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Research Article

Intratumoral TLR-4 Agonist Injection Is Critical for Modulation of Tumor Microenvironment and Tumor Rejection

Fabio Luiz Dal Moro Maito,[1] Ana Paula Duarte de Souza,[1] Luciana Pereira,[1] Megan Smithey,[2] David Hinrichs,[2] Archie Bouwer,[2] and Cristina Bonorino[1]

[1] *Faculdade de Biociências e Instituto de Pesquisas Biomédicas, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Avenida Ipiranga 6690, 2° Andar, 90680-001 Porto Alegre, RS, Brazil*
[2] *Departments of Molecular Microbiology and Immunology, Veterans Affairs Medical Center, Oregon Health and Science University, Portland, OR 97239, USA*

Correspondence should be addressed to Ana Paula Duarte de Souza, ana.duarte@pucrs.br and Cristina Bonorino, cbonorino@pucrs.br

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The tumor microenvironment shelters a complex network of mechanisms that enables local Immunosuppression to support tumor growth. In this study we found that, B16F10 melanoma growth is inversely correlated with peritumoral infiltrate cell number and with cell numbers in draining lymph nodes. Tumor growth ensued even when a foreign antigen was expressed by B16F10 cells in the presence of naïve specific $CD8^+$ T cells. Treatment with TLR agonists has shown to sometimes result in tumor regression, however, not always with long-lasting effects. We compared the relevance of different injection regimens of lipopolysaccharide (LPS). Tumor growth was arrested only by intratumoral LPS injection after the tumor was already established. This result was accompanied by a dramatic change in DC activation inside the tumor. Intratumoral LPS also enhanced antigen presentation and tumor-specific $CD4^+$ T cell production of IFN-γ. Injection of LPS before tumor challenge or codelivery of tumor cells and LPS did not have any effect on tumor progression. Our results suggest that an efficient antitumor immune response leading to tumor regression can be achieved with proper TLR4 activation inside the tumor tissue, impacting the tumor microenvironment. These findings are relevant for the design of treatment for patients with malignant melanomas.

1. Introduction

Tumor immunity includes both innate and adaptive immune responses against malignant cells. Infiltration of immune effector cells occurs early in the course of tumor growth, and in some cases cell infiltration correlates with disease outcomes [1–3]. However, the immune system is not always able to circumvent tumor growth. Besides the fact that most tumor antigens are in fact self-antigens, the additional lack of stimulatory signals provided by tumor cells, does not favor proper activation of dendritic cells (DCs) [4] necessary to initiate a T cell response. In addition, tumors have been shown to create an immunosuppressive environment. Several mechanisms have been described by which tumors can suppress the immune system, including secretion of cytokines, alterations in antigen-presenting cell subsets, expression of costimulatory and coinhibitory molecules and changes in ratios of regulatory T cells (Tregs) to effector T cells, and it is abundantly documented that these mechanisms of immunosuppression can impair tumor specific immune responses [5].

Different studies have shown that Toll-like-receptors (TLR) activation is a powerful stimulus to overcome tumor specific T cells tolerization [6]. This is an observation that can be traced to the work of the surgeon William Coley in 1910 [7], back when neither TLRs nor DCs were known. Several studies have focused on using different TLRs agonist, such as TLR9, TLR3, TLR7, and TLR4 ligands, alone or in association with other immune-stimulatory molecules, in order to control tumor grown [8–10] lipopolysaccharide

(LPS), a TLR-4 ligand, has been known for decades to have antitumor effects when administered systemically to tumor-bearing rodents or human patients with cancer [11–14]. Early studies have shown that complete tumor regression after LPS administration requires an immune response involving T cells [11], suggesting even then that its effects were not merely due to activation of cytotoxic mechanisms in macrophages. However, systemic administration of LPS, as performed in several studies, is far from an ideal treatment, due to the cytokine storm it generates, with various side effects. Recent studies have demonstrated that LPS has antitumor properties when administered intratumorally in a subcutaneous murine glioma model [15–17]. It is still unclear, though, if the effect is mediated mainly by the activation of tumor-specific cells in the draining lymph nodes or by the modulation of the cells infiltrating the tumor.

In this study we investigated the effect of intratumoral LPS injection in the control of murine melanoma B16F10. We verified that even when the tumor is expressing a foreign antigen (OVA or EaRFP), antitumor T cell responses can be antagonized, leading to tumor growth. Our results suggest that this process can be reversed by activation of tumor infiltrating cells using a TLR4 agonist, but not by previous activation of local or lymph node cells. These results are relevant for the design of novel antimelanoma therapies.

2. Materials and Methods

2.1. Mice and Tumor Cell Lines. Six-to eight-week-old C57BL/6 (H-2b) mice were purchased from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS), Porto Alegre, RS, Brazil. OT-I TCR transgenic mice encoding a T-cell receptor (TCR) specific for the OVA epitope (SIINFEKL-H2K^b) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained in specific pathogen-free conditions at 22°C under controlled light (12 h light/12 h darkness), and allowed free access to water and food. B16F10 melanoma cells, a kind gift from Dr. Peter Henson (National Jewish Center for Immunology, Denver, CO, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM—Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS—Gibco, Carlsbad, CA, USA), Gentamicin 80 mg/L (Novafarma, Anápolis, Brazil), Fungisone-Anfotericin B, 5 mg/L (Bristol Myers Squibb, New York, NY, USA), 55 μM 2-Mercaptoethanol (Sigma, St. Louis, MO, USA), 2 mM L-Glutamine (Sigma), and 1X MEM amino acid solution (Invitrogen Corporation, Carlsbad, CA, USA). The OVA-expressing melanoma cell line (B16OVA) was maintained in DMEM plus 10% fetal calf serum (FCS) and 100 μg/mL G-418 (Geneticin-Invitrogen, Carlsbad, CA, USA). The current study was approved by the Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Brazil and of Veteran Affairs Medical Center. Experimental procedures followed the recommendations for Animal Care and Use by the Ethics Committee. Some experiments were performed at PUCRS, Brazil and some at the Veteran Affairs Medical Center, Portland, OR, USA.

TEa mice expressing a transgenic specific TCR for the Ea58-62:I-A^b complex obtained from Dr. Marc Jenkins (University of Minnesota, Mn), TEa CD4$^+$ T cells express Thy1.1 (CD90.1) and Vβ6 and recognize the I-E$^d\alpha$-chain peptide 52–68 (pEa) presented by I-A^b. B16F10 cells were transfected with plasmid pDsRedEa encoding the fusion protein DsRed and Ea peptide (pEa) using lipofectamine 2000 (Invitrogen, Carlsbad, CA.). Transfectants were selected and maintained with 500 μg/mL of G418 (Geneticin-Invitrogen).

