The effects of 5-azacytidine on the function and number of regulatory T cells and T-effectors in myelodysplastic syndrome

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Online Supplementary Appendix

RNA isolation and quantitative real-time polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) primers were designed for specific genes according to the protocol for the Universal Probe Library (Roche). qPCR reactions were carried out in an ABI 7900 HT Sequence Detection System, using FastStart Taqman qPCR mastermix. mRNA transcript levels for *STAT1*, *STAT3*, *STAT5*, *STAT6*, *ROR-Yt*, *p16*^{INK4A}, T-bet and FOXP3 were normalized to GAPDH mRNA. One of the experimental samples (GAPDH normalized) was designated as the calibrator and given a relative value of 1.0. All quantities (GAPDH normalized) were expressed as n-fold relative to the calibrator. An unpaired *t* test was used to assess the significance of the difference between the median values.

Methylation assay

Our methylation assay provides the gene methylation status as percentages of unmethylated, hypermethylated and intermediate methylated fractions of total input genomic DNA. The total input genomic DNA is determined by q-PCR with the mock digest as template. Hypermethylated DNA is digested by Enzyme A, while unmethylated DNA is digested by Enzyme B. Results of the q-PCR reactions with these two digests as templates can be compared with results from the mock digest and the difference provides the percentages of unmethylated and hypermethylated DNA, respectively. The percentage of intermediate methylated DNA is then calculated as 100 minus percentage of unmethylated and hypermethylated DNA.

Functional analysis of regulatory T cells

Treg were first sorted based on their CD25 and CD127 expression ($CD3^+CD4^+CD25^{high}$ $CD127^{low}$) on a BD FACS Aria. After 48 h of stimulation with CD3/CD28 beads, 5-azaC was added to Treg at a dose of 2 μM, every 24 h for 96 h. After 96 h, fresh effector T cells ($CD3^+CD4^+CD25^{low}CD127^{high}$) from the same donor were sorted and labeled with 1 μM CFDASE (CellTrace CFSE Cell Proliferation Kit, Life Technologies Ltd, Paisley, UK) according to the manufacturer's instructions. These cells were then combined in a 1:1 ratio with 5-azaC-treated Treg or control Treg, also labeled with 1 μM Violet Proliferation Dye 450 (Becton Dickinson, San Jose, CA, USA), and cultured for 72 h in the presence of CD3/CD28 beads for further stimulation. For this experiment, only Treg were pre-treated with5-aza C and a proliferation assay was carried out on both Treg and T-effector cells at 72 h.

After the co-culture cells were harvested, stained for dead cells with Live/Dead Fixable Dead Cell Stain Kit (Life Technologies Ltd, Paisley, UK) and assayed for proliferation on the appropriate channels on a BD FACS Canto II instrument.

