Tasquinimod Modulates Suppressive Myeloid Cells and Enhances Cancer Immunotherapies in Murine Models

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Abstract

A major barrier for cancer immunotherapy is the presence of suppressive cell populations in patients with cancer, such as myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAM), which contribute to the immunosuppressive microenvironment that promotes tumor growth and metastasis. Tasquinimod is a novel antitumor agent that is currently at an advanced stage of clinical development for treatment of castration-resistant prostate cancer. A target of tasquinimod is the inflammatory protein S100A9, which has been demonstrated to affect the accumulation and function of tumor-suppressive myeloid cells. Here, we report that tasquinimod provided a significant enhancement to the antitumor effects of two different immunotherapies in mice models of cancer: a tumor vaccine (SurVaxM) for prostate cancer and a tumor-targeted superantigen (TTS) for melanoma. In the combination strategies, tasquinimod inhibited distinct MDSC populations and TAMs of the M2-polarized phenotype (CD206+). CD11b+ myeloid cells isolated from tumors of treated mice expressed lower levels of arginase-1 and higher levels of inducible nitric oxide synthase (iNOS), and were less immunosuppressive in vivo when these cells were co-injected with tumor cells. Tumor-specific CD8+ T cells were increased markedly in the circulation and in tumors. Furthermore, T-cell effector functions, including cell-mediated cytotoxicity and IFNγ production, were potentiated. Taken together, these data suggest that pharmacologic targeting of suppressive myeloid cells by tasquinimod induces therapeutic benefit and provide the rationale for clinical testing of tasquinimod in combination with cancer immunotherapies.

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Introduction

Immunotherapies have gained momentum in cancer therapeutics following the recent approvals of drugs for the treatment of prostate cancer and melanoma. Sipuleucel-T dendritic cell (DC) vaccine is now available for treatment of patients with asymptomatic or minimally symptomatic, metastatic, and castration-resistant prostate cancer (1). Clinical observations have indicated that melanoma is an immunogenic tumor (2), and extended survival data have led to the approval of the immune checkpoint inhibitor ipilimumab for the treatment of metastatic melanoma (3). However, despite these clinical advances, immunotherapies for these diseases and solid tumors, in general, benefit only a subset of patients, as intrinsic or acquired tumor immune tolerance remains a major hurdle.

A significant barrier in vaccine therapy is the presence of immunosuppressive soluble and cellular components, including myeloid-derived suppressor cells (MDSC; ref. 4) and tumor-associated macrophages (TAM; ref. 5), which are induced by tumor- and stroma-secreted inflammatory mediators (6–8). MDSCs facilitate tumor progression by impairing T-cell and natural killer (NK)–cell activation (9) and by modulating angiogenesis. Preclinical data have suggested a role for MDSCs in suppressing T-cell responses and inducing tolerance against tumor-associated antigens (TAA; ref. 9). In addition, by secreting IL10 and TGFβ, MDSCs induce the accumulation of other immunosuppressive cell populations such as regulatory T cells (Treg; refs. 10–12). Similarly, the presence of TAMs in the tumor microenvironment (TME) may inhibit the immune response (13). Taken together, there is strong evidence indicating that targeting immunosuppressive MDSCs and TAMs and modifying the TME can improve the efficacy of immunotherapy.

Tasquinimod, a quinoline-3-carboxamide analogue, is in clinical development for treatment of prostate cancer and other solid tumors. In a placebo-controlled, phase II randomized trial, tasquinimod doubled the median progression-free survival (PFS) period and prolonged survival of patients with metastatic prostate cancer (14). Here, we report that tasquinimod provided a significant enhancement to the antitumor effects of two different immunotherapies in mice models of cancer: a tumor vaccine (SurVaxM) for prostate cancer and a tumor-targeted superantigen (TTS) for melanoma. In the combination strategies, tasquinimod inhibited distinct MDSC populations and TAMs of the M2-polarized phenotype (CD206+). CD11b+ myeloid cells isolated from tumors of treated mice expressed lower levels of arginase-1 and higher levels of inducible nitric oxide synthase (iNOS), and were less immunosuppressive in vivo when these cells were co-injected with tumor cells. Tumor-specific CD8+ T cells were increased markedly in the circulation and in tumors. Furthermore, T-cell effector functions, including cell-mediated cytotoxicity and IFNγ production, were potentiated. Taken together, these data suggest that pharmacologic targeting of suppressive myeloid cells by tasquinimod induces therapeutic benefit and provide the rationale for clinical testing of tasquinimod in combination with cancer immunotherapies.
castration-resistant prostate cancer (14, 15). A phase III clinical trial to test the effect of tasquinimod in the same patient population is ongoing (NCT01234311). Tasquinimod has been shown to inhibit prostate cancer growth and metastasis in animal models (16–18). Results from these studies have suggested that the antiangiogenic property of this molecule may be responsible for its antitumor activity, because tumor growth inhibition was associated with reduced microvessel density, increased expression, and secretion of the angiogenesis inhibitor thrombospondin-1 (TSP-1), and downregulation of VEGF and hypoxia-inducible factor-1α (HIF1α; refs. 19, 20).

More recent data have suggested that tasquinimod may affect mechanisms other than impairing angiogenesis may play an important role in the antitumor and antimetastatic activities of tasquinimod. S100A9, a Ca 2+ -binding inflammatory protein, has been identified as a potential target of tasquinimod. S100A9 interacts with proinflammatory receptors Toll-like receptor 4 (TLR4) and this interaction is inhibited by the specific binding of tasquinimod to S100A9 (22, 23). These receptors are expressed on the surface of multiple myeloid-cell populations, including MDSCs, macrophages, DCs, and endothelial cells. Functionally, S100A9 regulates the accumulation of MDSCs and inhibits DC differentiation (24, 25), which may lead to suppression of immune responses and tumor progression. Therefore, by targeting S100A9, tasquinimod has immunomodulatory activity and the potential to regulate multiple myeloid populations.

