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Targeting BTN2A1 Modulates Anti-tumor Activity of Vγ9Vδ2 T Cells

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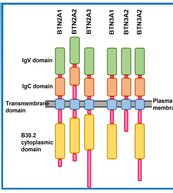
Background:

Vγ9Vδ2 T cells constitute the predominant subset (60 to 95%) among γδ T cells in peripheral blood (1). Their infiltration into malignant tissues is associated with a favorable prognosis (2). Their anti-tumor activity is triggered by the binding to the intracellular domain of butyrophillin 3A (BTN3A) of intracellular organic phosphoantigens (pAgs) accumulated during tumorigenesis (3). Recently, BTN2A1 was shown to act jointly with BTN3A to initiate the pAg sensing of Vγ9Vδ2 (4). Indeed, BTN2A1 interacts with the Vγ9+ TCR chain, which constitutes the first step of the immune synapse between Vγ9Vδ2T cells and cancer cells that triggers Vγ9Vδ2T cell anti-tumor responses (4, 5). In this study, antagonist and agonist mAbs against BTN2A1 were generated and their ability to modulate Vγ9Vδ2 cell cytotoxicity against cancer cell lines and primary cancer cells was evaluated *in vitro*.

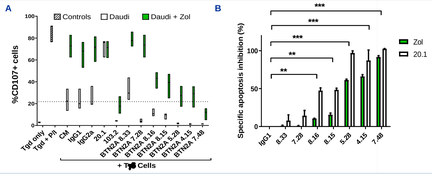
Methods:

Mouse anti-human BTN2A1 mAbs were generated by mouse immunization using recombinant human BTN2A1-Fc fusion protein. The effect of anti-BTN2A1 mAb on Vγ9Vδ2T T cell degranulation (as depicted by the percentage of CD107+ Vγ9Vδ2T cells), secretion of IFNγ and TNFα, and target cell killing (as depicted by caspase 3/7 cleavage), were tested in co-cultures with Daudi (Burkitt lymphoma) and HL-60 (acute myeloblastic leukemia) cell lines, and primary acute myelocytic leukemia (AML, n=12) blasts or primary acute lymphocytic leukemia (ALL, n=11) blasts in the presence or absence of zoledronate or the anti-BTN3A mAb (20.1) as activators of γδT cell responses. Endometrial cancer spheroids were used to assess the ability of anti-BTN2A1 antagonist 7.48 mAb to inhibit Vγ9Vδ2 T cell killing of cancer cells.



Butyrophilins (BTN) belong to the Immunoglobulin superfamily and share structural homology to the B7 family. Only BTN2A and BTN3A sub-families are shown (adapted from Redwan et al. (6)). BTNs are immune regulators with either stimulatory or inhibitory effects on immune cells (7).

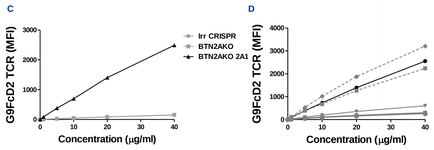
1) Anti-BTN2A1 antagonist mAbs Inhibit Vγ9Vδ2 T cell cytotoxicity against cancer cell lines



Seven Vγ9Vδ2 T cell antagonists were identified. Anti-BTN2A mAb 7.48 has the strongest antagonist effect

(A) Vγ9Vδ2 T cell degranulation against Daudi cells with or without zoledronate (Zol), and with or without the indicated anti-BTN3A (20.1 and 103.2) and anti-BTN2A mAbs;
(B) Vγ9Vδ2 T cells were co-cultured with HL-60 cells (E:T is 5:1) in presence of zoledronate (50 µM) or 20.1 mAb (10 µg/mL) with or without anti-BTN2A mAbs or control isotype. Apoptosis inhibition was estimated by flow cytometry analysis of caspase 3/7 cleavage in HL-60 cells.