2.2. Tumor Injections and OT-I and TEa Cell Transfer. Naive C57BL/6 mice were inoculated subcutaneously with 5×10^5 B16F10 melanoma cells in the right thigh, in 100 μL of phosphate-buffered saline (PBS), and 100 μL of PBS at the left thigh as a negative control. For all animal experiments, tumor size was measured every 3 days. Mice were killed in a CO_2 chamber at days 3, 6, 9, and 12 after tumor injection in order to harvest the draining inguinal LNs. For the adoptive transfer experiments, CD8$^+$ OT-I cells were obtained from spleen of OT-I mice and purified by negative selection with EasyStep Magnet Kit (StemCell Technologies, Vancouver, BC, Canada). Twenty-four hours before tumor injection, 2×10^4 CD8$^+$ OT-I cells were transferred i.v. into C57Bl/6 mice, and mice received either 5×10^5 B16FOVA cells subcutaneously on both thighs, or 5×10^5 B16F10 cells. To test the importance of lipopolysaccharide (LPS, Sigma, St. Louis, MO, USA) activation of DCs, mice did not receive any cells, but were divided in five groups: PBS only injection; B16F10 cells in PBS; coinjection of B16F10 cells with 500 ng LPS; LPS (500 ng) injection at the tumor site 18 hours before tumor injection; intratumoral LPS (500ng) injection, 5 days after tumor injection. Alternatively, mice were adoptively transferred with 5×10^4 TEa cells and 24 h later injected with 5×10^5 B16EaRed cells or PBS.

2.3. Histology. Tumor samples were either snap-frozen in Tissue-Tek OCT (Sakura Finetechnical Co. Ltd., Tokyo, Japan) or fixed in formaldehyde 10% for 24 hours and embedded in paraffin wax. Paraffin embedded tissue blocks and frozen samples were cut semiserially, 7 μm-thick slides at 200–500 μm intervals. Standard procedure for haematoxylin and eosin (H/E) staining was done in paraffined sections and immunofluorescence staining was performed in sections of frozen samples. For immunostaining, the commercially available signal amplification technique that is effective for amplifying the fluorescent signal (TSA Evaluation kit, PerkinElmer, USA) was used. Briefly, slides were fixed in acetone, rehydrated with PBS, and the endogenous peroxidase was blocked for 10 minutes with 3% H_2O_2 + 0,1% azide in PBS. Fc receptors were blocked with 5% sheep serum in PBS 1X for 15 minutes. Avidin/biotin blocking was done using the respective buffers provided by TSA Kit, 10 minutes each. At this time, the sections were incubated with biotinylated anti-CD4, anti-CD8, anti-CD11c (Bekton Dickinson, Hyalea, FL, USA), and anti-Foxp3 (e bioscience) diluted in TNB buffer provided in the TSA Kit during 30 minutes. Streptavidin, also from TSA Kit, was incubated for 30 minutes. To amplify the signal, CY3 was added for 10 minutes.

(Cyanine 3 System, which combines the high fluorescence of cyanin dyes with the detection sensitivity of TSA technology). Nuclear staining was performed with Hoescht 33342 (Invitrogen Corporation, Carlsbad, CA, USA), for 2 minutes and the slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Between steps, sections were rinsed 4 times with PBS 1X.

2.4. Quantification of Tumor Area and Cell Subsets. H/E and immunofluorescence sections were analyzed at 400x magnification, and tumor area and peritumoral infiltrate were quantified, capturing 8 fields equidistantly surrounding the whole tumor section. H/E sections were photographed with a Sony (Tokyo, Japan) CCD-Iris camera, connected to an Olympus (Tokyo, Japan) BX50 microscope. Infiltrating lymphocytes were identified by morphology according to an experimented pathologist and all of them were counted in each analyzed microscopic field. The software used to capture and count the cells was the Image Pro-plus version 4.1.5 (Media cybernetics, Bethesda, MD, USA). Immunofluorescence sections were captured by ZEISS (Oberkochen, Germany) Axioskope 40 microscopic equipped with a CoolSNAP-PROef color camera coupled to Image Pro-Plus software.

2.5. Lymph Node or Tumor Analysis by Flow Cytometry. Cell suspensions were prepared from tumor draining lymph nodes (LNs) or tumor mass by mechanical disruption on 70 μm nylon cell strainers and incubated with collagenase D (Roche, Basel-Switzerland). Fc receptors were blocked (24G2 cell supernatant supplemented with 5% mouse serum and 5% rat serum) for 15 minutes and cells were stained for 30 minutes on ice with antibodies anti-CD4 PE, anti-CD8PacBl, anti-CD11c APCCy7, anti-CD86 FITC, and anti-B220 PE-Cy5.5, in 100 μL of PBS containing 1% FBS, 0.1% sodium azide. All the data were collected on FACScan or LSR II (BD Biosciences, San Diego, CA, USA) and analyzed with FlowJo software (TreeStar, San Carlos, CA, USA). TEa cells were stained with anti-CD90.1, anti-CD4, anti-INF-γ, and anti-CD44$^+$ cells. The peptide-MHC II complex derived from processing of the Ea antigen by dendritic cells was visualized using the YAe antibody.

2.6. RT PCR. RNA was extracted from 10^6 B16F10 cells using Trizol (Gibco) according manufacturer's guidelines recommendation. cDNA was synthesized using SuperScript III kit (Invitrogen). PCR reaction was performed using *Taq* DNA Polimerase (Invitrogen) and the oligonucleotides forward 5′ ATATGCATGATCAACACCACAG 3′ and reverse 5′ TTTCCATTGCTGCCCTATAG 3′ (Sigma).

2.7. Statistical Analysis. The Two-Way ANOVA test was used to compare differences among more than two groups. Differences between two groups were tested by t-tests. Statistical analysis and graphs were performed using GraphPad Prizm version 4.0 (GraphPad Software, San Diego, CA). Values of $^*P < 0.05$ were considered statistically significant.

