The IMMENSE study: the Interplay between iMMune and ENdothelial cells in mediating cardiovascular risk in Systemic lupus Erythematosus.

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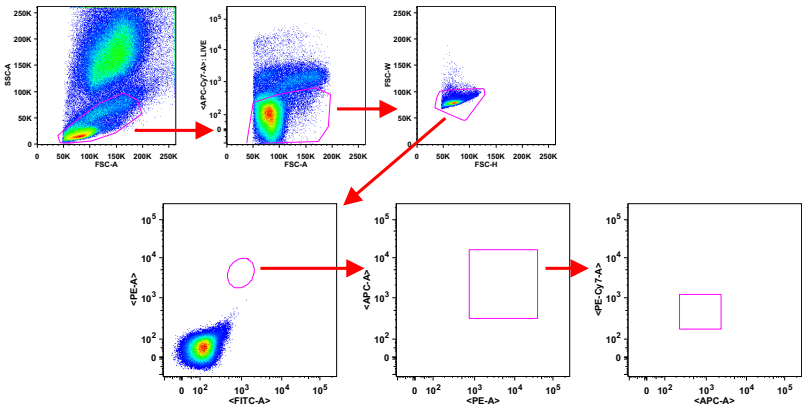
Supplementary Information

Tang:
CD3 FITC [REA613]
CXCR4 PE [REA649]
CD31 PerCP-Vio700 [REA730]
CD4 PE-Vio770 [REA623]
CD28 APC [28.2]
LIVE/DEAD eFluor 780
CD8 VioGreen [REA734]

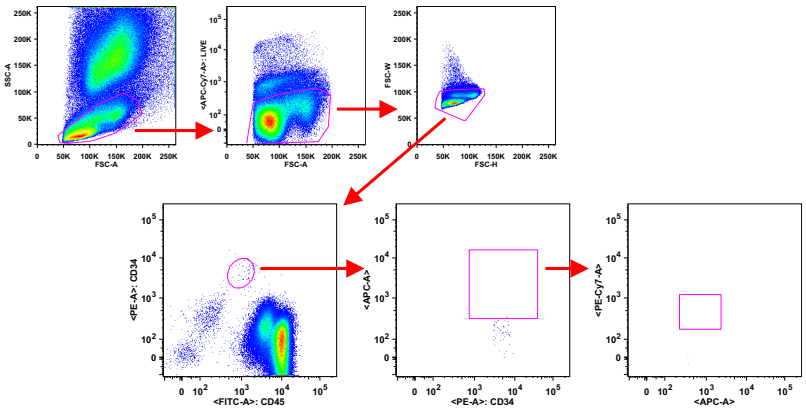
EPC:
CD45 FITC [REA747]
CD34 PE [AC136]
VEGFR2 PE-Vio770 [REA1116]
CD133 APC [AC133]
LIVE/DEAD eFluor 780

Supplemental material 1. Panels with antibody and fluorochromes used for the flow cytometric analysis.

