Breast cancer induces a tolerogenic state of healthy activated CD4+ T-cells, characterized by reduced PI3K, NFκB, JAK-STAT, Notch, and increased TGFβ pathway activity.

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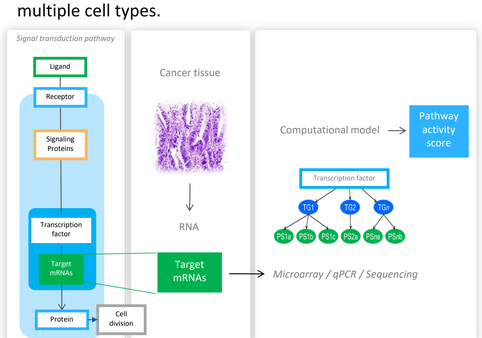
Introduction

Tumor cells can induce immunotolerance, which can be reversed by checkpoint blockade immunotherapy in some patients, although response prediction remains a challenge. CD4+ T cells play an important role in activating adaptive immune responses with their conversion to a suppressed state impairing anti-tumor immune responses. CD4+ T cells function by activating and controlling various signal transduction pathways.

A novel method for quantitative measurement of activity of signaling pathways, e.g., PI3K-FOXO, TGFβ, NFκB, JAK-STAT1/2, STAT3, and Notch signal transduction pathways, was *biologically validated to measure functional activity status of various immune cell types, and is here used to measure the immune suppressive effect of tumor cells on lymphocytes.*

Assays for quantitative measurement of signal transduction pathway activity in cancer and immune cells

Activity levels of different signal transduction pathways (for this study: PI3K-FOXO, NFκB, Notch, JAK-STAT1/2 type I interferon (IFN), JAK-STAT1/2 type II IFN, STAT3, and TGFβ) are assessed by measuring mRNA expression of downstream target genes of pathway transcription factors. Knowledge-based Bayesian models translate mRNA expression levels into a quantitative pathway activity score as described in Cancer Res 2014;74(11):2936-45. Pathway model-based assays are calibrated on one cell type-dataset, models are frozen, and assays are validated on independent datasets for multiple cell types.



Pathway analysis method references

1. Verhaegh and A. Van de Stolpe, *Oncotarget*, vol. 5, no. 14, pp. 5196–5197, Jul. 2014.
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3. H. van Ooijen *et al.*, *Am. J. Pathol.*, vol. 188, no. 9, pp. 1956–1972, Sep. 2018.
4. A. van de Stolpe *et al*, *Sci. Rep.*, vol. 9, no. 1, p. 1603, Feb. 2019.
5. A. van de Stolpe, *Cancers*, vol. 11, no. 3, p. 293, Mar. 2019.

Conclusion

A soluble factor(s) from breast tumor tissues increases TGFβ and reduces effector immune pathway activity in activated CD4+ T cells and thereby can induce an immunotolerant state. Investigation into the nature of this soluble factor(s) is in progress. These data demonstrate that signaling pathway assays can be used to quantitatively measure the functional state of immune responses in CD4+ T lymphocytes with potential applications for predicting and monitoring immunotherapy responses and identifying novel drug targets that can reverse tumor-induced immunosuppression.

Signaling pathway activity in immune cells

Signaling pathway analysis was performed on datasets from the GEO database

Different immune cell types isolated from peripheral blood of healthy volunteers, either untreated or cultured as indicated. PI3K-FOXO, NFκB, Notch, JAK-STAT1/2 type I interferon (IFN), JAK-STAT1/2 type II IFN, STAT3, and TGFβ signaling pathway analysis was performed on Affymetrix microarray data.

Indicated is the log2odds result of the pathway analyses, from blue to red indicates the range from inactive to active pathway.
Note: FOXO is the reverse of PI3K pathway activity (in the absence of oxidative stress, van Ooijen, Am.J.Pathol, 2018).