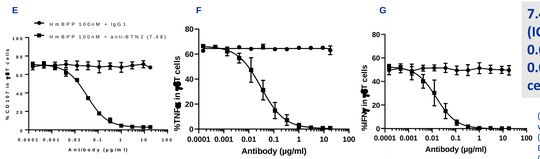
2) Anti-BTN2A1 clones 7.48, 4.15, 5.28 and 8.15 block BTN2A1/Vγ9Vδ2 TCR interaction



Blockade of BTN2A1 interaction with Vγ9Vδ2 TCR is observed with most potent antagonist anti-BTN2A1 mAbs

HEK-BTN2AKO cells and HEK-BTN2AKO cells transduced to restore BTN2A1 were incubated with anti-BTN2A1 mAbs (2 µg/mL) prior to incubation with a recombinant Vγ9Vδ2 TCR(G118)-Fc fusion protein revealed with a goat anti-human-PE Fc probe. (C) Binding curves of recombinant Vγ9Vδ2 TCR to HEK-BTN2AKO+2A1 and HEK-BTN2AKO cells. (D) Binding curves of recombinant Vγ9Vδ2 TCR to HEK-BTN2AKO+2A1 cells, following saturation with a panel of anti-BTN2A1 antibodies compared to isotype control (black).

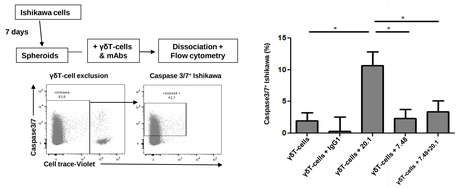
3) Anti-BTN2A1 7.48 inhibits pAg-induced Vγ9Vδ2 T cell cytotoxicity against Daudi cells



7.48 abrogates Vγ9Vδ2 T-cell degranulation (IC50= 0.033±0.0003 µg/mL), TNFα (IC50= 0.03±0.006 µg/mL) and IFNγ (IC50= 0.015±0.004 µg/mL) secretion against Daudi cells in presence of HMBPP

(E) Vγ9Vδ2 T cell degranulation against Daudi cells in presence of HMBPP w/o anti-BTN2A1 7.48 (10 µg/mL);
(F) TNFα secretion against Daudi cells in presence of HMBPP w/o anti-BTN2A1 7.48 (10 µg/mL);
(G) IFNγ secretion against Daudi cells in presence of HMBPP w/o anti-BTN2A1 7.48 (10 µg/mL); *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

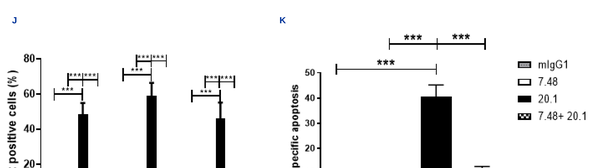
4) Anti-BTN2A1 antagonist 7.48 inhibits Vγ9Vδ2 T-cells cytotoxicity in conditions mimicking the tumor setting

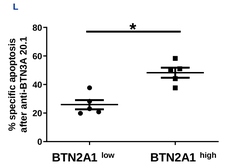


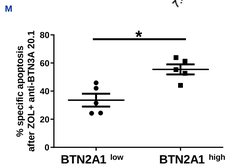
Vγ9Vδ2 T cells killing of Ishikawa endometrial adenocarcinoma spheroids was inhibited with anti-BTN2A1 7.48

(H) Ishikawa endometrial adenocarcinoma cell line was cultured to obtain 3D spheroids. CTV-stained Vγ9Vδ2 T-cells were then added to the spheroids under the following conditions: Vγ9Vδ2 (γδT-cell alone, γδT-cells + mIgG1, γδT-cells + 7.48, γδT-cells+20.1 or γδT-cells + 7.48 and 20.1. After overnight culture with caspase3/7 staining, spheroids were dissociated and processed for flow cytometry.
(I) Bar chart represents the percentage of Caspase 3/7+ Ishikawa cells after culture conditions detailed in (H). *p-value < 0.05. (n=4 Vγ9Vδ2 T-cells donors)

