Supplementary Information for: Microenvironment tailors nTregs structure and function.

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SUPPLEMENTARY TEXT

Materials and Methods.

1) Human blood samples: Peripheral Blood samples were obtained either from healthy donors through Etablissement Français du Sang (EFS, Paris, France) or from patients with amyopathic dermatomyositis (DMa, n=12), rheumatoid arthritis (Rha, n=18) and relapsed acute myeloblastic leukemia (AML) after hematopoietic stem cell transfer (HSCT) (AML, n=10). AML patients were enrolled in the European clinical Trial EUDRACT number 2012-005535-90. Umbilical cord blood (UCB) samples were obtained from normal term deliveries, after maternal informed consent and stored in the cord blood bank according to approved institutional guidelines (Cellular therapy unit, Saint Louis hospital, Paris, Assistance Publique–Hôpitaux de Paris, France). Blood cells were collected using standard procedures. The study was performed according to the Helsinki declaration, and the study protocol was reviewed and approved by the local Ethics Committee. All samples were de-identified prior to use in this study.

2) Cell purification and culture. Peripheral blood mononuclear cells (PBMCs) and UCB were isolated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia, St Quentin en Yvelines, France). PBMCs were used either as fresh cells or stored frozen in liquid nitrogen. CD4+ T cell subsets and T cell-depleted accessory cells (ΔCD3 cells) were isolated from either fresh or frozen PBMCs or UCB. All CD4+ T cells were positively selected with a CD4+ T cell isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany), yielding CD4+ T cell populations at a purity of 96–99%. Subsequently, selected CD4+ T cells were labelled with anti-CD4 (RPA-T4)-BV510 (Biolegend), anti-CD25 (B1.49.9)-PC5.5 (Beckman Coulter), and anti-CD127 (R34.34)-PE (Beckman Coulter), anti-CD45RA (REA562)-FITC (Miltenyi), anti-CD39 (A1)-PC7 (Biolegend) and anti-CD26 (Ba5b)-PC5 before being sorted into nTreg (CD4+CD127-/lowCD25high) and Tconv (CD4+CD127+CD25neg/dim) subpopulations using a FACSARIAII Cell Sorter (Becton Dickinson, Le Pont Claix, France). NTregs can be further subdivided into 5 major subpopulations based on CD39 and CD26 markers: naive N1 (CD45RA+ CD26+ CD39-), memory RA- CD26+ CD39- (M1), RA- CD26- CD39- (M2), RA- CD26+ CD39+ (M3) and RA- CD26- CD39+ (M4). Tconvs can be subdivided into 2 major subpopulations: nTconv (RA+ CD25-), mTconv (RA- CD25-). Postsort analysis confirmed that the purity for each cell type was routinely greater than 90% and that more than 90 % of sorted nTreg expressed FOXP3. T cell-depleted accessory cells (ΔCD3 cells) were isolated by negative selection from PBMCs by incubation with anti-CD3-coated Dynabeads (Dynal Biotech, Oslo, Norway) and were irradiated at 5000 rad (referred to as ΔCD3-feeder). CD8+ T cells were isolated with a Miltenyi MACS kit using positive selection, following the manufacturer's protocol.
Immature DCs (iDCs) were generated from MACS-isolated CD14+ human monocytes by 6-day cultivation with 20 ng/mL of GM-CSF and IL-4. Their maturation (mDC) was induced by stimulation with LPS (100 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) for an additional 48h.
Purified nTreg and Tconv cell subsets were cultured either separately or cocultured in IMDM medium containing 2 mM L-glutamine, 100 U/mL penicillin-streptomycin, 1 mM sodium pyruvate and 10% human AB serum, (referred to as complete medium) (Invitrogen, Cergy-Pontoise, France) in either 96-well U-bottom plates or in 1.0-µm pore size 96-well HTS transwell plates (Falcon/Becton Dickinson). All cultures were incubated at 37°C with 5% CO2 and 95% air. Cells were stimulated with plate-bound or soluble anti-human CD3

(OKT3) mAb (eBioscience, San Diego, CA) at the specified concentrations of 0.5 or 4 µg/mL, in the presence or absence of ΔCD3-feeder. For plate-bound CD3 stimulation, 100 µL of the anti-CD3 mAb diluted into PBS (Invitrogen) were added to each culture well, placed at 4°C for 16 h, and then washed twice with PBS. In some cultures, recombinant human IL-2 (Proleukine, Chiron, Amsterdam), soluble anti-human CD28 (CD28.2) mAb (Becton Dickinson, 0.5-4 µg/mL), plate-bound anti-human CD46 Ab (clone E4.3, Becton Dickinson, 10 µg/mL) were added. Stimulated nTreg subsets were irradiated (2000 rad) before their coculture with Tconv cells when indicated.

3) Flow cytometry analysis:

a) **mAb labelling**. A multicolor immunophenotyping approach was used for the identification and analysis of different lymphocyte subpopulations. Immunophenotypic studies were performed on fresh or frozen samples, using 11 to 18-colour flow cytometry. Six common membrane markers and a viability dye were constantly present in all aliquots: CD4, CD45RA, CD25, CD14, CD26 and CD39. These antibodies were used as a stand-alone combination or as a common marker backbone to which we have added the following additional antibodies for specific questions : FOXP3-PECF594 (236A/E7), CD15s-BV711 (CSLEX1), CD45RA-BV650 (HI100), CTLA4-BV421 (BNI3), HLADR-BV786 (G46.6), HLADR-BUV395 (G46.6), PD1-BUV737 (EH12.1), CD3-BV711 (UCHT1), CD3-BB515 (UCHT1), CD45-BV711 (HI30), CD25-BV786 (M-A251), AnnexinV-PE, FCRL3-biotin (H5), streptavidin-BUV737, CD45RO-BV786 (UCHL1), CD95-FITC (DX2), CD80-PE (L307), IL10-PE (JES3-19F1) and pSTAT5-PE (47/Stat5) were obtained from BD Biosciences ; TIGIT-PerCPeF710 (MBSA43), Ki67-PE (SolA15) and CD4-PerCPeF710 (SK3) were obtained from eBiosciences ; GARP-PE (REA166), CD25-APC (REA570) and CD25-PE (REA570) were obtained from Miltenyi ; IL17-BV711 (BL168) was obtained from Biolegend ; CD127-APCAF700 (R34.34) was obtained from Beckman Coulter and IL17 mRNA-AF488 (type 4) and IL10 mRNA-AF647 (type 1) were obtained from Thermofisher. Cells were stained for membrane markers (at 4°C in the dark for 30 min) using cocktails of Ab diluted in PBS containing BSA/NaN3 (0.5% BSA, 0.01% NaN3) (FACS buffer). FOXP3 intracellular staining was performed according to the manufacturer's instructions. Appropriate isotype control Abs were used for each staining combination. Samples were acquired on BD LSR-Fortessa flow cytometer using FACSDiva software (Beckton Dickinson). Flow data were analysed using FlowJo software (FlowJo, LLC) and the cloud-based software cytobank viSNE (Cytobank.org). viSNE analysis were performed on cells gated on CD3+ CD4+ FOXP3+ CD127-/low T cells with the following settings: 5000 iterations, perplexity 50 and theta 0.25. Markers used as the clustering channels were FOXP3, CD45RA, CD25, CD26 and CD39.

b) **CFSE staining**. nTregs or Tconvs were stained with 1 µM CFSE (CellTrace cell proliferation kit; Molecular Probes/Invitrogen) in PBS for 8 min at 37°C at a concentration of 1 X 10^6 cells/mL. The labelling was stopped by washing the cells twice with RPMI-1640 culture medium containing 10% FBS. The cells were then re-suspended at the desired concentration and subsequently used for proliferation assays.

c) **7-AAD staining**. Apoptosis of stimulated CFSE-labelled or unlabelled nTregs and Tconvs was determined using the 7-AAD assay (1). Briefly, cultured cells were stained with 20 µg/mL nuclear dye 7-amino-actinomycin D (7-AAD; Sigma-Aldrich, St-Quentin Fallavier, France) for 30 minutes at 4°C. FSC/7-AAD dot plots distinguish living (FSChigh/7-AAD-) from apoptotic (FSChigh/7-AAD+) cells and apoptotic bodies (FSClow/7-AAD+) and debris ((FSClow/7-AAD-). Living cells were identified as CD3+ 7-AAD− FSC+ cells.

d) **Calcium mobilization**. Purified populations of CD4+ T cells were loaded with the Ca2+ indicator indo-1 and then stained. Samples were stained with the usual backbone of antibody

combinations with anti-CD127 Ab and 0.5 or 5.0 µg of biotinylated anti-CD3ϵ. Baseline calcium levels were established for 8 min prior to addition of 20 µg/mL of streptavidin (crosslinking agent) (arrow). Calcium mobilization was detected by the analysis of the Indo-1 violet-blue fluorescence ratio collected for 20 min by flow cytometry using FACSARIAII analyzer (BD) and the percentage of responding cells in the population was determined.
e) **Cell cycle status through costaining of Ki-67/DNA**. For cell cycle analysis with Ki67 intracellular staining, CD4+ T cells were stained with the usual backbone of antibody combinations, then fixed and permeabilized using the transcription factor staining buffer set (eBioscience) and stained with anti-Ki67 (eBioscience, clone SolA15) and anti-FOXP3 mAbs. 1 µL DAPI (FxCycleTM Violet Stain, Life Technologies) was added directly prior to FACS analysis. The simultaneous analysis of proliferation specific marker (Ki-67) and cellular DNA content discriminate resting/quiescent cell populations (G0 cell) and quantify cell cycle distribution (G1, S, or G2/M), respectively.
f) **Simultaneous detection of RNAs and their encoded protein using the PrimeFlow RNA assay**. Purified CD4+ T cells were stimulated with PMA/ Iono for 5 h in presence of brefeldin during the last two hours. Stimulated CD4+ T cells were stained with the usual backbone of antibody combinations fixed and permeabilized using the transcription factor staining buffer set (eBioscience) followed by intracellular staining with anti-IL-10 and anti-IL-17 and anti-FOXP3 mAbs. The PrimeFlow RNA assay (Ebioscience) was then performed according to the manufacturer's recommendations to detect IL-17 and IL-10 transcripts.
g) **pSTAT5 Analysis**. Purified CD4+ T cells were stimulated with 10 IU/mL of human recombinant IL-2 (Peprotech EC ltd., London). Samples were stimulated for 15 min at 37°C to measure pSTAT5 level in nTreg subsets with the PerFix EXPOSE (Beckman Coulter). Staining was performed with usual backbone of antibody combinations and anti-pSTAT5-PE (Becton Dickinson) and anti-FOXP3 (259D)-PECF594 antibodies. The MFI of the pSTAT5 specific signal in each subset was measured and fold changes in phosphorylation were calculated as the ratio of MFI in stimulated versus unstimulated cells.

