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Limited Infiltration of Exogenous Dendritic Cells and Naive T Cells Restricts Immune Responses in Peripheral Lymph Nodes

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Primary CD8 T cell responses in lymph nodes (LN) and protective immunological tumor control are quantitatively limited following immunization with exogenous peptide-pulsed dendritic cells (DC). This arises from two constraints. First, LN are saturated by relatively small quantities of exogenous DC. Second, circulation of new naive T cells into DC-infiltrated LN during the functional lifespan of the DC is negligible. Limits on DC and T cellularity in, and flux through, LN constrain the magnitude of both primary and subsequent recall responses. Enhanced immune responses and tumor control can be achieved using maneuvers to augment LN retention of DC or availability of naive T cells to Ag-presenting DC. These data offer an increased understanding of LN function in general and provide a practical basis for improvements in tumor immunotherapy. *The Journal of Immunology*, 2006, 176: 4535–4542.

Exogenous dendritic cells (DC) bearing specific Ag have been evaluated as adjuvants for the induction of immune responses. Exogenously administered DC can prime T cells capable of recognizing and killing tumors in an Ag-specific manner in animal models (1–4), and have been subsequently incorporated into clinical trials for immunotherapy of cancers (5–11). Unfortunately, clinical efficacy has been variable. It is clear that DC are complex reagents, and that additional insight into the factors that control their ability to induce effective immunity is needed.

An important aspect of immunization using exogenous DC is that their cellular nature and migratory phenotype restrict lymphoid distribution as compared with other types of soluble immunogens (12, 13). I.v.-injected DC efficiently enter spleen (13, 14), but fail to cross high endothelial venules, and are excluded from peripheral lymph nodes (LN) (13, 15). s.c. injected exogenous DC efficiently access peripheral LN via afferent lymphatics in a CCR7-dependent manner (16–18). However, infiltration is restricted to only those nodes in the injection site drainage (13, 15, 19–21).

We have previously shown that the constrained distribution of exogenous DC significantly impacts immunologic control of tumor outgrowth (13). Control of s.c. growing tumors requires that DC prime Ag-specific CD8 T cells in peripheral skin-draining LN. T cells primed to the same Ag by DC that infiltrate the spleen are capable of tumor recognition and lysis ex vivo and infiltration of lung metastatic-like lesions in situ, yet are incapable of controlling s.c. growing tumors. DC-based therapies may therefore lead to restricted distributions of memory cells in different compartments and/or the induction of restricted tissue-homing patterns on activated effectors. In addition, there is an apparent limitation on the magnitude of immune response that can be induced in LN. We observed steadily improving control of tumor in lung, associated with splenic immune response, as the number of i.v. injected DC was increased, up to at least 10⁶ cells. In contrast, control of s.c. growing melanoma could not be improved by increasing the number of s.c. injected DC above 10⁵ cells. In this study, we examined the quantitative relationship between LN infiltration by exogenous DC and both the magnitude of immune response and control of tumor outgrowth. Our data demonstrate that tumor control is linked to the overall magnitude of the primary immune response, which is directly correlated with, and constrained by, infiltration of both DC and Ag-specific naive T cells into individual LN. We also demonstrate strategies for circumventing both of these limitations that are applicable in the further optimization of immunotherapeutic approaches for tumor treatment involving exogenous DC.

**Materials and Methods**

**Animals**

Transgenic mice on the C57BL/6 background expressing a chimeric MHC class I composed of the α1 and α2 domains of HLA-A*0201 and the α3 domain of H2-D1 (AAD) have been described previously (22). C57BL/6 mice were obtained from Charles River Laboratories. Animals were maintained in pathogen-free facilities, and protocols were approved by the University of Virginia Institutional Animal Care and Use Committee.