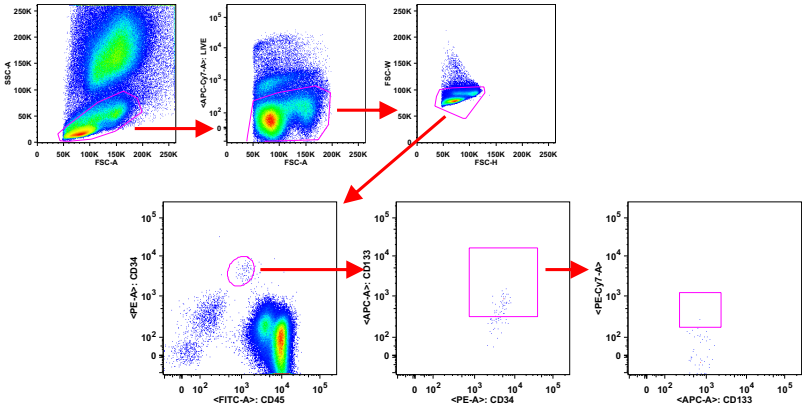
Viability Dye eFluor™ 780



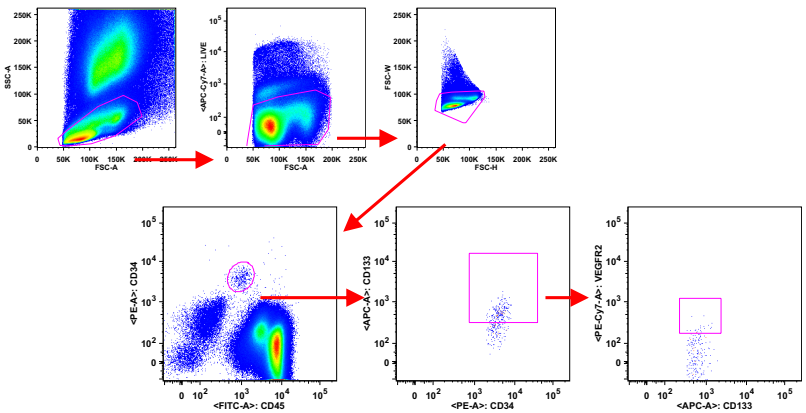
CD45 FITC
CD34 PE
Viability Dye eFluor™ 780



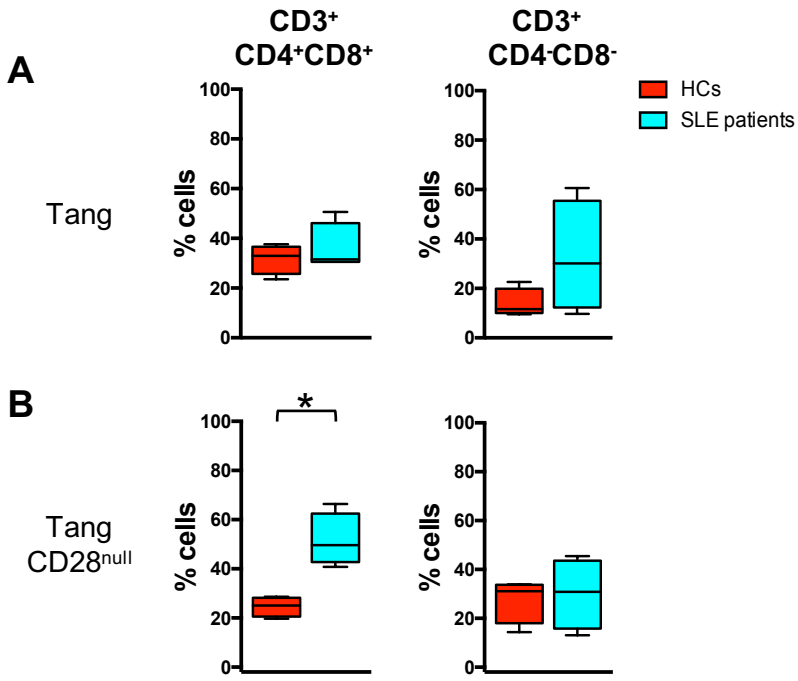
CD45 FITC
CD34 PE
CD133 APC
Viability Dye eFluor™ 780



CD45FITC
CD34 PE
CD133 APC
VEGFR2 PE-Vio770
Viability Dye eFluor™ 780



Supplemental figure 1. Example of Fluorescence Minus One [FMO] staining.
WBCs isolated from one representative healthy donor were analyzed for the expression of EPCs by flow cytometry.
The axis scales for fluorescence are reported as log, the axis scales for SSC, FSC are reported as linear.



Supplemental figure 2. Analysis of double positive [$CD4^+CD8^+$] and double negative [$CD4^-CD8^-$] Tang subpopulations.

WBCs isolated from HCs and SLE patients were analyzed for the differences in the percentage of Tang cells and CD28null cells in Tang subpopulations using flow cytometry.

