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Article

Effects of vascular normalizing agents on immune marker expression in T cells, dendritic cells, and melanoma cells

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SUMMARY

Tertiary lymphoid structures (TLS) are lymph node-like structures that form at sites of inflammation, and their presence in cancer patients is predictive of a better clinical outcome. One significant obstacle to TLS formation is reduced immune cell infiltration into the tumor microenvironment (TME), resulting from aberrant vasculature within the TME. Recent studies have shown that low doses of vasculature normalizing (VN) agents may override this defect, leading to improved tissue perfusion and increased immune cell entry into the TME. While the effect of VN agents on vascular endothelial cells has been well documented, their effects on immune cell and tumor cell phenotype remain understudied. We hypothesized that treating immune cells with VN agents would induce a pro-inflammatory phenotype in T cells and dendritic cells (DCs), while treating tumor cells would reduce their immunosuppressive phenotype and promote production of chemokines that recruit immune cells and foster TLS formation. To test this, a mouse melanoma cell line, primary murine T cells, and DCs were treated overnight with VN agents. The next day, treated and control cells were harvested for analyses to measure transcript levels of target genes as well as levels of surface markers. Overall, VN agents were observed to have differential but predominantly immune-supportive effects on immune and tumor cell phenotypes. These findings will guide future experiments which may result in an effective clinical treatment.

INTRODUCTION

As the number of diagnosed cancer cases continues to rise each year, cancer remains one of the leading causes of death in the United States (1). Many novel approaches to cancer treatment have emerged, and at the forefront of these approaches is immunotherapy (2), where the immune system itself is supported and stimulated to fight against cancer. Immunotherapies are often used in advanced-stage metastatic cancers when traditional methods such as surgery are ineffective. However, since immunotherapies usually target one pathway or molecule, their efficacy in the clinical setting has been unpredictable, and positive results are observed in only a minority of patients. For example, patients whose tumors are PD-L1-negative, meaning they lack the PD-L1 protein which is a target of checkpoint blockade immunotherapy, have been shown to respond worse to checkpoint blockade therapy (3). Therefore, recent studies have attempted to combine therapies in order to achieve synergistic therapeutic effects (4).

In this work, we focused on exploring vascular normalizing (VN) agents to better inform the development of effective combination immunotherapies. VN agents act to normalize the aberrant vasculature that exists within the tumor microenvironment (TME). The TME consists of nonmalignant cells, including vascular cells, that can foster cancer growth through various pathways and factors (5). The faulty tumor vasculature, which is created by excessive angiogenesis (the formation of new blood vessels), promotes a highly immunosuppressive TME due to its poor perfusion of tumor tissue, limiting immune cell entry into the TME (6). In fact, tumors with higher levels of angiogenic factors have been shown to be more aggressive (7). VN agents override this defect by preventing aberrant angiogenesis and/or promoting the maturation of blood vessels (8). These effects serve to facilitate entry of immune cells into the TME, which in turn may lead to increased formation of tertiary lymphoid structures (TLS). TLS are lymph node-like structures that form directly at the site of inflammation and produce more immediate and localized immune responses to the tumor (9). The presence of TLS in cancer patients has been correlated with a better prognosis (10). Normalized vasculature may also increase efficacy of immunotherapies because most immunotherapies focus on increasing activation or functionality of anti-tumor immune cells. However, if these cells cannot easily access the TME, the efficacy of the treatment is greatly diminished. A combination therapy consisting of a VN agent and an immunotherapy to treat solid tumor cancers has been actively pursued, but so far with limited success (11).

The VN agents analyzed in this study include Aduro, Dasatinib, and Bevacizumab. These three agents are in distinct molecule classes and act on different targets, suggesting that their effects on immune and tumor cell phenotypes will also be distinct. Aduro activates the STimulator of INterferon Genes (STING) pathway, which has been shown to promote maturation of vascular endothelial cells (12). Meanwhile, Dasatinib inhibits tyrosine kinases, notably platelet-derived growth factor receptor beta (PDGFRβ), which is a receptor that can contribute to the aberrant vasculature in the TME as well as promote proliferation of tumor cells (13). Finally,

