C24D
A paradigm shift in immuno-oncology

CD45
a novel PTPR immuno-oncology target

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Tel Aviv University, Sackler School, of Medicine.

Background on C24D

- **Our drug-candidate:** C24D is a proprietary therapeutic peptide, derived from the placental immunomodulatory ferritin (PLIF).
- **The receptor:** CD45 is the predominant transmembrane tyrosine phosphatase in leukocytes and plays a critical role in the efficient induction of T cell receptor signaling and activation. C24D binds to the CD45 receptor and resets the functionality of the immune system on immune-suppressed leukocytes.
- **Dr. Annat Raiter:** CTO of our company, Annat is the head of Head The Breast Cancer Research Lab at the Felsenstein Medical Research Center, connected to Tel Aviv University (TAU) and to the Rabin Medical Center (RMC).

Significant milestones C24D

- **Immune-modulated, specific tumor cell killing:**

  In vitro: MCF-7 (ER+) and T47D (ER+) breast cancer cells, Neoplasia, 16(9), 2014

  MDA-MB-231 and MDA-MB-468 triple negative breast cancer cells, OncoImmunology, 10(1), 2021.

  CAL 33 tongue and FaDu hypopharynx squamous cell carcinomas.

  Ex vivo: Immune-modulated killing of patient derived breast cancer tumors in an autologous setting.

  In vivo: MCF-7 [Neoplasia, 16(9), 2014] and MDA-MB-231 [OncoImmunology, 10(1), 2021].

  Mechanism of Action:

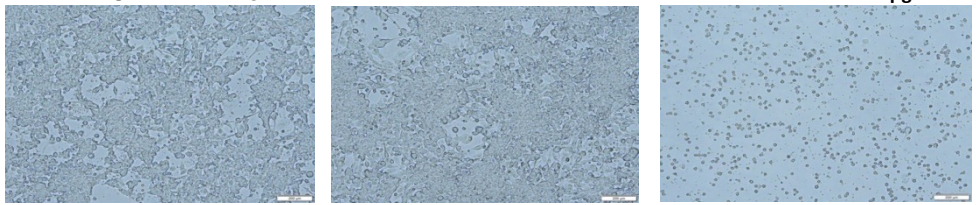
  - C24D binds to the CD45 transmembrane tyrosine phosphatase in leukocytes
  - Discovery of a heretofore unreported tumor escape pathway
  - Elucidation of the molecular mechanism via which the C24D technology overcomes immune suppression [OncoImmunology, 10(1), 2021].
  - Deciphering the TNBC immunosuppression mechanism
  - Design of new effective and specific peptides.

- **Preliminary studies:** Toxicity and Pharmacokinetics

In vitro: Immune-modulated killing of TNBC cells

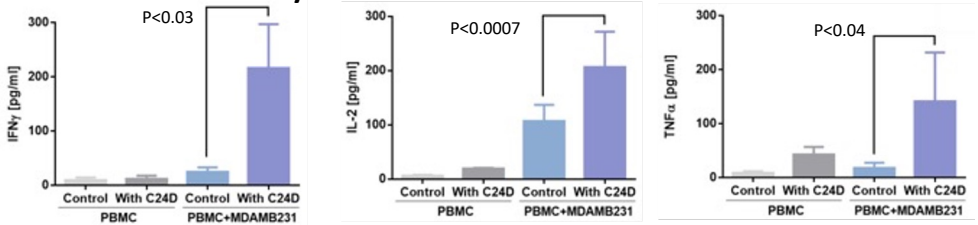
Microscopic observation (x20)

PBMC + MDA-MB-231 | PBMC + MDA-MB-231+ 10µg S-C24D | PBMC + MDA-MB-231 10µg C24D



MDA-MB-231 (TNBC) cells were co-cultured with PBMC from healthy female donors and treated with C24D (10µg/ml). Negative controls – minimal effect on cells treated with: PBMC alone or PBMC + scrambled peptide (S-C24D). No effect with C24D on cancer cells (not shown). Massive killing with PBMC +MDA-MB-231 cells + 10µg of C24D. Figures depict cells 4 days after treatment.

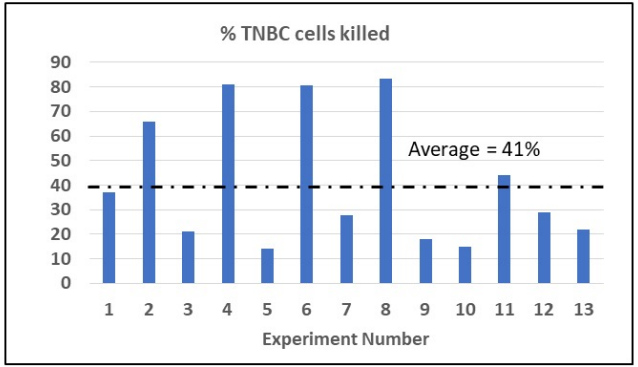
Cytokines secretion

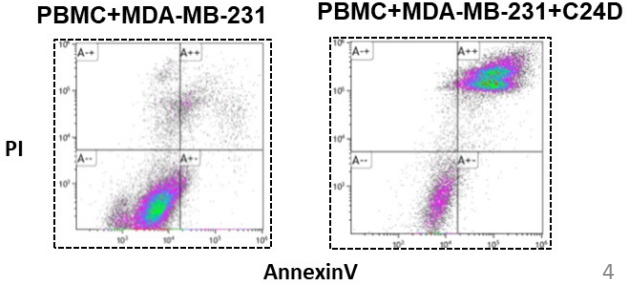


MDA-MB-231 (TNBC) cells were co-cultured with PBMC from healthy female donors and treated with C24D (10µg/ml). Negative controls – minimal effect on cells treated with: PBMC alone or PBMC + scrambled peptide (S-C24D). Significant inflammatory cytokines secretion with PBMC + cells + 10µg of C24D for 4-6 days (measured by ELISA).