4) **RNA sequencing experiments**
RNA extraction: total RNA was extracted from 250,000-1,000,000 sorted cells from N1, M1 and M4 nTregs populations using the miRNeasy Micro Kit (Qiagen) on a QIAcube robotic workstation (Qiagen) according to the manufacturer's instruction. RNA quantification was performed on a Nanodrop 2000c (Thermo Fischer Scientific) and the integrity of the RNA assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, France).
RNA sequencing: total stranded RNA sequencing of 10 nTreg samples was performed by GIGA-Genomics Core Facility (University of Liège, Belgium). Library preparation was performed using 400 ng of total RNA (RIN>8) of each sample with the TruSeq stranded total RNA kit (Illumina) according to manufacturer's instruction. Each barcoded library was diluted and pooled in equimolar ratio on 4 lanes of a single flowcell. Next generation sequencing was performed on a NextSeq 550 (Illumina) and generated ~400 million of 2x75 bp paired-end reads.
Data analysis: FASTQ data were obtained for each sample from demultiplexed and adapter-trimmed RNA-seq raw data. The quality of the sequenced reads was assessed using FastQC (v0.11.5) (2). The resulting reads were then trimmed using Trimmomatic (v0.36) (3) (LEADING=30, TRAILING=30, SLIDINGWINDOW=4:30). The cleaned reads were all mapped to the *Homo sapiens* genome (GRCh37.2) using bowtie2 (v2.2.2) (4) and tophat2 (v2.1.1) (5) with "--max-multihits" parameter set to 1, i.e. each aligned read is mapped at a unique locus in the genome. The resulting bams were deduplicated and sorted by position using picard-tools (v2.8.2) (MarkDuplicates and SortSam) (6). The newly obtained bams were then processed using HTSeq (v0.11) (htseq-count) (7) in order to get raw read counts per

gene. TPM (transcripts per kilobase million) values were then calculated using an in-house script in order to obtain normalized gene expression levels. PCA analysis was performed with "R" software using TPM data. 2D-heatmap representation and hierarchical clustering (Euclidean distance and complete method) were performed with "R" software using log2-transformed TPM data. DESeq2 (v1.18.1) (8) was used to detect differentially expressed genes for each pair of groups (M4 vs M1, N1 vs M1 and M4 vs N1) with an adjusted (BH) P-value < 0.05 and with a minimum fold change of 2. The intersection of these three gene lists allowed us to get the list of genes which had an overall distinct expression level patterns for the three groups. Venn diagram was generated using Venny's online tools (v2.1) (9).

5) **Functional assays**:

a) **T cell activation**. Cell surface expression of CD25 was detected by FACS on CD3+ 7-AAD- CFSE+ stimulated nTreg subsets or Tconvs at the end of the culture. The CD25 expression level per cell (Median Fluorescence Intensity (MFI)) was measured by flow cytometry.

b) **T cell proliferation**. T cell proliferation was assessed by CFSE-dilution assays. At coculture completion, stimulated CFSE-labelled nTregs or Tconvs were harvested, co-stained with anti-CD3 mAb and 7-AAD, and the percentage of proliferating cells (defined as CFSE low fraction) in gated CD3+ 7-AAD- cells was determined by flow cytometry.

c) **nTreg subset cell culture**. To evaluate the effect of the microenvironment on nTreg subset physiology, CFSE-stained nTregs (4 X 10^4/well) were cultured alone or cocultured at a 1:1 cell ratio with Tconvs in the presence of ΔCD3-feeder (4 X 10^4/well) and plate-bound anti-CD3 Ab (0.5 µg/mL). IL-2 (100 IU/mL), soluble anti-CD28 mAb (4 µg/mL) with or without plate-bound anti-CD46 mAb (10 µg/mL) were added where indicated. nTreg cell activation and proliferation were evaluated with the T cell activation and CFSE dilution assays as described above by flow cytometry.

d) **Polyclonal nTreg cell-contact mediated suppression**. CFSE-labelled Tconvs (4 X 10^4/well), used as responder cells, were cultured with ΔCD3-feeder (4 X 10^4/well) in the presence or absence of defined amounts of nTregs (0.4 X 10^4 to 4 X 10^4 cells/well) for 4-5 days. Cultures were performed in round bottom wells coated with 0.5 µg/mL anti-CD3 mAb in 200 µL of complete medium. Varying concentrations of soluble anti-CD28 mAb were added when indicated. Results are expressed either as the percentage of proliferating CFSE low T cells or as a percentage of suppression calculated as follows: (100 × [(percentage of Tconv CFSE low cells -- percentage of Tconv CFSE low in coculture with nTregs)/percentage of Tconv CSFE low cells]).

e) **Ag-specific HLA-DR-restricted nTreg suppressive assay**. Pre-activated CFSE-labelled Tconv cells (4 X 10^4/well) with soluble anti-CD3 (4 µg/mL) and anti-CD28 (4 µg/mL) were stimulated with autologous iDC or LPS mDC (10^5 cells/well of each cell type) in the presence or absence of defined amounts of autologous nTreg subsets (0.4 X 10^4 to 4 X 10^4 cells/well) for 4-5 days.

f) **IL-10-secreting Tr1 suppression.** For assessing Tr1 suppressive activity mediated by soluble factors, the test was performed in 96-well transwell plates with stimulated nTregs or Tconvs at sufficient concentrations as described (10). Briefly nTregs or Tconvs (3 X 10^5 cells/well) were cultured in the bottom chamber of transwell plates in the presence of ΔCD3-feeder (1 X 10^5/well) and plate-bound anti-CD3 mAb (4 µg/mL). Soluble anti-CD28 mAb (4 µg/mL), plate-bound anti-CD46 mAb (10 µg/mL), IL-2 (20 IU/mL) were added when indicated. 24 hours later, cells were washed and fresh complete medium (300 µL) with or without 20 IU/mL IL2 was added as well as transwell inserts in which pre-activated CFSE-labelled Tconv cells (4 X 10^4/well) with soluble anti-CD3 (4 µg/mL) and anti-CD28 (4 µg/mL) and ΔCD3-feeder (4 X 10^4/well) were loaded. Cells were cultured for another 5 days.

Pre-stimulated responder cells were analyzed for CFSE dilution in CD3+ 7-AAD– cells at the end of the culture.
g) **TCR-stimulating DC**: **nTregs interplay assay**. Autologous iDC or LPS DC (5 X 10^4/well) were cultured without or with either nTreg subsets (2 X 10^5/well) pre-activated with soluble anti-CD3 (4 µg/mL), anti-CD28 (4 µg/mL) and IL-2 (100 IU/mL) or anti-CTLA4 Ab (5 ug/mL) for 2 days. When indicated, iDC were stimulated with LPS (100 ng/mL) at the time of nTreg subsets addition. CD80 expression on DC was analyzed by flow cytometry.

6) Cytokines and other cellular proteins quantification.
IL-17, IL-10 and IL-12 levels in cell culture supernatants (SN) were determined by luminex technology. For the IL-2 quantification, a high sensitivity ELISA kit was used (Merck Millipore, Darmstadt, Germany).

7) Bisulfite pyrosequencing.
Primer Design: All primers used in this study are listed and were purchased from Eurofin Genomics. Reverse PCR primers were biotinylated for downstream pyrosequencing experiments. For *FOXP3* upstream enhancer 1 and proximal promoter regions, the pyrosequencing primers were designed using the SNP Primer design software (Qiagen).
Bisulfite conversion: Bisulfite conversion of DNA was performed on DNA from 5,000-100,000 fixed cell using the EZ DNA Methylation -DirectTM Kit (Zymo Research) according to the manufacturer's instruction in an elution volume of 12-20 µL.
PCR amplification: For each region, PCR reactions were performed using 1 µL of bisulfite treated DNA as template in a 20 µL PCR mix including 200 nM of each primer, 1x HotStar Taq DNA polymerase Buffer, 1.6 mM of additional MgCl2, 200 µM of each dNTPs, and 2 U of HotStar Taq DNA polymerase. The reaction was performed in a Mastercyler Pro S (Eppendorf) and the cycling conditions included an initial denaturation step performed for 10 min at 95°C, followed by 50 cycles of 30 sec denaturation at 95°C, 30 sec annealing at Ta and 30 sec elongation at 72°C. The final step included 5 min elongation at 72°C. The optimal primer annealing temperatures were determined for each assay using the same PCR and cycling conditions except for the annealing step performed using a gradient temperature program ranging from 50°C to 70°C, followed by the analysis of 5 µl of the PCR reaction by electrophoresis on a 2% agarose gel.
DNA methylation analysis by pyrosequencing: 10 µL of PCR product were supplemented with 2 µL of Sepharose beads, 40 µL of binding buffer (10 mM Tris–HCl, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20; pH 7.6) and 28 µL of water and incubated under constant mixing (1400 rpm) for 10 min at room temperature. PCR products were then purified and rendered single-stranded using the PyroMark Q96 Vacuum Workstation (Qiagen) after three successive baths of 70 % ethanol, 0.2 M NaOH denaturing solution and 1x washing buffer (10 mM Tris–acetate; pH 7.6). Final elution was performed in a pyrosequencing plate (PyroMark Q96 Plate Low, Qiagen) including 4 pmol of the pyrosequencing primer and 12 µL of annealing buffer (20 mM Tris–acetate, 2 mM Mg–acetate; pH 7.6). DNA methylation analysis was performed using PyroMark Gold SQA Q96 Kit (Qiagen) on a PyroMark Q96 MD (Qiagen) and analyzed with PyroMark CpG software (Qiagen).

