Supplementary material

Title: Primary 4T1 tumor resection provides critical “window of opportunity” for immunotherapy

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Authors: Anahit Ghochikyan, Arpine Davtyan, Armine Hovakimyan, Hayk Davtyan, Anna Poghosyan, Alexander Bagaev, Ravshan I Ataullakhanov, Edward L. Nelson, Michael G. Agadjanyan.

Corresponding Authors:

Michael G. Agadjanyan,
The Institute for Molecular Medicine,
16371 Gothard Street, Suite H,
Huntington Beach,
CA 92647, US.
Tel: 714-596-7821.
Fax: 714-596-3791.
E-mail: magadjanyan@immed.org.

Anahit Ghochikyan,
The Institute for Molecular Medicine,
16371 Gothard Street, Suite H,
Huntington Beach,
CA 92647, US.
Tel: 714-596-3981.
Fax: 714-596-3791.
E-mail: aghochikyan@immed.org.

Supplementary Table 1. a) The absolute numbers of total splenocytes, MDSC, Treg cells in spleens and b) the absolute numbers of pulmonary mononuclear cells, MDSC and Treg cells in lungs of mice bearing tumors of different sizes.

a)

Absolute number of Suppressor cells in spleen (x10^3)	Tumor-free mice	Tumor-bearing mice		
		2-3 mm	3.5-5mm	10-15 mm
MDSC	779±4	1141±235^{ns}	4529±994*	41916±1861**
Tregs	656±26.7	1397±105***	1343±258*	912±54*
Absolute number of of splenocytes	30000±1000	54600±4600	56000±11049	99375±4204

b)

Absolute number of Suppressor cells in lungs (x10^3)	Tumor-free mice	Tumor-bearing mice		
		2-3 mm	3.5-5mm	10-15 mm
MDSC	11±3.7	37±7.3*	92±16**	1477±149***
Tregs	0.7±0.1	0.54±0.14^{ns}	1.1±0.36^{ns}	2.34±0.62*
Absolute number of PMC in lungs	614±127	672±76.7	654±61.3	2266±207

*Statistical significance is calculated against group of **tumor-free** mice using two-tailed t-test (*P≤0.05, **P≤0.01, ***P≤ 0.001)*

Supplementary Table 2. The absolute numbers of total and activated $CD4^+$ and $CD8^+$ T cells in a) spleens and b) lungs of mice bearing tumors of different sizes.

a)

Absolute number of cells in spleen ($x10^3$)	**Tumor-free**	**Tumor-bearing mice**		
		2-3 mm	**3.5-5mm**	**10-15 mm**
$CD4^+$	7345±95	13426±1127**	$14321±2854^{ns}$	9614±558*
$CD69^+CD4^+$	246±21.5	438±48*	$242±43.6^{ns}$	$198±18^{ns}$
$CD8^+$	4655±305	6465±541*	$7059±1344^{ns}$	3379±227**
$CD69^+CD8^+$	137±38.5	$150±14^{ns}$	$143±36^{ns}$	72±9***

b)

Absolute number of cells in lungs ($x10^3$)	**Tumor-free mice**	**Tumor-bearing mice**		
		2-3 mm	**3.5-5mm**	**10-15 mm**
$CD4^+$	35±4.8	18±2.9**	$30±4.6^{ns}$	$30±3.2^{ns}$
$CD69^+CD4^+$	0.52±0.16	$0.32±0.21^{ns}$	$1.2±0.29^{ns}$	$0.43±0.054^{ns}$
$CD8^+$	11±1.9	$6.0±1.3^{ns}$	$7.3±1.4^{ns}$	$7.9±1.0^{ns}$
$CD69^+CD8^+$	0.14±0.05	$0.2±0.1^{ns}$	$0.47±0.15^{ns}$	$0.27±0.068^{ns}$

*Statistical significance is calculated against group of **tumor-free** mice using two-tailed t-test (*P≤0.05, **P≤0.01, ***P≤ 0.001)*

Supplementary Table 3. The absolute numbers of splenocytes, suppressor cells and total, as well as, activated $CD4^+$ and $CD8^+$ T cells in spleens of mice at indicated days after surgical removal of tumor.

