Thrombomodulin-expressing monocytes are associated with low-risk features in myelodysplastic syndromes and dampen excessive immune activation

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Supplementary methods

Patient and control samples

IPSS-R risk groups of 92 patients could be calculated. In total, 20 patients were considered very low risk, 34 low risk, 22 intermediate, 8 high and 8 very high risk. The 2016 World Health Organization (WHO) classification was available for 142 patients. Five patients could be classified as MDS with single lineage dysplasia (MDS-SLD), 48 as MDS with multilineage dysplasia (MDS-MLD), 8 as MDS with ringed sideroblasts with single lineage dysplasia (MDS-RS-SLD), 27 as MDS with ringed sideroblasts with multilineage dysplasia (MDS-RS-MLD), 22 as MDS with excess blasts-1 (MDS-EB-1) and 20 patients had MDS with excess blasts-2 (MDS-EB-2). Furthermore, 12 patients were assigned to the group involving isolated deletion of chromosome 5q (Table 1).

Flow cytometric analysis was performed on total white blood cells after erythrocyte lysis. Furthermore, BM samples were centrifuged on high speed programs for the collection of platelet-poor BM-derived plasma. These samples were stored at -30°C until they were used for cytokine analyses.

Flow cytometry

PB and BM cells were analyzed on a flow cytometer (FACSCanto™, BD Biosciences) after incubation with a panel of monoclonal antibodies (mAb) consisting of M-DC8-FITC and CD303-FITC (both Miltenyi Biotec, Utrecht, The Netherlands), CD16-PE (Beckman Coulter, Brea, USA), CD11c-PerCP-Cy5.5 (BD Biosciences), CD1c-Pe-Cy7 (eBioscience, San Diego, USA), CD141-APC (Miltenyi Biotec), CD14/CD19-APC-H7, HLA-DR-V450 and CD45-KO (all, BD Biosciences).

Frequencies of classical, intermediate and non-classical monocytes were calculated as percentage of CD45+ mononuclear cells (MNC). Median fluorescence intensity (MFI) levels of TM (CD141/BDCA-3) and HLA-DR were measured on all three subsets and percentages of monocytes positive for TM were calculated as percentage of total monocytes as well as of CD45+ MNC. Furthermore, TM expression on MDS-derived monocytes was evaluated after overnight stimulation and compared to TM expression at baseline (Figure S2). For further functional assays, only stimulated monocytes were used and no unstimulated condition was included (also because of limited cell numbers). Without stimulation co-stimulatory molecules, mainly CD80, were not up-regulated as compared to stimulated conditions (Figure S2).

Fluorescence in situ hybridization (FISH)

Frozen vials with MNC were rapidly thawed and stained with a monoclonal antibody cocktail containing M-DC8, CD1c, CD11c, CD14, CD19, CD34, CD45, CD141 and HLA-DR. TM+ monocytes, CD34+ myeloid progenitor cells and CD19+ B cells were flow cytometrically sorted (BD FACSAria^{TM}). Cells were collected in small Eppendorf tubes and further processed for interphase FISH analysis. They were fixed with 3:1 methanol/acetic acid and transferred to a microscopic slide. FISH was performed on each sorted cell sample according to the manufacturer's protocol using probes LSI EGR1(5q31)/D5S23,D5S721(5p15.2) Dual Colour Probe Set, LSI D7S486(7q31)/CEP7, and LSI CEP8 (D8Z2) (all probes from Abbott Molecular, Des Plaines, IL). For each probe at least 100 cells were investigated. In samples with less than 100 cells on the slide, all cells present were evaluated.

T cell cultures and multidimensional mass cytometry

TM- and TM+ monocytes from fresh samples of two MDS patients (MDS-RS-MLD) were flow cytometrically sorted (Figure S4). They were incubated and stimulated overnight with the TLR-4 ligand LPS (100 ng/ml, Sigma-Aldrich, St. Louis, USA). Next, total CD4+ T cells were derived from a healthy donor by magnetic isolation (Miltenyi Biotec, Utrecht, The Netherlands). Thereafter, T cells were co-cultured with either TM- or TM+ MDS monocytes on a plate pre-coated with anti-CD3 in a ratio of 1:10 for 5 days. T cells from day zero, cultured in the presence of monocytes, or cultured alone were stained with an extensive antibody panel containing cell surface markers, transcription factors and cytokines. Each antibody was tagged to a metal isotope (Table S1) and data was acquired on a Helios mass cytometer (Fluidigm).

