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Tumor-infiltrating dendritic cells exhibit defective cross-presentation of tumor antigens, but is reversed by chemotherapy

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Abbreviations: DC, dendritic cell; HA, hemagglutinin; PD-1, Programmed Death receptor 1; TiDC, tumor-infiltrating dendritic cell; TDLN, tumor-draining lymph node;

ABSTRACT

Cross-presentation defines the unique capacity of an antigen presenting cell to present exogenous antigen via MHC class I molecules to CD8$^+$ T cells. Dendritic cells (DCs) are specialized cross-presenting cells and as such have a critical role in anti-tumor immunity. DCs are routinely found within the tumor microenvironment, but their capacity for endogenous or therapeutically enhanced cross-presentation is not well characterized. In this study, we examined the tumor and lymph node DC cross-presentation of a nominal marker tumor antigen, hemagglutinin (HA), expressed by the murine mesothelioma tumor AB1-HA. We found that tumors were infiltrated by predominantly CD11b$^+$ DCs with a semi-mature phenotype that could not cross-present tumor antigen, and therefore, were unable to induce tumor-specific T-cell activation or proliferation. Although tumor-infiltrating DCs were able to take up, process and cross-present exogenous cell-bound and soluble antigens, this was significantly impaired relative to lymph node DCs. Importantly, however, systemic chemotherapy using gemcitabine reversed the defect in antigen cross-presentation of tumor DCs. These data demonstrate that DC cross-presentation within the tumor microenvironment is defective, but can be reversed by chemotherapy. These results have important implications

for anti-cancer therapy, particularly regarding the use of immunotherapy in conjunction with cytotoxic chemotherapy.

INTRODUCTION

Tumor antigens derived from solid tumors can be presented by host APCs to naïve CD8^{+} T cells in a process known as cross-presentation. Professional APCs, such as dendritic cells (DCs), play a key role in cross-presentation of tumor antigens in draining lymph nodes, a process that is critical for inducing tumor-specific cytotoxic T lymphocytes (CTLs) [1, 2]. Several studies, including our own, have demonstrated the pivotal role of DCs in the cross-priming of tumor specific T-cell responses [3-7]. Furthermore, the most effective anti-tumor immunotherapies are associated with improving the response to cross-presented antigens [1, 2].

When studying how DCs cross-present tumor antigens, most attention has focused on the priming events occurring in the tumor draining lymph node. However, there is evidence that DC cross-presentation within tissues is also an important component of memory and even naïve CD8^{+} T-cell responses [8, 9]. Additionally, it has been shown that tumor-infiltrating DCs (TiDCs) may be functionally impaired and/or unable to migrate to tumor-draining lymph nodes (TDLNs) [10].

In this study, we examined the capacity of TiDCs to cross-present cell-associated tumor antigen using the murine malignant mesothelioma line, AB1-HA, where influenza virus hemagglutinin (HA) is a membrane bound tumor neo-antigen. We characterized TiDCs in terms of their phenotype, antigen uptake and cross-presenting capacity during normal

tumor growth and during regression following systemic cytotoxic chemotherapy with gemcitabine.

RESULTS

Tumor antigen-specific T-cell activation does not occur in the tumor microenvironment

In order to determine if tumor-specific $CD8^+$ T cells recognize antigen presented in the tumor environment, we directly injected CFSE-labeled, HA-specific (CL4) T cells into the tumor site and measured their proliferation in the tumor as well as the spleen and tumor draining (TDLNs) and non-draining lymph nodes (NDLNs) (Fig. 1A). Following intratumoral transfer, CFSE-labeled CL4 T cells made their way into the lymphatics and underwent robust proliferation in the TDLNs. The proportion of CL4 T cells proliferating in the draining axillary lymph node was greater than 60% at all time-points examined (Fig. 1B). Similarly, the proportion of proliferating CL4 cells was greater than 60% in the draining inguinal lymph node by day 14 and as high as 85% by day 21. In contrast, CL4 T-cell proliferation in the NDLNs and spleen was less than 10% at all time points. Intriguingly, there were no proliferating CL4 cells in NDLN when cells were injected directly into the tumor site (Fig. 1B). Proliferating CL4 T cells were never observed within the tumors at any time point throughout tumor growth (<5%), indicating that tumor antigen-specific T-cell activation does not occur in the tumor microenvironment either because of lack of antigen presentation or because of local suppression of proliferation.

Tumor-infiltrating DCs display a semi-mature phenotype

We hypothesized that the failure of transferred antigen-specific T cells to proliferate directly in the tumor site might be due to a failure of DCs within the tumor to restimulate infiltrating T cells, which has been termed 'post-licensing' [11]. For this reason, we first confirmed the presence of DCs within the tumor microenvironment using CD11c. DCs accounted for 1-3% of all cells in the tumor (Fig. 2A). We found that tumors were predominantly infiltrated by DCs that were positive for the myeloid and dermal/interstitial DC marker CD11b, and negative for the plasmacytoid DC marker CD45RA, the resident lymphoid organ DC marker CD8α and the myeloid DC marker CD4 (Fig. 2B). The majority of TiDCs were CD11b$^+$CD205$^+$ (>75%), which may partly relate to the subcutaneous location of the tumor. These cells could be further separated into CD11b$^+$CD205^{hi} and CD11b$^+$CD205^{lo} populations (Fig. 2B), representing dermal CD11b$^+$ DCs and Langerhans cells, respectively [12].

Importantly, TiDCs constitutively expressed MHC class I, MHC class II and the co-stimulatory molecules CD80 and CD86 (Fig 2C), although they were able to further upregulate expression after overnight culture in the absence of specific additional stimulation (Fig. 2D-E and Supporting Information Fig. 1). TiDC also expressed the positive and negative co-stimulatory molecules CD40, CD70, PD-L1/B7-H1 and PD-L2/B7-DC (Fig. 2C and Supporting Information Fig. 1). CD70 expression is generally a feature of activated DCs, and is a powerful co-stimulator of CD8$^+$ T cells upon interaction with its ligand, CD27. This suggests that TiDCs could have the capacity to activate CD8$^+$ T cells in the tumor microenvironment. However, expression of the programmed death receptor (PD-)1 ligands,

PD-L1 and PD-L2, molecules known to inhibit T-cell mediated immunity [13], may counteract the potentially positive signal provided by CD70. Overall, these results indicate that TiDCs display a semi- mature phenotype but they have the capacity to spontaneously mature upon ex vivo culture to express T-cell stimulatory molecules, suggesting that there is no intrinsic functional defect, rather the cells are regulated by locally produced factors. What was then critical to understand was whether the observed lack of cross-priming by TiDCs was due to lack of cross-presented antigen or a dominant inhibitory effect from negative costimulators.