5) Anti-BTN2A1 7.48 antagonist mAb supresses Vγ9Vδ2 T-cells cytotoxicity against primary acute myloblastic leukemia (AML) blasts







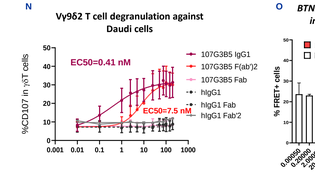
Anti-BTN2A1 7.48 mAb efficiently inhibits Vγ9Vδ2 T-cell degranulation, TNFα and IFNγ production, as well as killing of primary AML blasts upon 20.1 and zoledronate activation

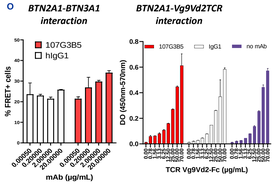
J) Fraction of CD107ab+, TNFa+ and IFNγ+ Vγ9Vδ2 T cells (n=3) after a 4-hour co-culture (E:T ratio 1:1) with primary AML blasts (n=6) with isotype control or anti-BTN2A1 7.48 mAb and/or anti-BTN3A 20.1 (10 µg/mL).
K) Caspase 3/7 expression on primary AML blasts (n=12) after coculture with Vγ9Vδ2 T cells (n=3) with an E:T ratio 15:1.
(J and K) AML blasts were preincubated O/N in medium. Results from 6 independent experiments are depicted, expressed as mean +/- SEM, non-parametric paired Anova repeated measures test with Bonferonni post-test. ***$p < 0.001$.

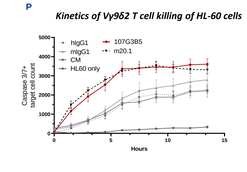
(L and M) Caspase 3/7 expression on primary AML blasts (n=10) after a coculture with Vγ9Vδ2 T cells (E:T 15:1). Cells were treated with anti-BTN3A 20.1 agonist (10µg/mL) with and without zoledronate, as indicated. BTN2A1 expression was assessed by flow cytometry and expressed as Median Fluorescence Intensity (MFI). The statistical significance was established using the Mann-Whitney test. *$p < 0.05$.

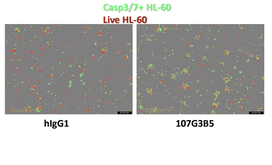
6) Anti-BTN2A1 agonist mAb (107G3B5) enhances Vγ9Vδ2 T cell cytotoxicity against cancer cell lines

Anti-BTN2A1 107G3B5 mAb increases Vγ9Vδ2 T cell degranulation and killing of cancer cell lines *in vitro*, in a Fc-independent manner. This antibody also enhances BTN2A1/BTN3A1 interaction