In this study, we tested the effect of tasquinimod on immunosuppressive myeloid-cell populations and investigated its immunomodulatory activity. We conducted preclinical studies of tasquinimod in combination with two different immunotherapeutic approaches in mouse models of prostate cancer and melanoma. Our results suggest that treatment with tasquinimod affects the TME by modulating suppressive myeloid-cell populations, leading to augmented immune responses and enhanced antitumor effects of immunotherapies.

Materials and Methods

Tumor cells

The development of castration-resistant Myc-CaP cell line has been reported previously (26). Castration-resistant Myc-CaP cell line was cultured in DMEM (Mediatech, Inc.) with 10% FBS. The 5T4-tranfected murine B16-F10 melanoma cell line (B16-h5T4; ref. 27) was kindly provided by Peter Stern (Paterson Institute for Cancer Research, Manchester, UK) and was cultured in R10 medium (RPMI-1640 with Ultra glutamine (BioWhittaker/Lonza); supplemented with 10% FBS (Fisher Scientific), 1 mM/L sodium pyruvate, 10 mM/L HEPES, 0.1 mg/mL gentamicin sulfate, and 50 μM/L β-mercaptoethanol). The castration-resistant Myc-CaP and B16-h5T4 cell lines were tested to be Mycoplasma free; no other authentication assay was performed.

In vivo tumor growth

The animal protocols were approved by the Institutional Animal Care and Use Committee at Roswell Park Cancer Institute (Buffalo, NY; protocol 1137 M), or by the Bioethics Committee in Lund, Sweden (M60-10), as indicated, and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Castration-resistant Myc-CaP cells (1 × 10⁶) were inoculated subcutaneously (s.c.) in the right flank of castrated male FVB mice. Animals were killed immediately after the 4 weeks treatment. Mice were given 100 μg of SurVaxM peptide and 100 ng of GM-CSF by s.c. injection, once per week. The tumor size was measured by a caliper twice a week. At the end of the 3- to 4-week experiment, tumors and spleens were collected and analyzed. B16-h5T4 cells were cultured as described above, counted, resuspended, and maintained in ice-cold Matrigel (BD Biosciences) at a concentration of 0.3 × 10⁶ cells/mL. Tumor cells were implanted s.c. into the hind flank of C57Bl/6 mice on day 0 in a volume of 0.1 mL. Matrigel. Mice were treated with tasquinimod (30 mg/kg/d in drinking water) either from day 0 or 1 after tumor inoculation and throughout the experiments. For tumor-targeted superantigen (TTS) treatment, mice were given daily injections of 5T4-Fab-SEA (25 μg/kg) on days 3 to 6, or on days 9 to 11 for analysis of TTS-reactive T cells in the tumors. Experiments were terminated between days 16 and 21. Tumor sizes were measured twice a week and tumor volumes were calculated as volume = L × W² × 0.4, where L is the length (mm) and W (mm) is the width of the tumor (L > W; ref. 28). Animal experiments and correlative studies in the castration-resistant Myc-CaP and the B16-h5T4 models were conducted at Roswell Park Cancer Institute and Active Biotech AB, respectively.

Splenocytes and tumor suspension preparation

For isolation of splenocytes, spleens were harvested, mashed on, and passed through a 70-μm strainer. These cell suspensions were centrifuged at 300 × g for 10 minutes at 4°C. Cell pellets were treated with ACK lysis buffer (Biosource). Splenocytes were then resuspended and cultured in complete medium [RPMI supplemented with 10% FBS, 1 mM/L sodium pyruvate, 100 mM/L nonessential amino acid, 2 mM/L L-glutamine, Pen (100 U/mL)–Strep (100 mg/mL), and 55 μM/L β-mercaptoethanol]. Single-cell suspensions were prepared from tumors with mouse tumor dissociation kit (Miltenyi Biotec). Brieﬂy, tumors were cut into small pieces and incubated in an enzyme-cocktail solution for 40 minutes at 37°C with agitation, followed by meshing the tumors in a 70-μm cell strainer. Alternatively, the tumors were cut into small pieces and incubated in 0.5 mg/mL collagenase IV ( Worthington Biochemical Corporation) and 0.1% DNase (Sigma-Aldrich) for 45 minutes at 37°C, followed by meshing the tumors in a 70-μm cell strainer.

Cell staining and flow cytometry

Splenocytes, tumor single-cell suspensions, or peripheral blood cells were washed with flow buffer (PBS with 1% of FBS and 2 mM/L of EDTA), then incubated with an Fc-blocking antibody (anti-mouse CD16/CD32 mAb 2.4G2; BD Biosciences), and stained with fluorescence-conjugated antibodies against surface markers. Cells were then fixed in Fix/Perm buffer (eBioscience) and stained with antibodies against intracellular proteins. The following fluorochrome-labeled antibodies were used: Gr1 (clone RB6-8C5), CD11b (clone M1/70), Ly6G (clone 1A8), Ly6C (clone AL-21), F4/80 (clone BM8), CD206 (clone C068C2), Arg 1 (polyclonal antibody; R&D Systems; Cat: IC5868A), iNOS
FCS Express (De Novo Software).

The whole-cell population with Dio label. Cell events were calculated to determine dead cells with Dio label compared with the control. Cell cytotoxicity was analyzed by using the FACS Canto II, or a LSR II flow cytometer (BD Biosciences). Data analysis was performed using the FCS Express (De Novo Software) or FACS Diva software (BD Biosciences).

**IFNγ induction assay**

Splenocytes (1 × 10⁶) were cultured with stimulation of PMA (Sigma; 20 ng/mL) and ionomycin (Sigma; 1 μg/mL) for 5 hours. Brefeldin A (Sigma) was added to the cultures to block protein secretion. Cells were harvested and stained for surface markers, then fixed and stained for intracellular IFNγ (eBioscience), and analyzed by flow cytometry.

