Introduction

One of the critical questions in tumor immunology is how the host senses the presence of the many potential antigens that are present in each tumor. It was initially thought that the host remained ignorant of the presence of a tumor unless the tumor cells themselves metastasize to the lymph nodes, and thus engage directly with host T cells. In line with this, tumor-specific T cells have been detected in secondary lymphoid organs where tumor cells are present. However, this view has changed over the past few years and it now appears that tumor antigens are efficiently “cross presented” to host T cells in a process that is almost entirely restricted to the lymph node that drains that tumor. Migratory dendritic cells (DCs) are the principle cell type responsible for delivering antigens from peripheral tissues to lymph nodes, carrying with them not just antigen, but information regarding the “context” of the antigen, such as the presence of “danger” signals. DC infiltration of solid tumors is well documented in tumor-bearing animals and patients. Thus, generation of tumor-specific responses might be expected to involve migration of DCs from the tumor site to the tumor draining lymph nodes (TDLN). However, whether this process occurs is uncertain, and indeed several studies have suggested that DC-function and LN migration may be impaired in cancer due to the immunosuppressive nature of the tumor microenvironment. In such situations the ability of DCs in the TDLN to cross-present tumor antigen may be dependent on the migration of tumor cells themselves.

Sentinel lymph node metastases are well documented in human cancers and are also found in several murine models of solid tumor. It is therefore possible that DCs in the TDLN acquire antigen from metastatic tumor cells for cross-presentation to naïve T cells. In line with this, tumor specific T cells can be detected in secondary lymphoid organs where tumor cells are present. It has been postulated that generation of effective tumor specific CTL requires small numbers of tumor cells reaching the secondary lymphoid organs early during tumor development, thus, strictly extra-lymphatic tumors would be ignored by the host immune system, resulting in tumor outgrowth. However, subsequent studies have shown that tumor specific T-cell responses exist in the absence of detectable lymph node metastasis, and tumors progress despite the presence of tumor cells in secondary lymphoid compartment. The majority of these studies examined the ability of tumor cells to directly prime tumor specific CD8+ T cells in secondary lymphoid organs, and not their capacity to act as a source of antigen for cross-presentation.

Normal tumor progression is associated with rapid proliferation of viable tumor cells and differing levels of tumor cell death in the form of apoptosis and necrosis. In addition, tumor cells are known to secrete soluble proteins and antigen carrying exosomes. However the form of tumor antigen that is captured by DCs for cross-presentation has not been fully elucidated.

It is widely accepted that generation of tumor specific CD8+ T-cell responses occur via cross-priming; however the source of tumor antigen for this event is unknown. We examined the source and form of tumor antigen required for cross-presentation in the local lymph node (LN) using a syngeneic mouse tumor model expressing a marker antigen. We found that cross-presentation of this model tumor antigen in the LN is dependent on continuous traffic of antigen from the tumor site, but without any detectable migration of tumor resident dendritic cells (DCs). Instead, small numbers of tumor cells metastasize to local LNs where they are exposed to a localized CTL attack, resulting in delivery of tumor antigen into the cross-presentation pathway.

Keywords: cross-presentation, dendritic cells, tumor, lymph nodes, cytotoxic T cell
node environment, and while they are important for the development of effective DC immunotherapy protocols, they cannot predict the natural source of tumor antigen for cross-presentation in vivo.