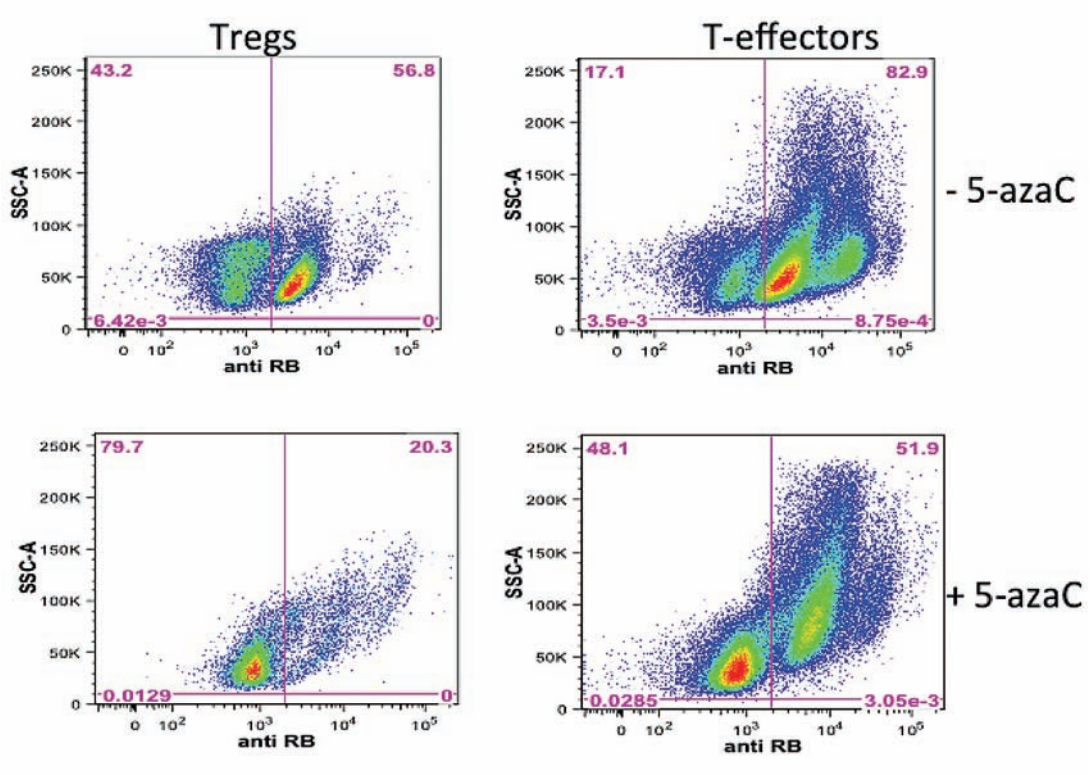
To investigate the function of 5-azaC-treated Treg, at least $1x10^9$ peripheral blood mononuclear cells from a healthy donor were separated and divided into two parts. Half the cells were frozen and stored on liquid nitrogen and the remaining cells were sorted for $CD4^+CD25^{high}CD127^{low}$ Treg. Sorted Treg (at least $2x10^6$) were stimulated for 48 h with CD3/CD28 beads and 5-azaC was added every 24 h to half of them (test sample) for 96 h. The viability of Treg in the presence of 2 μM of 5-azaC was checked by flow cytometry. At the end of 96 h, the remaining cells were thawed and sorted for $CD4^+CD25^{low}CD127^{high}$ (T-effectors). Treg were then stained with Violet Proliferation Dye (VPD) and T-effector cells with CFSE. T-effectors were then co-cultured at a 1:1 ratio with either 5-azaC treated or untreated Treg and stimulated with CD3/CD28 beads for another 72 h. The proliferation of Treg and T-effectors was assessed by flow cytometry in both conditions. Supernatants were also collected after 72 h of stimulation.

Online Supplementary Table S1. IPSS cytogenetic risk group of patients: IPSS cytogenetic risk groups were good risk (n=22, 32%) (21 patients had normal cytogenetics and one patient had 5q- and –Y and del20q), intermediate risk (n=6, 9%), poor risk (n=40, 59%) and cytogenetics failed in one patient. The poor risk cytogenetic category consisted of isolated monosomy 7(-7/del 7q), -16, monosomy 7 with one or more aberrations, -23 and complex karyotype (3 or more) without abnormal chromosome 7 in one patient.

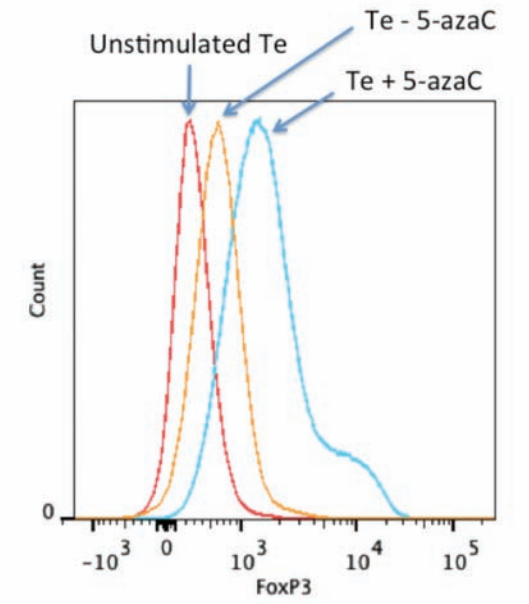
Risk group	Number	Percentage
Good risk	22	32
Intermediate risk	6	9
Poor risk	40	59

Online Supplementary Table S2. WHO classification of the cohort of patients studied. AML with MDS features: all these patients had antecedent MDS and included five with AML with blasts counts between 20-30%. Eighty-five percent of patients were evaluable for IPSS risk category. Of the 56 patients, 48 belonged to intermediate-2 or high-risk groups. The median bone marrow blast percentage was 11% (range 1-30%).

