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Dasatinib promotes the expansion of a therapeutically superior T-cell repertoire in response to dendritic cell vaccination against melanoma

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Abbreviations: CTLA-4, cytotoxic T-lymphocyte antigen 4; DAS, dasatinib; DC, dendritic cell; DGKα, diacylglycerol kinase α; Fmoc, 9-fluorenylmethyloxycarbonyl; GM-CSF, granulocyte-macrophage colony-stimulating factor, IACUC, Institutional Animal Care and Use Committee; IL, interleukin; Lck, lymphocyte-specific protein tyrosine kinase; MACS, magnetic-activated cell sorting; MART1, melanoma antigen recognized by T cells 1; MDSC, myeloid-derived suppressor cells; OVA, ovalbumin; PD-1, programmed cell death protein 1; PDGFR, platelet-derived growth factor receptor; STAT, signal transducer and activator of transcription; TCR, T cell receptor; TDLN, tumor-draining lymph node; TIL, tumor-infiltrating lymphocyte; Treg, regulatory T cell; VAC, DC vaccine

Dasatinib (DAS) is a potent inhibitor of the BCR-ABL, SRC, c-KIT, PDGFR, and ephrin tyrosine kinases that has demonstrated only modest clinical efficacy in melanoma patients. Given reports suggesting that DAS enhances T cell infiltration into the tumor microenvironment, we analyzed whether therapy employing the combination of DAS plus dendritic cell (DC) vaccination would promote superior immunotherapeutic benefit against melanoma. Using a M05 (B16.OVA) melanoma mouse model, we observed that a 7-day course of orally-administered DAS (0.1 mg/day) combined with a DC-based vaccine (VAC) against the $OVA_{257–264}$ peptide epitope more potently inhibited tumor growth and extended overall survival as compared with treatment with either single modality. The superior efficacy of the combinatorial treatment regimen included a reduction in hypoxic-signaling associated with reduced levels of immunosuppressive $CD11b^+Gr1^+$ myeloid-derived suppressor cells (MDSC) and $CD4^+Foxp3^+$ regulatory T (Treg) populations in the melanoma microenvironment. Furthermore, DAS + VAC combined therapy upregulated expression of Type-1 T cell recruiting CXCR3 ligand chemokines in the tumor stroma correlating with activation and recruitment of Type-1, vaccine-induced $CXCR3^+CD8^+$ tumor-infiltrating lymphocytes (TILs) and $CD11c^+$ DC into the tumor microenvironment. The culmination of this bimodal approach was a profound "spreading" in the repertoire of tumor-associated antigens recognized by $CD8^+$ TILs, in support of the therapeutic superiority of combined DAS + VAC immunotherapy in the melanoma setting.

Introduction

A broad range of small molecule kinase inhibitors have been developed as anticancer therapies due to their abilities to antagonize signaling pathways associated with tumor growth, survival, and metastasis, as well as those regulating tumor-associated neo-angiogenesis.[1–5] More recently, a number of these inhibitors have also been observed to modulate stromal cell populations, including immune cells, present within the tumor microenvironment, hence expanding appreciation of the clinical utility of these drugs as immune modulators.[6–16]

Dasatinib (DAS) is a broad-spectrum multi-kinase inhibitor that exhibits modest single-agent clinical efficacy in patients afflicted with diverse forms of cancer, including melanoma.[3–5,17,18] Interestingly, although DAS has been reported to inhibit T-cell activation via blockade of lymphocyte-specific protein tyrosine

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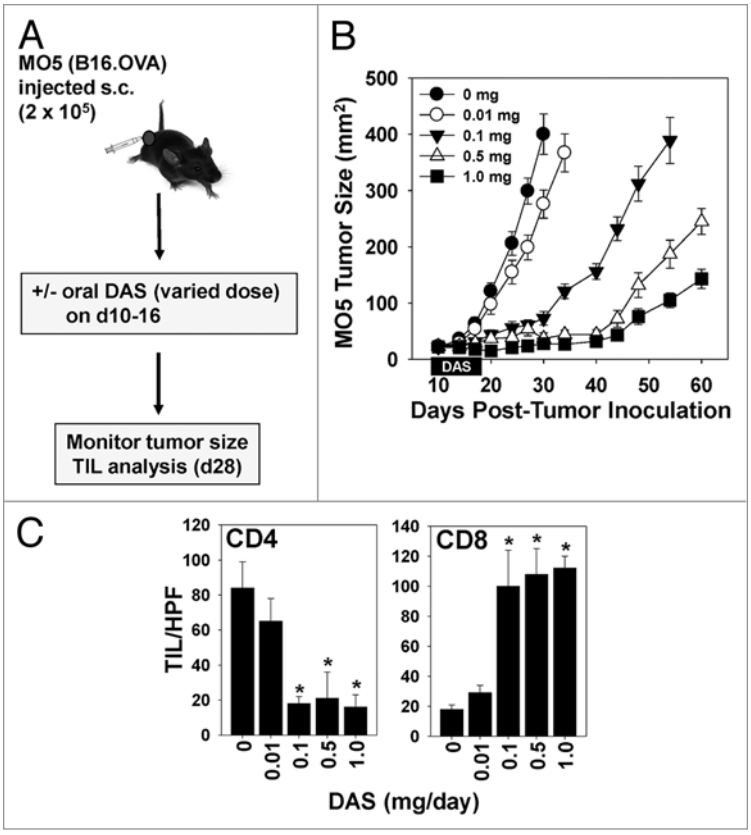

