Listeria monocytogenes (*Lm*)-LLO Immunotherapies Reduce the Immunosuppressive Activity of Myeloid-derived Suppressor Cells and Regulatory T Cells in the Tumor Microenvironment

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Summary: Myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg) are major components of the immune suppressive cells that potentially limit the effectiveness of an immunotherapy-based treatment. Both of these suppressive cell types have been shown to expand in tumor models and promote T-cell dysfunction that in turn favors tumor progression. This study demonstrates that *Listeria monocytogenes* (*Lm*)-LLO immunotherapies effect on the suppressive ability of MDSC and Treg in the tumor microenvironment (TME), resulting in a loss in the ability of these cells to suppress T cells. This alteration of immunosuppression in the TME was an inherent property of all *Lm*-LLO immunotherapies tested and was independent of the tumor model. The virtually total loss in the suppressive ability of these cells in the TME was linked to the reduction in the expression of arginase I in MDSC and IL-10 in Treg. The results presented here provide insight into a novel mechanism of *Lm*-LLO immunotherapies that potentially contributes to therapeutic antitumor responses.

Key Words: immunotherapy, tumor microenvironment, immunosuppression, Listeria

(*J Immunother* 2013;36:468–476)

Live-attenuated *Listeria monocytogenes* (*Lm*)-LLO immunotherapies secreting nonhemolytic listeriolysin O-tumor associated antigen (tLLO-TAA) fusion protein have been extensively investigated as a platform for cancer immunotherapy.[1–3] As a bacterium, *Lm* strongly activates innate immunity and has the ability to replicate in the cytosol of antigen-presenting cells (APC) after escaping from the phagolysosome, which requires the virulence factor listeriolysin O (LLO).[4,5] Recently, a nonhemolytic detoxified form of LLO protein has been reported to act as pathogen-associated molecular pattern-like molecule and an effective adjuvant for tumor immunotherapy.[6] Because of its unique life cycle, *Lm* induces potent cytotoxic CD8 and Th1 CD4 T-cell–mediated responses to TAA.[7,8] In preclinical studies, the therapeutic regression of tumors with *Lm*-LLO immunotherapies secreting a tLLO-TAA fusion protein, has been correlated with an increase in the infiltration of T cells (TILs) and reduction in the frequency of regulatory T cells (Treg) in the tumor microenvironment (TME).[3,9,10] However, the effect of *Lm*-LLO immunotherapy on another suppressor population known as myeloid-derived suppressor cells (MDSC) has not been reported previously.

MDSC are a large group of myeloid cells consisting of immature macrophages, granulocytes, DCs as well as myeloid cells at earlier stages of differentiation.[11,12] MDSC are one of the major components responsible for T-cell defects in cancer and contribute to tumor progression.[13] These cells are defined by the expression of different markers in mice and humans. In mice, MDSC express both the myeloid lineage differentiation antigen Gr-1 (Ly6G and Ly6C) and α_M integrin CD11b. Two major populations of MDSC are characterized as: granulocytic $CD11b^+$ $Ly6G^+$ $Ly6C^{low}$ (gMDSC) and monocytic $CD11b^+$ $Ly6G^{low}$ $Ly6C^{high}$ (mMDSC).[14] In humans, MDSC are defined as cells that express CD11b, the myeloid marker CD33, but lack the expression of markers of mature myeloid and lymphoid cells and MHC class II HLA-DR.[15] Published reports show a positive correlation between elevated levels of the suppressor cells and tumor burden.[15] In addition, MDSC can modulate the de novo development and induction of Treg in the TME.[16] This study provides evidence that *Lm*-LLO immunotherapies reduce the frequency and suppressive ability of both MDSC and Treg in the TME. A virtually complete loss of suppressive activity in these cells was found to be associated with the reduced expression of arginase I in MDSC and IL-10 in Treg. The results presented here highlight an important mechanism of action of *Lm*-LLO immunotherapies. Currently, an *Lm*-LLO immunotherapy (ADXS11-001) is being evaluated in clinical studies for the treatment of HPV-associated dysplasia and cancer (cervical intraepithelial neoplasia-NCT01116245, cervix cancer-NCT01266460, head and neck cancer-NCT01598792, and anal cancer-NCT01671488). The mechanistic evidence provided here may have an effect on the future design of clinical studies using *Lm*-LLO immunotherapies and the potential to combination with other cytotoxic therapies.

METHODS

Mice and Tumor Models

All animal experiments were approved by Animal Care and Use Committee of Advaxis Inc. C57BL/6 male and Balb/c female mice (6–8 wk old) were obtained from Jackson Laboratories. Two different tumor models

Received for publication May 8, 2013; accepted September 26, 2013.
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TPSA23, syngenic for C57BL/6 mouse and 4T1, syngenic for Balb/c mouse were used in the studies.[9,17] TPSA23 cells were grown and maintained in the medium Dulbecco's modified Eagle's medium with 4 mM glutamine and adjusted to contain 1.5 g/L of sodium bicarbonate, 4.5 g/L glucose, 5 μg/mL insulin, 10 nM dehydroisoandrosterone, 5% fetal bovine serum, and 5% Nuserum IV (BD biosciences) at 37°C and 5% CO_2. 4T1 cells were cultured in RPMI containing 10% FBS and 1× antibiotic antimycotic solution (Sigma) at 37°C and 5% CO_2. For the generation of tumors, 1×10^6 TPSA23 cells were inoculated subcutaneously in the right flank of male C57BL/6 mice (8 mice per group) or 1×10^4 4T1 cells were inoculated in the mammary fat-pad of female Balb/c mice (8 mice per group). Depending on the tumor model, mice were immunized either on days 7 and 14 (TPSA23) or days 4 and 10 (4T1) with 1×10^8 CFU of different *Lm*-LLO immunotherapies.

Preparation and Storage of *Lm*-LLO Immunotherapies

Different immunotherapies used in this study include: *Lm*-secreting tLLO-E7 (*LmddA134*) or *Lm*-secreting tLLO-carbonic anhydrase 9 (CA9) as an irrelevant immunotherapy, *Lm*-secreting tLLO-PSA (PSA) as the relevant immunotherapy for TPSA23 and *Lm*-secreting tLLO-chimera-Her2 (Her2) as the relevant immunotherapy for 4T1.[3,18] A single colony from each vaccine was used for growth in an overnight culture in brain heart infusion medium. This culture was further expanded for ~4–5 hours in a shaking incubator at 37°C and grown until the microbial density reached 0.6–0.8 OD_{600}, at which time the microbes were aliquoted into cryogenic vials and frozen at −80°C until use. To quantify CFU in each vial the stocks were titred. Each lot was checked for fusion protein expression by Western blot with an anti-LLO and anti-TAA-specific Ab. For each dose, 1 vial was selected, thawed, and washed twice in 1× PBS before dilution and used at a dose of 1×10^8 CFU per mouse.