3. Results

3.1. Tumor Growth Correlated Inversely with Infiltrate at the Tumor Site. The presence of tumor infiltrate has been associated with good [2] or poor [1] prognosis, nonetheless it is now understood that it is the nature and state of activation, rather than number of infiltrating cells that actually influences the outcome. When we evaluated B16F10 melanoma growth, tumor histology showed that the subcutaneously injected B16F10 cells formed a considerable tumor mass, presenting a clear borderline dividing between tumor and surrounding connective tissue containing abundant infiltrate by immune cells (Figure 1(a)), which was absent in the control thigh (sham treated) (Figure 1(b)). Figure 1(c) is an H/E stain of the frozen tumor, basis for the immunofluorescence stain, which revealed that the tumor was infiltrated by DCs (CD11c$^+$ cells, Figure 1(e)), CD8$^+$ cells (Figure 1(d)), and CD4$^+$ cells (Figure 1(f)). We also analyzed the Foxp3$^+$ cells inside the tumor. The results shows that the number of Foxp3$^+$ cells increases over time as tumor grows, demonstrated on Figure 1(g) (6 days of tumor growth) and Figure 1(h) (9 days of tumor growth). Tumor mass started to grow faster on day 6 after injection (Figure 1(i)). This growth was inversely correlated with peritumoral infiltrate (Figure 1(j)). Altogether, these results indicated an immune response taking place at the tumor site, however that response seemed to gradually decrease, while the tumor mass increased in size.

3.2. Tumor Growth correlated with Decreased CD86 Expression by Lymph Node DCs. To further investigate the apparently inefficient response at the tumor site we analyzed immune cell subpopulations at different times in the tumor draining lymph node (TDLN). The results, shown in Figure 2, revealed that the frequency of all investigated cell types seemed to be decreasing to levels approaching normal in the TDLN as the tumor grew in size (Figures 2(a)–2(e)), this including B cells, CD4$^+$ and CD8$^+$ T cells, and DCs (CD11c$^+$ cells). A marked down regulation in CD86 levels in TDLN DCs, however, was observed to correlate with tumor growth (Figure 2(f)). Such alterations were not observed in the control lymph node, where PBS had been injected at the corresponding site (Figures 2(a)–2(f)). These results suggested that an antitumor immune response had been activated at the draining lymph node, but was subsiding at the same time the tumor mass increased in size. They also pointed to an important effect of tumor cells on the activation state of DC in the TDLN, as the same time that there appears to be a gradual cessation of cell proliferation in, or cell traffic to, the TDLN.

3.3. Tumor Growth Occurred regardless of the Generation of a Specific Cytotoxic T Cell Response. Previous studies have attributed the inefficiency of immune responses against B16F10 tumors to a low immunogenicity of this melanoma [18]. Melanoma tumors are known to express antigens that usually induce weak immune responses of short duration [19]. Indeed, due to the self-nature of most tumor antigens, individuals would be not be expected to mount strong

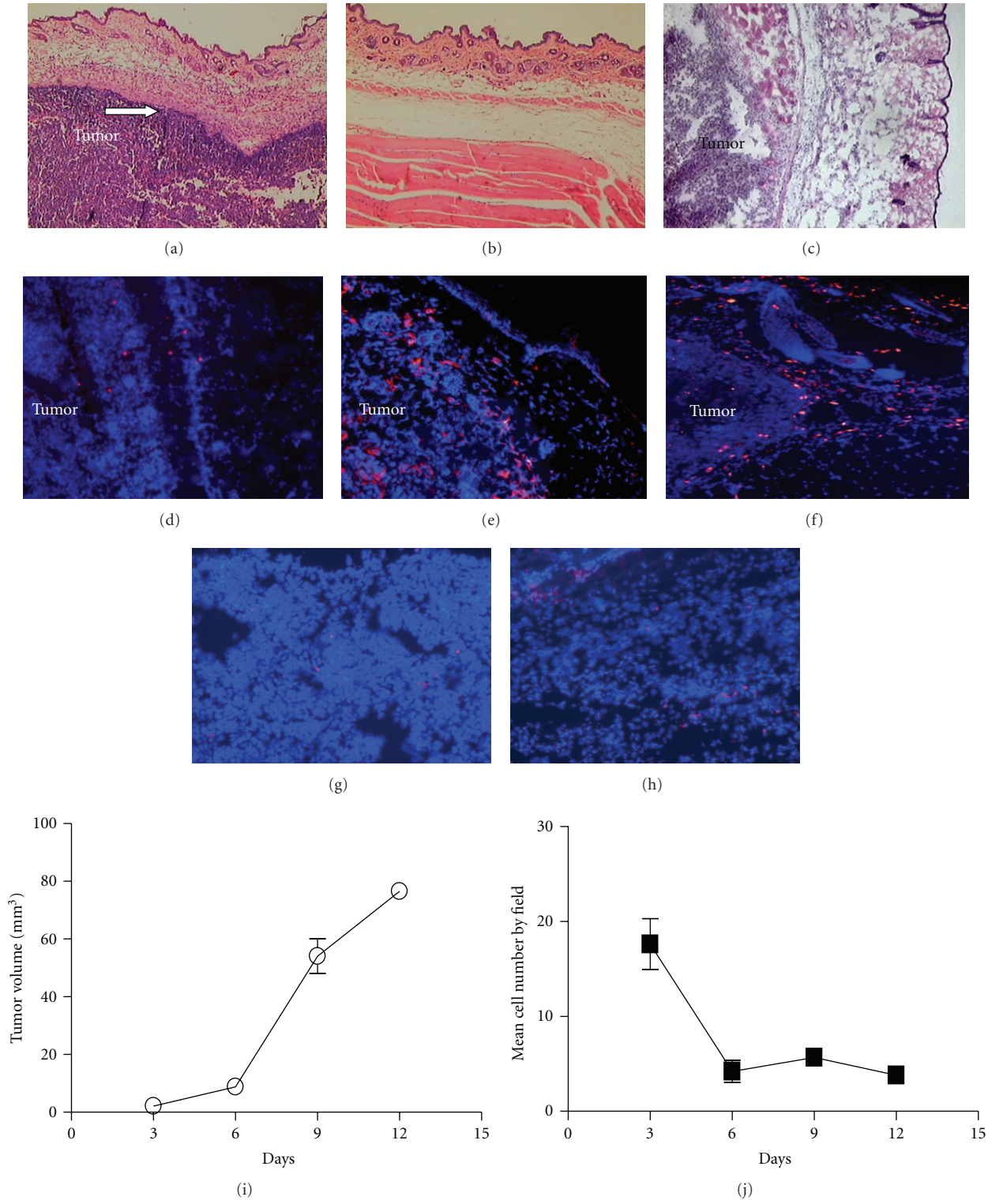

Figure 1: Peritumoral infiltrate in mice subcutaneously injected with B16F10. (a) H/E paraffin skin section of B16F10, day 9 of tumor grown in mice. (b) H/E section of PBS injection site in control mice without tumor. (c) H/E frozen section of injected tumor, used as basis to immunofluorescence. (d), (e), and (f) Immunofluorescence (Red) staining of frozen tumor to CD8 (d), CD11c (e), and CD4 (f); counterstained whit Hoechst. (g) and (h) Immunoflouresce staining of frozen tumor to Foxp3, day 6 of tumor grown (g) and day 9 of tumor grown. (i) Tumor measurements over time. (j) Number of lymphocytes per field, at each tumor measurement timepoint. Data are representative of five different experiments, with $N = 4$ mice per group.

