The purpose of this study was to determine whether tumor draining lymph nodes (TDLNs) contain the necessary components to process and present tumor-rejection antigens. The secondary objective was to determine whether short term ex vivo culture of TDLNs could generate peptide-reactive T cells with specific anti-tumor effector function in vivo.

Methods: 4T1 and RENCA cancer cell lines were transfected with an expression plasmid containing HER2. These cell lines were then inoculated into the mammary fat pads of BALB/c mice to generate TDLNs. The antigen-experienced CD62L^hi T cell subpopulation of TDLNs was isolated and activated ex vivo with anti-CD3 and expanded in interleukin (IL)-2 prior to determining in vitro and in vivo activity.

Results: Of nine HER2 nonameric peptides synthesized using the SYFPIETHI prediction model, culture activated cells derived from TDLNs from HER2-bearing 4T1 cells (4T1.2) secreted significant levels of interferon-γ in response to the highest affinity peptide TYLP TNASL. Furthermore, culture-activated cells derived from 4T1.2 TDLNs secreted significant levels of interferon-γ when co-cultured with either 4T1.2 or HER2-transfected RENCA cells, but not RENCA transfected with control plasmid. Additionally, adoptive transfer of culture activated cells derived from 4T1.2 TDLNs cured mice bearing 4T1, 4T1.2 and RENCA-HER2 but not RENCA transfected with control plasmid.

Conclusions: Short-term culture of TDLNs ex vivo results in generation of peptide-reactive T cells which can be expanded and cure mice bearing HER2 transfected tumors in vivo. These cell lines were then inoculated into the mammary fat pads of BALB/c mice to generate TDLNs. The antigen-experienced CD62L^hi T cell subpopulation of TDLNs was isolated and activated ex vivo with anti-CD3 and expanded in interleukin (IL)-2 prior to determining in vitro and in vivo activity.

Tumor draining lymph node | T cells | Adoptive immunotherapy | HER2

INTRODUCTION

Adoptive immunotherapy (AIT) entails the transfer of activated immune cells into a cancer-bearing host. Several AIT approaches have demonstrated success in human clinical trials, including the use of tumor-infiltrating lymphocytes (TIL) and chimeric antigen receptor (CAR) modified T cells. TIL therapy in combination with high-dose intravenous interleukin (IL)-2 has demonstrated significant durable response rates in patients with metastatic melanoma [1, 2]. Additionally, CAR T cells have demonstrated significant clinical responses in patients with refractory leukemia [3]. In both instances, the transferred T cells are either clonal or target a limited number of antigens, which appear to correlate with strong antitumor activity in vivo but rely on epitope spreading to broaden the immune response. In addition, complexities of generating T cells clones limit the applicability of these approaches to highly-specialized centers.

In contrast, our group and others are focused on the use of tumor-draining lymph node (TDLN) cells as a platform for AIT[4-16]. The use of TDLN for AIT is predicated on the notion that tumors shed antigens into the lymphatic system or antigen presenting cells (APCs) migrate from the tumor site, with tumor antigens ultimately being recognized and processed within TDLNs. TDLNs draining established tumors contain antigen-specific pre-effector T cells which may have been sensitized to tumor antigens. It has been demonstrated that TDLNs contain T cells which are primed against neoantigens that are unique to individual patient tumors and these are presented in the context of MHC to naïve T cells in vivo [17]. The ability of TDLN derived T cells to recognize multiple antigens is one of its potential advantages over strategies which only target single antigens. In addition, culture of TDLNs in vitro allows for not only expansion of antigen-primed T cells, but also generation of newly primed T cells when APCs interact with naïve T cells in vitro. Importantly, these pre-effector cells acquire tumor-specific effector function against tumor in vivo only after undergoing ex vivo activation with anti-CD3 and culture with IL-2 [18]. AIT using TDLN has shown significant therapeutic activity against a variety of murine tumors [5, 6, 8, 10, 15, 16, 18-22]. It has been observed in animal models that effector T cells derived from TDLNs have tumor-specific antitumor effects in vivo, which seem to correlate with secretion of interferon-γ (IFN-γ) in response to tumor cells in vitro[20, 23-26]. Previous studies have also demonstrated that antigen-experienced T cells reside within the CD62L (L-selectin)^hi subset of T cells from TDLNs, and ex vivo culture of this subpopulation generates potent CD4+ and CD8+ effector cells with significant in vivo activity [27].

Our lab has previously demonstrated that T cells derived from TDLN in human melanoma patients, when activated and expanded ex vivo, have demonstrated melanoma-specific cytotoxicity in vivo and in vitro and contain melanoma peptide-specific T cells [24]. However, to our knowledge no study to date has demonstrated the ability of TDLNs to process and present a tumor neoantigen to generate T cells that not only are peptide-specific but also recognize tumor expressing the neoantigen in vivo and improve mouse survival in an adoptive immunotherapy model.