**Cell lines**

Murine B16-F1 melanoma transfected to stably express genes for AAD and for G418 resistance (B16-AAD) has been described previously (4). B16-F1 expressing a cytoplasmic-restricted construct of hen OVA (B16-cOVA) was provided by Dr. T. N. J. Bullock (University of Virginia Health System, Charlottesville, VA).

**Immunization with exogenous DC**

CD40L-activated bone marrow-derived DC were generated as described previously (4, 23). Activated DC were CD70<sup>high</sup>CD80<sup>high</sup>CD86<sup>high</sup> and produced IL-12 p70 (data not shown). DC were pulsed for 3 h with the indicated peptides in the presence of human β2 microglobulin, as described previously (13). Mice received DC in 200 μl of saline by s.c. injection into the scapular fold or flank, as indicated, or i.v. injection into the dorsal tail vein. For analysis of recall responses, DC-immunized mice received injections i.v. with 10⁷ PFU of hTyrVac, a recombinant vaccinia virus encoding human tyrosinase (24).

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DC distribution in vivo
CD40L-activated DC were labeled with CFSE (Molecular Probes) or SNARF-1 (Molecular Probes) and injected s.c. or i.v. At the indicated times, cells from spleen and LN were collected and stained with anti-CD11c (BD Pharmingen). The number of infiltrating DC (CD11c+CFSE+ or CD11c+SNARF-1+ cells) was quantified by flow cytometry.

Ex vivo analysis of Ag-specific T cells
Lymphocytes were obtained from spleen and peripheral LN by homogenization. In some experiments, CD8+ T cells were enriched from spleens and LN of immunized mice using a StemSep column and reagents (StemCell). Preparations were consistently 85–95% CD8+ as assessed by flow cytometry. Total lymphocytes or enriched CD8+ T cells were directly assessed for Ag specificity by staining with anti-CD8 mAb (eBioscience) and either HLA-A2-tetramer folded with Tyr369Y (tyrosinase 369–377) or gp100270-280 M peptides (National Institutes of Health Tetrarm Core Facility, Emory University Vaccine Center, Atlanta, GA) or Kb-tetramer folded with OVA257-264.

Tumor induction and measurements
s.c. tumors or lung metastases were established by injection of 4 × 104 B16-AAD in 200 μl of saline via s.c. and i.v. routes, respectively (4). All tumor measurements were performed in a blinded manner. Tumor cells were 100% viable by trypan blue exclusion and >98% AAD+ by flow cytometric analysis on the day of injection.

Statistics
Statistical comparisons were performed by Student’s t test using SigmaStat 3.01 software (Systat); all data sets were evaluated for normality of distribution before comparison by t test. Unless noted, data are presented as means ± SD of three replicates, and one experiment is shown from three to six independent trials.

Results
Peripheral LN have a limited capacity to retain exogenous DC, which limits primary and secondary immunity
Using mice expressing a recombinant human HLA-A*0201 class I MHC molecule (referred to as AAD), we previously demonstrated injection route-specific compartmentalization of immune responses using exogenous DC pulsed with the human melanoma peptide Ag, Tyr369Y (13). Using this system, we examined the association between the number of exogenous DC that infiltrated lymphoid tissue and both the size of the primary immune response and the extent of immunologic tumor control. We first injected AAD+ mice with 103-activated DC by either i.v. or s.c. routes. After i.v. injection, exogenous DC maximally infiltrated spleen after 4 h (Fig. 1A), although only ~30% of the number injected were recovered from this organ. However, exogenous DC were not detected in any peripheral LN over at least 24 h (Fig. 1A and data not shown). Using graded numbers of Tyr369Y-pulsed DC, the number of exogenous DC that infiltrated spleen increased in proportion to the number injected over a range from 102 to 106 cells (Fig. 1B). The number of infiltrating DC correlated directly over this entire range with the size of the primary immune response, measured as the percentage of CD8+ tetramer+ cells (Fig. 1B).