TiDCs do not cross-present tumor antigen at the effector site

In order to further assess the direct T-cell priming potential of TiDCs, we purified them from tumors and co-cultured them with CL4 T cells, comparing responses to those by DCs purified from TDLNs in the same animals. Although purified DCs extracted from TDLNs induced robust CL4 proliferation, as previously demonstrated [14], TiDCs were not able to do this (Fig. 3A). Importantly, the CL4 T cells exposed to TiDCs also showed no signs of activation in terms of CD69 expression or IFN-γ production (Fig. 3A).

In order to determine if this failure of TiDCs to activate CL4 T cells was due to active inhibition, we added exogenous CL4 peptide to purified DCs – we anticipated that if inhibitory influences were dominant, the response to this peptide would be blunted relative to LNDCs (Fig 3B). There was no significant difference in the ability of TiDCs to present HA-peptide on their surface compared with normal LNDCs. Titration of HA peptide concentration revealed that the limit of sensitivity for both TiDCs and normal DCs was

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similar; at 10 pg/mL. These results strongly suggest that TiDCs have a defect in the antigen cross-presentation pathway, rather than being generically paralysed or being unable to co-stimulate T cells.

Ex vivo maturation does not rescue the capacity of TiDCs for cross-presentation

One possible explanation for these findings could be the semi-mature phenotype of TiDCs, given that there is a close association between DC maturity and antigen processing and presentation [15]. Semi-mature DCs might be expected to take up antigen but fail to complete the processing of antigen and/or subsequent MHC loading. Therefore we extracted TiDCs, exposed them ex vivo to agents that are known to potently mature/activate DCs [16], and then assessed their capacity to cross-present tumor antigen. Ex vivo maturation of TiDCs with either LPS, polyI:C, activating anti-CD40 antibody (FGK45) or IFN-γ was unable to promote activation and proliferation of CL4 T cells ex vivo (Fig. 4A-B and Supporting Information Fig. 2). In contrast, polyI:C and FGK45 stimulation of HA-peptide pulsed-DCs, significantly increased proliferation of CL4 T cells compared with unstimulated DCs (Fig. 4A and B). These data suggest that TiDCs have an intrinsic defect in their capacity to process antigen that is not directly related to maturation.

TiDCs can take up and process exogenous cell-bound and protein antigen

Since the abrogated capacity of TiDCs to activate T cells could be restored by adding exogenous antigen, we next examined the capacity of TiDCs to take up cell-bound material, to determine if their poor cross-presentation at the tumor site was due to a failure to uptake antigens from tumor cellular material. We did this by co-culturing TiDCs with CFSE-labeled living, UV-irradiated or heat-killed tumor cells, respectively. We found that TiDCs could take up exogenous cell-bound material, as evidenced by acquisition of a positive CFSE signal after co-culture with labeled tumor cells (Fig. 5A).

To determine if the block in DC cross-presentation was due to a reduced ability to process captured antigen, we next examined the capacity of TiDCs to take up and proteolytically cleave exogenous proteins, using the DQ-OVA system. This consists of the protein OVA conjugated with the self-quenching fluorescent dye, BodipyFL. Upon proteolysis the resulting peptides are highly fluorescent, allowing a quantitative assessment of processing by flow cytometry. Following incubation with DQ-OVA, TiDCs displayed a marked, temperature-dependent shift in green fluorescence (Fig. 5B) with an increase in MFI from 26.3 to 1492 when comparing culture at 4°C (no processing) to 37°C (active processing). This was more than fifteen times that observed in normal LN and splenic DCs which showed MFIs of 80 and 63 at 37°C, respectively (Fig. 5B), indicating that TiDCs are efficient at internalizing and processing soluble protein antigen.

Given that neither uptake nor processing were defective, we next examined the capacity of TiDCs to load cross-presented soluble protein antigen into MHC class I molecules, using

recombinant HA protein. TiDCs and normal LNDCs were pulsed with HA protein prior to co-culture with CFSE-labeled CD8$^+$ CL4 T cells. TiDCs were able to process and cross-present HA protein, as demonstrated by proliferation and IFN-γ production (Fig 5C-D). However, proliferation induced by TiDCs was significantly less than that by normal LNDCs but only at the highest concentration of HA protein (Fig. 5C; p=0.019). Altogether these results indicate that although TiDCs can take up and process soluble protein antigen, their ability to cross-present is impaired relative to LNDCs.

The reduced capacity of TiDCs to cross-present antigen is reversed by chemotherapy

Given the potential importance of cross-presentation by TiDCs in restimulating TILs, and the block in this process described above, we reasoned that immunogenic chemotherapies which induce strong CD8-dependent effector responses in vivo might reverse this block. We have previously shown that immunogenic anti-cancer chemotherapy with the cytotoxic drug, gemcitabine, can lead to an increase in HA-specific CD8$^+$ T cells within the tumor tissue [5]. Given this, we examined the effect of systemic gemcitabine chemotherapy on TiDC cross-presentation capacity. Whereas TiDCs from saline treated mice were defective in their capacity to induce CL4 T-cell proliferation, TiDCs enriched from gemcitabine treated animals spontaneously induced proliferation of CL4 T cells (Fig. 6A). Furthermore, this proliferation was associated with increased IFN-γ production (Fig. 6A). This effect was limited to the tumor site as gemcitabine treatment did not alter the capacity of DCs in the TDLN to cross-present HA tumor antigen (Fig. 6B). To further investigate a possible effect of gemcitabine on TiDC composition and activation state, we measured expression of DC

subtype and maturation markers (Fig. 6C-D). We found no change in the proportion of CD4$^+$ or CD8α$^+$ DC subsets, but a slight decrease in the CD11b$^+$CD205^{hi} dermal DCs. There was no increase in activation markers upon gemcitabine treatment. In fact, MHC class II expression was slightly decreased (Fig. 6D). In addition, gemcitabine did not affect the ability of TiDCs to take up a particulate antigen at the tumor site (Fig 6E). Thus, the level of tumor antigen cross presentation observed following gemcitabine therapy is not due to changes in TiDC subsets, maturation state or antigen capture. These results support the idea that gemcitabine increases the cross-presentation of tumor antigens by tumor-infiltrating dendritic cells and increases our understanding of why some chemotherapy drugs are immunogenic and others are not [17, 18].