Conflict of Interest: No conflicts declared.

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The purpose of this study was to determine whether lymph nodes draining a murine tumor transfected with a surrogate neoantigen human epidermal growth factor receptor-2 (HER2) would contain HER2 peptide-specific T cells and demonstrate antitumor effects after ex vivo culture and adoptive transfer that were HER2 specific. Our results confirm that TDLNs have the capacity to not only generate peptide-specific T cells to tumor antigens, but also can be used as a source for ex vivo culture and neoantigen specific adoptive immunotherapy of cancer.

METHODS

**Transfection of tumor cell lines**

In order to generate HER2 expressing tumor cell lines which were syngeneic in BALB/c mice, we transfected the murine carcinoma cell lines 4T1 (mammary) and RENCA (renal) with a murine stem cell virus (MSCV) vector containing the full-length coding region of HER2 downstream of a cytomegalovirus (CMV) promoter. Following transfection and selection in G418 containing media, tumor cells underwent one round of immunoselection based upon HER2 cell surface expression using immunomagnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and subsequently analyzed by FACS to confirm expression of HER2 and MHC Class I (H-2K\(^d\)).

**Generation of tumor-draining lymph nodes (TDLNs)**

All mouse studies were performed on an IACUC-approved protocol and all procedures were performed in accordance with institutional guidelines. The general experimental overview for adoptive immunotherapy using TDLNs is illustrated in Figure 1. 8-12 week old BALB/c mice (Jackson Labs, Bar Harbor, ME) were inoculated subcutaneously in the mammary fat pad bilaterally with 5 x 10\(^5\) 4T1 or 4T1.2 tumor cells. TDLNs were harvested 9 days after tumor cell inoculation. The CD62L\(^{low}\) TDLN subpopulation, was then isolated using immunomagnetic columns. CD62L\(^{low}\) TDLN cells were then activated with immobilized anti-CD3 for 48 hours followed by culture in the presence of IL-2 at 25 IU/ml for 72 hours prior to either in vitro peptide or tumor-stimulated cytokine release assay or in vivo adoptive transfer experiments.

**Adoptive immunotherapy experiments**

4T1 and 4T1.2 TDLN cells were adoptively transferred intravenously via tail vein into BALB/c recipient mice bearing 3-day established tumors. Mice underwent a conditioning regimen of whole body irradiation (500 cGy), followed by a single injection of 5 x 10\(^6\) TDLN cells in 1 mL HBSS. Control treatment mice received HBSS injection via tail vein at the same volume as the T cell injections.

**Development of HER2 peptides**

The SYFPIETHI epitope prediction model was used to identify nonamer HER2 peptides which could bind MHC Class I H-2K\(^d\). The top nine HER2 immunodominant peptides were synthesized for stimulation of TDLNs to determine the presence of reactive T cells using cytokine release assays.

**Cytokine release assays**

TDLN cells were culture-activated with anti-CD3/IL-2 for 5 days as described above, washed and then co-cultured with either irradiated tumor cells (RENSA or 4T1.2) or irradiated splenocytes pulsed with synthesized HER2 immunodominant peptides without additional IL-2. Supernatants were harvested at 24 hours and analyzed for IFN-\(\gamma\) using cytometric bead assay (Becton, Dickinson and Company, East Rutherford, NJ, USA).
Figure 2. HER2 and MHC Class I expression of HER2 transfected 4T1.2 tumor cells. After transfection of 4T1 cell lines with HER2, FACS analysis demonstrated high levels of expression of HER2 and MHC class I (H-2K^d).

**Statistics**
Survival analysis was performed and differences between groups estimated by log rank test.

**RESULTS**

**HER2 and MHC Class I expression of HER2 transfected 4T1.2 tumor cells**
In order to confirm successful HER2 transfection and cell surface protein expression, FACS analysis of 4T1.2 tumor cells was performed following transfection with HER2 MSCV vector and immunomagnetic selection using anti-HER2 magnetic beads. 4T1.2 maintained in G418 containing selective media demonstrated high levels of expression of HER2 as well as MHC Class I (H2-K^d) (Figure 2). These results demonstrate that a high percentage of tumor cells were successfully transfected and expressed extracellular components of the HER2 protein.