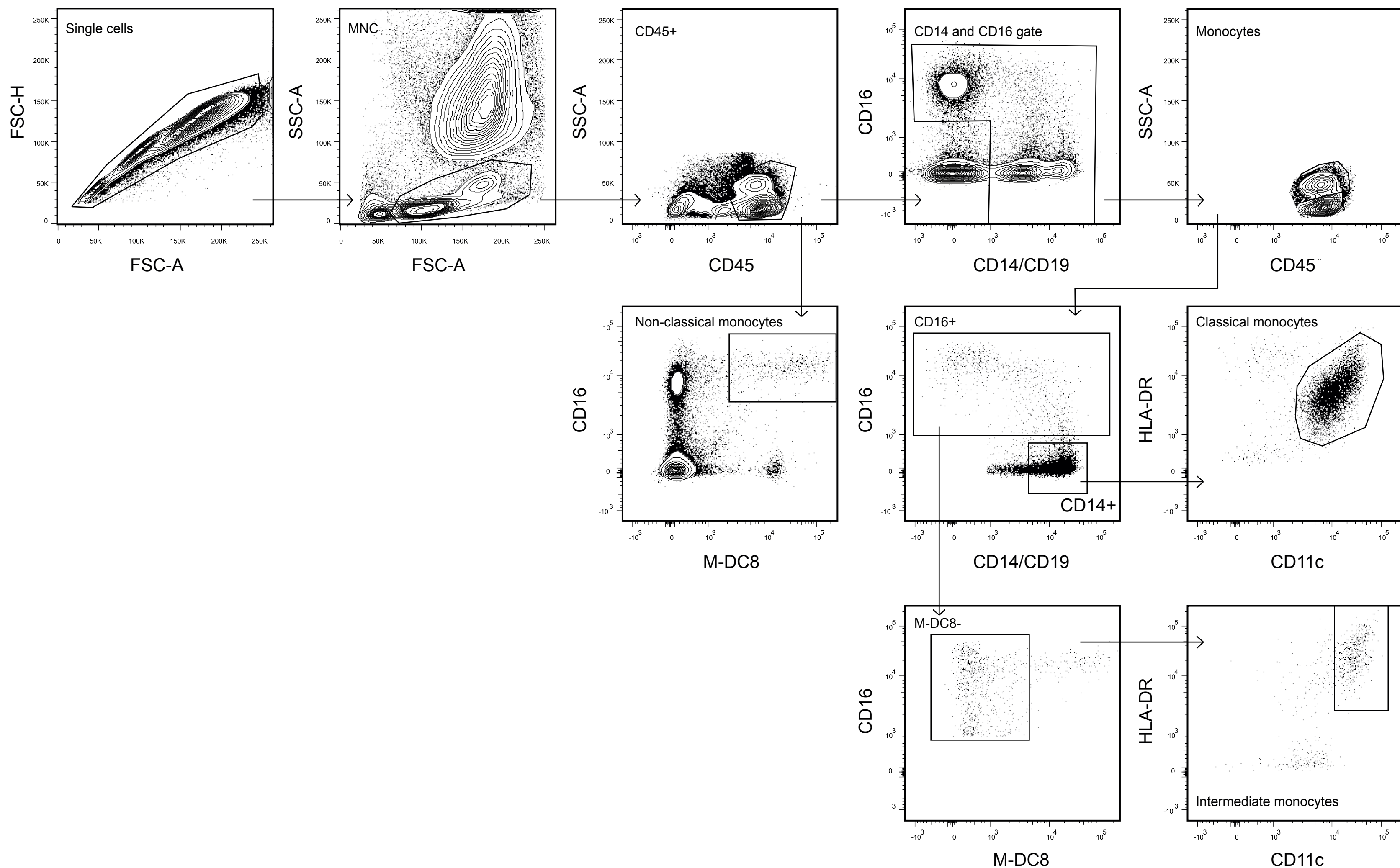
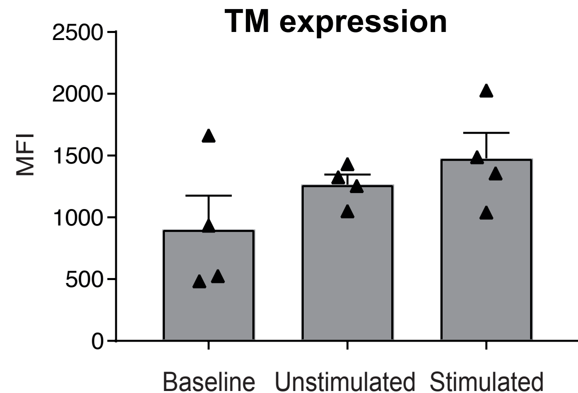