MDSC Isolation from Tumors and Spleen

Tumors and spleens were harvested from each mouse either on day 21 (TPSA23) or day 14 (4T1). In the control-naive group, mice were left untreated. The preparation of a single-cell suspension from tumor tissues was carried out by mincing and digesting with a collagenase/DNAse solution as described previously.[19] The processing of spleens to prepare a single-cell suspension has also been reported previously.[9] Alternatively, the GENTLEMACS dissociator was used along with a tumor dissociation kit (Miltenyi Biotec). Different suppressive populations such as gMDSC, mMDSC, and Treg were purified from single-cell suspension of tumor or spleen by using mouse MDSC-isolation and Treg-isolation kit (Miltenyi Biotec) in conjunction with the AutoMACS pro or manual purified with magnetic columns. The cells were purified according to the manufacturer specifications. The purified gMDSC, mMDSC, Treg, or conventional T cells (Tcon) were used in a carboxyfluorescein succinimidyl ester (CFSE)-based proliferation assays to determine their suppressive function, and also stored in RNA later at −80°C for subsequent RNA purification.

CFSE-Proliferation Assays

Five days before harvesting the tumor or spleen for MDSC or Treg isolation, non–tumor-bearing mice were vaccinated with either Her2 (4T1) or PSA (TPSA23) immunotherapy to be used as responder T cells depending upon the tumor model used for the isolation of suppressor cells. The spleens of these mice were processed to prepare responder T cells, and these cells were then labeled with CFSE. To set-up a proliferation assay, responder T cells were plated together at a 2:1 ratio to gMDSC, mMDSC, Treg, or Tcon at a density of 1×10^5 T cells per well in 96-well plates. Responder

TABLE 1. Primers Used in 1-Step RT-PCR or Real-Time PCR-based Assays

Primers	Genes	DNA Sequence	Amplicon Size (bp)
Forward	β-actin	CCTTCGTTGCCGGTCCACACC	220
Reverse	β-actin	TCTTGCTCTGGGCCTCGTCAC	
Forward	Arginase I	CCCCTGACAACCAGCTCTGGGA	163
Reverse	Arginase I	CCCCAGGGTCTACGTCTCGCA	
Forward	IDO	AGAGGACACAGGTTACAGCG	134
Reverse	IDO	GAGAGCTCGCAGTAGGGAAC	
Forward	NOS II	AGACCTCAACAGAGCCCTCA	305
Reverse	NOS II	GCAGCCTCTTGTCTTTGACC	
Forward	TGF-β	CTTTAGGAAGGACCTGGGTT	258
Reverse	TGF-β	CAGGAGCGCACAATCATGTT	
Forward	Foxp3	TCGTAGCCACCAGTACTCAG	386
Reverse	Foxp3	ATCTACGGTCCACACTGCTC	
Forward	IL-6	CCTACCCCAATTTCCAATGCTCTCC	151
Reverse	IL-6	GGCATAACGCACTAGGTTTGCCG	
Forward	IL-10	TACCTGGTAGAAGTGATGCC	252
Reverse	IL-10	CATCATGTATGCTTCTATGC	
Forward	IL-12a	CCATCGATGAGCTGATGCAG	340
Reverse	IL-12a	ATGCTGAGGTAGCTGTGCCA	
Forward	IL-12b	CAGAAGCTAACCATCTCCTGGTTTG	396
Reverse	IL-12b	TCCGGAGTAATTTGGTGCTTCACAC	
Forward	IL-23a	CCAGCAGCTCTCTCGGAATC	123
Reverse	IL-23a	CACTGGATACGGGGCACATT	

IDO indicates indoleamine-2,3-diioxygenase; IL, interleukin; NOS II, nitric oxide synthase II.

T cells were then stimulated with the appropriate peptide for PSA (HCIRNKSVIL), Her2 (PDSLRDLSVF),[20,21] or non-specifically with PMA/ionomycin (PMA/I). The cocultured cells were incubated in the dark for 2 days at 37°C with 5% CO_2. After 48 hours, the cells were harvested, labeled with fluorescent surface markers, and then the division of the CFSE-labeled cells were analyzed on a FACS Calibur.

Flow Cytometry

The characterization of different populations of cell types in the tumors, spleen, and blood were performed by staining with cell-specific markers, which includes; Treg: $CD4^+$ $CD25^+$ $Foxp3^+$; MDSC: Gr^+ $CD11b^+$; PSA-specific T cells: $CD8^+$ $CD62L^{Low}$ PSA^{tet}. The antibodies used for staining of different markers were CD8-FITC, CD45-PerCP, CD4-PE, $CD25^+$-APC, CD11b-APC, CD62L-APC, and Gr-1-FITC (BD Biosciences, eBioscience). The Foxp3-PE staining of Treg were performed after permeabilization of cells stained with surface markers using the recommended protocol (Miltenyi Biotec). A total of 1×10^6 cells were stained with fluorochrome-labeled antibodies after blocking Fc receptors on the cell surface as described previously.[9] In the CFSE-proliferation assays, cells were stained for the surface markers using the following antibodies: CD8-PE and CD3-APC. The different populations of cells were analyzed on 4-color FACS Calibur using Cell Quest software.