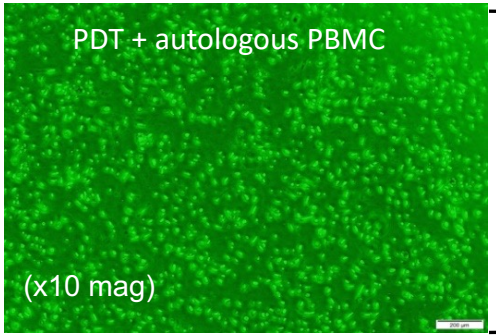
% killing of MDA-MB-231 cells, determined by FACS analysis

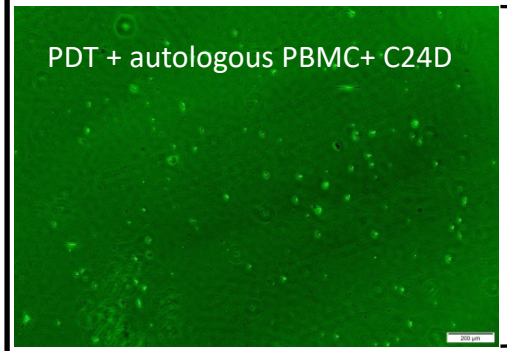
(AnnexinV/PI positive cells in 13 experiments)





Ex vivo: massive tumor cell killing in patient-derived tumor (PDT) biopsies





Patient-derived tumors

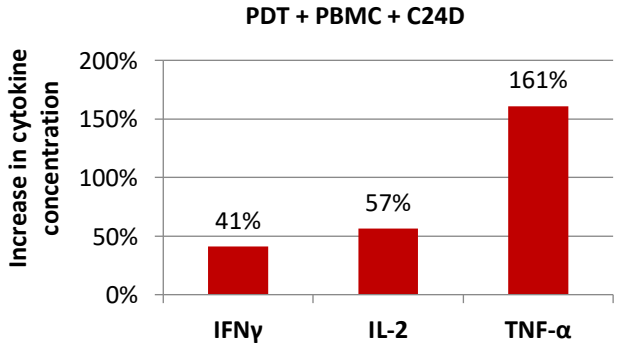
Primary cultures obtained from breast cancer patients' biopsies, before any treatment, were placed in growth medium, until establishment (2 – 3 weeks).

Fresh autologous PBMCs, with and without C24D, were subsequently added to the cultures for 4 – 6 days.

Results:

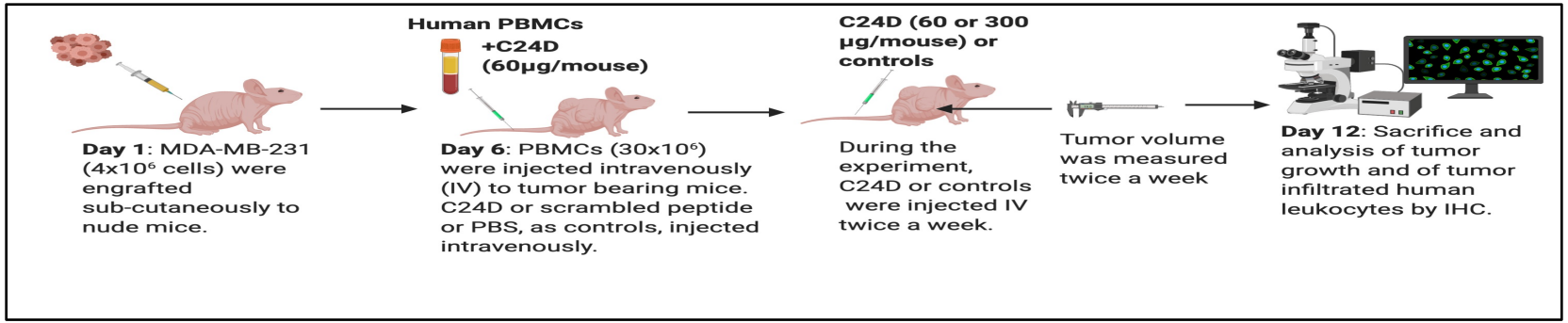
- **Massive death of tumor cells**.
- Secretion of inflammatory cytokines
- No effect without C24D.

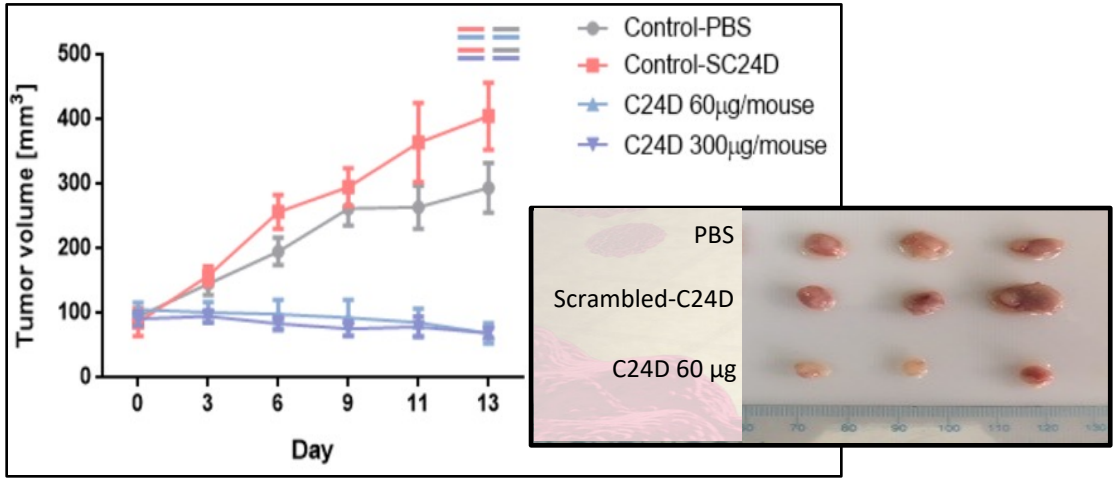
Cytokine Secretion

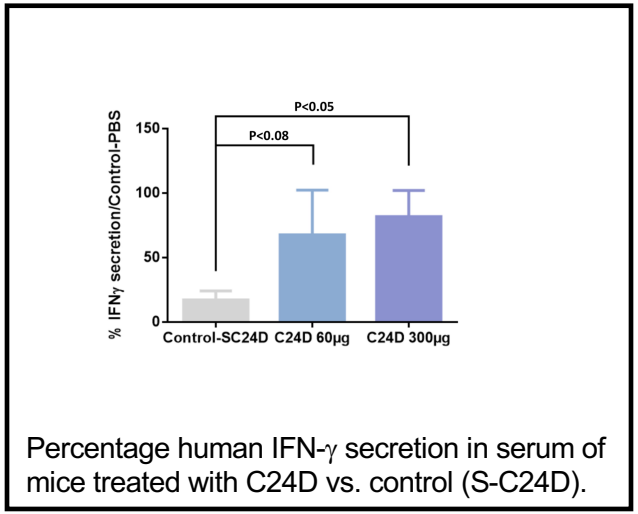


Fresh PDTs (primary cultures) and autologous PBMCs + C24D resulted in massive tumor cell killing, demonstrated by increased IFN**γ** secretion.

In vivo: TNBC tumor volume decreased significantly in C24D treated mice.

