Supplementary Information for

Change and predictive ability of circulating immunoregulatory lymphocytes in long-term outcomes of acute ischemic stroke

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Supplementary Tables

Supplementary Table 1. Age and gender differences between patients and controls

	Patients (n=210)	Controls (n=87)	P value
Age, years, mean ± SD	66.86 ± 14.48	64.24 ± 14.21	0.3010
Sex, Male, n (%)	131 (62.38%)	50 (57.47%)	0.4362

Mann Whitney test was employed to examine the differences in age and sex between controls and patients. SD: standard deviation.

Supplementary Table 2. Linear regression of lymphocytes vs. admission neurological assessments.

(/Lymphocyte, %)	Infarct volume (n=185)		NIHSS (n=210)		mRS (n=210)	
	Spearman r	P Value	Spearman r	P Value	Spearman r	P Value
Admission						
B cells	0.10	0.18	-0.01	0.87	-0.04	0.55
Bregs	0.09	0.23	-0.03	0.62	-0.07	0.32
T cells	0.08	0.30	-0.04	0.54	-0.09	0.19
CD4$^+$ T cells	-0.01	0.90	-0.09	0.21	-0.06	0.38
CD4$^+$ Tregs	-0.02	0.84	-0.21	0.002	-0.19	0.01
CD8$^+$ T cells	-0.02	0.82	-0.04	0.58	0.00	1.00
CD8$^+$ Tregs	0.02	0.82	0.02	0.79	0.11	0.10
DNTs	0.16	0.03	0.11	0.11	-0.04	0.58
Discharge						
B cells	0.03	0.76	-0.05	0.62	-0.18	0.07
Bregs	0.16	0.13	0.059	0.56	-0.13	0.20
T cells	-0.02	0.83	0.10	0.31	0.10	0.35
CD4$^+$ T cells	-0.11	0.29	0.18	0.08	0.13	0.21
CD4$^+$ Tregs	-0.18	0.09	0.03	0.74	0.001	0.99
CD8$^+$ T cells	-0.09	0.42	-0.02	0.82	0.03	0.80
CD8$^+$ Tregs	-0.05	0.65	-0.04	0.66	0.07	0.48
DNTs	0.13	0.23	0.03	0.80	-0.07	0.51

1-month						
B cells	-0.04	0.79	-0.29	0.04	-0.14	0.34
Bregs	0.10	0.53	-0.004	0.98	-0.09	0.53
T cells	0.11	0.47	0.03	0.86	-0.08	0.60
CD4$^+$ T cells	0.11	0.46	0.17	0.23	-0.03	0.82
CD4$^+$ Tregs	0.17	0.26	0.18	0.22	-0.05	0.71
CD8$^+$ T cells	0.02	0.91	-0.05	0.71	0.01	0.95
CD8$^+$ Tregs	-0.01	0.95	0.22	0.13	0.31	0.03
DNTs	0.10	0.54	-0.05	0.74	-0.05	0.72
3-months						
B cells	0.19	0.14	0.15	0.24	-0.01	0.91
Bregs	0.11	0.40	0.17	0.17	0.11	0.36
T cells	-0.25	0.06	-0.02	0.88	0.01	0.97
CD4$^+$ T cells	-0.06	0.64	0.01	0.91	-0.09	0.49
CD4$^+$ Tregs	-0.14	0.27	-0.11	0.37	-0.14	0.25
CD8$^+$ T cells	-0.15	0.25	0.02	0.86	0.10	0.43
CD8$^+$ Tregs	0.06	0.66	-0.02	0.89	0.12	0.32
DNTs	-0.06	0.67	0.01	0.93	0.09	0.46

Data were analyzed using Spearman correlation analysis. NIHSS: National Institutes of Health Stroke Scale; mRS: modified Rankin Scale.

Supplementary Table 3. Linear regression of lymphocytes vs. discharge and 3 months neurofunction.