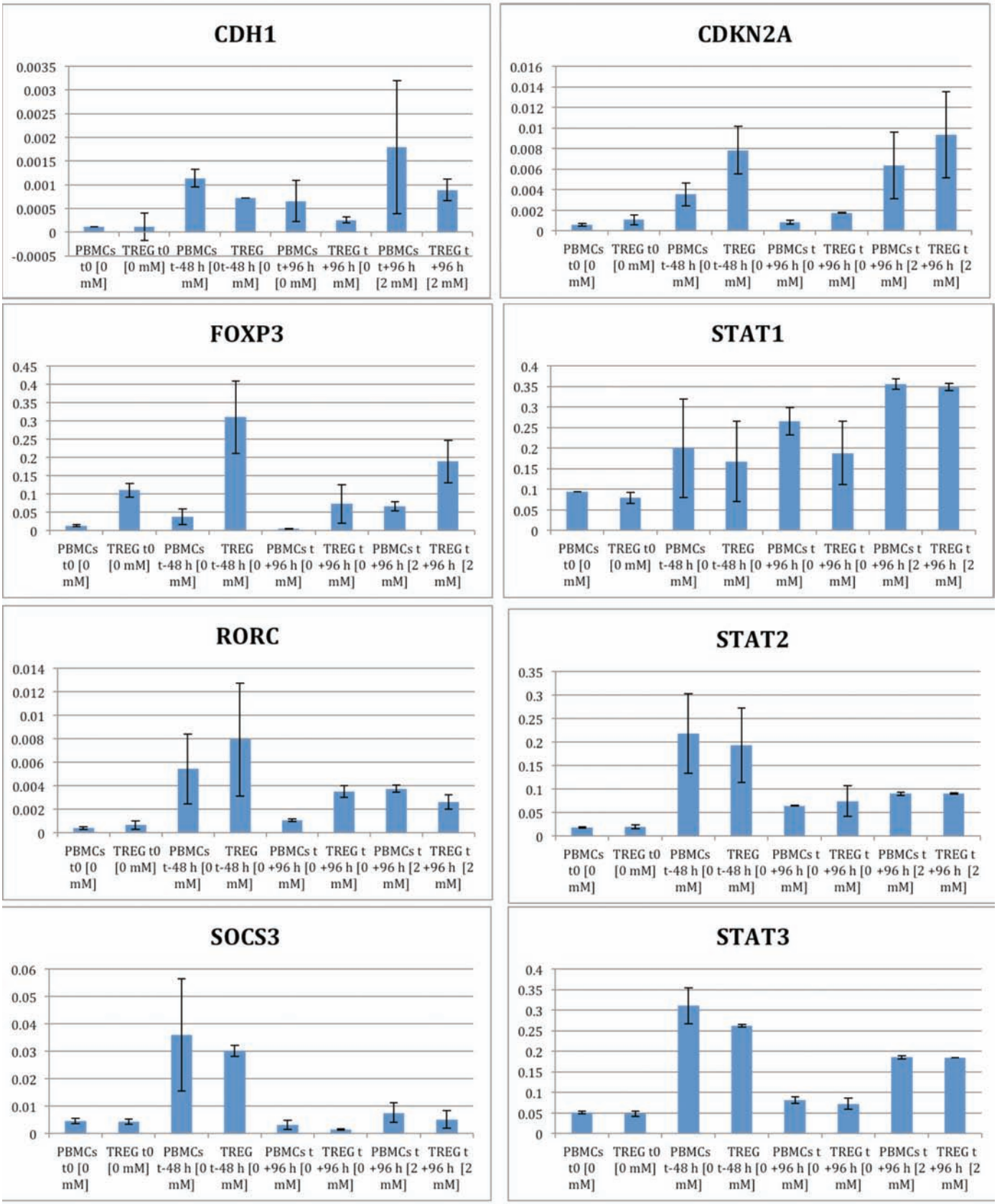
Disease	Number
Refractory anemia	3
Refractory cytopenia with multilineage dysplasia (RCMD)	6
Refractory anemia with excess blasts 1 (RAEB-1)	17
Refractory anemia with excess blasts 2 (RAEB-2)	25
AML evolved from MDS (secondary AML)	12
MDS/myeloproliferative neoplasm	5