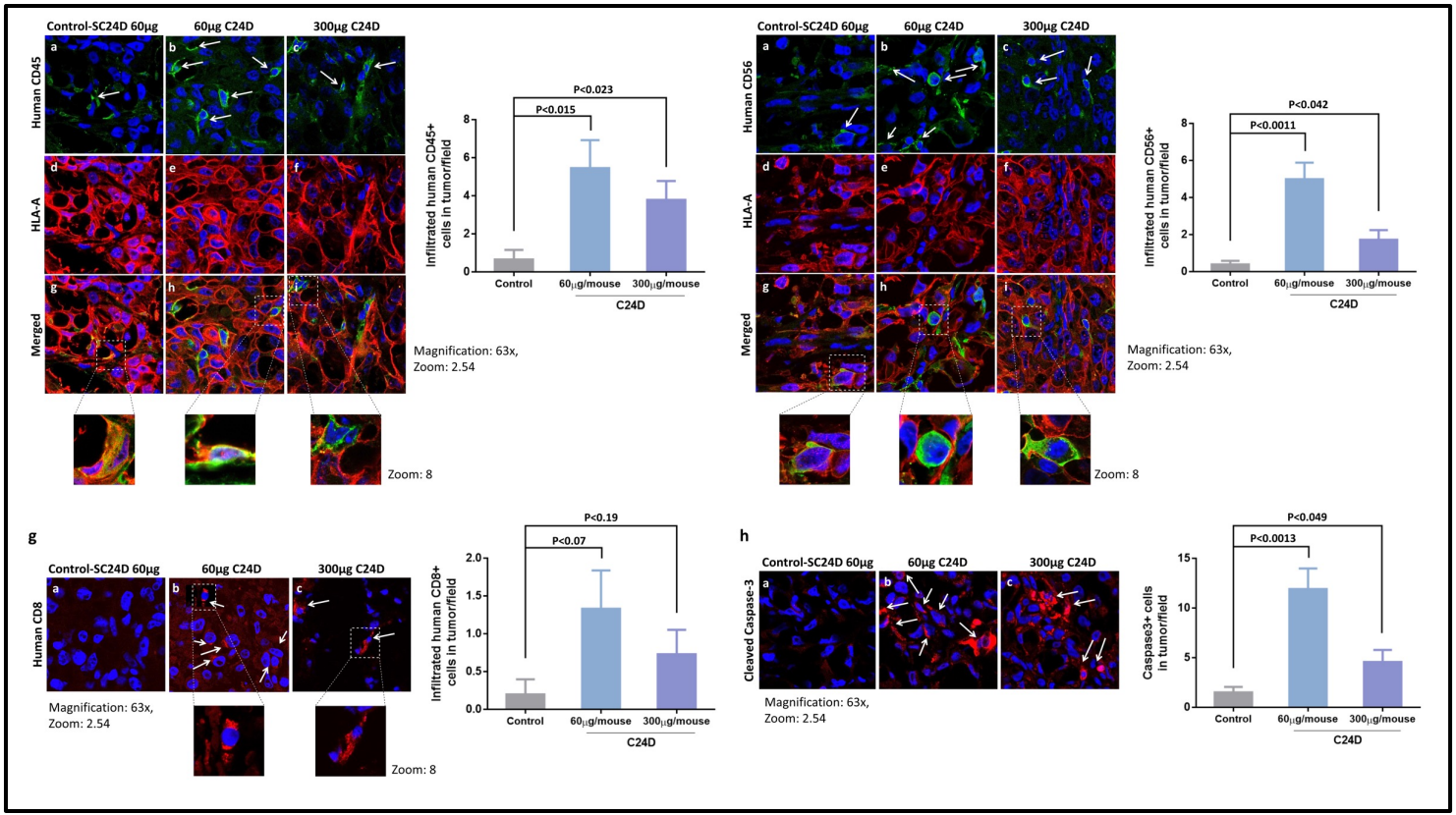






Percentage human IFN-γ secretion in serum of mice treated with C24D vs. control (S-C24D).

Tumor infiltrating cells induce apoptosis



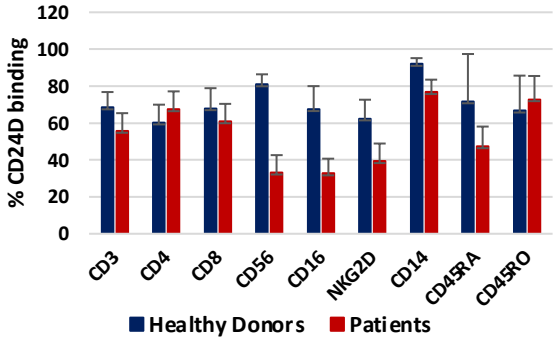
Tumor cell killing by tumor infiltrating T and NK cells

Immunofluorescence analysis and quantification of tumor (red) infiltrating human CD45+ cells (green), T cells (CD8+, red) and NK cells (CD56+, green) in mice treated with C24D, compared to S-C24D treated mice. Tumor infiltrating T and NK cells induce tumor cell apoptosis (caspase 3+ cells).

C24D binds to the CD45 receptor on human leukocytes

Extensive testing, with human samples, revealed that C24D binds specifically to the extracellular domain of CD45

C24D binds to all tested PBMC's sub-populations (FACS analysis)

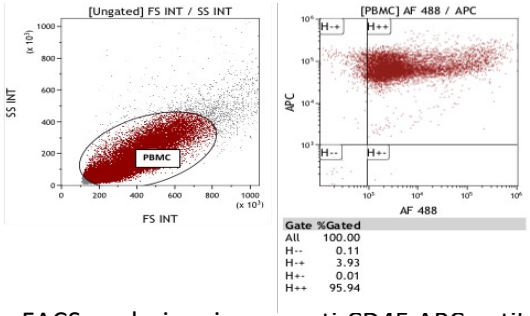


C24D binds to sub-populations of PBMCs.

C24D binding to CD45+ cells

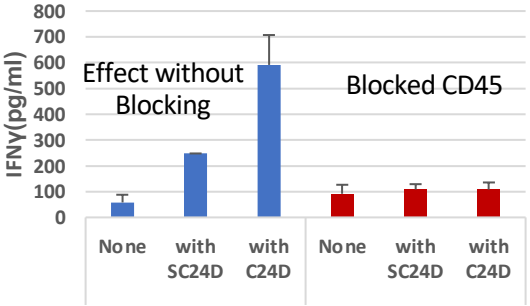
PBMC isolated from healthy female donor

Double staining of CD45-APC + cells with C24D-FITC



FACS analysis using an anti-CD45 APC antibody and C24D-FITC peptide.

