

Bavituximab Activates CD8+ Tumor Infiltrating Lymphocytes in a 3D Ex Vivo System of Lung Cancer Patients



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ABSTRACT

Background: Bavituximab is a chimeric monoclonal antibody that targets the membrane phospholipid phosphatidylserine (PS) exposed on endothelial cells and cancer cells in solid tumors. Bavituximab blocks PS-mediated immune suppression in the tumor microenvironment.

Methods: Fresh tumor tissues from consented patients with adenocarcinoma of the lung extracted at the time of surgical resection were

utilized in a proprietary 3D ex vivo tumor microsphere assay to assess the immunomodulatory effects of bavituximab and potential immunosuppressive mechanisms such as expression of PD-1, CTLA-4, LAG3, TIM3, BTLA, and adenosine A2A receptor on the CD4+ and

Results: BaxinuVx induces activation of CD3 + ex vivo tumor microscope models of lung cancer, as evaluated by a significant increase in IFN- γ , INF- α , and GM-CSF secretion. Flow cytometry analysis revealed that, this effect was associated with PCP-12-induced CD4+ tumor infiltrating T-cells. A 3D combination microscope was prepared and cells were treated *ex vivo* with baxinuVx, bavatuiximab, doceataxel, and docetaxel for 36 hours within an intact tumor microenvironment. Flow cytometry analysis was performed to evaluate treatment-mediated activation of TILs and changes in CD4, CD8 and Treg (CD25+CD127+) subpopulations. A multiplex human cytokine assay was used to simultaneously analyze the distribution of cytokines. Additionally, a NanoString platform containing probes to quantitate 770 human function genes was used to determine potential positive or negative associations between expression of immune function genes and TIL activation by baxinuVx.

Conclusions: Our preliminary data support the use of bevacizumab as an immunomodulatory treatment in adenocarcinoma of the lung by expression on CD8 cells, but did not correlate with expression of other immune inhibitory molecules.

conclusions, our preclinical data support the use of bavituximab as an immunotherapy treatment in non-small-cell lung cancer by enhancing the activation of CD8+ TIL that correlates with increased cytokine production by lymphoid and myeloid cells. We identified PD-L1 and PD-1 expression as a potential biomarker of response to bavituximab treatment, suggesting that the interruption of the PD-1/PD-L1 axis may enhance the bavituximab effect in lung cancer.

BAVITUXIMAB MECHANISM OF ACTION

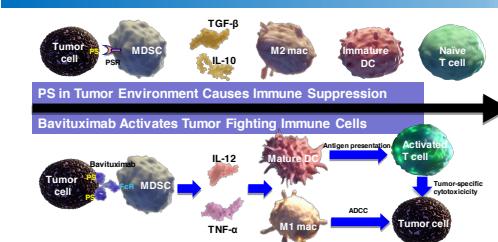


Figure 3 IHC analysis of fresh tumor tissue from each patient for H&E, CD4, CD8, CD68 (MΦ), CD163 (M2) and PD-L1 expression levels. The Allred scoring (AS) system was used to evaluate the percentage of PD-L1-positive stained cells and staining intensity. A proportion score (PS) was assigned representing the proportion of positively stained tumor cells ($P = 0$; none; $1 < 1/100$; $2 = 1/100$ to $< 1/10$; $3 = 1/10$ to $1/3$; $4 = 1/3$ to $2/3$, and $5 = > 2/3$). Average intensity of staining in positive cells was assigned as an intensity score ($0 = \text{none}$; $1 = \text{weak}$; $2 = \text{intermediate}$; and $3 = \text{strong}$).

Table 1: Patient Demographics and Tumor Type

Patient Derived	Gender	Smoker	Tumor Type	Stage
Tumor 1	Female	No	Adenocarcinoma	T1aN0M0
Tumor 2	Female	Yes	Adenocarcinoma	T2aN0M0
Tumor 3	Female	Yes	Adenocarcinoma	T2bN2M0
Tumor 4	Female	Yes	Adenocarcinoma	T2aN0M0
Tumor 5	Female	No	Adenocarcinoma	T1bN2M0
Tumor 6	Female	Yes	Adenocarcinoma	T2aN0M0

Table 2: Immune Checkpoint Proteins Expression at Baseline Profile

Patient Derived	PD-L1 IHC tissue	PD-1 CD8+ cells (FACS)	Lag3 CD8+ cells (FACS)
Tumor 1	Positive	20.7%	0.8%
Tumor 2	Negative	6.8%	62.6%
Tumor 3	Positive	25.8%	62.8%
Tumor 4	Negative	35.0%	1.2%
Tumor 5	Positive	4.9%	83.1%

Figure 1. Summary: Bevutiximab Activates Tumor Immunity. Bevutiximab blocks the immunosuppressive effects of phosphatidylserine (PS) in the tumor microenvironment. PS blockade with bevutiximab reduces the level of M1Cs, M2 macrophages and Tregs and increases the tumor-infiltrating function of mature dendritic cells. M1Cs represent an anti-tumor and anti-tumor T cells.