Primers used for the DNA methylation analysis are the following:
IL-2 (11): IL2_F: GGAGGAAAAATTGTTTTATATAGAAGG; IL2_R: Biotin-CCTCTTTATTACATTAACCCACACT; annealing temperature 56°C; IL2_pyro_F : AAATTGTTTTATATAGAAGG;

FOXP3 TSDR (12): FOXP3_TSDR_F: TGTTTGGGGGTAGAGGATTT, FOXP3_TSDR_R : Biotin-TATCACCCCACCTAAACCAA, annealing temperature 56°C; FOXP3_TSDR_pyro_F : GATGTTTTTGGGATATAGATTA;

8) Adenosine and inosine measurement by a UPLC-HRMS method. nTreg subsets (1 X 10^5 cells/well) were stimulated with plate-bound anti-CD3 mAb (0.5 µg/mL) in presence of Tconvs (1 X 10^5/well) and ΔCD3-feeder (4 X 10^4/well) for 1 day. Cells were then washed twice in AIM V medium and incubated (120 min, 37 °C) in AIM V medium with 100 µM ATP. Cell supernatants were collected, centrifuged for 2 min at 6000 g, boiled for 2 min to inactivate ADO-degrading enzymes and stored at −80C° for subsequent analysis. The determination of adenosine monophosphate (AMP), adenosine and inosine in the supernatants of cell culture was performed using a liquid chromatography coupled to high resolution mass spectrometry detection (UPLC-HRMS) method, adapted from a published method dedicated to the quantification of urinary modified nucleosides (13)**.** Briefly, a 10 µL aliquot of supernatant (diluted or not) was directly injected in the LC-HRMS system. The absolute quantification of the compounds of interest was performed in positive ionization full scan mode using adenosine monophosphate-13C10 - 15N5, adenosine-13C5 and inosine-15N4 as internal standards. AMP, inosine and adenosine retention times were around 0.9, 1.4 and 2.25 min, respectively, in a total analysis runtime of 6.7 minutes.
Owing to its high polarity, adenosine triphosphate (ATP) was quantified using an ion pairing reverse phase liquid chromatography-tandem mass spectrometry detection (LC-MS/MS) method adapted from a published method addressed to the quantification of intracellular active triphosphorylated forms of nucleoside reverse transcriptase inhibitors (14). The quantification of ATP was carried-out in negative ionization multiple reaction mode using adenosine triphosphate-13C10 - 15N5 as internal standard. ATP retention time was around 6.3 min in a total analysis runtime of 11 minutes.

9) Statistical analysis.
Difference between groups was assessed using Student's T-Test. Values that were not normally distributed were evaluated by the Mann-Whitney U test. For comparisons of median from multiple groups against one control group the Kruskal-Wallis with Dunn's multiple comparison post-test analysis was performed. Error bars on graphs represent either s.e.m. or interquartile range. Statistical analysis was performed using GraphPad Prism. P values under or equal to 0.05 were considered as statistically significant. In the figures, P values are displayed according to the following representation: * $P< 0.05$, ** $P<0.005$, *** $P<0.001$.

SUPPLEMENTARY FIGURES

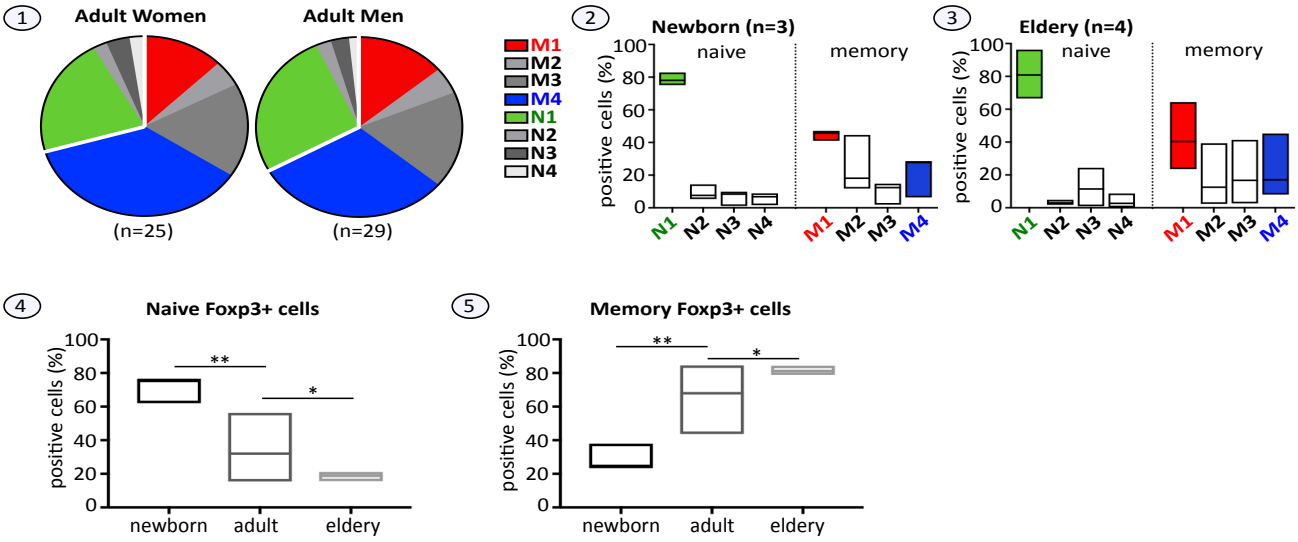

Fig. S1. FOXP3 nTreg heterogeneity in healthy human PBMCs analysis. Human nTreg subsets distribution based on their expression of CD39 and CD26 in naive and memory compartments according to gender **(1)** and age **:** newborn **(2)** and elderly **(3)**. Boxplots illustrating the distribution of naive **(4)** and memory nTregs **(5)** in newborn, adult and elderly. Data shown are expressed as mean +/- SEM. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001.

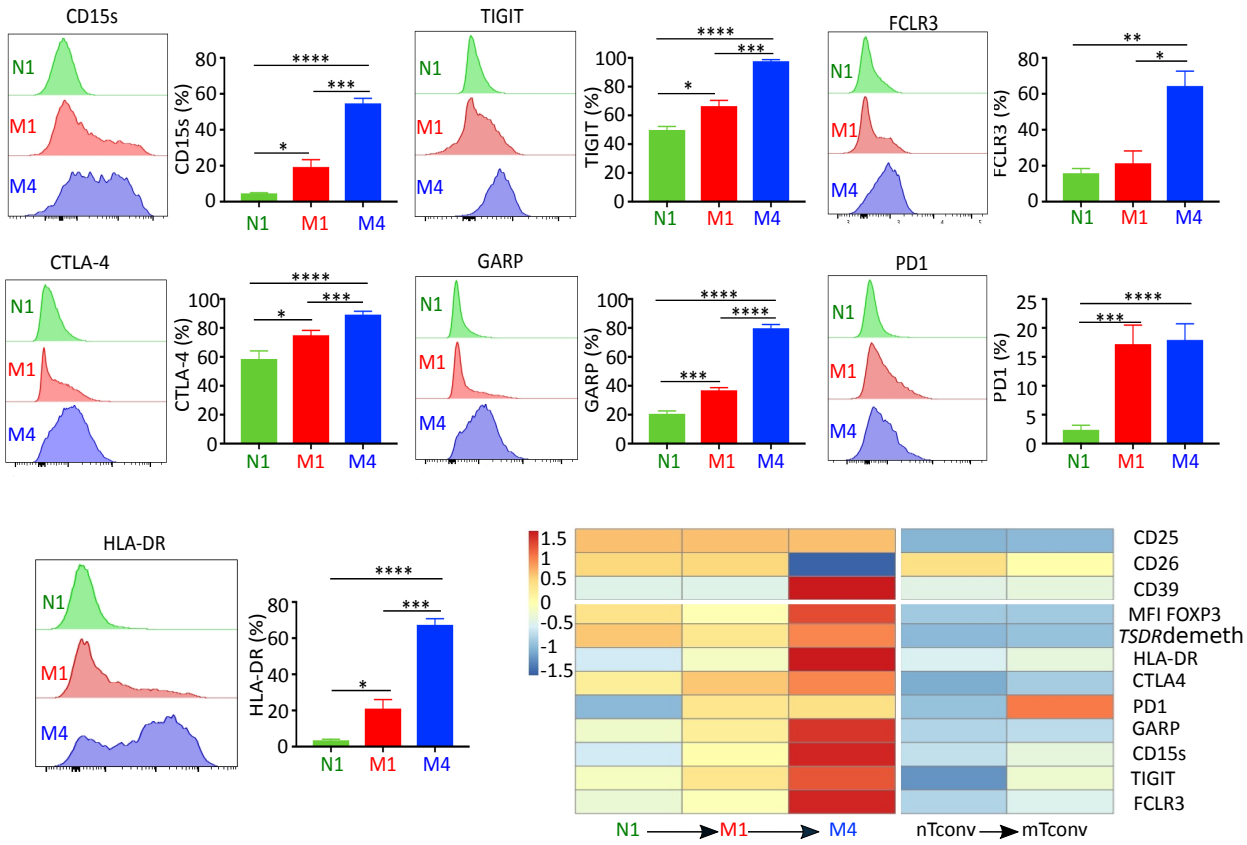

Fig. S2. Expression of regulatory markers is correlated to nTreg cell cycle evolution. Representative dot plot of FACS analysis shows the frequency of regulatory markers in nTreg subsets N1, M1 and M4. Regulatory markers, i.e. TIGIT (n=4), GARP (n=4), PD1 (n=10), HLA-DR (n=4), CD15s (n=4), CTLA4 (n=29) and FCLR3 (n=3) expression was determined and expressed as mean % ± SEM in N1, M1 and M4 T cells. Heatmap representation of the expression of CD25, CD26, CD39, MFI of FOXP3 and the demethylation percentage of the CNS2 locus of *FOXP3* as well as regulatory markers. nTreg: naturally induced T regulatory cell; FACS: fluorescent assay cell sorting; TIGIT: T cell immunoreceptor with Ig and ITIM domains; GARP: Glycoprotein A repetitions predominant; HLA: human leucocyte antigen; PD1: programmed death 1; CTLA4: cytotoxic T-lymphocyte–associated antigen 4; FCLR3: Fc like receptor 3; SEM: standard error of the mean. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001.