Blocking of the CD45 receptor prevents C24D binding, resulting in inactivity, determined by IFNγ secretion



Blocking was done with a commercially available anti-CD45 antibody and a commercially available CD45-binding peptide.

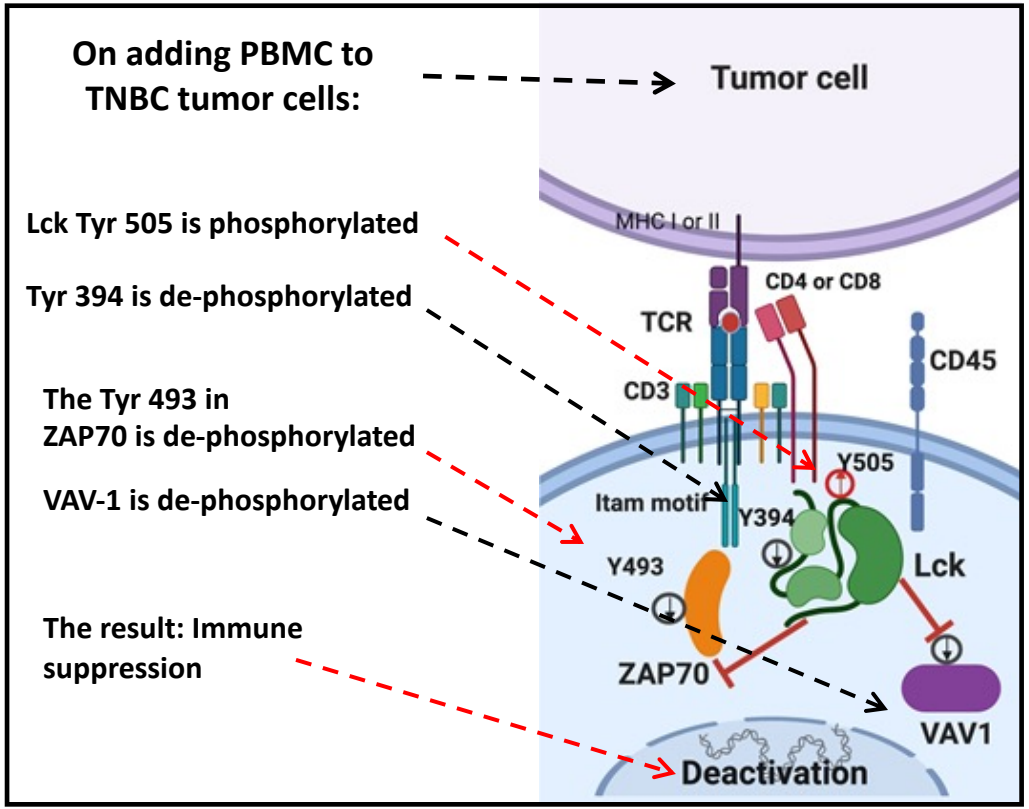
IFN **γ** was measured by ELISA in supernatants of MDA-MB-231 cells co-cultured with PBMCs ± C24D or a scrambled C24D (SC24D, as control)

C24D binding to CD45+ cells was established by mass spectrometry (with PBMCs from 8 different donors (6 healthy + 2 from breast cancer patients)

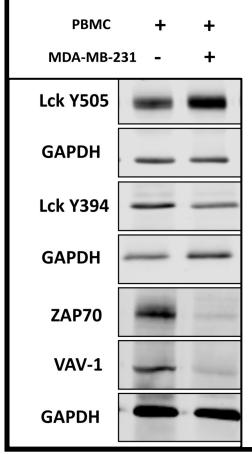
X6R433	Receptor-type tyrosine-protein phosphatase C OS=Homo sapiens GN=PTPRC PE=1 SV=1 - [X6R433_HUMAN]	PTPRC

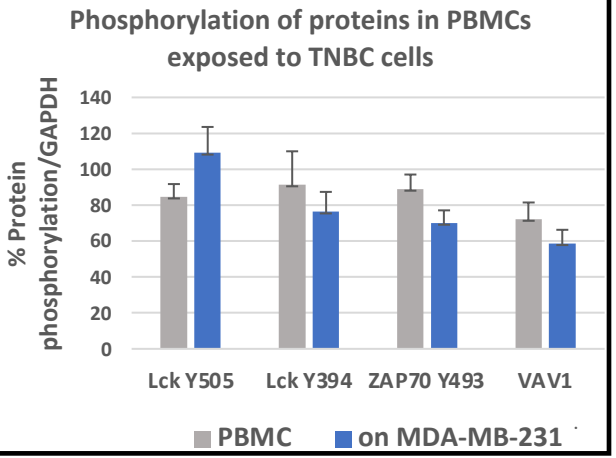
Smoler Proteomics Center, Technion

Tumor escape pathway involves the Src family tyrosine kinases

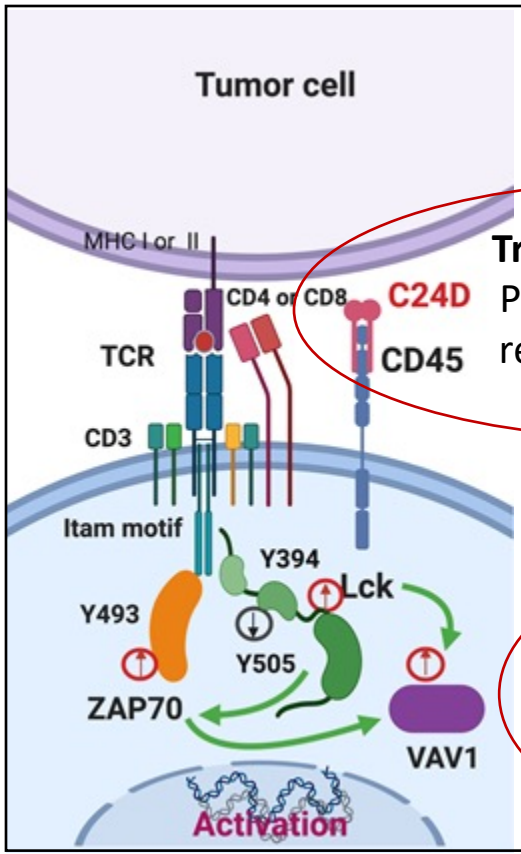


Lck Y505, Y394, ZAP-70 Y493 and VAV-1 Y173 phosphorylation were evaluated by Western blot and FACS analysis





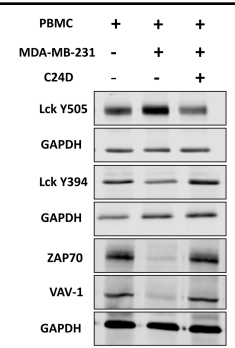
On binding to CD45, C2D reverses TNBC-induced immune suppression