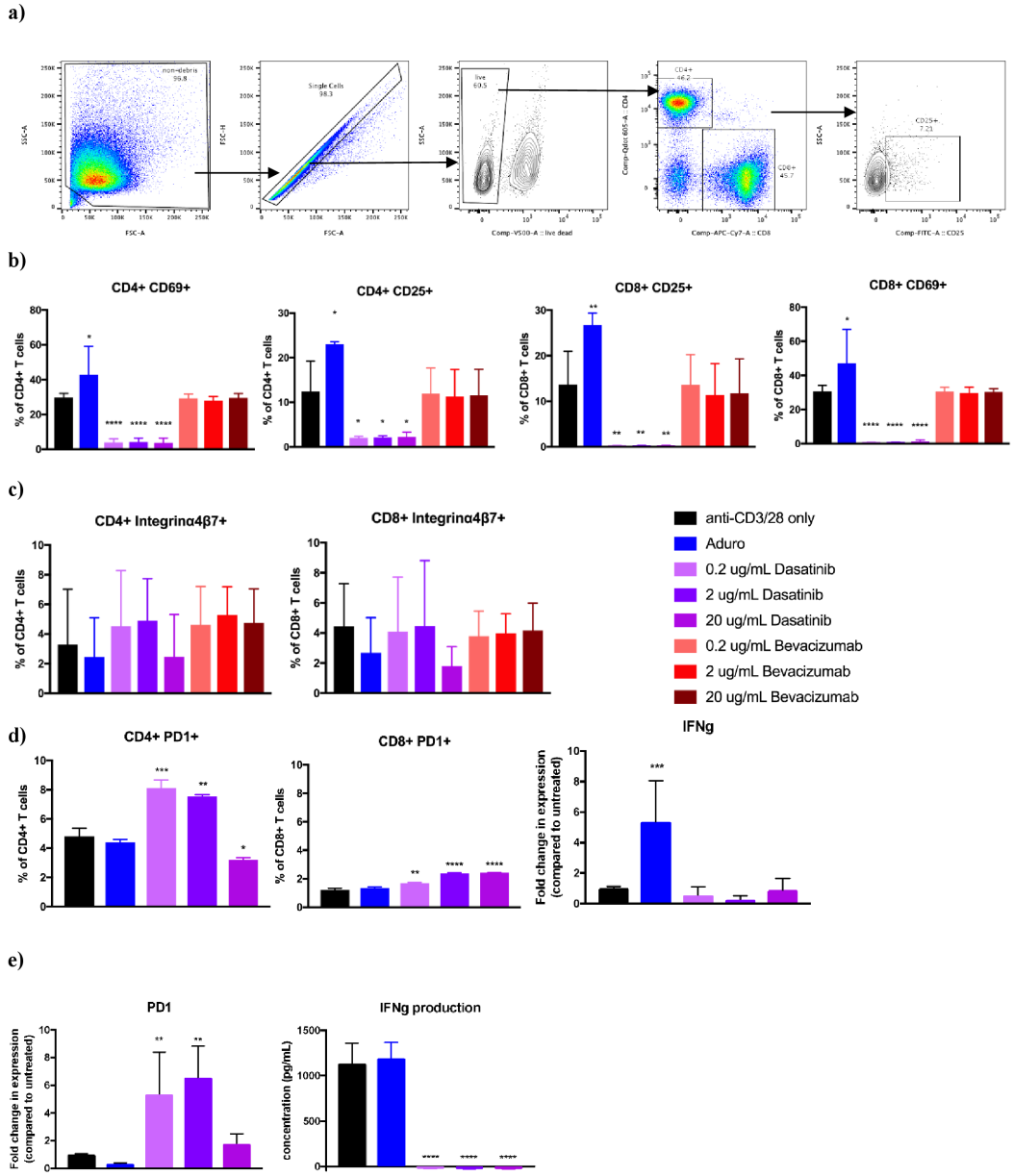

Figure 1: Effects of VN agents on T cells. T cells were isolated from mice and cultured in the indicated doses of VN agents. Figures 1b-1e show average immune marker expression with the error bars representing standard deviation. a) Example of the flow cytometry gating strategy used. b) Flow cytometry results of activation of CD4+ and CD8+ T cells (n=4). c) Flow cytometry results of Integrin α4β7 expression (n=4). d) Flow cytometry and ELISA results for protein levels of PD1 and IFNγ, respectively (n=2 and n=3, respectively). e) qRT-PCR results of transcript levels of PD1 and IFNγ (n=4). PD1, Programmed cell death protein 1; IFNγ, Interferon gamma. *p<0.05, **p<0.01, ***p<0.001, ****p=0.0001

Bevacizumab binds to vascular endothelial growth factor (VEGF), thus preventing it from binding to its receptor. VEGF is a potent promoter of angiogenesis and is upregulated in many cancers (14).