DISCUSSION

When studying how DCs cross-present tumor antigens, most attention has focused on priming events in the local draining lymph node. However, there is some evidence that cross-presentation within tissues may also be an important component of CD8$^+$ T-cell responses contributing to effector function or tolerance within the tumor [19, 20]. Therefore in this study, we aimed to characterize the immunogenic potential and in particular the cross-presenting capacity of TiDCs. We found several key features of TiDC to be defective when compared to LNDCs and importantly, showed how these defects could be reversed.

Firstly, our data show that tumor antigen-specific T-cell activation does not occur in the tumor microenvironment, even when CD8$^+$ T cells are delivered directly into the tumors and persist. This suggests that either tumor-specific CD8$^+$ T cells do not engage with antigen

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within the tumor environment or that local suppression of proliferation is dominant. This may help to explain why anti-tumor CD8^{+} effector cells fail to eradicate tumors. This feature is not seen in all models, which may explain why different responses to immunotherapy are observed between models. For example in a mouse model of melanoma, cross-presenting APCs were sufficient to induce the activation, proliferation and effector differentiation of naïve CD8^{+} T cells in the tumor tissue [8]. A failure of local cross-presentation, as observed in our model, may contribute to a general failure of anti-cancer immunotherapies that manage to achieve a strong CTL response in lymphoid organs but fail to control tumor growth due to a lack of necessary signals from APCs at the effector site to maintain a damaging CTL phenotype.

The semi- mature phenotype of these DCs could contribute to the inability of antigen-specific T cells to proliferate in the tumor site secondary to inadequate post-licensing by TiDCs, i.e. a failure of tumor infiltrating T cells to be re-stimulated by DCs within the tumor. In cancer patients and tumor-bearing animals, DCs infiltrating tumor tissue bear an immature phenotype [21] and several studies have shown that tumors can actively recruit immature DCs to the tumor site then impede their differentiation [22]. A number of studies have shown that TiDCs have poor antigen presenting function in vitro and in vivo [22-24]. For example, melanoma and thymoma tumors are infiltrated by a large number of partially activated CD11b^{+} DCs [24, 25] that are inefficient at MHC class II presentation due to a poor capacity for intrinsic protein uptake and they display defective migration to TDLN [14, 24]. Thus, as TiDC in cancer patients display a semi-mature phenotype [3, 7, 26-28], the tumor microenvironment may fail to promote full maturation and activation of resident DCs.

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Secondly, although TiDCs had an inhibited potential to activate antigen-specific T-cells, the fact that this could be fully restored when exogenous MHC class I peptide was added indicated that the cross-presentation pathway was hampered rather than T-cell priming. Our findings that spontaneous maturation occurs upon ex vivo culture prior to co-incubation with antigen-specific T cells but addition of LPS, polyI:C, IFN-γ or anti-CD40 agonist antibody to ex vivo TiDCs could not overcome their inability to cross-present tumor antigen shows that the defect was not one of maturation. In a similar study, DCs isolated from B16 melanomas were previously shown to be defective in their ability to induce proliferation of TAA-specific CD8^{+} or CD4^{+} T cells, even when stimulated with LPS [29, 30]. A similar effect was observed upon ex vivo culture of TiDCs from NSCLC patients with several TLR ligands [26]. This refractory state of TiDCs has been thought to be induced by a variety of tumor-derived soluble factors such as IL-10, PGE-2 and TGF-β, which have been shown to inhibit DC function [31-34]. A study by Vicari et al., showed that blocking the IL-10R on TiDCs in combination with CpG stimulation led to priming of tumor-specific T-cell responses in vitro and in vivo [35]. Furthermore, tumor cells transfected to express GMCSF and CD40L favored the recruitment of mature DCs that induced IFN-γ production by CTL, indicating the potential of targeting TiDCs for cancer immunotherapy [36].

Following our observation that TiDCs have poor T-cell stimulatory potential and our finding that the uptake and proteolytic cleavage of exogenous antigen is normal, we conclude that the failure to cross-prime is distal to antigen uptake and processing. As TiDCs were able to cross-present exogenous HA protein to specific T cells, albeit with reduced efficiency, the

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problem may temporally occur after the processes of uptake and proteolytic cleavage, in the MHC class I-loading pathway. The failure of cross-presentation of cell-derived tumor antigens combined with an intact ability to process and cross-present soluble tumor antigen is consistent with a defect in the processing pathway between intracellular compartments and the cytoplasm. Future studies will need to address where this block occurs, determine which molecular events create the block and how they can be overcome.

Lastly, we tested whether gemcitabine, a chemotherapy agent known to be immunogenic and to facilitate the accumulation of T cells in tumors, could reverse the failure of TiDCs to cross-present antigens. The fact that it did so, could be explained by either a quantitative change, i.e. it increased antigen load consequent upon increased cell death, or it induced a qualitative change e.g. through overcoming inhibition of cross-presentation or by changing DC populations or phenotype within the tumor. It has previously been suggested that chemotherapy acts to alter the immunogenicity of the dying tumor cell or regulate immune suppression through inhibition of checkpoint molecules or depletion of suppressive immune cells [17, 37, 38]. Ma and colleagues demonstrated that anthracycline chemotherapeutics induced recruitment of CD11c$^+$CD11b$^+$Ly6^{hi} DCs to the tumor, which were able to take up dead tumor cells, resulting in enhanced cross-presentation [39]. Our data show that gemcitabine, a drug that is widely used in breast, pancreatic, ovarian and lung cancers also exerts a beneficial effect on the cross-presenting potential of TiDCs.