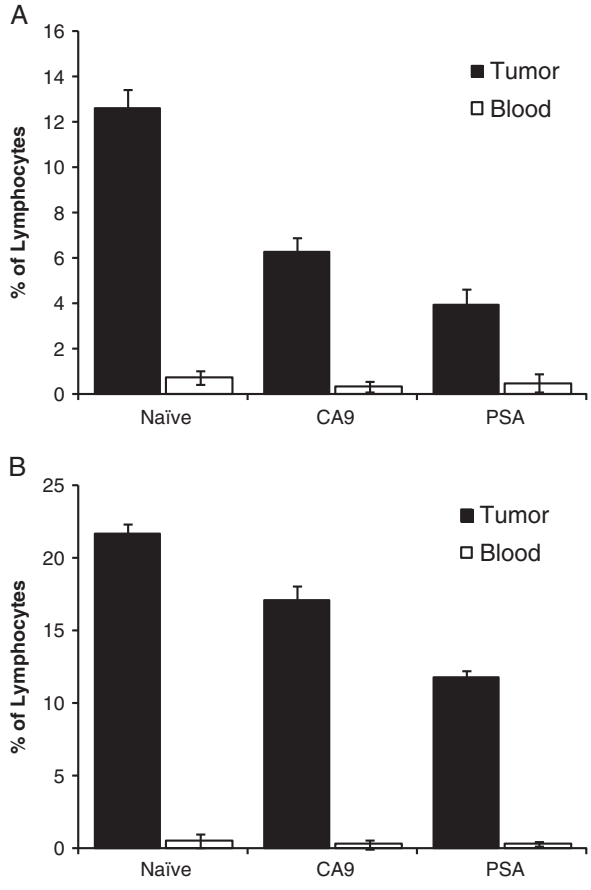

FIGURE 1. Decrease in regulatory T cells (Treg) and myeloid-derived suppressor cell (MDSC) after *Lm*-LLO treatment. Both Treg and MDSC frequencies decrease as a percentage of total lymphocytes after treatment with *LmddA181* (CA9) and *LmddA142* (PSA) in the TPSA23 tumors. A, Treg. B, MDSC.

RT and Real-Time Polymerase Chain Reaction (PCR) Assays

RNA was extracted from MACS-isolated gMDSC, mMDSC, Treg, and Tcon that had been previously purified and stored in RNA later at −80°C, using the RNA isolation kit (Qiagen). DNA sequences of the primers used for the detection of different cytokines and proteins are given in Table 1. Expression levels of different mRNA such as arginase I, interleukin (IL)-12, IL-23, nitric oxide synthase (NOS) II, and indoleamine-2,3-diioxygenase (IDO) in both gMDSC and mMDSC populations were analyzed in cDNA prepared by using Sensiscript II reverse transcriptase (Qiagen) and further quantified by real-time PCR using SYBR mix. The relative expression of arginase I, IL-12, IL-23, IDO, and NOS II in both gMDSC and mMDSC populations was quantified by $\Delta\Delta C_T$ analysis. In $\Delta\Delta C_T$ analysis, the reference control RNA was obtained from gMDSC and mMDSC populations from spleens of normal non–tumor-bearing Balb/c mice and β-actin expression was used as an endogenous control.

The expression of various cytokines mRNA such as TGF-β, IL-6, IL-10, and transcription factor Foxp3 in either Treg or Tcon populations from spleens or tumors were determined by reverse-transcriptase PCR using 1-step RT-PCR kit (Qiagen). The DNA sequences of different primers used for the amplification of these cytokines in Treg are given in Table 1.

Statistical Analysis

All the studies were repeated at least twice. Statistical analysis was done by the Student *t* test using Microsoft excel version (2010). Statistical significance was based on the *P* values listed ($P < 0.01$).

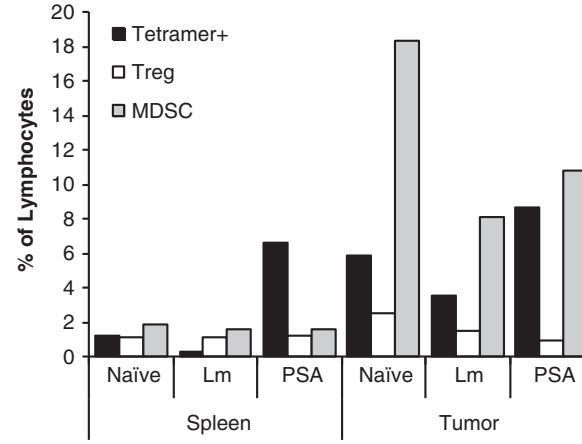

FIGURE 2. Antigen-specific $CD8^+$ T cells versus regulatory T cells (Tregs) and myeloid-derived suppressor cell (MDSC) in the tumor microenvironment (TME). In the TPSA23 tumor model, antigen-specific $CD8^+$ T cells increase in the spleen and TME after *LmddA142* (PSA) vaccination. However, there was a decrease in MDSC and Treg frequency with both irrelevant control *LmddA181* (Lm) and *LmddA142* (PSA), specifically in the TME.