We have previously shown that cell-associated tumor antigen is cross-presented in the local TDLNs by both CD8α+ and CD8α− DCs. The ability of CD8α− DC to cross-present tumor antigen led us to speculate that migration of DCs from the tumor site was required for cross-presentation of tumor antigen in the TDLN. We now show that cross-presentation of tumor antigen in the TDLN is dependent on the continuous traffic of antigen from the tumor site to the local node, let alone prove them as a source of tumor antigen. Indeed, given that tumor cells can metastasize to lymph nodes, cross-presentation of tumor antigen in the draining node could be explained more directly by the presence of a viable metastatic tumor deposit. To investigate this scenario in more detail we first confirmed that only CD11c+ cells, (DCs), enriched from the TDLN were able to induce proliferation of tumor-specific CD8+ T cells and, importantly, that the CD11c− population, which contained all other cells such as macrophages and tumor cells, did not induce proliferation (Fig. 1A). Then, to determine whether antigen presentation in the TDLN required continuous traffic of antigen from the tumor site, or instead, is sustained by local antigen production, such as metastatic tumor growth, we examined the kinetics of tumor antigen presentation in vivo after surgically removing the tumor mass (and hence the primary source of tumor-derived antigens and tumor-infiltrating DCs; Fig. 1B). Complete tumor resection resulted in the cessation of tumor antigen specific T-cell proliferation in vivo whereas sham surgery had no effect (pre-op = 40.17% ± 15.95% proliferation compared with day of surgery = 43.22% ± 13.70%, p = 0.266). Reduced but significant proliferation could still be detected at day 7 post-surgery (14.72% ± 6.22%, p = 0.014). The observed kinetics of tumor antigen-presentation confirms that (1) the proliferating tumor-specific T-cells observed in the TDLN are not being activated in the tumor and arriving in the DLN as already-proliferating cells and (2) strongly argues against the presence of a metastatic tumor deposit and demonstrates that cross-presentation of tumor antigen in the TDLN requires continuous traffic of antigen from the tumor site.

Which cell delivers antigen to the TDLN? As HA is a transmembrane, rather than secreted, protein we reasoned that it must traffic to the TDLN from the tumor site in a cell associated form. This, taken together with (1) cross-presentation of tumor antigen by TDLN DCs and (2) the dependence of in vivo antigen presentation on the presence of a solid growing tumor, suggests a role for migratory DCs in this process. We therefore examined the capacity of tumor infiltrating DCs (TiDC) to traffic to the TDLN by following the fate of injected fluorescent beads. AB1HA tumor bearing mice received an i.t. injection of Fluoresbrite YG beads (1 μM; Polysciences). As a control for normal migration of skin DCs, naïve Balb/c received a s.c. injection of the same amount of beads into the equivalent flank. Tumor tissue and draining lymph nodes were collected, stained for CD11c expression and analyzed by flow cytometry. In this setting, the presence of fluorescence in the cellular gate indicates bead capture (Fig. 2A). The beads were captured by TiDCs within 24 h following injection.
with greater than 900 bead$^{+}$CD11c$^{-}$ cells detected per $10^5$ tumor cell suspension, and captured beads were still detectable 3 d after injection (Fig. 2B). However, we found no evidence for migration of tumor DCs to the TDLN, as there were no bead-positive cells detected in the axillary or inguinal TDLNs at any time point (Fig. 2C). In contrast, skin DCs in naïve Balb/c showed normal migration of DC, with peak numbers of bead$^{+}$CD11c$^{-}$ cells present in the DLNs at day 3 post injection (Fig. 2C).

The observed absence of migratory DCs in TDLNs combined with the seemingly paradoxical requirement for continuous transfer of antigen from the tumor environment, led us to revisit the role of metastatic tumor cells as a source of antigen for cross-presentation in the TDLN. AB1-HA tumor cells were labeled with the lipophilic tracer, DiI (Molecular Probes, Invitrogen), and injected s.c. into naïve mice. TDLNs were harvested on days 1, 3, 8 or 14 following injection, stained for CD11c expression and analyzed by flow cytometry for the presence of metastatic DiI-positive tumor cells (Fig. 3). The majority of the DiI signal was associated with CD11c$^{+}$ cells in the TDLN (Fig. 3A), presumably DCs, as AB1HA tumor cells do not express CD11c (data not shown). The number of DiI$^{+}$ DCs in the axillary TDLN peaked at day 1 post injection (39.6 DiI$^{+}$ CD11c$^{+}$ cells per $10^5$ total LN cells) and steadily declined to background (naïve Balb/c, day 0) by day 14 (Fig. 3B). In contrast, the number of DiI$^{+}$ DCs in the inguinal TDLN rose steadily over days 1 and 3 to peak at day 8 post injection (18.3 DiI$^{+}$ CD11c$^{+}$ cells per $10^5$ total LN cells) (Fig. 3C). Detection of DiI$^{+}$ nonDCs (DiI$^{+}$ CD11c$^{-}$) was less than 4 per $10^5$ total lymph node cells at all time points in both TDLNs. These results indicate that despite the apparent lack of DC migration from the tumor site, tumor antigen is associated with DCs in the TDLN, in the absence of any detectable tumor cell metastasis. This raises the possibility of continuous migration of AB1HA to the TDLN, but with apparent control of metastatic outgrowth.