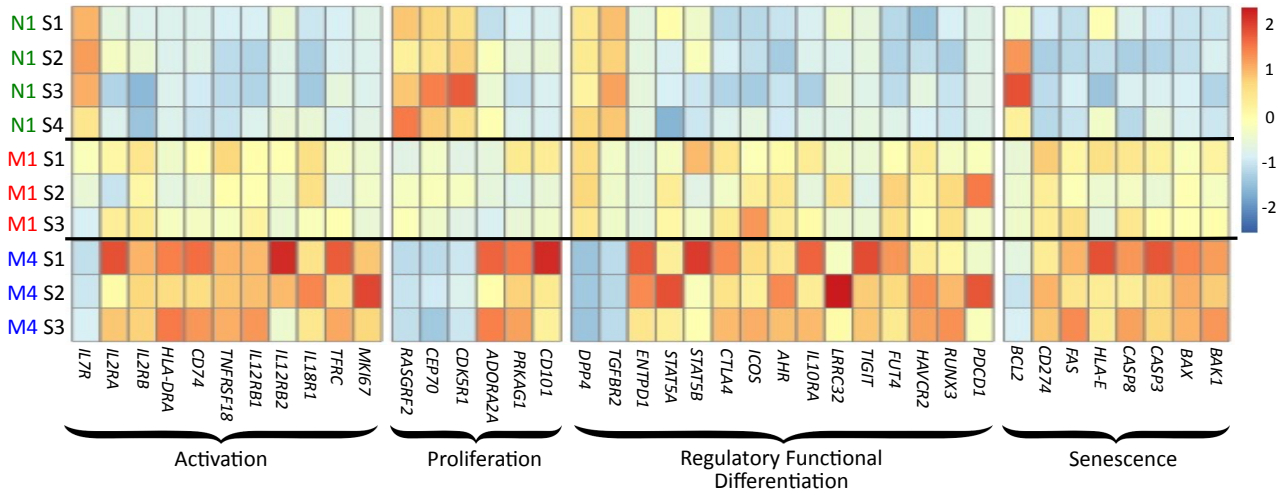

Fig. S3. Supervised analysis of gene expression levels implicated in nTreg life cycle. Heatmap representation of 40 relevant markers**.** The raws represent the cell subsets, N1, M1 M4. The color of each column represents the fold change of the expression of the marker compared to the mean expression level, the degree of change is shown in the scale.

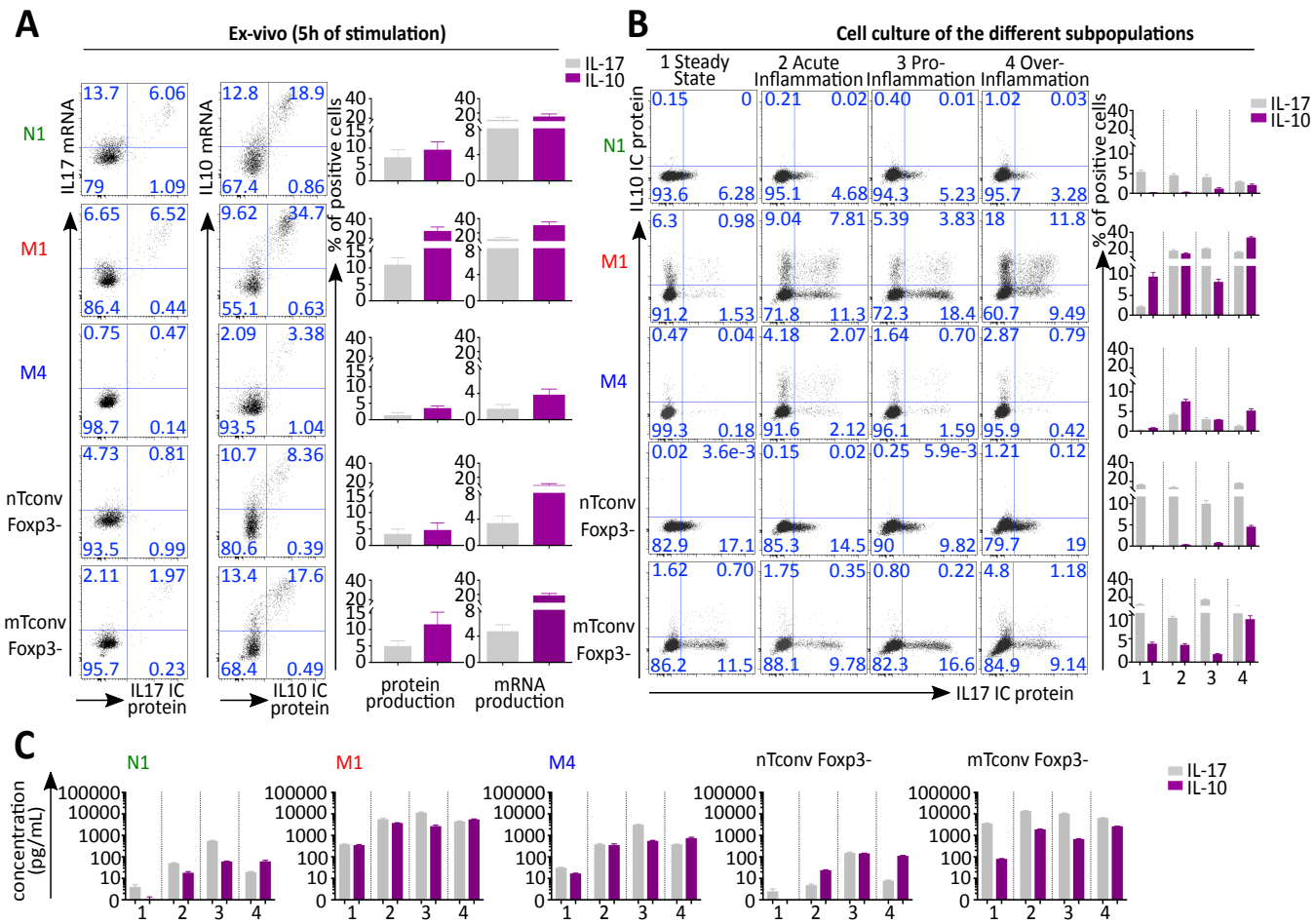

Fig. S4. The microenvironmental context dictates the nTreg subset functional differentiation as assessed by their production of cytokines. (A) nTreg subsets (N1, M1, M4) and conventional T cells (naive (nTconv) or memory (mTconv)) were stimulated *ex-vivo* for 5h with PMA and ionomycin. **(A1)** Representative FACS showing the expression of mRNA and intracellular protein of IL-17 and IL-10 in nTreg subsets and conventional T cells. **(A2)** Histograms indicating the percentage of mRNA and intracellular protein IL-10 (n=6 for mRNA and n=3 or more for protein) and IL-17 (n=5 for mRNA and n=3 for protein) expressed by the three nTreg and the two Tconv subsets. **(B)** nTreg subsets and Tconv cells were stimulated (100 000 per well) with platebound anti-CD3 mAb (pbαCD3) in presence of irradiated ΔCD3-feeders (100 000 per well) according the following conditions : (1) steady state: pbαCD3 0.5µg/mL and IL-2 2 IU/mL; (2) Acute and (3) Pro inflammation: pbαCD3 4µg/mL, soluble anti-CD28 mAb (sαCD28) 4µg/mL and IL-2 100 IU/mL with inflammatory cytokine cocktail for the pro-inflammation; (4) Over-inflammation: pbαCD3 4µg/mL, sαCD28 4µg/mL, platebound anti-CD46 mAb 5µg/mL and cytokines. After 5 days, cells and culture supernatants were harvested. Cells were restimulated with PMA/ionomycin for 5 h, fixed, and stained for intracellular cytokines. **(B1)** Representative dot plots showing expression of intracellular IL-10 and IL-17 protein by the nTreg and Tconv cells. **(B2)** Histograms indicating the percentage of intracellular IL-10 and IL-17 expressed by the nTreg and Tconv cells (n=3)**. (C)** Culture supernatants collected were analyzed for cytokine levels. Histograms showing the concentration of IL-10 and IL-17 protein in culture supernatant from nTreg subsets and Tconv cells (n=3).

All histograms are represented as mean ± SEM, each color represent a different cytokine: IL-10 in purple and IL-17 in grey. nTreg, naturally induced T regulatory cell; PMA, phorbol 12-myristate 13-acetate; FACS, fluorescent assay cell sorting; SEM, standard error of the mean.

