Quality of CD8(+) T cell immunity evoked in lymph nodes is compartmentalized by route of antigen transport and functional in tumor context

M.J. O'Melia, Georgia Institute of Technology
N.A. Rohner, Georgia Institute of Technology
M.P. Manspeaker, Georgia Institute of Technology
D.M. Francis, Georgia Institute of Technology
Haydn Kissick, Emory University
S.N. Thomas, Georgia Institute of Technology

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**INTRODUCTION**

Melanoma remains a substantial clinical problem, representing the sixth most common cancer worldwide, with only ~20% of patients surviving at least 5 years following diagnosis of advanced disease and ~41 to 55% of patients demonstrating recurrent disease (1). Immunotherapy, which focuses on reinvigorating antitumor immune responses, particularly on the part of T cells, has emerged as the most promising previously unidentified approach to increase patient survival through its potential to both treat advanced disseminated disease and protect against recurrence. However, response rates in advanced melanoma are disappointingly low: Only ~10 to 33% of patients respond to immune checkpoint blockade (ICB) therapy (2). Enhanced survival is independently associated with several features of a robust antitumor CD8$^+$ T cell immune response including the number of these cells within the tumor (3). More specifically, higher levels of CD8$^+$ T cells expressing granzyme B (GzmB), a marker of degranulation, and interferon-γ (IFN-γ), an inflammatory cytokine, along with immune checkpoint and antigen (Ag) experience marker programmed cell death 1 (PD1) within the tumor are associated with improved survival (4–6). The abundance of CD8$^+$ T cells expressing PD1 in combination with transcription factor Tcf1, which marks a stem-like cell subtype with high proliferative potential and capacity to differentiate into cells with antitumor lytic functions (7, 8), is also a prognostic indicator in melanoma (5, 6). Thus, the generation of both high-quantity and high-quality Ag-primed T cells in the tumor microenvironment (TME) is an important step in inducing effective antitumor immunity, the holy grail to broaden the benefits afforded by cancer immunotherapy.

To this end, the generation of a large and robust pool of tumor-specific CD8$^+$ T cells, the target of ICB and many other immunotherapeutic strategies, relies on priming of T cells by Ag-presenting cells (APCs) (9). The activation status and Ag presentation by APCs, which include dendritic cells (DCs) and macrophages, affect the differentiation of naïve T cells and, thus, their resulting functionality. To facilitate Ag sensing by rare cognate T cells, encounters with APC-presented Ag are tightly orchestrated within lymphocyte-rich secondary lymphoid organs. These include lymph node (LNs), whose structure is specialized to facilitate both tight interactions between immune cell subsets (10) and Ag dispersal among cell subtypes that are locally distributed in a spatially prescribed manner. Specifically, Ag derived from peripheral tissues accesses LNs by lymphatic vessel-mediated lymph drainage to the downstream, draining LN (dLN) (10–12). This lymph enters the LN at the subcapsular sinus (SCS), a barrier lined with fibroblastic reticular cells, APCs, and lymphatic endothelial cells (10). From the SCS, lymph-borne solutes diffuse into the LN cortex where B cells, other APCs, and intrafollicular T cells reside (10, 13, 14) or, in a highly size-restricted manner (10, 13, 15), enter conduits to reach the T cell zones of the LN paracortex. APCs also patrol peripheral tissues before migration to dLNs under both steady-state and inflamed conditions (11, 12, 16, 17) to disperse in a chemotactically driven manner among discrete LN zones (16–20). Ag access to strategically distributed APCs colocalized with LN-resident lymphocytes is, thus, dependent on a number of barriers that are sensitive to the mechanism of transport to the LN.

In response to disease, both the tumor and dLN remodel in cancer, with potential impacts on Ag sensing and the resulting magnitude and functions of the antitumor immune response. TME-localized immunosuppression has been widely recognized as a cancer hallmark (21) with consistent and advancing suppression occurring throughout tumor development and progression (22). In particular, T cell activation in the TME is impaired (3, 5, 6) and tumor-associated DCs show high PD1 ligand and B7 family expression, two features associated with impaired prognosis (6). Likewise, immunosuppressive tumor-associated macrophages (23) as well as regulatory T cells and myeloid-derived suppressor cells infiltrate the TME to high extents...
Constitutive transport barriers and mechanisms regulate access by LN-resident immune cells to skin-derived macromolecules

A panel of fluorescent tracers (table S1 and fig. S1) (11, 42) was co-infused into the skin of naïve animals and, at various times after administration, dLNs were analyzed for both total levels of tracer accumulation as well as tracer distribution profiles among resident leukocytes. Thirty- and 10-nm dextrans (500 and 40 kDa, respectively) versus 500-nm polystyrene spheres were implemented to compare the extents of lymphatic transport to the dLN, resulting from either of two modes of lymphatic transport from the periphery—passive lymph drainage versus active transport mediated by emigrating leukocytes. Thirty- and 10-nm dextrans (500 and 40 kDa, respectively) were found to be compartmentalized by route of lymphatic transport and to remain therapeutically functional in a melanoma mouse model. These results provide previously unknown insight into how immunotherapeutic strategies for melanoma might be improved by leveraging the unique microenvironment and Ag-sensing capabilities of TdLNs.

Within LNs, leukocyte distributions are tightly organized in a spatially defined manner, with B cells (the substantial majority of B220⁺ dLN-resident cells), DCs (predominantly CD11c⁺), and SCS macrophages (CD169⁺) versus T cells (CD3⁺) being more LN capsule proximal versus distal, respectively (Fig. 1E and fig. S2C). To determine the effects of lymphatic transport mechanism on patterns of leukocyte uptake, tracer uptake was flow cytometrically assessed using a cell extraction protocol that liberated leukocyte but not stromal cell populations of analyzed LNs (43, 44). Consistent with their distinct patterns of spatial distributions within dLNs, tracers of different sizes exhibited differential access to not only differing numbers of dLN-resident cells (Fig. 1D) but also differing leukocyte (CD45⁺) subtypes (Fig. 1F). In particular, 10-nm tracers were found to accumulate at all measured times most prodigiously in B220⁺ (B) cells, again consistent with their efficient transit into LN conduits after accumulation within the SCS (Fig. 1F, left) (13–15). In contrast, the 30-nm tracer, which, once transited to the LN, was more restricted to the SCS, was found within more CD11c⁺ (DC) rather than B220⁺ cells (Fig. 1F, middle). Further differentiation into more defined cell subtypes [B cells, T cells, plasmacytoid DCs (pDCs), conventional DCs (cDCs), SCS macrophages (SSMs), medullary sinus macrophages (MSMs), medullary cord macrophages (MCMs), dermal DCs (dDCs), and Langerhans cells (LCs) (fig. S3)] revealed the 30- but not the 10-nm tracer to be restricted to LN-resident phagocytic leukocytes positioned at the sinus, while the 500-nm tracer was primarily within skin migratory cells, e.g., dDCs and LCs (fig. S2D and Fig. 3). Constitutive barriers to lymphatic transport and transit into the LN parenchyma via conduits thus substantially influence the resulting distribution of tracers among LN-resident leukocytes.
Melanomas mature peripheral DCs and alter the dynamics of cell-trafficked Ag to dLNs, resulting in modified Ag presentation