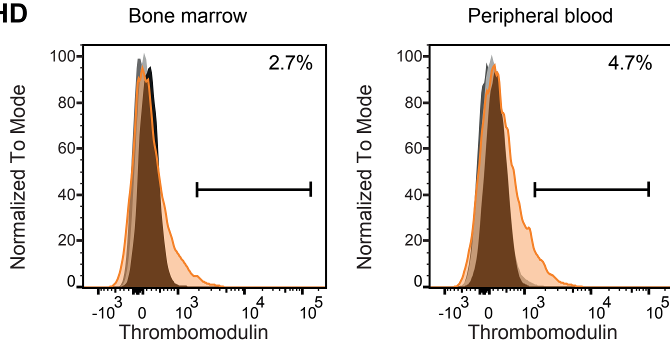
Figure S1. Gating strategy for different monocyte subsets. First, doublets were removed and the CD45+ mononuclear cell (MNC) fraction was selected. Monocytes were gated based on CD16/CD14 and CD45/SSC characteristics. Lymphocytes were removed. Then classical monocytes were identified using CD11c, HLA-DR and high expression of CD14. Intermediate monocytes were gated by using CD16, negative to intermediate expression of CD14 and negativity for M-DC8. The population was cleaned up by a HLA-DR/CD11c gate. CD16/M-DC8 was used for the identification of non-classical monocytes directly from the CD45+ MNC.

A



B

Figure S2. (A) Expression of thrombomodulin on MDS-derived monocytes at baseline and after overnight culture in which monocytes were either left unstimulated or were stimulated with TLR-ligands. (B) Expression of the activation markers CD80, CD86 and HLA-DR on MDS-derived monocytes upon stimulation and without stimulation.



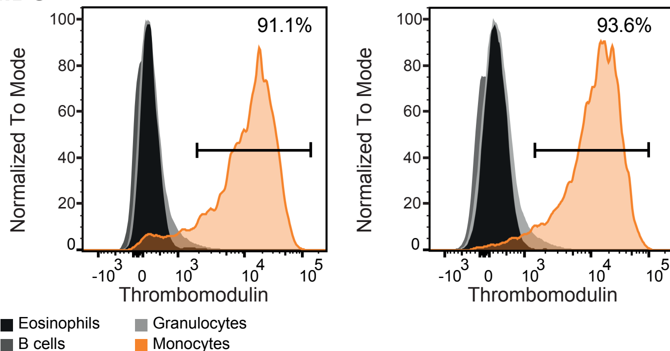


Figure S3. Expression of thrombomodulin on different cell types, including classical monocytes (orange histogram), in bone marrow and peripheral blood of a healthy individual (HD) and a MDS patient. The percentage of TM-positive classical monocytes is shown.

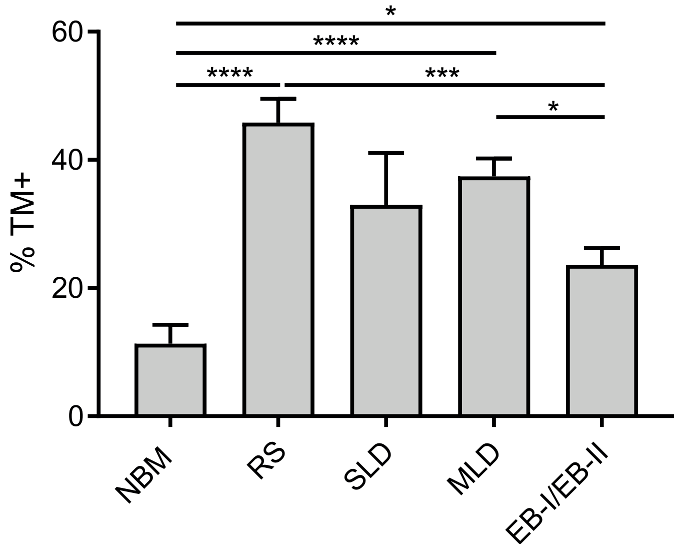


Figure S4. Percentages of TM+ monocytes in WHO 2016 subgroups with and without ring sideroblasts (RS). SLD, single lineage dysplasia; MLD, multilineage dysplasia; EB, excess blasts.