(A) The percentage of T_{ang} cells for each T cell subpopulation is represented as Tukey's boxplot.

(B) The percentages of $CD28^{null}$ cells within each T_{ang} cell subpopulation is represented as Tukey's boxplot. SLE patients show higher percentage of double positive $CD4^+CD8^+$ $CD28^{null}$ T_{ang} cell subpopulations than HCs.

The axis scales are reported as linear. Statistical analysis of the differences was performed by Mann-Whitney test. p values <0.05 were considered significant: * $p<0.05$

	% Tang $CD3^+$	% Tang $CD3^+CD4^+$	% Tang $CD3^+CD8^+$	% early EPC	% late EPC
HCs	48.6 [41.4 - 52.4] 47.1±7.1	42.0 [26.8 - 44.7] 39.6±7.4	62.3 [47.2 - 70.5] 61.6±9.1	0.038 [0.009 - 0.064] 0.044±0.030	0.002 [0.001 - 0.003] 0.002±0.001
SLE	40.2 [35.9 - 46.8] 41.0±9.2	35.8 [24.3 - 40.4] 32.7±10.3	53.0 [33.9 - 65.0] 54.8±13.9	0.014 [0.011 - 0.028] 0.020±0.016	0.001 [0.0006 - 0.0022] 0.001±0.0009

Data are presented as median [IQR] mean±SD

Supplemental Table 1. Increased percentage of Endothelial progenitor and T_{ang} cells in SLE patients.
The descriptive statistics of each distribution reported in Figure 2 is shown in the table.

	% $CD28^{null}$ in $CD3^{+}$	% $CD28^{null}$ in $CD3^{+}CD4^{+}$	% $CD28^{null}$ in $CD3^{+}CD8^{+}$	% $CD28^{null}$ in Tang $CD3^{+}$	% $CD28^{null}$ in Tang $CD3^{+}CD4^{+}$	% $CD28^{null}$ in Tang $CD3^{+}CD8^{+}$
HC	10.7 [3.5 - 17.1] 11.6±5.9	0.9 [0.2 - 2.4] 2.3±3.8	25.1 [14.2 - 37.7] 26.2±11.6	7.6 [2.8 - 11.6] 8.0±4.0	0.8 [0.2 - 1.5] 0.9±0.8	11.5 [7.8 – 24.0] 14.9±8.3
SLE	21.8 [10.47 - 36.6] 25.1±15.9	6.4 [1.2 - 12.1] 10.0±13.3	45.5 [20.1 - 59.9] 43.9±19.6	11.2 [8.5 - 21.6] 15.6±11.0	1.2 [0.5 - 2.6] 3.1±6.8	24.4 [13.2 – 34.2] 25.0±13.6

Data are presented as median [IQR] mean±SD

Supplemental Table 2. Senescent angiogenic T cells characterize SLE patients.
The descriptive statistics of each distribution reported in Figure 3 is shown in the table.

	SLEDAI-2K	ESR	CRP	Chol. [TOT]	Chol. [LDL]
% Tang $CD3^+CD4^+$	**x** = 28.5 [24.3 - 39.8] 31.6±9.9 **y** = 3.0 [2.0 - 4.0] 3.4±2.6	--	--	--	--
% early EPC	--	**x** = 0.01 [0.01 - 0.02] 0.02±0.01 **y** = 10.0 [5.0 - 16.0] 11.5±8.3	**x** = 0.01 [0.01 - 0.02] 0.02±0.01 **y** = 0.2 [0.1 - 0.5] 0.5±1.0	--	--
% late EPC	--	**x** = 0.001 [0.006 - 0.002] 0.001±0.0009 **y** = 10.0 [5.0 - 16.0] 11.5±8.3	--	--	--
% $CD28^{null}$ in Tang $CD3^+$	--	--	--	**x** = 10.9 [8.1 - 20.4] 15.1±11.1 **y** = 168.0 [138 - 184] 165.8±28.9	**x** = 10.5 [6.9 - 20.4] 15.1±12.4 **y** = 88.0 [74.0 - 108.0] 86.1±27.6
% $CD28^{null}$ in $CD3^+CD4^+$	--	--	--	**x** = 5.4 [1.0 - 12.4] 10.0±13.7 **y** = 168.0 [138.0 - 184.0] 165.8±28.9	**x** = 3.1 [0.7 - 12.4] 9.5±14.7 **y** = 88.0 [74.0 - 108.0] 86.1±27.6