Figure 1. Therapeutic administration of dasatinib monotherapy elevates the numbers of tumor-infiltrating CD8⁺ T cells and exhibits dose-dependent anti-melanoma efficacy. (**A–C**) M05 melanoma cells were injected sub-cutaneously into syngenic C57BL/6 mice and allowed to establish for 10 d, at which time animals were randomized by tumor size and cohorts (n = 7 mice/group) were left untreated (controls) or were treated with orally administered dasatinib (DAS, dose ranging from 0.01–1 mg/day as indicated) for 7 consecutive days. (**B**) Animals were monitored for tumor growth every 3–4 d using external calipers and estimated tumor volume calculated as the product of the orthogonal measurements. Data are the mean ± SD tumor volume per group. (**C**) On day 28, 2 mice/group were sacrificed and cryo-preserved tumor sections analyzed by immunofluorescence staining and fluorescence microscopy to assess the numbers of CD4⁺ and CD8⁺ tumor-infiltrating lymphocytes (TILs). The mean ± SD number of cells over 10 high-power fields (HPF) are reported. Representative data from 1 of 3 independent experiments are depicted. Statistical analysis was performed by 1-way ANOVA; *$P < 0.05$ vs. untreated or DAS 0.01 mg/day.

kinase (Lck)-mediated proximal T-cell receptor (TCR) signaling in vitro,[19,20] upon administration in vivo, DAS can profoundly enhance T effector cell activation, expansion, and function.[21–25] Indeed, pre-clinical modeling suggests that T cells play a dominant role in the antitumor activity of DAS treatment in vivo,[21] providing rationale for the development of potential immunotherapeutic applications. In this particular context, Yang et al.[21] have also recently reported that the treatment of BALB/c mice bearing P815 mastocytomas with DAS improved recruitment of T cells into the tumor and surrounding stroma, an immunological response also required to achieve the clinical benefit associated with therapeutic vaccines.[11,12,26] Consequently, we hypothesized a combinatorial protocol, comprising specific vaccination to activate and expand tumoricidal CD8⁺ T cells plus systemic administration of DAS to facilitate the refined targeting of vaccine-induced T effector cells to the tumor microenvironment, would be therapeutically superior to monotherapy.

In the current study, we utilized mice bearing established sub-cutaneous M05 (B16.OVA) melanoma to experimentally test this hypothesis. We report here that bimodal therapy with the combination of tumor-specific (OVA peptide) DC-based vaccine (VAC) + DAS provide superior antitumor benefit as compared with treatment using either single-agent modality alone. Our results provide an experimental basis for the clinical translation of combined DAS + VAC immunotherapies for the treatment of cancer, particularly melanoma.

Results

DAS monotherapy mediates substantial anti-melanoma activity in association with enhanced CD8⁺ T cell infiltration into the tumor microenvironment

Before assessing the potential immunologic benefits of combining DAS with vaccine-based immunotherapy, we first established an optimal dose of DAS monotherapy based on tumor growth suppression and the preferred immunologic endpoint of tumor-infiltrating lymphocyte (TIL) recruitment. C57BL/6 mice bearing established (implanted 10 d prior) subcutaneous M05 melanomas were left either untreated (controls) or were treated daily with DAS at doses ranging from 0.01–1.0 mg/day for 1 wk by oral gavage (**Fig. 1A**). We observed significant inhibition of melanoma growth at doses of DAS in excess of 0.01 mg/day (**Fig. 1B**, $P < 0.05$ for all time points past 17d post tumor-inoculation vs. the untreated control, ANOVA), which was associated with a dramatic rise in CD8⁺ (but not CD4⁺) TILs (**Fig. 1C**). Based on these data, we selected a DAS dose of 0.1 mg/day for our combinational therapies, as this was the minimal dose of single-agent drug yielding discernable, yet sub-optimal antitumor efficacy and a modest elevation in CD8⁺ TIL numbers, thereby permitting assessment of improved treatment outcome upon co-administering DAS together with a cancer-specific vaccine.

DAS potentiates the immunogenicity and therapeutic efficacy of peptide-based dendritic cell vaccine in vivo

We next sought to test the impact of DAS on vaccine efficacy in vivo. C57BL/6 mice bearing subcutaneous M05 melanomas established 10 d prior were left untreated or were treated with genetically modified dendritic cell (DC) VAC comprising $OVA_{257–264}$ peptide-pulsed DC overexpressing murine interkeukin-12 (IL-12) that we have previously shown to promote robust T-helper independent anti-OVA Type1 cytotoxic T (Tc) cell responses in C57BL/6 mice.[12] Experimental animals were administered either s.c. contralateral VAC on days 10 and 17, DAS (0.1 mg/day via oral gavage on days 10–16) alone, or a

combination of the s.c. VAC and oral DAS (**Fig. 2A**). While untreated animals displayed rapidly progressive disease that required euthanasia in accordance with IACUC guidelines by 34 d post-tumor inoculation, M05-bearing mice treated with either single modality (i.e., DAS or VAC) harbored tumors with a slower growth rate and exhibited an extended survival period of approximately 15–25 d relative to untreated control animals (**Fig. 2B**). In contrast, animals treated with combined DAS + VAC therapy exhibited profoundly reduced melanoma growth (**Fig. 2B**, $P < 0.05$ vs. all other cohorts after day 20).

Analyses of tumor-infiltrating immune cells on day 34 revealed significantly increased numbers of CD8$^+$ T lymphocytes and CD11c$^+$ DC in the tumors of mice treated with DAS, VAC, or DAS + VAC, with a statistically elevated level of CD8$^+$ T effector cells in mice receiving the combination therapy (**Fig. 2C**, $P < 0.05$ for DAS + VAC) in comparison to all other cohorts. In contrast, the levels of CD4$^+$ T cells in all treatment groups were found to be significantly decreased relative to those in untreated controls. As shown in **Figure 2D** a corresponding RT-PCR analysis of total tumor mRNA extracted from representative tumors revealed that the combination therapy appeared to stimulate the highest expression of transcripts encoding pro-inflammatory cytokine and chemokines. These include interferon-γ (IFNγ) and leukocyte trafficking regulatory proteins chemokine (C-X-C motif) ligand variants 9–11 (CXCL9–11), as well as their corresponding chemokine receptor CXCR3, immunoregulatory molecules known to be expressed by Type1 effector T cells (**Fig. 2D**). In addition, purified CD11c$^+$ DC isolated from tumor digests subsequently challenged with lipopolysaccharide ex vivo also exhibited higher production of IL-12p70 and reduced production of IL-10 in cell preparations from the DAS + VAC cohort vs. all other cohorts (**Fig. S1**, $P < 0.05$, ANOVA). Taken together, these data support the notion that combined treatment with DAS + VAC fosters superior pro-inflammatory Type1 CD8$^+$ effector T cell and DC infiltration into the therapeutic tumor microenvironment. Furthermore, based on antibody-mediated T-cell subset depletion studies in vivo, we demonstrate that the superior antitumor efficacy associated with combined DAS + VAC therapy is largely CD8$^+$ T-cell dependent (**Fig. S2**), whereas depletion of CD4$^+$ T cells has no effect.