The aim of our study was to determine the effects of VN agents on murine dendritic cells (DCs), T cells, and melanoma cells. DCs and T cells are immune cells that play a critical role in the anti-cancer immune response, and many immunotherapies such as DC vaccines and CAR T cell therapies utilize or target these cells (15,16). These cell types have also been previously explored as targets in immunotherapy approaches integrating select VN agents. However, the impact of VN agents on these cell types is relatively understudied and gaining a better understanding of these effects will inform future design of more effective combinatorial immunotherapies using VN agents. The effect of VN agents on DCs, T cells, and melanoma cells was analyzed through expression of certain anti-tumor and TLS-promoting cell surface proteins and cytokines. The specific molecules that were assessed are shown in Table 1. We

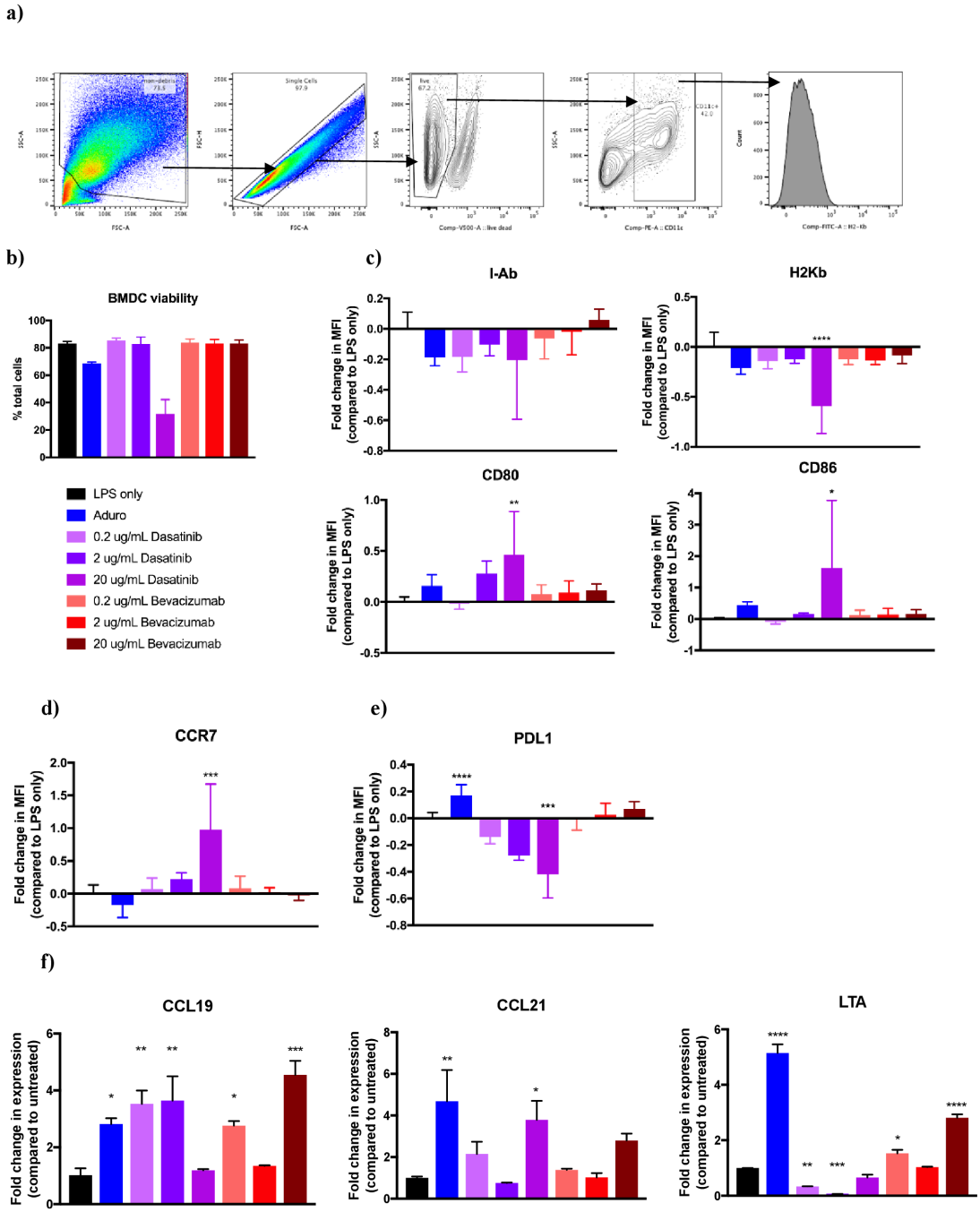

Figure 1: Effects of VN agents on DCs. DCs were isolated from mice and cultured in the indicated doses of VN agents. Figures 2b-2e show averages with the error bars representing standard deviation. a) Example of the flow cytometry gating strategy used. b) Cellular viability data (n=4). c) Flow cytometry results of MHC and co-stimulatory molecule expression (n=4). d) Flow cytometry results of CCR7 expression (n=4). e) Flow cytometry results of PDL1 expression (n=4). f) qRT-PCR results of transcript levels of TLS promoting factors including CCL19, CCL21, and LTA (n=2). MHC, major histocompatibility complex; CCR7, C-C chemokine receptor type 7; PDL1, Programmed death-ligand 1; CCL19, chemokine ligand 19; CCL21, chemokine ligand 21; LTA, lymphotoxin alpha. *p<0.05, **p<0.01, ***p<0.001, ****

hypothesized that treating immune and tumor cells with VN agents would lead to an upregulation of immune-supportive markers, including co-stimulatory, major histocompatibility complex (MHC), adhesion, immune recruitment and activation molecules, and downregulation of immune checkpoint molecules. Furthermore, we expected increased production of inflammatory and TLS-promoting cytokines. After experimentation, VN agents were observed to have more differential and varied effects on immune and tumor cell phenotypes than hypothesized.