Absolute number of cells in spleen ($x10^3$)	**Tumor - free mice**	**Days Post-surgery**						
		0	**2**	**4**	**6**	**13**	**17-20**	**30-33**
MDSC	779±4	4529±994*	2274± 361*	1070 ±89*	781 ±170^{ns}	ND	ND	21890±10626 ^{ns}
Treg	656±26.7	1343±258*	642±46 ^{ns}	726±60 ^{ns}	539±106 ^{ns}	ND	ND	1594±373 ^{ns}
$CD4^+$	7345±95	14321±2854 ^{ns}	8063±592^{ns}	6694±532 ^{ns}	5354±872 ^{ns}	ND	ND	15661±2432*
$CD69^+CD4^+$	246±21.5	242±43 ^{ns}	252±13.9 ^{ns}	243±24.5 ^{ns}	206±34 ^{ns}	ND	ND	397±81 ^{ns}
$CD8^+$	4655±305	7059±1344^{ns}	4275±384^{ns}	3624±323^{ns}	2875±437*	ND	ND	7503±1123^{ns}
$CD69^+CD8^+$	137±38.5	143±36 ^{ns}	103±9.5 ^{ns}	94.9±12.2 ^{ns}	81.4±12.2 ^{ns}	ND	ND	139±34.3 ^{ns}
Total	30000±10	56000±11049	26125±2263	28100±3104	19166±3600	ND	ND	95888±27293

*Statistical significance was calculated against group of **tumor-free** mice using two-tailed t-test (*P≤0.05, **P≤ 0.01, ***P≤ 0.001)*

Supplementary Table 4. The frequency of suppressor cells in spleens of mice at indicated days after surgical removal of tumor.

(%) of suppressor cells in spleen	Tumor - free mice	Days Post-surgery						
		0	2	4	6	13	17-20	30-33
MDSC	2.4±0.17	8.3±0.38***	7.2 ±0.63 ***	5.4± 0.91 ***	3.5±0.26***	5.5±0.5***	12±2.3***	15±4***
Treg	9.5±0.3	9.5±0.23^{ns}	8.5±0.4 ^{ns}	10.1±0.42 ^{ns}	9.8±1.4 ^{ns}	8.7±0.3 ^{ns}	9.9±0.37 ^{ns}	9.5±0.82 ^{ns}