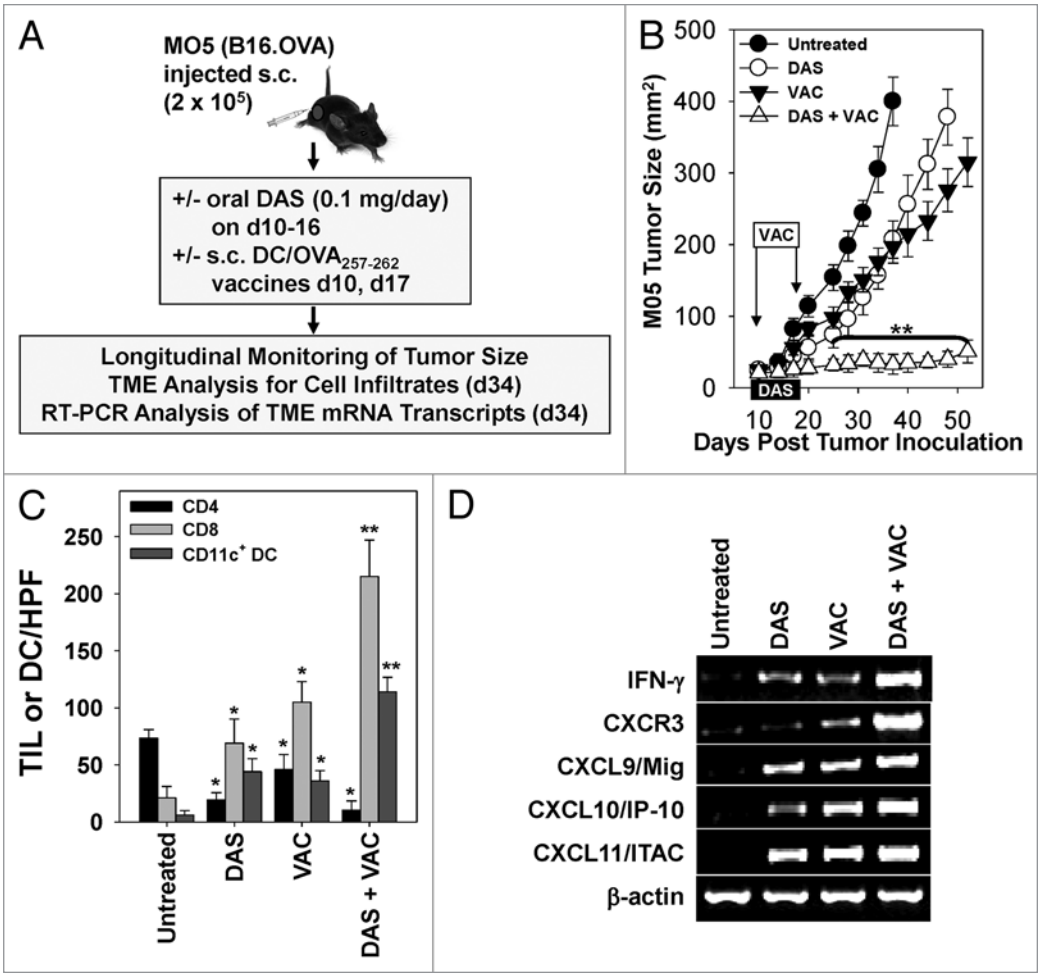

Figure 2. Combination dasatinib + OVA peptide-based dendritic cell vaccination therapy yields superior antitumor efficacy and immune cell recruitment into the tumor microenvironment vs. either monotherapy. (**A–D**) C57BL/6 mice bearing subcutaneous M05 melanomas (n = 7 mice/group) established 10 d prior were left untreated or were treated with either 0.1 mg/day dasatinib (DAS) administered by oral gavage for 7 consecutive days, contralateral s.c. vaccination (VAC) consisting of 10^6 $OVA_{257–264}$ peptide-pulsed dendritic cells (DCs) genetically modified to overexpress IL-12, or a combination of DAS + VAC. (**B**) Animals were monitored for tumor growth every 3–4 d using external calipers and estimated tumor volume calculated as the product of the orthogonal measurements. Data are the mean ± SD tumor volume per group. (**C**) On day 34, 2 mice/group were sacrificed and cryo-preserved tumor sections analyzed by immunofluorescence staining and fluorescence microscopy for CD4$^+$ T cell, CD8$^+$ T cell, and CD11c$^+$ DC content. The numbers of CD4$^+$ and CD8$^+$ tumor-infiltrating lymphocytes (TILs) or DC are reported as the mean ± SD of the indicated cells over 10 high-power fields (HPF). (**D**) Total tumor mRNA purified from representative tumors from the indicated treatment group was analyzed by RT-PCR for expression of the indicated Type-1-associated cytokine, chemokine, or chemokine receptor transcript. Representative data from 1 of 3 independent experiments are depicted. Statistical analyses were performed by Student's *t* test or 1-way ANOVA; *$P < 0.05$ vs. untreated (*t* test) and **$P < 0.05$ vs. all other groups (ANOVA).