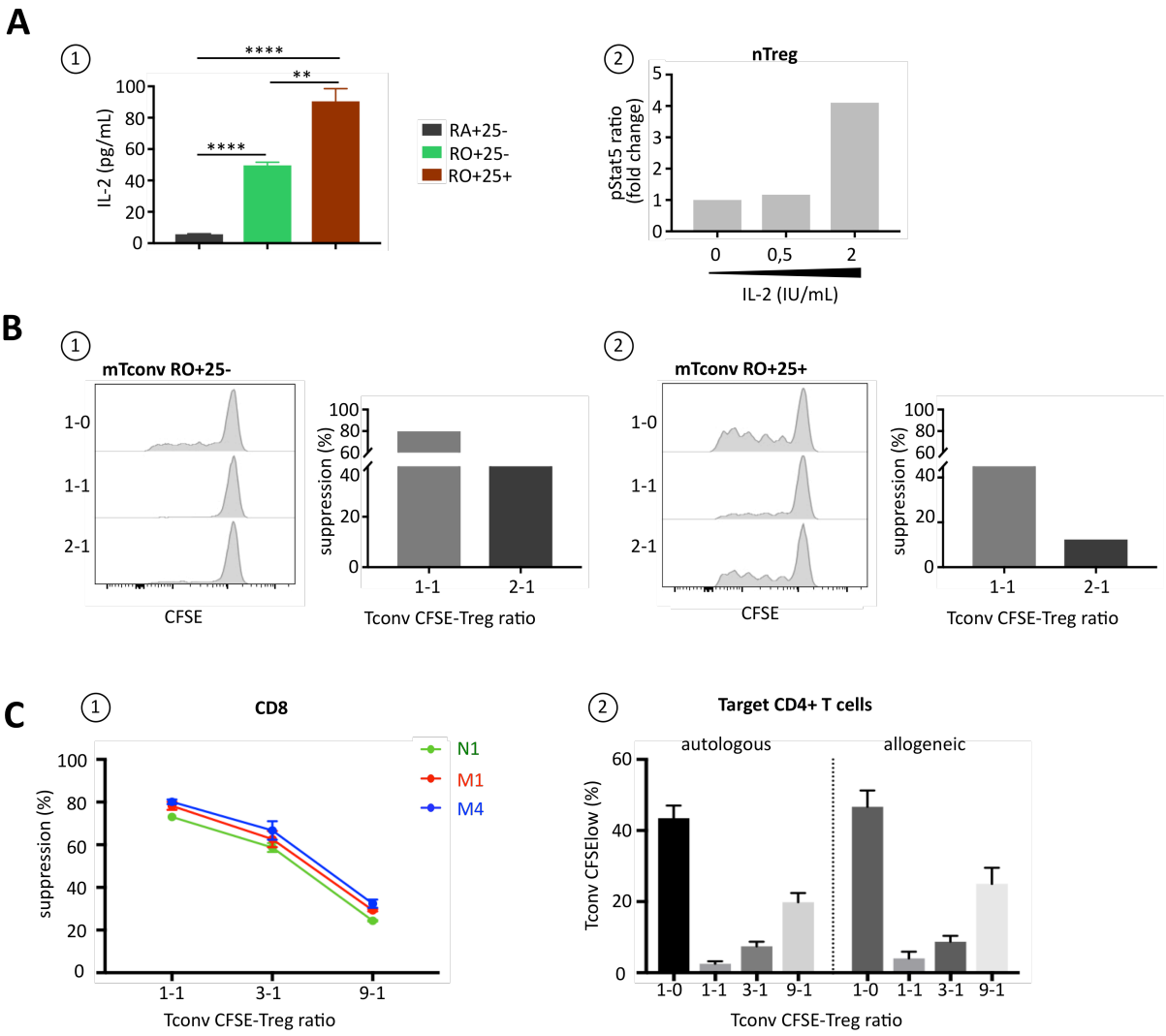

Fig. S5. Target cells for the current suppressive nTreg assay. (A1) Measure of IL-2 production in culture supernatant from 40h-stimulated Tconv cells subset (Tconv RA+25-, Tconv RO+25- and Tconv RO+25+) by ELISA. Mean ± SEM of IL-2 concentration (n=3). **(A2)** Representative pSTAT5 ratio (MFI at 15 min / MFI at baseline) in nTregs stimulated with the indicated amount of IL-2 for 15 min. **(B)** Ability of nTregs to suppress CFSE-labelled mTconv CD25- **(B1)** or CD25+ **(B2)** measured by the current nTreg suppressive assay, at varying Tconv/Treg ratios (1-0, 1-1 and 2-1). Proliferation of $Tconv^{CFSE}$ was measured by the CFSE dilution assay. Representative FACS histograms and mean ± SEM of nTregs suppressive activity are represented. **(C)** Cell-contact suppressive activity of nTreg is neither cell type specific nor HLA restricted. **(C1)** Capacity of nTreg subsets to suppress CFSE-labelled CD8+ ($CD8+^{CFSE}$) T cells proliferation was measured by the current nTreg suppressive assay, slightly modified. 4 X 10^4 $CD8+^{CFSE}$ T cells were cocultured with nTreg subsets at different ratios in presence of irradiated feeder and 4 X 10^4 irradiated prestimulated Tconv. Graph represents the mean percentage of suppression ± SEM (n=2). **(C2)** Ability of M4 cells to suppress the proliferation of autologous or allogeneic responder cells. CD4+ $Tconv^{CFSE}$ proliferation in absence (1-0) or presence of nTreg M4 cells is indicated. Graph shows mean of $Tconv^{CFSE}$ dilution ±SEM. FACS: fluorescence assay cell sorting; MFI: mean fluorescence intensity; SEM: standard error of the mean. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001.

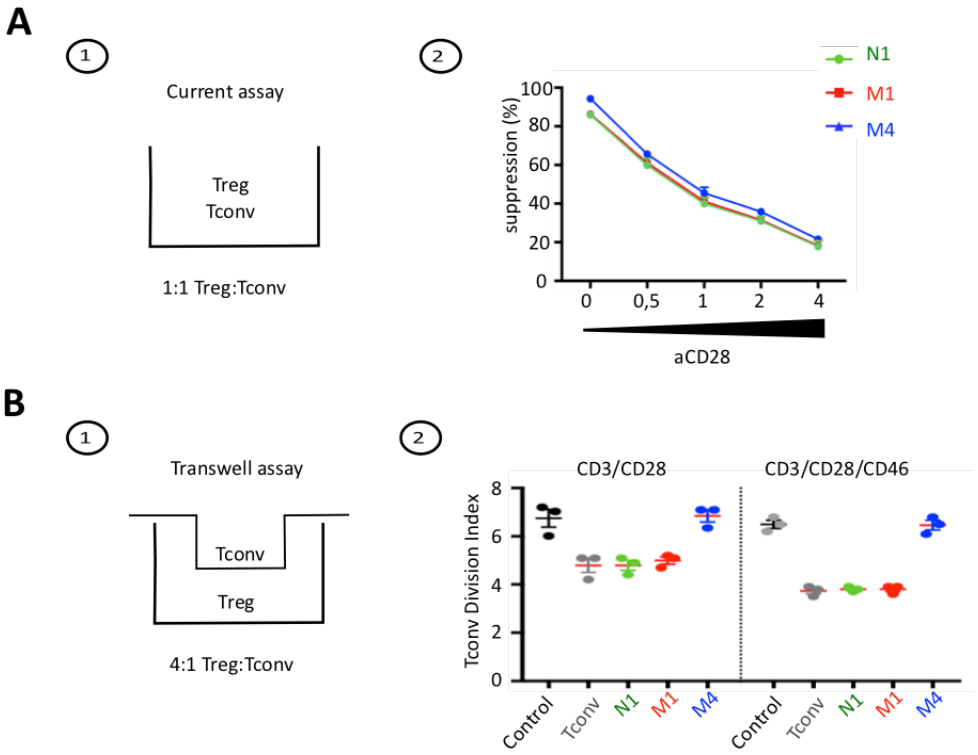

Fig. S6. Suppressive activity of nTreg depends on microenvironmental context. nTreg subsets suppressive activity under inflammatory context was assessed with two different suppressive assays as described in Mat and meth : the current suppressive nTreg assay, where 4 X 10^4 $Tconv^{CFSE}$ cells and 4 X 10^4 nTreg subsets were in direct contact and stimulated with a low dose of pbαCD3 (0.5 µg/mL) and a transwell assay where CD3/CD28-prestimulated $Tconv^{CFSE}$ cells (4 X 10^4) were physically separated from 3 X 10^5 nTreg subsets stimulated with a high dose of pbαCD3 (4 µg/mL) in the presence of IL-2 (20 IU/mL) and when indicated, the additional costimulatory signals sαCD28 mAb (4 µg/mL), pbαCD46 mAb (10 µg/mL). **(A1)** Schema of the current suppressive nTreg assay. **(A2)** Graph showing the nTreg subsets cell contact suppressive activity in the presence of increasing concentrations of soluble anti-CD28 mAb. The graph represents the mean percentage of suppression +- SEM (n=3). **(B1)** Schema of the transwell suppressive nTreg assay. **(B2)** $Tconv^{CFSE}$ proliferation in absence (Control) or presence of Tconv and nTreg subsets stimulated with either pbαCD3 mAb (4 µg/mL) and sαCD28 mAb (4 µg/mL)) (mimicking acute inflammation) or pbαCD3 mAb (4 µg/mL), sαCD28 mAb (4 µg/mL) and pbαCD46 (10 µg/mL) mAb) (mimicking overinflammation). Scatterplot shows mean of Tconv division index +- SEM (n=3).