**Granzyme B induction assay**

Splenocytes (1 × 10⁶) were cultured with stimulation of CD3 (eBioscience; 1 μg/mL) and CD28 (0.5 μg/mL) for 72 hours. Brefeldin A (Sigma) was added to the cultures during the last 5 hours of culture to block protein secretion. Cells were harvested and stained for surface markers, then fixed and stained for intracellular Granzyme B (eBioscience) and analyzed by flow cytometry.

**T-cell suppression assays**

T cells (1 × 10⁵), isolated with a Pan T cell isolation kit; Miltenyi Biotec were cultured in plates coated with CD3 (eBioscience; 1 μg/mL) and CD28 (0.5 μg/mL) for 72 hours. Different numbers of magnetic beads-purified CD11b⁺ cells from tumors were added to the culture at the beginning. ³H-thymidine (1 μCi) was added to the culture for the last 12 hours. Cells were then harvested and the incorporated ³H-thymidine was detected with scintillation counting.

Alternatively, CD11b⁺ cells were added to CFSE-(Vybrant CFDA SE Cell Tracer Kit; Molecular Probes)-labeled T cells (isolated from naïve spleens using a Pan T cell isolation kit; Miltenyi Biotec) activated by anti-CD3/anti-CD28-coated beads (Dynabeads; Dynal) and incubated for 72 hours. The frequencies of divided CD4⁺ and CD8⁺ T cells were determined by FACS analysis.

**Splenocyte- and CD8 T cell-mediated cytotoxicity assay**

Cytotoxicity assay was performed by using LIVE/DEAD cell-mediated cytotoxicity kit (Invitrogen). Castration-resistant MycCaP cells were labeled with Dio and cultured in complete medium. Splenocytes or isolated CD8⁺ T cells were added to the culture in different ratios to tumor cells. After 5-hour incubation, all cells in culture were harvested and propidium iodide (PI) staining was performed to detect dead cells. Cell cytotoxicity was analyzed by calculating percentage of dead cells with Dio label compared with the whole-cell population with Dio label. Cell events were acquired using LSRII and FACSDiva. Data were analyzed with FCS Express (De Novo Software).

**Antigen-specific tetramer binding assay**

Blood samples (100 μL) and splenocytes (1 × 10⁶ cells) were incubated for 30 minutes with 10 μL of iTag MHC Class I Murine H2-Kᵇ–Tetramer-SA-PE bound by MFCFKEL peptide with specificity for SurVaxM (Beckman Coulter) or iTag MHC Class I Murine H2-Kᵇ–Tetramer-SA-PE bound by SIINFEKL ovalbumin peptide to represent negative control (Beckman Coulter). Samples were also labeled with 10 μL of anti–CD8-ITC (clone 33.6.7; BioLegend). Following incubation, 1 μL of iTag MHC Tetramer Lyse Reagent (Beckman Coulter) supplemented with 25 μL of iTag MHC Tetramer Fix Reagent (Beckman Coulter) was added to the samples, which were then incubated for 10 minutes at room temperature, subsequently washed with PBS, and resuspended in 400 μL of FluoroFix Buffer (BioLegend).

**Immunofluorescence staining of tumor sections**

Snap-frozen tumors were sliced into 8-μm frozen sections and fixed in cold acetone for 10 minutes, before fluorescence labeling. Primary antibody, rat anti-mouse CD31 (BD, Mec 13.3; 1:1,000) and secondary antibody, goat anti-rat Alexa Fluor 555 (Invitrogen; AF555; 1:500) in PBS (5% and 2% mouse serum, respectively) were used, and slides were washed in PBS and mounted with fluorescein mounting medium (Dako; S3023). The sections were analyzed in a Leica DMRX-E microscope. Representative photos were taken and the density of CD31-positive cells (fluorescence) was measured with Leica QWin image analysis system.

**Immunohistochemistry staining**

Tissue specimens were fixed for 24-hour, paraffin-embedded and 4-μm sections were prepared. Sections were deparaffinized and rehydrated through graded alcohol washes. Antigen unmasking was achieved by boiling slides in sodium citrate buffer (pH 6.0). Sections were further incubated in hydrogen peroxide to reduce endogenous activity. Then tissue sections were blocked with 2.5% horse serum (Vector Laboratories) and incubated overnight in primary antibodies against CD31 (1:100; Dianova). Following anti-CD31 incubation, tissue sections were incubated in horseradish peroxidase-conjugated anti-rat antibody according to the manufacturer’s protocol (Vector Laboratories) followed by enzymatic development in diaminobenzidine (DAB) and counterstained in hematoxylin.

Sections were dehydrated and mounted with cytoseal 60 (Thermo Scientific). Corresponding isotype negative controls were used for evaluation of specific staining. Stained sections were analyzed under bright field using the Zeiss Axio microscope. The number of positive cells was determined in a blinded fashion by analyzing four random 20× fields per tissue and quantified using ImageJ software.