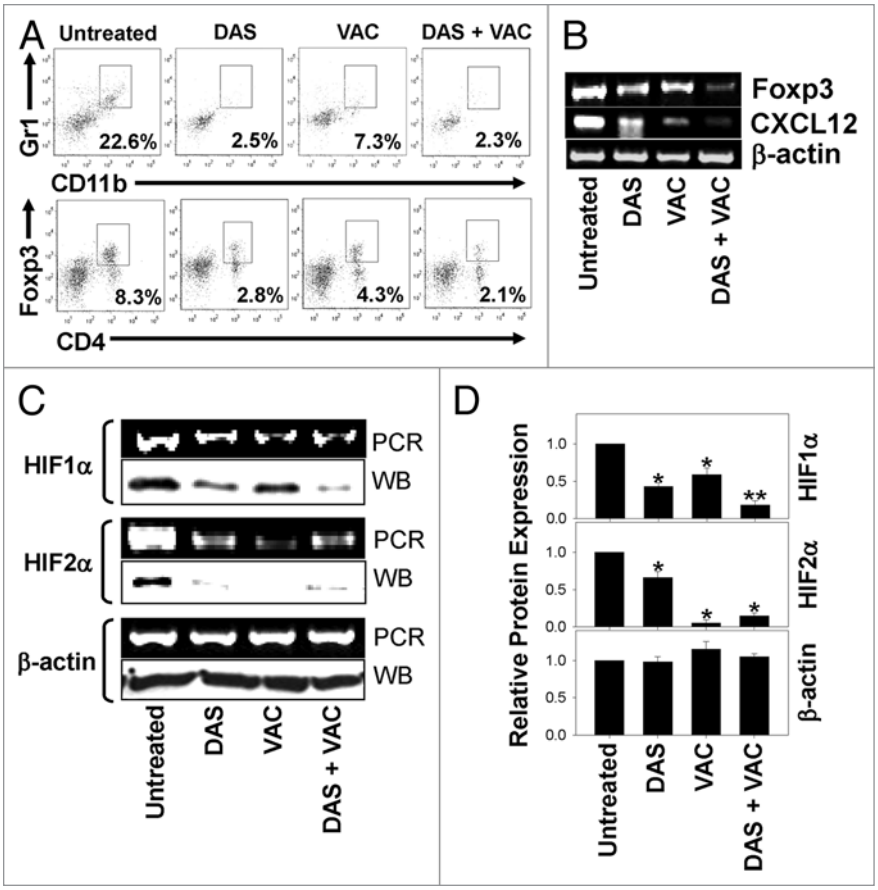

Figure 3. Combinatorial DAS + VAC therapy reduces immunoregulatory cell populations and alters hypoxia-mediated signaling in the tumor microenvironment. (**A–D**) Day 34 M05 melanomas were isolated from host mice that were left untreated or were treated (starting on d10 post tumor cell s.c. injection) with either 0.1 mg/day dasatinib (DAS) orally administered for 7 consecutive days, or contralateral s.c. vaccination (VAC) consisting of 10^6 $OVA_{257–264}$ peptide-pulsed dendritic cells (DCs) genetically modified to overexpress IL-12, or a combination of DAS + VAC. (**A**) Dissociated tumors from the various treatment groups were analyzed for their content of myeloid-derived suppressor cells (MDSC; $CD11b^+Gr1^+$) and regulatory T cells (Tregs, $CD4^+Foxp3^+$) by immunofluorescence staining and flow cytometry. The percentage of cells bearing the specified phenotype is reported in panel insets. (**B and C**) RT-PCR analysis of the levels of Foxp3, CXCL12, HIF1α, HIF2α, and β-actin mRNA transcripts (panels **B and C**) and western blot analysis for expression of HIF1α, HIF2α, and β-actin protein (panel **C**). (**D**) Densitometric analysis of western blots (shown in **C**) scanned to determine comparative protein levels in each tumor cohort relative to the untreated control that was assigned an arbitrary value of 1.0. Representative data from 1 of 3 independent experiments is depicted. Statistical analyses were performed by Student's *t* test or 1-way ANOVA; $^*P < 0.05$ vs. untreated (*t* test), $^{**}P < 0.05$ vs. all other groups (ANOVA).

Combinatorial DAS + VAC therapy reduces immunoregulatory cell populations and alters hypoxia-mediated signaling in the tumor microenvironment

Considering that protective Type1 $CD8^+$ effector T cells would likely mediate more robust anticancer function under conditions that are unopposed by regulatory cell populations,[12] we next examined tumors for therapy-associated changes in suppressor cell subsets, i.e., $CD11b^+Gr1^+$ myeloid-derived suppressor cell (MDSC) and $CD4^+Foxp3^+$ regulatory T (Treg) cells. As shown in **Figure 3A**, cytofluorometric analysis revealed that progressive and rapidly growing, untreated day 34 M05 tumors accrue high frequencies of immune cells bearing regulatory phenotypes. In contrast, levels of these suppressor cell populations were dramatically reduced in melanomas harvested from animals treated with either single agent modality or with the combination DAS + VAC immunotherapy (**Fig. 3A**). Notably, among $CD4^+$ TILs, $CD4^+Foxp3^+$ Tregs appeared to be preferentially reduced relative to untreated control levels, whereas the frequency of $CD4^+Foxp3^-$ T cells seemed comparable in all treatment cohorts (**Fig. 3A**). Corresponding RT-PCR analyses revealed loss of the Foxp3 and CXCL12, a hypoxia-responsive chemokine known to recruit MDSC and Treg cells,[27,28] encoding transcripts in the tumors of treated mice, particularly those receiving the DAS + VAC combined therapy (**Fig. 3B**). Furthermore the canonical markers of hypoxia, hypoxia-inducible factor 1 α (HIF-1α) and hypoxia-inducible factor 2 α (HIF-2α) were found to be reduced in the treated tumors at both the RNA transcript and protein level as a consequence of therapeutic intervention (**Fig. 3C and D**, $P < 0.05$ vs. untreated controls, Student's *t* test). Densitometry revealed that the greatest reduction of HIF1α, specific protein expression occurred in response to combined DAS + VAC treatment (**Fig. 3D**, $P < 0.05$ vs. all other cohorts, ANOVA). When taken together, these data suggest that the DAS + VAC combination immunotherapy may recondition the melanoma microenvironment in a manner that reduces hypoxia-driven recruitment and accumulation of regulatory cell populations which may allow for the improved antitumor functionality mediated by vaccine-induced tumor-infiltrating Type-1 $CD8^+$ T cells.