Gemcitabine-induced apoptotic destruction of tumor cells potentially exposes the immune system to large amounts of tumor antigen. However this alone is typically not

enough to initiate a protective immune response as tumors start to grow again at the end of treatment [5, 38]. Instead, gemcitabine has been shown to prime the host immune system for adjuvant immunotherapy. Treatment of tumor-bearing mice with activating anti-CD40 antibody following gemcitabine chemotherapy induced long term cures in >80% of mice [40]. This effect was not solely due to the debulking effects of the drug as surgical resection did not augment the effects of immunotherapy. Our results provide further evidence for this immune priming effect of gemcitabine and suggest how this immunogenic property can be further exploited, for example through combination therapies with immune checkpoint blocking antibodies (43).

Recent studies indicate that cognate interactions between antigen experienced T cells and tissue DCs can enhance T-cell proliferation and function [8, 9, 41]. Based on our data, we speculate that gemcitabine acts to modulate TiDCs, thus enabling the potential reactivation of HA-specific T cells at the effector site. This is supported by previous findings in the AB1HA model indicating that gemcitabine acted to augment tumor specific T-cell responses only at the tumor site [5]. While the number of HA-specific $CD8^+$ T cells was not altered in the TDLNs or spleen of gemcitabine treated animals, there was an increase in HA-specific $CD8^+$ T cells at the tumor site as determined by tetramer staining. This correlated with an increase in CTL activity observed only at the tumor site, while the total number of infiltrating $CD8^+$ T cells remained the same [5]. To further evaluate the importance of post-licensing by TiDCs, it would be necessary to deplete DCs in a temporal and spatially specific manner, i.e. in the tumor during the effector phase. Future studies will further address this question.

In conclusion, the data supports a model where gemcitabine chemotherapy increases cross-presentation of tumor antigens by tumor resident DCs to cross-arm tumor-infiltrating CD8$^+$ T cells for full effector function; a process which can be further exploited in the immunotherapy of cancer.

Materials and methods

Animals

BALB/c (H-2^d) mice were obtained from the Animal Resources Centre (Canning Vale, Western Australia) and maintained under specific pathogen free conditions. Clone 4 (CL4) TCR-transgenic mice, which express a TCR specific for the H-2^d-restricted peptide IYSTVASSL (residues 518-526) of A/PR/8/34 (H1N1) influenza virus hemagglutinin (HA), were generated and screened as previously described [5, 42]. Animal experiments were conducted according to The University of Western Australia Animal Ethics Committee guidelines.

Antibodies and reagents

Specific anti-mouse mAb against the following molecules were used: CD8α PECy5 (53-6.7, BD Biosciences), CD8α PE (53-6.7, BD Biosciences), CD4 PE (RM4.5, BD Biosciences), CD205 PE (NLDC-145, Cedarlane), CD45RA PE (14.8, BD Biosciences), GR-1 PE (RB6-8C5, eBiosciences), IFN-γ Allophycocyanin (XMG1.2, eBiosciences), CD11c Allophycyanin (N418, BioLegend), CD11b Alexa Fluor 488 (M1/70, BioLegend), CD69 (H1.2F3, eBiosciences) MHC class II PECy5 (M5/114.15.2, BioLegend), CD80 PE (16-10A1, eBiosciences), CD86 PE (GLI, eBiosciences), CD40 PECy5 (ICI0, BioLegend). Anti-mouse MHC class I IgG (TIB126) was detected using FITC-conjugated goat anti-rat IgG (Life Technologies). HA peptide (IYSTVASSL) was synthesized by the Centre for Cell and Molecular Biology (University of Western Australia, Perth, Australia). CFSE and DQ-OVA were purchased from Molecular Probes (Life Technologies). Fluoresbrite YG microbeads were purchased from Polysciences (Warrington, PA, US).

Tumor cells and inoculation

AB1-HA (H-2^d) 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 [5]. AB1HA tumors were grown subcutaneously (s.c.) following injection of 5x10^5 viable cells into the left flank. In some experiments tumors were resected on day 16 following inoculation.

Gemcitabine treatment

For some experiments, mice were treated with gemcitabine (Gemzar, Lilly) 120 mg/kg intraperitoneally (i.p.) or saline, once on day 19 after tumor inoculation. At day 21 tumor tissue was pooled from 9 mice and DCs were enriched and tested for their T-cell stimulatory potential as described below.

Collagenase-DNase digestion of lymph nodes and tumor tissue

Lymph nodes and tumor tissue were digested with 1 mg/mL collagenase type II (Worthington Biochemicals) and 1 μg/mL (LNs) or 100 μg/mL (tumor) bovine pancreatic DNase I grade II (Roche Applied Science cat.10104159001) for 20 min (LN) or 60 min (tumor) at room temperature. EDTA was added for a final concentration of 10 mM and the suspension digested for a further 5 min. Cells were washed and resuspended for subsequent procedures in Hanks balanced salt solution (Invitrogen Life Technologies) supplemented with 5 mM EDTA and 5% FBS (EDTA-BSS-FBS).

Staining of tissues for flow cytometry

Axillary and inguinal lymph nodes were pooled for the tumor flank (TDLNs) and for the contralateral flank (NDLNs) unless otherwise stated. Single cell suspensions were generated by enzymatic digestion. Staining for surface markers was carried out at 4°C for 30 min in EDTA-BSS-FBS. Cells were washed and fixed using 1% paraformaldehyde prior to flow cytometry. Staining for DC subset and phenotype was preceded by incubation at 4°C for 15 min in blocking solution containing 5% (w/v) bovine serum albumin (BSA) and 1/250 Fc

Block (anti-CD16/32 IgG). In some experiments, intracellular cytokine staining was performed following a 4 h re-stimulation in the presence 5 μg/mL brefeldin A (Sigma) with or without 5 μg/mL HA peptide (IYSTVASSL). Following staining for surface molecules, cells were fixed and permeabilized using BD Cytofix/Cytoperm™ buffer (BD Biosciences) before incubation with anti-IFN-γ−Allophycocyanin. Data was acquired using CellQuest Software on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar Inc.).