Both of these parameters also correlated with the extent of B16-AAD melanoma tumor control in lung (Fig. 1B). We have previously shown this control to be dependent on splenic immunity (13), and it likely reflects reactivation of memory T cells after tumor injection. These results establish a direct correlation between the number of DC injected i.v. over a three-log range, the number of splenic-infiltrating DC, and the size of primary and recall CD8 T cell responses in that organ.

After s.c. injection, exogenous DC rapidly infiltrated the draining LN, achieving a maximal number within 30 min, and this value was maintained for several hours (Fig. 1C). DC injected via this route also infiltrated lungs (Fig. 1D) and spleen (Fig. 1C), and achieved a maximal value in spleen only after 4 h. Again, however, DC were not observed in peripheral LN noncontiguous to the injection site drainage at any time (Fig. 1C and data not shown), consistent with entry of excess DC into circulation via efferent lymphatics (25). DC infiltration of draining LN was very efficient; s.c. delivery of as few as 104 DC led to ~3400 cells in the LN draining the injection site. However, injecting as many as 106 DC did not increase this number of infiltrated cells further at any time up to 72 h (Fig. 1E and data not shown), although it did increase the number of cells found in the spleen (Fig. 1F). As with i.v. injection, the number of exogenous DC in spleen was directly proportional to the number injected over the entire range of 102–106 cells (Fig. 1F).

Both the primary immune response in the draining LN and the control of a s.c. growing solid tumor, which we previously showed to be dependent upon an immune response in peripheral LN (13), reached a plateau upon injection of as few as 104 DC (Fig. 1E). In contrast, the number of Ag-specific activated CD8+ T cells in spleen and control of metastatic-like lesions in lungs correlated directly to the number of DC that infiltrated this organ after s.c. injection (Fig. 1F). These results demonstrate that peripheral LN have a limited capacity to retain exogenous DC, which limits the magnitude of primary and secondary immune responses in that compartment.

Induction of immune responses in peripheral LN by exogenous DC is not limited by competition for DC access or capacity to support T cell activation
We also evaluated whether immune responses to exogenous DC in individual LN were further limited by T cell competition for DC or soluble factors within the LN microenvironment. To test these possibilities, we asked whether the magnitude of one Ag-specific CD8+ T cell response was diminished by a simultaneous response in the same LN, directed against a second Ag presented by the same exogenous DC. AAD+ mice were immunized s.c. with 106 DC that had been pulsed with equimolar concentrations of either Tyr369Y or gp100270-280 (a modified epitope from the melanocyte differentiation protein gp100), or both Ags together. DC pulsed with both peptides stimulated primary responses against each epitope that were comparable to those observed using DC pulsed with either peptide alone (Fig. 2, A and B). In addition, the outgrowth of B16-AAD tumors was delayed in animals immunized with Tyr369Y- and gp100270-280-pulsed DC as compared with single epitope-immunized littermates (Fig. 2C), demonstrating an additive secondary response. Median survival of dual epitope-immunized mice was also significantly increased by 17.8 ± 2 days (χ2 test; p < 0.005) as compared with survival following single epitope (Tyr369Y) immunization (Fig. 2D). Thus, the stimulation of two distinct primary responses in the same peripheral LN did not compromise the magnitude of either, nor did it limit the development of protective antitumor immunity. Collectively, these results suggest that the primary immune response in peripheral LN after exogenous DC immunization is not limited either by T cell competition for DC, or by the availability of factors within the LN microenvironment necessary for T cell activation other than the number of DC.

Availability of naive Ag-specific T cells for activation by exogenous DC also limits immune responses in peripheral LN
Because there appeared to be no intrinsic limit on T cell activation within an individual LN, we next explored whether the immune response was limited by the availability of naive T cells during the time the DC were functional. To do this, we took advantage of the fact that primary immune response induced by exogenous DC in peripheral LN is limited to those nodes draining the injection site
Therefore, we asked whether the immune response in one DC-infiltrated node was limited by a simultaneous immune response occurring in a noncontiguous LN, because both would compete for naive T cells from the recirculating pool. AAD/H11001 mice were immunized with a total of 10^5 Tyr369Y-pulsed DC as follows: a single i.v. injection; a single s.c. injection; equally distributed between two noncontiguous s.c. sites; or i.v. and a single s.c. site. Although the number of DC injected into each of the two s.c. sites (50,000) was only half as many as injected in a single-site schema, it was still well above the number required to saturate the draining LN (Fig. 1E).