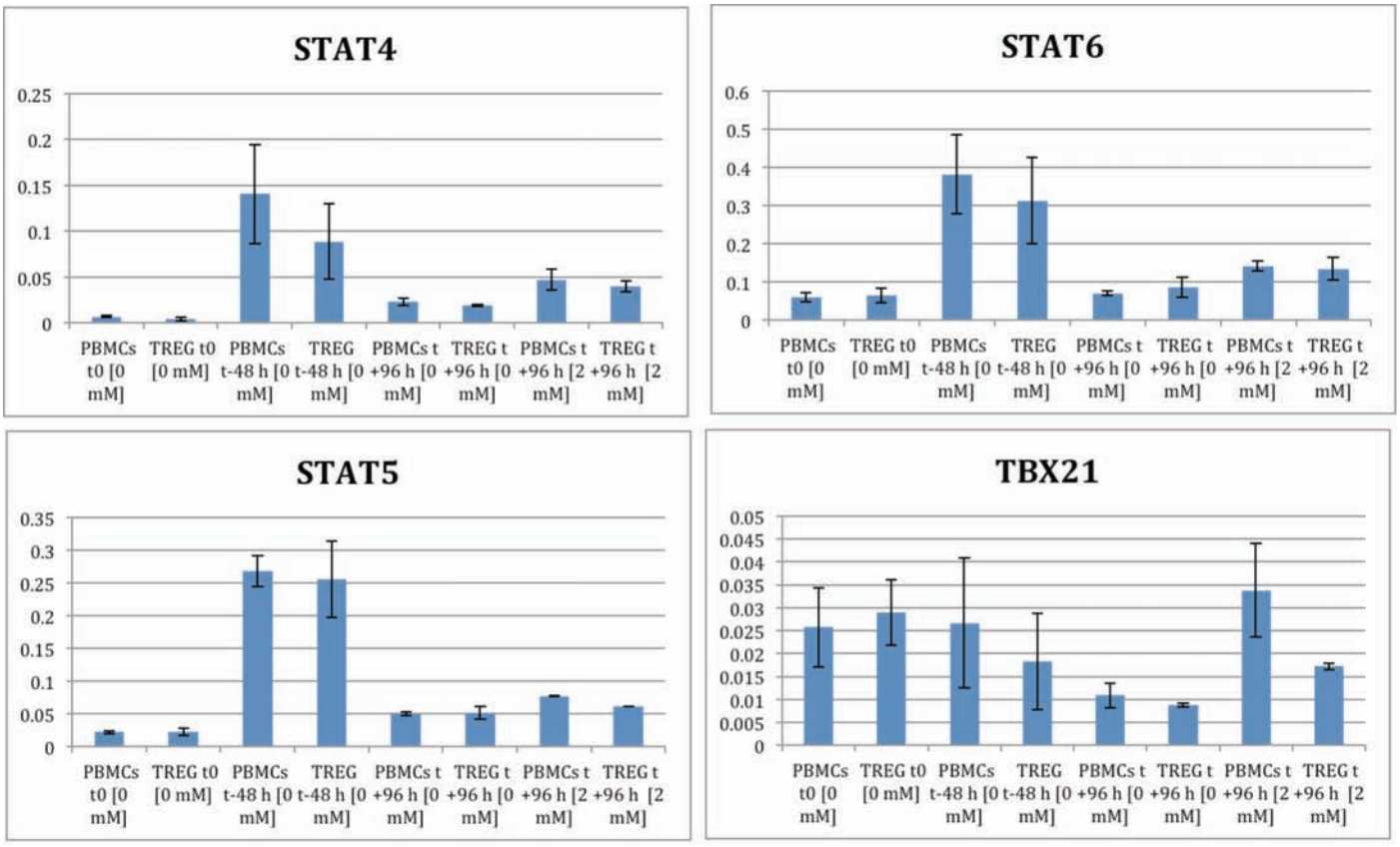
Online Supplementary Figure S1. Cell cycle arrest following *in-vitro* treatment with 5-azaC. Following treatment with 5-azaC (2 μM) both Treg and T effectors expressed lower levels of retinoblastoma (BR) protein phosphorylated as $S^{807/811}$.



