antibody (Ab) panels (related to Figure 3). The antibody panels used for staining are shown in the table below:

Table 2: Flow Cytometry Antibodies:

Antibody	Color	Supplier	Clone	Catalog #
CD4	FITC	Biolegend	OKT4	317408
CD8	APC/Cy7	Biolegend	RPA-T8	301016
CD279 (PD-1)	APC	Biolegend	EH12.2H7	329908
CXCR3	PerCPCy5.5	Biolegend	G025H7	353714
CD28	PE/Cy7	Biolegend	CD28.2	302926
LAG3	FITC	Biolegend	11C3C65	369308
TIGIT	PE/Cy7	Biolegend	VSTM3	372714

Table 3: Bioplex 40-plex Human Analytes:

Number	Bioplex Analytes
1	Hu IL-1b (39)
2	Hu IL-2 (38)
3	Hu IL-4 (52)
4	Hu IL-6 (19)
5	Hu IL-8 (54)
6	Hu IL-10 (56)
7	Hu Eotaxin (43)
8	Hu GM-CSF (34)
9	Hu IFN- γ (21)

10	Hu IP-10 (CXCL10) (48)
11	Hu MCP-1(MCAF) (53)
12	Hu MIP-1a (55)
13	Hu TNF- α (36)
14	Hu 6CKine/CCL21 (12)
15	Hu BCA-1/CXCL13 (74)
16	Hu CTACK/CCL27 (72)
17	Hu ENA-78/CXCL5 (73)
18	Hu Eotaxin-2/CCL24 (30)
19	Hu Eotaxin-3/CCL26 (65)
20	Hu Fractalkine/CXCL1 (77)
21	Hu GCP-2/CXCL6 (15)
22	Hu GRO-a/CXCL1 (61)
23	Hu GRO-b/CXCL2 (78)
24	Hu I-309/CCL1 (20)
25	Hu IL-16 (27)
26	Hu I-TAC/CXCL11 (25)
27	Hu MCP-2/CCL8 (57)
28	Hu MCP-3/CCL7 (26)
29	Hu MCP-4/CCL13 (28)
30	Hu MDC/CCL22 (29)
31	Hu MIF (35)
32	Hu MIG/CXCL9 (14)
33	Hu MIP-1d/CCL15 (66)
34	Hu MIP-3a/CCL20 (62)
35	Hu MIP-3b/CCL19 (76)
36	Hu MPIF-1/CCL23 (37)
37	Hu SCYB16/CXCL16 (64)
38	Hu SDF1a+b/CXCL12 (22)
39	Hu TARC/CCL17 (67)
40	Hu TECK/CCL25 (46)