Combination DAS + VAC therapy promotes a broader therapeutic $CD8^+$ T cell repertoire in the tumor-draining lymph node and the tumor microenvironment

Given the superior enrichment of both Type-1 $CD8^+$ T cells and $CD11c^+$ DC within melanomas treated with bimodal DAS + VAC therapy, we hypothesized that this treatment may reinforce continuous longitudinal cross-priming of anticancer $CD8^+$ T-cell responses in the tumor-draining lymph node (TDLN) against tumor-associated antigens unrelated to the vaccine OVA peptide. To test the possibility that a broader spectrum of cancer cell-specific $CD8^+$ T cells are present in the TDLN or tumor microenvironment in DAS + VAC treated mice, $CD8^+$ T cells were isolated from day 34 TDLN and dissociated tumors and analyzed for their ability to produce IFNγ when stimulated in vitro with intact M05 (OVA^+) or B16 (OVA^-) melanomas or EL4 thymoma cells +/– $H\text{-}2^b$ class I-presented peptides derived from OVA, the

melanoma-associated antigens gp100 or tyrosinase-related protein 2 (TRP2),[29] or the melanoma stromal cell-associated antigen ephrin A2 (EphA2).[30] As shown in **Figure 4**, $CD8^+$ T cells from the TDLN as well as those infiltrating the tumor isolated from either untreated or DAS alone treated mice displayed weak or no reactivity against any targets evaluated, except for the DAS-treated cohort that exhibited a significant degree of OVA-specific $CD8^+$ T cell reactivity in comparison to T cells from untreated M05 tumors. In marked contrast, $CD8^+$ T cells from the TDLN and TIL population isolated from mice treated with VAC alone or DAS + VAC registered strong anti-OVA responses (i.e., differential reactivity against M05 vs. B16 and EL4 + OVA peptide vs. EL4 alone, $P < 0.05$, Student's *t* test). Notably, the DAS + VAC treatment cohort was unique in developing robust Type1 $CD8^+$ T cell responses among T cells present in both TDLN and TIL against non-vaccine tumor-associated antigens, such as EphA2, gp100 and TRP2 (**Fig. 4**).

Discussion

Our major finding is that the multi-kinase small molecule inhibitor DAS serves as an effective adjuvant to peptide-based DC vaccination in the treatment of subcutaneous M05 murine melanoma in vivo. DAS appears to mediate this vaccine-potentiating function via multiple immunological mechanisms (**Fig. 5**). These include the treatment-associated erosion of immunosuppressive cells populations such as MDSCs and Tregs within the tumor microenvironment. DAS also enhanced the stimulation of anticancer $CD8^+$ T cells in the tumor periphery and promoted the recruitment of therapeutic Type-1 $CXCR3^+CD8^+$ T cells via locoregional production of the CXCR3 ligand chemokines CXCL9, CXCL10, and CXCL11. Furthermore, we found that $CD8^+$ T cells are critical for the enhanced efficacy of combinatorial DAS + VAC treatment, as evinced by antibody-based depletion studies.

Some of the adjuvant-like qualities associated with DAS are consistent with a recent report by Yang et al.[21] and similar to our prior findings regarding alternative tyrosine kinase inhibitors such as axitinib[11] and sunitinib.[12] These data suggest that DAS treatment benefits may be associated with its potent inhibitory action on the receptor tyrosine kinase c-KIT known to play a supportive role in MDSC immunobiology.[13,14,21] It is also conceptually possible that the normalizing of immunologic responses (toward protection) stems from the ability of DAS to inhibit SRC family kinases that act to reinforce STAT3 signaling implicated in Treg immunosuppressive function, effector T cell and DC dysfunction and the pro-angiogenic tumor milieu.[31–33] Our findings suggesting that DAS co-treatment attenuates hypoxic signaling in the tumor micrenvironment may also be salient in this context as hypoxia reinforces immune dysfunction and promotes a state of cancer cell resistance to T cell-mediated cytotoxicity.[34–38] In the latter scenario, malignant cell escape of cytotoxic immunosurveillance is believed to be regulated by hypoxia-responsive microRNAs[34] or the process of autophagy.[36]