**Quantitative real-time PCR**

mRNA was extracted from CD11b⁺ cells that were isolated as anti-CD11b⁺ magnetic bead fractions from single-cell suspensions of B16-h514 tumors. mRNA extraction was performed using the RNeasy Mini Kit (Qiagen) and RNA concentration and purity was determined through measurement of A260/A280 ratios with a NanoDrop ND-1000 spectrophotometer. cDNA was prepared using the iScript Kit (Bio-Rad) and qPCR was performed using a CFX384 real-time PCR detection system (Bio-Rad) with a three-step PCR protocol (95°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds, and 58°C for 30 seconds) using SYBR Green (SsoFast EvaGreen; Bio-Rad) as fluorophore and expression levels were calculated (CFX Manager software; Bio-Rad) as normalized ΔCt expression values between the target gene and the two “housekeeping” genes β-actin and Ywhaz. Data were presented as fold-induction (2ΔΔCt) levels of treated tumors compared with control tumors (ΔΔCt). The primers used...
for target genes were β-actin_fv 5'-ATG CTC CCC GGG CTG TAT-3', β-actin_rev 5'-CAT AGT CCT TCT GCC CCA TTC-3', YwHaz_fv 5'-AAC AGC TIT CCA AGA AGC CAT-3', YwHaz_rev 5'-TCC GTA TCC CAT GTC CCC AAT-3', CD206_fv 5'-CCA AAT GGA GCC GTC TGC GTC-3', CD206_rev 5'-CTC GTC GAT CTC CGT GAC AC-3', Arg1_fv 5'-GTC-3', AAG AAC CCA CGG TCT GT-3', Ang1-1_rev 5'-CTG GTT GTG AGG GGA GTT TT-3', iNOS_fv 5'-TGG TGG TGA CAA GCA CAT TT-3', iNOS_rev 5'-AAG GCC AAA CAC AGC ATA CC-3', Cxcl9_fv 5'-TCA ACA AAA GAG CTG CCA AA-3', Cxcl9_rev 5'-GCA GAG GCC AGA AGA GAG AA-3', Ccl10_fv 5'-TCTGACTCTCTGCTCTACGTG-3', Ccl10_rev 5'-CCCTGGGAAGATGTTGTTA-3', Cxcl11_fv 5'-TCTT CCT CCC CCA ATA TCA CG-3', Cxcl11_rev 5'-CAG CCA TCC CTA CCA TCC AT-3', Ccr2_fv 5'-ACT TIT CCG AAG CAC CGT CT-3', Ccr2_rev 5'-GTA ACA GCA TCC GCC GGC AGT TT-3', Ccl2_fv 5'-CAGTTCCCTGCCATCTGCT-3', Ccl2_rev 5'-GTCAGCACAGAC CTCTCTCT-3', S100A8_fv 5'-ATG CC-3', S100A8_rev 5'-CAG CCA-3', S100A9_fv 5'-GCT CCG TCT TCA-3', S100A9_rev 5'-GCT CCG TCT TCA-3', Ccr2_fv 5'-CTC GTG GAT TTT ACT CGA-3', Ccr2_rev 5'-ATG CTC CCC GGG CTG TAT-3', Ccl2_fv 5'-CTC GTG GAT TTT ACT CGA-3', Ccl2_rev 5'-ATG CTC CCC GGG CTG TAT-3', Ywhaz_fv 5'-CCC ACT CTT CTT CTT CTT-3', Ywhaz_rev 5'-AGA GAG AA-3'.

Nitrergic synapse by assay activity
The assay was performed with an ultra-sensitive assay for nitrergic synapse (NOS) from Oxford Biomedical Research (Cat: NB78). Brie
tly, lysates from isolated CD11b cells were first incubated with substances and cofactors. Then, the mixtures were incubated with nitrate reductase to transform nitrate to nitrite, and mixed with coloring reagent to quantify total end-product concentration. These reactions were performed in a 96-well plate and absorbance was read at 540 nm.

Statistical analysis
The difference in tumor weight between treatment groups was statistically evaluated by the nonparametric Mann–Whitney U test. Differences between experimental groups were tested by either the Student t test or for variances by ANOVA.

Results
Tasquinimod enhances immunotherapy in castration-resistant prostate cancer and melanoma models
Results from previous studies in experimental tumor models indicated that immunomodulatory effects of tasquinimod may contribute to its antitumor activity (23). To investigate the potential immunomodulatory activities of tasquinimod, we tested this agent in combination with a survivin peptide vaccine (SurVaxM) in a survivin-expressing castration-resistant Myc-PCa prostate cancer model and with a TTS in a B16 melanoma model. Survivin is an intracellular TAA expressed in several solid tumors, including prostate cancer (29). SurVaxM is a modified survivin peptide vaccine SVN53-67/M57-KLH (30) that we have tested previously in multiple tumor models (31). FVB mice were inoculated with castration-resistant Myc-PCa cells s.c. Tumor-bearing mice were divided into four groups and treated with vehicle, SurVaxM (1 dose/wk), tasquinimod (10 mg/kg/d in drinking water), or the combination of SurVaxM and tasquinimod. In the castration-resistant Myc-PCa model, SurVaxM and tasquinimod single treatments displayed modest antitumor effect but did not induce significant change in tumor growth (Fig. 1A, left). However, the combination of SurVaxM and tasquinimod significantly inhibited tumor growth (58% reduction; combination vs. vehicle; P = 0.0002). The combination treatment also significantly inhibited tumor growth compared with that of single treatment groups (tasquinimod vs. combination, P = 0.009; survivin vs. combination, P = 0.017). Similarly, both SurVaxM and tasquinimod single treatments induced modest, but not statistically significant, reductions of tumor weight at the endpoint of the study, whereas the combination induced more than additive effect, a 65% reduction from vehicle level (Fig. 1A, right; vehicle vs. combination; P = 0.0002).

In parallel, we tested tasquinimod in combination with a different immunotherapy approach, TTS in a transplantable B16 melanoma model. TTS immunotherapy activates and directs T lymphocytes to attack tumor cells by means of fusion proteins between bacterial superantigens, such as staphylococcal enterotoxin A (SEA) and Fab-fragments of tumor-reactive monoclonal antibodies (mAb; ref. 32). Superantigens activate a high number of CD4+ and CD8+ T cells expressing particular T-cell receptor (TCR)-Vβ chains (33). In this study, B16-h5T4-expressing tumors were treated with tasquinimod (30 mg/kg/d in drinking water), the TTS fusion protein 5T4Fab-SEA at a suboptimal therapeutic dose (25 μg/kg), or the combination. Tasquinimod treatment began the day after tumor-cell inoculation, and 5T4Fab-SEA was administered on days 3 to 6. Although both TTS and tasquinimod single-agent treatments elicited substantial antitumor effects; the combination regimen led to a significant reduction in tumor size at the endpoint (>75% reduction; vehicle vs. combination; P < 0.0001; Fig. 1B). Thus, the combination of tasquinimod with two different immunotherapeutic strategies resulted in a significant enhancement of antitumor effects.