Tumors locally modulate the functions of APCs, which are necessary for the effective generation of antitumor immunity (fig. S4). As such, tumor effects on APCs within TdLNs were evaluated. DCs in both skin/tumor and dLNs of day 10 B16F10 melanoma-bearing mice were found to exhibit both higher CD86 and lower CD206 levels [by both number of cells and mean fluorescence index (MFI) of positive cells] among DEC205+ DCs (both dDCs and LCs) compared to naïve animals (Fig. 2, A and B). Thus, melanomas induce maturation and decreased tolerogenicity among skin-derived DCs in both the tumor and the dLN, with potential effects on DC trafficking, phagocytosis, and Ag presentation.

In addition to the immunological microenvironment, tumors also induce local remodeling of the tissue interstitium and vasculature, hallmarks of the disease (21) likely to influence lymphatic transport and resulting Ag access within the TdLN (fig. S4). B16F10 melanomas, for example, exhibit increased lymphatic vascularization and T cell infiltration compared to the naïve skin (fig. S5), recapitulating spontaneous melanoma models (35). As such, the tracer panel (Fig. 1 and fig. S1) was implemented to probe lymphatic transport effects on Ag access and how this is affected by disease (fig. S4). After injection into day 7 B16F10 tumors, 500-nm tracers were transported to TdLNs (Fig. 2C) and were associated with dLN-resident cells at extents greater than those seen in LNs draining the naïve skin, as determined by flow cytometric measurement of tracer fluorescence (Fig. 2, D and E). Of the migratory cells assessed, numbers of 500-nm+ dDCs but not LCs were increased (Fig. 2, D and F). Numbers of 500-nm+ cDCs, an LN-resident cell subtype, were also increased (Fig. 2, D and F), presumably due to tracer handoff from immigrating 500-nm+ cells (18, 45). As mature APCs tend to be more migratory (20, 46), this is in line with changes in APC maturation induced by the tumor (Fig. 2, A and B). When evaluated on a per-cell basis, more cells within dLNs were 500 nm+ overall (Fig. 2E), although among all CD45+ 500-nm+ cells, 500-nm tracer MFI was decreased at both 24 and 72 hours after injection in tumor-bearing compared to naïve animals (Fig. 2G). Likewise, the 500-nm MFI trended downward within individual cell subtypes (Fig. 2H). These data can be interpreted in two ways. As tumors induce APCs to mature and become more migratory, they may also become less phagocytic, bringing less Ag per cell to the dLN. It is also possible that
decreases in measured per-cell tracer MFIs result from tracer dilution within TME because of the rapidly growing tissue mass. Nevertheless, these data demonstrate that, overall, APC trafficking from the skin is sustained within melanomas.

As alterations in APC maturation, total migrating cell numbers, and their carried payload are likely to affect Ag presentation within dLNs, animals were subjected to antigenic challenge via an engineered synthetic Ag system (Fig. S4). In this configuration, 500-nm particles [microparticles (MPs)] were covalently attached via a disulfide linkage that is reversible within the reducing intracellular environment of APCs to the H-2K\(^b\) peptide of chicken albumin (OVA\(_{257-264}\)) with an N-terminal cysteine (fig. S6) (47, 48). These COVA\(_{257-264}\)-MPs were injected into the skin of naïve or tumor of B16F10 melanoma–bearing animals, and Ag presentation was assessed 72 hours later through measurement of H-2K\(^b\):OVA\(_{257-264}\) staining (Fig. 2I) (49). Consistent with the known transfer of lymphatic-transported Ag between LN-resident cells (18, 45), most cells presenting Ag within dLNs were found to not contain the 500-nm sphere (Fig. 2I). Examining all H-2K\(^b\):OVA\(_{257-264}\)\(^+\) cells, numbers of lymph-migrating dDCs and LCs along with LN-resident B cells presenting Ag were found to be increased by the tumor (Fig. 2J). However, Ag presentation by dLN-resident cDCs or pDCs was not modified by the presence of the tumor (Fig. 2J). Simultaneously, the amount of Ag presented by each H-2K\(^b\):OVA\(_{257-264}\)\(^+\) dDC (flow cytometrically assessed per cell MFI) was lower in LNs draining Ag-MP–injected tumors compared to LNs draining Ag-MP–injected skin (Fig. 2K). LCs and B cells, on
the other hand, exhibited Ag presentation MFI that was unchanged in the tumor compared to the naïve context (Fig. 2K). The increase in the number of Ag-presenting LCs, which interact primarily with CD8⁺ T cells (16, 19, 50), thus suggests an increase in presentation of tumor-derived Ag to LN-resident CD8⁺ T cells in melanoma-bearing animals. dDCs, on the other hand, are reported to prime/interact with CD4⁺ T cells and B cells (16, 19, 50). The diminished ratio of Ag-presenting MP⁺ cells (Fig. 2L) is suggestive of dDCs transferring Ag for presentation by B cells (Fig. 2, I and L), thus providing a mechanism for enhanced presentation of peripheral tissue-derived Ag to B cells of the dLN, a hypothesis that requires further experimentation to confirm. These results overall suggest that changes in APC maturation and migration induced by the tumor result in enhanced access of 500-nm spheres to TdLN cells as well as increased Ag presentation by dDCs, LCs, and B cells within TdLNs.