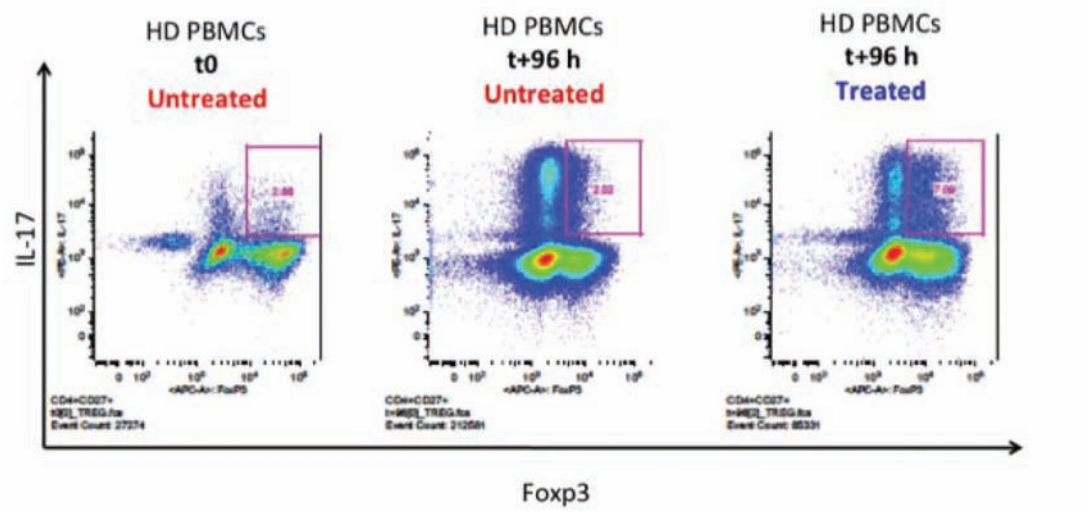
Online Supplementary Figure S2. FOXP3 expression by T-effector cells following *in-vitro* culture with 5-azaC. To investigate FOXP3 expression by T-effector (Te) cells following *in-vitro* exposure to 5-azaC, Te cells were sorted by FACS based on their surface markers ($CD3^+CD4^+CD25^{low}CD127^{high}$). Sorted Te cells were stimulated for 48 h by CD3/CD28 beads followed by another 96 h of stimulation with or without 2 μM of 5-azaC. Stimulated cells were harvested and after fixation and permeabilization, stained intracellularly for FOXP3 as described in the *Design and Methods* section. The expression level of FOXP3 was markedly higher in 5-azaC-treated cells than in untreated cells and unstimulated Te cells.



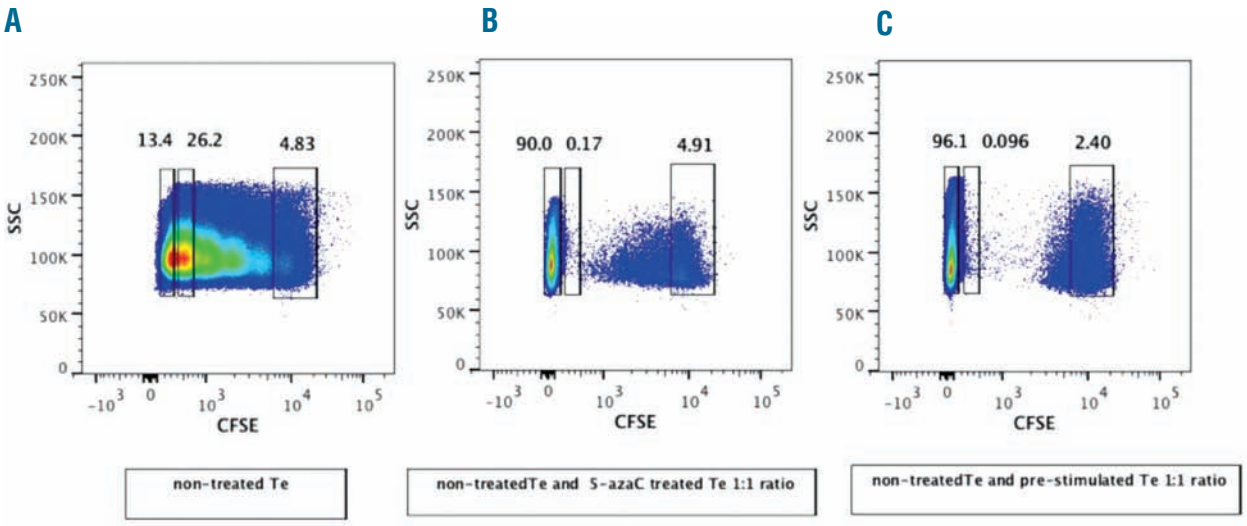
Online Supplementary Figure S3. Continued on the next page.