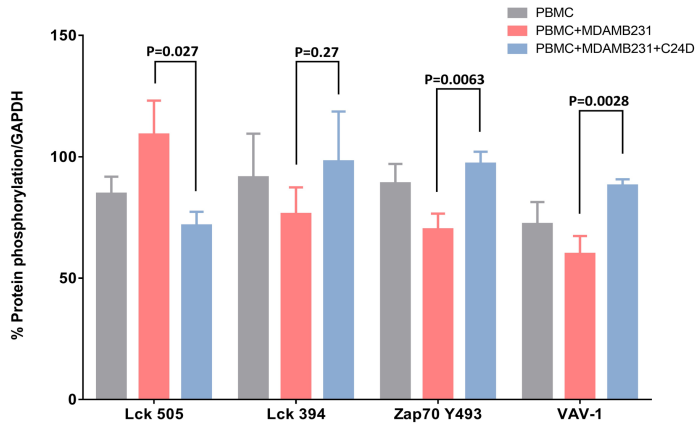


Treatment with C24D on PBMC/C24D co-cultures reverses tumor immune suppression.

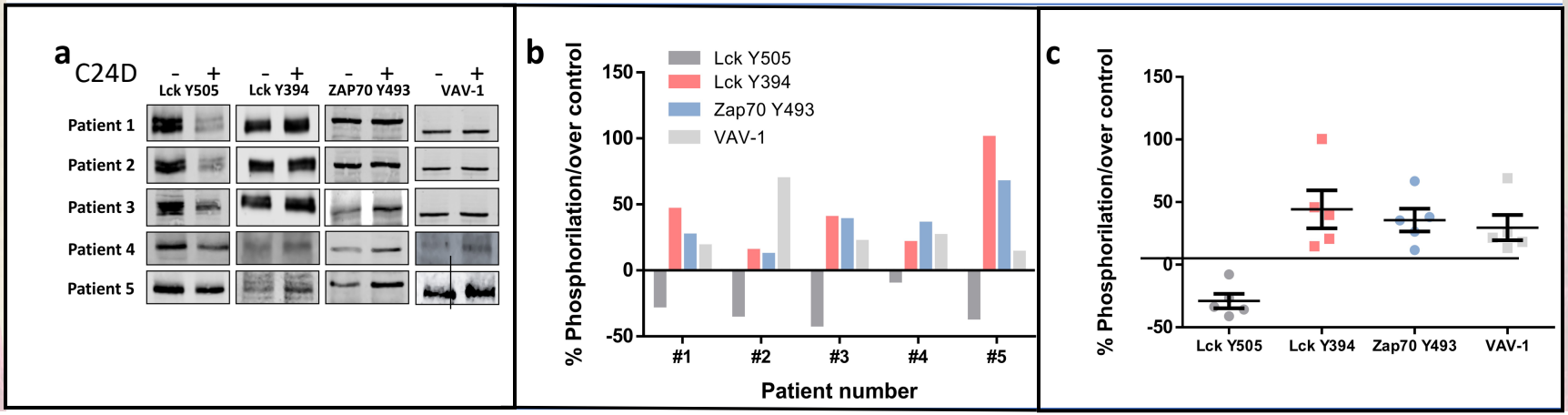
The result: Reactivation and massive immune-modulated tumor killing.

- De-phosphorylation of the Lck's inhibitory Tyr505
- Phosphorylation of Tyr394
- Phosphorylation of Tyr493 of ZAP-70
- Phosphorylation of Tyr 174 in VAV-1





C24D binding to CD45 reverses immunosuppression in PBMCs from TNBC patients



a. PBMCs from 5 metastatic TNBC patients were incubated with the C24D peptide for 5 to 60 minutes. Cells were lysed and subjected to SDS PAGE. Individual western blot analysis of lysed cells blotted with antibodies to detect the protein phosphorylation.

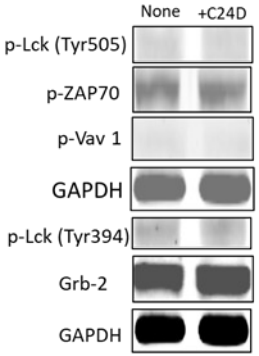
b. Statistical analysis, per TNBC patient, of western blots, depicting phosphorylation of proteins, normalized to control.

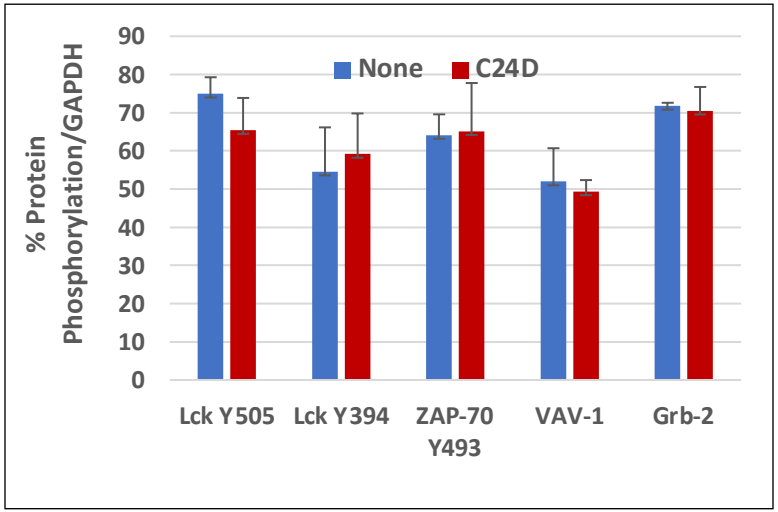
c. Mean (%) ± SD of phosphorylation of the Lck, ZAP-70 and VAV-1 proteins in the 5 TNBC patients, normalized to control.