	Discharge (n=210)				3 months (n=210)			
	NIHSS		mRS		NIHSS		mRS	
(/Lymphocyte, %)	Spearman r	P Value	Spearman r	P Value	Spearman r	P Value	Spearman r	P Value
Admission								
B cells	-0.04	0.57	-0.01	0.86	0.03	0.66	-0.14	0.04
Bregs	-0.07	0.34	0.001	0.99	0.03	0.64	0.04	0.60
T cells	-0.19	0.006	-0.06	0.36	-0.01	0.91	0.12	0.08
CD4$^+$ T cells	-0.17	0.01	-0.12	0.08	-0.01	0.92	0.12	0.09
CD4$^+$ Tregs	-0.23	0.001	-0.23	0.001	-0.03	0.65	0.14	0.04
CD8$^+$ T cells	-0.07	0.29	0.03	0.68	-0.04	0.52	0.08	0.23
CD8$^+$ Tregs	0.07	0.34	0.18	0.01	0.02	0.72	0.07	0.33
DNTs	0.02	0.73	-0.01	0.93	-0.03	0.66	0.01	0.91
Discharge								

B cells	-0.09	0.40	-0.10	0.34	-0.09	0.36	-0.12	0.22
Bregs	0.06	0.56	0.003	0.98	0.05	0.63	0.18	0.07
T cells	0.12	0.24	0.001	1.00	-0.04	0.72	0.03	0.73
CD4$^+$ T cells	0.07	0.50	-0.05	0.61	-0.01	0.96	-0.06	0.56
CD4$^+$ Tregs	-0.04	0.70	-0.19	0.06	0.00	0.98	-0.02	0.86
CD8$^+$ T cells	-0.03	0.74	0.14	0.17	-0.007	0.94	0.13	0.20
CD8$^+$ Tregs	0.04	0.72	0.04	0.73	-0.07	0.52	0.02	0.83
DNTs	0.12	0.24	-0.15	0.13	-0.15	0.14	0.07	0.49
1-month								
B cells	-0.12	0.40	-0.01	0.96	-0.17	0.24	-0.05	0.76
Bregs	0.17	0.24	0.16	0.28	-0.05	0.71	0.07	0.64
T cells	-0.10	0.49	0.014	0.93	-0.19	0.18	-0.37	0.01
CD4$^+$ T cells	0.09	0.52	0.12	0.43	0.07	0.62	-0.24	0.10
CD4$^+$ Tregs	0.08	0.61	0.09	0.53	0.10	0.50	-0.08	0.56
CD8$^+$ T cells	-0.06	0.66	0.06	0.70	-0.19	0.19	-0.21	0.15
CD8$^+$ Tregs	0.10	0.51	0.17	0.25	-0.18	0.20	-0.05	0.72
DNTs	-0.08	0.58	-0.16	0.29	0.002	0.99	0.11	0.46
3-months								
B cells	0.001	0.99	-0.05	0.69	0.25	0.04	-0.05	0.67
Bregs	0.16	0.21	0.08	0.53	0.10	0.43	-0.09	0.49
T cells	0.04	0.76	0.12	0.33	-0.24	0.05	-0.05	0.71
CD4$^+$ T cells	0.04	0.76	0.04	0.75	0.02	0.87	0.07	0.57
CD4$^+$ Tregs	-0.04	0.77	-0.02	0.87	0.02	0.86	-0.02	0.86
CD8$^+$ T cells	0.10	0.43	0.10	0.42	-0.15	0.24	-0.06	0.61
CD8$^+$ Tregs	0.15	0.24	0.005	0.97	-0.14	0.27	0.11	0.40
DNTs	0.01	0.93	0.01	0.96	-0.15	0.23	-0.006	0.96

Data were analyzed using Spearman correlation analysis. NIHSS: National Institutes of Health Stroke Scale; mRS: modified Rankin Scale.

Supplementary Table 4. Relationships between admission regulatory lymphocytes and clinical risks.