Ex vivo antigen presentation assay

DCs were enriched from TDLNs and tumor tissue of AB1HA bearing mice and isolated as previously described [4]. Briefly, single cell suspensions were generated by collagenase-DNase digestion and DCs purified using CD11c microbeads (Miltenyi Biotec cat.130-052-001) according to the manufacturer's instructions. 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 (cat. 130-095-236) according to the manufacturer's instructions. Enriched cells were routinely >90% CD8$^+$ T cells. CFSE-labeling of T cells was performed as described previously [4]. 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 1-2x10^7 CFSE-labeled HA-specific CL4 TCR-transgenic splenocytes were injected intratumorally (i.t.) into recipient mice. TDLNs and tumor tissue were harvested 3 days following adoptive transfer and counter-stained with anti-CD8-PECy5.5 (53-6.7, BD Biosciences) prior to flow cytometry (FACS Calibur, BD Biosciences).

DC and tumor cell co-culture

Lymph nodes, spleens and tumor tissue were harvested, single cell suspensions generated by enzymatic digestion and DCs isolated as described above. CFSE-labelled AB1-HA tumor cells were left untreated, subjected to UV light for 10 min followed by 2 h recovery at 37°C, or heat killed by incubation at 65°C for 1 hour. DCs and tumor cells were cultured alone or were mixed together at a 1:1 ratio and incubated at 37°C for 24 or 0 h then washed and counter stained with anti-CD11c-Allophycocyanin or hamster IgG-Allophycyanin isotype control. The amount of CFSE label acquired by DCs following 24 h co-culture was determined by flow cytometry.

In vitro uptake of DQ-OVA

Lymph nodes, spleens and tumor tissue were harvested, single cell suspensions generated by enzymatic digestion and DCs isolated as described above. DC preparations from each tissue were incubated with 100 μg/mL DQ-OVA for 2 h at 37°C or 4°C, washed extensively and stained for CD11c expression prior to FACS acquisition (BD FACS Calibur). The amount of

DQ-OVA taken up and processed by DCs at 37°C was determined by increased fluorescence in the green fluorescence (FL-1) channel compared to background at 4°C.

Incubation with HA peptide or protein

Purified DCs were resuspended at 1×10^6 per mL in R10 and incubated at 37°C with the desired concentration of HA peptide or recombinant HA protein (Protein Sciences Corporation, Meriden, CT, USA) for 30 min or 2 h, respectively. Cells were then washed three times with warm R10 through an FCS cushion and cell number determined by trypan blue exclusion.

In vivo uptake of Fluoresbrite microspheres

AB1-HA tumor-bearing mice were injected i.t. with 2×10^7 Fluoresbrite YG microbeads (Polysciences, Warrington, PA, US) in 20 uL of saline. Tumors were harvested 24 h later and single cell suspension generated by enzymatic digestion as discussed above. Cells were stained with anti-CD11c-Allophycocyanin (N418, BioLegend, San Diego, CA, US) and then analyzed by flow cytometry.

Statistical Analysis

Statistical significance was calculated using GraphPad PRISM (San Diego, CA). Statistical significance between individual treatment groups was determined using the student's t-test or

One-way ANOVA non-parametric test with Dunn's multiple comparisons post-test. Differences were considered significant if the p value was less than 0.05.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

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Figure 1. Tumor antigen-specific T-cell activation is inhibited in the tumor microenvironment. (A and B) CFSE-labeled CL4-transgenic lymphocytes were transferred by i.t. injection at days 4, 11, 18 and 25 into AB1HA-bearing mice. Lymph node, spleen and tumor cells were harvested 3 days after adoptive transfer and analyzed by FACS for T-cell proliferation. (A) Flow cytometry gating strategy. (B) Percentages of gated proliferating CFSE⁺CD8⁺ CL4 T cells in lymph nodes, tumor and spleen. Data are shown as mean ± SEM (n=3-6 mice/group) and are representative of two independent experiments. *p<0.05, **p<0.01, One-way ANOVA non-parametric test; Dunn's multiple comparisons post-test.