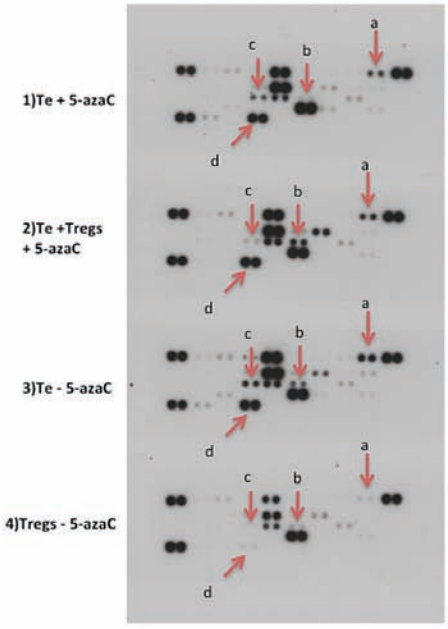
Online Supplementary Figure S3. The expression of each gene (*CDKN2A*, *FOXP3*, *RORC*, *SOCS3*, *STAT1*, *STAT2*, *STAT3*, *STAT5B*,*STAT6*, *STATB4*, *TBX21*) is represented as a Δ-CT value relative to an internal GAPDH control for the PBMC (n=2) and Treg (n=2) at each timepoint, pre and post *in-vitro* addition of 5-azaC in two healthy donors. The Y-axis is fold change (ΔCT). Error bars represent standard error of mean.

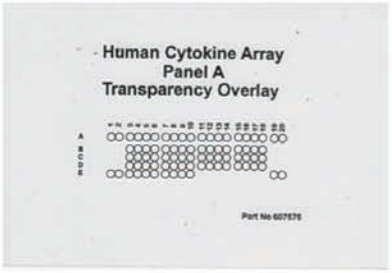


Online Supplementary Figure S4. Effect of 5-azaC on IL-17 and FOXP3 expression. PBMC from healthy donors (HD) were initially stimulated for 48 h and then stimulated for another 72 h in the presence of 5-azaC (2 μM). The PBMC were stained and gated for $CD3^{+}CD4^{+}CD25^{high}FOXP3^{+}IL\text{-}17^{+}$. The percentage of $CD3^{+}CD4^{+}CD25^{high}FOXP3^{+}IL\text{-}17^{+}$cells was markedly increased in 5-azaC-treated samples. The experiment was repeated on four separate samples and the difference was statistically different, *P*=0.02)



Online Supplementary Figure S5. The suppressive function of 5-azaC-treated $CD4^+$ effector T cells. To investigate the function of 5-azaC-treated $CD4^+$ effector T cells (Te), $2x10^8$ PBMC from a healthy donor were separated and divided into two parts. Half the cells were frozen and stored on liquid nitrogen and the remaining cells were sorted for $CD4^+CD25^{low}CD127^{high}$ Te. Sorted Te cells ($5x10^6$) were stimulated for 48 h with CD3, CD28 beads and then 5-azaC was added every 24 h to half of them (test sample) for 96 h. Viability of Te cells in the presence of 2 μM of 5-azaC was checked by flow cytometry. At the end of 96 h, the remaining cells were thawed and sorted for $CD4^+CD25^{low}CD127^{high}$. Sorted Te cells were then stained with CFSE. Te cells from a second sorting were then co-cultured at a 1:1 ratio with either 5-azaC treated or untreated Te cells and stimulated with CD3, CD28 beads for another 72 h. The proliferation of Te cells in the presence and absence of 5-azaC treated and untreated Te cells was assessed by flow cytometry in both conditions. (A) After 72 h of stimulation with CD3, CD28 beads, about 39.6% of the Te cells proliferated in the absence of 5-azaC-treated Te cells. (B, C) Co-culture of Te cells with pre-stimulated Te cells (in the absence of 5-azaC) leads to proliferation of Te cells. Co-culture with 5-azaC-treated Te had no inhibitory effect on the proliferation of untreated Te cells.