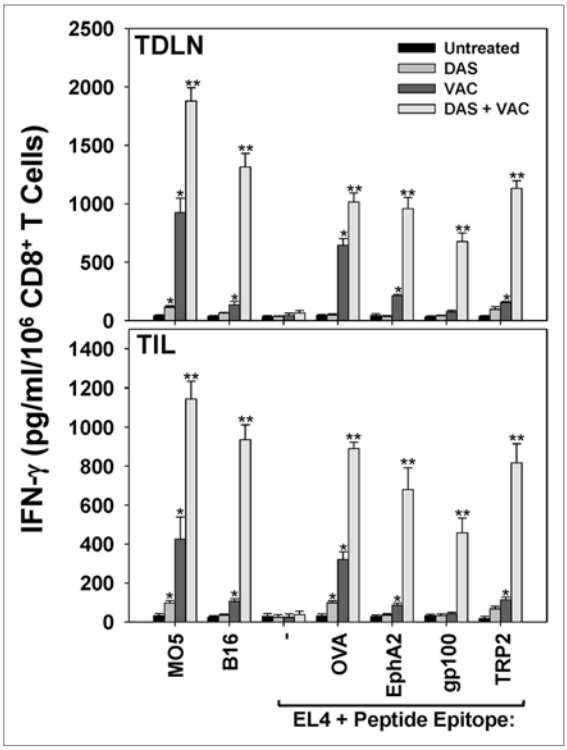

Figure 4. Combined DAS + VAC therapy promotes a broader therapeutic $CD8^+$ T cell repertoire in the tumor-draining lymph node and the tumor microenvironment. M05 melanoma bearing mice were left untreated or were treated (starting on d10 post tumor cell s.c. injection) with either 0.1 mg/day dasatinib (DAS) orally administered for 7 consecutive days, or contralateral s.c. vaccination (VAC) consisting of 10^6 $OVA_{257–264}$ peptide-pulsed dendritic cells (DCs) genetically modified to overexpress IL-12, or a combination of DAS + VAC. Tumors and tumor-draining lymph nodes (TDLN) were harvested on day 34 and $CD8^+$ T cells were isolated via anti-CD8 antibody labeling and magnetic beads. CD8+ effector cells were stimulated for 48h with M05 (OVA^+) melanoma cells, B16 (OVA^{neg}) melanoma cells, EL4 thymoma control cells or EL4 cells pre-pulsed (for 4h at 37 °C) with $CD8^+$ T cell peptide epitopes derived from other tumor-associated antigens (at an effector-to-target cell ratio of 10:1) as indicated. Cell-free supernatants were then recovered from each culture well and analyzed for IFNγ content by specific ELISA. Data are reported as the mean ± SD from triplicate ELISA determinations. Representative data from 1 of 3 independent experiments is depicted, Statistical analyses were performed by Student's *t* test or 1-way ANOVA; $*P < 0.05$ vs. untreated (*t* test), $**P < 0.05$ vs. all other groups (ANOVA).

Interestingly, combined DAS + VAC therapy also stimulated elevated $IL\text{-}12p70^{hi}IL\text{-}10^{low}CD11c^+$ DC recruitment and accumulation into the tumor microenvironment. This influx of antigen-presenting $CD11c^+$ DCs may coordinate an increase in effector T cell cross priming, thus giving rise to a broader repertoire of $CD8^+$ T cells in the TDLN. Of note, this immunologic manifestation was also reflected within the tumor-infiltrating effector T cell population. This "spreading" in the $CD8^+$ T cell repertoire is presumed to occur secondarily to the initial wave of vaccine-induced TIL-mediated anticancer cytotoxicity. The TIL cancer cell killing would permit the uptake of cellular debris, including tumor-associated antigens, by the recruited $CD11c^+$ DC. These tumor-antigen presenting DCs may then migrate

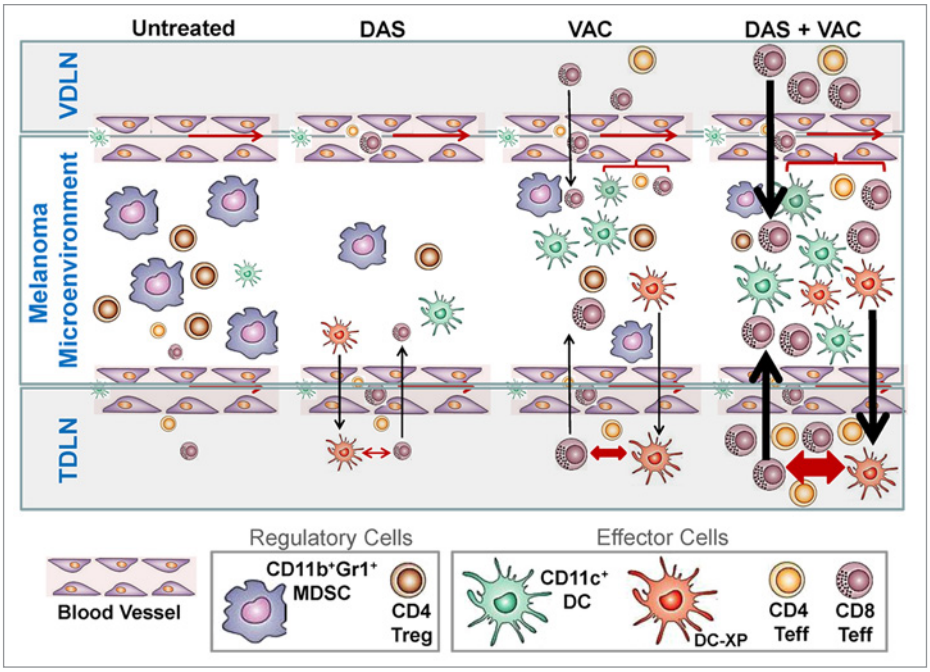

Figure 5. Proposed mechanism for the superior antitumor action of combined DAS + VAC immunotherapy. We propose that DAS monotherapy exerts an antagonistic effect on tumor-associated myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) manifesting in reduced numbers of such regulatory cells in the melanoma microenvironment. This may be the result of reduced recruitment of MDSCs or Tregs due to reduced levels of relevant recruiting chemokines such as CXCL12, or due to dasitinib (DAS)-mediated inhibition of MDSC or Treg expansion and differentiation within the tumor microenvironment. Removal of such suppression may allow for improved recruitment and function of $CD11c^+$ dendritic cells (DCs) and the effector T (Teff) cells that are cross-primed (by DC-XP) in the tumor-draining lymph node (TDLN). Vaccination (VAC) monotherapy drives anti-OVA $CD8^+$ T cell responses in the vaccine-site draining lymph node (VDLN) and the subsequent trafficking of such effector cells into the tumor area results in locoregional production of IFNγ that stimulates production of $CXCR3^+$ chemokines (such as CXCL9–11) allowing for the corollary recruitment of additional Type-1 $CXCR3^+$ tumor-infiltrating lymphocytes (TILs), a portion of which are the result of cross-priming events in the TDLN. Treatment with DAS + VAC provides an optimal combination therapy that stimulates Type-1 immunity (both T cell and DC) and mitigates immunosuppression associated with MDSCs and Tregs. Re-iterated cycles of $IL\text{-}12^{hi}IL\text{-}10^{low}CD11c^+$ DC cross-priming of $CD8^+$ T cells in TDLN results in the rapid broadening of the antitumor T cell repertoire, allowing for effective immune-mediated control of both melanoma cells and tumor-associated stromal cell populations, such as $EphA2^+$ vascular endothelial cells, and superior therapeutic efficacy. Note: Larger symbol sizes and bolder lines infer greater numbers or strength of responses.