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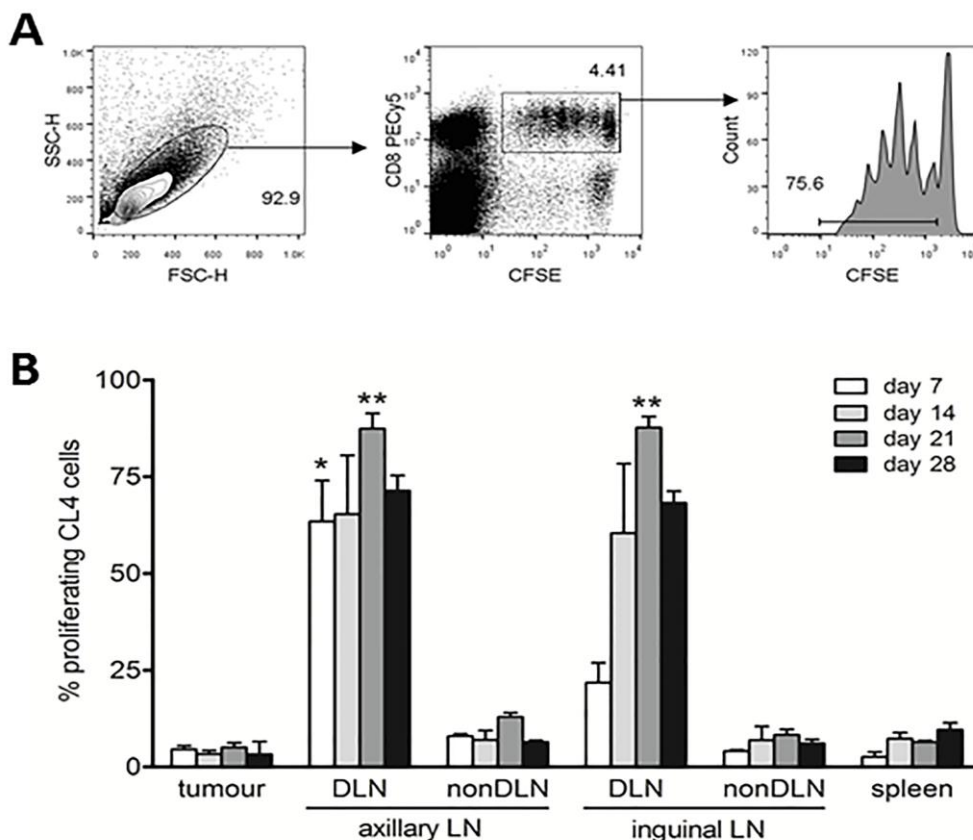
Figure 2. TiDCs display a semi-mature phenotype. AB1HA tumors were harvested on day 21 post inoculation, pooled and digested. (A) Tumor cells were gated on CD11c$^+$ DCs. (B) CD11c$^+$ cells were analyzed for the expression of CD11b, CD8α, CD4, CD205 and CD45RA by FACS. Numerical values within regions represent percentages of total CD11c-positive cells. Data are representative of five independent experiments (n= 5 mice/samples per experiment). (C) TiDCs were analyzed for expression of MHC class I, MHC class II, CD80, CD86, CD40, CD70, B7DC and B7H1 specific antibodies (filled histograms) or isotype controls (dotted line). Data are representative of three experiments with similar findings (n=5 mice/samples per experiment). (D) DCs were enriched from tumor cell suspensions using CD11c-microbeads, cultured overnight, then analyzed for expression of MHC class I, MHC class II, CD80 and CD86. Data are representative of two experiments with similar findings (n=5 mice/samples). (E) Normalized MFIs of indicated markers, comparing naïve LNDCs, TDLN DCs, TiDCs directly ex vivo, and following 24 h in culture. Data shown are from a single experiment (n= 5 mice/samples).
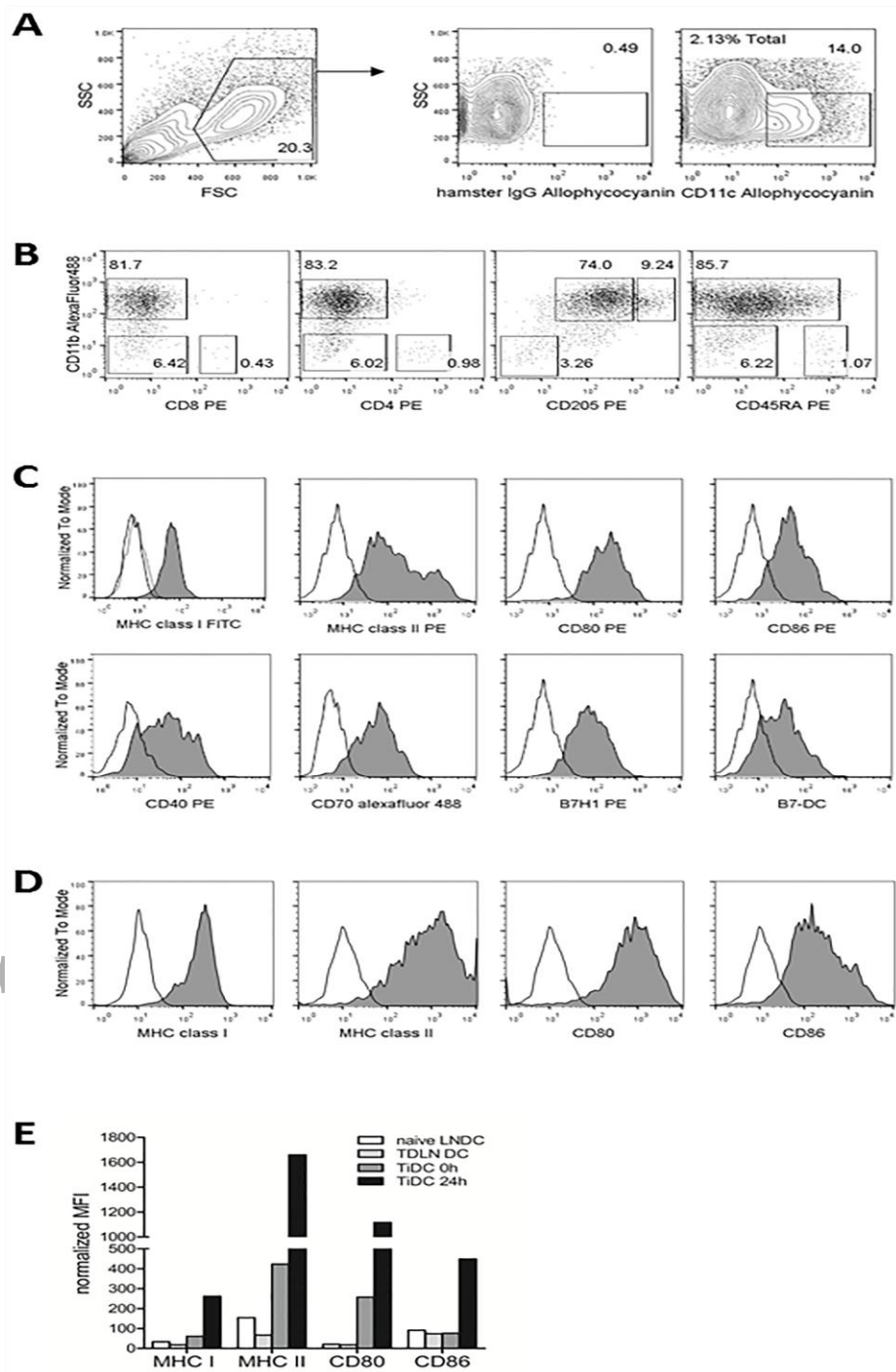
Figure 3. TiDCs do not cross-present tumor antigen at the effector site. (A) CFSE-labeled CD8^{+} CL4 T cells were co-cultured ex vivo with purified CD11c^{+} cells from tumor tissue (upper) and TDLNs (lower) of day 21 AB1HA-bearing mice. Following a 60 h incubation, CD8^{+}CFSE^{+} CL4 T cells were analyzed for proliferation (left), CD69 expression (middle) and IFN-γ production (right). Data are representative of three individual experiments (n= 10-20 mice/experiment). (B) DCs were enriched from tumor tissue (day 21) and normal LNs, using CD11c microbeads and pulsed with increasing concentrations of HA peptide prior to co-culture with CFSE-labeled CL4 CD8^{+} T cells for 60 h and analyzed by FACS. Percentage of gated proliferating CD8^{+} CL4 T cells are plotted according to the concentration of HA peptide used to pulse normal LNDC (■) or TiDC (▲). Data are presented as mean ± SEM (n= 8 mice) and are representative of three independent experiments.
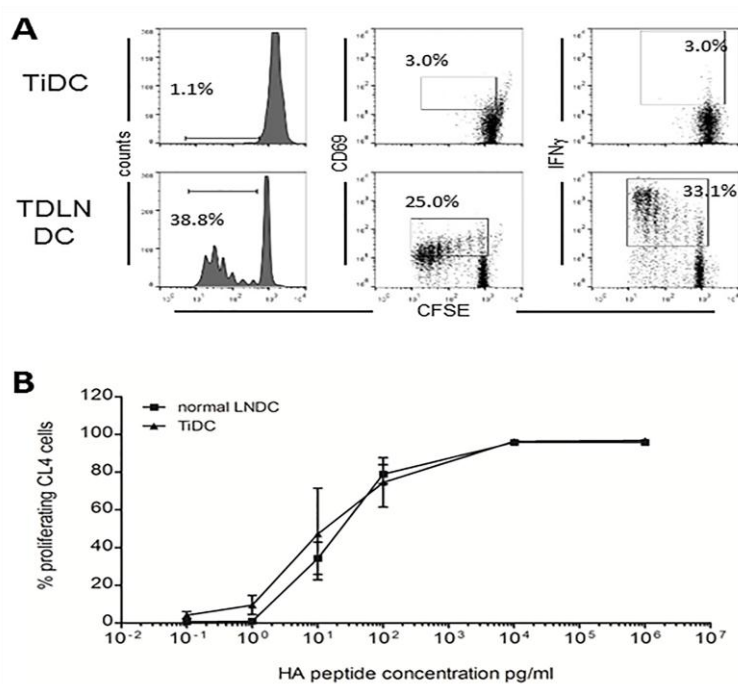
Figure 4. Ex vivo maturation does not restore the capacity of TiDCs to cross-present