Injection of DC at a single s.c. site, or equally distributed between i.v. and a single s.c. site, stimulated primary responses of comparable magnitude in the LN draining the s.c. injection site (Fig. 3A and data not shown). These primary immune responses in peripheral LN were only detected following s.c. and not i.v. immunization. Furthermore, they were limited to the draining LN, and not found in LN of two noncontiguous drainages. Importantly, injection of DC into two noncontiguous s.c. sites resulted in primary responses of comparable magnitude in the LN draining both injection sites, and the response in each LN was also comparable to that observed in LN of mice receiving a single s.c. injection of DC (Fig. 3A). Collectively, these data demonstrate that the immune responses induced in two individual LN by peptide-pulsed DC do not influence one another, and demonstrate that the total number of cognate naive cells available for activation is far greater.
than the number activated in any individual DC-infiltrated node. As a corollary, this result shows that the size of the primary immune response in an individual LN is limited by entry of cognate naive T cells into it during the time that the exogenous DC remain functional.

We next evaluated tumor control following induction of immune responses in single or multiple LN drainages. Interestingly, mice immunized at two s.c. sites controlled s.c. growing tumor significantly ($p < 0.001$) better than those immunized with an equivalent number of cells delivered at a single site (Fig. 3B). In contrast, no difference was observed in control of lung metastatic lesions following either single site or dual site s.c. immunization, and neither of these immunization strategies was as effective as injection solely by the i.v. route (Fig. 3C). This is in keeping with our previous observation that control of lung metastases is dependent upon splenic immunity and occurs in proportion to the magnitude of the primary response (13).

The enhanced control of tumor following dual site s.c. injection of DC was intriguing, given that s.c. growing tumors cause T cell activation only in the draining LN (K. M. Hargadon and V. H. Engelhard, unpublished observation). We hypothesized that this enhanced control was due to induction of a larger number of memory T cells that were distributed among all peripheral LN. Therefore, we evaluated the location of early stage recall responses activated by secondary immunization with tyrosinase-expressing vaccinia virus (13). Secondary immunity was not confined to the LN in which DC priming had occurred, but was also evident in noncontiguous LN (Fig. 3C). This occurred only after primary immunization by s.c. and not i.v. injection of exogenous DC, indicating that these recall responses were dependent upon memory T cells that had been induced in LN and not in spleen. Most importantly, primary immunization by injection of DC into two noncontiguous lymphoid drainages led to an approximate doubling of the recall response in a third noncontiguous drainage, when compared with the response in mice immunized at a single s.c. site. These results suggest that, as with naive T cells, the recall response is limited by the number of memory T cells that reside in the draining LN. In the context of exogenous DC immunization, this number of memory cells is proportional to the size of the preceding primary response, which is in turn restricted by the small capacities of the priming LN for both exogenous DC and naive Ag-specific T cells.

**LN conditioning enhances DC infiltration and magnitude of immune responses in individual LN**

The previous data demonstrated that recall immunity in individual draining LN could be enhanced by increasing the number of systemically distributed memory T cells that had been activated in multiple drainages. The functional characteristics of these memory