RESULTS

Molecule Group	Molecules	Cells observed	Function
Co-stimulatory (Cell surface)	CD80 CD86	Tumor, DCs	Assists in T cell activation (immune-supportive) (17)
MHC I/II (Cell surface)	I-Ab H-2Kb	Tumor, DCs	Presents antigens to T cells; activates T cells (immune-supportive) (17)
Adhesion (Cell surface)	Integrin α4β7	T cells	Allows T cells to enter tissue from blood (immune-supportive) (18)
Immune Recruitment (Cell surface)	CCR7	All	Directs migration of immune cells to lymph nodes/TLS; receptor for CCL19 and CCL21 (immune-supportive) (19)
Immune Checkpoints (Cell surface)	PDL1	DCs, Tumor	Induces T cell exhaustion/death (immunosuppressive) (17)
	PD1	T cells	Marker of T cell exhaustion (immunosuppressive) (17)
TLS promoting factors (chemokines)	CCL19 CCL21 LTA	DCs, Tumor	Promotes TLS formation (immune-supportive) (20)
Inflammatory (cytokines)	IFNγ	T cells	Pro-inflammatory (immune-supportive) (21)
Activation (cell surface)	CD25 CD69	T cells	Markers of T cell activation (immune-supportive) (22)

Table 1: Immune markers analyzed. The effect of VN agents on the following molecule groups was explored. Column 2 shows the specific molecule that was tested, and Column 3 shows the cells that express those molecules. Their basic function is shown in Column 4.

The effects of VN agents on murine DCs, T cells melanoma cells were explored. We first cultured murine DCs and T cells. These cells, along with murine melanoma cells, were then treated with VN agents (Aduro, Dasatinib, and Bevacizumab), and the effects of these VN agents on the anti-tumor response and TLS formation were analyzed via expression of cell surface proteins and cytokines **(Table 1)**. The phenotypic effects of the agents were quantitatively measured using flow cytometry (extracellular markers) and qRT-PCR (cytokine expression). Finally, a one-way ANOVA test was used to determine statistical significance. It is important to note that due to the low viability of T cells treated with Bevacizumab, those treatment groups were not available to be used for qRT-PCR.