C24D specificity - immune system re-activation is specific and occurs only in the presence of tumors

Specificity: Signal Transduction

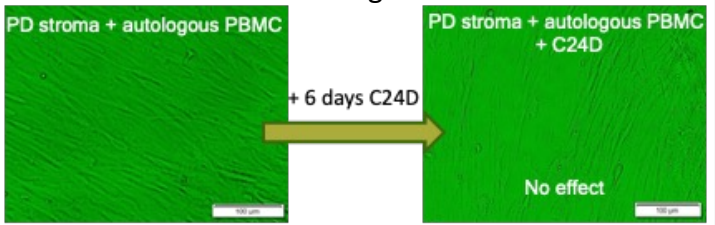
In the absence of tumors, C24D on PBMCs from healthy human donors does not induce TCR activation (N= 4 - 10)





Specificity: Cell Killing

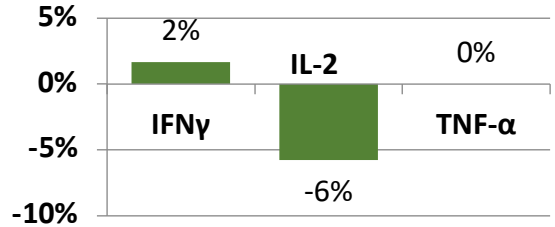
C24D has no effect on stroma of PDT in co-cultures with autologous PBMCs



PD = patient-derived

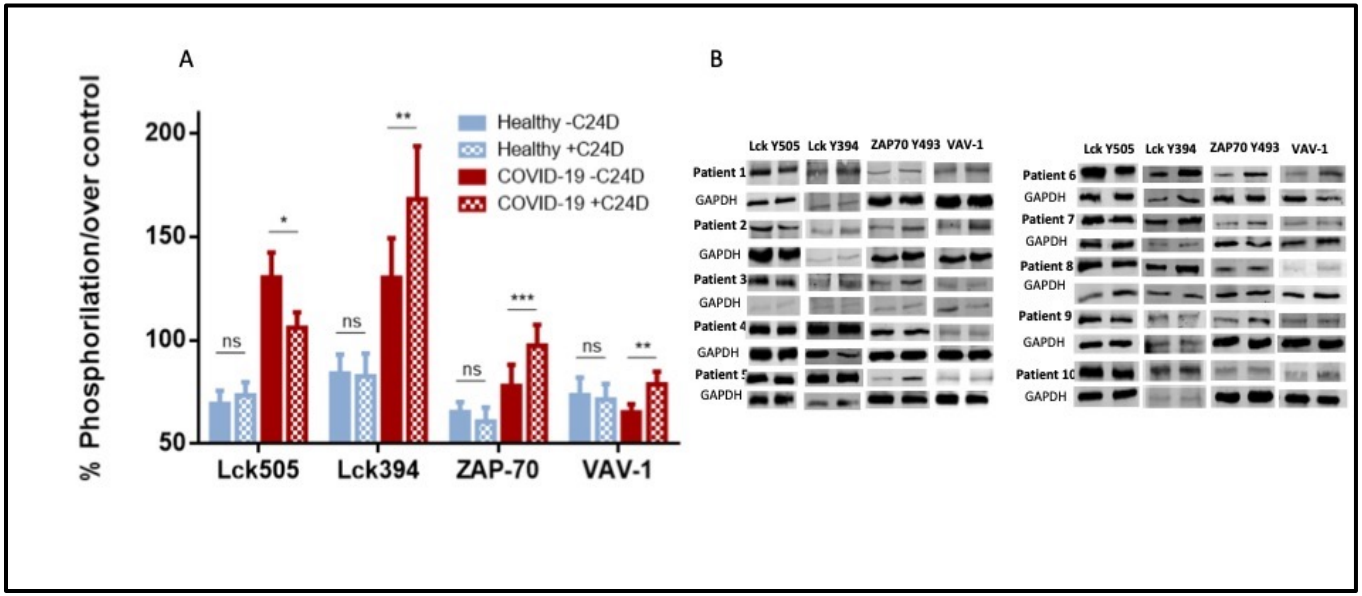
Cytokine secretion

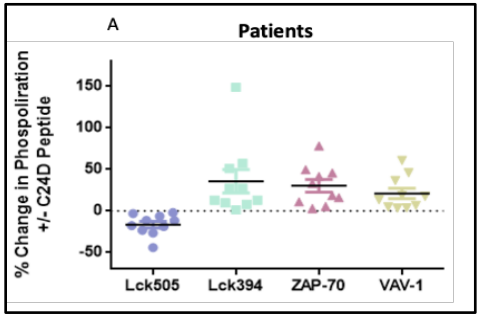
Human PBMCs seeded onto MCF-10A normal breast cells with the addition of C24D.

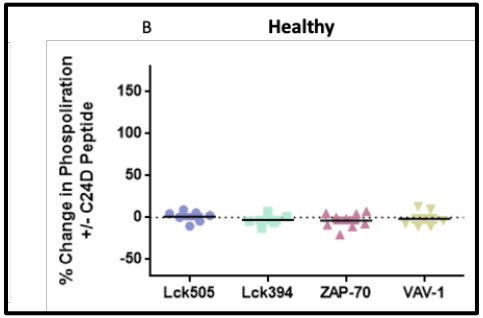


N = 2 Normalized to control without C24D

C24D reverses CD45 suppressive signals in PBMCs from COVID-19 patients

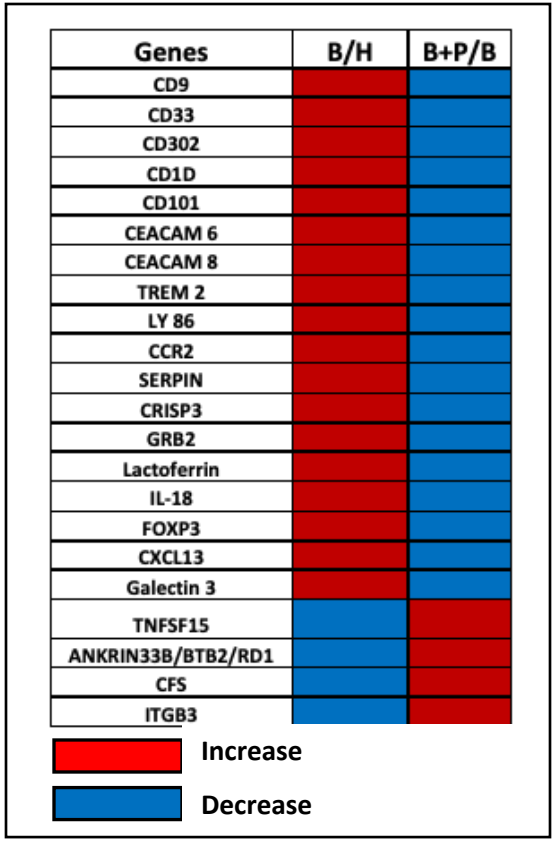






C24D reversed CD45 immune suppression signaling only in COVID-19 patients: (A). Percentage change of Lck, ZAP-70 and VAV-1 phosphorylation in PBMCs from each of the 10 patients, after addition of C24D for 5 to 60 minutes, normalized to control without C24D. (**B)** Percentage change of Lck, ZAP-70 and VAV-1 phosphorylation in PBMCs from healthy donors, after addition of C24D for at least 24 hours, normalized to control without C24D.