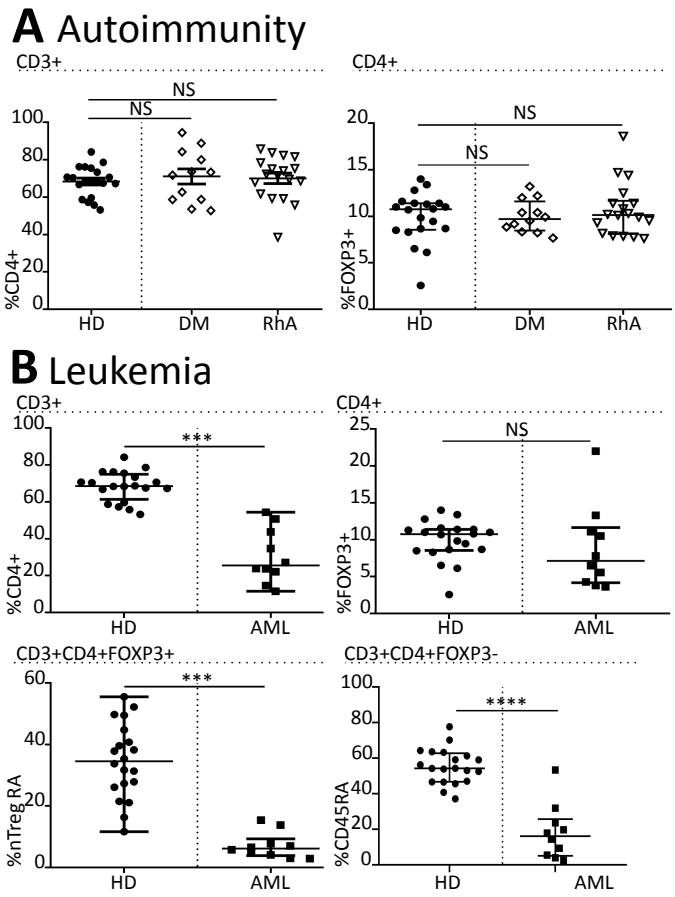

Fig. S7. Distribution of blood CD3+ sub-populations in auto-immunity and cancer. (A) ***Auto-immunity*.** Scatter plots of the percentage of CD4+ cells among CD3+ and of FOXP3+ cells among CD4+ in healthy donors (HD) compared to dermatomyositis (DM) and rheumatoid arthritis (RhA). **(B)** ***Acute Myeloid Leukemia (AML)*.** Scatter plots of the percentage of CD4+ cells among CD3+ and of FOXP3+ cells among CD4+ in HD compared to AML. Scatter plots of the percentage of nTreg RA among CD3+CD4+FOXP3+ and of the percentage of CD45RA+ among CD3+CD4+FOXP3-. Data in scatter plots are presented as median with interquartile range in HD (n=20) compared to DM (n=12), rheumatoid arthritis (RhA) (n=18) and AML (n=10). P values were calculated using Wilcoxon-Mann-Whitney test. P values < 0.05 were considered significant. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. NS, non-significative; HD, healthy donor; DM, dermatomyositis; RhA, rheumatoid arthritis; AML, acute myeloid leukemia; FOXP3, forkhead box P3.

SUPPLEMENTARY TABLES

Table S1 : Additional FOXP3 lineage conventional regulatory T cells.

	Phenotypage	Frequence (% of CD4+)	Frequence (% of CD4+ FOXP3+)	MFI Foxp3 ratio	TSDR demethylation	il2 demethylation
FOXP3 nTreg variants	CD4+ CD8+ DP	0.12+/-0.04 (n=8)	1.25+/-0.46 (n=8)	8.38 +/- 1.7 (n=6)	76.3 +/- 5.13 (n=3)	51 +/- 1 (n=3)
	CD4+ CD127- CD25- CD39+	0.5 +/- 0.36 (n=8)	4.82 +/- 2.35 (n=8)	9.71+/- 5.68 (n=4)	91.41 +/- 5.96 (n=4)	59.27 +/- 3.11 (n=4)
activated FOXP3+ T cells *	CD4+ CD127+	1.2+/-0.56 (n=8)	12.37+/-3.96 (n=8)	7.545 +/- 1.97 (n=4)	26.412 +/- 8.56 (n=5)	89.318 +/- 6 (n=5)

* Note that activated FOXP3+ T cells expressing a low TSDR and high demethylation level of CpG site 1 in the IL-2 promoter are not nTregs by contrast to FOXP3 nTreg variants.

Table S2 : mRNA expression levels of markers implicated in nTreg life cell cycle.

	Gene name	Protein name	H48			F28			H43			H26
			N1	M1	M4	N1	M1	M4	N1	M1	M4	N1
Activation	*MKI67*	Ki-67	0.15	1.04	4.74	0.01	1.25	7.99	0.03	0.62	4.32	0.23
	TFRC	CD71	53.19	84.16	192.12	55.91	63.71	134.05	68.63	97.15	157.59	55.43
	IL18R1	IL-18R1	10.23	30.55	29.46	6.69	30.27	41.13	5.3	20.55	28.69	15.89
	IL12RB2	IL-12Rβ2	3.06	7.83	32.95	0.73	4.29	19.3	0.4	3.37	3.9	2.77
	IL12RB1	IL-12Rβ1	14.46	22.39	31.72	11.16	22.23	30.95	11.03	24.5	34.47	15.1
	TNFRSF18	GITR	4.45	35.18	40.57	0.34	21.94	38.33	0.12	16.68	41.6	1.74
	CD74	HLA-DR invariant chain	147.76	436.07	1097.81	181.05	259.96	719.98	89.52	353.38	952.02	80.76
	HLA-DRA	HLA-DR chaine α	2.31	59.96	330.91	0.55	19.34	206.44	2.69	75.62	339.29	3.58
Proliferation	*IL2RB*	IL-2Rβ	105.55	182.46	213.44	122.53	158.19	196.62	60.07	175.88	200.29	69.62
	IL2RA	IL-2Rα	77.75	107.3	177.16	92.55	63.28	105.24	55.88	115.75	139.35	72.72
	IL7R	IL-7R	524.02	335.24	294.74	550.57	277.44	212.31	530.97	231.3	231.56	438.75
	CD101	IGSF2	0.22	31.29	79.5	8.11	8.27	32.51	0.32	11.92	28.8	1
	PRKAG1	AMPK-g1	40.03	60.75	78.67	44.55	42.25	67.1	37.95	45.46	73.09	41.53
	ADORA2A	A2A-R	4.51	6.1	13.87	7.44	6.14	8.15	6.55	5.31	13.09	7.75
	CDK5R1	p35	57.68	15.06	5.56	64.74	29.82	12.32	96.53	15.74	6.08	57.62
	CEP70	CEP70	22.88	13.66	7.27	20.75	14.99	8.71	29	13.35	5.05	23.46
	RASGRF2	RASGRF2	25.73	5.76	0.18	17.67	11.2	1.03	25.48	12.38	0.81	33.57
Regulatory Functional Differentiation	*PDCD1*	PD-1	0.48	5.81	3.6	0.19	23.31	26.38	0.67	4.88	5.78	0.12
	RUNX3	RUNX3	61.76	98.86	122.96	35.76	160.83	176.91	52.69	154.73	198.89	56.55
	HAVCR2	TIM-3	1.62	5.36	5.2	2.37	4.87	7.21	3.28	4.49	6.92	2.8
	FUT4	CD15s	2.85	4.37	6.35	2.75	5.63	5.53	2.9	5.37	5.12	3.45
	TIGIT	TIGIT	43.92	49.65	300.97	34.2	23.82	189.84	23.97	52.97	194.1	34.29
	LRRC32	GARP	8.52	10.39	13.7	0.88	31.58	78.18	6	8.87	20.88	1.77
	IL10RA	IL-10-Rα	291.78	512	783.09	300.6	333.34	518.29	198.5	453.64	642.69	309.92
	AHR	AhR	57.33	109.15	129.72	44.46	129.49	175.2	58.76	124.52	152.14	63.04
	ICOS	ICOS	112.56	315.59	470	134.33	400.73	393.09	89.03	585.28	553.49	220.18
	CTLA4	CTLA-4	150.98	553.99	765.41	223.64	351.32	569.31	140.75	534.35	677.11	248.19
	STAT5B	STAT5B	127.83	180.5	221.65	134.85	117.38	136.16	106.15	129.01	141.06	106.3
	STAT5A	STAT5A	119.46	113.67	125.93	102.64	126.51	157.56	101.2	121.17	125.59	86.26
	ENTPD1	CD39	0.76	0.53	22.89	0.59	0.8	19	0.88	0.79	11.23	0.6
	TGFBR2	TGFb-R2	397.57	196.68	66.15	376.43	186.6	64.52	433.33	187.5	72.06	388.77
	DPP4	CD26	30.65	33.73	1.49	29.82	35.34	2.42	26.68	30.68	1.3	35.6
Senescence	*BAK1*	BAK1	20.24	54.11	83.64	25.32	41.38	72.58	14.23	48.67	86.17	20.71
	BAX	BAX	23.01	30.55	44.81	22.59	31.22	41.67	24.24	31.5	41.49	24.71
	CASP3	CASP3	37.17	58.92	87	32.27	45.24	64.8	38.58	54.12	67.36	42.2
	CASP8	CASP8	261.27	506.41	669.6	168.01	360.63	482.52	253.26	499.47	647.18	185.13
	HLA-E	HLA-E	1547.97	1736.04	2086.66	1280.4	1460.43	1674.62	1188.83	1418.32	1687.32	1488.16
	FAS	FAS	18.1	161.89	265.59	7.82	125.93	201.51	38.9	215	307	21
	CD274	PD-L1	1.73	6.21	4.93	0.88	4.85	6.73	1.26	4.89	6.32	1.13
	BCL2	Bcl-2	41.77	36.24	34.39	37.17	58.92	87	84.91	39.28	29.43	52.12

Table S3 : Clinical characteristics of patients included in this study.