Melanomas alter access of lymph-derived nanoscale solutes and DC phenotype within LNs

Disease influences on lymph-draining Ag accumulation and uptake by cells within dLNs were next interrogated. Tracers 30 and 10 nm dextran and Ag-NPs (30 nm) injected intradermally accumulate within axillary (white circle) and brachial (yellow circle) dLNs 24 hours after intradermal injection (positive pixels as collected by IVIS imaging are red-yellow, negative background is gray scale), quantified in (F). Flow cytometry histograms of Ag presentation (2SD1.16 staining for H-2Kb:SIINFEKL) among CD45⁺ cells within dLNs (G), number of dLN cells presenting Ag relative to the naïve condition (H), and as MFI of H-2Kb:SIINFEKL signal of positive dLN cells (I) 24 hours after intradermal injection of Ag-NP. CD206 signal (J) among pDCs and cDCs in the skin/tumor and dLN of naïve and day 10 B16F10-bearing animals, quantified in (K), normalized to values in naïve skin and dLN. * indicates significance by one-way ANOVA with Tukey's post hoc comparison (* indicates \( P \lt 0.05 \), ** indicates \( P \lt 0.01 \)); \( n = 5 \) to 6 animals; (A) to (F) are representative of at least two independent experiments.
in hydrodynamic diameter simultaneously administered as one injection into the day 7 B16F10 melanomas accumulated within TdLNs over 72 hours at reduced levels compared to that seen in dLNs after injection in naïve skin (Fig. 3A). Nevertheless, levels of both 30- and 10-nm tracer association with B cells within TdLNs remained high with respect to per-tracer positive cell MFI over 72 hours (Fig. 3B, left, and Fig. 3C). With respect to pDCs, which reside more distant from the LN SCS within the LN parenchyma and are thus more restricted from lymph access (51), tracer association was increased within tumor compared to naïve skin dLN (Fig. 3B, right, and Fig. 3C). This is consistent with the increased permeability of the LN fibroblastic reticular cell–lined conduits within LNs draining melanomas (11, 35), suggesting that 30-nm tracers in TdLNs are available to be sampled by previously inaccessible cell types within LNs draining the naïve (tumor-free) skin. These results suggest that despite disease-induced alterations in lymphatic drainage, TdLN remodeling sustains access of lymph-draining solutes to resident leukocytes.

The influence of these changes in lymph-borne nanoscale solute access by TdLN cells on Ag presentation was evaluated using the synthetic Ag system instead composed of COVA257-264 disulfide tethered to lymphatic-draining nanoparticles (NPs) composed of Pluronic-stabilized poly(propylene sulfide) NPs (figs. S4 and S6) (12, 47, 48, 52–54). By virtue of their size (Fig. 3D), these NPs transit the interstitium, drain into lymphatic vessels, and accumulate rapidly within the dLN (Fig. 3E) for prolonged times after injection (Fig. 3F) in a manner similar to that of the fluorescent dextran tracers (Fig. 3D and fig. S1). Administration of these lymph-draining Ag-NPs thus allows cross-presentation by APCs of lymph-accessed Ag to be assessed through measurement of H-2K b :OVA257-264 staining. Lymph drainage of Ag tethered to the NPs administered intratumorally did not result in increased numbers of cross-presenting total (Fig. 3G) or individual cell subtypes within TdLNs (Fig. 3H), nor was the mean per-cell MFI of H-2K b :OVA257-264 staining altered relative to naïve dLNs (Fig. 3I). Consistent with this, CD206 expression, which is associated with DC tolerogenicity (55), was enhanced among pDCs within both the tumor and the TdLN and among cDCs of the tumor (Fig. 3, J and K). This suggests that despite increased tracer access (Fig. 3, B and C), cross-presentation of lymph-borne Ag by LN-resident APCs, which overall exhibit higher tolerogenicity, is unaffected by the tumor. This is in opposition to trends in Ag presentation resulting from Ag-MP treatment phenotype (Fig. 2, A and B), further motivating the elucidation of the ramifications of these lymphatic transport mechanism–specific changes on T cell Ag sensing in the tumor context.

Melanoma dLNs are niches that support survival and Ag experience by tumor Ag–specific T cells

Changes in Ag presentation and phenotype of APCs induced by melanomas have the potential to substantially affect Ag sensing and responses by T cells. As such, CD8 + T cell immunity that develops against endogenous tumor Ag in various immune microenvironments of melanoma-bearing animals was interrogated. Both the tumor and TdLNs were found to be highly enriched in presented tumor Ag in animals bearing day 7 B16F10 melanomas that expressed OVA (Fig. 4, A and B, and fig. S7). Accordingly, proliferation 72 hours after transfer of tumor Ag–specific (OT-I) CD45.2 cells into recipient CD45.1 mice bearing B16F10-OVA tumors 7 days after implantation (figs. S8 and S9) was extensive in both the tumor and its TdLNs but not the NdLN or spleen (Fig. 4C). With respect to the density of live tumor Ag–specific donor cells per total live cells, proliferating OT-I donor cells were concentrated in the TdLN but not other analyzed tissues (Fig. 4D). Notably, however, proliferating donor CD8 + T cells within the tumor exhibited low or poor viability, in contrast to cells within TdLNs (and, to a lesser extent, NdLNs and spleens) that were largely viable (Fig. 4, E and F). Frequencies of PD1 but not CD39 expressing donor CD8 + T cells, which have previously been observed Ag but did not become exhausted (56, 57), within TdLNs were also high, in contrast to donor cells measured within tumors, spleens, and non-draining lymph nodes (NdLNs) (Fig. 4, G and H). Densities of a stem-like subtype of PD1-expressing donor CD8 + T cells that are CXCR5 + CD39 +, which provide the proliferative burst in response to coPD1 ICBlow (7, 8), were greater in TdLN compared to the tumor, spleen, and NdLN (Fig. 4I). Thus, the TdLN supports the enhanced proliferation and survival of tumor Ag–specific T cells, a subset which is stem-like (7, 8), in contrast to the tumor and other lymphoid tissues, and could act as a source of tumor Ag–specific T cells to regulate antitumor immunity and response to immunotherapy.