Tumor tissue was harvested from day 21 AB1HA-bearing mice, pooled and digested. Axillary and inguinal lymph nodes were harvested from naïve BALB/c mice to serve as normal controls. DCs were enriched from tumor and lymph node suspensions using CD11c microbeads and incubated in the presence of 1 μg/mL LPS, 50 μg/mL pI:C, 10 μg/mL FGK45, 20 ng/mL IFN-γ or without stimulation for 24 h. Normal LNDCs were pulsed with 0.1 ng/mL HA-peptide. DCs (2.5×10^4) were then co-cultured with 5×10^5 CFSE-labeled CD8$^+$ CL4 T cells for 60 h. (A) Representative CFSE profiles of proliferating CD8$^+$ CL4 T cells. Gated on CFSE$^+$CD8$^+$ cells. (B) Percentage of gated proliferating CD8$^+$ CL4 T cells induced by individual stimuli are plotted against the DC fraction. Data are shown as mean ± SEM (n= 6-8 samples) and are pooled from three independent experiments. **p<0.01; Student's t-Test (paired).
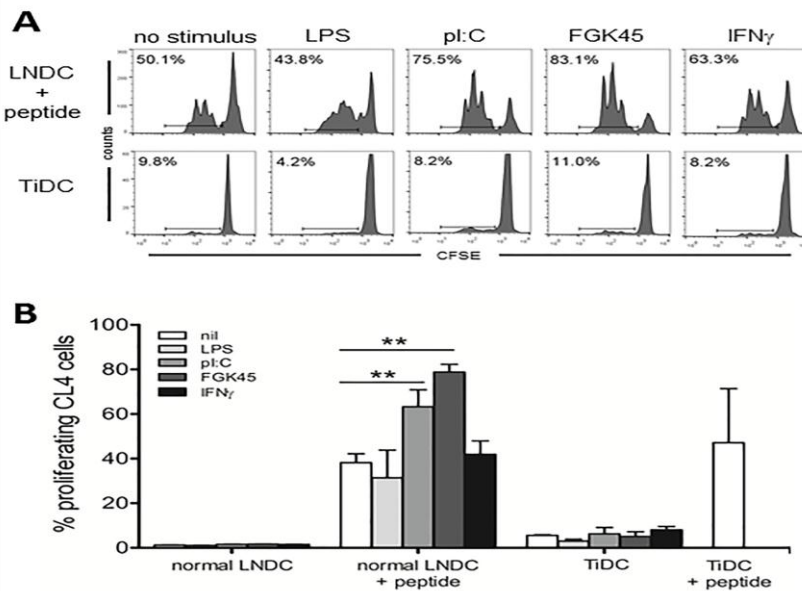
Figure 5. TiDCs are able to take up and process exogenous cell-bound and soluble protein antigen Tumor tissue was harvested from day 21 AB1HA-bearing mice, pooled and digested. TDLNs were collected from the same AB1-HA-bearing mice, pooled and digested. Lymph nodes and spleens collected from naive BALB/c mice served as normal control tissue. DCs were enriched from TDLNs, tumor tissue, normal LNs and spleens using CD11c microbeads and co-cultured for 0 or 24 h with CFSE-labeled live, UV-treated or heat-killed AB1HA tumor cells. Cells were washed extensively and stained for CD11c expression before FACS analysis. (A) DCs were gated on CD11c as indicated. Histograms show acquisition of CFSE label by DCs at 24 h (filled) compared with 0 h (solid line). Shown are representative histograms depicting acquisition of CFSE label from live, UV-treated and heat-killed AB1HA tumor cells by the different DC groups. Data shown are representative of three individual experiments, each with ten pooled mice. (B) DCs were enriched from tumor tissue, normal LNs and normal spleen then incubated with or without 100 μg/mL DQ-OVA for 2 h at 4°C (upper panel) or 37°C (lower panel). Cells were washed and analyzed by flow cytometry. Histograms are gated on CD11c$^+$ cells. Data are representative of three independent experiments (n= 10 mice/experiment). (C) DCs were enriched from tumor tissue and normal LNs then pulsed with increasing concentrations of HA protein prior to co-culture with CFSE-labeled CD8$^+$ CL4 T cells. Percentage of gated proliferating CD8$^+$ CL4 T cells are plotted according to the concentration of HA-protein used to pulse normal LNDCs (■) or TiDCs (▲). Data are shown as mean ± SEM (n= 8 mice/sample) and are representative of two independent experiments. *p<0.05; Students t-test (unpaired). (D) Representative FACS plots of two independent experiments (n= 8 mice) showing the proportion of proliferating