Deciphering the TNBC immunosuppression mechanism

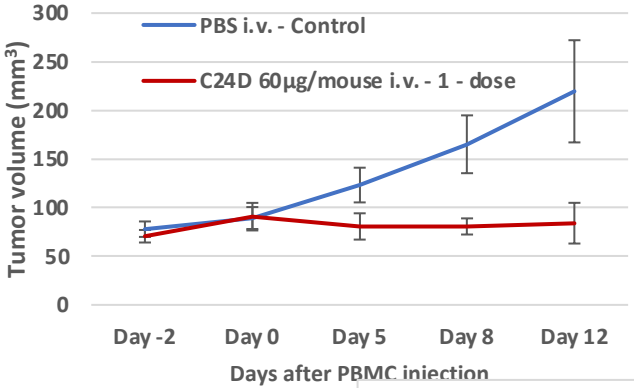


- We compared the transcriptome profiling of PBMCs from 4 metastatic TNBC patients with PBMCs from 4 healthy female volunteers, by RNA-seq.
- "Ingenuity Pathway Analysis" of the mRNA signatures of the 2 populations showed a significantly different expression of certain genes (**identified-genes**).
- We found that a number of the **identified-genes** related to exosomes – including tumor derived exosomes – were overexpressed in the PBMCs of TNBCs.
- Treatment with C24D of PBMCs from TNBC patients reverted the expression of the **identified-genes**.
- Some of the **identified-genes** are connected to the CD45 pathway**.**

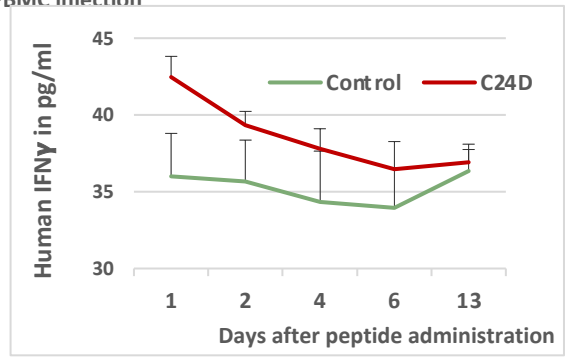
We submitted a new manuscript based on the TNBC mechanism of immunosuppression exerted by exosomes expressing Galectin 3 binding protein which form a complex with Galectin 3 through the CD45 receptor.

Preliminary studies: Pharmacokinetics

Nude mice engrafted subcutaneously with MDA-MB-231. After 2 weeks, mice were transfused intravenously with PBMCs and **one dose** of C24D (60μg/mouse).



Mice were bled and sacrificed at different times for determination of human INFγ in serum.



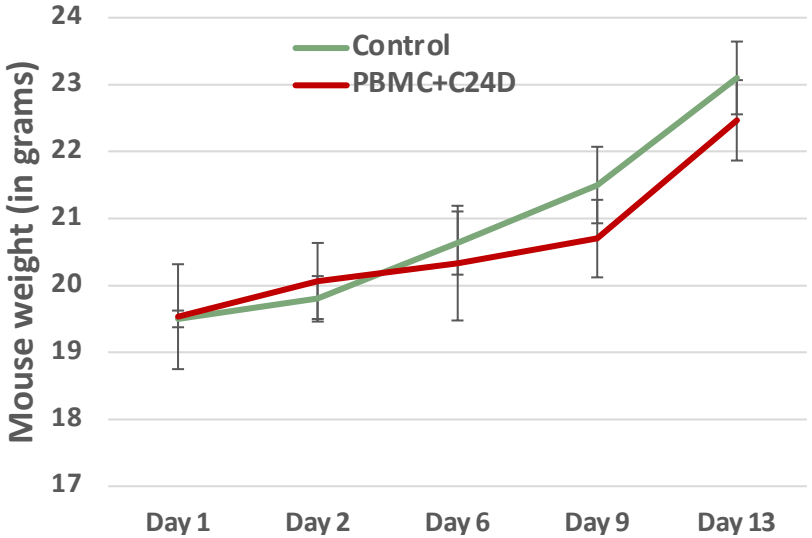
C24D has a clinically viable half-life

- The extra-cellular domain of human CD45 is very different from that of most species. C24D binds only to the human CD45 and not to the rodent protein.
- Therefore, results from traditional rodent PK and PD studies would have very little clinical significance.
- Nonetheless, in an attempt to get a first-order approximation of the stability of C24D, we ran a "Whole Blood Stability Study" via a CRO (Chempartner).
- The $T_{1/2}$ of C24D in human whole blood was 165.03 minutes (2¾ hours).

Test Article	Species		Percent Remaining (%)							$T_{1/2}$ (minute)
			0 min	5 min	15 min	30 min	45 min	60 min	120 min	
Procaine	human	Mean	100.00	1.34	BQL	BQL	BQL	BQL	BQL	**0.80**
		RSD of Area Ratio	0.02	0.02	N/A	N/A	N/A	N/A	N/A	
Procaine	mouse	Mean	100.00	56.84	16.14	2.17	BQL	BQL	BQL	**5.40**
		RSD of Area Ratio	0.01	0.01	0.02	0.13	N/A	N/A	N/A	
C24D	human	Mean	100.00	68.48	66.03	85.46	78.26	52.58	51.63	**165.03**
		RSD of Area Ratio	0.02	0.06	0.02	0.01	0.06	0.05	0.13	
C24D	mouse	Mean	100.00	92.00	90.23	90.51	91.63	96.28	87.86	**1,318.65**
		RSD of Area Ratio	0.02	0.02	0.01	0.01	0.00	0.01	0.05	