	Mann-Whitney U test			Lineal regression		
	Group		p value	vs.	Spearman r	p value
	male	sex female	p value		age	
Controls						
Breg (/Lymphocytes, %)	1.766 ± 1.468	2.104 ± 1.181	0.2532		-0.7239	< 0.0001

DNT (/Lymphocytes, %)	3.484 ± 2.242	3.03 ± 2.313	0.3595	-0.2642	0.0134
CD8+ Treg (/Lymphocytes, %)	0.8288 ± 0.4026	0.8478 ± 0.3353	0.8167	-0.003985	0.971
CD4+ Treg (/Lymphocytes, %)	2.386 ± 0.6411	2.372 ± 0.5512	0.911	0.01234	0.9097

Patients at Admission

Breg (/Lymphocytes, %)	3.115 ± 1.825	2.655 ± 1.587	0.0649	-0.3998	< 0.0001
DNT (/Lymphocytes, %)	2.784 ± 1.73	3.092 ± 2.002	0.2141	-0.1619	0.0189
CD8+ Treg (/Lymphocytes, %)	1.542 ± 0.8931	1.515 ± 0.8985	0.834	0.183	0.0079
CD4+ Treg (/Lymphocytes, %)	2.339 ± 0.6986	2.082 ± 0.5675	0.0062	-0.2864	<0.0001
NIHSS	3.962 ± 3.687	3.899 ± 3.249	0.9002	0.09663	0.163
mRS	2.122 ± 1.209	2.025 ± 1.132	0.5655	0.1612	0.0194

	drinking			LDL	
	no	yes	p value		
Patients at Admission					
Breg (/Lymphocytes, %)	2.972 ± 1.778	2.71 ± 1.524	0.4914	0.1403	0.0448
DNT (/Lymphocytes, %)	2.838 ± 1.732	3.378 ± 2.514	0.176	-0.02164	0.7581
CD8+ Treg (/Lymphocytes, %)	1.547 ± 0.9015	1.414 ± 0.8334	0.4915	-0.06429	0.3598
CD4+ Treg (/Lymphocytes, %)	2.238 ± 0.6274	2.237 ± 0.8465	0.9925	0.05775	0.4108
NIHSS	3.871 ± 3.44	4.458 ± 4.139	0.4431	0.01951	0.7813
mRS	2.129 ± 1.183	1.75 ± 1.113	0.1386	-0.05338	0.4472

	smoking		
	no	yes	p value
Patients at Admission			
Breg (/Lymphocytes, %)	2.954 ± 1.798	2.891 ± 1.554	0.8372
DNT (/Lymphocytes, %)	2.908 ± 1.802	2.866 ± 2.004	0.8957
CD8+ Treg (/Lymphocytes, %)	1.574 ± 0.8841	1.358 ± 0.9197	0.1637
CD4+ Treg (/Lymphocytes, %)	2.2 ± 0.643	2.42 ± 0.7205	0.0557
NIHSS	3.988 ± 3.602	3.732 ± 3.194	0.6766
mRS	2.148 ± 1.173	1.829 ± 1.181	0.1208

	hypertension		
	no	yes	p value
Patients at Admission			
Breg (/Lymphocytes, %)	3.177 ± 1.962	2.834 ± 1.64	0.1882
DNT (/Lymphocytes, %)	2.966 ± 1.83	2.87 ± 1.848	0.7252
CD8+ Treg (/Lymphocytes, %)	1.41 ± 0.8722	1.588 ± 0.9	0.1814
CD4+ Treg (/Lymphocytes, %)	2.23 ± 0.6326	2.249 ± 0.6783	0.8483
NIHSS	3.515 ± 3.273	4.132 ± 3.622	0.2384
mRS	1.851 ± 1.118	2.196 ± 1.194	0.0478

	diabetes		
	no	yes	p value
Admission			
Breg (/Lymphocytes, %)	2.869 ± 1.725	3.088 ± 1.803	0.394
DNT (/Lymphocytes, %)	3.069 ± 1.986	2.562 ± 1.454	0.0595
CD8+ Treg (/Lymphocytes, %)	1.505 ± 0.8963	1.587 ± 0.8905	0.5823
CD4+ Treg (/Lymphocytes, %)	2.224 ± 0.6757	2.28 ± 0.6396	0.568
NIHSS	4.121 ± 3.696	3.571 ± 3.133	0.2669
mRS	2.021 ± 1.202	2.214 ± 1.128	0.2678

Data were analyzed using Mann-Whitney U test and lineal regression. In Mann-Whitney U test, data were presented as mean ± Standard Deviant. LDL: low density lipoprotein.

