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Characterization of Butyrophilin 3A Expression Across Multiple Tumor Types to Support Target Patient Population Selection in the EVICTION Study with ICT01, an Anti-BTN3A Monoclonal Antibody that Selectively Activates Vγ9Vδ2 T Cells.

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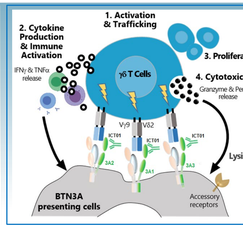
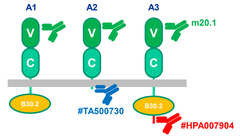
Background: Vγ9Vδ2 T are key players of innate and adaptive anti-tumor immunity (1). Butyrophilin 3A1 (BTN3A1) plays a key role in their activation through sensing of intracellular phosphoantigens by its cytoplasmic domain (2)(6). Butyrophillin 3A (BTN3A) three isoforms (3A1/3A2/3A3) are widely expressed on a variety of tumors (4). Vγ9Vδ2 T cell infiltration into tumor tissues is associated with a positive prognosis across multiple cancers (3), making BTN3A an interesting target for enhancing Vγ9Vδ2 anti-tumor activity.
ImCheck Therapeutics is developing ICT01, an anti-BTN3A mAb that specifically activates Vγ9Vδ2 T cells. ICT01 is currently in an international, multi-center Phase 1/2a clinical trial (NCT04243499, EVICTION Study). The level of BTN3A expression required for a clinical response to ICT01 is not known. Therefore, we developed novel immunohistochemistry (IHC) methods to enable a precision-medicine based approach to target population selection for dose escalation and potentially guide patient selection in the expansion cohorts of the ongoing EVICTION study.

Methods: A panBTN3A IHC staining that recognizes the three isoforms was developed on Fresh frozen (FF) tissues, while BTN3A2 and BTN3A3 specific stainings were developed on formalin-fixed paraffin embedded (FFPE) tissues. BTN3A1-specific staining is still under development. Transfected knock-out/knock-in cell lines and positive tissues were used to assess antibody specificity. BTN3A expression was then analyzed on both normal and associated tumor tissue using tissue microarrays (TMA) and selected frozen blocks from tumor biopsies. FACS analyses were also performed on dissociated lung and pancreatic cancer biopsies to determine BTN3A (3 isoforms) membrane expression on tumor-infiltrating immune cells and cancer/stromal cells.



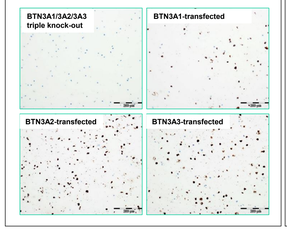
ICT01 MOA
Binds to all three BTN3A isoforms triggering:
1) γ9δ2 T-cell activation and trafficking from the circulation within 30 minutes, 2) Production of IFNγ and TNFα leading to immune cell activation, 3) Proliferation in the presence of cytokines, and 4) Cytotoxic attack of malignant cells that express a 2nd necessary signal.

1) Three anti-BTN3A antibodies are validated for IHC stainings

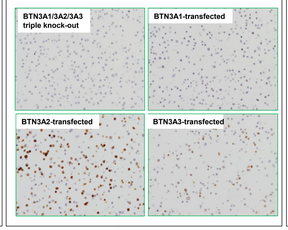


Immunohistochemistry was performed on the Benchmark XT automated platform (Ventana-Roche). Fresh Frozen samples sections were fixed and then stained with anti-BTN3A mAb (clone #20.1). FFPE samples sections were pretreated and then incubated with anti-BTN3A2 (TA500730, Thermo) or anti-BTN3A3 (HPA007904, Sigma) antibodies.
Antibodies epitopes, when known, are depicted on the scheme (left). Specificity was demonstrated by staining a BTN3A1/BTN3A2/BTN3A3 triple knock-out HEK cell line, transfected for each respective isoform-(below).

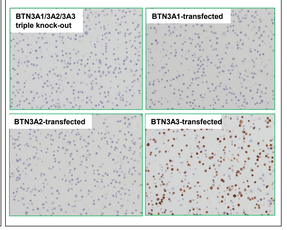
A- #20.1 recognizes all three BTN3A isoforms, as confirmed by staining of the transfected-triple knock-out cell lines below.