Data are presented as median [IQR] mean±SD

Supplemental Table 3. Impaired percentage of circulating EPCs and T_{ang} is disease activity related.
The descriptive statistics of each distribution reported in Figure 4 is shown in the table.

	IL-8 of Low % Tang CD3	IL-8 of High % Tang CD3	IL-8	MMP-9
	47.0 [30.5 - 69.5] 65.2±65.5	263.0 [135.8 - 1195] 576.5±577.4	--	--
% Tang CD3$^+$	--	--	**x** = 42.2 [37.6 - 49.6] 43.0±8.9 **y** = 158 [45.2 - 382.5] 323±415.6	**x** = 42.2 [37.6 - 49.6] 43.0±8.9 **y** = 495.0 [312.5 - 721.5] 546.6±309.1
% Tang CD3$^+$CD4$^+$	--	--	**x** = 33.2 [26.3 - 42.6] 34.3±9.8 **y** = 158 [45.2 - 382.5] 323±415.6	--
% CD28nul in Tang CD3$^+$	--	--	**x** = 10.5 [6.8 - 16.28] 13.0±9.9 **y** = 158 [45.2 - 382.5] 323±415.6	--
% CD28^{null} in Tang CD3$^+$CD4$^+$	--	--	**x** = 1.23 [0.5 - 2.1] 2.4±5.6 **y** = 158 [45.2 - 382.5] 323±415.6	--
% CD28nul in Tang CD3$^+$CD8$^+$	--	--	**x** = 18.35 [10.9 - 30.4] 21.7±12.9 **y** = 158 [45.2 - 382.5] 323±415.6	--
% CD34$^+$	--	--	--	**x** = 0.05 [0.04 - 0.09] 0.06±0.03 **y** = 493 [309.0 - 706.0] 519.9±277.2

Data are presented as median [IQR] mean±SD

Supplemental Table 4. Circulating proangiogenic factors reflect angiogenic circulating cells related compartments.
The descriptive statistics of each distribution reported in Figure 5 is shown in the table.

	<33th percentile		>66th percentile	
	HCs	SLE	HCs	SLE
% Tang CD3	56.0 [51.3 - 56.1] 54.4±2.7	54.4 [49.9 – 55.0] 52.8±3.3	36.5 [34.9 – 43.1] 38.1±4.3	34.3 [25.6 – 75.3] 31.8±6.3
% Tang $CD3^+CD4^+$	48.8 [43.4 – 49.3] 47.1±3.2	43.3 [36.4 – 49.7] 43.1±7.3	**31.8 [26.8 – 32.4]*§** **30.3±3.0**	**25.5 [21.0 – 26.6]*§** **23.0±4.9**
% Tang $CD3^+CD8^+$	69.8 [65.3 – 73.8] 69.6±4.2	73.2 [55.5 – 78.9] 68.4±13.9	52.9 [47.2 – 55.6] 51.9±4.2	46.7 [34.9 – 57.1] 46.8±10.8
% $CD28^{null}$ in Tang $CD3^+$	5.2 [4.4 – 8.7] 6.1±2.2	8.1 [6.2 – 10.7] 8.3±2.3	11.1 [7.8 – 15.4] 11.4±3.8	14.5 [6.9 – 28.3] 20.3±16.4
% $CD28^{null}$ in Tang $CD3^+CD4^+$	0.2 [0.2 – 2.3] 0.9±1.2	0.5 [0.3 – 1.6] 0.8±0.7	1.3 [1.3 – 2.1] 1.6±0.4	1.7 [0.6 – 4.3] 6.1±11.2
% $CD28^{null}$ in Tang $CD3^+CD8^+$	9.2 [8.0 – 16.1] 11.1±4.3	13.5 [9.5 – 21.7] 15.2±6.6	25.2 [12.1 – 29.6] 22.3±9.1	31.2 [13.2 – 40.4] 31.2±17.4
% $CD28^{null}$ in CD3	9.5 [5.7 – 12.0] 9.0±3.1	7.5 [6.7 – 24.6] 14.0±11.7	16.0 [12.6 – 20.6] 16.4±4.0	24.1 [14.9 – 42.0] 29.1±20.7
% $CD28^{null}$ in $CD3^+CD4^+$	0.2 [0.2 – 1.9] 0.7±0.9	0.7 [0.2 – 4.2] 1.9±2.2	4.1 [1.4 – 12.8] 6.1±5.9	8.1 [1.9 – 26.4] 15.8±18.7
% $CD28^{null}$ in $CD3^+CD8^+$	22.0 [13.9 – 28.2] 21.3±7.1	17.3 [14.6 - 48.1] 28.5±18.2	36.4 [29.8 – 41.9] 36.0±6.0	45.8 [44.1 – 66.0] 50.4±19.6