Enhanced immunotherapy is associated with induction of effector T cells and increased antitumor immune responses
To determine whether the observed inhibition of tumor growth induced by the combination strategy was associated with improved immune responses, we examined CD8+ T cells harvested at the end of the experiment. First, using a survivin vaccine-specific peptide-MHC class I tetramer binding assay, we showed that the survivin vaccine, as a single treatment or in combination with tasquinimod, induced antigen-specific CD8+ T cells (Supplementary Fig. S1). We also tested the cellular expression of IFNγ and Granzyme B, which are critical for CD8+ effector T-cell functions. Splenocytes were isolated from differentially treated mice, stimulated, and then stained for cell-surface markers and intracellular proteins. IFNγ expression was increased slightly in CD8+ T cells from combination-treated animals as compared with vehicle group (Fig. 2A), while no significant changes were observed in CD8+ T cells from single agent–treated animals. Similarly, when compared with vehicle- and single agent–treated groups, Granzyme B expression in CD8+ T cells from combination-treated animals was significantly higher (Fig. 2B).

To determine whether the changes in specific CD8+ T cells were associated with an improvement in cytotoxic T lymphocyte (CTL) activity, we tested ex vivo the ability of splenocytes and purified...
CD8+ T cells to kill castration-resistant Myc-CaP tumor cells. Consistent with enhanced antitumor activity observed following combination treatment, splenocytes from these mice displayed significantly improved tumor-cell killing capacity as compared with those from other treatment groups (Fig. 2C, left). Interestingly, when purified CD8+ T cells were used ex vivo in the same assay, tumor-cell killing capacity was equal in all treatment groups (Fig. 2C, right). These results thus suggest that the combination therapy does not enhance CTL activity per se but rather inhibits T cell–suppressing factor(s) in the cultured splenocytes.

In the B16-h5T4 melanoma model, analysis of tumor-infiltrating cells showed that the combination treatment significantly increased accumulation of CD4+ and CD8+ T cells measured at the endpoint as compared with those of control and single-agent treatments (Fig. 2D). To address the influence of tasquinimod on the activation of TTS-reactive T cells, B16-h5T4 tumors were allowed to grow until day 9 before giving three daily injections of ST4Fab-SEA. Tumor-infiltrating cells were analyzed at different days (day 12–16) to follow the kinetics of specific T-cell expansion. Tasquinimod significantly enhanced and prolonged tumor infiltration of TTS-reactive TCR-Vβ3+ CD8+ T cells induced by ST4Fab-SEA (Fig. 2E). The TTS-nonreactive TCR-Vβ8+ CD8+ T cells were only marginally affected by the treatment (Fig. 2F). In contrast, the TCR-Vβ3+ CD4+ T-cell population was less enhanced by the combination (Fig. 2G).

Tasquinimod has been reported to display antiangiogenic activity in prostate cancer models (19, 34). To determine whether the antiangiogenic effect of tasquinimod was involved in enhancing the antitumor effects of immunotherapy, we assessed the microvasculature density (CD31 expression) in the harvested tumor tissue by either immunofluorescence or immunohistochemistry analysis in the two therapeutic strategies, respectively. The results showed that tasquinimod treatment reduced microvasculature density in B16 tumors (Fig. 3A), but it did not change tumor vasculature in the castration-resistant Myc-CaP model (Fig. 3B). In summary, these results suggest that the immunomodulatory effects of tasquinimod may be dissociated from its antiangiogenic activity, and in the B16-h5T4 tumor model, the tasquinimod-induced inhibition of tumor blood vessel formation may account at least in part for its antitumor effect in this model.

Infiltration of suppressive myeloid-cell populations is reduced by tasquinimod treatment in immunotherapy
S100A9 is an inflammatory protein that affects the accumulation of immunosuppressive myeloid cells, including MDSCs (24, 25). Tasquinimod binds to S100A9, inhibiting its downstream signaling, and thus has the potential to affect myeloid cells. To investigate the mechanism of immune-promoting activity of tasquinimod in combination with immunotherapy, we analyzed the peripheral and tumor-infiltrating myeloid-cell populations.

In the castration-resistant Myc-CaP tumor model, blood samples were taken from differentially treated mice after 2 weeks of treatments and subjected to immunofluorescence staining and FACS analysis. We observed three different CD11b+ cell populations in the blood distinct by their expression levels of the Gr1 marker: Gr1neg, Gr1low, and Gr1high (Supplementary Fig. S2A). Tasquinimod did not affect the number of either Gr1low CD11b+ cells or Gr1high CD11b+ MDSCs in the blood, but decreased the Gr1neg CD11b+ population (Supplementary
Similarly, the number of MDSCs in the spleen did not change following treatments (Supplementary Fig. S2C). In addition, tumors were harvested from differentially treated mice and processed into suspension. Interestingly, tasquinimod significantly reduced the number of tumor-infiltrating MDSCs when given as a single agent or in combination with the vaccine (Fig. 4A). Further analysis of MDSC subpopulations present in the blood and tumors revealed a striking dominance of the granulocytic CD11b+Ly6CintLy6G+ population (Supplementary Fig. S2B and S2D).

A similar analysis of CD11b+ cells and MDSC subpopulations was performed in the B16-h5T4 model (C57Bl/6 strain). The frequency of tumor-infiltrating CD11b+ cells was not altered following tasquinimod treatment (Supplementary Fig. S3A), whereas a significant reduction of the number of CD11b+ cells was observed in the spleen (Supplementary Fig. S3B). In contrast to the castration-resistant Myc-CaP model, the majority of MDSCs in untreated B16-h5T4 tumors were of the CD11b+Ly6Cint/Ly6G+ monocytic subtype (Fig. 4B, left). Moreover, a significant reduction of the CD11b+Ly6Cint/Ly6G+ monocytic...
A subpopulation was observed while the proportion of CD11b<sup>+</sup> Ly6<sup>c</sup><sup>hi</sup>Ly6G<sup>-</sup> granulocytic MDSCs increased in tumors upon tasquinimod treatment (Fig. 4B). A comparable picture was also seen in the spleen (Supplementary Fig. S3C). Interestingly, the tumor-infiltrating CD11b<sup>+</sup> Ly6<sup>c</sup><sup>hi</sup>Ly6G<sup>-</sup> MDSCs expressed high levels of the angiopoietin receptor Tie2 (data not shown), which plays a key role in tumor angiogenesis (35). Thus, the decrease in microvasculature density by tasquinimod in the B16 model could be the consequence of reducing proangiogenic monocytic cells within the tumors.