Aduro has immune-supportive effect while Dasatinib has immunosuppressive effects on immune marker expression in T cells

First, we evaluated the effects of Aduro, Dasatinib, and Bevacizumab on the T cell phenotype by flow cytometry **(Figure 1a)**. We found that Aduro significantly increased activation (measured by CD69 and CD25 expression), Dasatinib significantly decreased activation, and Bevacizumab had no effect on activation of CD4+ and CD8+ T cells as compared to the untreated control **(Figure 1b)**. In both CD4+ and CD8+ T cells, VN agents had no statistically significant effect on expression of Integrin α4β7 as compared to the untreated control **(Figure 1c)**. After observing the changes in T cell activation with Aduro and Dasatinib, we next wanted to evaluate the functionality of the T cells by measuring the expression levels of PD-1 and IFNγ. We observed that cells treated with Dasatinib had increased PD1 transcript levels with the lower two dosages and no change in IFNγ transcript levels compared to the control **(Figure 1d).** In both CD4+ and CD8+ T cells, Aduro had no effect on PD1 surface expression while the two lower dosages of Dasatinib increased surface expression. However, the highest dosage of Dasatinib decreased PD1 expression in CD4+ cells but had the opposite effect on CD8+ cells. Supernatants from cultured T cells were tested in an IFNγ ELISA to confirm that the changes in transcript levels of IFNγ were also observed at the protein level. Treatment with Dasatinib significantly decreased the production of IFNγ by T cells **(Figure 1e)**. These results indicated that Aduro improves T cell activation while Dasatinib has an inhibitory effect on T cells.

VN agents have modest effects on immune marker expression in DCs.

We next evaluated the effects of Aduro, Dasatinib, and Bevacizumab on DC cell phenotypes by flow cytometry **(Figure 2a)**. After culturing, however, it is important to note that the viability of DCs was decreased significantly by the highest dose of Dasatinib and decreased slightly by Aduro **(Figure 2b)**. We found that VN agents, regardless of the type, had no discernable effect on expression of co-stimulatory and MHC molecules in DCs with the exception of the highest dose of Dasatinib, which increased expression of both CD80 and CD86 but decreased expression of H-2Kb **(Figure 2c).** Furthermore, when observing the effects of VN agents on CCR7 expression, the highest dosage of Dasatinib increased expression, while the rest of the VN agents had little effect **(Figure 2d)**. We found that Aduro upregulated while the highest dose of Dasatinib downregulated PD-L1 expression **(Figure 2e)**. Next, we analyzed the effects of VN agents on TLS-promoting factors using qRT-PCR to measure transcript levels. Aduro upregulated expression of all three tested factors (CCL19, CCL21, and LTA). Bevacizumab had little effect on expression of CCL21 while the lowest and highest dose of Bevacizumab upregulated expression of CCL19 and LTA. Dasatinib had varied effects **(Figure 2f)**. Although results were differential, the highest dose of Dasatinib has predominantly immune-supportive effects on DCs, while Aduro and Bevacizumab have immune-supportive effects on TLS-promoting factor expression in DCs.

Dasatinib has immune-supportive effects while Aduro and Bevacizumab have little effect on immune marker expression in melanoma cells.

Finally, we evaluated the effects of Aduro, Dasatinib, and Bevacizumab on melanoma cell phenotype by flow cytometry **(Figure 3a).** Similar to the DCs, we saw that the

T cells	Antibody:	CD4 BV605	CD8 APC-eFluor780	CCR7 eFluor 450	Integrin α4β7 APC	CD25 FIT-C	CD69 PE-Cy7	FACS Buffer	
	Volume (µL):	10	10	25	25	5	2	910	
DCs	Antibody:	CD86 BUV395	CCR7 eF450	I-Ab PerCP-eFluor710	CD11c PE	PD-L1 APC	CD80 APC-eFluor 780	H-2kb FITC	FACS Buffer
	Volume (µL):	6	15	6	4.8	12	6	12	540
Melanoma Cells	Antibody:	CD86 BUV395	CCR7 eF450	I-Ab PerCP-eFluor710	PD-L1 APC	H-2kb FITC	CD80 APC-eFluor 780	FACS Buffer	
	Volume (µL):	5	12.5	5	10	10	5	448.5	

Table 2: Flow cytometry antibody cocktails. Antibody cocktails for each cell type were made by combining the following antibodies (column 1) at the shown volumes (column 2). The target molecule and fluorochrome identifies each antibody.