Data are presented as median [IQR] mean±SD

Supplemental Table 5. Percentage of Tang subsets of SLE patients and HCs stratified by the tertile of the percentage of circulating T_{ang} $CD3^+$ cells (low, <33th percentile; high, >66th percentile).
§ Statistical analysis of the differences among HCs vs SLE was performed by Mann-Whitney test. p values <0.05 were considered significant: * p<0.05.

	SLE	HCs	p-value
IL-6, pg/ml	2.30 [2.30-5.95]	9.25 [4.37-20.13]	**0.01***
IL-8, pg/ml	66.00 34.75-192.80]	607.5 [206.5-1090]	**0.001****
MMP-9, ng/ml	441.0 [259.8-531.0]	706.0 [510.3-802.0]	**0.007****
IFN-γ, pg/ml	13.0 [13.0-14.75]	13.0 [13.50-15.0]	0.784

Data are presented as median [IQR] mean±SD

Supplemental Table 6. IL-6, IL-8, MMP-9 and IFN-γ serum levels from patients and controls.
Statistical analysis of the differences was performed by Mann-Whitney test. p values <0.05 were considered significant: * p<0.05, ** p<0.001.
List of abbreviations: HCs, healthy controls; IFN, interferon; IL, interleukin; IQR, interquartile range; MMP, matrix metalloproteinase; SLE, systemic lupus erythematosus.

	IL-6 mRNA	IL-6 protein expression	ICAM-1 mRNA	ELAM-1 mRNA	MMP-9 protein expression
Low % Tang CD3	3.14 [2.19 - 4.8] 3.3±1.4	0.3 [0.07 - 0.6] 0.5±0.6	2.0 [1.3 - 2.1] 1.8±0.4	2.0 [1.1 - 3.2] 2.1±1.0	
High % Tang CD3	1.6 [1.0 - 1.8] 1.4±0.4	0.1 [0.0 - 0.1] 0.07±0.07	0.7 [0.4 - 0.9] 0.7±0.2	0.5 [0.3 - 0.8] 0.5±0.2	
% Tang $CD3^+$					**x** = 44.7 [33.2 - 54.3] 43.1±12.3 **y** = 1.4 [0.6 - 3.8] 2.4±2.7
% $CD28^{null}$ in Tang $CD3^+$					**x** = 9.3 [5.2 - 23.6] 15.6±15.2 **y** = 1.4 [0.6 - 3.8] 2.4±2.7
% $CD28^{null}$ in Tang $CD3^+CD4^+$					**x** = 1.1 [0.2 - 3.2] 4.3±9.6 **y** = 1.4 [0.6 - 3.8] 2.4±2.7
% $CD28^{null}$ in Tang $CD3^+CD8^+$					**x** = 25.1 [9.3 - 36.2] 25.2±18.3 **y** = 1.4 [0.6 - 3.8] 2.4±2.7