Supplementary Table 5. Multivariable linear regression of CD4$^+$ Treg/lymphocytes, age, sex, tPA treatment with neurological scores.

Admission	Admission		Discharge		3 months	
	Coef.	P Value	Coef.	P Value	Coef.	P Value
vs. mRS						
CD4$^+$ Tregs (/lymphocytes, %)	-0.18	0.012	-0.21	0.003	0.17	0.024
Age, year	0.14	0.061	0.15	0.039	0.06	0.405
Sex, Male	0.12	0.095	0.12	0.083	0.04	0.629
tPA treatment	-0.08	0.224	-0.01	0.856	0.02	0.777
vs. NIHSS						
CD4$^+$ Tregs (/lymphocytes, %)	-0.28	0.000	-0.22	0.002	-0.02	0.747
Age, year	0.04	0.538	0.08	0.286	0.06	0.428
Sex, Male	0.08	0.275	0.06	0.425	0.00	0.958
tPA treatment	0.30	0.000	0.04	0.562	0.09	0.209

Data were analyzed using multivariable linear regression analysis. NIHSS: National Institutes of Health Stroke Scale. mRS: modified Rankin Scale; tPA: tissue plasminogen activator; Coef.: Standardized Coefficient.
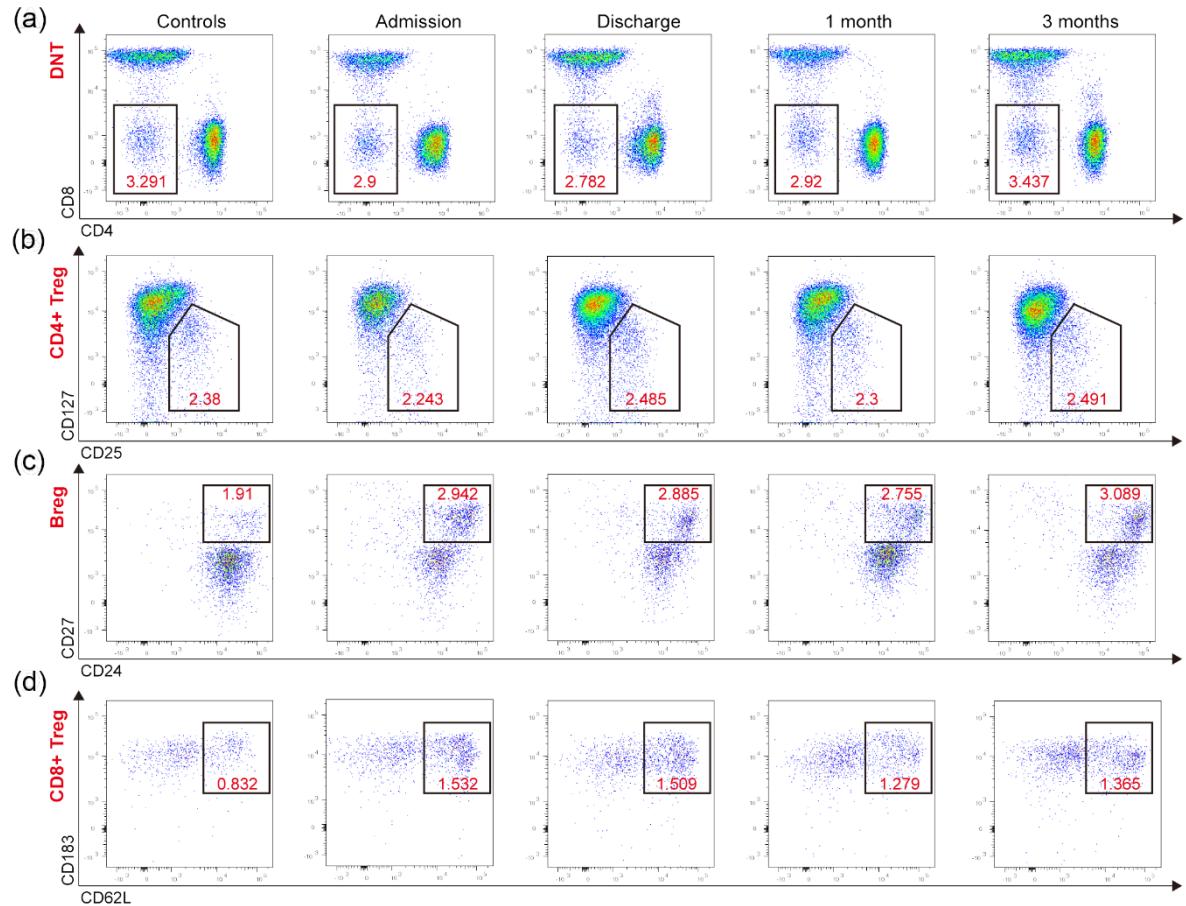
Supplementary Figure 1. Representative flow cytometry plot of DNTs, CD4+ Tregs, Bregs, and CD8+ Tregs in peripheral blood. (a) DNTs of controls and patients in admission, discharge, 1 month and 3 months groups. (b) CD4+ Tregs of controls and patients in admission, discharge, 1 month and 3 months