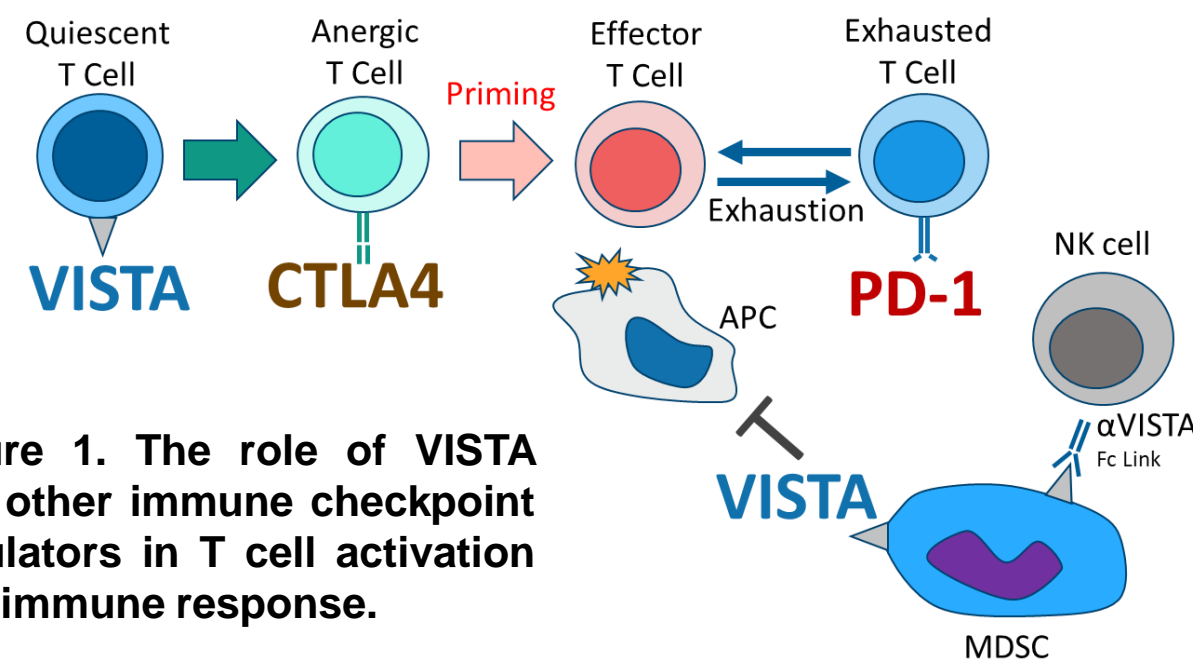


## Background

V-domain immunoglobulin suppressor of T cell activation (VISTA) is a negative checkpoint regulator of immune cells. It has been demonstrated that VISTA is expressed in resting T cells affecting the earliest phase of response to tumor antigen (1). In T cells, VISTA is the key regulator of quiescence whereas CTLA-4 inhibits priming and PD-1 regulates effector function of T cells (Fig. 1). VISTA has been recognized as a potential mediator of resistance to anti-PD-1 and anti-CTLA-4 immunotherapies in cancer patients. Targeting the VISTA signaling pathway is a promising approach for overcoming resistance to current immune checkpoint therapies. Curis is testing CI-8993, an anti-VISTA therapeutic antibody, in a Phase 1 trial in patients with solid tumors (NCT04475523). The goal of this study was to develop a VISTA-centric tumor immunophenotyping assay to explore potential tumor biomarkers for anti-VISTA therapeutics.



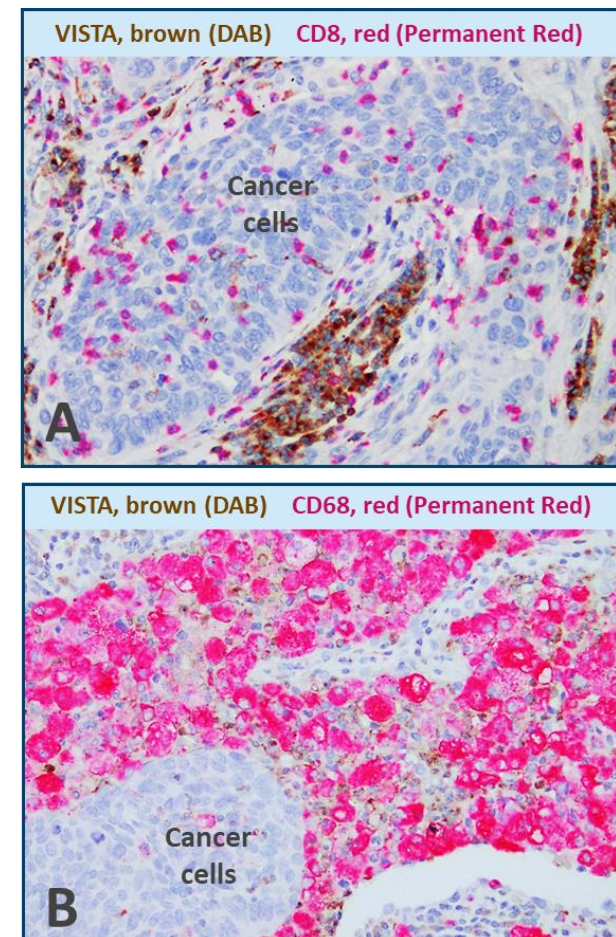
**Figure 1. The role of VISTA and other immune checkpoint regulators in T cell activation and immune response.**