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**FIGURE 2.** T cell competition does not limit activation in individual LN following immunization with exogenous DC. Mice were immunized s.c. with $10^5$ DC that had been pulsed with either Tyr369Y, gp100209M, or an equimolar mixture of both peptides. A, Primary (day 7) immune responses to Tyr369Y and gp100209M in axillary LN determined by costaining with specific HLA-A2-tetramer reagents and anti-CD8α. Numbers in plots indicate percentage of tetramer$^+$ CD8$^+$ cells in the lymphocyte forward scatter/side scatter gate. B, Total Ag-specific CD8$^+$ cells in immunization site-draining LN, calculated by multiplying the percentages of tetramer$^+$ CD8$^+$ lymphocytes by the total number of lymphocytes isolated from each LN. Data are mean of five separate animals ± SD. Data from one of three similar experiments are shown. C, Outgrowth of melanoma and (D) Kaplan-Meier survival data in mice challenged with $4 \times 10^5$ B16-AAD 21 days after immunization. Untreated; ○, unpulsed DC; ▼, Tyr369Y-pulsed DC; ▲, gp100209-pulsed DC; ■, Ag-copulsed DC. Data represent 10 animals per experimental grouping.
Discussion

In this study, we explored the basis for the limitation on primary immune responses and secondary antitumor immunity following s.c. immunization with peptide-pulsed DC. We have demonstrated that this limitation results from constrained infiltration of peripheral LN by exogenous DC and limited availability of Ag-specific naive T cells to the DC during the initiation of the primary response. The limited primary response in turn determines the size of the recall response in tumor-draining LN. To our knowledge, this is the first demonstration of a direct relationship between exogenous DC infiltration of a lymphoid compartment and the magnitude of both primary and recall immunity. We also define maneuvers that circumvent these limitations, leading to enhanced primary and recall immune responses and prolonged control of tumor outgrowth.

The factors that limit the infiltration of exogenous-activated DC into draining LN are unknown. The observation of a substantial portion of s.c. injected DC in spleen that is directly correlated with the number injected demonstrates that LN infiltration is not limited by sequestration of cells at the injection site. DC enter LN exclusively viaafferent lymphatics, emptying into the subcapsular sinus before at least a portion of the cells percolate into the T cell zones of the node (25). DC infiltration of LN may be limited by saturation of the conduits through which DC transit from the subcapsular sinus into the T cell areas of the LN. Alternatively, the T cell area of LN may accept only a limited number of exogenous cells before either no more cells are admitted or some infiltrating cells emigrate.

In contrast to the LN, we observed no limitation on DC infiltration into spleen over the range of DC numbers evaluated. The direct relationship between the number of infiltrating exogenous DC and the magnitude of the splenic primary response discounts the possibility that the high capacity of the spleen reflects simply circulating or red pulp-resident cells. Furthermore, even though circulating mature endogenous DC are rare (27, 28), activated exogenous DC efficiently enter splenic white pulp directly from circulation after i.v. injection (D. W. Mullins, unpublished observation). However, we reason that injection of exogenous DC in sufficient number would in fact lead to saturation of spleen. Using total T cell counts from spleen and axillary LN as a reference, we might expect splenic retention of exogenous DC to be 35-fold greater than LN (~120,000 cells). In our studies, injection of 10⁶ DC resulted in ~10⁻³-infiltrating DC in spleen. Although we have not achieved a saturation in the spleen, our data demonstrate that this compartment is infiltrated by exogenous DC in numbers significantly greater than observed in individual peripheral LN.

Two previous studies have examined the number of DC-infiltrating popliteal LN after injection in the footpad (19, 26). They failed to find a saturation limit on infiltration into this compartment, despite injection of up to 3-fold more DC than used in our study. On the contrary, they observed a positive cooperative relationship in which the fraction of injected DC retained in the LN increased as the number of injected cells was increased. However,
they also observed significantly slower migration to the draining LN: whereas we observed maximal DC infiltration into the axillary LN within ~30 min after s.c. injection, maximal numbers of DC in popliteal LN are not achieved until 2–4 days (19, 26). Therefore, we suggest that injection into the footpad results in initial sequestration and slow efflux of DC from this tissue, such that DC that enter the lymphatics and LN at early times lead to conditioning and acceptance of a larger number of later immigrants. Likewise, tissue retention of DC at the site of intradermal injection may account for the reported increase in LN infiltration by DC injected intradermally as compared with s.c. (29). Alternatively, the capacity of different LN for DC retention may be significantly different, although we have observed infiltration limits in inguinal LN comparable to those reported for axillary LN (our unpublished observation). Therefore, without prior conditioning, infiltration of exogenous DC into peripheral LN is strictly limited, and conditioning of draining lymphatics with mature DC significantly enhances DC infiltration and subsequent immune responses.