highest dose of Dasatinib lowered cell viability as compared to the control **(Figure 3b)**. With the exception of the highest dosage of Dasatinib, which significantly increased expression of I-Ab and CD86, VN agents had little observable effect on expression of co-stimulatory and MHC molecules in melanoma cells **(Figure 3c)**. Similarly, all VN agents had no effect on the expression of CCR7 and PDL1 except for the highest dosage of Dasatinib, which increased expression **(Figure 3d,e)**. Afterwards, we wanted to observe the effects of VN agents on the transcript levels of TLS-promoting factors. Aduro and Bevacizumab had no significant effect on CCL19/21 mRNA, while the higher dosages of Dasatinib significantly upregulated transcript levels of TLS promoting factors **(Figure 3f)**. In general, the highest dose of Dasatinib seemed to have immune-supportive effects on melanoma cells while all other VN agents and dosages had almost no effect.

DISCUSSION

The purpose of this study was to describe the effects of VN agents on T cells, DCs and melanoma cells. Overall, the T cell results showed Aduro has immuno-supportive effects, Dasatinib has immunosuppressive effects, and Bevacizumab has little effect on immune phenotype. This is likely due to Aduro's action as a STING agonist and this pathway being highly expressed in T cells. However, the STING pathway has mainly been researched in macrophages and DCs (23). This data suggests that stimulation of the pathway leads to increased T cell activation as well as production of IFNγ. Furthermore, Dasatinib had nearly the opposite effects of Aduro on T cells, which is likely caused by Dasatinib's ability to inhibit the Src kinase, which is known to be vital to T cell activation (24). This effect seems to be consistent with the current literature. As a result, reduced activation could contribute to the overall immunosuppressive effect. However, in IFNγ expression, there was a notable discrepancy between the ELISA and qRT-PCR results, specifically, the agents had nearly opposite effects on IFNγ transcript levels as on IFNγ protein levels. This signifies that the transcriptional changes are not reflected in protein levels. This finding requires further exploration, as the increase in IFNγ transcript levels from Aduro seems consistent with its action as a STING agonist while the decrease in IFNγ transcript levels from Dasatinib is consistent with the lowered activation described earlier. Lastly, Bevacizumab's mild effects is likely due to the lack of VEGF in the culture system we employed.

The DC cell surface proteins that we measured had very little change in expression in response to VN agents with the exceptions of the highest dose of Dasatinib, which had predominantly immune-supportive effects, and Aduro, which upregulated expression of PDL1. These largely uniform effects are unexpected as we predicted that the unique nature of the three VN agents would lead to variable effects on immune markers as seen with the T cell results. Furthermore, the TLS promoting factor transcript levels suggest that Aduro and the highest and lowest dosage of Bevacizumab have immune-supportive effects in DCs. Aduro's effect was expected considering the relevancy of the STING pathway in DCs, while Bevacizumab's effect is consistent with the ability of VEGF to inhibit maturation and differentiation of DCs (25).

Finally, the effect of VN agents on expression of melanoma cell surface markers were similarly minimal except for the effects observed using Dasatinib at its highest dose, which led to upregulated expression of CCR7, CD86, I-Ab, and PDL1. The mild effect of Aduro is more than likely due to defects in the STING signaling pathway in tumors (26). Research into the nature of this disruption is ongoing, but it is highly possible that a corrective interventional strategy in melanoma cells would allow Aduro to have an immune-supportive effect on tumor cells, similar to what we observed for DCs and T cells. Furthermore, VN agent effects on tumor cell expression of TLS promoting factors were similar to those on cell surface markers, although the effects of Dasatinib treatment were also observed with the medium dosage and seemed more pronounced and consistent.

Future research will include the study of additional immune and cancer cell lines. Furthermore, other VN agents, VN agent concentrations, or markers could be explored, as