## Materials and Methods

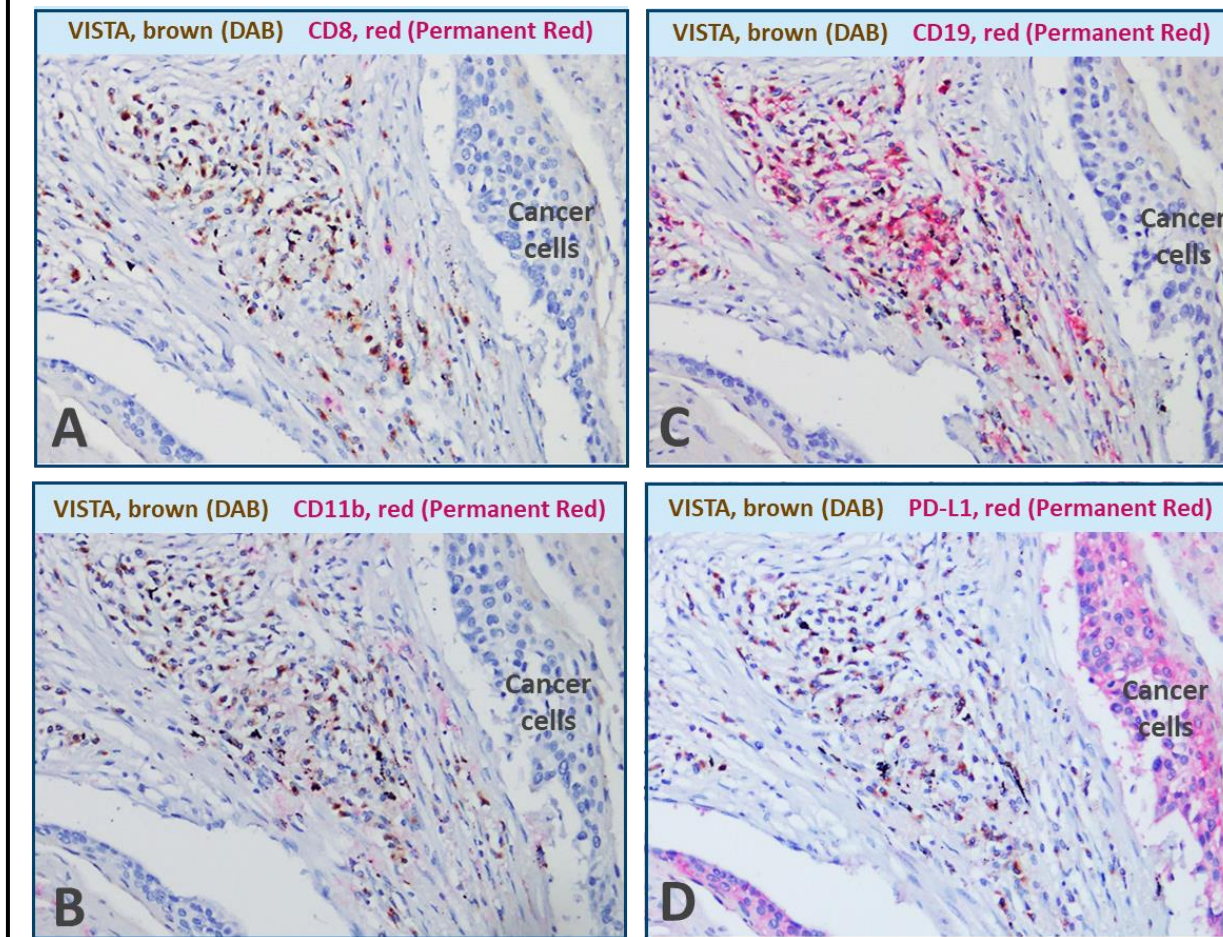
Formalin-fixed paraffin embedded (FFPE) tumor tissue sections from 10 cases of non-small cell lung carcinoma (NSCLC) were purchased from NovoVita Histopath Laboratory (Boston, MA). Serial tumor tissue sections were double-immunostained with VISTA combined with CD8 (cytotoxic T cell marker), CD4 (T helper cell marker), CD11b (myeloid cell marker), CD68 (monocyte/macrophage marker), CD56 (NK cell marker), CD19 (B cell marker) or Programmed Death-Ligand 1 (PD-L1).