Mechanism of lymphatic transport regulates Ag presentation and T cell sensing within the LN

To assess how the mechanism of lymphatic transport of Ag from peripheral tissues to dLNs influences responses by Ag-specific CD8 + T cells localized to within dLNs (Fig. 5A), the synthetic Ag system that leverages either an MP or NP carrier to restrict lymphatic transit to dLN via cell-mediated versus passive lymph drainage, respectively (Fig. 5B, S6), was administered into naïve animals. Responses 3 days after administration by both recipient CD8 + T cells and donor CD45.2 OT-I CD8 + cells (fig. S8) adoptively transferred 1 day before Ag challenge (Fig. 5B, i) or endogenous CD8 + T cells 7 days after administration (Fig. 5B, ii) were subsequently monitored (figs. S6, S8, and S9). As would be expected given programmed proliferation by CD8 + T cells in response to Ag experience (58), the extent of proliferation by responding T cells within dLNs 3 days after challenge was equivalent between animals challenged with NPs versus MPs for both responding donor and recipient cells (Fig. 5C and fig. S10). However, NP-mediated Ag challenge resulted in sustained high levels of CD8 + dLN cells, whereas the number of CD8 + cells after MP challenge was reduced at 7 days after challenge (Fig. 5D). This suggests that CD8 + T cells primed via NPs versus MPs exhibit differences in their dLN recruitment, retention, and/or viability during response to Ag priming. Consistent with the substantially higher levels of dLN accumulation, NP-primed cells resulted in higher numbers of PD1 + cells within dLNs compared to MP-primed cells 3 and 7 days after Ag challenge were also Tcf1 + Tim3 + (Fig. 5F), indicating that antigenic priming via drained NPs results in greater expansion of the stem-like CD8 + T cell pool within dLNs compared to cell-trafficked MP-tethered Ag. The number of recipient/endogenous CD25 + T cells was somewhat higher 3 but not 7 days after MP challenge, a difference not seen in donor cells (Fig. 5, E and F). More PD1 + donor cells 3 days and endogenous cells 7 days after Ag challenge were also Tcf1 + Tim3 + (Fig. 5F), indicating that antigenic priming via drained NPs results in greater expansion of the stem-like CD8 + T cell pool within dLNs compared to cell-trafficked MP-tethered Ag. The number of recipient/endogenous CD25 + T cells (both endogenous and donor) within dLNs resulted from treatment with both Ag-conjugated NPs and MPs (fig. S10). Functional responses assessed by ex vivo restimulation with OVA257-264 revealed more GzmB - and IFN- γ -producing donor cells 3 days after challenge in response to MP- as opposed to NP-mediated Ag challenge.
Numbers of GzmB-expressing cells within dLNs were also higher in response to Ag challenge using MPs compared to NPs both 3 and 7 days after treatment, and IFN-γ–producing cells were higher in OT-I cells responding to MP challenge at day 3 (Fig. 5, G and H). Numbers of tumor necrosis factor–α (TNFα)– and interleukin-2 (IL-2)–producing cells remained constant (fig. S10).

Together, these results reveal that Ag sensing and elicitation of functional cytotoxic CD8+ T cells are compartmentalized between the mechanism of Ag transport to dLNs. Specifically, lymphatic transport to dLNs via trafficking APCs leads to the induction of T cells with more cytotoxic functions. However, lymph drainage of Ag results in higher overall levels of Ag experience with dLNs, effects reflected by higher numbers of induced PD1+ cells and the generation of more stem-like CD8+ T cells. Both of these cell subsets have the potential to induce protective benefits against skin Ag and tumor development.

**Therapeutically functional antitumor T cell responses in the TME result from Ag priming of CD8+ T cells within TdLNs**

The ramifications of altered APC Ag access, maturation, and Ag presentation within TdLNs as a result of melanoma lymphatic drainage with respect to elicited functional T cell responses were assessed using the synthetic Ag system (figs. S4 and S6). Specifically, Ag-MPs or -NPs were administered intratumorally on day 0 into animals bearing parental (non–OVA-expressing) B16F10 melanomas 7 days after implantation. End point analyses of both donor CD45.2 OT-I CD8+ cells (fig. S8) adoptively transferred 1 day before Ag challenge and recipient CD8+ T cells 3 days after administration (i) or endogenous CD8+ T cells 7 days after administration (ii) were performed. Similar to responses observed in tumor-free animals (Fig. 5), proliferation of donor Ag-specific T cells within TdLNs 3 days after treatment was roughly equivalent regardless of the mechanism of lymphatic transport of Ag and irrespective of the presence of the day 7 B16F10 melanoma (fig. S10). In contrast to...
responses seen in naïve animals (Fig. 5C), CD8+ T cell numbers within TdLNs in response to MP- and NP-tethered Ag were also roughly equivalent (Fig. 6A). However, when evaluating the quality of responses elicited, subtle differences were noted. Ag-MP treatment resulted in an increase in PD1+ and PD1+Tcf1+Tim3− CD8+ T cell numbers within TdLNs 7 days but not 3 days after administration (Fig. 6B). However, with Ag-NP treatment, the levels of both PD1+ and PD1+Tcf1+Tim3− Ag–specific donor and PD1+ recipient CD8+ T cells were decreased within TdLNs relative to LNs draining tumor-free skin 3 days after administration (Fig. 6B). CD25+ and CD39+ cell numbers showed less consistent trends across time in Ag-NP–treated tumor-bearing animals, with a decrease in CD25+ cells 3 days and endogenous CD39+ cells 7 days after treatment. However, Ag-MP treatment resulted in diminished levels of CD25+ cells 3 days after treatment but increases in both CD25+ and CD39+ cells 7 days after treatment (Fig. S10). With respect to cytotoxic CD8+ T cell immunity, levels of cytokine-producing cells (both donor and endogenous) within TdLNs elicited by Ag-MP treatment, on the other hand, were largely unchanged, save a reduction in IL-2–expressing cells 3 days after treatment (Fig. S10). Ag-NP treatment resulted in diminished IFN-γ– and IL-2–expressing CD8+ T cells within TdLNs 7 days after administration (Fig. S10). However, numbers of GzmB–expressing CD8+ T cells, which are degranulated and can directly kill tumor cells, elicited by Ag-MP, but not Ag-NP, treatment were diminished within TdLNs at both 3 and 7 days after administration compared to LNs draining tumor-free skin (Fig. 6C). This decrease within the TdLN coincided with a substantial increase in GzmB+ CD8+ T cells in the TME 7 days after treatment compared to saline-treated tumors (Fig. 6D). In contrast, although stem-like (PD1+ Tcf1+Tim3−) CD8+ T cells were expanded within TdLNs by both synthetic Ag systems, their numbers within the TME remained relatively unchanged irrespective of treatment type (Fig. 6E). However, numbers of PD1+Tcf1+Tim3− cells, which are derived from stem-like PD1+Tcf1+Tim3− cells, as they transition into an effector phenotype (7, 8), were substantially increased within the TME day 7 after administration of either Ag-MP or -NP, although more so with Ag-NP treatment (Fig. 6E). Moreover, ~20 to 30% of tumor-infiltrating CD8+ donor cells were found to be viable in response to either Ag-MP or -NP treatment (Fig. 6F), in sharp contrast to endogenous, tumor Ag–specific CD8+ cells infiltrating the tumor that are largely (>90%) nonviable (Fig. 4, E and F) (59). Overall, Ag-NP/MP treatment resulted in higher total CD8+ T cell
infiltration into tumors (Fig. 6G), a characteristic that has been independently associated with increased survival in patients with melanoma (3). These results suggest that priming of CD8+ T cell immunity within TdLNs results in improved quality and quantity of TME-infiltrating CD8+ T cells.