TAMs are important components of the immunosuppressive TME. Immature monocytes and monocytic MDSCs migrate to the tumor in response to inflammatory mediators released from the TME. When infiltrating the tumor tissue, these cells adapt to the environment and differentiate into TAMs by losing Gr1 marker expression and gaining an even more immunosuppressive M2 macrophage phenotype (36–38). Therefore, we assessed the effect of tasquinimod on TAMs. Results from the castration-resistant Myc-CaP model showed that tasquinimod treatment led to a reduction of infiltrating CD206<sup>+</sup> M2 TAMs (Fig. 4C). Similarly, analysis of macrophages in B16-hST4 tumors also revealed a strong reduction of this subpopulation in tasquinimod-treated mice (Fig. 4D).
In addition to MDSCs and TAMs, we also investigated whether tasquinimod treatment affects immune-promoting activities of other myeloid and lymphoid cells. Tasquinimod did not impair T-cell expansion upon activation either in T cells isolated from differentially treated mice (Supplementary Fig. S4A) or when tasquinimod was added in culture (Supplementary Fig. S4B). Tregs represent an immunosuppressive lymphocyte population whose accumulation can be regulated by MDSCs. In both castration-resistant Myc-CaP (Supplementary Fig. S4C, left) and B16-h5T4 (Supplementary Fig. S4C, right) models, tasquinimod increased the accumulation of Tregs. DC differentiation has been shown to be regulated by the S100A9 protein (24). Although tasquinimod slightly reduced the number of DCs in the spleen (Supplementary Fig. S5A), drug treatment did not impair the capacity of DCs to stimulate T cells (Supplementary Fig. S5B). These data suggest that immunosuppressive myeloid cells, such as MDSCs and TAMs, but not other myeloid or lymphoid populations, are the potential cellular targets of tasquinimod and they may be responsible for the immune-promoting activity of tasquinimod in combination with immunotherapies.

Tasquinimod inhibits immunosuppressive functions of tumor-associated myeloid cells and modulates relevant gene expression

So far, we have shown that tasquinimod significantly reduced the numbers of distinct MDSCs and altered the TAM populations in two different tumor models, suggesting that tasquinimod may affect the accumulation/trafficking of immunosuppressive myeloid cells into the tumors. To investigate the mechanisms by which tasquinimod regulates these cells, we measured the immunosuppressive capacity of intratumoral CD11b⁺ myeloid cells on T-cell activation. CD11b⁺ cells were purified from tumors and cultured with purified, stimulated T cells. As expected, CD11b⁺ cells from tumors inhibited T-cell proliferation (Fig. 5A). However, CD11b⁺ cells isolated from tasquinimod-treated castration-resistant Myc-CaP tumors showed significantly less suppression on T-cell proliferation compared with that of the controls (Fig. 5A). Similarly, CD11b⁺ cells purified from tasquinimod-treated B16-h5T4 tumors were also less suppressive (Fig. 5B). In this experiment, a CFSE-based method was used to detect CD4⁺ and CD8⁺ T-cell proliferation. Inhibition of T-cell division by CD11b⁺ cells was significantly lower following

Figure 4.
Tasquinimod modulates MDSC and TAM populations in the castration-resistant Myc-CaP and B16-h5T4 tumor models. A, FACS analysis of tumor-cell suspensions from the castration-resistant Myc-CaP model for CD11b⁺Gr1⁺ MDSCs after tasquinimod treatment. B, FACS analysis of tumor-cell suspensions from the B16-h5T4 model for granulocytic Ly6G⁺Ly6C⁺ and monocytic Ly6G⁻Ly6C⁻ GR1⁻ MDSCs. FACS analysis of tumor-cell suspensions for F4/80⁺ macrophages and for CD206 expression from castration-resistant Myc-CaP tumors (C) and B16-5T4 tumors (D). FACS plots and quantifications are depicted throughout (*, P < 0.05; **, P < 0.01; ***, P < 0.001, t test; error bars indicate SEM).
Tasquinimod treatment. Taken together, these results suggest that tasquinimod modulates not only the infiltration but also the suppressive capacity of tumor-infiltrating myeloid-cell populations.

As shown in Fig. 4C, the majority of the tumor-infiltrating myeloid cells in the castration-resistant Myc-CaP model are macrophages, and tasquinimod treatment reduced CD206^+ immunosuppressive M2 macrophages (Fig. 4C and D). This observation led us to investigate the expression of two mechanistically relevant genes, arginase-1 (Arg1) and inducible NOS (iNOS) in the tumor-infiltrating myeloid cells (Fig. 5C–E). Previous studies have shown that Arg1 expression is critical to the...
suppressive function of MDSCs and TAMs. It has been reported that Arg1 gene expression can be regulated by the TLR4 pathway (39), which is a target receptor for S100A9. The iNOS marker can be used to differentiate cytotoxic M1 macrophages from immunosuppressive M2 macrophages. Intracellular staining and flow cytometry analysis of castration-resistant Myc-CaP tumors showed that tasquinimod reduced Arg1 expression in myeloid cells and induced significant iNOS expression, which indicates an increase in immune-promoting M1 macrophages (Fig. 5C). In the B16-5T4 model, mRNA analysis also indicated that tasquinimod shifted an M2 macrophage (immunosuppressive) gene expression signature into an M1 macrophage signature (Fig. 5D). FACS analysis confirmed the reduction of Arg1 expression and the induction of iNOS in tumor-infiltrating Ly6Chigh monocytic cells, although not as dramatically as those in the castration-resistant Myc-CaP model (Fig. 5E).