Data are presented as median [IQR] mean±SD

Supplemental Table 7. Low percentage of circulating T_{ang} cells induces pro-inflammatory endothelial phenotype. Correlation between the percentage of cell subpopulations and the expression of factors at mRNA and protein level, after HUVECs' stimulation with the sera from patients and controls.
The descriptive statistics of each distribution reported in Figure 6 is shown in the table.

	SLE	HCs	p-value
IL-6, fold change [RQ]	1.91 [1.27-3.14]	1.82 [1.80-3.50]	0.904
MMP-9, fold change [RQ]	0.81 [0.36-1.20]	1.12 [1.10-1.22]	0.143
IFN-γ, fold change [RQ]	N.D.	N.D.	N.P.
ICAM-1, fold change [RQ]	1.00 [0.60-2.15]	0.70 [0.60-0.80]	0.476
ELAM, fold change [RQ]	0.90 [0.35-2.90]	0.70 [0.60-0.80]	0.667

Data are presented as median [IQR] mean±SD

Supplemental Table 8. IL-6, MMP-9, IFN-γ, ICAM-1 and ELAM-1 mRNA expression levels in HUVECs treated with serum from patients and controls.
Statistical analysis of the differences was performed by Mann-Whitney test.
List of abbreviations: ELAM, endothelial cell leukocyte adhesion molecule; HCs, healthy controls; ICAM, inter-cellular adhesion molecule; IFN, interferon; IL, interleukin; MMP, matrix metalloproteinase; N.D., not detectable; N.P., not performed;SLE, systemic lupus erythematosus.

Relative protein level	SLE	HCs	p-value
IL-6	0.10 [0.0-0.30] 0.12±0.13	0.45 [0.12-1.67] 0.75±0.87	0.079
IL-8	N.D.	N.D.	N.P.
MMP-9	1.20 [0.70-1.80] 1.36±0.90	3.30 [1.72-8.02] 4.35±3.48	**0.034***
IFN-γ	0.85 [0.50-1.05] 0.85±0.38	0.55 [0.50-1.12] 0.73±0.39	0.606

Data are presented as median [IQR] mean±SD

Supplemental Table 9. IL-6, IL-8 and MMP-9 protein expression levels in HUVECs treated with sera from patients and controls.
Statistical analysis of the differences was performed by Mann-Whitney test. p values <0.05 were considered significant: * $p<0.05$
List of abbreviations: HCs: healthy controls; IFN: interferon; IL: interleukin; IQR: interquartile range; MMP: matrix metalloproteinase; N.D.: not detectable; N.P.: not performed; SLE: systemic lupus erythematosus.

	IL-8	% early EPC
% proliferation	**x** = 128.0 [115.0 - 168.5] 142.9±42.4 **y** = 118.0 [42.0 - 273.0] 277.4±393.1	**x** = 128.0 [115.0 - 168.5] 142.9±42.4 **y** = 0.01 [0.008 - 0.02] 0.02±0.01

Data are presented as median [IQR] mean±SD

Supplemental Table 10. Serum IL-8 correlates with endothelial progenitor cells and HUVECs proliferation.
The descriptive statistics of each distribution reported in Figure 7 is shown in the table.

	SLE	HCs	p-value
MTT 24 h, %	62.0 [28.0-75.0]	81.0 [61.50-86.0]	0.189
MTT 72 h, %	117.0 [115.0-152.0]	158.0 [114.8-193.0]	0.479

Data are presented as median [IQR] mean±SD

Supplemental Table 11. Proliferation rate of HUVECs treated with sera from patients and controls.
Statistical analysis of the differences was performed by Mann-Whitney test.
List of abbreviations: HCs: healthy controls; h: hours; SLE: systemic lupus erythematosus.