To assess the impacts of enhanced tumor infiltration of effector-like PD1+Tcfc1−Tim3− and effector GzmB+CD8+ T cells on therapeutic responses, two classes of tumor immunotherapy were evaluated. First, we evaluated the effects of monoclonal antibody (mAb)-mediated blockade of PD1 signaling, which results in a proliferative...
tumor infiltration by PD1+ Tcf1− Tim3+ CD8+ cells resulting generally found within TdLNs compared to tumors irrespective of maligns (Fig. 6K). In contrast, earlier generations of donor cells were in higher numbers of circulating donor cells in later (G4 to 8) generations compared to saline or intraperitoneally ICN-treated animals (Fig. 6K). In contrast, earlier generations of donor cells were generally found within TdLNs compared to tumors irrespective of treatment (Fig. 6K). Together with observations of increased tumor infiltration by PD1+ Tcf1+ Tim3+ CD8+ cells resulting from Ag-MP/NP treatment (Fig. 6E), this is consistent with stem-like PD1+ Tcf1+ Tim3+ cells expanded within TdLNs being mobilized and accumulating in their differentiated state as effector-like cells within the TME. This is also consistent with reports of T cells disseminating from the dLN subsequently losing Tcfl expression (61). Augmented immunotherapeutic effects from TdLN-directed ICN thus appear to be associated with mobilization of stem-like and responding CD8+ T cells from TdLNs into the circulation to result in TME infiltration and antitumor effects.

Second, the potential for the synthetic Ag-MP/NP system that expands the effector CD8+ T cell pool to protect against tumor growth was evaluated. To assess the effectiveness of the different subpopulations of T cells primed by each Ag system at controlling tumor growth, Ag covalently tethered to either MPs or NPs was administered intratumorally into a day 7 B16F10 (non-OVA expressing) melanoma. Three days after synthetic Ag administration, the primary tumor was excised. In so doing, animal survival was determined not by the treated primary tumor but instead by the growth of a secondary, OVA-expressing B16F10 tumor that was implanted in the contralateral dorsal skin. Irrespective of conjugation to either MPs or NPs, treatment with the synthetic Ag resulted in retardation of growth of the contralateral B16F10-OVA melanoma (Fig. 6L), effects that prolonged animal survival (Fig. 6M). In these studies, the excision of the primary tumor (receiving the Ag-MP/NP injection) left only primed cells within TdLNs in addition to those that had already disseminated from the treated TME. As cells expanded within the TME exhibit low viability (Fig. 4, E and F), therapeutic effects can, thus, be interpreted to be dLN-mediated. This implicates tumor lymphatic transport of passively drained or cell-trafficked Ag as inducing CD8+ T cell immunity that has antitumor functionality. While both the MP and NP treatment resulted in decreased tumor growth and enhanced survival, both synthetic Ag systems provided similar survival benefits, indicating that expansion of either stem-like or effector cells within the dLN can enhance antitumor responses to a similar degree. Together, these data support the concept that the TdLN represents a niche for expansion of therapeutically relevant CD8+ T cell subsets implicated in the efficacy of tumor immunotherapy.

**DISCUSSION**

Long overlooked for other than their prognostic value, LNs represent increasingly attractive therapeutic targets in cancer therapy applications (12, 54, 60, 62). LNs function as “transit hubs” of the adaptive immune system that facilitate co-mingling of APCs and lymphocytes within microenvironments conducive to the generation of adaptive immune responses. We show here two engineered material systems inspired by the way Ag is transported to LNs via the lymphatic vasculature to delineate in a quantitative fashion how lymphatic transport mechanism influences LN-localized signaling to CD8+ T cells and the impact of malignancy on these processes. By applying these biomaterials tools to the integrated analysis of lymphatic physiology effects on CD8+ T cell immunity relevant to tumor immunotherapy, this work expands upon the currently limited understanding of how Ag transport and interactions between the tumor and LN-resident cells, as mediated by the lymphatic system occurring in melanoma. Our findings elaborate the potential for nanoengineering approaches to harness various lymphatic transport mechanisms to optimize melanoma immunotherapy in eliciting and mobilizing various classes of antitumor CD8+ T cell immunity.

A fluorescent tracer panel designed to quantify lymphatic transport as mediated by migrating APCs versus lymph drainage revealed that alterations in cellular uptake and LN remodeling compensate to sustain access of tumor-derived solutes by LN-resident cells. Specifically, skin-resident DCs become more migratory, reaching the dLN in higher numbers. This is in line with reports of enhanced migratory and decreased phagocytic capacity of activated DCs (20, 46). Others have shown that CD11b+ dDCs that express major histocompatibility complex II (MHC-II) access the melanoma dLN at similar rates to the naïve skin dLN (63); however, by examining all dDCs and LCs, we demonstrate enhanced mobility of the skin DC population as a whole. Evaluating this entire cell population facilitates examination of CD8+ T cell responses in this context, instead of CD4+ T cell responses elicited by MHC-II+ DCs. In addition, lymph-derived, nanoscale solutes are also accessible to cells normally restricted from sampling lymph by the SCS and other structural barriers within the LN. The melanoma dLN has been shown to remodel (35), enhancing intra-LN access to tracers of this size range. Thus, these findings are in line with current understandings of melanoma-induced remodeling locally and in the TdLN. Despite its simplicity, limitations in the tracer system used herein include the comparisons between dextran and spheres, which differ in physical and chemical properties (42).

