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Complete List of Authors:	Kehrmann, Jan; Institute of Medical Microbiology, University Hospital Essen, University of Duisburg-Essen Tatura, Roman; Institute of Medical Microbiology, University Hospital Essen, University of Duisburg Essen Zeschnigk, Michael; Institute of Human Genetics, University Hospital Essen, University of Duisburg-Essen Probst-Kepper, Michael; Institute for Clinical Transfusion Medicine, Städtisches Klinikum Braunschweig gGmbH Geffers, Robert; Genome Analytics, Helmholtz Centre for Infection Research Steinmann, Jörg; Institute of Medical Microbiology, University Hospital Essen, University of Duisburg Essen Buer, Jan; University Hospital Essen, Medical Microbiology
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Impact of 5-Aza-2`-deoxycytidine and Epigallocatechin-3-gallate for induction of human regulatory T cells

Jan Kehrmann[1], Roman Tatura[1], Michael Zeschnigk[2], Michael Probst-Kepper[3], Robert Geffers[4], Jörg Steinmann[1], Jan Buer[1]

1 Institute of Medical Microbiology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, **2** Institute for Human Genetics, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, **3** Institute for Clinical Transfusion Medicine, Städtisches Klinikum Braunschweig gGmbH, Braunschweig, Germany, **4** Genome Analytics, Helmholtz Centre for Infection Research, Braunschweig, Germany

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Corresponding author:

Jan Kehrmann, MD

Institute of Medical Microbiology, University Hospital Essen, University of Duisburg-Essen

Hufelandstrasse 55, 45122 Essen, Germany

Telephone: +49-201-72385913

Fax: +49-201-7235602

E-mail: jan.kehrmann@uk-essen.de

Summary

The epigenetic regulation of transcription factor genes is critical for T cell lineage specification. A specific methylation pattern within a conserved region of the lineage specifying transcription factor gene *FOXP3*, the *Treg-specific demethylated region* (*TSDR*), is restricted to regulatory T (Treg) cells and required for stable expression of FOXP3 and suppressive function. We analyzed the impact of hypomethylating agents 5-Aza-2`-deoxycytidine and Epigallocatechin-3-gallate (EGCG) on human $CD4^+CD25^-$ T cells for generating demethylation within *FOXP3-TSDR* and inducing functional Treg cells. Gene expression, including lineage specifying transcription factors of the major T cell lineages and their leading cytokines, functional properties and global transcriptome changes were analyzed. *FOXP3-TSDR* methylation pattern was determined by using deep amplicon bisulfite sequencing.

5-Aza-2`-deoxycytidine induced *FOXP3-TSDR* hypomethylation and expression of Treg cell specific genes *FOXP3* and *LRRC32*. Proliferation of 5-Aza-2´deoxycytidine treated cells was reduced, but the cells did not show suppressive function. Hypomethylation was not restricted to *FOXP3-TSDR* and expression of master transcription factors and leading cytokines of Th1 and Th17 cells were induced. EGCG induced global DNA hypomethylation to a lower extent than 5-Aza-2´deoxycitidine, but no relevant hypomethylation within *FOXP3-TSDR* or expression of Treg cell specific genes.

Both DNA methyltransferase inhibitors did not induce full functional human Treg cells. 5-Aza-2´deoxycitidine treated cells resemble Treg cells, but they did not suppress proliferation of responder cells, which is an essential capability to be used for Treg cell transfer therapy. Using a recently developed targeted demethylation technology might be a more promising approach for generation of functional Treg cells.

Introduction

Regulatory T (Treg) cells play an important role for establishing peripheral immune tolerance. Functional defects of Treg cells or their low percentage to all T cells may cause or worsen inflammatory or autoimmune diseases including inflammatory bowel disease, rheumatoid arthritis, type I diabetes, multiple sclerosis or Graft-versus-Host Disease (GvHD).[1, 2] Adaptive Treg cell based immune therapies are therefore considered for treatment of these diseases.[3, 4] Due to the need of high numbers of Treg cells and their limited availability, an *in vitro* induction of Treg cells from $CD4^+CD25^-$ T cells could overcome these limitations.

A reliable and sufficient *in vitro* or *in vivo* induction of human Treg cells with stable suppressive function is lacking so far. Stability of immunosuppressive phenotype is a critical parameter for Treg cells and required for safe therapeutic application[5] to exclude harmful effects developing through conversion into proinflammatory T cells *in vivo*. The most specific feature of Treg cells, ensuring stable Treg cell phenotype with stable FOXP3 expression and suppressive function, is the existence of an unmethylated *FOXP3 Treg specific demethylated region (TSDR),*[6, 7] which does not occur in other major blood cells, including other T cells.[7]

TGFβ treatment has been a promising approach for *in vitro* induction of human Treg cells. TGFβ induced cells express Treg cell marker molecules but do not show a Treg cell phenotype and suppressive function *in vivo*.[8] In addition, TGFβ-induced human Treg cells lose FOXP3 expression and suppressive activity and do not exhibit hypomethylated *FOXP3-TSDR*.[9] Another approach has been the overexpression of Treg cell specific master transcription factor FOXP3 in $CD4^+CD25^-$ T cells. These cells show a partial unstable Treg cell phenotype[10, 11] and also do not exhibit Treg cell specific hypomethylation within *FOXP3-TSDR*.[12]

In this study we analyze the potency of the two hypomethylating agents 5-Aza-2`-deoxycytidine (5-Aza-dC) and Epigallocatechin-3-gallate (EGCG) for *in vitro* induction of functional Treg cells through generation of a hypomethylated *FOXP3-TSDR*. We analyzed the expression of Treg cell specific genes and the functional properties of treated $CD4^+CD25^-$ T cells. 5-Aza-dC is a derivative of 5-Azacytidine. Both substances are inhibitors of DNA

methyltransferases (DNMTs) and are used for therapy of patients with myelodysplastic syndrome and acute myeloid leukaemia.[13] In these patients, 5-Azacytidine has been reported to augment regulatory T cell expansion in blood.[14-16] EGCG is the most abundant catechin of green tea and has been reported to have cardio protective, anti-cancer, anti-infective properties[17] and protective effects on autoimmune diseases.[18] EGCG has also been described to be a potent inhibitor of DNMTs[19, 20] and to induce FOXP3 in Jurkat T cell line.[21]

Materials and Methods:

Ethics statement and study group

The study was approved by the Ethics Committee at the University Hospital of Essen (Northrine Westfalia, Germany, no.: 13-5546-BO). Buffy coats were provided from the Institute of Transfusion Medicine of the University Hospital Essen. Blood was taken from healthy male blood donors, who gave their written informed consent.

Antibody Staining

For immune staining, FITC-, APC-, PB and PE-conjugated mAbs against CD4 (clone RPA-T4), CD25 (clone BC96, 4E3), CD127 (clone eBioRDR5) and FOXP3 (clone PCH101) were used (all from eBioscience, San Diego, USA). For measurement of proliferation activity, cell staining was performed with eFluor670 proliferation dye (eBioscience, San Diego, USA).