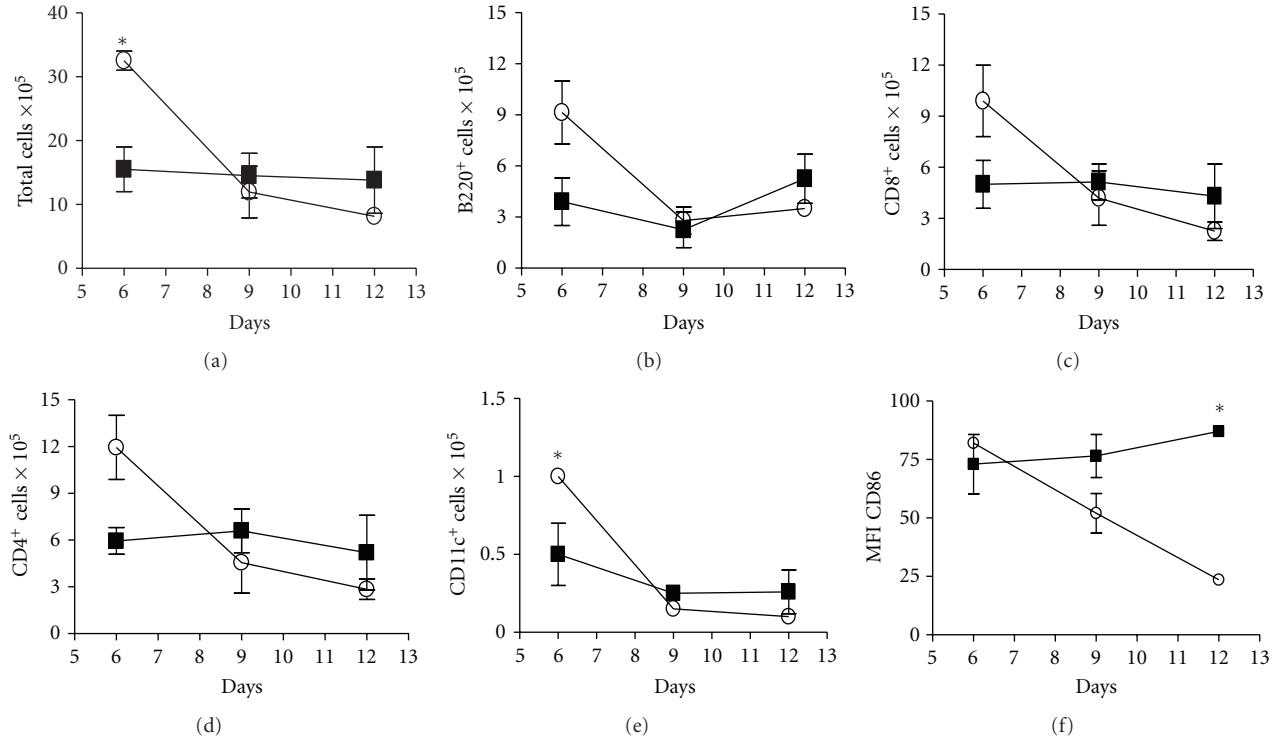

Figure 2: Flow cytometry analysis of draining LN 6, 9, and 12 days after B16F10 and PBS injection. Lymph nodes cells were stained with antibodies for B220, CD8, CD4, and CD11c, and CD86 (in $CD11c^+$ cells). (a) Total lymph node cells were counted. (b)–(e) Total number of positive cells for each staining (f) Mean CD86 fluorescence in $CD11c^+$ cells. Tumor, (◯) and PBS, (■) lymph nodes. Values of $^*P < 0.05$ were considered statistically significant. Data representative of three different experiments, $N = 3$.

antitumor responses, due to being centrally tolerized against them [20]. In order to test whether or not tumor elimination could be achieved by an increase in the precursor frequency of antitumor cells, we adoptively transferred 2×10^4 naive OVA-specific OT-I cells into C57BL6 mice. One day later, mice were subcutaneously injected with either B16F10 cells or B16OVA cells. Tumor growth was recorded every other day, and OT-I cells analyzed for differentiation into an effector phenotype.

The results are shown in Figure 3. As expected, the B16F10 tumor grew in spite of the OT-I cells (Figure 3(a)). B16OVA tumor growth, however, was clearly slowed down by the naïve OT-I cells, however the tumor resumed growth at the same time OT-I cells contracted in the LN—day 9 (Figures 3(a) and 3(b)). Analysis of surface markers in OT-I cells revealed that CD44 was upregulated in these cells (Figure 3(c)), suggesting their differentiation into an antigen experienced phenotype. Taken together the results suggested that although an increase in precursor frequency of tumor specific $CD8^+$ T cells could delay tumor growth and lead to antitumor effector cell differentiation, these cells were not capable of stopping tumor growth.

3.4. Intratumoral LPS Injection after 5 Days of Tumor Growth Can Lead to Tumor Rejection. One of the main difficulties in generating anti-tumor responses is that they do not possess endogenous ligands that stimulate toll-like receptors (TLR) the same way pathogens do. Other studies have described that injection of TLR ligands can enhance antitumor responses [6], however systemic injections of such ligands can have undesired systemic effects. Therefore, we asked if a local injection of TLR4 agonist LPS could activate a local antitumor response, which could have a negative impact on tumor growth. We questioned if it would be more effective to activate immune cells before the tumor was present, at the same time tumor was injected, or after the tumor was already established. We thus injected B16F10 cells subcutaneously in mice and provided LPS either together with the tumor (in the same injection), 18 h before the tumor was injected, or intratumorally, on day 5 after tumor injection, when tumor started to grow faster and tumor mass was clearly established as histologically demonstrated in Figure 1. To our surprise, the only treatment that resulted in the interruption of tumor growth was the intratumoral injection of LPS on day five after tumor injection (Figure 4(a)). It resulted in complete elimination of the tumor, 12 days after tumor injection. Interestingly, intratumoral LPS injection on day 5 was also the only treatment able to lead to cell number increase in the TDLN (Figure 4(b)). We also confirmed that B16F10 express TLR4 by RT PCR as demonstrated in Figure 4(c).