A synthetic Ag system was used to elaborate the influence of disease on Ag presentation by LN-localized cells resulting from Ag delivered to dLNs via different lymphatic transport mechanisms. Despite sustained access by LN-resident cells to lymph-draining Ag, the extent of Ag presentation by dLN-resident cells was unchanged in the melanoma context, suggesting that Ag presentation is locally suppressed. In contrast, presentation of cell-transported Ag, which is taken up and whose presentation occurs first within the periphery or during intralymphatic transit (17, 64), was increased within TdLNs. We hypothesize that differences in tumor effects between these mechanisms are due to the phenotypes of DCs differentially accessed by each lymphatic transport mechanism. Passively draining Ag was presented primarily by pDCs and cDCs, which exhibit a tolerogenic phenotype, while actively transported Ag was presented by dDCs and LCs, which are more activated in the tumor context. These results are consistent with previous reports demonstrating that both
infiltration of functional, effector T cells into the TME, which has been independently associated with survival (3, 4). With the advent of bioengineering approaches in cancer immunotherapy and the emerging appreciation of LNAs as druggable, therapeutically relevant tissues, lymphatic-directed drug delivery innovations therefore represent novel approaches to improve translational management of melanoma.

MATERIALS AND METHODS

Experimental design

The objectives of these studies were to understand how cells of the LN access skin- and melanoma-derived Ag on the basis of size and mechanism of transport, how this Ag is presented, how T cells sense Ag in both the tumor and naive context, and how this influences immunotherapeutic outcomes in mouse models. Experiments were designed with appropriate controls (e.g., saline injected intradermally and intratumorally) and appropriate timelines to assess these outcomes. Sample size was determined using power analysis. End points were predetermined as time points for all nonsurvival studies. End point for survival studies was set at the humane end point (tumors reaching 1.5 cm in any direction or ulcerating and actively bleeding). Animals were randomized to receive different treatments using a random number generator and mixed among cages, and researchers were blinded for survival studies. Survival studies were replicated for a total of two separate studies to confirm results.

Cell culture

B16F10 and B16F10-OVA mouse melanoma cells (obtained from Swartz Laboratory, EPFL, Lausanne, Switzerland) were cultured in Dulbecco’s modified Eagle’s medium (Gibco, Thermo Fisher Scientific Inc., Waltham, MA) with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO) and 1% penicillin/streptomycin/amphotericin B from Life Technologies (Carlsbad, CA, USA). Cells were passaged at ~80% confluency and maintained at 37°C with 5% CO₂ in a standard incubator.

Animal tumor models

C57Bl/6 or B6 CD45.1 mice were purchased at 6 weeks of age from the Jackson Laboratory (Bar Harbor, ME, USA). All protocols were approved by the Institutional Animal Care and Use Committee (IACUC). For tumor-bearing cohorts, 0.5 × 10⁶ melanoma cells were intradermally implanted into the left dorsal skin of 6- to 8-week-old mice. Tumor dimensions were measured with calipers in three dimensions and reported as an ellipsoidal volume.

Fluorescent tracers

Five hundred–nanometer yellow-green and red fluorescent (505/515 and 580/605 excitation/emission, respectively) carboxylate-modified microspheres were purchased from Thermo Fisher Scientific Inc. Tetrarmethylrhodamine isothiocyanate (TRITC) dextran (40,000 Da) was purchased from Sigma-Aldrich. Amino-dextran (500,000 Da) (Thermo Fisher Scientific Inc.) was covalently labeled by incubation with Alexa Fluor 647 or 700 NHS (N-hydroxysuccinimide) ester dyes (Thermo Fisher Scientific Inc.) in 0.1 M NaHCO₃ at pH 8.4 for 4 hours on a tube rocker. AF647 and AF700 dextran-dye conjugates were purified from unreacted free dye by Sepharose CL-6B gravity column chromatography after conjugation. Purified dextran-fluorophore conjugates were further confirmed free of unconjugated dye by a second Sepharose CL-6B column analysis (11).
All reagents were used and maintained under sterile conditions. Hydrodynamic sizes were confirmed before injection by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK).

Tracer injections
Fluorescent tracers suspended in saline were co-infused by a syringe pump at a rate of ~300 nL/s directly into the center of the tumors for tumor-bearing groups or into the dermal layer of the skin for naïve groups, using a 27- to 31-gauge needle (Becton Dickinson, Franklin Lakes, NJ) while mice were under anesthesia via isoflurane. For biodistribution experiments, 500-nm red or yellow-green fluorescent microspheres (19 pM), 30-nm AF700 or AF647 dextran (4.8 μM), and 10-nm TRITC dextran (4.8 μM) were co-infused in 10 μL of saline total. Mice were euthanized via CO₂ asphyxiation (fig. S1). Cells were maintained in sterile conditions before adoptive transfer.

IVIS imaging
Animals were injected intradermally in the left dorsal skin with AF647-conjugated NPs or 500-nm yellow-green microspheres (MP) as described above. Twenty-four hours after NP injection or 72 hours after MP injection, animals were euthanized via CO₂ asphyxiation in accordance with AVMA and local IACUC guidelines. Animals were then dissected to expose axillary and brachial LNs and imaged using a PerkinElmer IVIS (in vivo imaging system) Spectrum CT (Waltham, MA). LNs were then dissected and placed on a black plastic and imaged using a PerkinElmer IVIS Spectrum CT.

Tumor and LN immunohistochemistry and imaging
Tumor, skin, and dLNs were frozen in optimum cutting temperature compound (Sakura Finetek USA Inc., Torrance, CA, USA) in 2-methylbutane (Sigma-Aldrich) chilled by liquid nitrogen and frozen tissue blocks immediately stored at ~80°C. For imaging of tracer dLNs, coverslips were mounted onto LN slices (10-μm thickness) using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) with no prior wash steps and within 30 min of imaging. Otherwise, LN, skin, and tumor tissue sections were fixed with pre-chilled acetone for 10 min at 4°C and subjected to standard immunofluorescence protocols using the following antibodies, which were obtained from Thermo Fisher Scientific Inc. unless otherwise specified: fluorescein isothiocyanate (FITC)–conjugated rat anti-mouse CD31 (1:50), rabbit anti-mouse Lyve-1 (1:250), Alexa Fluor 633 goat anti-rabbit (1:300), Armenian hamster anti-mouse CD3E (1:50), AF647 goat anti-hamster (1:300; Abcam plc., Cambridge, MA, USA), AF488-conjugated rat anti-mouse CD169 (1:100; BioLegend Inc., San Diego, CA, USA), biotinylated rat anti-mouse F4/80 (1:200; Life Technologies), streptavidin-AF555 (1:400; Life Technologies), biotinylated rat anti-mouse B220 (1:250), and AF488-conjugated Armenian hamster anti-mouse CD11c (1:50; BioLegend Inc.). Blocking and antibody dilutions were performed with 10% donkey serum (Sigma-Aldrich) in Dulbecco’s phosphate-buffered saline (D-PBS). Slides were washed with 0.1% Tween 20 (Sigma-Aldrich) in D-PBS for washing steps, counterstained with DAPI (4’,6-diamidino-2-phenylindole) (VWR International Inc.), and imaged using a 710 NLO confocal microscope (Carl Zeiss Microscopy Ltd., Jena, Germany) with a 20× magnification objective.