## Results

Immunohistochemical analysis revealed the presence of CD8+ cells (9/10 cases), CD4+ cells (3/10 cases), CD11b+ cells (10/10 cases), CD68+ cells (10/10 cases), CD56+ cells (4/10 cases) and CD19+ cells (8/10 cases) in lung tumors (Fig. 2-4). Using double IHC staining, we found that VISTA was expressed in CD8+ cells (5/9 tumors), CD11b+ cells (5/10 tumors) and CD19+ cells (5/8 tumors), whereas VISTA was hardly detectable in CD4+, CD68+ or CD56+ cells in NSCLC tumors analyzed (Fig. 2-4). VISTA was not expressed in CD8+ cells infiltrating the tumor parenchyma (Fig. 2A). Expression of PD-L1 was detected in cancer cells in 6/10 tumors, whereas VISTA-positive cancer cells were revealed in 1/10 tumors. We developed an algorithm for evaluation of VISTA-centric tumor immunophenotyping and demonstrated that every tumor has a unique cell-type-specific pattern of VISTA expression which could serve as a potential biomarker (Fig. 4).



**Figure 2. Representative images of double IHC staining in NSCLC tumors.** A, VISTA is not expressed in CD8-positive cells infiltrating tumor parenchyma. B, VISTA is not expressed in CD68-positive cells.



**Figure 3. Representative images of VISTA expression in serial sections of NSCLC tumor (A-D).** Double IHC staining of tumor serial sections shows that VISTA is not expressed in CD8-positive cells (A), CD11b-positive cells (B) or PD-L1-positive cells (D). VISTA (brown) expression was detected in CD19-positive (red) cells in the stroma of the tumor (C). Expression of PD-L1 (red) was detected in cancer cells (D).

## Results

NSCLC, Case #	Immune cells						Cancer cells	
	CD8	CD68	CD11b	CD19	CD4	CD56	PD-L1	VISTA
1	Positive single staining, marker	Negative staining	Positive single staining, marker	Positive double staining, marker and VISTA are expressed in the same cells	Negative staining	Negative staining	Negative staining	Negative staining
2	Positive double staining, marker and VISTA are expressed in the same cells	Negative staining	Negative staining	Negative staining	Negative staining	Negative staining	Negative staining	Negative staining
3	Positive double staining, marker and VISTA are expressed in the same cells	Negative staining	Negative staining	Negative staining	Negative staining	Negative staining	Negative staining	Negative staining
4	Positive double staining, marker and VISTA are expressed in the same cells	Negative staining	Positive double staining, marker and VISTA are expressed in the same cells	Positive double staining, marker and VISTA are expressed in the same cells	Negative staining	Negative staining	Negative staining	Negative staining
5	Positive double staining, marker and VISTA are expressed in the same cells	Negative staining	Negative staining	Negative staining	Negative staining	Negative staining	Negative staining	Negative staining
6	Positive single staining, marker	Negative staining	Positive double staining, marker and VISTA are expressed in the same cells	Negative staining	Negative staining	Negative staining	Negative staining	Negative staining
7	Positive single staining, marker	Negative staining	Positive double staining, marker and VISTA are expressed in the same cells	Positive double staining, marker and VISTA are expressed in the same cells	Negative staining	Negative staining	Negative staining	Negative staining
8	Positive double staining, marker and VISTA are expressed in the same cells	Negative staining	Positive double staining, marker and VISTA are expressed in the same cells	Positive double staining, marker and VISTA are expressed in the same cells	Negative staining	Positive single staining, marker	Negative staining	Positive double staining, marker and VISTA are expressed in the same cells
9	Positive single staining, marker	Negative staining	Positive double staining, marker and VISTA are expressed in the same cells	Positive single staining, marker	Negative staining	Negative staining	Negative staining	Negative staining
10	Positive single staining, marker	Negative staining	Positive double staining, marker and VISTA are expressed in the same cells	Positive double staining, marker and VISTA are expressed in the same cells	Negative staining	Negative staining	Negative staining	Negative staining

**Figure 4. VISTA-centric immunophenotyping of 10 NSCLC tumors.**

## Conclusions

Our results demonstrate that comprehensive VISTA-centric immunophenotyping enables spatially resolved and cell-type-specific characterization of VISTA expression in solid tumors and can serve as an applicable bioanalytical approach for identification of potential biomarkers to guide anti-VISTA therapeutic treatment decisions.

## References

- ElTanbouly MA, Zhao Y, Nowak E et al. VISTA is a checkpoint regulator for naïve T cell quiescence and peripheral tolerance. *Science* 2020;17:367(6475).

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