Isolation and cultivation of $CD4^+CD25^-$ T cells, $CD4^+CD25^{high}CD127^{low}$ T cells and dendritic cells

Peripheral blood mononuclear cells were isolated from human buffy coat using Biocoll (Biochrom, Berlin, Germany) separating solution. 100 ml of buffy coat was diluted with 100 ml phosphate buffered saline buffer (PBS). 20 ml of the suspension was carefully layered

over 20 ml Biocoll separating solution and centrifuged for 25 min at 1800 x rpm. The lymphocyte layer was transferred into a new tube and washed with PBS once. For long term storage $1.2x10^8$ cells were suspended in 2 ml medium (IMDM, 10% FCS, 25µM β-mercaptoethanol, Penicillin/Streptomycin and 10% DMSO) and stored in liquid nitrogen. $CD4^+$ T cells were enriched by autoMACS using the $CD4^+$ T cell isolation Kit (Miltenyi Biotech, Bergisch-Gladbach, Germany). Cells were stained with CD4-, CD25- and CD127-antibodies for 15 min at 4°C and washed with FACS buffer. Cell sorting of $CD4^+CD25^-$ and $CD4^+CD25^{high}CD127^{low}$ T cells was performed using a FACS Aria II (BD, Franklin Lakes, USA). Purity was >95 % as controlled by FACS. FACS-sorted lymphocytes were cultivated in RPMI 1640 (Life Technologies, Carlsbad, USA) medium with 1 µg/ml anti-CD3, 1 µg/ml anti-CD28 (both Miltenyi Biotec, Bergisch Gladbach, Germany), 100 U/ml IL2 (eBioscince) and Penicillin/Streptomycin at 37°C, 5% CO_2 in an incubator (Thermo Scientific Heracell 150i, Waltham, USA). $2,5x10^6$ cells were cultured with two different concentrations of 5-Aza-dC (5 µM and 1 µM) or EGCG (50 µM and 5 µM), which were added every other day (both Sigma-Aldrich, St. Louis, USA). Both substances were diluted in 1% DMSO / 99% IMDM medium. Control cells were stimulated with 1% DMSO diluent. After 4 days of incubation, medium was replaced by fresh medium including hypomethylating agents, as required. One part of the cells was used for RNA extraction, DNA extraction and FACS analysis. Three days later, residual cells were subjected to RNA- and DNA-extraction or FACS analysis. All experiments were performed at least in triplicate with cells from different blood donors.

$CD11c^+$ dendritic cells were isolated from PBMCs of the same blood donor, using biotin CD11c antibodies (BioLegend, San Diego, USA) at 4°C. After incubation and washing with FACS buffer, anti-Biotin beads (Miltenyi Biotech, Bergisch-Gladbach, Germany) were added for 15 min at 4°C. Labeled cells were washed, suspended in 500µl FACS buffer and used for magnetic separation by autoMACS.

mRNA-Expression

RNA-isolation was performed using the QIAamp RNA-easy Kit (Qiagen, Hilden, Germany) according to the manufacturer´s guidelines. RNA concentration was measured by NanoDrop ND-1000 spectrophotometer (peqLAB, Erlangen, Germany) and stored at -80°C. For cDNA-synthesis, up to 8 µg RNA was mixed with oligo dT Primers/random hexamers and incubated for 10 minutes at 70°C. The synthesis was performed for 60 minutes at 42°C with MML-V RT (Promega, Madison, USA). Afterwards, the enzyme was heat inactivated at 95°C for 5 min. Quantitative real-time PCRs were performed using SYBR Green (Thermo Fisher Scientific, Waltham, USA). Each reaction contained 2,5 µl of each primer, 5 µl cDNA-template and 10 µl 2x SYBR Green MasterMix. RNA expression was measured using following primers:

FOXP3_fw: 5-GAACGCCATCCGCCACAACCTGA-3

FOXP3_rev: 5-CCCTGCCCCCACCACCTCTGC-3

GARP_fw: 5-TTCCAGGGCCCCAGCTAACTAATG-3

GARP_rev: 5-GGGGCCACTTCCTGTCCACTT-3

IL-10_fw: 5-CCCTAACCTCATTCCCCAACCAC-3

IL-10_rev: 5-CCGCCTCAGCCTCCCAAAGT-3

TGFβ_fw: 5-TGGCTGTATGAGCACCGTTA-3

TGFβ_rev: 5-TGGATCTTTGCCATCCTTTC-3

TBX21_fw: 5-ACGCTTCCAACACGCATATC-3

TBX21_rev: 5-ATCTCCCCCAAGGAATTGAC-3

IFNγ_fw: 5-TGACCAGAGCATCCAAAAGA-3

IFNγ_rev: 5-CTCTTCGACCTCGAAACAGC-3

GATA3_fw: 5-GTCCTGTGCGAACTGTCAGA-3

GATA3_rev: 5-GGGGAAGTCCTCCAGTGAGT-3

IL-4_fw: 5-GCCACCATGAGAAGGACACT-3

IL-4_rev: 5-ACTCTGGTTGGCTTCCTTCA-3

RORγT_fw: 5- AGGGCTCCAAGAGAAAAGGA-3

RORγT_rev: 5-CTTTCCACATGCTGGCTACA-3

IL-17_fw: 5-ACCAATCCCAAAAGGTCCTC-3

IL-17_rev: 5-GGGGACAGAGTTCATGTGGT-3

RPS9_fw: 5-CGCAGGCGCAGACGGTGGAAGC-3

RPS9_rev: 5-CGAAGGGTCTCCGCGGGGTCACAT-3

Primers specific for the ribosomal protein S9 (*RPS9*) gene were used for normalization of real-time quantitative PCR data. Real-time-PCR was carried out using a 7500 Fast Real Time PCR system (Applied Biosystems, Carlsbad, USA) using the following parameters: denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, 56°C (IL-10: 58°C) for 60 sec and 72°C for 60 sec.

DNA Microarray Hybridisation and Analysis

Quality and integrity of the total RNA was controlled on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). 500 ng of total RNA were applied for Cy3-labelling reaction using the one color Quick Amp Labeling protocol (Agilent Technologies; Waldbronn, Germany). Labeled cRNA was hybridized to Agilent´s human 4x44k microarrays for 16h at 68°C and scanned using the Agilent DNA Microarray Scanner. Expression values were calculated by the software package Feature Extraction 10.5.1.1 (Agilent Technologies; Waldbronn, Germany). Statistical analysis of the expression data was

performed using the Gene Spring Software package (Agilent Technologies; Waldbronn, Germany). The array data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE53448 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53448)

DNA extraction and DNA methylation analysis

DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. Global methylation analysis was done using MethylFlash Methylated DNA Quantification Kit (Epigentek, Farmingdale, USA) with 100 ng of extracted DNA. $CD4^+CD25^-$ T lymphocytes from healthy subjects were cultured with 5-Aza-dC or EGCG for four days. Global DNA methylation was compared with that of $CD4^+CD25^-$ T lymphocytes cultured without 5-Aza-dC or EGCG. DNA methylation of cultured $CD4^+CD25^-$ control cells was set 100%. Fluorescence was measured by GENios Microplate Reader (Tecan, Männedorf, Switzerland).

For quantification of *FOXP3-TSDR* methylation, bisulfite DNA was prepared using BisulFlash DNA Modification Kit (Epigentek, Farmingdale, USA) according to the manufacturer's guidelines. Analysis was performed with *FOXP3-TSDR* QAMA (Quantitative analysis of methylated alleles) assay, described elsewhere.[22]

For deep amplicon analysis of *FOXP3-TSDR* using next generation sequencing (NGS), bisulfite treated DNA was amplified with tagged primers (shown below, FOXP3_AMP5-fw and FOXP3_AMP5-rev) using AmpliTaq Polymerase (Life Technologies, Carlsbad, USA) and following settings: 5 min denaturation at 95°C, first 14 cycles touchdown from 63°C to 56°C following 40 cycles with 95°C for 20 s, 56°C for 1 min, 72°C for 1 min and a final elongation for 5 min at 72°C.