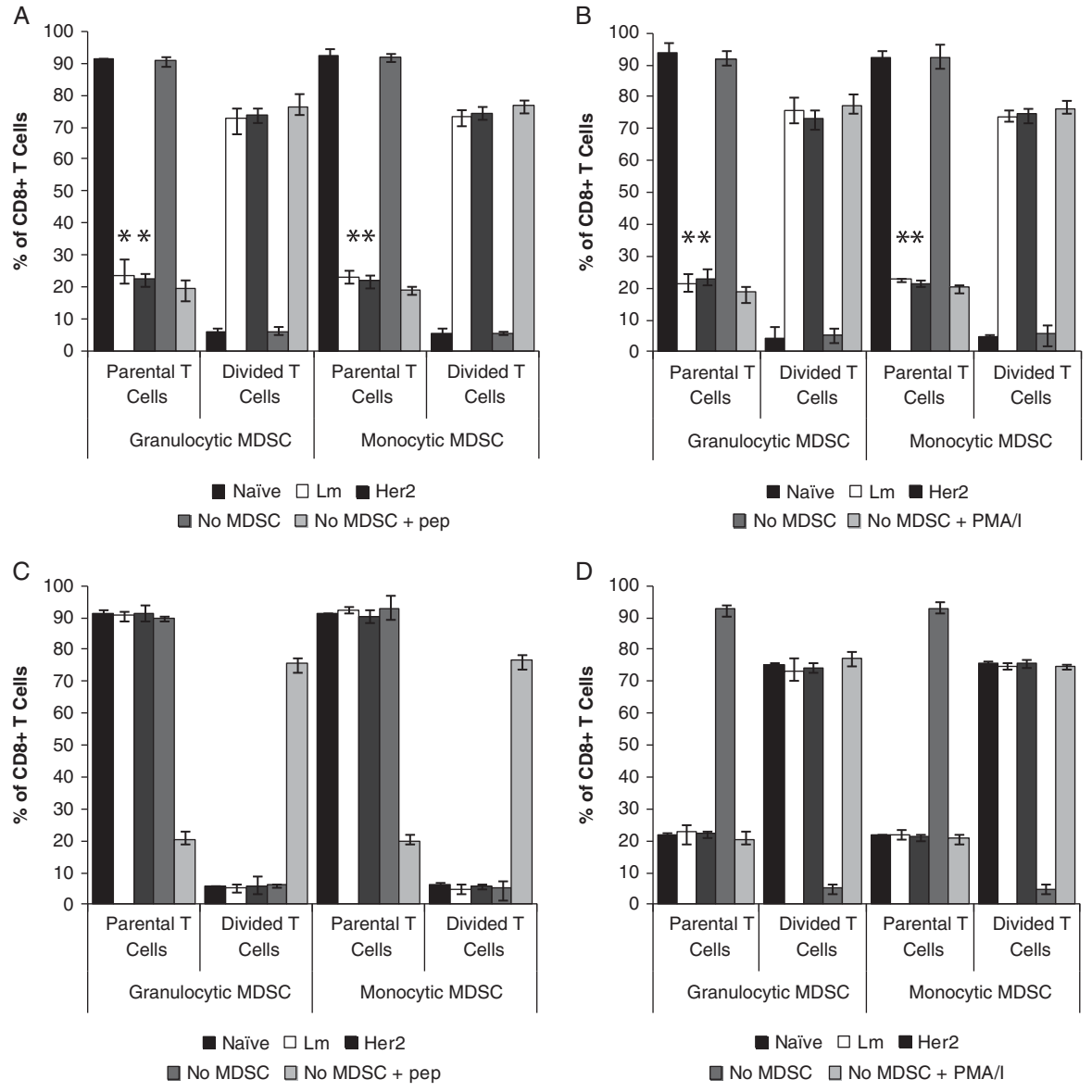

FIGURE 3. Both gMDSC or mMDSC isolated from the tumor microenvironment (TME) lose virtually all suppressive activity after *Lm*-LLO treatment. Myeloid-derived suppressor cell (MDSC) were isolated from the tumors of Balb/c mice with 4T1 tumors implanted in the mammary fat-pads treated with *LmddA164* (Her2), *LmddA134* (Lm) or naive group on day 14. In the suppression assay, carboxyfluorescein succinimidyl ester-labeled responder and MDSC were plated in the ratio of 2:1 and stimulated either with Her2-peptide or nonspecifically with PMA/I for 48 hours. As a control responder T cells were left either unstimulated (no MDSC) or stimulated with Her2-peptide (no MDSC + pep) or PMA/I (no MDSC + PMA/I). A, MDSC isolated from tumors and cocultured with Her2-peptide-stimulated responder T cells. B, MDSC isolated from tumors and cocultured with nonspecifically stimulated responder T cells. C, MDSC isolated from the spleens of tumor-bearing mice and cocultured with Her2-peptide-stimulated responder T cells. D, MDSC isolated from the spleens of tumor-bearing mice and cocultured with nonspecifically stimulated responder T cells. $^{**}P < 0.01$.

RESULTS

Lm-LLO Immunotherapies Cause a Reduction in the Frequency of Suppressor Cells in the TME

Previously, we showed that therapeutic treatment of tumors with *Lm*-LLO immunotherapies reduces the frequency of Treg specifically in the TME.[3,9] As MDSC have been reported to promote the induction of Treg in the TME,[16] we hypothesized that this immunotherapy also affects MDSC in TME. In mice bearing established (~5 mm) TPSA23 tumors we observed that treatment with *Lm*-LLO immunotherapies caused a reduction in the frequency of MDSC (~2-fold) and Treg (~2- to 3-fold) in the TME (Fig. 1). In contrast, the frequency of MDSC or Treg after *Lm*-LLO immunotherapy treatment was unchanged in the peripheral blood (Figs. 1A, B). As, both control and relevant *Lm*-LLO immunotherapies caused a reduction in the frequency of MDSC or Treg within the