to the TDLN where they may in turn activate antigen-specific $CD8^+$ T cells that may subsequently circulate via the peripheral blood into melanoma sites (**Fig. 5**, and ref.[39]). Notably, in our experiment we observed therapeutic cross-priming against both melanoma tumor-associated antigens (i.e., gp100, TRP2), as well as, the EphA2 protein expressed by tumor-associated vascular endothelial cells but not B16 melanoma cells themselves.[27,40] This provides an operational paradigm in which DAS co-therapy promotes the expansion of a qualitatively distinct Type-1 $CD8^+$ T cell repertoire that is initiated against a limited set of vaccine-associated antigens (i.e., OVA) but ultimately culminates in reactivity against both tumor and tumor stromal cell populations, thereby providing optimal therapeutic benefit. Although our studies did not address the functional avidity of the various antigen-specific $CD8^+$ T cell populations harvested from each treatment cohort, Wölfl et al. have recently reported that at clinically-relevant dosages (i.e., up to 90 nM), DAS pretreatment in vitro enhances DC immunostimulatory activity leading to higher avidity T cells such as those targeting the melanoma antigen MART1.[41] The development of complex antigen-specific T cell responses would further aid in immune recognition of heterogeneous populations of melanoma cells that may contain subpopulations with defects in antigen processing and presentation,[42] such that only high avidity anti-melanoma T cells may be capable of curative therapeutic impact.[41,43]

There are theoretical treatment concerns for combinatorial therapies employing DAS and cancer vaccines comprising antigens that are non-mutated gene products overexpressed by the tumor relative to the normal counterpart. By removing regulatory immunity while enhancing specific Type-1 T effector cell function using DAS, one may potentially promote autoimmune pathological responses (colitis, diabetes mellitus, rheumatoid arthritis, among others) as previously observed in clinical trials evaluating immunotherapeutic antibodies such as those targeting cytotoxic T-lymphocyte antigen 4 (CTLA4) or programmed cell death 1 (PD-1).[44,45] Although there have been rare case reports of DAS-associated colitis in the setting of leukemia,[46,47] in extensive clinical trials so far DAS appears to be a reasonably well-tolerated agent for the treatment of solid tumors, including melanoma.[17,48] Indeed, to date, we have not observed any evidence of autoimmune pathology (including vitiligo) in our M05 murine melanoma model.

Further clinically relevant concerns include the known activity of DAS as a reversible inhibitor of Lck, an important mediator of proximal TCR-mediated signaling. This pharmacological activity can transiently inhibit T cell proliferation and IFNγ production (without affecting T cell viability) in vitro.[49] While at face value this is potentially problematic, in the cancer setting, chronic Lck activation occurs in antitumor TIL, a molecular phenotype that may lead to phosphorylation of anergy-associated diacylglycerol kinase α (DGKα) in T cells.[50] As a consequence, transient interruption of TCR signaling by DAS might actually prevent T effector cell tolerance, potentially re-awakening dysfunctional TILs for therapeutic benefit. We are currently investigating such possibilities in ongoing murine modeling studies that integrate longitudinal monitoring of therapy-associated alterations in T-cell and DC immune function. This should allow for

the development of a more detailed mechanistic paradigm underlying the acute vs. sustained antitumor benefits associated with therapeutic administration of DAS monotherapy or combinatorial treatment in conjunction with VAC.

When taken together, our results provide rationale for the clinical translation of combined DAS + VAC protocols for the treatment of melanoma patients. Indeed, we have recently begun evaluating this combination treatment modality in advanced stage melanoma patients at the University of Pittsburgh Cancer Institute (UPCI 12–048, NCT01876212, "A Randomized Phase II Pilot Study of Type I-Polarized Autologous Dendritic Cell Vaccines Incorporating Tumor Blood Vessel Antigen (TBVA)-Derived Peptides in Combination with Dasatinib in Patients with Metastatic Melanoma.")

Materials and Methods

Mice

Female 6–8 wk old C57BL/6 (H-2^b) mice were purchased from the Jackson Laboratory. Animals were maintained in micro-isolator cages and handled under aseptic conditions per an Institutional Animal Care and Use Committee (IACUC)-approved protocol.

Cell lines and culture

The M05 (B16.OVA, H-2^b) and wild-type B16 (H-2^b) melanoma cell lines[12] and the EL4 thymoma (H-2^b, American Type Culture Collection, ATCC) were free of *Mycoplasma* contamination and cultured in complete media (CM) consisting of RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 μM L-glutamine (all from Life Technologies). Cells were cultured at 37 °C with 5% CO_2. Expression of the OVA transgene in M05 cells was maintained under culture selection with G418 (Life Technologies).

Peptide

The H-2^b class I-presented $OVA_{257–264}$ (SIINFEKL),[12] murine $EphA2_{682–689}$ (VVSKYKPM),[30] murine $gp100_{25–33}$ (EGSRNQDWL)[29] and murine $TRP2_{181–188}$ (VYDFFVWL)[29] peptides were synthesized to > 96% purity by 9-fluorenylmethoxycarbonyl (Fmoc) chemistry by the University of Pittsburgh Cancer Institute's Peptide Synthesis Facility (a Shared Resource).

VAC generation

DC were generated from the bone marrow of tibias/femurs from naïve C57BL/6 mice as previously described.[11,12] Briefly, bone-marrow-derived cells were cultured in CM over a period of 5 d with 1000 U/mL of recombinant murine (rm)GM-CSF and rmIL-4 (both from Peprotech). $CD11c^+$ cells were subsequently purified using magnetic-activated cell sorting (MACS) bead positive selection (Miltenyi Biotec) and infected at a multiplicity of infection = 50 with an adenoviral vector encoding murine IL-12p70 (Ad.mIL-12p70) as previously described.[11] After a 48h culture in the presence of rmGM-CSF and rmIL-4, the IL-12 gene-modified DC (DC.IL12) were loaded with 10 μM $OVA_{257–264}$ peptide for 4 h at 37 °C. After washing with phosphate-buffered saline (PBS), these antigen-loaded cells constituted our VAC formulation.