We also tested the enzymatic activities of NOS and Arg1 in infiltrating myeloid cells. Tasquinimod treatment in vivo led to a significant increase of NOS activity, as compared with vehicle treatment (Supplementary Fig. S6). The arginase activity assay did not reveal a significant change between these two conditions (data not shown).

**Tasquinimod treatment reduces the ability of suppressive myeloid cells to support tumor growth**

In the therapeutic studies, tasquinimod treatment enhanced immune responses and vaccine effects (Figs. 1 and 2 and Supplementary Fig S1). We hypothesized that suppressive myeloid cells, including MDSCs and TAMs and not the other populations, are potential targets of tasquinimod immunomodulatory activity (Figs. 4 and 5 and Supplementary Figs. S4 and S5). To test this hypothesis, castration-resistant Myc-CaP cells were inoculated into FVB mice as described. When tumor growth was established, mice were randomized into two groups receiving either vehicle or tasquinimod treatment for 4 weeks. CD11b+ myeloid cells, isolated from tumors that were harvested from different treatment groups, were mixed with fresh castration-resistant Myc-CaP cells and inoculated into recipient FVB mice receiving SurVaxM vaccine therapy. As shown in Fig. 6, inoculations containing tasquinimod-treated tumor-derived myeloid cells induced significantly slower tumor growth, as compared with those containing vehicle-treated tumor-derived myeloid cells. These data indicate that tasquinimod directly impairs the tumor-promoting activity of immunosuppressive myeloid cells.

**Discussion**

The aim of immunotherapy is to induce durable and effective immune responses. MDSCs and TAMs contribute to immune tolerance in the TME and consequently affect the efficacy of immunotherapies. Our study provides evidence supporting the development of tasquinimod as a novel approach to target the immunosuppressive TME and facilitate immunotherapy. The data were generated in parallel in two different laboratories, providing evidence for reproducibility of our observations.

We tested two different immunotherapeutic strategies in combination with tasquinimod in two murine tumor models, and observed a similar immunomodulating effect by tasquinimod coupled to modulation of tumor-infiltrating MDSCs and TAMs. These myeloid populations express receptors for S100A9 and are likely cellular targets for tasquinimod. Furthermore, we demonstrated that the adoptively transferred tasquinimod-treated myeloid cells were sufficient to delay tumor growth in vaccinated animals, as compared with tumor inoculates with vehicle-treated myeloid cells (Fig. 6). There were differences in the subpopulations of tumor-induced myeloid cells in the two models, possibly due to the different tumor origins. Granulocytic MDSCs are prevalent in the castration-resistant Myc-CaP model on C57Bl/6 background. Upon tasquinimod treatment, the Ly6G+Ly6C− monocytes were reduced in the vaccines treated animals, as compared with those containing vehicle-treated tumor-derived myeloid cells. These data indicate that tasquinimod directly impairs the tumor-promoting activity of immunosuppressive myeloid cells.

**Figure 6.**

Tasquinimod treatment reduces the ability of suppressive myeloid cells to support tumor growth. CD11b+ cells were isolated from tumors collected from either vehicle or tasquinimod-treated donor mice, mixed with fresh castration-resistant Myc-CaP cells (mixture contained 1.5 × 10^6 castration-resistant Myc-CaP cells and 0.75 × 10^6 CD11b+ cells), and inoculated s.c. into recipient mice receiving SurVaxM vaccine. Recipient mice received two doses of the vaccine before inoculation and two additional doses were administered after tumor-cell inoculation. Left, tumor growth curves by serial caliber measurements. Right, end of treatment tumor weights (*, P < 0.05, Mann-Whitney U test; error bars indicate SEM).
B16-h5T4 tumors (Fig. 4B), and the total number of Gr1−CD11b− MDSCs was reduced in castration-resistant Myc-CaP tumors (Fig. 4A). At peripheral sites, tasquinimod treatment led to depletion of Gr1−CD11b− monocytes in castration-resistant Myc-CaP tumor-bearing mice (Supplementary Fig. S2A), and a significant reduction of the CD11b+/Ly6ChighLy6G− and CD11b+/Ly6Cin−Ly6G− monocytic populations in the B16-h5T4 tumor-bearing animals (Supplementary Fig. S3 and data not shown). These observations suggest that immature monocytes are potential targets for tasquinimod. Because monocyteic MDSCs or immature monocytes can be precursors of TAMs (38), the reduction of monocytes at peripheral sites could lead to an altered profile of TAMs observed in both models (Fig. 4C and D).

As shown in Fig. 2C, spleenocytes from mice treated with the combined regimen of vaccine and tasquinimod presented increased tumor-cell killing ex vivo, compared with that of vehicle and single treatment groups. However, the purified CD8+ effector T cells from mice treated with the combined regimen did not show a significant difference in cytotoxicity against tumor cells. This result suggests that the combined treatment does not affect effector T-cell functions directly but instead it relieves the immunosuppression present in the cultures, such as the inhibition by immunosuppressive MDSCs. We observed no inhibition of T-cell proliferation ex vivo or when tasquinimod was added to culture at high concentrations (Supplementary Fig. S4). The effect of combination treatment on specific T-cell activation in tumors was addressed in the B16-h5T4 mouse model. Tumor-infiltrating effector T cells by TCR-Vβ expression demonstrated increased proliferation and prolonged presence of TTS-activated CD8+ T cells in tumors following tasquinimod cotreatment, further supporting the induction of a less immunosuppressive environment. A similar increase in tumor-infiltrating CTLs in B16 tumors was reported recently following TTS therapy in combination with anti–CTLA-4 checkpoint blockade (40).