NIOGEN'S DRUG DISCOVERY PLATFORM

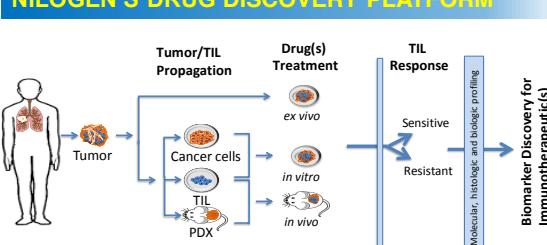


Figure 2. Nilogen's ex-vivo model for immunotherapy drug discovery. Freshly resected patient samples were used to produce a 3D ex-vivo model in which tumor response to different drugs could be elucidated. After collection, the tumor sample was divided into three. Sample 1 was processed and mounted onto slides for IHC analysis. Sample 2 was disaggregated to characterize the tumor microenvironment. The tumor microenvironment was characterized by the presence of CD45+ cells. The tumor and the tumor microphones were disaggregated into single-cell suspensions, and the immune components (CD3, CD4, CD8 and Treg) were characterized. Arbitrarily, matched TIL and cancer cell lines as well as patient-derived xenograft (PDX) models were generated for

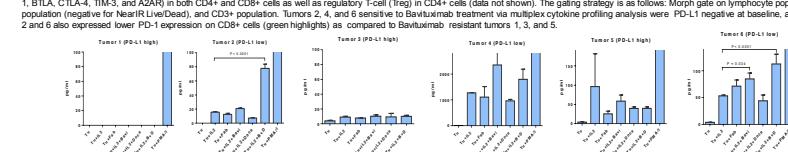


Figure 4. Mean IFN- γ expression levels of *ex vivo* drug treated 3D microspheres. Culture media obtained from 36 hrs *ex vivo*-treated 3D microspheres were analyzed using the Milliplex Immunofluorescence Assay for cytokine secretion. All experiments were performed in triplicate wells, and the means and standard deviations were plotted. Combination of Phorbol myristate acetate (PMA) and $\text{CD}40$ (monocyte), (L) was the positive control to activate TLR-4. Statistical analysis by 1-way ANOVA.

Patient Derived	IFN- γ	GM-CSF	TNF- α	IL-6	IL-10	TGF- β 1	IL1- α	IL-1B
Tumor 1	No Activity	↑ 9.5 Fold	↑ 7.4 Fold	↑ 2.1 Fold	↑ 2.2 Fold	No Change	↑ 11.8 Fold	↑ 3.3 Fold
Tumor 2	↑ 5.0 Fold	↑ 2.0 Fold	↑ 5.3 Fold	↑ 6.1 Fold	↓ 2.4 Fold	No Change	↓ 3.5 Fold	↓ 5.5 Fold
Tumor 3	No Change	No Change	No Change	No Change	No Change	No Change	No Change	No Change
Tumor 4	↑ 1.4 Fold	↑ 1.8 Fold	↓ 2.0 Fold	No Change	↑ 2.0 Fold	No Change	No Change	↓ 3.1 Fold
Tumor 5	↓ 2.1 Fold	No Change	No Change	No Change	No Change	No Change	No Change	↓ 2.2 Fold
Tumor 6	↓ 2.6 Fold	↑ 4.5 Fold	↑ 6.9 Fold	↑ 2.0 Fold	No Change	No Change	No Change	No Change

Table 3. Cytokine profile of Bavituximab and Docetaxel in the presence of IL-2 versus IL-2 control in adenocarcinoma of the lung patient samples. 3D microspheres were seeded in a 24-well plate and treated with IL-2 (600 U/ml), Bavituximab (Bav, 1.5g/m²) or docetaxel (Docetx, 3μM) for 36 hours. After treatment, the supernatants were collected and analyzed via multiplex analysis. Induction of cytokine fold increase (≥ 2.0) or decrease (≤ 0.50) mediated by bavituximab and docetaxel combination treatment as compared to the IL-2 control was illustrated above. Favable vs non-favorable immune responses