CD8$^+$ CL4 T cells producing IFN-γ following 60 h co-culture with TiDCs and LNDCs incubated with increasing concentrations of recombinant HA protein as indicated.
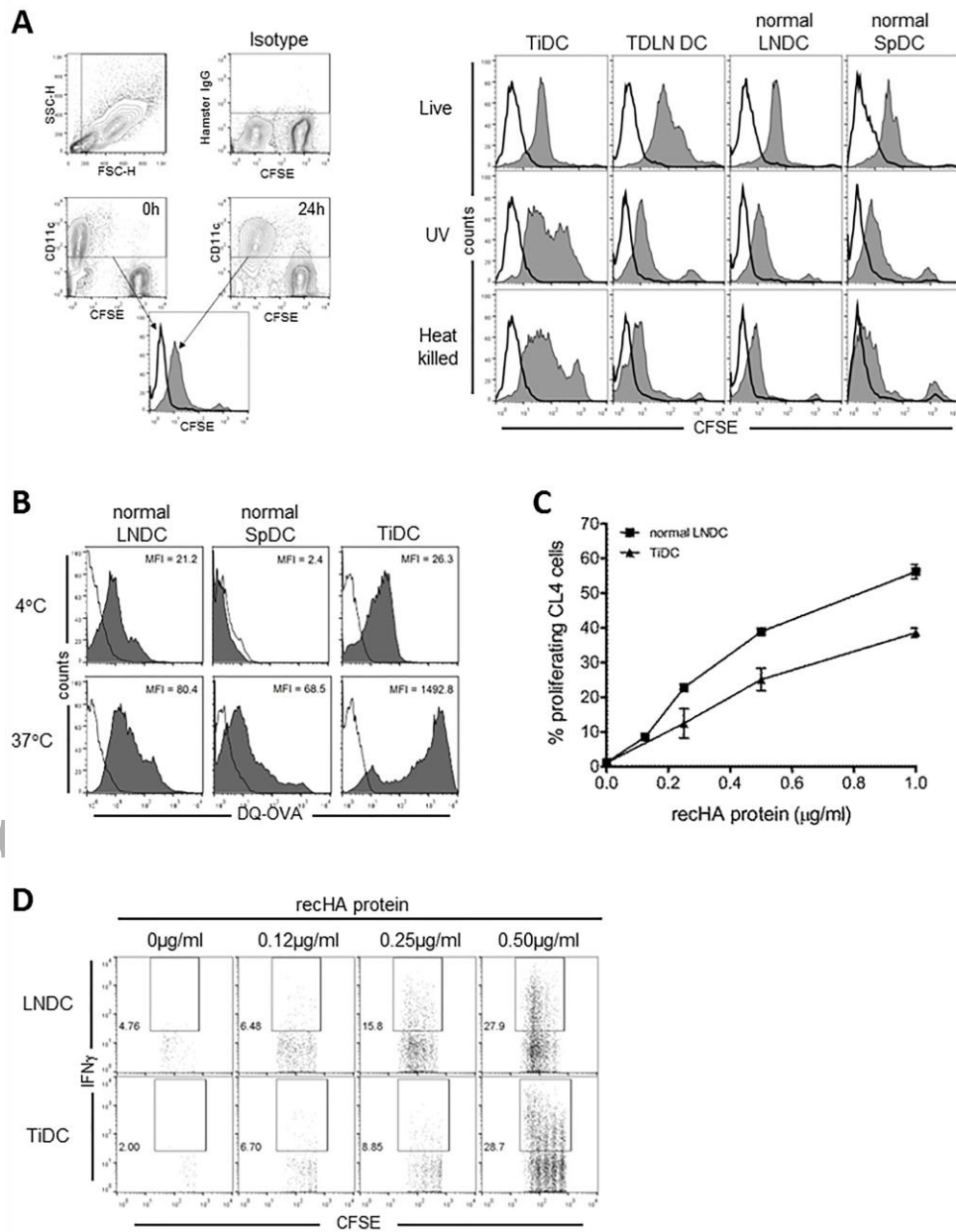
Figure 6. Gemcitabine chemotherapy reverses the reduced capacity of TiDCs to cross-present Mice were inoculated with AB-1-HA tumor cells and treated with one dose of saline or 120 mg/kg gemcitabine i.p. on day 19 of tumor growth. On day 21 tumor tissue was pooled and DCs were enriched. TiDC were then incubated with CFSE-labeled CD8^{+} CL4 T cells. (A) Following 60 h incubation, CD8^{+}CFSE^{+} CL4 T cells were analyzed for proliferation (upper panel), and IFN-γ production (lower panel). (B) Same experiment using DCs from TDLN. (C-D) Tumor cell suspensions of day 21 tumors from gemcitabine- or saline-treated mice were stained for (C) CD11c, CD11b, CD8α, CD4, CD205 and (D) CD11c, MHC class I, MHC class II, CD80 and CD86 expression and analyzed by flow cytometry. (A-D) Data are from one representative experiment of two independent experiments performed ((A-B) n=9 or (C-D) n=8 mice/sample per experiment). (E) Mice were inoculated with AB1-HA cells and treated with one dose of saline or 120 mg/kg gemcitabine i.p. on day 19 of tumor growth. At day 20 post-inoculation 2×10^7 fluorescent beads (1μm) were injected i.t. and 24 h later tumors were harvested and analyzed by flow cytometry for CD11c^{+} bead^{+} cells. Representative plots of CD11c expression versus bead fluorescence (left). Gates indicate the percentage of CD11c^{+} bead^{+} and CD11c^{-} bead^{+} cells. The absolute number of CD11c^{+} bead^{+} cells were counted and normalized by 1×10^5 tumor cells (right). Data are shown as mean ± SEM (n= 3 mice/group) and are representative of two independent experiments.

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A TiDC

saline   GEM
1.93   16.8

5.84   15.2

B Axillary TDLN   Inguinal TDLN

saline   GEM   saline   GEM
24.5   16.8   26.9   22.0

18.5   11.8   21.4   20.2

C
saline
85.1   82.5   73.6   6.45
7.28 0.29   9.14 0.60   7.03

GEM
71.0   69.4   71.2   0.96
21.3 1.85   21.2 2.16   20.2

CD8 PE   CD4 PE   CD205 PE

D
MHC class I   MHC class II

CD80   CD86

E
saline
0.28   1.38

GEM
0.25   0.98