FOXP3_AMP5_fw: 5-*CTTGCTTCCTGGCACGAG*TGTTTGGGGGTAGAGGATTT-3

FOXP3_AMP5_rev: 5-*CAGGAAACAGCTATGAC*TATCACCCCACCTAAACCAA-3

PCR products were purified by QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany) using the standard protocol. Afterwards, sample-specific sequences [(MIDs) multiplex identifiers] and universal linker tags (454 adaptor sequences, A- or B-primer) were added in a second PCR, with the same setting as described above.

PCR products were purified using QIAEX II Gel Extraction Kit and DNA concentration was measured with the NanoDrop ND-1000 Spectrophotometer (ThermoScientific, Wilmington, USA). Amplicons were purified using the Agencourt AMPure XP Beads (Beckman Coulter, Krefeld, Germany) system according to the protocol recommended by the manufacturer (Roche Amplicon Library Preparation Method Manual) and quantified by NanoDrop ND-1000 Spectrophotometer. The bisulfite amplicons were diluted, pooled, clonally amplified in an emulsion PCR (emPCR) and sequenced on the Roche/454 GS junior system according to the manufacturer's protocol (Roche emPCR Amplification Method Manual—Lib-A and Roche Sequencing Method Manual). Methylation analysis with NGS was not performed on cells treated with 50 µM EGCG due to strong toxic effects after 7 days of incubation.

Cell viability

Cultured cells were centrifuged for 5 min at 1200 rpm, resuspended in 200 µl FACS buffer and supplemented with 2,5 µl of 7-AAD viability staining solution. 7-AAD measurement was performed after 5 min incubation in the dark by FACS.

Proliferation and suppression assay

For assaying proliferation, $CD4^+CD25^-$ T cells ($2x10^5$) treated with 5-Aza-dC and EGCG for four days were stained with eFluor670 and cultured for three more days in the presence of 1 µg/ml soluble anti-CD3 and $5x10^4$ $CD11c^+$ dendritic cells of the same blood donor. Analysis of cell proliferation was performed in 96- well flat-bottom plates filled up to a final volume of

200 µl with IMDM medium (Life Technologies, Carlsbad, USA) containing 10% fetal calf serum using FACS.

For analysis of suppressive function, $2x10^5$ 5-Aza-dC and EGCG treated cells were co-cultured with eFluor670 stained freshly sorted $CD4^+CD25^-$ responder T cells at a ratio of 1:1 and $CD11c^+$ dendritic cells in presence of anti-CD3 antibody (1 µg/ml). Analysis was performed after 72 h of cultivation.

Statistical Analysis

Statistical analysis was performed with GraphPadPrism 5.0 software (Graph Pad Software, La Jolla, CA). $p<0.05$ was constituted as significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

Analysis of global DNA methylation

5-Aza-dC treatment significantly reduced global DNA-methylation to about 60% of the untreated cells at both concentrations, ($p<0.001$ for 5 µM 5-Aza-dC and $p<0.01$ for 1 µM 5-Aza-dC). 50 µM EGCG treatment reduced global DNA methylation to 80% of the untreated cells ($p<0.05$). No global hypomethylation was detected for the lower EGCG concentration of 5 µM (Figure 1).

Methylation status of *FOXP3-TSDR*

Treg cells with stable suppressive function are characterized by an unmethylated *FOXP3-TSDR* and stable expression of FOXP3.[9] Therefore, we analyzed the potency of these hypomethylating agents for induction of a Treg specific methylation pattern within this crucial gene region. Freshly isolated or untreated cultured human $CD4^+CD25^-$ T cells are completely methylated within *FOXP3-TSDR* as quantified by methylation-sensitive *FOXP3-TSDR QAMA* assay, while Treg cells are almost completely unmethylated (Figure 2a). Four days of culture with 1 µM and 5 µM 5-Aza-dC significantly reduced DNA methylation within *FOXP3-TSDR* by

8-9% using ($p<0.01$ for both concentrations) as quantified by QAMA. Incubation of cells for seven days led to further reduction of *FOXP3-TSDR* DNA-methylation of 15-20 % depending on the 5-Aza-dC concentration, with $p<0.001$ for both concentrations. In contrast to 5-Aza-dC, the 5 µM and 50 µM EGCG was not sufficient for inducing relevant hypomethylation within *FOXP3-TSDR*.

We used deep amplicon next generation sequencing (NGS) to determine the methylation pattern of *FOXP3-TSDR* induced by 5-Aza-dC. NGS showed a scattered methylation profile of the CpGs within *FOXP3-TSDR* within individual sequence reads, demonstrating that 5-Aza-dC does not induce demethylation of the entire region in most cells (Figure 2b). The average demethylation of 5-Aza-dC treated cells was 25-50% within this region as determined by NGS. The discrepant results obtained by both methods can be explained by the presence of the scattered methylation pattern. The QAMA assay was originally designed for the quantification of physiologically existing cells which are either completely unmethylated (Treg cells) or fully methylated (other human $CD4^+$ T cells) within this region and does not adequately capture scattered methylation patterns. [22] NGS also confirmed the failure of the low EGCG concentration for induction of hypomethylation within *FOXP3-TSDR*.

Cell viability

Since hypomethylating agents may have toxic effects, we analyzed viability of cultured cells using 7-AAD staining. Both concentrations of 5-Aza-dC and 5 µM EGCG showed fewer toxic effects compared to 50 µM EGCG, which showed strong toxic effects with about 97% of 7-AAD positive cells after seven days of culture. 5 µM EGCG showed about 66% 7-AAD positive cells compared to about 20% AAD-positive cells of untreated $CD4^+CD25^-$ T cells after four and seven days of culture (Figure 3).

Analysis of Treg cell specific gene expression

Unmethylated *FOXP3-TSDR* has been described to be required for stable expression of FOXP3 [23]. We analyzed 5-Aza-dC and EGCG treated $CD4^+CD25^-$ T lymphocytes for mRNA

expression of FOXP3. Additionally, we analyzed gene expression of the Treg cell specific molecule GARP (glycoprotein A repetitions predominant (GARP or *LRRC32*)), which has been described to be specific for activated human Treg cells [24] and important Treg cytokines IL-10 and TGFβ. FOXP3 mRNA expression was significantly higher in 5-Aza-dC cultured cells, which was 3-4 fold higher after four days of culture and 7-10 fold higher after 7 days of culture (Figure 4). EGCG cultured cells did not show a substantial increase in FOXP3 expression.

5-Aza-dC treatment increased GARP mRNA expression 6-7 fold, (1 µM 5-Aza-dC $p<0.05$), while EGCG did not have relevant effects on GARP expression. IL-10 mRNA was strongly upregulated by 5-Aza-dC (20-fold for 1 µM and 270-fold for 5 µM). Protein expression of FOXP3 was clearly upregulated in 5-Aza-dC treated cells after four and seven days compared to EGCG and untreated T cells.

Besides hypomethylation of $CD4^+CD25^-$ T cells, incubation with 5-Aza-dC induces Treg cell phenotype in these cells by strong expression of FOXP3 and GARP as well as of IL-10 and TGFβ. EGCG does not induce Treg cell phenotype from $CD4^+CD25^-$ T cells.