**Immune cell subsets of 4T1 and 4T1.2 TDLNs**
Immune cell subsets of 4T1 and 4T1.2 TDLN cells were compared prior to culture and showed similar quantities of Thy 1.2, CD4, CD8, L-selectin, and B220 (Figure 3). Thy 1.2(CD90.2), a T lymphocyte marker, was expressed by 60% of cells in both populations. The ratio of CD4^+ helper T cells to CD8^+ cytotoxic T cells was consistent at 3:2. L-selectin (CD62L) is low, confirming successful depletion to obtain our antigen-primed T cell population. B220, a B lymphocyte marker was between 35-50%. These data demonstrate that although HER2 is a foreign protein, the processing and presentation of this neoantigen does not result in significant differences in the percentages of immune cell subsets. Thus, any differences in therapeutic activity between activated 4T1 and 4T1.2 TDLN cells is not likely due to initial differences in TDLN immune cell subsets.

**TDLN naturally prime T cells against immunodominant tumor-associated peptide antigens**
Subcutaneous tumors of either 4T1 or 4T1.2 were established for 3 days in BALB/c mice. Adoptive immunotherapy of 3-day established 4T1 and 4T1.2 subcutaneous tumors using 4T1 TDLNs or 4T1.2 TDLNs was performed as described with a single injection of activated T cells following conditioning whole body irradiation of the mice. 4T1 and 4T1.2 TDLN cells cured mice bearing the identical tumors from which they were derived. Of note, 4T1 TDLN cells cured 50% of mice bearing 4T1.2 tumors (Figure 4A). These findings suggest that although the 4T1.2 tumor cells express shared antigens with 4T1, the transfection of HER2 conferred some resistance to 4T1 TDLN but not 4T1.2 TDLN adoptive therapy. This could be explained by a differing biology of 4T1.2 after transfection or a change in surface expression of 4T1 tumor rejection antigens after transfection with HER2.

For the next series of experiments, the H-2Kd MHC matched RENCA was transfected with HER2. Subcutaneous tumors of RENCA or RENCA-HER2 tumors were then established for 3 days in BALB/c mice. Administration of 4T1 TDLNs or 4T1.2 TDLNs showed no therapeutic effects against RENCA tumors. However, RENCA-HER2 tumors were eradicated by 4T1.2 TDLNs but not 4T1 TDLNs (Figure 4B). This result demonstrated that 4T1.2 TDLNs had attained effector activity that specifically targeted the HER2 antigen, as the 4T1 TDLNs had no therapeutic effect and thus there were likely no shared tumor-rejection antigens between 4T1 and RENCA.
Adoptive Immunotherapy of 3-day established 4T1 and 4T1.2 subcutaneous tumors. 4T1 and 4T1.2 TDLN cells cure mice bearing the identical tumors. 4T1 TDLN cells cured 50% of mice bearing 4T1.2 tumors. There was a significant improvement in survival in mice bearing 4T1.2 tumors treated with lymph nodes draining 4T1.2 tumors (p=0.015 log-rank test).

Adoptive Immunotherapy of 3-day established RENCA and RENCA-HER2 tumors using 4T1 and 4T1.2 TDLNs. RENCA-HER2 tumors are eradicated by 4T1.2 TDLNs (p=0.006, log-rank test) but not 4T1 TDLNs (p=n.s. log-rank test), confirming the activity of HER2 specific effector cells in vivo.

Analysis of HER2 peptide-specific reactivity of TDLN T cells
The next series of experiments examined the IFN-γ secretion from T cells when stimulated by HER2 specific peptides. Nine H-2Kd HER2 nonameric peptides were synthesized using the SYFPEITHI prediction model (http://www.syfpeithi.de) (Figure 5). Irradiated BALB/c splenocytes, a source of autologous APCs, were pulsed with the four highest affinity H-2Kd HER2 peptides as well as a control peptide (mesothelin). Culture activated cells derived from lymph nodes draining 4T1.2 tumors secreted significant levels of IFN-γ in response to the highest affinity immunodominant HER2 peptide TYLPTNASL (Figure 6A). 4T1 and 4T1.2 whole tumor cells stimulated IFN-γ release when co-cultured with either 4T1 and 4T1.2 TDLN cells. However, co-culture of RENCA cells with either 4T1 or 4T1.2 TDLN cells did not stimulate IFN-γ release, again demonstrating the antigen-specific nature of the TDLN response (Figure 6B). To further elucidate the interactions involved in stimulated IFN-γ release, irradiated 4T1.2 tumor cells were incubated with blocking antibodies prior to co-culture with 4T1.2 TDLN cells. IFN-γ secretion by 4T1.2 TDLN cells was abrogated by MHC Class I blocking antibody, suggesting that CD8+ T cells are necessary for IFN-γ secreted in this model system (Figure 6C).