Tumor therapy

Mice were challenged subcutaneously (s.c., right flank) with 2×10^5 M05 melanoma cells, and tumors were allowed to progress 10 d before randomization into groups of 5 animals each with comparable mean tumor sizes/group. Animals then were left untreated, or received VAC (s.c., left flank) with 1×10^6 OVA peptide-pulsed DC.IL12 cells on days 10 and 17 post-M05 challenge. DAS (0.01–1 mg, BMS-354825/Sprycel, Bristol-Myers Squibb) was dissolved in 50 μl Labrasol (Gattefossé Canada) and delivered via oral gavage daily, alone or in combination with VAC, for one week beginning on day 10. Tumor growth was monitored every 3–4 d and tumor dimensions measured using Vernier calipers. M05 tumor size was recorded in mm^2 (mean ± SD) based on the product of orthogonal measurements.

Fluorescence Microscopy

Tumor samples were prepared and sectioned as previously reported.[12,40] Briefly, tumor tissues were harvested and fixed in 2% paraformaldehyde (Sigma–Aldrich) at 4 °C for 1h, then cryoprotected in 30% sucrose for 24 h. Tumor tissues were then frozen in liquid nitrogen and 6 μm cryosections prepared. For analysis of T cell subsets, sections were first stained with purified rat anti-mouse CD8α, purified rat anti-mouse CD4 or purified hamster anti-mouse CD11c (all from BD-PharMingen) mAbs for 1h. After washing, sections were stained with PE-conjugated goat anti-rat or anti-hamster secondary antibody (Jackson ImmunoResearch). After washing, sections were then covered in Gelvatol (Monsanto) and a coverslip applied. Slide images were acquired using an Olympus 500 scanning confocal microscope (Olympus America). The positively stained cells were quantified by analyzing the images at a final magnification of × 20. The number of cells in sections with a given fluorescence marked immunophenotype was quantitated using Metamorph Imaging software (Molecular Devices) and data are reported as the mean ± SD number of cells over 10 high-power fields.

Analyses of Type-1 OVA-specific $CD8^+$ T cell responses in TIL and TDLN

At time points ranging from 28–34 d post M05 tumor inoculation, the constituent CD8+ T cells among the TILs and present in the TDLN were harvested from euthanized mice. Single cell suspensions were generated from tumors by enzymatic digestion using DNase I, collagenase, and hyaluronidase (all from Sigma-Aldrich), and from TDLNs by mechanical disruption, with $CD8^+$ T cells subsequently isolated from each tissue preparation using anti-CD8-MACS beads (Miltenyi Biotec), according to the manufacturer's protocol. In all cases, $CD8^+$ T cells were pooled between 3 animals per group and stimulated for 5 d in vitro with irradiated M05 tumor cells at an effector-to-target (E:T) ratio of 10:1. M05 tumor cells were irradiated prior to the experiment with 100 Gy at room temperature from a ^{137}Cs irradiator (Gammacell40, Atomic Energy of Canada Limited) at a dose rate of 0.87 Gy/min. To assess antigen-specific responses, T cells were then transferred to 96-well round bottom plates (Corning) and incubated with syngenic antigen-loaded EL4 cells (pre-pulsed for 4h at 37 °C with the $OVA_{257–264,}$ $EphA2_{682–689}$, $gp100_{25–33}$ or $TRP2_{181–188}$ peptides or no peptide) at a 10:1 effector-to-target cell ratio for

48 h. Cell-free supernatants were then harvested and assessed for mIFNγ content using a specific ELISA kit (BD Biosciences, lower detection limit = 31.3 pg/mL). Data are reported as mean IFNγ levels (pg/mL) over control based on triplicate determinations ± SD.

Flow cytometry

Single cell suspensions from tumor digests were stained with the following directly-conjugated fluorescein isothiocyanate (FITC) and phycoerythrin (PE) labeled anti-mouse antibodies (all from BD Biosciences): FITC-anti-CD8, FITC-anti-CD4, FITC-anti-CD11b, FITC-anti-CD11c, PE-anti-Gr1, and PE-anti-Foxp3. For Foxp3-specific staining, cells were first labeled with the FITC-anti-CD4 antibody before incubation with the PE-anti-Foxp3 antibody using an Intracellular Fixation and Permeabilization Kit as recommended by the manufacturer (eBioscience). Fluorescence cytometric analysis was performed using Cell Quest software and a FACscan flow cytometer (Becton Dickinson), with FlowJo software (Tree Star) used for data analysis.

RT-PCR

Total RNA was extracted from tumor tissues on day 34 post M05 inoculation using TRIzol reagent (Life Technologies). cDNA was generated using the MuLV reverse transcriptase with random hexamers (both from Applied Biosystems), and gene-specific PCR was performed with AmpliTaq DNA polymerase (Applied Biosystems) and primer pairs for IFNγ, CXCR3, CXCL9, CXCL10, CXCL11, CXCL12, HIF-1α, HIF-2α, Foxp3, and β-actin as previously described.[12] Cycling conditions were as followed: initial denaturation at 94 °C for 2 min, denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 1 min a total of 35–40 cycles followed by a final elongation at 72 °C for 5 min. RT-PCR products were resolved on 2% ethidium bromide stained agarose gels (Sigma–Aldrich), and gel images were captured and analyzed using a GDS 8000 bio-imaging system and Labworks software (UVP, LLC).

Western blotting

Single cell digests derived from tumors harvested on d34 were incubated in cell lysis buffer, with cell-free lysate protein preparations resolved by sodium-dodecyl sulfate PAGE (SDS-PAGE) prior to electro-transfer onto polyvinylidene difluoride membranes, that were subsequently probed with polyclonal rabbit anti-mHIF-1α, anti-mHIF-2α, or β-actin primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody reagents (all from Santa Cruz Biotechnology). Probed blots were visualized using the Western Lighting chemiluminescence detection kit (Perkin–Elmer) and exposed to X-Omat film (Eastman Kodak).

Statistical analysis

Statistical analyses between groups were performed using a 2-tailed Student's *t* test or 1-way ANOVA with post hoc analysis, as indicated. All data were analyzed using SigmaStat software, version 3.5 (Systat Software). Differences with a P value < 0.05 were considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental materials may be found here:
http://www.landesbioscience.com/journals/oncoimmunology/article/27589

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