Metastasizing tumor cells as the source of cross presented tumor antigen. Although the results thus far suggest that low numbers of metastasizing tumor cells might be the source of antigen in the local TDLN, PCR analysis of TDLNs to search for occult tumor cells was negative for tumor antigen (HA) expression (Fig. 4A). Therefore, in order to establish the threshold of detection of tumor cells in lymphocyte populations we performed PCR analysis of tumor cells which had been titrated into normal lymph node populations. This showed that the lowest level of detection of tumor cells was 10 tumor cells per $10^5$ lymph node cells (Fig. 4B). This suggested that the number of metastatic tumor cells in the TDLN in our model was below $10:10^7$ and therefore below the reasonable limits of histological and PCR detection. One reason that these low numbers of metastatic tumor cells in TDLNs might not progress is that they are controlled by a local CTL response. In line with this, examination of endogenous CD8$^{+}$ T cells by the in vivo CTL assay showed that HA-specific CTLs were detected solely in the TDLNs of tumor bearing mice (Fig. 4B). In vivo CTL activity was measured by injecting CFSE-labeled HA peptide-coated and uncoated targets into tumor-bearing mice and measuring their recovery 18 h later. The percentage of T-cell killing in the TDLN was significantly greater than that observed in the nonDLN (9.48% ± 4.42 v 4.64% ± 1.14, p = 0.006) or spleen (9.48% ± 4.42 v 4.82% ± 1.87, p = 0.009). To test if this might be restricting the growth of the few metastatic tumor cells. TDLNs were left intact or depleted of CD3$^{+}$ cells and cultured ex vivo in selective media to determine if an outgrowth of viable AB1HA tumor cells occurred—indeed, re-isolation of viable tumor cells from the TDLN was seen, and was dependent on the removal of CD3$^{+}$ cells (Fig. 4C). These data suggest that in the absence of DC migration, small numbers of tumor cells may be the source of tumor antigen for cross-presentation in the TDLN. This implies that T cells residing in the TDLN may play a role in controlling metastatic spread.

Discussion

It is widely accepted that generation of tumor specific CD8$^{+}$ T-cell responses occurs via cross-priming; however the source of tumor antigen for this event is unknown. It has been postulated that the immune system is ignorant of extralymphatic tumors and that generation of an anti-tumor T-cell response is dependent on tumor cell metastasis to secondary lymphoid organs.1,37 Despite this, several studies have shown generation of tumor-specific
tumor antigen in the TDLN is dependent on the continuous traffic of antigen from the tumor site, in the absence of DC migration. Based on the data we propose that cross-presentation of HA tumor antigen is mediated by DCs in the TDLN following acquisition of antigen from small numbers of metastatic tumor cells.

This result is in contrast with dogma suggesting that DCs migrate from peripheral tissues and deliver antigen to resident DCs for cross-presentation. This view has been predominantly generated from models of viral infection whereby tissue resident DCs capture the invading pathogen and following interaction with pathogen associated signals, such as PAMPs, are programmed for migration and terminal differentiation. While our observations show that CD11c+ DCs in TDLNs are responsible for the cross presentation of tumor antigens, and antigen presentation kinetics following surgical resection of the tumor were consistent with the in vivo life span of DCs in lymph nodes, this model may not properly represent the generation of tumor-specific T-cell responses in the steady-state for several reasons. First, tumor growth is associated with a lack of pro-inflammatory signals and pathogen by-products that may interact with tumor-resident DCs resulting in their activation and subsequent migration to the TDLN. Second, tumors produce a variety of immunosuppressive factors that inhibit DC maturation and differentiation, leading to the recruitment and accumulation of immature DCs at the tumor site. Third, several studies have shown that DC migration is impaired in the presence of a solid growing tumor. In line with this fluorescent bead tracking experiments showed that DCs infiltrating AB1-HA tumors were not detected in the TDLNs. Finally, sentinel lymph node metastasis is a hallmark of human disease and represents a mechanism whereby tumor antigen is delivered directly to the TDLN. While migration of tumor-resident DCs cannot be definitively ruled out it is important to note that since cross-presentation is favored by high doses of antigen it seems improbable that small numbers of DCs (below the level of detection by fluorescent bead transfer) carrying tumor antigen to the TDLN would be sufficient to induce a detectable tumor-specific CD8+ T-cell response. Thus in the absence of DC migration, small numbers of tumor cells may be the source of antigen for cross-presentation in the TDLN. Consistent with this finding, tumor cells have been detected in TDLNs where DCs mediate cross-presentation of tumor antigen.