DISCUSSION
In this study using the HER2 transfected neoantigen tumor model, we demonstrate that TDLNs have the ability to prime naïve T cells against tumor rejection antigens. Activated T cells from TDLN 4T1.2 tumors did not demonstrate therapeutic activity in vivo against wild-type RENCA. However, those same TDLN draining T cells were able to cure mice bearing RENCA-HER2 tumors. These findings demonstrate that TDLNs can process and present neoantigens and are a useful source of antigen-specific T cells for adoptive immunotherapy.
Adoptive immunotherapy is a therapeutic approach in which immune cells are activated in the laboratory and transferred into a tumor-bearing host. The ability of T cells to recognize and kill tumor cells over expressing antigens, which can vary by as little as a single amino acid change is very appealing due the potential for a markedly specific therapy [28]. As such, multiple methods have been and are currently being explored to harness T cells, including TILs, lymphokine activated killer cells, vaccine primed lymphocytes, and CAR T cells [29]. In addition to the above mentioned methods of T cell adoptive immunotherapy, TDLNs have drawn interest as a source of T cells. Potential advantages of TDLN derived T cells include the possibility to recognize all tumor associated antigens, including neoantigens which may be due to unique mutations within a patient’s individual tumor. By using CD62L\text{low} T cells in the current study, a population of highly reactive antitumor pre-effector cells are isolated prior to activation and expansion with anti-CD3 and IL-2 to become effector cells [15, 16]. The findings in this study demonstrate that TDLNs have the cellular components to recognize and process an immunodominant antigen, and infusion of the CD62L\text{low} effector T cells which target the dominant antigen lead to therapeutic effects in vivo.

Figure 5. Results of SYFPIETHI prediction model for H-2K\text{d} HER2 nonameric peptides.

Figure 6. (A) Peptide-stimulated IFN-gamma release by 4T1 and 4T1.2 culture-activated TDLN. (B) Tumor-stimulated IFN-gamma release by 4T1 and 4T1.2 culture-activated TDLN. (C) Tumor-stimulated IFN-gamma release by 4T1.2 culture-activated TDLN cells when co-cultured with tumor cells and when MHC blocking antibodies are added. P1 = TYLPTNASL; CV = positive control; IaD = irradiated.
Targeting HER2 has translational relevance to many cancers, particularly breast cancer and gastric cancer. HER2 is a cancer progenitor cell antigen that is correlated with poor clinical outcomes and is overexpressed in approximately 25% of all breast cancers [30]. As such, prior studies have targeted HER2 using multiple strategies including TDLNs [31]. However, to our knowledge, this is the first study to show that specific tumor antigens can be processed by TDLNs to generate peptide-specific anti-tumor effector cells.

While previous studies have shown that TDLN cells have therapeutic activity against HER2 expressing tumors, our study is the first to show that the TDLN cells act by targeting particular antigens. The improved in vivo efficacy of 4T1.2 TLDNs over 4T1 TDLNs to treat 4T1.2, as well as their ability to target RENCA-HER2 tumors, but not RENCA tumors suggests an activity specifically against HER2 expressing tumors. In our study, we attempted to link the specificity seen in our in vivo experiments with our understanding of MHC related antigen presentation by using a SYFPIETHI prediction model in conjunction with cytokine release assays. Interestingly, we found increased IFN- secretion by TDLN cells in response to only the predicted highest affinity immunodominant peptide TYLPTNASL. This parallels a recent study, which reported reactivity of ex vivo activated human melanoma draining lymph node cells to known known melanoma antigens [32]. The in vivo specificity and in vitro antigen reactivity shown in our study suggests the presence of antigen processing and presentation within TDLNs.

Although our study did not look at the processes within the TDLN, we hypothesize that HER2 expressing tumor cells are taken up by APCs with subsequent breakdown of the HER2 protein and presentation of compatible antigens on MHC class I molecules. In the context of a TDLN, they are then recognized by a subset of T cells, which are then primed. Our culture methods allow for ex vivo expansion of this T cell population, which can subsequently be adoptively transferred to target tumor cells expressing HER2.

This study has demonstrated the ability of TDLNs to process a novel antigen and generate peptide specific T cells in a predictable manner. When the specific mutations or overexpressed proteins are known, DNA transfected tumor cell vaccines have been used in an attempt to generate peptide specific effector cells. More recently, these tumor specific antigens have been targeted using artificially generated chimeric antigen receptor (CAR) T cell therapy [33]. While many common tumor rejection antigens are known, even more unidentified neoantigens exist and are unique to each cancer. These neoantigens are potential targets for immunotherapeutic strategies, but are currently difficult to rapidly identify and target [34]. One advantage of TDLN based adoptive immunotherapy is the ability to generate effector cells towards many, if not all, of these tumor specific neoantigens. In summary, our results show that TDLNs generate immunodominant peptide-specific T cells. This study provides proof of concept for the use of TDLNs to generate peptide-specific effector cells for use in adoptive immunotherapy.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Wayne Aldrich and Pierre Triozzi from the Cleveland Clinic Foundation for supplying the plasmid vector for HER2 transfection.