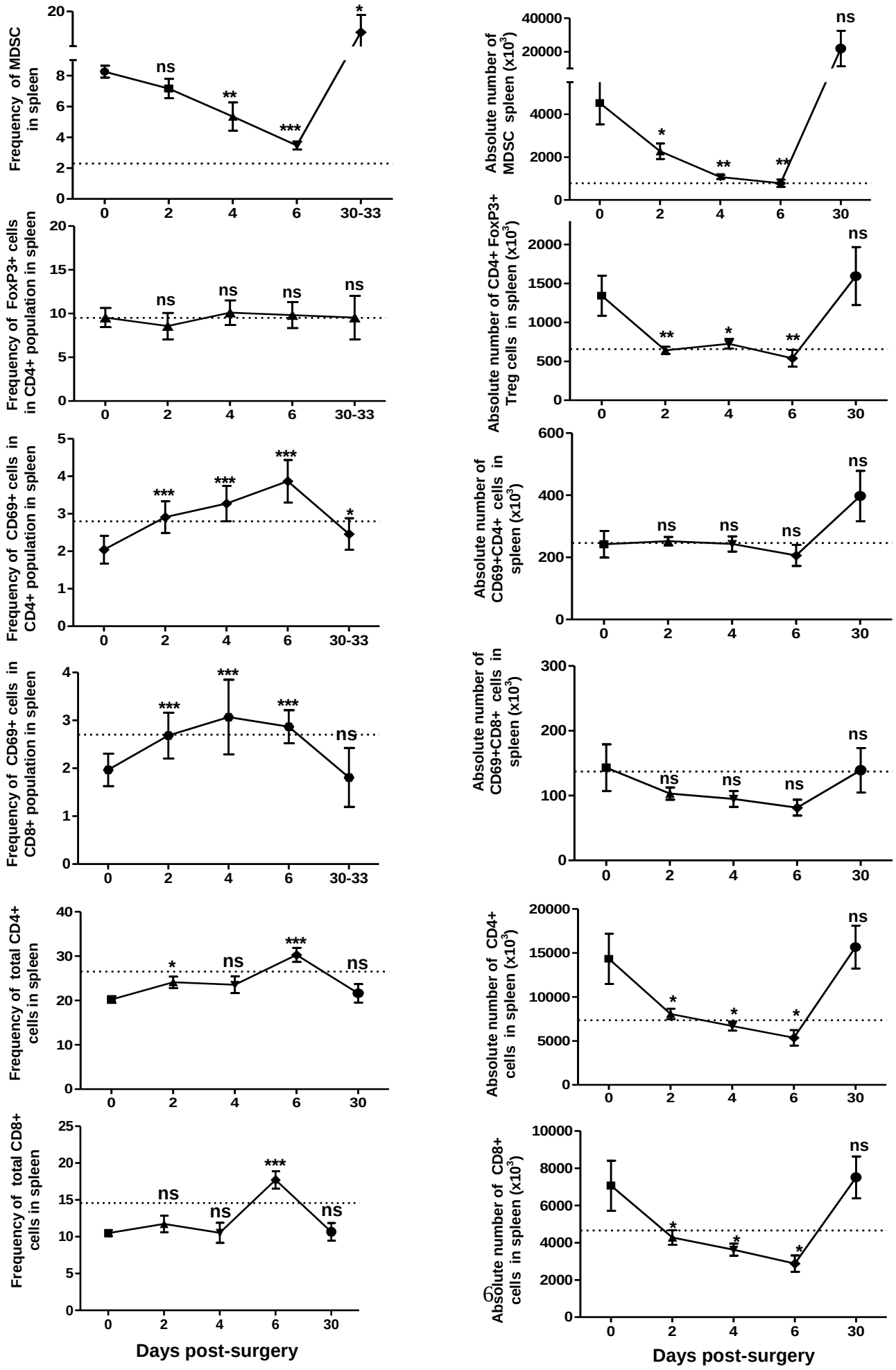
*Statistical significance was calculated against group of **tumor-free** mice using two-tailed t-test (*P≤0.05, **P≤ 0.01, ***P≤ 0.001).*

Supplementary Table *5*. The percentage of activated $CD4^+$ and $CD8^+$ T cells in spleens of mice at indicated days after surgical removal of tumor.

(%) of activated effector cells in spleen	Tumor free mice	Days Post-surgery						
		0	2	4	6	13	17-20	30-33
CD4+	2.9±0.15	2.0±0.085***	2.9±0.11 ^{ns}	3.3±0.14 ^{ns}	3.9±0.23**	3.9±0.13***	3.6±0.25*	2.5±0.14 ^{ns}
CD8+	2.6±0.13	2.0±0.078***	2.7±0.13 ^{ns}	3.1±0.23**	2.9±0.14 ^{ns}	3.0±0.098 ^{ns}	2.5±0.11 ^{ns}	1.8±0.2**

*Statistical significance was calculated against group of **tumor-free** mice using two-tailed t-test (*P≤0.05, **P≤ 0.01, ***P≤0.001).*

Supplementary Figure 1



Supplementary Fig.1 Longitudinal modulations in frequency and absolute numbers of suppressor and effector cell subsets in spleens of mice after surgical removal of primary tumor. Statistical significance was calculated against day 0 (day of surgery) using two-tailed t-test (**P≤0.05, **P≤ 0.01, ***P≤ 0.001, ns - non-significant*). The mean level of indicated cells in spleens of tumor-free mice is shown in dotted line.