Our observation that small numbers of tumor cells in TDLNs can escape detection by microscopy or sensitive PCR methods suggests that such methods may miss the presence...
of antigen-bearing tumor cells as a source of antigen delivery to nodes. This is consistent with a number of studies showing that isolated tumor cells can migrate to other sites in the body without forming solid metastatic deposits.44-47

One way that this could happen would be for those micrometastases to be controlled by local CTL mechanisms. Indeed we show that effector CTL are generated in TDLN and remain localized in that location. This observation, along with our finding that occult tumor cells only become manifest when T cells are depleted from the TDLN population, suggests that these locally generated CTL control the growth of these tumor cells while delivering sufficient antigen to the cross presentation pathway.

These data have several important implications. First, lymph nodes that are considered by pathologists to be tumor negative (“NO disease”),48 may actually have barely detectable numbers of tumor cells which are actually delivering antigen and cross priming tumor-specific T cells. Second, the localized TDLN CTL response may be controlling the spread of tumor cells to distal sites, that is, the presence of low-level metastasis to TDLNs may not per se be a negative indicator but would be the source of tumor-restricting CTL. Interestingly, these cross primed CTL in the lymph node would have been missed if systemic CTL activity, measured in peripheral blood or spleen, had been relied upon as the readout for T-cell priming. Third, given the above, the TDLN is likely to be a “checkpoint” for immunoselection of tumor cells that are resistant to CTL lysis, by, for example, loss of Class I expression or production of T-cell suppressive molecules. Indeed studies of metastatic deposits compared with primary sites support this notion.49 Of course such selection would only apply to those tumors that spread via the DLNs rather than directly via the bloodstream, as has been described.50 Finally, TDLN removal is occurring daily in thousands of cancer patients and our data showing that the TDLN is active in cross priming and limiting tumor spread support the notion that such removal cannot be a null event and requires further investigation.

In conclusion, these studies suggest that tumor antigens are delivered to TDLNs not by migratory DCs, which appear to be arrested by the tumor, but by extremely low levels of tumor cells themselves which are controlled by locally induced CTL. These results have implications for our understanding of the selection of immuno-resistant tumor variants and for cancer lymph node surgery.

Materials and Methods

Animals. BALB/c (H-2b) mice were obtained from the Animal Resources Centre (Canning Vale) and maintained under specific pathogen free conditions. Balb/c Clone 4 (CL4) TCR-transgenic mice, which express a TCR specific for the H-2d-restricted peptide IYSTVASSL (residues 518–526) of A/PR/8/34 (H1N1) influenza virus hemagglutinin (HA), were generated and screened as previously described.21 Animal experiments were conducted according to The University of Western Australia Animal Ethics Committee guidelines.

Tumor cells and inoculation. AB1-HA (H-2d) is a mouse mesothelioma cell line expressing the hemagglutinin (HA) molecule of influenza virus A/PR/8/34 (H1N1) and were maintained in culture as previously described.3 AB1HA tumors were grown subcutaneously (s.c.) following injection of 5 × 10⁵ viable cells into the left flank. In some experiments tumors were resected on day 16 following inoculation.