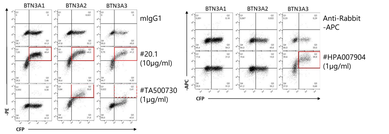


B- #TA500730 strongly recognizes BTN3A2 and displays weak staining on BTN3A3.



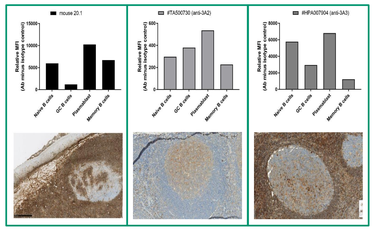
C- #HPA007904 recognizes only BTN3A3.





Antibodies specificities were confirmed by intracellular Flow Cytometry on the same Knock-out/Knock-in HEK cell lines described above. No staining was observed on BTN3A1/3A2/3A3 triple knockouts (not shown). Mouse #20.1 stains all three isoforms. anti-3A2 (#TA500730) stains mostly BTN3A2-transfected cells, with weak staining observed in BTN3A3-transfected cells. anti-BTN3A3 (#HPA007904) stains only BTN3A3 transfected cells

2) Further validation of anti-BTN3A antibodies specificity by Flow Cytometry and IHC staining on human tonsil



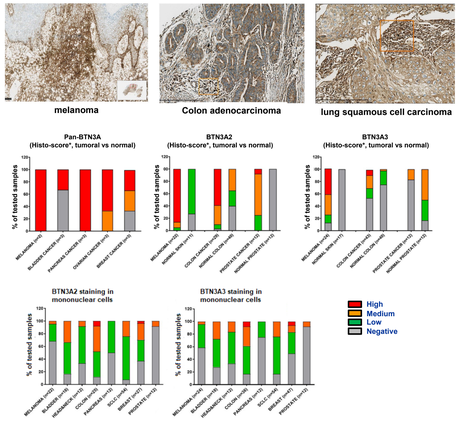
Antibodies specificities were further evaluated by comparing staining pattern obtained on human tonsil tissue.

First, human tonsil tissue was dissociated and FACS-stained with the three anti-BTN3A antibodies and a panel allowing the identification of four B cells subpopulation: naive B cells, germinal center B cells, plasmablasts and memory B cells.

As depicted (left, top), mouse #20.1 mAb stained mostly naive B cells and plasmablast, the anti-BTN3A2 mAb stained germinal centers B cells and plasmablast (germinal centers) and the anti-BTN3A3 stained mostly naive B cells and plasmablasts.

Interestingly (left, bottom), a coherent staining pattern was obtained by IHC staining human tonsil, with #20.1 and anti-BTN3A3 staining mostly interfollicular areas and anti-BTN3A2 staining mostly germinal centers B cells and plasmablast.

3) In solid tumors, BTN3A is upregulated in malignant tissue when compared to normal tissue, with inter-patient heterogeneity

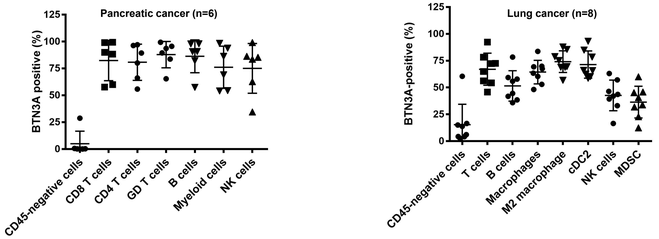


Melanoma (n=2), bladder cancer (n=3), pancreas cancer (n=3), ovarian cancer (n=3) and breast cancer(n=3) frozen blocks were screened for panBTN3A staining (clone #20.1, Benchmark XT, frozen tissues).

Normal and malignant Tissue microarrays (TMAs, Bladder cancer BL631, Head and neck HN241b, Colon and rectum carcinoma CO1002b, Lung cancer LC10010c, Skin malignant melanoma ME482a, Pancreas cancer PA241d, Prostate cancer PR243c, Breast cancer BRM961a and normal tissues arrays) were screened for BTN3A2 and BTN3A3 stainings (#TA500730 and #HPA007904, Benchmark XT, FFPE tissues).