Supplimentary Materials and Methods

Detialed mathematical analysis

Our experimental data showed that after the surgical resection of primary 4T1 tumor the percent of MDSC in splenocytes were significantly decreased while the percent of activated $CD69^+CD4^+$ T cells and $CD69^+CD8^+$ T cells in $CD4^+$ and $CD8^+$ cell populations, respectively, were significantly increased. Measurements of the percentage of these cell types were performed on days 0, 2, 4, 6, 13, 17-20 and 30-33. Day 4, 6 and 13 percents of MDSC were significantly lower than those of day 0 ($p<0.05$). Based on these experimental data, one can conclude that the time interval from day 4 until day 13 represents a “window” wherein the MDSC counts are statistically (significantly) lowered. Similarly, experimental data concerning increase in frequency of activated T cells showed that $CD69^+$ counts were significantly increased between day 2 and day 30-33 for $CD4^+$, and between day 2 and day 17-20 for $CD8^+$ T cells. The overlay of the three considered parameters, i.e. MDSC, $CD69^+CD4^+$ and $CD69^+CD8^+$, reveals the common “window of opportunity” between day 4 and day 13 after resection, actually restricted by the time-frame of significantly lowered MDSC.

To define exact borders of the time interval wherein the MDSC counts are significantly lowered and the activated T cell counts are significantly elevated, our experimental data were

mathematically analyzed. Experimental data in the region of interest (from day 0 to day 30-33) were approximated by polynomial function in the form: $\sum_{i=0}^{N} a_i x^i$

where coefficients were defined using the method of Ordinary Least Squares with weights estimated as follows: $weigths = \frac{1}{SD^2}$

SD –standard deviation of experimental data. A basis up to the third order was used (N=3).

Results of approximation of the function. For parameters search Mathematica 8.0 (Wolfram reseach, USA) and standard *LinearModelFit* function were used.

Function form $a_0 + a_1 x \quad + a_2 x^2 + a_3 x^3$

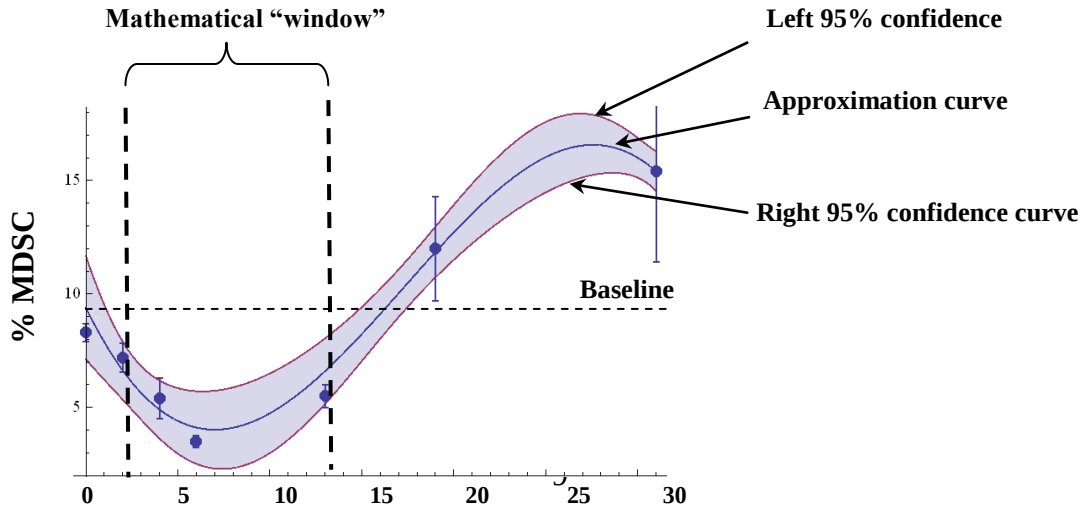
Optimization of the function using the form:

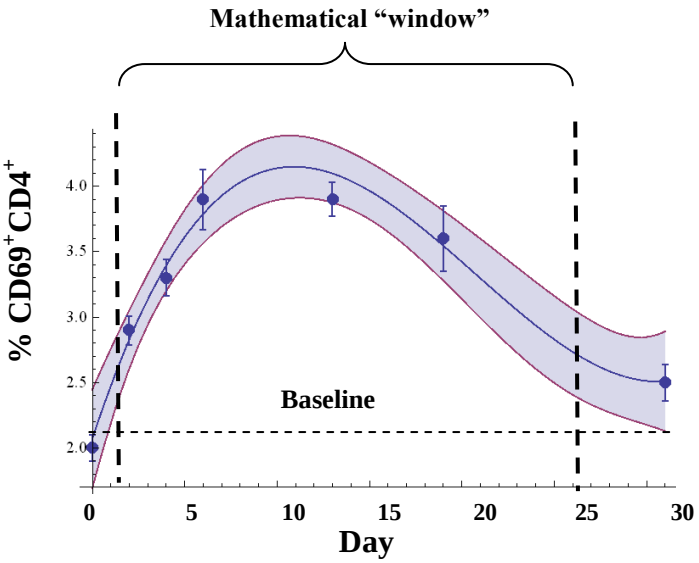
$$\sum_{i}^{N} \left[\sum_{k=0}^{3} a_k \left(t^i\right)^k - Data(t^i) \right]^2 \frac{1}{SD^2(t^i)}$$