Functional characterization

A reduced proliferative capacity and suppressing the function of $CD4^+CD25^-$ T effector cells represent characteristic features of Treg cells. Gaining a Treg cell specific phenotype with expression of Treg cell specific genes, we asked if 5-Aza-dC induced cells show Treg cell specific function. Proliferative capacity and suppressive effect on responder cells was analyzed after 4 days of cultivation with hypomethylating agents. Proliferation was lower in 5-Aza-dC treated cells but not in 5 µM EGCG treated cells measured after 3 days (Figure 5). There was no suppressive effect by inhibiting T cell proliferation of $CD4^+CD25^-$ responder T cells for both substances after 3 days of cultivation (Figure 6).

Gene expression of lineage-specifying factors of Th1, Th2, Th17 and Treg cells and their leading cytokines

Apart from studying specific Treg cell molecules induced by 5-Aza-dC and EGCG, we analyzed gene expression of lineage-specifying transcription factors of Th1, Th2 and Th17 cells in 5-Aza-dC treated cells. None of the master transcription factors of these cell types was significantly differently expressed compared to untreated control cells, although mean expression of TBX21 and RORγT was higher after 5-Aza-dC treatment compared to untreated cells (Figure 7). The leading cytokines of the different $CD4^+$ T cell subtypes are IFNγ for Th1, IL-4 for Th2 and IL17a for Th17 cells. The gene expression of the analyzed cytokines is shown in Figure 8. IL-10, which is produced by Treg cells as well as of Th1 and Th2 cells, IL-4, produced by Th2 cells and IL-17, expressed by Th17 cells, were significantly upregulated. IFNγ was also expressed to a higher extent.

Analysis of global Treg-specific gene expression by microarray analysis

To get deeper insights into relevant gene expression changes induced by 5-Aza-dC and EGCG, we performed global transcriptome microarray analysis. We used freshly isolated $CD4^+CD25^-$ T and Treg cells, activated $CD4^+CD25^-$ T and Treg cells, and activated $CD4^+CD25^-$ T cells treated with either 5-Aza-dC at 1 µM or 5 µM and EGCG. Genes were excluded from further analysis if expression level was judged as “not detected” by the software in all T cell subsets analyzed. Relevant regulated Treg-specific genes were defined by an at least 2-fold signal change between freshly isolated Treg and $CD4^+CD25^-$ T cells and/or activated Treg and $CD4^+CD25^-$ T cells. Analysis showed 10745 differentially expressed genes. Compared to activated Treg cells as reference, global gene expression of Treg-regulated genes of activated $CD4^+CD25^-$ T cells is clearly separated from Treg cells (Figure 9). Expression of Treg-regulated genes in $CD4^+CD25^-$ T cells treated with 5-Aza-dC is obviously in-between activated Treg and $CD4^+CD25^-$ T cells, indicating a partial switch from Th towards Treg cells. Gene expression of EGCG-treated $CD4^+CD25^-$ T cells overlaps for most genes compared to untreated activated $CD4^+CD25^-$ T cells.

Discussion

The epigenetic status of *FOXP3-TSDR* is crucial for stable expression of FOXP3, the lineage specifying transcription factor of Treg cells.[6, 23] The *FOXP3-TSDR* is completely unmethylated in Treg cells with stable FOXP3 expression and suppressive function, whereas it is methylated in all other major blood cells, including non-regulatory T cells.[7]

We performed an *in-vitro* study to evaluate the capability of demethylating agents to hypomethylate *FOXP3-TSDR* and transform $CD4^+CD25^-$ T cells into Treg cells. We used two different DNMT inhibitors, 5-Aza-dC and EGCG. 5-Aza-dC is a derivative of 5-Azacytidine. While 5-Aza-dC is incorporated only into DNA, 5-Azacytidine is also incorporated into tRNA[25], additionally inhibits tRNA methyltransferases and interferes with tRNA methylation and processing.[26]

In 5-Aza-dC treated cells we found both, *FOXP3-TSDR* hypomethylation and strong FOXP3 expression. Furthermore, *LRRC32*, encoding GARP, was strongly upregulated upon treatment. GARP is a receptor for latent TGFβ and is specifically expressed in activated human regulatory T cells.[24] In contrast to FOXP3, which is transiently also expressed by activated non-regulatory T cells, GARP has been described as genuine marker of activated human Treg cells.[27] Two further characteristic features of Treg cells, expression of the cytokine IL-10 and reduced proliferation, were also found in 5-Aza-dC treated cells. However, we cannot exclude, that reduced proliferation is at least in part a consequence of toxic effects of 5-Aza-dC. In summary, 5-Aza-dC treated cells show *FOXP3-TSDR* hypomethylation, expression of Treg cell specific marker molecules and reduced proliferation rates thus resembling typical features of Treg cells.

On the other hand, 5-Aza-dC treated cells did not suppress responder T cells, which is a crucial functional characteristic of Treg cells. This is in line with a report of Costantini et al. who did not find a suppressive effect of 5-Azacytidine treated T cells on T effector cells.[16]

The incomplete transformation into Treg cells is also indicated by increased expression of Th1 and Th17 master transcription factor genes *TBX21* and *RORγt* and their leading cytokines *IFNγ* and *IL-17*. *TBX21* and *IFNγ* have been shown to exhibit DNA-

hypomethylation in Th1 cells, *RORγt* and *IL17A* in Th17 cells.[28] As we showed that 5-Aza-dC induces global DNA hypomethylation within the genome, it is reasonable to assume, that the increased expression of these genes might be a result of 5-Aza-dC treatment. The incomplete transformation into Treg cells is also corroborated by only a partial switch from the Th-like global gene expression profile of Treg regulated genes towards the Treg-specific expression pattern, as determined by microarray analysis. Summarized, 5-Aza-dC treated human $CD4^+CD25^-$ T cells resemble Treg cells, but incomplete Treg cell phenotype with increased expression of TBX21 and RORγt and lack of the suppressive function do not justify to classify them as Treg cells.

EGCG, the second hypomethylating agent used for *in-vitro* induction of Treg cells in this study, has been reported to upregulate FOXP3 expression in $CD4^+$ Jurkat T cells.[21] We confirmed increased FOXP3 expression in Jurkat cells upon treatment (data not shown), but did not detect considerable expression of FOXP3 in EGCG treated human $CD4^+CD25^-$ T cells. In our study, EGCG did not reduce *FOXP3-TSDR* methylation and failed to induce a Treg cell phenotype in human sorted $CD4^+CD25^-$ T cells as relevant expression of *FOXP3, LRRC32* and *IL-10* was not displayed and treated cells did not show suppressive function. In line with these results, EGCG obviously did not change Th-like global gene expression of Treg-regulated genes as analysed by microarray. Although both concentrations of EGCG used did not generate hypomethylation within *FOXP3-TSDR*, the toxic effect was much more pronounced compared to 5-Aza-dC. This suggests that EGCG does not qualify for induction of human Treg cells.