3.5. Intratumoral DCs, but Not TDLN DCs Are Activated after 6 Hours of Intratumoral LPS Injection. We had previously observed (Figure 2(f)) that CD86 expression in TDLNs decreased from day 5 (mean = 82) to day 12 (mean = 23.5).

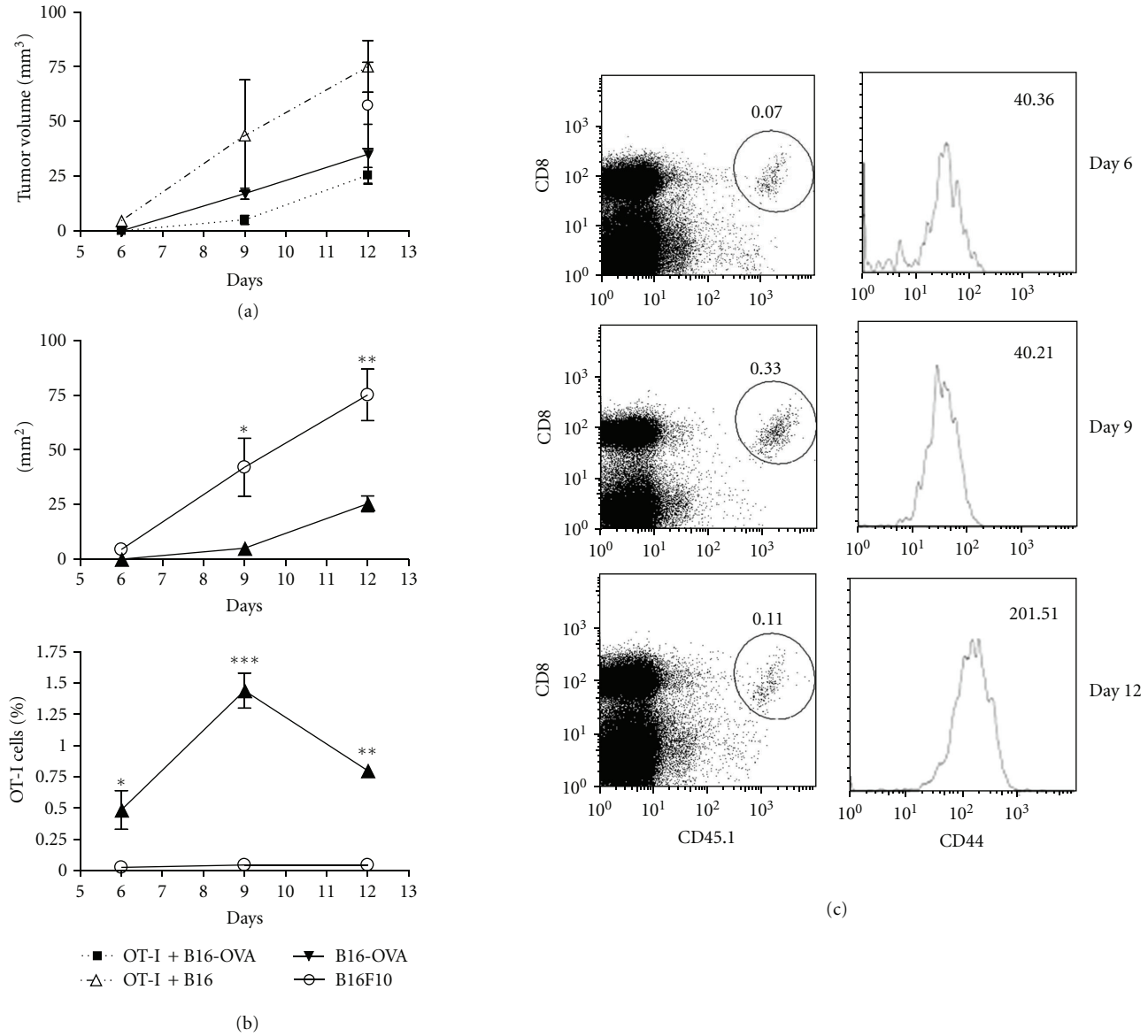

Figure 3: B16OVA tumor growth and OT-I T cell differentiation. (a) B16F10 (◯) and B16OVA (▼) tumor measurements in OT-I injected mice. (b) Percentage of cells recovered from LN of OT-I transferred mice and injected with B16OVA (▼) and B16F10 (◯) cells. (c) CD44 expression in OT-I cells recovered from LNs. Numbers on the upper right corner of histograms represent mean fluorescence ($n = 3$) for each marker. Values of $^*P < 0.05$ were considered statistically significant. Data representative of two different experiments, $N = 4$ mice per group.

It was possible that intratumoral LPS could act modulating activation of DCs inside the tumor or in the draining lymph node. To investigate this hypothesis, we repeated the intratumoral LPS injection on day 5 of B16F10 growth, and analyzed the response in the draining lymph node. Upregulation of CD86 in splenic DCs after intravenous LPS injection is observed after six hours, declining after 18 h [21]. In our experiment, six hours after LPS injection, the tumor mass and the draining lymph node were removed, and single cell suspensions were obtained for analysis. We verified that the major alteration in MFI of CD86 following LPS injection occurred in $CD11c^+$ cells inside the tumor mass rather than the ones in the lymph node (Figures 5(a) and 5(b)). This increase in MFI of CD86 occurred only in $CD11c^+$ cells, suggesting that DCs become activated by LPS rather than macrophages ($CD11c^-$ cells), that could also express CD86 (Figure 5(a)). When we compared the number of T cells inside the tumor, we found a two-fold increase in $CD4^+$ and $CD8^+$ T cells in the group that received LPS compared to control group inside the tumor but not in TDLN (Figures 5(c) and 5(d)). These results suggested that intratumoral injection of LPS leads to a rapid activation of DCs and infiltration of T cells inside the tumor, correlating with subsequent tumor rejection.