groups. (c) Bregs of controls and patients in admission, discharge, 1 month and 3 months groups. (d) CD8+ Tregs of controls and patients in admission, discharge, 1 month and 3 months groups. Numbers shown are the percentage of circled cells to total lymphocytes.
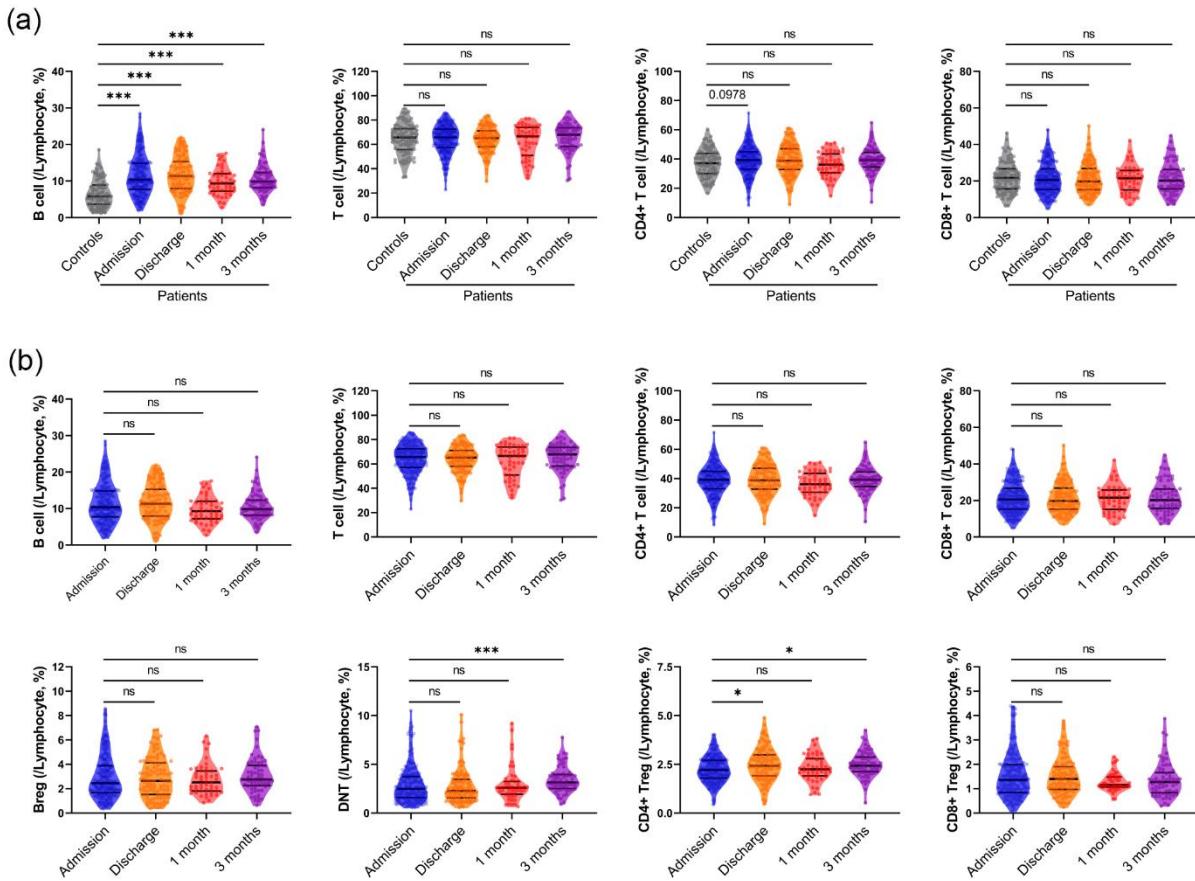
Supplementary Figure 2. Changes of cell proportion between controls and different time points after acute ischemic stroke. (a) Violin plots with median, 25 and 75% quartiles of the percentage of B cells, T cells, CD4$^+$ T cells, and CD8$^+$ T cells within lymphocytes of Controls (grey, n=87) and different time points after acute ischemic stroke (Admission: blue, n=210; Discharge: orange, n=100; 1 month: red, n=49; 3 months: purple, n=66). Mann-Whitney test was employed to examine the difference between Controls and each time point. (b) Violin plots of the percentage of B cells, T cells, CD4$^+$ T cells, CD8$^+$ T cells, Bregs, DNTs, CD4$^+$ Tregs, and CD8$^+$ Tregs within lymphocytes of progress of acute ischemic stroke (Admission: blue, n=210; Discharge: orange, n=100; 1 month: red, n=49; 3 months: purple, n=66). Kruskal-Wallis test and Dunn's multiple comparisons test were employed to examine