5-Aza-dC and EGCG treated cells both did not suppress proliferation of responder cells, which hinders also 5-Aza-dC *in-vitro* generated cells to be considered for Treg cell therapy. There is concern, that T cells mimicking Treg cells phenotypically, but do not have stable suppressive functions, may dampen the protective effect of transferred cells or may have harmful effects with respect to GvHD. Which cells to transfer and how many cells are needed

for sufficient prevention of GvHD, is unclear,[29] but there is consent, that Treg cells with stable suppressive function are needed. As FOXP3 is also expressed by non-regulatory T cells following T cell activation, its sole expression does not assure suppressive function. An unmethylated *FOXP3-TSDR* is an even more specific feature of Treg cells with suppressive function.[7] But as shown in this work, the *in-vitro* hypomethylation of *FOXP3-TSDR* as induced by 5-Aza-dC in $CD4^+CD25^-$ T cells does not mediate suppressive function. However, most 5-Aza-dC treated cells did not show completely unmethylated *FOXP3-TSDR*, as found in natural Treg cells. It is worth analysing, if the Treg cells, expanding in 5-Azacytidine treated patients with acute myeloid leukaemia are fully functional Treg cells or if they just mimic these cells without having suppressive function.

Although not all genes epigenetically regulated by DNA methylation are known so far, the differences of the DNA methylation status within lineage specifying transcription factors and leading cytokines of different T cell lineages implies that hypomethylating agents, inducing nonspecific hypomethylation within the genome may affect transcriptional activity of these genes. Just recently, a targeted DNA demethylation of specific CpGs in human cells using fusions of engineered transcription activator-like effector (TALE) repeat arrays and the TET1 hydroxylase catalytic domain has been reported.[30] This method, applied to crucial genomic regions of Treg cells might be a more specific and promising approach for the *in vitro* generation of Treg cells.

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RT, JK, RG and MZ performed the experiments, JK, JB and JS designed the study, JK, RT and MPK analysed the data, JK and MZ wrote the paper.

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Disclosures:

None of the authors has any potential financial conflict of interest related to this manuscript.

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Figure legends

Figure 1: Analysis of global DNA methylation in 5-Aza-dC and EGCG cultured $CD4^+CD25^-$ T cells. Cells were cultured with two different concentrations of 5-Aza-dC and EGCG and stimulated with anti-CD3 and anti-CD28 mAb before cultivation. DNA-methylation was normalized to anti-CD3 and anti-CD28 stimulated $CD4^+CD25^-$ T cells, not treated with hypomethylating agents. The DNA-methylation of these cells was set 100%.

Figure 2a: *FOXP3-TSDR* methylation anaylsis by methylation sensitive qRT-PCR. Quantification of methylated and unmethylated DNA using specific Taqman probes in QAMA qRT-PCR. $CD4^+CD25^-$ T cells stimulated with DNMT inhibitors for 4 days (white bars) and 7 days (black bars).

Figure 2b: *FOXP3-TSDR* methylation by Next Generation Sequencing (NGS). Quantification of methylated and unmethylated DNA using NGS, blue color indicates unmethylated CpG, red color indicates methylated CpG. Mean methylation of all CpGs and sequence reads is shown besides.

Figure 3: Cell viability

$CD4^+CD25^-$ T cells were cultured with 5-Aza-dC and EGCG and were analyzed for viability after 7-AAD staining.

A Gate setting is shown from one representative experiment

B Cell viability after 4 days (white bars) and 7 days (black bars)

Figure 4 Expression analysis of Treg cell specific genes and FOXP3 protein expression

Expression of Treg cell specific genes and FOXP3 protein expression was analyzed after four (white bars) and seven (black bars) days of stimulation with hypomethylating agents 5-Aza-dC and EGCG.

Figure 5: Proliferation of $CD4^+CD25^-$ T cells. 5-Aza-dC and EGCG treated $CD4^+CD25^-$ T cells were stained with eFluor670 proliferation dye, stimulated with anti-CD3 and dendritic cells, incubated for 72 hours and analyzed by FACS.

Figure 6: Suppression assay. 5-Aza-dC and EGCG stimulated $CD4^+CD25^-$ T cells were co-cultured with responder T cells (stained with eFluor670 proliferation dye) and dendritic cells in the presence of CD3 mAb for 3 days and analyzed by flow cytometry. Unstimulated $CD4^+CD25^-$ T cells co-cultured with responder T cells were used as control population. Degree of inhibition was determined by measuring proliferative capacity of cultured cells compared to the control population.

Figure 7 Effects of demethylating agents on T cell lineage-specifying transcription factor gene expression

mRNA expression was quantified by real-time PCR (black bars) and gene-array analysis (grey bars) after stimulation with 5-Aza-dC and EGCG for 7 days. Array data did not include GATA3.

Figure 8 Effects of demethylating agents on leading cytokine expression

Expression was quantified by real-time PCR (black bars) and by mRNA expression by array analysis (grey bars) after stimulation of CD4+CD25- T cells with 5-Aza-dC and EGCG for 7 days.

Figure 9 Effects of demethylating agents on expression profiling of Treg-regulated genes. Gene expression microarray analysis of $CD4^+CD25^-$ T cells treated with either 5-Aza-

dC at a final concentration of 5 μM [5-AZA (5 μM), blue triangle] or 1 μM [5-AZA (1 μM), pink triangle] and EGCG [EGCG (5 μM), black triangle]. Genes with at least 2-fold change of expression intensity between regulatory T cells and $CD4^+CD25^-$ T cells are considered. Gene expression intensity for Treg cell genes is shown on x-axis, gene expression intensity of the comparative cell on y-axis. This results in a bisecting red line for Treg cells as comparative population. Green diamonds show the $CD4^+CD25^-$ T cells [$CD4^+CD25^-$ (d7)], compared with Treg cells.