CD8⁺ T cell isolation
OT-I animals were purchased from Charles River Laboratories (Lyon, France) and bred in-house. OT-I animals were euthanized, and the spleens were harvested and disrupted with 18G needles (Becton Dickinson) followed by washing with D-PBS. Cells were suspended through a sterile 70-μm cell strainer (Greiner Bio-One, Monroe, NC, USA), washed, and incubated with ACK Lysing Buffer (Lonza Group AG, Basel, Switzerland) for 60 s at room temperature, quenched with D-PBS, washed, and resuspended for counting. Cells were resuspended at 10⁶ cells/ml buffer (2% bovine serum albumin in D-PBS), blocked with normal rat serum, and mixed with CD8⁺ T cell isolation antibody cocktail (STEMCELL Technologies, Vancouver, Canada), followed by streptavidin-coated magnetic beads (STEMCELL Technologies). Buffer was added to the mixture and placed in a magnet (STEMCELL Technologies, Vancouver, Canada), and the supernatant was collected. Cells were then counted and resuspended in carboxyfluorescein diacetate succinimidyl ester (CFSE; Life Technologies, Carlsbad, CA, USA) and then quenched with ice-cold RPMI medium containing >10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA, USA). Purity, viability, and CFSE loading were confirmed via flow cytometry on a customized BD LSRFortessa flow cytometer before adoptive transfer (fig. S1). Cells were maintained in sterile conditions before adoptive transfer.

Adoptive transfer
Isolated CD8⁺ T cells were suspended in sterile saline at a concentration of 2 × 10⁶ cells per 200 μL of sterile saline. After induction of isoflurane anesthesia, the hair over the neck of mice was removed using depilatory cream and cleaned using warm water and ethanol wipes, and suspended cells were injected intravenously via the jugular vein.

Flow cytometry analysis
Auxillary and brachial dLNs were pooled and incubated with collagenase D (1 mg/ml) (Sigma-Aldrich) in D-PBS with calcium and magnesium for 1 hour at 37°C, passed through a 70-μm cell strainer (Greiner Bio-One, Monroe, NC, USA), washed, and resuspended in a 96-well plate (VWR International Inc.) for staining. Lung tissues were treated with the same procedure as LNs. Spleen capsules were disrupted using 18G needles, and the cell suspension was passed through a 70-μm strainer, pelleted and then incubated with blood cell lysis buffer (Sigma-Aldrich) for 7 min at room temperature, diluted with D-PBS, washed, and resuspended. Liver tissues were disrupted with 18G needles, passed through a 70-μm cell strainer, and centrifuged at 60g for 1 min to remove large debris, and the supernatant was collected into a different tube and centrifuged at 300g for 5 min. The pellet was resuspended, layered onto lymphocyte separation medium (Thermo Fisher Scientific Inc.), and centrifuged for 20 min at 400g. The mononuclear cell layer was recovered, incubated with red blood cell lysis buffer as before, washed, and resuspended for staining. All antibodies for flow cytometry were from BioLegend Inc. unless otherwise stated. Cells were blocked with anti-mouse CD16/CD32 (clone, 2.4G2) (Tonbo Biosciences, San Diego, CA, USA) for 5 min on ice, washed, and then stained with a fixable viability dye eFluor 455UV (1:1000; eBioscience, San Diego, CA, USA) for 15 min on ice or fixable viability dye Zombie Aqua (1:100; BioLegend Inc., San Diego, CA, USA) for 30 min at room temperature, before quenching with 0.1% bovine serum albumin in D-PBS.
(flow cytometry buffer). Antibodies were prepared in flow cytometry buffer at the following dilutions on the basis of preliminary titrations: phycoerythrin (PE) anti-mouse CD45.2 (1.25:100), AF700 anti-mouse CD25 (0.5:100), BV785 anti-mouse PD1 (1.25:100), AF647 anti-mouse CXCR5 (0.5:100), PerCP anti-mouse CD3 (2.5:100), APC-Cy7 anti-mouse CD8 (2.5:100), PE-Cy7 anti-mouse CD39 (5:100), and BV421 anti-mouse CD44 (5:100) for T cell distribution analyses (Fig. 1); BV711 anti-mouse CD45.1 (1.25:100), BV605 anti-mouse CD3 (2.5:100), APC-Cy7 anti-mouse CD8 (2.5:100), and BV786 anti-mouse PD1 (1.25:100), PerCP anti-mouse CD69 (1.25:100), or PerCP anti-mouse CD45 (0.5:100) for T cell restimulation analysis (Figs. 3 and 6); PerCP anti-mouse CD45 (0.625:100), BV711 anti-mouse CD3 (1.25:100), FITC anti-CD8 (0.3125:100), BV786 anti-mouse PD1 (1.25:100), PE-Cy7 anti-mouse CD39 (2.5:100), and anti-mouse CD25 (1:100) for T cell vaccination response studies (Figs. 3 and 6); PE-Cy7 anti-mouse CD11b (0.625:100), BV421 anti-mouse CD11c (5:100), BV605 anti-mouse CD169 (5:100), BV650 anti-mouse B220 (2:100), BV711 anti-mouse CD3 (1.25:100), BV785 anti-mouse F4/80 (2.5:100), and PE anti-mouse H-2Kb:SIINFEKL (5:100; clone D5-1.16) for Ag presentation experiments; or PerCP anti-mouse CD45 (1.25:100), AF700 anti-CD11b (1.25:100), PE-Cy7 anti-CD11c (1.25:100), APC-Cy7 anti-mouse MHC-II (2.5:100), BV605 anti-mouse CD206 (5:100), PE anti-mouse CD86 (5:100), APC anti-mouse DEC205 (1:100), and BV711 anti-mouse B220 (1.25:100) for DC phenotyping experiments. Cells were fixed with 4% paraformaldehyde (VWR International Inc.) for Ag presentation and biodistribution studies. For intracellular staining, cells were incubated with FoxP3/Transcription Factor Fixation/Permeabilization solution (eBioscience, Thermo Fisher Scientific Inc.) for 60 min on ice in the dark. Cells were then incubated with PE anti-mouse Tcf1 (1.25:100; Becton Dickinson), APC anti-GzmB (2.5:100), and BV605 anti-Ki67 (1.25:100) in FoxP3/Transcription Factor Fixation/Permeabilization buffer (eBioscience, Thermo Fisher Scientific Inc.) for 75 min on ice in the dark. For ex vivo cytokine staining, cells were suspended in IC Fixation Buffer (eBioscience, Thermo Fisher Scientific Inc.) for 60 min at room temperature in the dark. Cells were then incubated with APC anti-mouse GzmB (2.5:100), PE anti-mouse IFN-γ (1.25:100), AF700 anti-mouse IL-2 (0.5:100), and PE-Cy7 anti-mouse TNF-α (1.25:100) in IC Permeabilization Buffer (eBioscience, Thermo Fisher Scientific Inc.) for 60 min at room temperature in the dark. Cells were then resuspended in FACS (fluorescence-activated cell sorting) buffer and kept at 4°C until analyzed with a customized BD LSRFortessa flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Compensation was performed using AbC, ArC, or UltraComp compensation beads (Thermo Fisher Scientific Inc.), and data were analyzed using FlowJo software version 10 (FlowJo LLC, Ashland, OR).