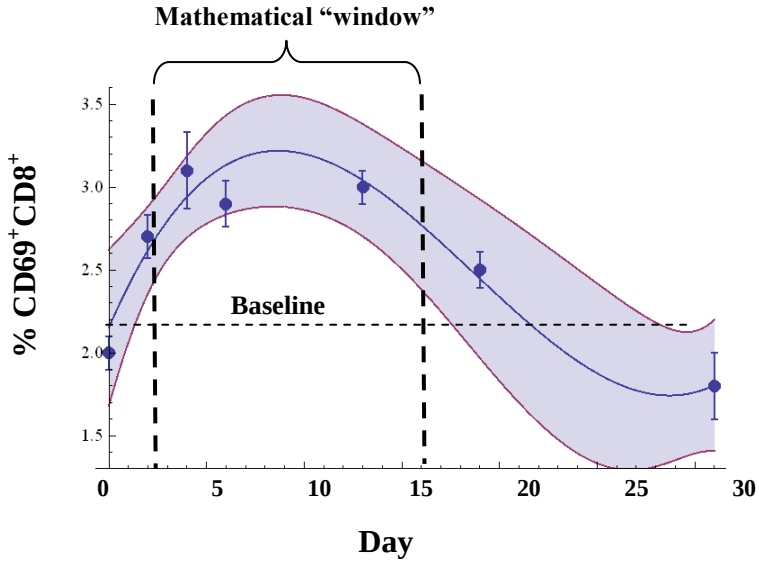
Data		***Best fit parameters***	***Parameters error***
% MDSC	a_0	9.405	0.968
	a_1	-1.677	0.368
	a_2	0.149	0.0278
	a_3	-0.00288	0.000549
% $CD69^+CD4^+$	a_0	2.0678	0.161
	a_1	0.4325	0.0549
	a_2	-0.0268	0.0043
	a_3	0.000431	0.000088
% $CD69^+CD8^+$	a_0	2.1512	0.2002
	a_1	0.2737	0.0737
	a_2	-0.02055	0.000366
	a_3	0.000366	0.00013

The time interval wherein the function is significantly decreased or increased (mathematical analysis based on approximation). Approximation of the function was made according to the form in above, and the curve of approximated function was obtained using Mathematica 8.0 plugins (Wolfram reseach, USA). The day 0 value of the approximated function was taken as a theoretical baseline. The 95% confidence interval of the approximated fuction and its errors were drawn as grey bands. Then the X-axis (days) positions for the beginning and ending of the bend of the approximated function were found as the earliest or latest points wherein the Y-axis values (% MDSC or % $CD69^+CD4^+$, or % $CD69^+CD8^+$) differed from the theoretical baseline value with the 95% confidence. The beginning and ending of the bend is marked with the vertical dashed lines specifying the theoretical borders of the "window" (see Table and Figures below).

The function	***The extreme value (position on X-axis, day)***	***The function decrease or increase interval (95% confidence)***
% MDSC data	7.0	(2.4 - 13.3)
% $CD69^+$ $CD4^+$	10.9	(1.8 - 26.1)
% $CD69^+$ $CD8^+$	8.6	(2.1 - 16.8)
Global overlap region	-	(2.4 - 13.3)




Mathematical “window”
% CD69+CD4+
4.0
3.5
3.0
2.5
2.0
Baseline
0
5
10
15
20
25
30
Day


Mathematical “window”
% CD69+CD8+
3.5
3.0
2.5
2.0
1.5
Baseline
0
5
10
15
20
25
30
Day