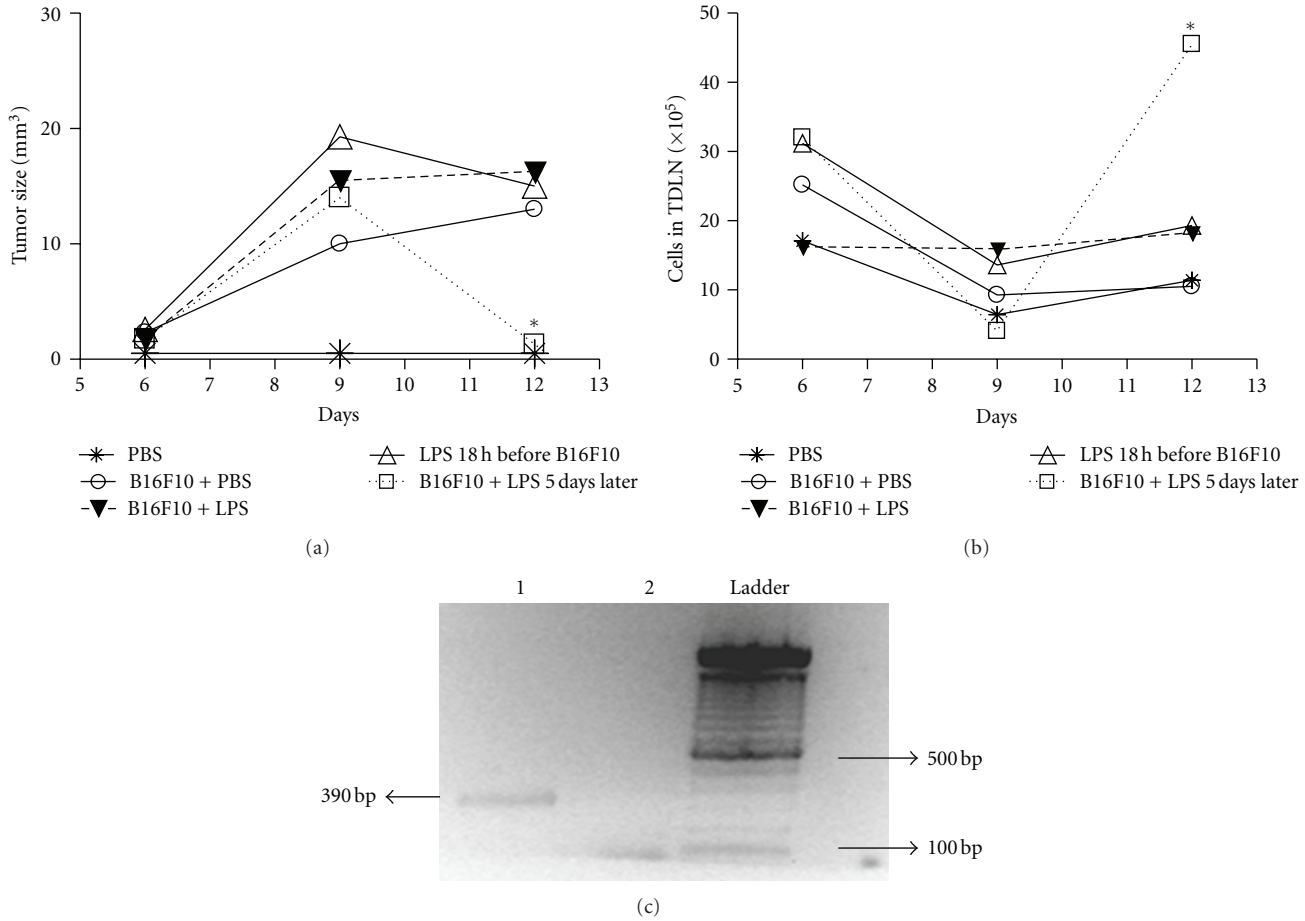

Figure 4: Effects of LPS injection. (a) Tumor measurements on different timepoints. (b) Total lymph node cells in mice with B16F10 injection (◯), Injection of LPS at the same time of B16F10 injection (▼); Injection of LPS 18 hours before B16F10 injection (△); Intratumoral LPS five days after B16F10 injection (□) and PBS injection (∗). Values of $^*P < 0.05$ were considered statistically significant. Data representative of three different experiments, $N = 4$ mice per group. (c) TLR4 expression on B16F10 cells by RT PCR. Agarose gel eletrophoresis analysis of PCR products Lane 1 B16F10, cDNA Lane 2 Negative control, Lane 3 Ladder 100 bp (Invitrogen).

3.6. Intratumoral LPS Injection Can Augment T Cell Response and Antigen Presentation. To investigate the impact of this treatment in the tumor specific T cell response, we performed adoptive transfers of TEa cells (5×10^4) in C57Bl/6 and 24 h later injected the with the B16EaRed tumor or PBS; on day 5, some of the mice injected with tumor received an intratumoral LPS injection, and 48 h later we analyzed the DLN. We observed that intratumoral LPS injection led to increased antigen presentation (Figures 6(a) and 6(b)) by APC, compared to the animals that only received tumor cells. It also resulted in a four-fold increase in INF-γ producing TEa cells (Figures 6(c), 6(d) and 6(e)), compared to what was observed for the B16EaRed tumor injected animals that were not injected with LPS.

4. Discussion

A major concern of cancer immunotherapy is the development of effective treatment protocols for established tumors that will induce strong immune responses and counteract local immunosuppressive mechanisms. We observed in this study that the kinetics of melanoma growth reproducibly correlated with reduction of intratumoral infiltrate as well as of draining lymph node cellularity, suggesting that the initial immune response leading to infiltration was gradually suppressed. Various mechanisms have been proposed to account for this phenomenon, such as loss of MHC class I expression by tumor cells, contributing to the ability of cancer to avoid recognition by T cell-mediated immunity [22], or yet alterations in cell traffic between the tumor and the tumor draining lymph node, as a mechanism of immunosuppression [23]. Ishida et al. [24] observed that Langerhans cells lost their ability to migrate to the draining LN in the presence of malignant tumors. DCs have been reported by other studies to be a major subpopulation infiltrating malignant tumors and to sometimes develop a regulatory phenotype, secreting immunosuppressive cytokines such as IL-10 and TGF-β [18]. These immunosuppressive cytokines can convert $CD4^+CD25^-$ T cells into Treg [25]. In our study, we found that only a small percentage of the abundant $CD4^+$ T cells infiltrating the tumor were $Foxp3^+$ (not shown), suggesting that this melanoma employs more than one