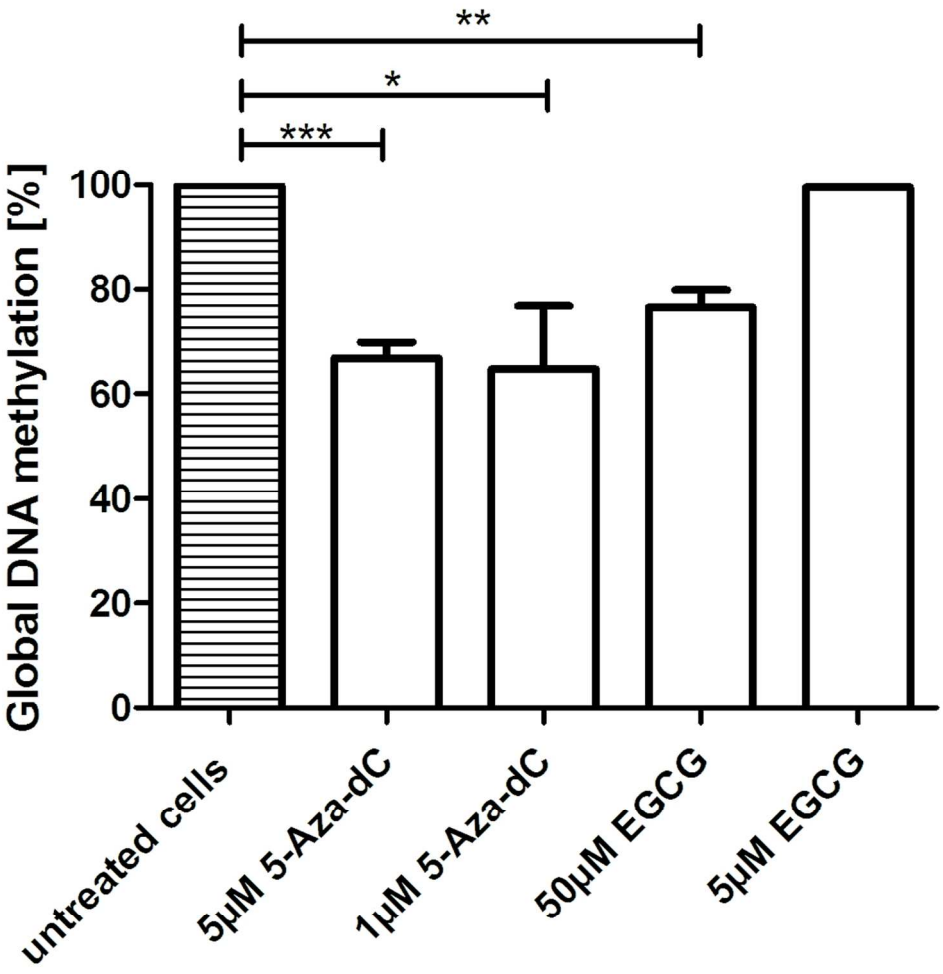

Figure 1

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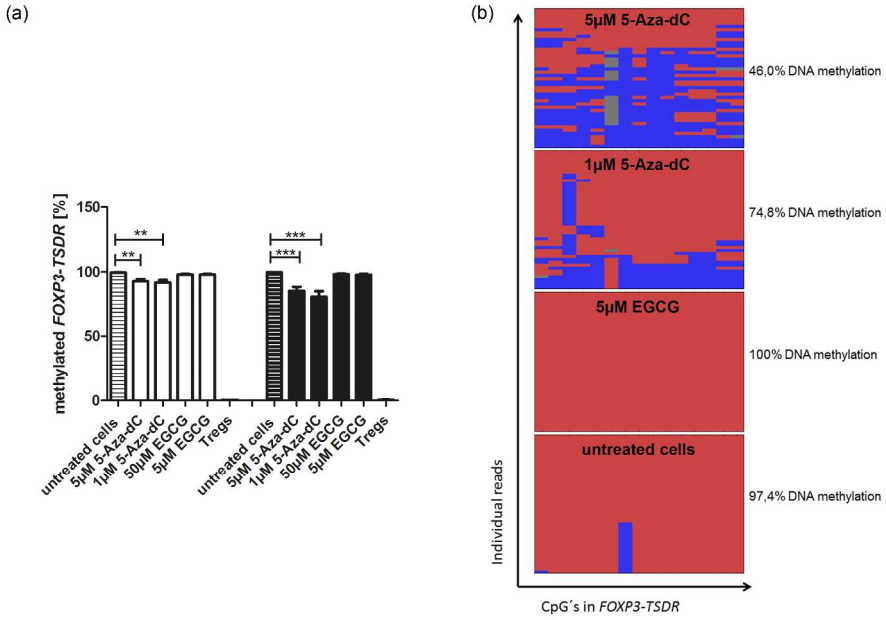

Figure 2

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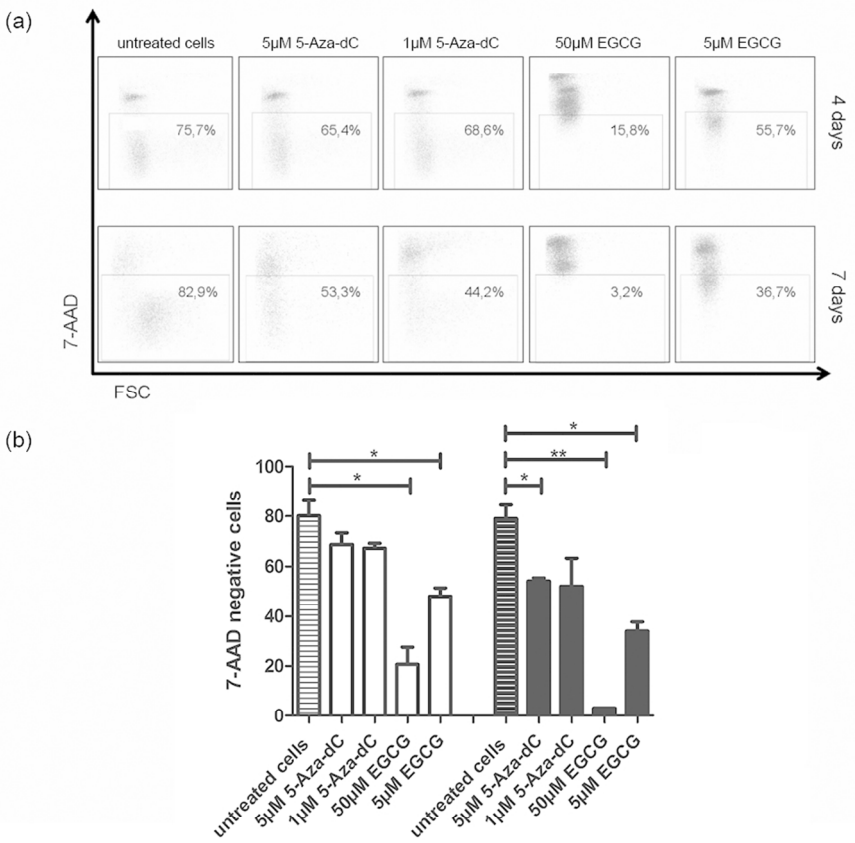

Figure 3

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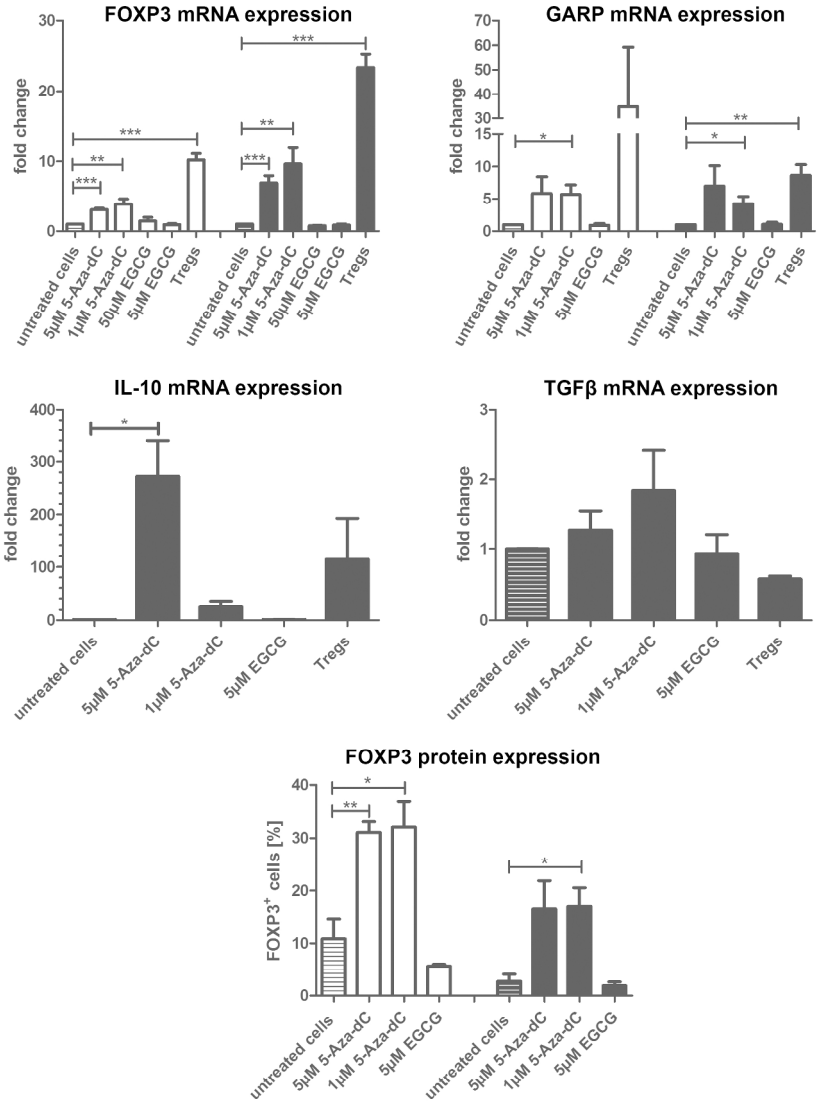