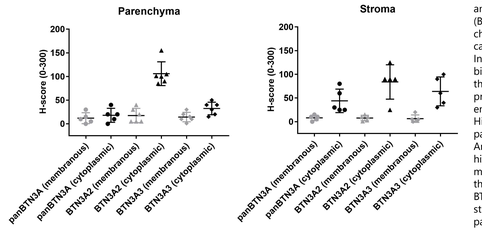
Tissues staining specificity was validated by a trained pathologist and panBTN3A, BTN3A2 and BTN3A3 staining intensity was assessed by a semi-quantitative method (Histology Score, Arbitrary Unit, 0-300, combination of staining intensity with the % of positive cells, in tumor parenchyma, stroma and mononuclear cells, for both cytoplasmic and membranous signals).

4) In pancreatic and lung cancer, BTN3A is detected at the membrane of immune cells, with lower level on stromal cells

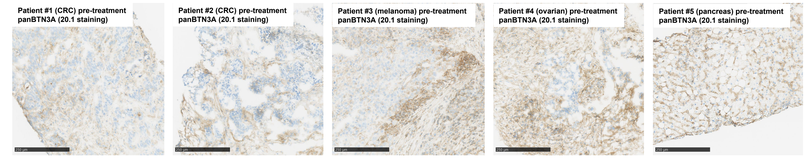


pan-BTN3A cell surface density was determined by FACS staining with the mouse #20.1 mAb on single cells suspension obtained from fresh tumor biopsies. Infiltrating immune cell populations were identifed using specific FACS panel.
BTN3A membrane expression was almost undetectable on stromal cells (defined as CD45-negative) in pancreatic cancer biopsies and low in lung cancer biopsies. BTN3A membrane expression was strong on all CD45+ cells.

5) BTN3A tumoral expression in first patients of EVICTION clinical trial



EVICTION ((NCT04243499) is a first–in-human, Phase I/IIa clinical trial evaluating ICT01, a first-in-class γδ2 T cell-activating monoclonal antibody (mAb) targeting the extracellular domain of Butyrophilin 3A (BTN3A) as monotherapy and in combination with an immune checkpoint inhibitor in patients with advanced, relapsed/refractory cancer, including both solid and hematologic tumors.
In order to evaluate the potential of BTN3A expression level as a biomarker for selecting patients that will benefit the most from ICT01 therapy, those IHC methods were used to assess BTN3A expression in pre- and post-treatment tumor biopsies from the first six patients enrolled in cohort 1 in the dose escalation phase.
Histo-scores (left) and panBTN3A representative IHC images (bottom panel) obtained in pre-treatment tumoral biopsies are presented.
Among those six patients, BTN3A expression levels are heterogenous. A higher BTN3A expression is observed in the cytoplasm in comparison to membrane expression. BTN3A2 expression seems to be up-regulated in the cytoplasm in parenchymal cells in comparison to pan-BTN3A and BTN3A3 expression, while no significant difference is observed in stromal cells. Those preliminary results will be confirmed in other patients and next cohorts.



6) Conclusions and Clinical Perspectives

1. Malignant cells tended to overexpress BTN3A, as compared to healthy tissue, although there was intra-patient variability.
2. Also, a significant percentage of Immune cells isolated from lung and pancreatic tumor biopsies expressed BTN3A.
3. These validated IHC methods are currently being used in the EVICTION trial (NCT04243499) in order to characterize specific cancers and identify patients that respond favorably to ICT01 therapy, a γ9δ2T cell-activating monoclonal antibody (mAb) targeting the extracellular domain of all 3 isoforms of BTN3A.
4. Results from the first 6 patients with solid tumors treated in Cohort 1 revealed higher cytoplasmic expression of BTN3A. Also, a higher cytoplasmic expression of BTN3A2 in parenchymal cells is observed in comparison to BTN3A3 or pan-BTN3A suggesting differential gene regulation mechanisms for the different BTN3A isoforms.
5. Additional data from EVICTION patients with solid tumors and hematologic malignancies are needed to confirm these preliminary results and help in understanding the expression of BTN3A, and identifying the right patients for treatment with and response to ICT01.

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