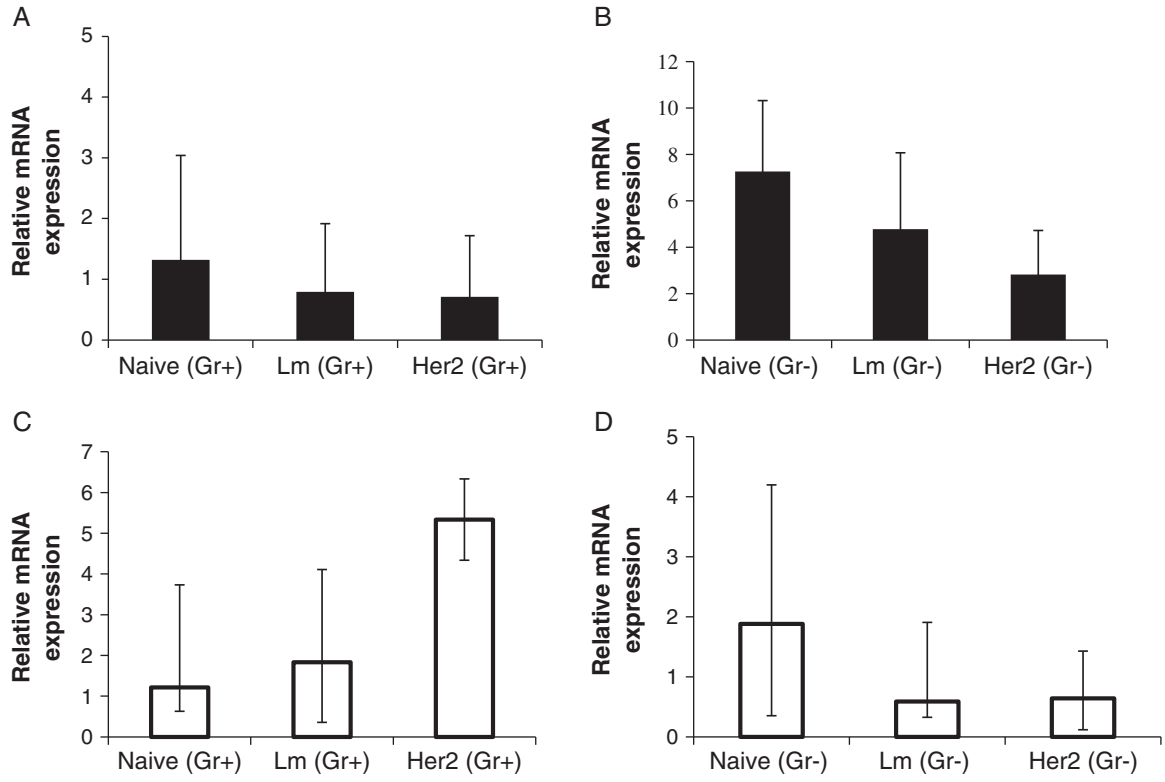

FIGURE 4. Relative expression of arginase I in the gMDSC and mMDSC cells populations in the tumor or spleen after *Lm*-LLO treatment. The myeloid-derived suppressor cell (MDSC) were purified from the tumor and spleen of 4T1 tumors treated with *LmddA134* (Lm) or *LmddA164* (Her2) or naive were processed for RNA isolation, cDNA conversion, and qPCR analysis with arginase I primers. The relative expression of arginase I mRNA in different samples was determined by $\Delta\Delta C_T$ analysis using normal non–tumor-bearing mouse spleen, MDSC as reference and β-actin as endogenous control. A, gMDSC tumor. B, mMDSC tumor. C, gMDSC spleen. D, mMDSC spleen.

TME, it was attributed to an inherent effect of *Lm*-LLO-induced immunity. We observed a similar reduction in the frequencies of suppressive cells in the TME after treatment with *Lm*-LLO immunotherapies in 2 different tumor models 4T1 and TPSA23 (data not shown), implicating that this result was independent of the tumor model. However, an increase in the infiltration of PSA-specific T cells in the TME were detected only after treatment with the antigen-specific (PSA) *Lm*-LLO immunotherapy (~9%) (Fig. 2). This suggests that the therapeutic regression of tumors induced by *Lm*-LLO immunotherapies is dependent on the generation of antigen-specific T cells combined with a reduction of suppressive cells in the TME.

MDSC Isolated from TME Fail to Inhibit T-Cell Proliferation Following *Lm*-LLO Immunotherapy

Next, we examined the suppressive function of MDSC in the tumors as well spleens after treatment with an *Lm*-LLO immunotherapy in a 4T1 model using a control (*Lm*) and a relevant (Her2) immunotherapy. Different populations of cell types such as gMDSC (Gr^{high} $Ly6G^+$), mMDSC (Gr^{dim} $Ly6G^-$), $CD4^+CD25^+$ (Treg), and $CD4^+CD25^-$ (Tcon), were purified from tumors and spleens using MACS-based cell sorting. The suppressive ability of gMDSC or mMDSC was examined by coculturing these cells with CFSE-labeled responder T cells and stimulating with either Her2-peptide (antigen-specific) or PMA/I (nonspecific). The individual division of responder cells cocultured with either gMDSC or mMDSC isolated from tumors and spleens is shown in Supplementary Figure 1 (Supplemental Digital Content 1, http://links.lww.com/JIT/A298). Both gMDSC and mMDSC isolated from the tumors of the *Lm*-LLO-treated group were found to lose their suppressive ability when compared with the MDSC isolated from the naive group to suppress antigen-specific or nonspecific T-cell proliferation (Figs. 3A, B). However, splenic gMDSC and mMDSC were suppressive to antigen-specific responder T cells, but had no effect on nonspecifically activated CD8 T cells in both naive as well as *Lm*-LLO-treated groups (Figs. 3C, D). This result indicates that treatment with an *Lm*-LLO immunotherapy results in the loss of suppressive ability of gMDSC as well as mMDSC populations with regards to CD8 T-cell function in the TME.

MDSC in the TME Exhibit a Reduction in Arginase I Expression Levels

To elucidate the mechanism of MDSC suppression by *Lm*-LLO immunotherapies, we examined the expression of different cytokines and enzymes such as IL-12, IL-23, NOS II, IDO, and arginase I in the mRNA of gMDSC and mMDSC populations by reverse transcription and real-time PCR. We detected very low and comparable relative levels of IL-12, IL-23, NOS II, or IDO in either gMDSC or mMDSC isolated from the tumors or spleens of *Lm*-LLO-treated or *Lm*-LLO-untreated groups (data not shown). However, *Lm*-LLO immunotherapy treatment resulted in a decrease in the relative