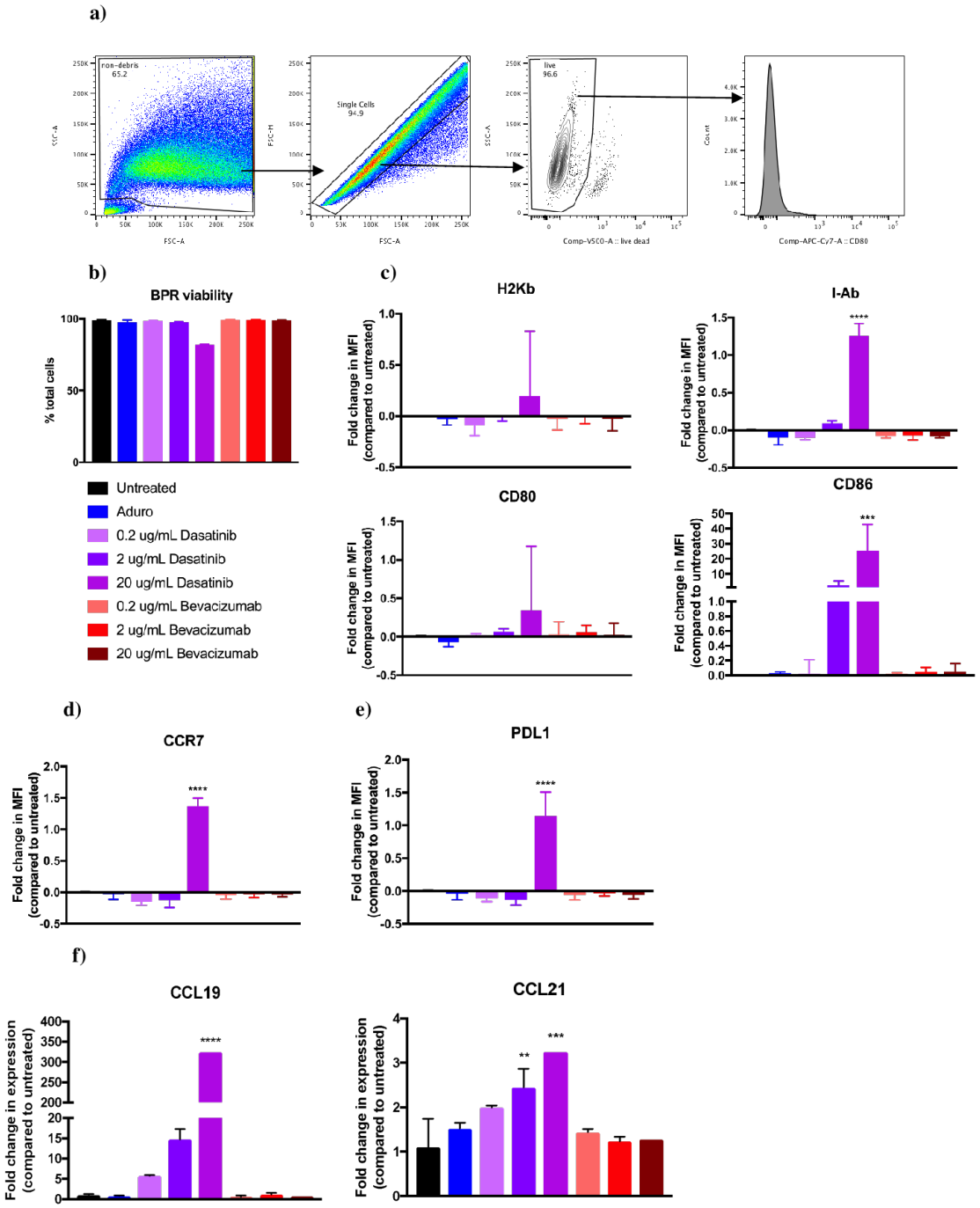

Figure 3: An Effects of VN agents on melanoma cells. A melanoma cell line was cultured in the indicated doses of VN agents. Figures 3b-3e show averages with the error bars representing standard deviation. a) Example of the flow cytometry gating strategy used. b) Cellular viability data (n=3). c) Flow cytometry results of MHC and co-stimulatory molecule expression (n=3). d) Flow cytometry results of CCR7 expression (n=3). e) Flow cytometry results of PDL1 expression (n=3). f) qRT-PCR results of transcript levels of TLS promoting factors including CCL19 and CCL21 (n=2). MHC, major histocompatibility complex; CCR7, C-C chemokine receptor type 7; PDL1, Programmed death-ligand 1; CCL19, chemokine ligand 19; CCL21, chemokine ligand 21. *p<0.05, **p<0.01, ***p<0.001, ****p=0.0001

well as drug effects on the proliferation and functionality of cells. Also, all VN agents will need to be reassessed in vivo since their primary function of affecting vasculature is not replicable in vitro, and thus their effect on immune and tumor cells can differ. Although it is too early to determine a combination immunotherapy for possible application in the clinical setting, this data certainly provides a starting point on which clinical trials could be based. For example, Aduro's immune-supportive effects on T cells could be combined with a traditional chemotherapy which, unlike Aduro, promotes

tumor cell death. This could create a synergistic effect by providing a superior source of tumor antigen for DC-mediated activation of anti-tumor T cells, which may be the ultimate goal of VN agent-based immunotherapies.