**Peptide-conjugated nanoparticle synthesis and administration**

Pyridyl disulfide–functionalized NPs (PDS-NPs) were prepared as previously described (76). Cysteine-modified OVA_{257-264} (CSIINFEKL) was dissolved in Milli-Q water at 1 mg/ml and added 1:1 to PDS-NPs (40 mg/ml). The disulfide displacement reaction proceeded overnight at room temperature with stirring. After reacting, OVA_{257-264}-NP was separated from unreacted peptide by size exclusion chromatography using a CL-6B column. Fractions containing peptide were identified by reacting with fluorescamine, and PEG (polyethylene glycol)–containing fractions (NPs) were determined using an iodine assay. Fractions containing OVA_{257-264}-NP were combined and concentrated to the appropriate dose using 30-kDa MWCO (molecular weight cutoff) spin filters and sterilized by filtration through a 0.22-μm syringe filter. OVA_{257-264}-conjugated NP or MP in sterile saline was injected intradermally in the center of the tumor or into the dermal layer of the skin (naïve animals) of C57Bl/6 mice.

**Tracer diffusion analyses**

Capillary tubes were loaded with rat tail collage type I solution (5.6 mg/ml) (Corning Inc., Corning, NY, USA) in D-PBS and allowed to gel overnight at 37°C. Yellow-green spheres (500 nm), FITC-conjugated 30-nm dextran, and TRITC 10-nm dextran were loaded in separate tubes, and fluorescence imaging was performed every 4 min for 2 hours at 37°C with a Zeiss AxioObserver Z1 inverted microscope (Carl Zeiss) with a 4× magnification objective. Image analysis was performed using ImageJ software (77).

**Tracer biodistribution analyses**

At 4, 24, and 72 hours after tracer injection, mice were euthanized, and the tumor-draining axillary and brachial LNs were harvested and homogenized in D-PBS using 1.4-mm acid-washed zirconium grinding beads with a FastPrep–24 automated homogenizer. Whole-tissue homogenate fluorescence was measured with a Synergy H4 BioTek plate reader (BioTek Instruments Inc., Winooski, VT, USA), compensation was applied, and fluorescent tracer amounts and concentrations were calculated from standard curves made by spiking individual naïve tissue homogenates with tracer solution. Tracer concentration within tissues was calculated by application of standard curves generated in tissue homogenates to the fluorescent readouts. To determine percent injection, this concentration was multiplied by a dilution factor to account for the PBS added to the tissue after excision and the portion of the tissue measured on the plate reader (tubes were weighed before tissue was added, after homogenization, and after platting to determine dilution factor). Percent injection was then defined as the amount of tracer divided by the known amount of tracer injected into the animal. We note that in these experiments, only total fluorescence is measured via end point analyses. As such, this methodology cannot distinguish between rates of lymphatic drainage versus LN accumulation.

**Ex vivo restimulation**

After cell isolation (as above, in flow cytometry), 30% of LN samples, 5% of spleen samples, or 5% of tumor cells were plated in a sterile 96-well U-bottom plate. SIINFEKL peptide (1 μg/ml) in 200 μl of IMDM (Iscove’s modified Dulbecco’s medium) with 10% heat-inactivated fetal bovine serum and 0.05 mM β-mercaptoethanol (Sigma-Aldrich) was added to each sample and then incubated for a total of 6 hours at 37°C with 5% CO₂. Three hours into the incubation period, brefeldin A (50 μg/ml) (Sigma-Aldrich) was added to each sample. Cells were then stained for flow cytometry as above.

**Tumor resection surgery**

Animals are anesthetized using isoflurane in oxygen and then given sustained-release buprenorphine (1 mg/kg) and ketoprofen (5 mg/kg) via intraperitoneal injection as analgescics. The animals are then placed on a warming bed, and a sterile drape is placed to expose only the tumor and surrounding skin. Povidone-iodine is applied to the skin three times to sterilize the surgical area. Sterile scissors are
then used to excise and remove the tumor. Sterile wound clips are used to close the wound. The animal is monitored throughout recovery and returned to its cage. Wound clips are removed 10 days after surgery, and animals are monitored every other day to ensure well-being and examined for infection or irritation surrounding the surgical site. All procedures are approved by the IACUC.

ICB therapeutic analysis
On day 7 of B16F10-OVA tumor growth in CD45.1 mice, isolated, CFSE-labeled CD8+ OT-I T cells (CD45.2+) were administered intravenously into the jugular vein. On day 9 of B16F10-OVA tumor growth, 150 μg of each of anti-PD1 mAb and anti-cytotoxic T lymphocyte Ag 4 mAb was administered either intraperitoneally or intradermally in the forelimb, which results in mAb accumulation within the TdLN but not NDLNs (60). Saline administered intradermally in the forelimb served as control. Animals were euthanized on day 11, and lymphocyte responses were analyzed as described above.

Statistical analysis
Data are represented as the means accompanied by SEM, and statistical analysis was calculated using Prism 6, 7, and 8 software (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was defined as P < 0.05, 0.01, and 0.001, respectively, unless otherwise specified. Area under the curve was calculated using the built-in Prism analysis tool.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at https://advances.sciencemag.org/cgi/content/full/6/50/eabd7134/DC1

View request a protocol for this paper from Bio-protocol.

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