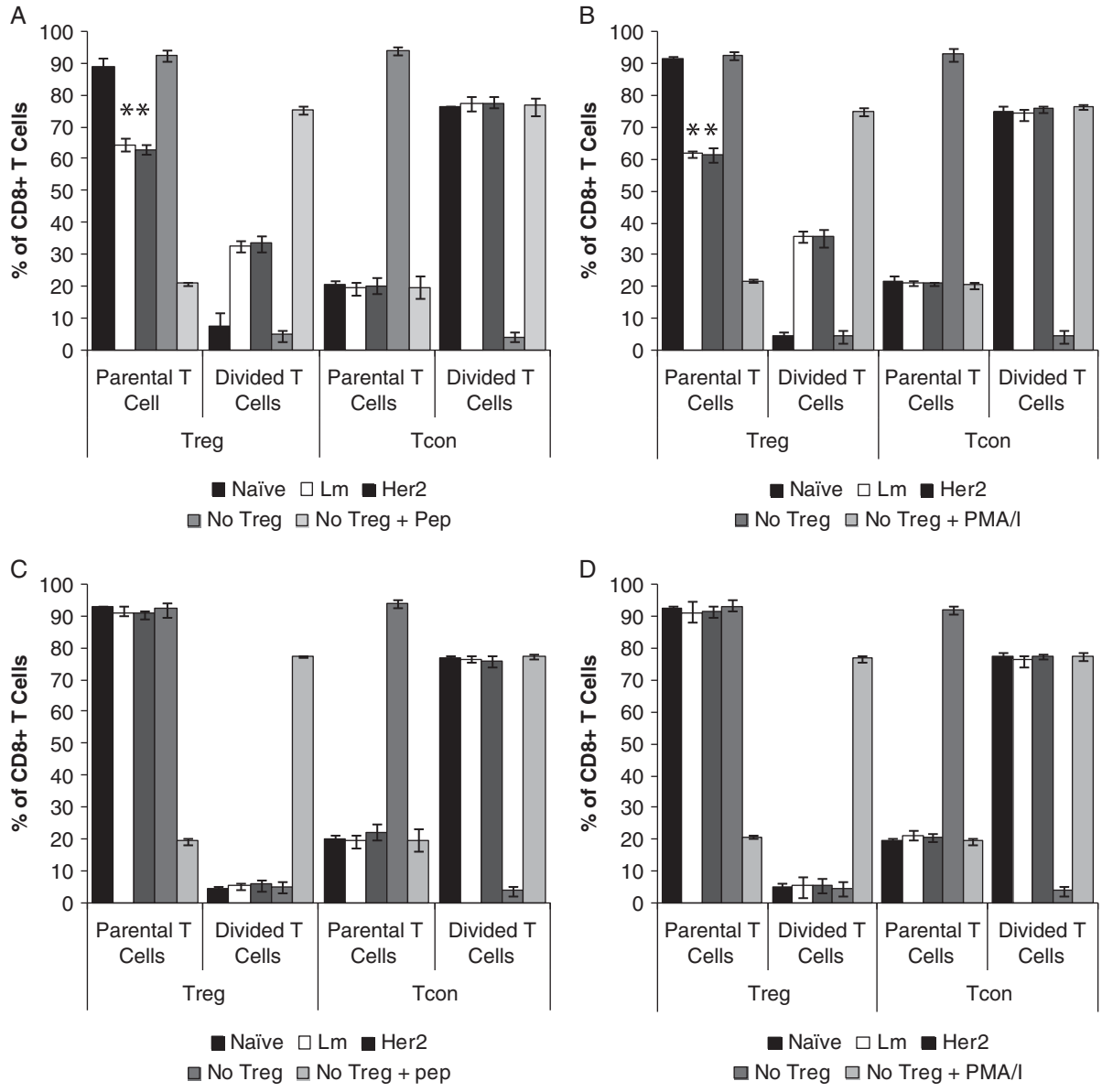

FIGURE 5. Regulatory T cells (Treg) isolated from the tumor microenvironment (TME) are less suppressive after *Lm*-LLO treatment. Treg were isolated from the tumors of Balb/c mice implanted with 4T1 tumors in the mammary fat-pads treated with either *LmddA164* (Her2), *LmddA134* (Lm), or untreated naïve group. Both carboxyfluorescein succinimidyl ester-labeled responder and suppressor cells were plated in the ratio of 2:1 and stimulated either with Her2-peptide or PMA/I for 48 hours. As a control responder T cells were left either unstimulated (no Treg) or stimulated with Her2-peptide (no Treg + pep) or PMA/I (No Treg + PMA/I). A, Treg and conventional T cells (Tcon) isolated from tumors and cocultured with peptide-stimulated responder T cells. B, Treg and Tcon isolated from tumors and cocultured with nonspecifically stimulated responder T cells. C, Treg and Tcon isolated from the spleens of tumor-bearing mice and cocultured with Her2-peptide-stimulated-responder T cells. D, Treg and Tcon isolated from the spleens of tumor-bearing mice and cocultured with nonspecifically stimulated responder T cells. $^{**}P < 0.01$.

mRNA of arginase I in both gMDSC and mMDSC populations in the TME (Fig. 4). This result suggests that the reduction in the expression of arginase I by MDSC in the TME is responsible for causing a loss in the suppressive ability of MDSC. In contrast, splenic gMDSC showed an increase in arginase I expression, supporting the retention in suppressive ability of these cells for antigen-specific T-cell proliferation. Surprisingly, the levels of arginase I were slightly decreased in splenic mMDSC after *Lm*-LLO treatment, indicating that these cells may suppress antigen-specific T-cell proliferation by utilizing additional biochemical mechanisms.

Reduction in Suppressive Activity of $CD4^+CD25^+$ Treg in the TME

We further investigated if *Lm*-LLO treatment had any effect on the suppressive activity of Treg isolated from tumor or spleen. A CFSE-based suppression assay was conducted using Treg or Tcon purified from the tumor (4T1) or spleen of