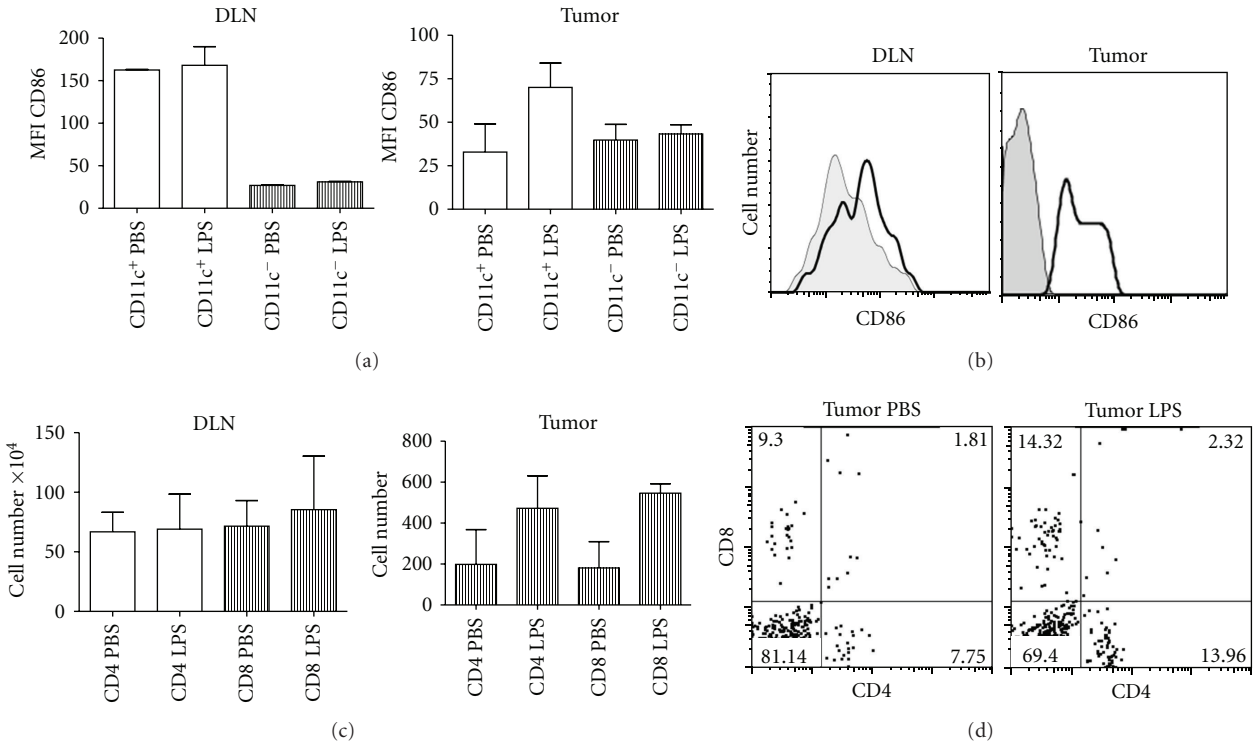

Figure 5: Effects of intratumoral LPS injection. B16F10 cells were subcutaneously injected into mice, and after 5 days of tumor growth LPS was intratumorally injected. Six hours after LPS injection the tumor mass and the tumor draining lymph nodes (DLN) were removed for analysis. One group received PBS instead LPS as a control. (a) MFI of CD86 in $CD11c^+$ cells or $CD11c^-$ cells, on tumor DLN (right graph) or tumor site (left graph), with or without LPS treatment (b) Histogram of CD86 expression by $CD11c^+$ cells in tumor DLN (right graph) or tumor site (left graph) of mice with PBS (gray filled histogram) or LPS treatment (black line histogram). (c) Numbers of $CD4^+$ or $CD8^+$ T cells on tumor DLN (right graph) or tumor site (left graph) with or without LPS treatment. (d) Dot plot showing intratumoral $CD4^+$ or $CD8^+$ cells after treatment with LPS or PBS. Data representative of two different experiments, $N = 3$ mice per group.

immunosuppressive strategy in order to grow. Infiltrating DCs may also modulate immune response by inhibiting production of other cytokines or surface molecules involved in cell-mediated responses [26]. We observed that the infiltrating DCs expressed virtually no CD86, and CD86 downregulation by DCs in the draining lymph nodes observed by us was also observed by others [24]. Our results support previous studies on local immunosuppression exerted by tumors on the host's immune response [5, 20, 26, 27].

Increasing the precursor frequency of naïve OT-I $CD8^+$T cells did slow down tumor growth, but it was not sufficient to prevent it completely. Other studies [28, 29] have not verified any impact of naïve OT-I cells transfer on B16OVA growth, concluding that tumor specific T cells needed to be preactivated in order to show any antitumoral effect *in vivo*. Their results could be related to the high number of T cells that were transferred. Adoptive transfers of high numbers of $CD8^+$ T cells lead to poor proliferation and differentiation when compared to low transfer numbers [30]. By transferring only 2×10^4 OT-I cells, a low frequency number more similar to physiological conditions, we might have favored a better antitumor immune response, even though not sufficient to permanently counteract tumor immunosuppressive mechanisms.

Interestingly, tumor growth could be reversed by intratumoral LPS injection, activating the infiltrating cells inside the tumor and increasing cell numbers in the tumor draining lymph node, suggesting that these are the effector cells in the antitumor response. Other studies have showed the importance of using a TLR4 agonist to achieve tumor regression [14, 31–33]. However, most of these studies used other immune-stimulatory molecules associated with TLR ligands [17], or rather used more than one dose of the agonist [15]. In our system, a single intratumoral injection, five days after the tumor was established, was able to result in tumor rejection, increasing local DCs activation and $CD4^+$ and $CD8^+$ T cells infiltration. Infiltrating DCs have been reported to be able to induce antitumor T cell responses when stimulated *ex vivo* with the antigenic peptide [34], rapidly acquiring a mature phenotype just by manipulation, and by intratumoral activation using CpG, a TLR9 agonist [35]. Although LPS is a potent activator of macrophages, in our study we did not verify an increase in CD86 in $CD11c^-$ cells, but rather observed increased CD86 expression by $CD11c^+$ cells, suggesting that the LPS effect was mediated mainly by DCs present in the tumor site. A recent study using CpG to reduce tumor growth showed that macrophages were not necessary for therapeutic effects of this TLR ligand [36].