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the difference between Admission and each other time point. Decimals represent p-value. *:
p<0.05, **: *p*<0.01, ***: *p*<0.001, ns: no significance.
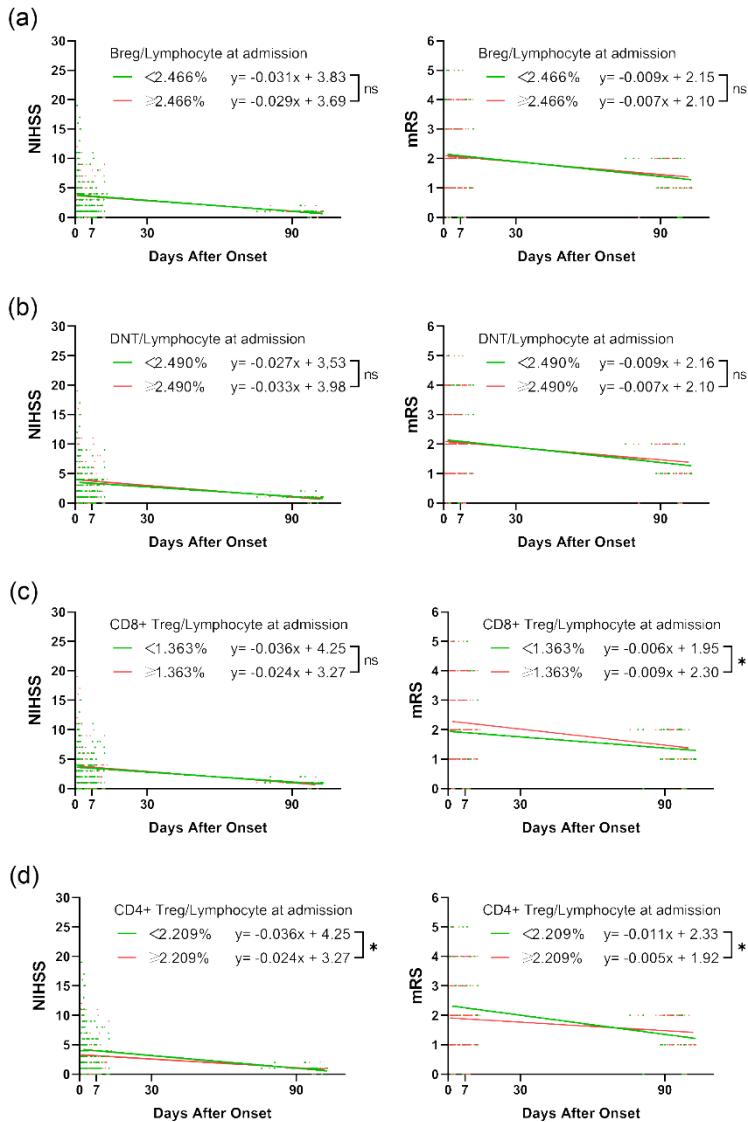
Supplementary Figure 3. Time-score lineal fitting curves of neurological score from admission to 3 months in different levels of regulatory lymphocytes. Line regression model of NIHSS (left) or mRS scores (right) with time after onset. Formulas represented the best-fit equation. F tests were employed to test significances between intercepts. NIHSS:

National Institutes of Health Stroke Scale. mRS: modified Rankin Scale. *: $p<0.05$, ns: no significance.
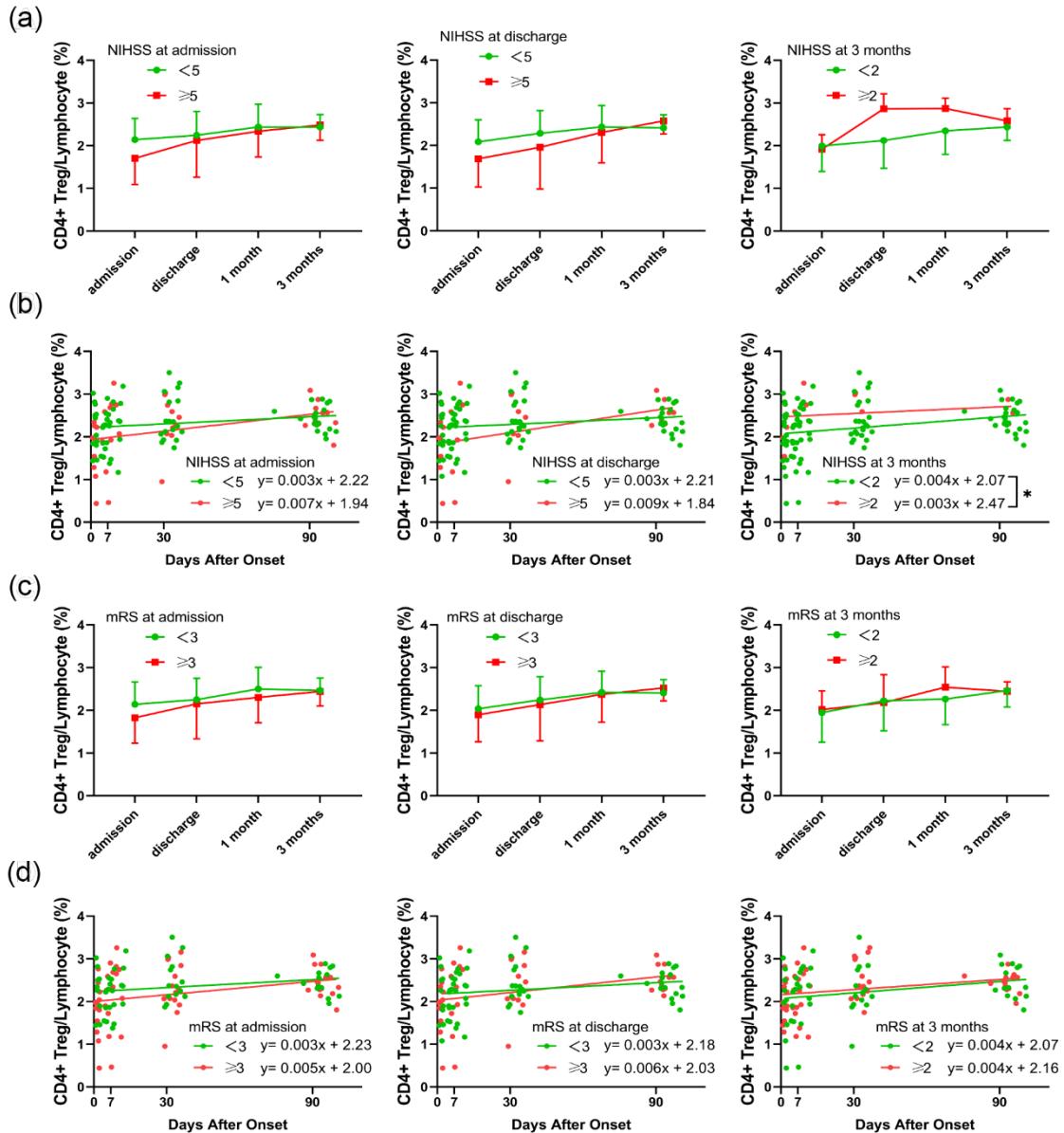
Supplementary Figure 4. Correlations between the continuous changes in the percentage of CD4$^+$ Tregs within lymphocytes and neurological scores. (a) Variation of the proportion of the percentage of CD4$^+$ Tregs within lymphocytes from admission to 3 months in patients with the poorer functional outcome (red square, NIHSS≥5 or 2) and