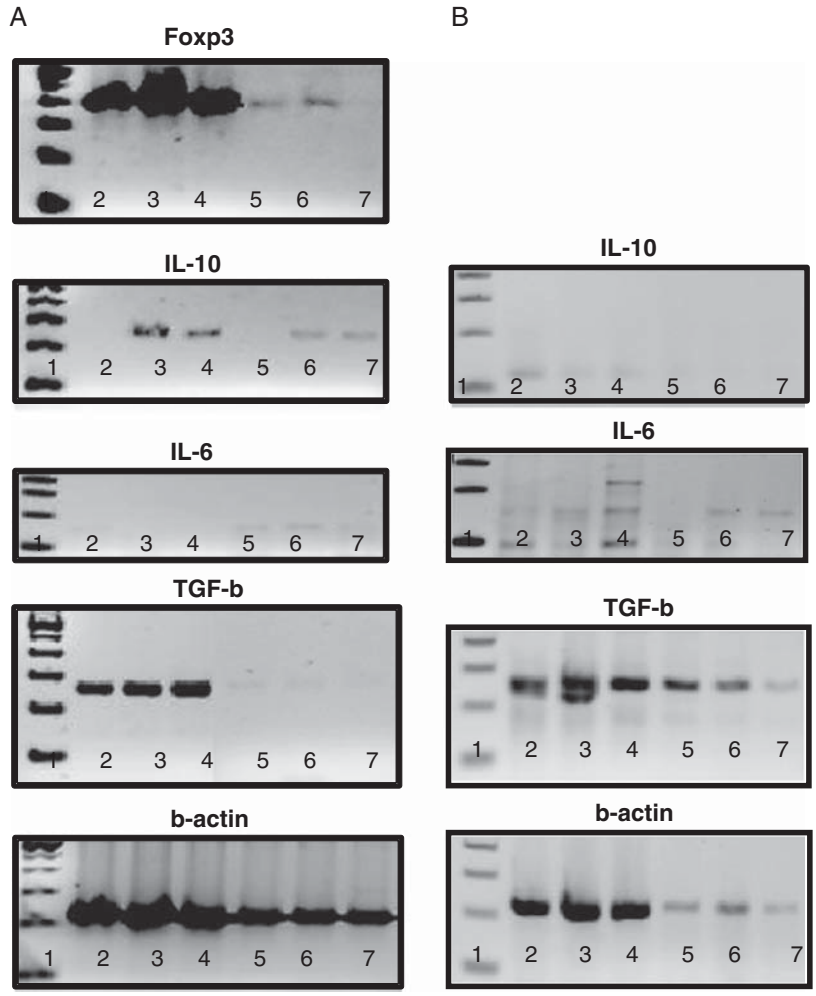

FIGURE 6. Expression of different cytokines in (A) regulatory T cells (Treg) and (B) conventional T cells (Tcon) present in the tumor or spleen after *Lm*-LLO treatment. The Treg and Tcon purified from the tumor and spleen were processed for RNA isolation and subsequently used in 1-step real-time polymerase chain reaction reaction for the expression of different cytokines or proteins. Naive mice were not treated, irrelevant control were immunized with *LmddA134* (Lm) and *LmddA*164 (Her2). The expression of Foxp3, interleukin (IL)-10, IL-6, TGF-β, and β-actin in the Treg purified from tumor (lanes 5–7) or spleen (lanes 2–4). Lane 1: 100 bp ladder; lanes 2, 5: naive; lanes 3, 6: Lm and lanes 4, 7: Her2. The expression of IL-6, TGF-β, and β-actin in Tcon purified from tumor (lanes 5–7) or spleen (lanes 2–4). Lane 1: 100 bp ladder; lanes 2, 5: naive; lanes 3, 6: Lm; and lanes 4, 7: Her2.

the naive (untreated) or *Lm*-LLO-treated mice, with responder cells that were stimulated with either Her2-peptide or PMA/I. The individual division of responder T cells cocultured with Treg in different stimulation conditions is shown in Supplementary Figure 2 (Supplemental Digital Content 2, http://links.lww.com/JIT/A299). In both antigen-specific and nonspecific T-cell proliferation assays, Treg isolated from tumors of *Lm*-LLO-treated mice were less suppressive when compared with the untreated naive group as indicated in Figures 5A and B. Again, the decrease in Treg suppressive function after *Lm*-LLO immunotherapies treatment was specifically detected in the cells isolated from the TME (Figs. 5C, D), supporting that this effect may be related to the reduction in MDSC frequency or suppressive activity. The control Tcon group showed no suppression of T cells when cocultured with Her2-peptide or nonspecifically stimulated responder T cells. Once again, loss in the suppressive ability of Treg was linked to the treatment with *Lm*-LLO immunotherapies and found to be independent of tumor model (data not shown).

Reduced Expression of IL-10 by Treg

To understand the mechanism responsible for causing the reduction in the suppressive activity of Treg, we examined the mRNA expression of different cytokines in untreated naive and *Lm*-LLO-treated groups. We detected a slight decrease in the mRNA expression of cytokines such as IL-10 and TGF-β in Treg isolated from tumors when compared with splenic Treg (Fig. 6A). In addition, there was an increase in the expression of IL-6 in Treg purified from tumors in comparison with splenic Treg. Surprisingly, Treg isolated from tumors of both naive and *Lm*-LLO-treated groups showed similar expression of IL-6 or TGF-β cytokines. Thus, a slight decrease in IL-10 expression by Treg after *Lm*-LLO treatment in the TME possibly affects its

suppressive activity. Control Tcon purified from tumors or spleens expressed similar levels of TGF-β or IL-6, but were found to completely lack the expression of IL-10 (Fig. 6B).

DISCUSSION

Elimination or reprogramming of the immune suppressive TME is one of the major challenges in the use of immunotherapy for the treatment of cancer. This is the first report to demonstrate that *Lm*-LLO immunotherapies drastically decrease the frequency of MDSC and lose their ability to impair T-cell function particularly in the TME. MDSC have been recognized as critical mediators of tumor progression in numerous solid tumors through their inhibition of tumor-specific immune responses.[22] Although the number of MDSC may not increase in certain models, their suppressive function clearly parallels increases in the tumor burden.[14] Furthermore, we demonstrate that Treg are also decreased in the TME and exhibit a reduced suppressive activity, which may be a direct or an indirect consequence of the loss of immunosupression by MDSC. Because, the modification in MDSC-based and Treg-based suppression in the TME was found to be an inherent effect of *Lm*-LLO immunotherapies and independent of the nature of TAA, this supports the theory that it may be a consequence of the innate immunity induced by *Lm*-LLO immunotherapies. Detoxified nonhemolytic LLO protein has been demonstrated to display pathogen-associated molecular pattern-like properties by stimulating the production of proinflammatory cytokines and inducing maturation of DCs.[6] In addition, it has been reported that *Lm*-LLO immunotherapies over expressing tLLO cause rapid and effective phenotypic and functional maturation of myeloid DCs.[23] It is highly likely that the overexpression of tLLO by *Lm*-LLO immunotherapies is responsible for the modulation of MDSC and Treg in the TME. We extended these studies using an appropriate *Lm* control immunotherapy that did not express tLLO and found that indeed tLLO overexpression had a effect on the alteration of the suppressive ability of these cells in the TME (data not shown).

MDSC have been shown to blunt antitumor responses through multiple mechanisms that includes arginine and tryptophan metabolism by factors such as the expression of arginase I, NOS II, and IDO.[24,25] The *Lm*-LLO-based reduction, both in quantity and functionally, of gMDSC and mMDSC leading to T-cell nonresponsiveness in the TME was found to be associated with the decreased levels of arginase I. In contrast, a relative increase in the expression of arginase I was observed in gMDSC in the spleen, supporting the fact that arginase I is the major mechanism that regulates the suppressive activity of these cells in the TME. The presence of reduced levels of arginase I mRNA in splenic mMDSC suggest that these cells have an alternate mechanism for causing antigen-specific T-cell suppression.[14] In this study, we looked at the relative expression of other cytokines and factors such as IL-12, IL-23, NOS II, and IDO, all of which were very low and comparable in both the gMDSC as well as mMDSC isolated from tumors or spleen of both naive and treated mice. However, it is possible that the expression of other cytokines or factors that were not included in this study may alter MDSC suppression. The reduction in the suppressive ability of Treg in the TME was found to be related to the reduced expression of IL-10, which is known to be essential for Treg suppressive activity.[26] This is consistent with the previously published data where increase in the efficacy of *Lm*-LLO-E7 immunotherapy was attributed to the reduction in the IL-10 expression by Treg.[10]

Previously, we have shown that the therapeutic regression of tumors by *Lm*-LLO immunotherapies are highly dependent on the generation of TAA-specific T cells, and subsequently their infiltration in the TME.[1,7,9] It is possible that modulation of suppressive cells in the TME along with increases in TILs is responsible for the overall therapeutic antitumor immunity induced by *Lm*-LLO immunotherapies. This modification of immunosuppression by *Lm*-LLO immunotherapies in the TME is highly intriguing and possibly could be utilized as a surrogate biomarker for response to *Lm*-LLO immunotherapy. Advaxis is currently conducting 5 different phase 1/2 clinical studies using an *Lm*-LLO immunotherapy that expresses HPV16-E7 as the TAA (cervical intraepithelial neoplasia, cervix cancer, head and neck cancer, and anal cancer). Investigations are underway to examine if the number and phenotype of human MDSC could be used as a potential biomarker to evaluate the efficacy of *Lm*-LLO immunotherapies, and also if this affects TAA-specific T-cell responses in the peripheral tissues as well as in tumors. This study highlights an important mechanism of action associated with the *Lm*-LLO immunotherapies. Additional investigation of MDSC and Treg function in clinical samples will provide more mechanistic details and will lead to the development of improved strategies to support the immunotherapy-based treatment of cancer.

CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

A.W. and I.M. are currently employed by Advaxis Inc. In addition, R.S. and I.M. are shareholders of Advaxis Inc.

REFERENCES

1. Maciag PC, Seavey MM, Pan ZK, et al. Cancer immunotherapy targeting the high molecular weight melanoma-associated antigen protein results in a broad antitumor response and reduction of pericytes in the tumor vasculature. *Cancer Res*. 2008;68:8066–8075.
2. Sewell DA, Shahabi V, Gunn GR 3rd, et al. Recombinant Listeria vaccines containing PEST sequences are potent immune adjuvants for the tumor-associated antigen human papillomavirus-16 E7. *Cancer Res*. 2004;64:8821–8825.
3. Wallecha A, Maciag PC, Rivera S, et al. Construction and characterization of an attenuated *Listeria monocytogenes* strain for clinical use in cancer immunotherapy. *Clin Vaccine Immunol*. 2009;16:96–103.
4. Gedde MM, Higgins DE, Tilney LG, et al. Role of listeriolysin O in cell-to-cell spread of *Listeria monocytogenes*. *Infect Immun*. 2000;68:999–1003.
5. Lee KD, Oh YK, Portnoy DA, et al. Delivery of macromolecules into cytosol using liposomes containing hemolysin from *Listeria monocytogenes*. *J Biol Chem*. 1996;271:7249–7252.
6. Wallecha A, Wood L, Pan ZK, et al. *Listeria monocytogenes*-derived listeriolysin O has pathogen-associated molecular pattern-like properties independent of its hemolytic ability. *Clin Vaccine Immunol*. 2013;20:77–84.
7. Wallecha A, Carroll KD, Maciag PC, et al. Multiple effector mechanisms induced by recombinant *Listeria monocytogenes* anticancer immunotherapeutics. *Adv Appl Microbiol*. 2009;66:1–27.

8. Weiskirch LM, Paterson Y. *Listeria monocytogenes*: a potent vaccine vector for neoplastic and infectious disease. *Immunol Rev*. 1997;158:159–169.
9. Shahabi V, Reyes-Reyes M, Wallecha A, et al. Development of a *Listeria monocytogenes* based vaccine against prostate cancer. *Cancer Immunol Immunother*. 2008;57:1301–1313.
10. Hussain SF, Paterson Y. CD4 + CD25 + regulatory T cells that secrete TGFbeta and IL-10 are preferentially induced by a vaccine vector. *J Immunother*. 2004;27:339–346.
11. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. *Cancer Res*. 2001;61:4756–4760.
12. Zea AH, Rodriguez PC, Atkins MB, et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res*. 2005;65:3044–3048.
13. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. 2009;9: 162–174.
14. Youn JI, Nagaraj S, Collazo M, et al. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J Immunol*. 2008;181:5791–5802.
15. Rodriguez PC, Ernstoff MS, Hernandez C, et al. Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. *Cancer Res*. 2009;69:1553–1560.
16. Schlecker E, Stojanovic A, Eisen C, et al. Tumor-infiltrating monocytic myeloid-derived suppressor cells mediate CCR5-dependent recruitment of regulatory T cells favoring tumor growth. *J Immunol*. 2012;189:5602–5611.
17. Pulaski BA, Ostrand-Rosenberg S. Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and B7.1 cell-based tumor vaccines. *Cancer Res*. 1998;58: 1486–1493.
18. Seavey MM, Pan ZK, Maciag PC, et al. A novel human Her-2/neu chimeric molecule expressed by *Listeria monocytogenes* can elicit potent HLA-A2 restricted CD8-positive T cell responses and impact the growth and spread of Her-2/neu-positive breast tumors. *Clin Cancer Res*. 2009;15:924–932.
19. Shahabi V, Seavey MM, Maciag PC, et al. Development of a live and highly attenuated *Listeria monocytogenes*-based vaccine for the treatment of Her2/neu-overexpressing cancers in human. *Cancer Gene Ther*. 2011;18:53–62.
20. Pavlenko M, Leder C, Roos AK, et al. Identification of an immunodominant H-2D(b)-restricted CTL epitope of human PSA. *Prostate*. 2005;64:50–59.
21. Singh R, Paterson Y. Vaccination strategy determines the emergence and dominance of CD8 + T-cell epitopes in a FVB/N rat HER-2/neu mouse model of breast cancer. *Cancer Res*. 2006;66:7748–7757.
22. Wang L, Chang EW, Wong SC, et al. Increased myeloid-derived suppressor cells in gastric cancer correlate with cancer stage and plasma S100A8/A9 proinflammatory proteins. *J Immunol*. 2013;190:794–804.
23. Peng X, Hussain SF, Paterson Y. The ability of two *Listeria monocytogenes* vaccines targeting human papillomavirus-16 E7 to induce an antitumor response correlates with myeloid dendritic cell function. *J Immunol*. 2004;172:6030–6038.
24. Rodriguez PC, Quiceno DG, Zabaleta J, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res*. 2004;64:5839–5849.
25. Rodriguez PC, Zea AH, DeSalvo J, et al. L-arginine consumption by macrophages modulates the expression of CD3 zeta chain in T lymphocytes. *J Immunol*. 2003;171:1232–1239.
26. Bollyky PL, Falk BA, Long SA, et al. CD44 costimulation promotes FoxP3 + regulatory T cell persistence and function via production of IL-2, IL-10, and TGF-beta. *J Immunol*. 2009; 183:2232–2241.