Previous reports have shown that MDSC-targeting strategies affect systemic or peripheral MDSC accumulation (25, 41). For example, mAbG8B3.1, an antibody against the carboxylated N-glycan on RAGE, reduced MDSC accumulation in blood, spleen, and lymph nodes in 4T1 tumor-bearing animals, but not in the metastatic site. However, this antibody treatment did not affect the suppressive function of MDSCs (25). In our castration-resistant Myc-CaP model, tasquinimod did not change the number of Gr1−CD11b− MDSCs at peripheral sites (Supplementary Fig. S2), which would suggest that tasquinimod does not affect the generation or expansion of MDSCs. However, tasquinimod reduced tumor-infiltrating MDSCs (Fig. 4A). This observation suggests that tasquinimod may inhibit MDSC trafficking/accumulation in the tumor, leading to modulation of the TME and relief of immune tolerance. In support of this finding, S100A9 signaling has been reported to regulate both expansion and migration of MDSCs (24, 25). It has also been shown that intracellular S100A9 expression in myeloid progenitor cells induces MDSC expansion (24, 42). However, extracellular (secreted) S100A9 protein binds to carboxylated N-glycan receptors (RAGE) that are expressed on the surface of MDSCs and promotes MDSC migration to the site of tumors (25, 43). Taken together, our results provide evidence supporting a mechanism of action by tasquinimod in blocking extracellular S100A9 and receptor signaling that may be critical to MDSC tumor infiltration via cell surface receptors such as TLR4 (23) and RAGE (22).

The notion of a cross-talk between different regulatory myeloid cells is well established (44). Aside from the reduction and modulation of tumor-associated MDSCs, tasquinimod treatment resulted in decreased numbers of CD206+ M2-polarized TAMs and reduced the suppressive function of CD11b-expressing TAMs (Figs. 4 and 5). Macrophages are categorized as either the classically activated, cytotoxic M1 macrophages, or the alternatively activated, suppressive M2 macrophages. The M2-polarized TAMs are enriched in hypoxic tumor areas with a superior proangiogenic activity in vivo, a limited capacity to present antigen, and the ability to suppress adaptive immune responses such as T-cell activation (38, 45). In the castration-resistant Myc-CaP and B16-h5T4 models, F4/80+ macrophages represent the major population of tumor infiltrates and a large component of these infiltrating macrophages are CD206+, M2-like type, which is significantly reduced upon tasquinimod treatment (Fig. 4C and D). The function of macrophages depends on the expression of Arg1 and iNOS. Although classically activated M1-polarized macrophages express both Arg1 and iNOS, suppressive TAMs only express Arg1, which is critical for the immunosuppressive function. As shown in Fig. 5, tasquinimod treatment reduced Arg1 expression in CD11b+ cells in both models (Fig. 5C–E), which could explain the reduced suppressive function of these cells (Fig. 5A and B). An important regulator of Arg1 gene expression is TLR4 signaling (39), which is a receptor for a tasquinimod-target protein, S100A9. Potentially, the S100A9–TLR4–Arg1 pathway may be involved in tasquinimod-induced changes of suppressive myeloid cells. Interestingly, in the castration-resistant Myc-CaP model, tasquinimod induced iNOS expression in CD11b+ cells (Fig. 5C). An assay testing NOS enzyme also showed that tasquinimod-treated CD11b+ cells had higher NOS activity (Supplementary Fig. S6). iNOS is mainly expressed in macrophages and monocytic MDSCs, whereas granulocytic MDSCs have low iNOS. Therefore, the increase of iNOS in CD11b+ cells is likely due to an increase of M1 macrophages in the tumor, rather than an induction of monocytic MDSCs because the vast majority of MDSCs in the tumors of this model are of the granulocytic type (Supplementary Fig. S2D).

Tasquinimod has pleiotropic effects that contribute to its antitumor activity, including antiangiogenesis, immunomodulation, and inhibition of metastasis. As demonstrated in this study, modulation of suppressive myeloid cells may represent a critical biologic mechanism of action of tasquinimod and the common target giving rise to the diverse effects. Immunosuppressive myeloid cells (MDSCs and TAMs) secrete multiple factors, including VEGF and MMP9, which promote angiogenesis (46). In a hypoxic microenvironment, myeloid cells can also recruit endothelial cells and their precursors (47). MDSCs have also been reported to promote tumor-cell dissemination (48) and cancer stemness (49). In addition, MDSCs and TAMs have the potential to prime distal sites to promote the seeding of metastatic tumor cells (50–52). Moreover, MDSCs have been found to promote cancer cell survival upon chemotherapy by producing certain chemokines (53). These findings suggest that suppressive myeloid populations represent key mediators of multiple critical aspects of cancer immune tolerance, metastasis, and drug resistance. The inhibitory effects of tasquinimod on tumor-infiltrating...
immunosuppressive myeloid cells, and, in particular, on the M2-polarized TAMs, have been observed in preclinical syngeneic tumor models. These biologic properties of tasquinimod support the further development of this agent for clinical combination strategies with immunotherapies such as vaccines and immune checkpoint inhibitors. On the basis of our preliminary data, a clinical trial of tasquinimod in combination with sipuleucel-T in patients with metastatic castration-resistant prostate cancer is planned to open in 2014.

In conclusion, tasquinimod is a small-molecule inhibitor with a potentially unique mechanism of action that targets the TME. Future preclinical and clinical testing of this agent will define its application in a wide range of therapeutic strategies including immunotherapies, antiangiogenic agents, and antimetastatic drugs.

Disclosure of Potential Conflicts of Interest
A. Sundstedt, M. Celander, H. Eriksson, T. Leanderson, A. Olsson have ownership interest (including patents) in Active Biotech. R. Pili reports receiving a commercial research grant from ActivelyBiotech AB and is a consultant/advisory board member for Ipsen. M. Ciesielski has ownership interest (including patents) in MimiVax, LLC. R. Fenstermaker is the founder of MimiVax LLC. No potential conflicts of interest were disclosed by the other authors.

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