DERMATOMYOSITIS								
Patient n°	**Age (year)**	**Sex [1]**	**Disease evolution (month)**	**CDASI [2]**	**Treatment [3]**	**Clinic**	**Serology [4]**	**Cancer associated**
1	49	F	2	10	None	Hypo myopathic	Mi2-antibody	No
2	30	M	1	10	None	Amyopathic	MDA5-antibody	No
3	49	F	6	37	None	Amyopathic	Mi2-antibody	No
4	51	F	1	34	None	Amyopathic	MDA5-antibody	No
5	45	F	4	18	Corticoids	Amyopathic	Negative	No
6	55	F	41	23	None	Amyopathic	Mi2-antibody	No
7	28	M	30	37	Methotrexate	Hypo myopathic	Negative	No
8	80	F	150	24	None	Amyopathic	Negative	No
9	44	M	4	15	None	Amyopathic	Negative	No
10	86	F	108	14	None	Amyopathic	Negative	No
11	41	F	1	29	None	Hypo myopathic	Negative	No
12	56	F	1	17	None	Hypo myopathic	Negative	No
ACUTE MYELOID LEUKEMIA								
Patient n°	**Age (year)**	**Sex [1]**	**Duration between HSCT [5] and relapse (days)**		**Type of HSCT [5] conditioning**	**Clinic [6]**	**Type of relapse [7]**	**HLA donor mismatch [8]**
1	67	M	1627		Reduced regimen	AML with multilineage dysplasia	Cyto+Immuno	1 antigen
2	57	M	509		Reduced regimen	AML M6 - Erythroleukemia	Immuno+Molecu	10/10 alleles
3	58	F	139		Standard regimen	AML	Cyto+cytogene	1 allele
4	68	M	1195		Reduced regimen	RAEB1	Cyto+Immuno	10/10 alleles
5	73	F	195		Reduced regimen	AML secondary to MDS	Cyto+Immuno	10/10 alleles
6	55	F	333		Standard regimen	AML	Cyto	10/10 alleles
7	57	M	90		Reduced regimen	Secondary AML	Cyto+Immuno	10/10 alleles
8	22	M	4520		Reduced regimen	RA	Cyto+Immnuo+Cytogene+ Molecu	1 antigen
9	48	M	75		Standard regimen	AML with minimal differentiation	Cyto	10/10 alleles
10	60	F	504		Reduced regimen	AML	Cyto+Immuno	1 antigen
RHEUMATOID ARTHRITIS								
Patient n°	**Age (year)**	**Sex [1]**	**DAS28 CRP [9]**	**Treatment**				
1	58	F	4.36	Methotrexate + Corticoids				
2	25	F	NA [10]	NA				
3	60	F	5.03	Methotrexate+corticoids				
4	54	F	5.4	Methotrexate + salazopyrin				
5	56	M	3.17	Leflunomide + cortidoids				
6	53	F	4.82	Methotrexate+corticoids				
7	50	M	3.5	Abatacepts + Corticoids				
8	60	M	2.2	Leflunomide				
9	29	F	6.12	Corticoids				
10	40	F	4.3	None				
11	50	F	1.44	Methotrexate+corticoids				
12	NA	M	5.06	Corticoids				
13	66	F	1.44	None				
14	60	F	2.6	Methotrexate				
15	63	F	1.9	Methotrexate				
16	45	F	1.28	None				
17	32	F	2.87	Methotrexate				
18	NA	F	1.8	Methotrexate+corticoids				

[1] F: female, M: male; [2] CDASI: cutaneous disease severity index activity score at inclusion; [3] Immunosuppressive treatment at inclusion, none stands for no treatment for the last 12 months; [4] MDA5: Melanoma differentiation associated gene [5] HSCT: Hematopoietic stem cell transplantation; 6 AML: acute myeloid leukemia, RAEB: Refractory Anemia with Excess Blasts, MDS: myelodysplasia, RA: Refractory Anemia; [7] Cyto: cytologic, immune: immunophenotypic, cytogene: cytogenetic, molecu: molecular; [8] HLA: Human Leucocyte antigen; [9] DAS28 CRP: disease activity score of the rheumatoid arthritis, [10] NA: non-available

Table S4 : Statistical analysis

Panel 1 : statistics of Figure 1.

C1	**Naive**						**Memory**					
	N1 vs N2	N1 vs N3	N1 vs N4	N2 vs N3	N2 vs N4	N3 vs N4	M1 vs M2	M1 vs M3	M1 vs M4	M2 vs M3	M2 vs M4	M3 vs M4
	<0.0001	**<0.0001**	**<0.0001**	0.0826	**0.0001**	**0.0351**	**<0.0001**	**<0.0001**	0.8621	0.7863	**0.0005**	**<0.0001**
D1	**Naive**											
	C vs N1	C vs N2	C vs N3	C vs N4	N1 vs N2	N1 vs N3	N1 vs N4	N2 vs N3	N2 vs N4	N3 vs N4		
	0.0002	**0.0002**	**0.002**	**0.0002**	>0.9999	0.1304	0.2345	0.1949	0.2345	0.3823		
D1	**Memory**											
	C vs M1	C vs M2	C vs M3	C vs M4	M1 vs M2	M1 vs M3	M1 vs M4	M2 vs M3	M2 vs M4	M3 vs M4		
	0.0002	**0.0002**	**0.0002**	**0.0002**	**0.0019**	**0.0002**	**0.0002**	**0.0281**	**0.0006**	**0.0104**		
D2	**Naive**	**Memory**										
	C vs N1	C vs M1	C vs M2	C vs M3	C vs M4	M1 vs M2	M1 vs M3	M1 vs M4	M2 vs M3	M2 vs M4	M3 vs M4	
	0.0571	**0.0043**	**0.0043**	**0.0043**	**0.0043**	**0.0079**	**0.0079**	**0.0079**	0.8413	0.1508	**0.0079**	
D3	**Naive**	**Memory**										
	C vs N1	C vs M1	C vs M2	C vs M3	C vs M4	M1 vs M2	M1 vs M3	M1 vs M4	M2 vs M3	M2 vs M4	M3 vs M4	
	0.0714	**0.0159**	**0.0159**	**0.0159**	**0.0159**	0.6857	0.0571	0.2	**0.0286**	0.3429	0.1143	
D4	**Naive**	**Memory**										
	C vs N1	C vs M1	C vs M2	C vs M3	C vs M4	M1 vs M2	M1 vs M3	M1 vs M4	M2 vs M3	M2 vs M4	M3 vs M4	
	0.0286	**0.0286**	**0.0286**	**0.0286**	**0.0286**	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	
D5	**CD25 MFI**						**CFSE (%)**					
	C vs N1	C vs M1	C vs M4	N1 vs M1	N1 vs M4	M1 vs M4	C vs N1	C vs M1	C vs M4	N1 vs M1	N1 vs M4	M1 vs M4
	0.0002	**0.0003**	**0.0001**	0.9414	**0.0032**	**0.0097**	**<0.0001**	**<0.0001**	**<0.0001**	>0.9999	>0.9999	>0.9999

Values represented P-values, evaluated by the Mann-Whitney U test.

Panel 2 : statistics of Figure 2.

B2	**% CD39**						**% CD26**					
	IL-2 vs PGE2	IL-2 vs TGFb	IL-2 vs TGFb+PGE2	PGE2 vs TGFb	PGE2 vs TGFb+PGE2	TGFb vs TGFb+PGE2	IL-2 vs PGE2	IL-2 vs TGFb	IL-2 vs TGFb+PGE2	PGE2 vs TGFb	PGE2 vs TGFb+PGE2	TGFb vs TGFb+PGE2
	0.0001	0.0781	**<0.0001**	**0.0003**	0.1651	**0.0002**	0.2208	**<0.0001**	**0.0016**	**0.0004**	**0.003**	**0.0091**
B2	**MFI CD39**						**MFI CD26**					
	IL-2 vs PGE2	IL-2 vs TGFb	IL-2 vs TGFb+PGE2	PGE2 vs TGFb	PGE2 vs TGFb+PGE2	TGFb vs TGFb+PGE2	IL-2 vs PGE2	IL-2 vs TGFb	IL-2 vs TGFb+PGE2	PGE2 vs TGFb	PGE2 vs TGFb+PGE2	TGFb vs TGFb+PGE2
	<0.0001	**0.0064**	**<0.0001**	**<0.0001**	0.1284	**<0.0001**	**0.0022**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0021**
C2	**IL-2 0**			**IL-2 2**			**IL-2 4**			**IL-2 8**		
	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4
	0.5614	0.5614	>0.9999	**0.0148**	**0.0006**	**0.0011**	0.1761	**0.0006**	**0.0005**	0.1094	**<0.0001**	**<0.0001**
C4	**IL-2 0**			**IL-2 2**			**IL-2 4**			**IL-2 8**		
	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4
	<0.0001	**<0.0001**	**<0.0001**	**0.0002**	**<0.0001**	**0.0017**	**0.0002**	**<0.0001**	**0.0083**	**0.0005**	**<0.0001**	**0.0011**

Values represented P-values, evaluated by the Student's T-Test.

Panel 3 : statistics of Figure 5.