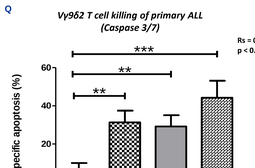


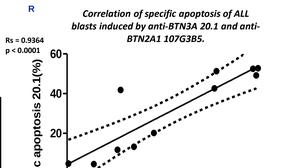




(N) Vγ9Vδ2 T cells from healthy donors (n=3) were expanded and co-cultured with Daudi cells (ratio 1:1) in presence of 107G3B5 or its control isotype. Vγ9δ2 T cell degranulation (%CD107ab+ cells) was assessed 4-hours post-incubation.
(O) Left: HEK-293T cells were transfected with plasmids encoding BTN2A1-CFP and BTN3A1-YFP, treated overnight with CHX+bafilomycin in presence of 107G8S or its isotype control. FRET+ cells percentages were assessed by cytometry. Right: Competition between anti-BTN2A mAbs and Vγ9δ2 TCR for BTN2A1 binding. ELISA was performed by coating recombinant human BTN2A1-His protein and using Vγ9δ2 TCR-Fc as analyte. Vγ9δ2 TCR (clone G118) binding was revealed using an HRP-conjugated anti-human Fc probe. Anti-BTN2A1 107G3B5 and control isotype (2 µg/mL) were pre-incubated on BTN2A1-His for saturation, prior to incubation with Vγ9δ2 TCR-Fc. 107G3B5 binding did not affect the interaction between BTN2A1 and the Vγ9Vδ2 TCR.
(P) Left: HL-60 cells stably expressing mKate (red) were co-cultured with expanded Vγ9δ2 T cells from healthy donors (n=3) in presence of CellEvent Caspase 3/7 Green Detection Reagent for 18h in an Incucyte device (Sartorius). Apoptosis of HL-60 cells (caspase 3/7 green+) was assessed every 90 min. Right: snapshots of HL-60 apoptosis after 6 hours co-cultures with Vγ9δ2 T cell.

7) Anti-BTN2A1 107G3B5 agonist mAb increases Vγ9Vδ2 T cell-driven apoptosis of primary acute lymphocytic leukemia (ALL) blasts

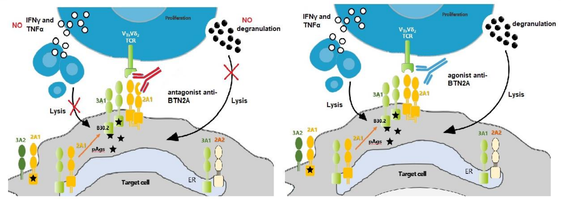




Killing of ALL blasts by Vγ9Vδ2 T cells is increased in presence of 107G3B5. The effect is comparable to the one obtained with anti-BTN3A 20.1 mAb

(Q) Caspase 3/7 expression on primary AML blasts (n=11 including T-ALL and b-ALL) after a coculture for 3h30 with Vγ9Vδ2 T cells (E:T 15:1) in presence of the indicated mAbs (10 µg/mL). Apoptotic ALL blasts were assessed by flow cytometry.
(R) Pearson correlations of specific apoptosis of ALL blasts triggered by anti-BTN3A 20.1 and anti-BTN2A1 107G3B5.

8) Conclusion and Perspectives



Vγ9Vδ2 T cell mediated cytotoxicity against cancer cells is a sequential process that requires the establishment of an immune synapse followed by the transduction of an intracellular phosphoantigen danger signal. By its binding to the Vγ9+ TCR chain BTN2A1 mediates the first step of recognition of cancer cells by Vγ9Vδ2 T cells (4). The binding of phosphoantigens to the intracellular domain of BTN3A1 triggers a conformational change on its ectodomain that is sensed by Vγ9Vδ2 T cells as an activation signal (3).
In this study, seven antagonist and one agonist mAbs to BTN2A1 were discovered. Anti-BTN2A1 7.48 potently inhibits Vγ9Vδ2 T-cells degranulation, cytokines (IFNγ and TNFα) secretion and killing of tumor cells, in both cell lines and primary AML blasts. Conversely, anti-BTN2A1 clone 107G3B5 enhances Vγ9Vδ2 T-cells responses as demonstrated by an increase in degranulation of Vγ9Vδ2 T cells and cancer cell apoptosis in co-cultures with cancer cell lines and primary ALL blasts. In addition, we identified an agonist anti-BTN2A1 mAb with the ability to enhance BTN2A1/BTN3A1 interaction.
This study demonstrates that anti-BTN2A1 antibodies constitute powerful tools for immunomodulating Vγ9Vδ2 T cell responses, which can be associated to the modulation of BTN2A1 interactions with the TCR or with BTN3A1. In summary, our study shows that BTN2A1 is a compelling target for the development of Vγ9Vδ2 T cell focused immunotherapies in oncology and potentially immuno-inflammatory diseases.

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