					STAT1/2			
Cell type	Replicate	FOXO	NFκB	Notch	IFN-II	IFN-I	STAT3	TGFβ
CD14+ monocytes	Replicate 1	9.0	2.1	-3.7	-5.4	-4.8	-8.6	-13.6
	Replicate 2	8.9	1.7	-2.8	-5.2	-4.5	-8.2	-12.6
	Replicate 3	8.6	0.8	-3.7	-5.4	-5.2	-8.2	-13.0
CD19+ B cells	Replicate 1	-0.5	0.3	-5.1	-5.5	-6.6	-4.4	-18.2
	Replicate 2	-0.8	-1.7	-6.5	-5.5	-6.7	-4.6	-18.3
	Replicate 3	-2.2	-0.2	-4.3	-5.0	-5.9	-5.2	-17.4
CD4+ T cells	Replicate 1	-2.8	-1.3	-3.5	-6.5	-5.8	-4.5	-13.5
	Replicate 2	-3.1	-0.6	-4.2	-6.1	-6.1	-5.0	-15.0
	Replicate 3	-0.8	-1.7	-4.6	-6.7	-6.5	-5.5	-15.2
CD56+ NK cells	Replicate 1	-1.8	-1.7	-7.1	-8.2	-6.9	-9.9	-11.7
	Replicate 2	-3.2	-1.3	-6.8	-7.6	-6.6	-9.0	-10.2
	Replicate 3	-2.1	-0.5	-6.9	-7.7	-6.4	-9.6	-11.4
CD8+ T cells	Replicate 1	-2.5	-3.9	-5.7	-7.4	-7.4	-8.6	-12.0
	Replicate 2	-1.7	-3.6	-4.9	-7.7	-8.1	-8.7	-12.0
	Replicate 3	-2.9	-3.3	-6.2	-8.0	-8.0	-9.9	-13.9
PMN	Replicate 1	18.1	9.6	-3.4	-4.5	-3.7	-1.8	-5.8
	Replicate 2	17.6	10.6	-5.8	-3.9	-3.6	0.9	-6.9
	Replicate 3	18.0	9.1	-3.6	-3.5	-3.5	0.0	-5.9

Conclusion: Pathway activity results are similar between different healthy individuals, but differ between immune cell types.

N=3 healthy individuals.
Study reference: Du et al. Genomics 2006 Jun;87(6):693-703

					STAT1/2			
Cell type	State	FOXO	NFκB	Notch	IFN-II	IFN-I	STAT3	TGFβ
NK cells	Resting	7.8	8.5	0.3	-6.3	-4.6	2.3	-2.9
	Stimulated with IL-2 (2 hrs)	1.5	2.5	-1.6	-6.7	-7.0	0.3	-1.0
	Stimulated with IL-2 (8 hrs)	-6.8	-0.4	4.5	-6.7	-8.5	-2.5	-8.4
	Stimulated with IL-2 (24 hrs)	-4.9	7.6	-5.1	-7.0	-8.4	5.4	-7.1

Conclusion: IL2 withdrawal in Natural Killer (NK), PBMCs and T-cells (lymphoblasts) results in lower PI3K, STAT3 and NFkB pathway activity.
In PBMCs IL2 withdrawal also results in lower STAT1/2 interferon type II and slightly higher TGFβ pathway activity.

					STAT1/2			
Cell type	State	FOXO	NFκB	Notch	IFN-II	IFN-I	STAT3	TGFβ
ALPS 1A PBMCs	0h after IL-2 withdrawal	-4.4	4.8	-3.2	-5.9	-9.3	-1.6	-21.0
	24h after IL-2 withdrawal	6.3	0.5	-3.3	-8.7	-8.8	-13.2	-17.2
Control PBMCs	0h after IL-2 withdrawal, biological replicate 1	-2.1	6.9	-2.9	-3.3	-7.5	3.3	-16.1
	24h after IL-2 withdrawal, biological replicate 1	7.9	0.3	-3.1	-7.3	-8.1	-10.5	-15.6
Control PBMCs	0h after IL-2 withdrawal, biological replicate 2	-4.0	3.2	-3.1	-5.7	-9.0	-0.6	-18.4
	24h after IL-2 withdrawal, biological replicate 2	3.7	0.6	-2.5	-8.1	-8.3	-11.5	-13.4
PS8 PBMCs	0h after IL-2 withdrawal	-5.6	2.2	-3.0	-5.5	-8.7	0.6	-16.3
	24h after IL-2 withdrawal	3.1	-0.3	-4.5	-5.5	-7.1	-11.8	-14.5

Study references:

- GSE8059, Blood-derived NK cells were cultured with IL2 at 100 IU/ml for 0, 2, 8 and 24 hours. REF: Dybkaer K, *et al. BMC Genomics* 2007 Jul 10;8:230.
- GSE7345, Blood-derived T cells (PBMCs) were cultured in 100 units/ml IL-2 for at least 6 days, and analyzed at 0 and 24 h after IL-2 withdrawal. REF: Oliveira JB, *et al. Proc Natl Acad Sci U S A*2007 May 22;104(21):8953-8.
- GSE 13909, primary lymphoblasts in IL-2-containing culture and 8-hrs after IL-2 withdrawal. REF: Chechlinska M, *et al. BMC Genomics* 2009 Jun 8;10:261

					STAT1/2			
Cell type	State	FOXO	NFκB	Notch	IFN-II	IFN-I	STAT3	TGFβ
T-Lymphocytes	IL-2-containing cultures, biological replicate 1	1.7	1.5	-4.6	-6.9	-7.7	3.6	-17.1
	After IL-2 withdrawal, biological replicate 1	6.5	-0.4	-5.2	-7.4	-8.1	-7.5	-18.2
	IL-2-containing cultures, biological replicate 2	-1.8	2.4	-3.9	-1.0	-3.1	3.9	-19.5
	After IL-2 withdrawal, biological replicate 2	3.6	-0.1	-4.4	-3.6	-7.5	-10.7	-20.3

Breast cancer tissue supernatant reduced PI3K, NFκB, JAK-STAT, and increased TGFβ pathway activity in CD4+ T-cells from healthy individuals.

Breast cancer tissue sections from fresh surgical specimens of primary untreated breast cancers (n=4) were mechanically dissociated in X-VIVO 20. Following activation with anti-CD3/CD28, CD4+ T cells from healthy donor blood were incubated with primary tumor supernatants and compared to controls. PI3K-FOXO, NFκB, Notch, JAK-STAT1/2 type I interferon (IFN), JAK-STAT1/2 type II IFN, STAT3, and TGFβ signaling pathway activities were measured in individual samples. *Study reference: J Clin Invest 2013;123(7):2873-92*, *GEO* dataset GSE36766).

Indicated is the log2odds result of the pathway analyses, from blue to red indicates the range from inactive to active.
Note: FOXO is the reverse of PI3K pathway activity (in the absence of oxidative stress).

						STAT1/2			
			FOXO	NFκB	Notch	IFN-II	IFN-I	STAT3	TGFβ
Memory CD4 T cells from healthy donor	Not activated	Control	-2.7	-10.9	-7.5	-7.7	-7.4	-5.5	-14.8
			-2.4	-10.8	-7.3	-8.2	-7.6	-5.4	-14.3
			-3.2	-11.1	-7.1	-8.2	-7.4	-6.3	-14.8
		+ cancer tissue supernatant	-1.7	-9.2	-6.8	-7.7	-7.1	-4.5	-11.7
			-2.0	-11.2	-7.2	-8.1	-7.4	-6.2	-13.2
			-2.6	-9.1	-7.4	-7.7	-7.3	-7.8	-10.1
			-2.4	-11.5	-7.8	-7.8	-7.2	-7.7	-10.3
	Activated (antiCD3/antiCD28)	Control	-7.5	3.5	-4.2	-5.0	-5.5	10.9	-15.8
			-7.2	2.3	-4.3	-4.7	-5.2	9.9	-16.4
			-7.5	3.1	-4.2	-4.8	-5.3	10.2	-17.0
		+ cancer tissue supernatant	3.5	-4.4	-7.3	-4.8	-6.0	5.7	-6.5
			5.9	-5.9	-5.8	-8.2	-7.6	4.0	-3.8
			9.1	-10.0	-4.8	-8.6	-8.0	2.6	-1.4
			8.5	-10.0	-4.3	-8.5	-7.7	2.9	-1.4

Conclusion: CD4+ T cell activation resulted in activation of PI3K, NFkB, JAK-STAT1/2, JAK-STAT3, Notch, and slight decrease in TGFβ pathway activities. Incubation with primary tumor supernatants did not affect pathway activity in non-activated CD4+ T cells, but reduced activity of PI3K, NFκB, JAK-STAT1/2, JAK-STAT3, while increasing activity of the immune suppressive TGFβ pathway in activated CD4+ T cells. A soluble factor is responsible for this immuno-suppressive effect on CD4+ T cells.