A	**ratio 1-1**			**ratio 3-1**			**ratio 9-1**					
	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4			
	0.9685	**0.0009**	**0.0018**	0.675	**0.0039**	**0.0031**	0.9626	0.1168	0.0599			
B1	**IL-2 0.5**			**IL-2 2**								
	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4						
	0.1863	0.102	0.0516	0.4287	0.2847	0.1396						
B2	**IL-2 0**			**IL-2 2**			**IL-2 4**			**IL-2 8**		
	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4
	0.9414	**0.0032**	**0.0097**	0.6179	0.1619	0.1906	**0.0104**	**0.0021**	0.1423	**0.0083**	0.0919	**0.0037**
B3	**ratio 1-1**			**ratio 3-1**								
	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4						
	0.6461	**0.0003**	**0.0007**	0.7181	**0.0005**	**0.0005**						
B4	**ratio 1-1**			**ratio 3-1**								
	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4						
	0.881	**0.0049**	**0.0007**	**0.0052**	**0.0002**	**0.0006**						
C	**ratio 1-1**			**ratio 3-1**								
	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4						
	0.0502	**0.0008**	**0.0115**	0.0961	**0.0002**	0.0481						
D3	**- PMA iono**			**+ PMA iono**								
	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4						
	0.8383	0.754	0.3291	0.0877	0.051	0.2566						
E3	**IL-12**						**IL-10**					
	0 vs 3	0 vs 10	0 vs 30	3 vs 10	3 vs 30	10 vs 30	0 vs 3	0 vs 10	0 vs 30	3 vs 10	3 vs 30	10 vs 30
	0.0052	**0.0005**	**0.0001**	**0.0012**	**<0.0001**	**0.001**	0.0725	**0.002**	**0.0019**	**0.0055**	**0.0029**	**0.017**

Values represented P-values, evaluated by the Student's T-Test.

Panel 4 : statistics of Figure 7.

cocktail = TGFb + PGE2 + RAPA

A2	IL-2 vs PGE2	IL-2 vs TGFb+RAPA	IL-2 vs cocktail	PGE2 vs TGFb+RAPA	PGE2 vs cocktail	TGFb+RAPA vs cocktail			
	0.0015	**0.0003**	**<0.0001**	**0.0018**	**<0.0001**	0.5635			
A3	IL-2 vs PGE2	IL-2 vs TGFb+RAPA	IL-2 vs cocktail	PGE2 vs TGFb+RAPA	PGE2 vs cocktail	TGFb+RAPA vs cocktail			
	0.0002	**<0.0001**	**<0.0001**	0.18	**<0.0001**	**<0.0001**			
B1	**ratio 1-1**			**ratio 3-1**			**ratio 9-1**		
	Tconv vs nTreg	Tconv vs iTreg	nTreg vs iTreg	Tconv vs nTreg	Tconv vs iTreg	nTreg vs iTreg	Tconv vs nTreg	Tconv vs iTreg	nTreg vs iTreg
	<0.0001	**<0.0001**	**0.0195**	**0.0002**	**<0.0001**	**0.0091**	**0.0006**	**0.0005**	**0.003**
B2	**ratio 1-1**			**ratio 3-1**			**ratio 9-1**		
	Tconv vs nTreg	Tconv vs iTreg	nTreg vs iTreg	Tconv vs nTreg	Tconv vs iTreg	nTreg vs iTreg	Tconv vs nTreg	Tconv vs iTreg	nTreg vs iTreg
	0.1905	**0.0002**	**<0.0001**	0.2314	**0.0001**	**0.0001**	**0.0228**	**0.0039**	0.1166
B3	Tconv vs nTreg	Tconv vs iTreg	nTreg vs iTreg						
	0.008	**<0.0001**	**<0.0001**						

Values represented P-values, evaluated by the Student's T-Test.

Panel 5 : statistics for Figure S1.

A2	**Naive**						**Memory**					
	N1 vs N2	N1 vs N3	N1 vs N4	N2 vs N3	N2 vs N4	N3 vs N4	M1 vs M2	M1 vs M3	M1 vs M4	M2 vs M3	M2 vs M4	M3 vs M4
	0.1	0.1	0.1	>0.9999	0.7	>0.9999	0.2	0.1	0.1	0.4	>0.9999	0.4
A3	**Naive**						**Memory**					
	N1 vs N2	N1 vs N3	N1 vs N4	N2 vs N3	N2 vs N4	N3 vs N4	M1 vs M2	M1 vs M3	M1 vs M4	M2 vs M3	M2 vs M4	M3 vs M4
	0.0286	**0.0286**	**0.0286**	>0.9999	0.8857	0.3429	0.1143	0.2	0.2	0.4857	0.4857	0.6857

Values represented P-values, evaluated by the Mann-Whitney U test.

Panel 6 : statistics of Figure S3.

A	IL-10 ARN	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		0.0187	**0.0128**	0.1703	0.4377	**0.0001**	**0.0011**	**0.0392**	0.0601	**0.0004**	**0.0179**
	IL-17 ARN	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		0.8468	**0.0497**	0.1092	0.1805	**0.003**	**0.0124**	**0.0265**	0.2071	**0.0312**	0.3873
	IL-10 protein	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		0.0035	**0.0361**	0.243	0.6402	**<0.0001**	**0.0068**	**0.039**	0.5157	**0.0174**	0.1839
	IL-17 protein	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		0.292	0.0739	0.249	0.473	**0.013**	**0.0443**	0.0875	0.2629	0.1164	0.546

B	Freq IL-10 1	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		0.0007	**0.0015**	**0.0027**	**0.0004**	**0.0009**	**0.0007**	**0.0055**	**0.0008**	**0.0008**	**0.0003**
	Freq IL-17 1	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		0.0022	**0.0003**	**<0.0001**	**0.0018**	**0.0003**	**<0.0001**	**0.0002**	**<0.0001**	**0.0001**	**0.007**
	Freq IL-10 2	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		<0.0001	**0.0001**	0.4918	**0.0002**	**0.0002**	**<0.0001**	**<0.0001**	**0.0001**	**0.0023**	**0.0002**
	Freq IL-17 2	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		0.0002	0.4917	**0.0002**	**0.0007**	**0.0001**	**0.0049**	**0.0007**	**0.0001**	**0.0004**	**0.0035**
	Freq IL-10 3	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		0.0004	**0.0038**	0.2482	0.1591	**0.0009**	**0.0003**	**0.0005**	**<0.0001**	**0.0013**	**0.004**
	Freq IL-17 3	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		<0.0001	0.2131	**0.0041**	**<0.0001**	**<0.0001**	**0.0004**	**0.0047**	**0.0015**	**<0.0001**	**0.0014**
	Freq IL-10 4	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		<0.0001	**0.0039**	**0.0034**	**0.0008**	**<0.0001**	**<0.0001**	**<0.0001**	0.3019	**0.0095**	**0.0041**
	Freq IL-17 4	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		<0.0001	**0.0048**	**<0.0001**	**0.0002**	**<0.0001**	0.1395	**0.0005**	**<0.0001**	**0.0001**	**0.0004**

C	SN IL-10 1	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		<0.0001	**<0.0001**	0.8349	**<0.0001**	**<0.0001**	**<0.0001**	**0.0002**	**0.0001**	**<0.0001**	**<0.0001**
	SN IL-17 1	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		<0.0001	**0.0003**	0.2684	0.2684	**<0.0001**	**<0.0001**	**<0.0001**	**0.0002**	**0.0002**	>0.9999
	SN IL-10 2	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		<0.0001	**0.0025**	0.1028	**<0.0001**	**<0.0001**	**<0.0001**	**0.0003**	**0.0027**	**<0.0001**	**<0.0001**
	SN IL-17 2	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		0.0005	**0.0002**	**<0.0001**	**<0.0001**	**0.0007**	**0.0005**	**0.0006**	**0.0001**	**<0.0001**	**<0.0001**
	SN IL-10 3	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		0.001	**0.0001**	**<0.0001**	**<0.0001**	**0.0023**	**0.0012**	**0.0028**	**0.0003**	0.0613	**<0.0001**
	SN IL-17 3	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		0.0002	**<0.0001**	**0.0003**	**<0.0001**	**0.0006**	**0.0002**	0.3119	**<0.0001**	**0.0001**	**<0.0001**
	SN IL-10 4	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		<0.0001	**0.0009**	**0.0057**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0003**	**0.0012**	**<0.0001**	**<0.0001**
	SN IL-17 4	N1 vs M1	N1 vs M4	N1 vs nTconv	N1 vs mTconv	M1 vs M4	M1 vs nTconv	M1 vs mTconv	M4 vs nTconv	M4 vs mTconv	nTconv vs mTconv
		<0.0001	**<0.0001**	**0.0008**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0039**	**<0.0001**	**<0.0001**	**<0.0001**

Values represented P-values, evaluated by the Student's T-Test.

Panel 7 : statistics of Figure S4.

C1	**ratio 1-1**			**ratio 3-1**			**ratio 9-1**		
	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4
	0.1529	0.0533	0.4732	0.4568	0.2367	0.552	**0.0149**	0.0547	0.262
C2	**ratio 1-1**	**ratio 3-1**	**ratio 9-1**						
	auto vs allo	auto vs allo	auto vs allo						
	0.2688	0.3613	0.1624						

Values represented P-values, evaluated by the Student's T-Test.

Panel 8 : statistics of Figure S5.

B1	**CD28 0**			**CD28 0.5**			**CD28 1**			
	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4	
	0.7673	**0.001**	**0.0016**	0.6243	0.0723	**0.0396**	0.6289	0.0956	0.1145	
	CD28 2			**CD28 4**						
	N1 vs M1	N1 vs M4	M1 vs M4	N1 vs M1	N1 vs M4	M1 vs M4				
	0.8096	**0.0314**	**0.0144**	0.8664	0.1363	0.161				
B2	**CD3/CD28**									
	C vs Tconv	C vs N1	C vs M1	C vs M4	Tconv vs N1	Tconv vs M1	Tconv vs M4	N1 vs M1	N1 vs M4	M1 vs M4
	0.0148	**0.01**	**0.0118**	0.8333	>0.9999	0.5844	**0.0063**	0.4818	**0.0032**	**0.0032**
	CD3/CD28/CD46									
	C vs Tconv	C vs N1	C vs M1	C vs M4	Tconv vs N1	Tconv vs M1	Tconv vs M4	N1 vs M1	N1 vs M4	M1 vs M4
	0.0002	**0.0001**	**0.0002**	0.9066	0.6433	0.6918	**0.0003**	>0.9999	**0.0002**	**0.0003**

Values represented P-values, evaluated by the Student's T-Test.

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