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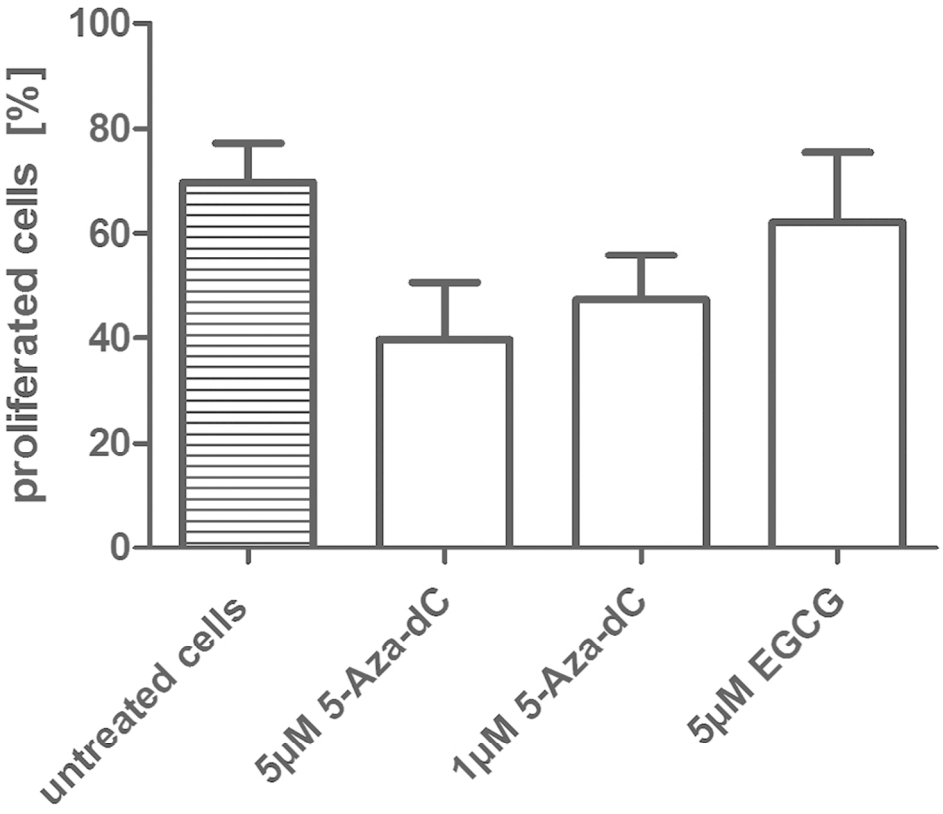

Figure 5

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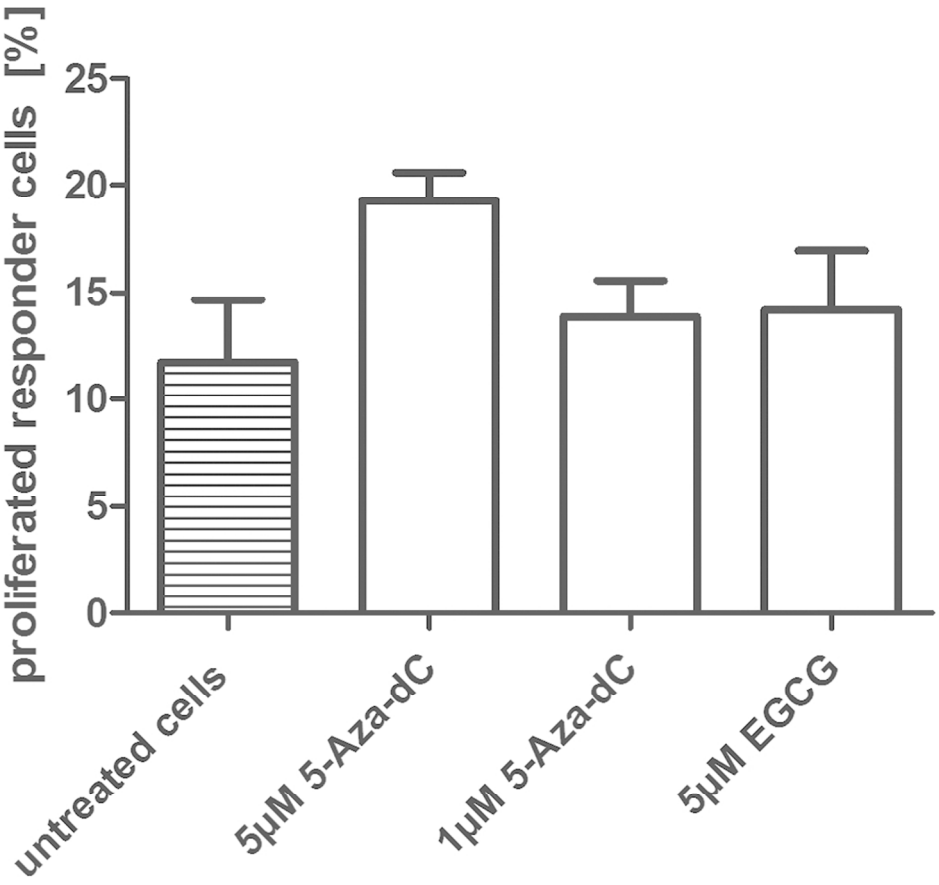