Ex vivo antigen presentation assay. DCs were enriched from TDLNs of AB1HA bearing mice and isolated as previously described.35 Briefly DCs were purified using CD11c microbeads according to the manufacturer’s instructions (Miltenyi Biotec). Enriched lymph node populations were routinely > 85% CD11c positive. HA-specific CD8⁺ T cells were purified from CL4 TCR transgenic mice using the Miltenyi Biotec CD8⁺ T-cell isolation kit according to the manufacturer’s instructions (Miltenyi Biotec). Enriched cells were routinely > 90% CD8⁺ T cells. CFSE-labeling of T cells was performed as described previously.52 Serial dilutions of purified DCs were incubated with CFSE-labeled CD8⁺ CL4 T cells for 60 h in vitro. Proliferation was analyzed by CFSE dilution using flow cytometry (FACS Calibur, BD Biosciences).
In vivo antigen presentation "Lyons-Parish" assay. A total of 1–2 × 10^7 CFSE-labeled HA-specific CL4 TCR-transgenic splenocytes were injected intravenously (i.v.) into recipient mice. TDLNs were harvested 3 d following adoptive transfer and counter-stained with anti-CD8-PECy5.5 (BD Biosciences) prior to flow cytometry (FACS Calibur, BD Biosciences).

In vivo CTL assay. Detection of HA-specific in vivo CTL was performed as previously described. Briefly, Balb/c splenocytes were divided into two populations, one of which was pulsed with 1 μg/ml HA peptide (IYS TVA SSL). Cells were labeled with CFSE using a final concentration of 5 μM CFSE for peptide-pulsed cells (CFSEhigh) and 0.5 μM for unpulsed cells (CFSElow). CFSElow and CFSEhigh cells were pooled in equal proportions and injected i.v. into recipients. Lymph nodes and spleens were harvested 18 h later and analyzed by FACS for recovery of CFSElow and CFSEhigh cells. The percentage killed was determined using the following formula: 100 × [1 – (CFSElow events/CFSEhigh events)].

DC tracking experiment. AB1HA tumor-bearing mice were injected i.t. with 2 × 10^7 Fluoresbrite YG microbeads (Polysciences) in 20 μL saline. Lymph nodes and tumors were harvested at the indicated time points, counter-stained with anti-CD11c-APC (N418, BioLegend) then analyzed by flow cytometry (FACS Calibur, BD Biosciences).

Tracking tumor cell migration. In vivo tracking of tumor cells, AB1HA cells were labeled with 1 μM of the lipophilic tracer, Dil (D282, Molecular Probes) according to the manufacturer’s instructions, and 5 × 10^6 Dil+ cells were injected s.c. Lymph nodes and tumors were harvested at the indicated time points and single cell suspensions were stained with anti-CD11c-APC specific antibody (N418, 117310; BioLegend) then analyzed by flow cytometry.

Tumor outgrowth experiment. TDLNs were harvested and pooled from 5–10 mice. T cells were depleted by incubation with anti-CD3 IgG (17A2, BD Biosciences) followed by sheep anti-rat Dynabeads (Invitrogen) and magnetic removal of positive cells. Intact or T-cell depleted TDLN cells were cultured at 1 × 10^6 cells/well in culture media with antibiotic selection (400 μg/ml genitin) and outgrowth of tumor cells evaluated by visual inspection.

PCR assay. Balb/c splenocytes were seeded with different numbers of AB1HA tumor cells ranging from 1 in 2 × 10^6 to 1 in 1 × 10^6 splenocytes. RNA was isolated from these samples using Trizol (Invitrogen) as per manufacturer’s instructions. HA expression, was assayed by real-time PCR using SuperScript III (Invitrogen), SYBR green (QIAGEN) and HA primers (5′-CAA TTG GGG TAT CAA TAA ATC GCC-3′ and 5′-AGC TTT GGG TAT CAA TAA ATC GCC-3′). Expression of the housekeeping gene GAPDH was also measured (5′-GAA GGT CGG TGT GAA AAA TGT AAC ATC GCC-3′ and 5′-AGC TTT GGG TAT CAA TAA ATC GCC-3′).

Statistical analysis. Statistical significance was calculated using GraphPad PRISM (San Diego, CA).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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