a= IFN-γ
b= IL-17
c= IL-13
d= TNF-α

Coordinate	Target/Control	Alternate Nomenclature
A1, A2	Positive Control	—
A3, A4	C5a	Complement Component 5a
A5, A6	CD40 Ligand	CD154
A7, A8	G-CSF	CSFβ, CSF-3
A9, A10	GM-CSF	CSFα, CSF-2
A11, A12	GROα	CXCL1
A13, A14	I-309	CCL1
A15, A16	sICAM-1	CD54
A17, A18	IFN-γ	Type II IFN
A19, A20	Positive Control	—
B3, B4	IL-1α	IL-1F1
B5, B6	IL-1β	IL-1F2
B7, B8	IL-1ra	IL-1F3
B9, B10	IL-2	—
B11, B12	IL-4	—
B13, B14	IL-5	—
B15, B16	IL-6	—
B17, B18	IL-8	CXCL8
C3, C4	IL-10	—
C5, C6	IL-12 p70	—
C7, C8	IL-13	—
C9, C10	IL-16	LCF
C11, C12	IL-17	—
C13, C14	IL-17E	—
C15, C16	IL-23	—
C17, C18	IL-27	—

Coordinate	Target/Control	Alternate Nomenclature
D3, D4	IL-32α	—
D5, D6	IP-10	CXCL10
D7, D8	I-TAC	CXCL11
D9, D10	MCP-1	CCL2
D11, D12	MIF	GIF, DER6
D13, D14	MIP-1α	CCL3
D15, D16	MIP-1β	CCL4
D17, D18	Serpin E1	PAI-1
E1, E2	Positive Control	—
E3, E4	RANTES	CCL5
E5, E6	SDF-1	CXCL12
E7, E8	TNF-α	TNFSF1A
E9, E10	sTREM-1	—
E19, E20	Negative Control	—

Online Supplementary Figure S6. Cytokine secretion by 5-azaC-treated Te cells. To investigate the effect of 5-azaC on cytokine profile of Te cells, sorted Te cells were stimulated by CD3,CD28 beads in the presence or absence of 5-azaC as described in the *Design and Methods* section. In condition one, only Te cells were cultured whereas in condition two, Te cells were co-cultured with Treg in a 1:1 ratio. Untreated Te cells and Treg were used as controls (conditions 3 and 4). Supernatants were collected after 96 h and cytokine levels measured using Proteome Profiler Antibody Arrays (R&D systems, Minneapolis, USA) as previously described.[1] The array allows detection of the following proteins: C5/C5a, CD40 ligand, G-CSF, GM-CSF, CXCL1, CCL1, sICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2,IL-4, IL-5, IL-6, CXCL8, IL-10, IL-12 p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, CXCL10, CXCL11, MCP-1, CCL2, MIF, MIP-1α, MIP-1β, serpin E1, RANTES, SDF-1, TNF-α and sTREM-1. The cytokine profile of 5-azaC-treated Te cells (condition 1) was different from that of stimulated, untreated Te cells and Treg (conditions 3 and 4). Most notably the amount of secreted IL-17 was higher in the presence of Treg (condition 2) than in the other conditions.

References

1. Wang QS, Xiang Y, Cui YL, Lin KM, Zhang XF. Dietary blue pigments derived from genipin, attenuate inflammation by inhibiting LPS-induced iNOS and COX-2 expression via the NF-kappaB inactivation. PloS one. 2012;7(3):e34122.