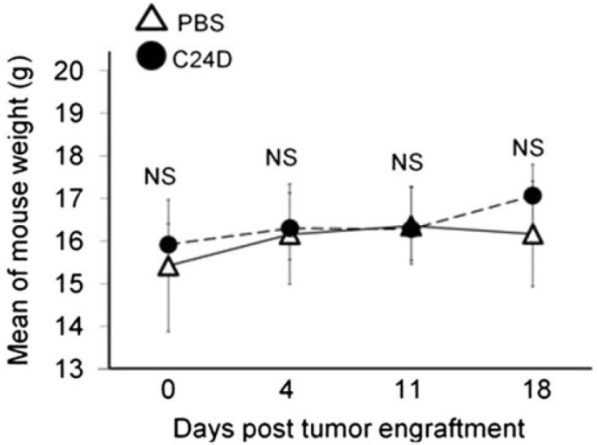
Preliminary studies: Toxicity

Body weight of mice did not change significantly after 4 IV doses of C24D



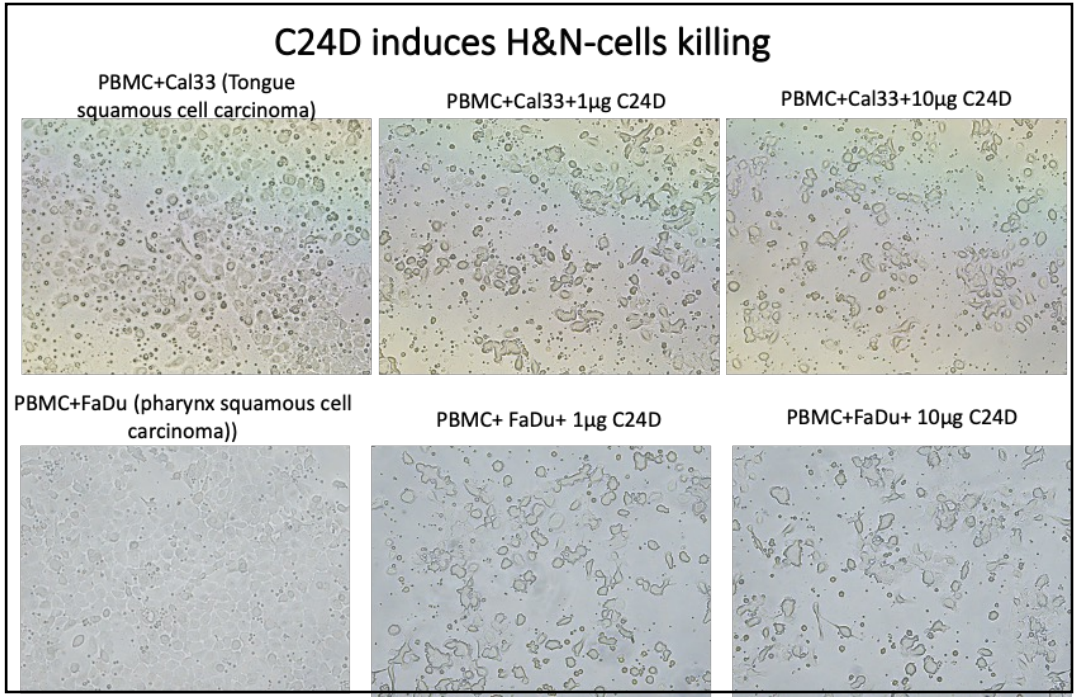
Nude mice engrafted subcutaneously with MDA-MB-231. After 2 weeks, mice were transfused intravenously with PBMCs and C24D (60μg/mouse) twice a week.

"C24D treatment did not affect the mouse weight compared to the PBS-treated control Group…"
Neoplasia, 16(9), 2014

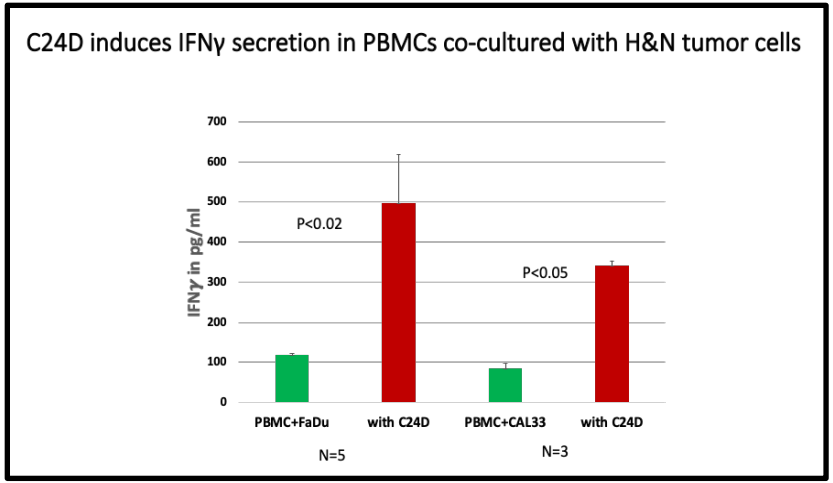


Nude mice were engrafted subcutaneously with MCF-7 tumor cells, transfused with human PBMCs, and treated by daily intraperitoneal injections of either C24D or PBS for 19 days.

Other Cancers: The effect of C24D on H&N tumors



Cal33 or FADU cells were co-cultured with PBMC from healthy female donors and treated with C24D (10µg/ml). Negative controls – minimal effect on cells treated with: PBMC alone. Massive killing with PBMC +MDA-MB-231 cells + 10µg of C24D. Figures depict cells 4 days after treatment.



Significant IFNγ secretion with PBMC + Cal33 or FADU tumor cells + 10µg of C24D for 4 days (measured by ELISA).

Competitive advantage of C24D over current immuno oncology strategies

Antibody Disadvantage	C24D Advantage
Blocking of checkpoints is unrelated to the presence or absence of tumors	Immune system re-activation only occurs in the presence of tumors
Not all patients benefit from checkpoint inhibitors	TNBC – an unmet medical need on which checkpoint inhibitor's effect had been demonstrated partially
Immune-based adverse effects are frequently observed.	C24D's specificity potentially averts many of the immune-related adverse events.
Given their long half-life, side effects cannot be easily combated	Reasonably short half-life. Rapid clearance. Peptides generally display low toxicity, fewer side effects and minimal drug-drug interaction.
Antibodies are difficult and expensive to produce	Easily and inexpensively produced
Combination therapy	MOA is complementary to that of many immune checkpoint and other cancer drugs

New peptides binding to a specific extracellular epitope on CD45

We are collaborating with Prof. Cyrille Cohen (Bar Ilan University), Prof. Rinat Yerushalmi (Davidoff Cancer Center, Rabin Medical Center) to increase the eficacy and specificity of peptides binding to the extracellular domain of CD45 protein.

Patent Status

Title	Country	Application Date	Status	Patent / Application #
PLIF MULTIMERIC PEPTIDES AND USES THEREOF	U.S.A.	March 10, 2013	Granted	9,334,308
– " –	Europe (Registered in Germany, France and UK)	March 10, 2013	Granted	13764023.1/2828280
– " –	U.S.A. / Continuation	March 10, 2013	Allowed	15/095,191
– " –	U.S.A. / Continuation	March 10, 2013	Filed	16/258,725
GENERATION OF CYTOTOXIC TUMOR SPECIFIC CELL LINES AND USES THEREOF	U.S.A.	February 3, 2014	Granted	10,000,737
– " –	U.S.A Divisional	February 3, 2014	Filed	16/010,560
– " –	Europe	February 3, 2014	Examination	14745808.7
TREATMENT OF DISEASES WITH MULTIMERIC PEPTIDES	U.S Provisional	February 21, 2019	Filed	62/808,307 and 62/808,319