MATERIALS AND METHODS

Cell Culturing

A mouse melanoma cell line with mutation (BRAFV600E)/deletion (PTEN-/-) common to human melanomas was obtained from the Wargo Laboratory at MD Anderson and were cultured in DMEM + 10% FBS. These cells were chosen due to the clinical relevance of the BRAF mutation, which is an indicator of more immune-resistant tumor cells that are often found in the clinical setting (27). Bone marrow and splenocytes were harvested from euthanized female C57BL/6J mice and were then cultured. T cells were isolated from the splenocytes using a Miltenyi CD3+ Isolation Kit (CD3 MicroBeads, human, Miltenyi Biotech) and cultured at 1×10^7cells/mL in RPMI media +10% FBS along with anti-CD3 and anti-CD28 antibody at 10 µg/mL. IL-4 and GM-CSF was added to the bone marrow culture at 1:1000 to induce DC differentiation immediately and again after 72 hours of incubation at 37°C. DCs were cultured at 2×10^6 cells/well in a standard six well plate in RPMI media + 10% FBS.

Treatment with VN agents

The cells were treated with VN agents. Aduro was added for a final concentration of 2.5 µg/mL while Dasatinib and Bevacizumab was added for final concentrations of 20, 2 and 0.2 µg/mL. All dosages were determined to be optimal from previous studies (28). For T cells, 100 µL of VN agents diluted in media were added to each well. For melanoma cells, all media was removed from the wells, and 2 mL of drug dilutions were added to each well. After 24 hours, the cells were collected and counted. For DCs, before adding drugs, cells were centrifuged. The cells were then resuspended in lipopolysaccharide (LPS) to induce further DC maturation/activation, and 1 mL was transferred back to each well. Next, 1 mL of drug dilutions were added to each well, and cells were incubated for 24 hours at 37°C. For all cell types, a portion of cells were designated as the control, and a volume of media equal to the volume of drug dilution was added to these cells. The control group for DCs is referred to as the LPS control as they were treated with LPS only, which can affect immune marker expression.

After all cells were treated with agents overnight, 3.5×10^5 cells from each treatment group were removed and placed in Eppendorf tubes. These tubes were then centrifuged, media was removed, cells were resuspended in 350 µL of Buffer RLT, which lyses the cell, and then frozen at -80°C to be used later for qRT-PCR. The rest of the cells were centrifuged and the media removed. For the cells and DCs, the remaining cells were resuspended in 200 µL PBS and transferred to a 96 well V-bottom plate. At this point, all cells were treated and in 96 well plates and thus ready for flow cytometry staining. The antibody cocktail for flow cytometry was made for each cell type according to the tables below **(Table 2)**.

Flow cytometry then proceeded, testing for cell surface marker expression in the 8 different experiment groups: 3 different dosages of Bevacizumab and Dasatinib, Aduro and the control. There were 10-minute room temperature incubations after the resuspension of cells in 100 µL of live/dead viability dye and later after the resuspension in 50 µL Fc block, as well as a 20-minute 4°C incubation after the addition of all stains. The FACS buffer used was made of 1x PBS and 2% fetal bovine serum. After all cells were stained, they were taken to the BD LSRFortessa™ Flow Cytometer where the aforementioned gating strategies were used. FlowJo software was then used for data processing and analysis (29).

qRT-PCR

RNA was first extracted from cells using a standard protocol (Universal SYBR Green qPCR Protocol, Millipore Sigma). cDNA was synthesized from RNA using an RNA-to-cDNA Kit from Applied Biosystems. Real Time PCR was then performed using SYBR green (30). Due to low viability of melanoma cells treated with 20 µg/mL, those treatments were not available to be used for PCR.

Statistical Analysis

Statistical analysis was performed on all data using a one-way ANOVA test to compare the mean values. Alpha cutoffs of $*p<0.05$, $**p<0.01$, $***p<0.001$, and $****p=0.0001$ were used.

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