We also demonstrated that restricted availability of naïve T cells in individual LN constrained immune responses. Although naïve T cells circulate widely throughout secondary lymphoid compartments (30), their residence time in individual LN has been estimated to be in the range of hours to days (31, 32). Indeed, we found that the size of the immune response in one LN was not affected by a response against the same Ag occurring in a second. Thus, these two populations are essentially independent of one another, and each represents a small portion of the repertoire for that Ag available in the individual animal. This limitation may be further constrained by the transience of the DC themselves. The capacity of exogenous DC to induce full T cell activation, both in vitro and in vivo, has been shown to persist for only a few hours following maturation (33). The DC used in our studies were activated in vitro before injection and may therefore retain the ability to induce in situ primary immune responses for only a limited time. Therefore, only T cells that engage Ag-presenting exogenous DC shortly following immunization would achieve full activation.

Given these limitations, together with the large capacity of spleen to retain DC and support activation of tumor Ag-specific T cells, it is reasonable to suggest that i.v. immunization with DC should be used in lieu of other routes. However, we have previously shown that cells activated in this compartment are unable to control the subsequent outgrowth of s.c. tumors (13). Instead, this control is mediated by cells in peripheral LN. In this study, we show that after a localized primary response to immunization with
exogenous DC, recall responses are distributed to multiple peripheral LN. Furthermore, the size of this distributed response is dependent on the size of primary responses occurring in one or more LN, and independent of the size of the splenic response. Our data suggest that these responses are based on memory cells resident in these nodes in advance of the response. We have also noted enhanced recall responses in the LN in which initial priming occurred, suggesting either that memory cells preferentially migrate into specific LN or that some memory cells are retained in the original priming compartment. Data consistent with the former possibility have been reported in an adoptive transfer model (34).

Finally, there is mounting evidence that activation of T cells in different LN compartments leads distinct tissue-homing characteristics (35–38). Therefore, targeted delivery of Ag-bearing DC to specific LN will be crucial for the induction of tumor Ag-specific memory and memory/effector T cells with appropriate migratory capabilities to enter lymphatic compartments where tumor Ag is presented and to infiltrate tumor itself upon reactivation, respectively.

Because of the limited ability of DC to engage naive T cells in peripheral LN, our work also offers practical insights to enhance the use of DC as immunotherapeutic reagents. First, conditioning of draining lymphatics by pretreatment with unpulsed, activated dendritic cells leads to enhanced primary immune responses and antitumor immunity. Our data are consistent with earlier work demonstrating that conditioning enhanced the magnitude of primary responses by increasing the number of Ag-bearing DC in the LN as well as the subsequent flux of naive T cells (26). However, we have extended it to demonstrate that this also leads to enhanced secondary immunity and associated tumor control. Second, tumor immunity can be enhanced by injection of DC simultaneously into multiple non-contiguous LN. In this case, the total number of DC and T cells in any individual LN remains unchanged. However, the total number of LN-infiltrating DC is increased, allowing greater access to the pool of naive T cells resident in, or circulating through, separate LN compartments. This leads to enhanced primary in local LN, but also a larger secondary immune response that is distributed more systematically among other peripheral LN. Finally, tumor control can be enhanced by immunization with DC presenting two different peptide Ag. We find no evidence of competition among T cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. J. Exp. Med. 183: 317–322.


References