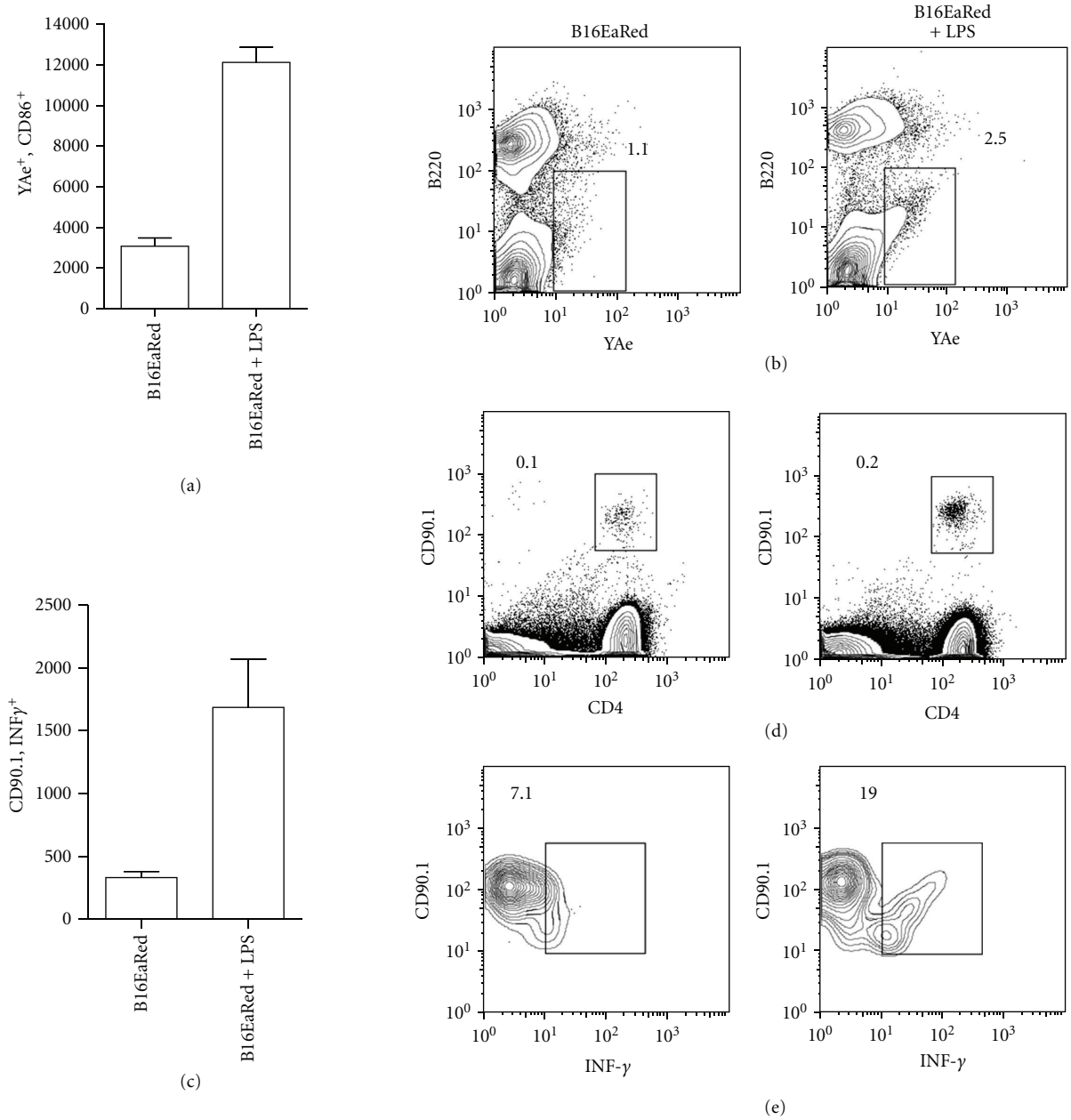

Figure 6: Antigen presentation and TEa differentiation in tumor draining lymph node (DLN) with LPS injection. Mice were adoptively transferred with 5×10^4 TEa cells and 24 h later injected with 5×10^5 B16EaRed cells or PBS. On day 5, some mice received intratumoral LPS injection and 48 h later the DLN were excised and analyzed by FACS. (a) Mean absolute number of YAe^+, $CD86^+$ cells in DLN (gated on $CD11c^+$, $B220^-$ cells). (b) Dot plot showing $CD11c^+$, $B220^-$ cells. (c) Absolute numbers of INF- producing cells (gated on $CD4^+$, $CD90.1^+$ cells). (d) Dot plot showing $CD4^+$, $CD90.1^+$ cells. (e) Dot plot showing cells $CD4^+$, $CD90.1^+$ $IFN\gamma^+$ cells. Numbers in the plots represent mean percentages in gates. Data representative of two different experiments, $N = 3$ mice per group. ($^*P < 0.05$).

Thus, it is likely that intratumoral DCs, rather than other infiltrating cells are a major target for TLR activation, and that the local suppression of tumor infiltrating DCs can be reversed by TLR ligation, which is critical for tumor elimination. Preactivation of immune cells or coimmunization with tumor and LPS were not sufficient to alter tumor development. This could be due to the fact that mature DCs downregulate their ability to take up newly encountered antigen, while they maintain their potential to produce cytokines and modulate surface molecules [37–39]. By injecting LPS before tumor cells, we probably induced the maturation of the DCs, which could no longer efficiently uptake tumor antigens.

It has been shown that tumor cells could express TLR4 [40], and it could be argued that LPS was toxic for the tumor cells. Although we know the tumor cells we used express TLR4, we believe that in our system LPS is probably acting on immune cells, particularly on the DCs that infiltrate

the tumor site, rather than on the tumor cells, because when LPS was given along with tumor cells, even though the B16F10 cells were in direct contact with LPS, the tumor still grew, indicating absence of direct toxic effects of LPS over the melanoma cells. A study that observed toxic effects of LPS over tumor cells attributed such effects to indirect toxicity over endothelial cells [41, 42] rather to direct effects over the tumor cells.

Finally, it is remarkable that in only six hours LPS could not only activate intratumoral DCs but also favor tumor infiltration by T cells. Such findings suggest that the local suppression on tumor infiltrating DC can be overcome by intratumoral LPS, increasing activation of DC presentation of the tumor antigen in DLN, efficiently stimulating anti-tumor $CD4^+$ T cells, which in turn acquire the ability to produce INF-γ. LPS is a pleiotropic agent, and it is likely that the intratumoral injection modulated the expression of local chemokines. Adenoviral delivery of CCL16 in tumors [43] has shown to promote recruitment of macrophages and DCs. Intratumoral injections of CCL20, with and without CpG, recruited circulating DCs and T cells to B16F10 and CT26 masses, leading to tumor rejection [35]. Our results support previous studies that point the tumor site, or tumor vincinity, as a preferred site for injection of activators of the immune response, it was demonstrated that peritumoral injection of CpG was more efficient for recruitment of DCs and tumor rejection than systemic routes [44]. Some others TLR4 agonists are currently under testing [45].

5. Conclusion

Based on the findings herein presented, we propose that an efficient antitumor response can be generated if infiltrating DCs from the tumor can be activated, leading to reactivation of an antitumoral T cell response that has been neutralized by the growing tumor. Tumors that are heavily infiltrated with DC may thus possibly offer a good prognosis for the patients, favoring immunotherapy that would generate antitumor memory, aiding in the prevention of cancer recurrence.

Authors' Contribution

Fabio Luiz Dal Moro Maito and Ana Paula Souza have equally contributed in the execution of the experiments reported in this paper.

Conflict of Interests

The authors declare that they have no conflict of interests.

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