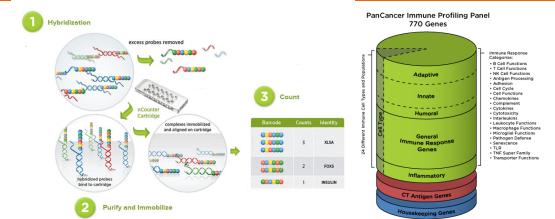


Figure 5. Schematic Illustration of Nanostrip gene expression assay using PanCancer Immune Profiling Panel. The **n**Counter PanCancer Immune Profiling Panel contains 770 genes including markers for 24 different immune cell types and populations. 30 commercial oligonucleotide probes are used to detect all categories of immune response including key checkpoint blockade genes. For each target gene, a unique colored reporter is hybridized with target mRNA, immobilized to cartridge and counted for quantification. All copy rights of the above image belong to Nanostrip.

	Tumor 4				
	IL2+Bavi+Doce vs IL2	IL2+Bavi+Doce vs IL2+Bavi	IL2+Bavi+Doce vs IL2+Douce	IL2+Bavi vs IL2	
Inflammation	PD-1 IL1-β	↓ 2.21 ↑ 2.48	No change ↑ 1.99	↓ 2.10 No change	No change No change
	TNFSF15	↑ 4.48	↑ 2.78	No change	No change
	M1-like macrophage	ICL3 CCL4 CXCL11	↑ 2.57 ↑ 2.19 No change	↑ 2.75 ↑ 2.35 No change	No change No change No change
M2-like macrophage	CCL24 CCL13 CCL23	↓ 2.23 ↓ 2.34 No change	↓ 2.52 No change No change	No change ↓ 1.94 ↓ 1.92	No change No change No change
	NSCLC prognosis	CXCL5 CCL22	↑ 2.65 No change	↑ 2.90 No change	No change No change
	Tumor 6				
	IL2+Bavi+Doce vs IL2	IL2+Bavi+Doce vs IL2+Bavi	IL2+Bavi+Doce vs IL2+Douce	IL2+Bavi vs IL2	
Inflammation	PD-1 IL1-β	No change ↑ 3.69	No change No change	↑ 3.98 ↑ 2.89	↑ 1.91 ↑ 3.37
	TNFSF15	↑ 1.84	No change	↑ 2.05	↑ 2.10
	M1-like macrophage	CCL3 CCL4 CXCL11	↑ 2.72 ↑ 2.63 No change	↑ 3.27 ↑ 2.90 ↓ 1.86	↑ 3.23 ↑ 2.63 ↑ 2.83
M2-like macrophage	CCL24 CCL13 CCL23	No change No change No change	No change No change ↑ 1.85	↓ 2.07 No change ↓ 1.89	↓ 2.49 ↓ 2.32 ↓ 2.98
	NSCLC prognosis	CXCL5 CCL22	↑ 1.99 ↑ 2.91	No change No change	↑ 2.52 ↑ 3.42
					↑ 2.05 ↑ 2.18

Table 4. Changes in gene expression profile induced by Bavituximab, Docetaxel or combination treatment in NSCLC patient samples. 3D microspheres generated from freshly resected patient tumor samples and were seeded in a 24-well plate with IL2 (6000 U/ml), Bavituximab (2.5μg/ml) and/or Docetaxel (Dose, 3μg/ml) for 36 hours. After treatment, total RNA was harvested and analyzed using Nanostring nCounter® PanCancer Immune Profiling Panel for Gene Expression. Induction of gene expression fold increase or decrease of gene z > 2.0 fold was highlighted, with favorable immune responses changes colored in green.

SUMMARY

- Nitogen's ex-vivo system is reliable to demonstrate drug combination effects on the tumor immune microenvironment of fresh patient samples.
 - Bavituximab, alone and in combination with Docetaxel, induces TIL activation as demonstrated by a significant increase in IFN- γ , TNF- α , and GM-CSF secretion.
 - Bavituximab's response appears to correlate with low PD-L1 expression in the tumor sample.
 - M1 polarization of tumor associated macrophages is likely involved in Bavituximab-mediated activation of tumor infiltrating lymphocytes.
 - Combination of Bavituximab with PD-1/PD-L1 inhibitors may enhance the immunomodulatory efficacy in cancer patients.

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