greater (green circle, NIHSS<5 or 2) at admission (Left, n=10 and 18), discharge (Middle, n=7 and 21), and 3 months (Right, n=3 and 25). All data are presented as mean ± SD. Two-way ANOVA and Bonferroni's multiple comparisons tests were employed to test significances. (b) Line regression model of the percentage of CD4$^+$ Tregs within lymphocytes from onset to 3 months in patients with the poorer functional outcome (red, NIHSS≥5 or 2) and greater (green, NIHSS<5 or 2) at admission (Left), discharge (Middle), and 3 months (Right). Formulas represented the best-fit equation. F tests were employed to test significances between intercepts. (c&d) Similar to figures above but with mRS≥3 or 2 and <3 or 2. Admission n=14 and 14, discharge n=11 and 17, 3 months n=14 and 14. NIHSS: National Institutes of Health Stroke Scale. mRS: modified Rankin Scale. *: $p<0.05$. no symbol: no statistical significance.

Ethical approved documents

上海市闵行区中心医院医学伦理委员会审查批件

审查会议日期	2017 年 6 月 23 日
审查会议地点	行政楼 330 会议室
课题名称	急性脑梗死患者远期预后的相关因素分析
研究科室	神经内科
主要研究者	赵 静
审查方式	会议审查
审查意见	本次伦理委员会共有 10 人参加会议，10 人有表决权，经对本课题申报书中涉及人的研究内容部分进行伦理审查。经伦理委员会审核，该项目研究内容和过程遵循国际及国家颁布的有关生物医学研究的伦理要求，同意该项目临床应用，跟踪审查频率为 12 个月。
正（副）主任委员签字	
伦理委员会盖章	
日 期	2017 年 6 月 28 日