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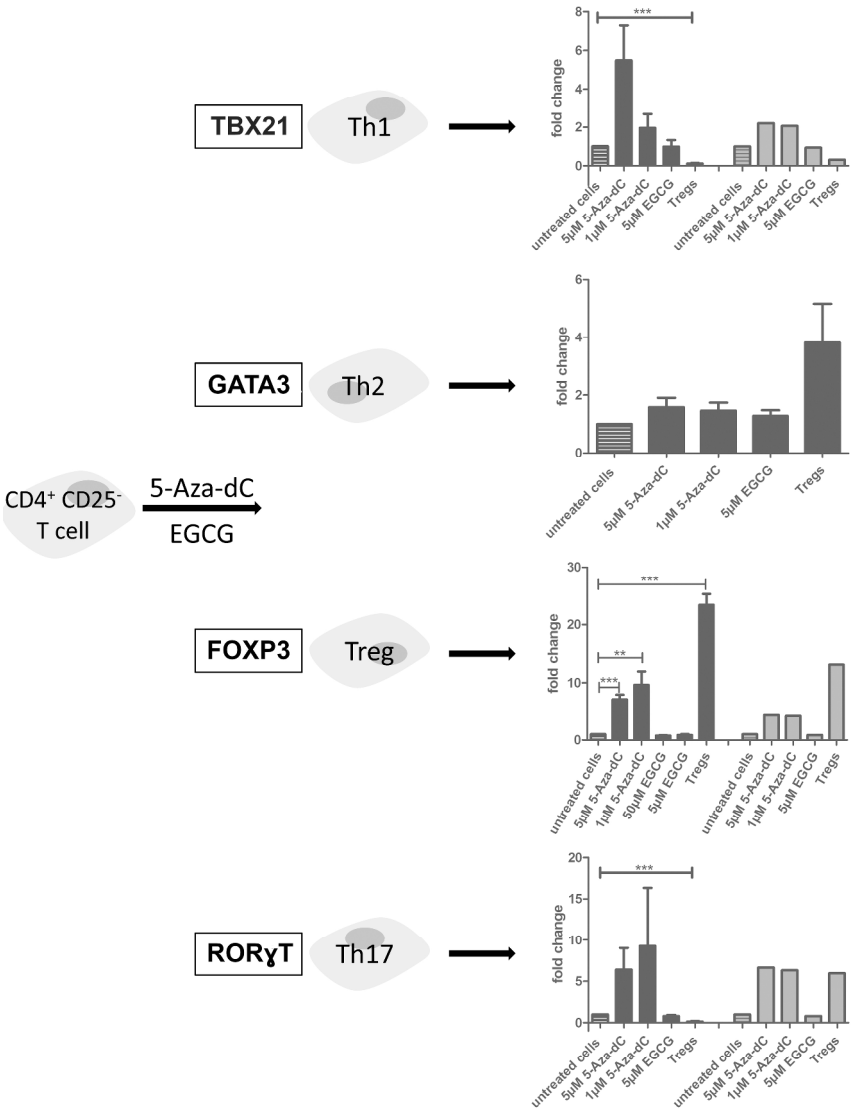

Figure 7

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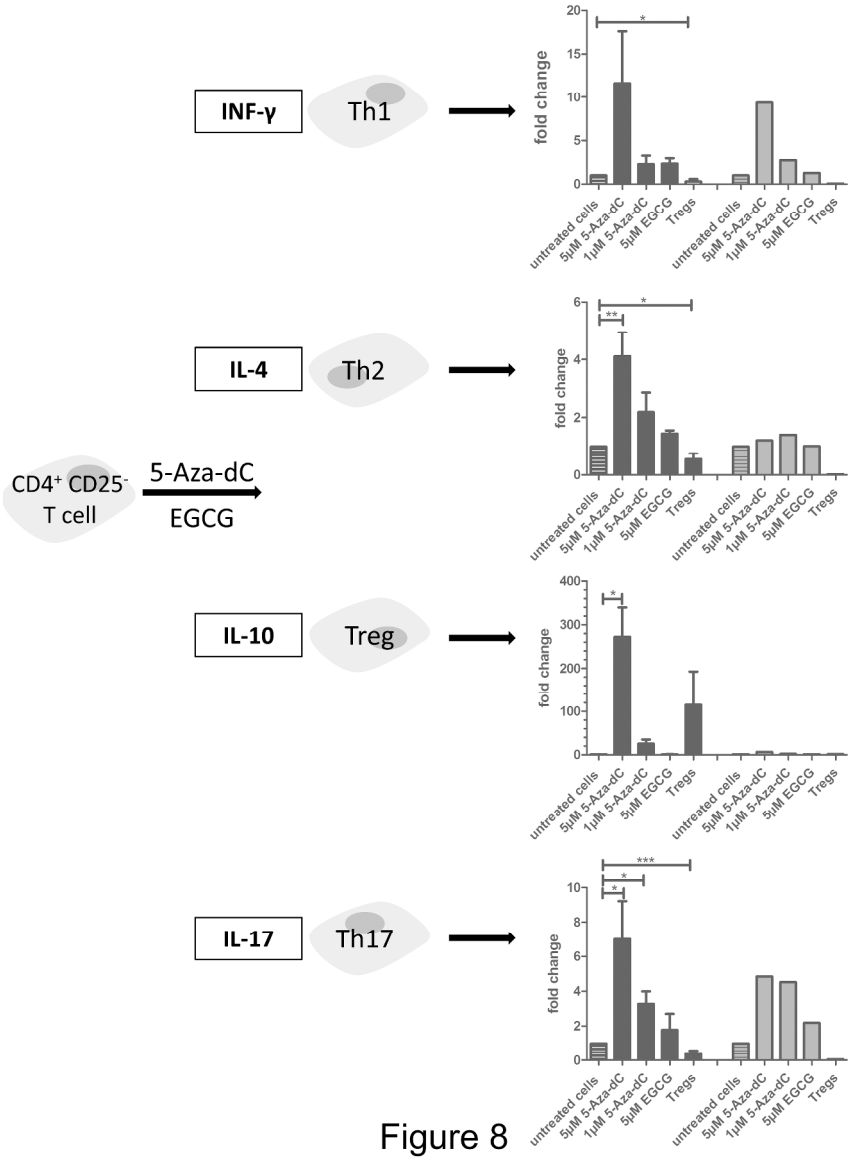

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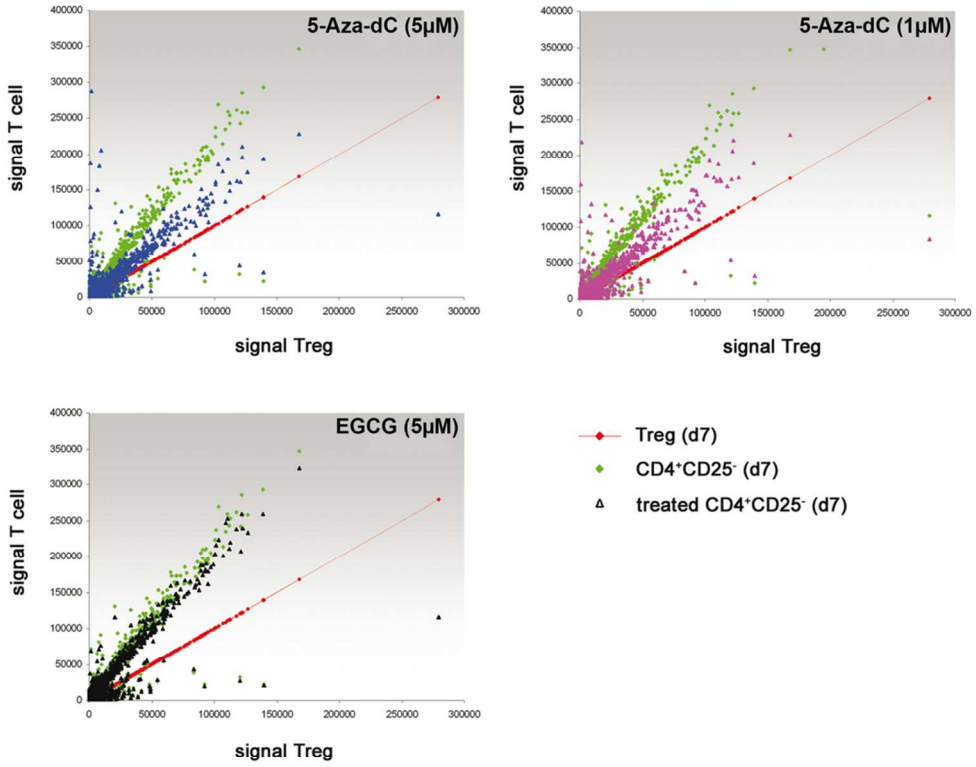

Figure 9

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