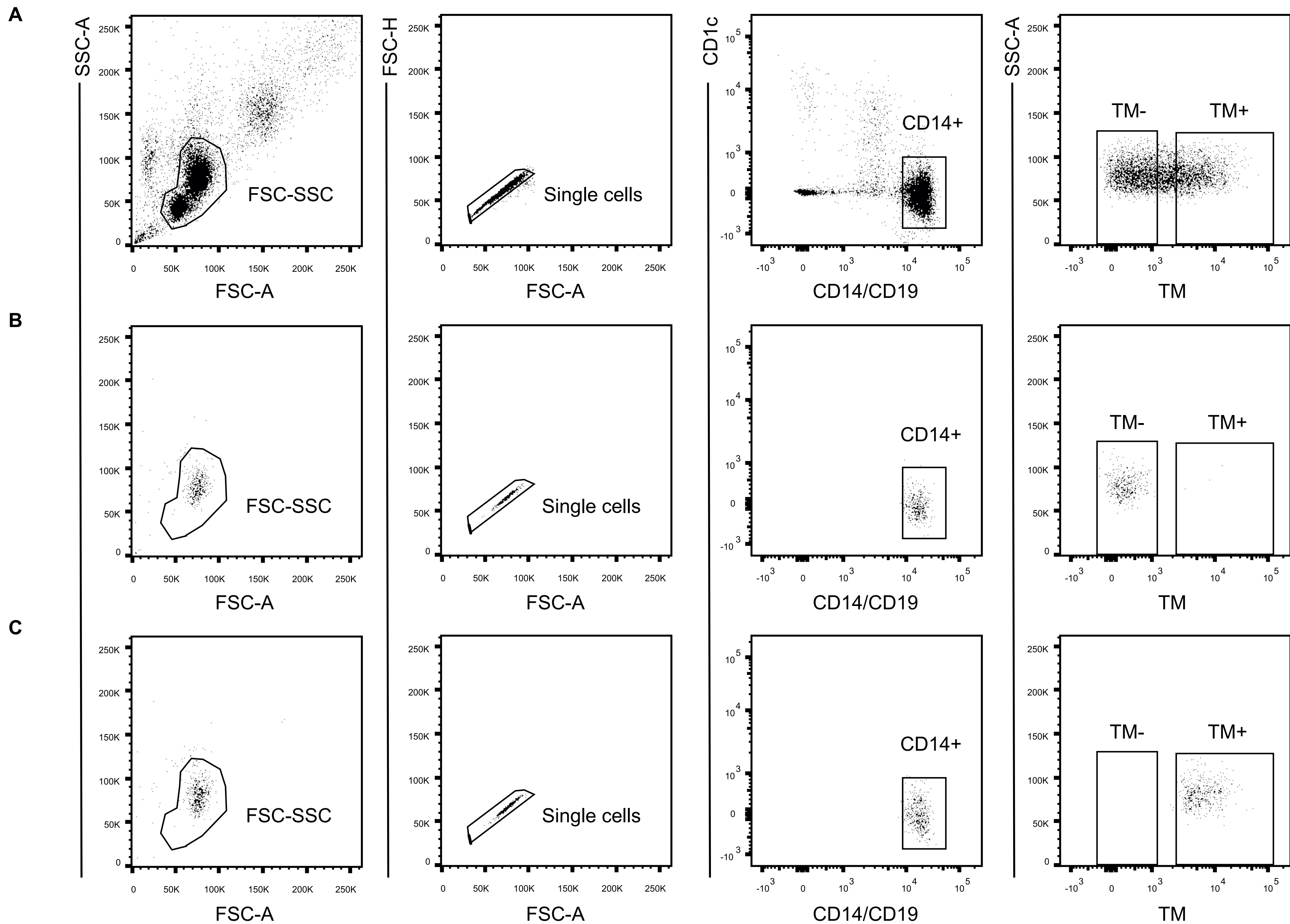
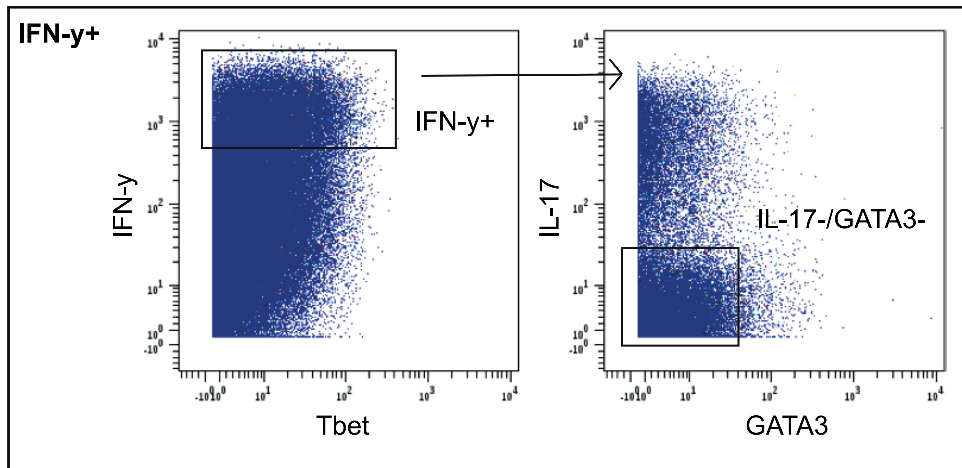
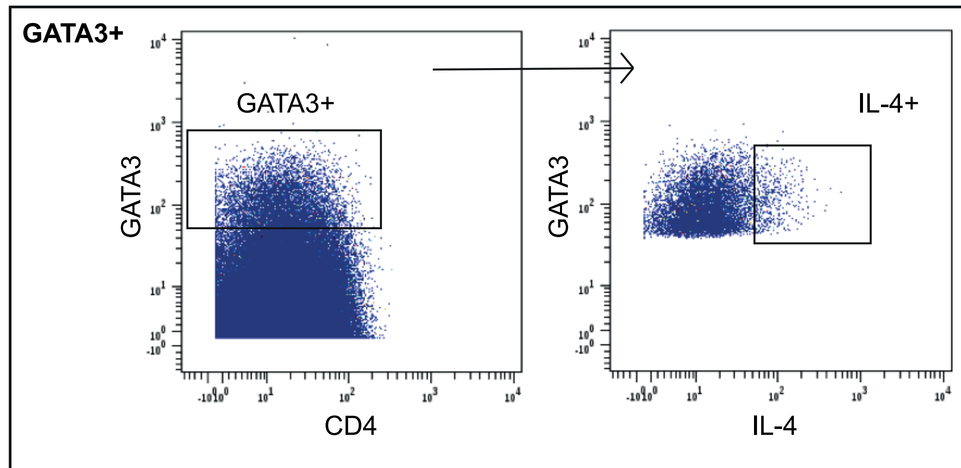
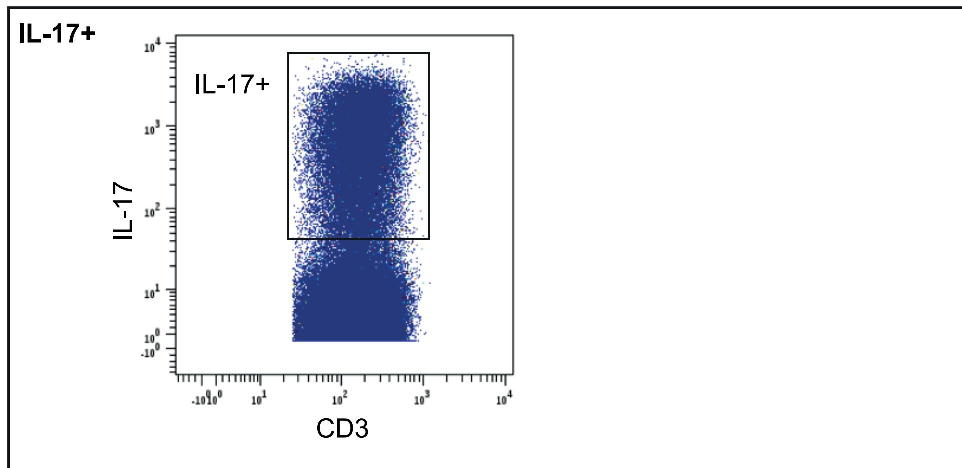


Figure S5. (A) Sort layout for CD14+ monocytes further subdivided in TM- and TM+ monocytes. Purity of sorted TM- (B) and TM+ (C) monocytes was verified afterwards.







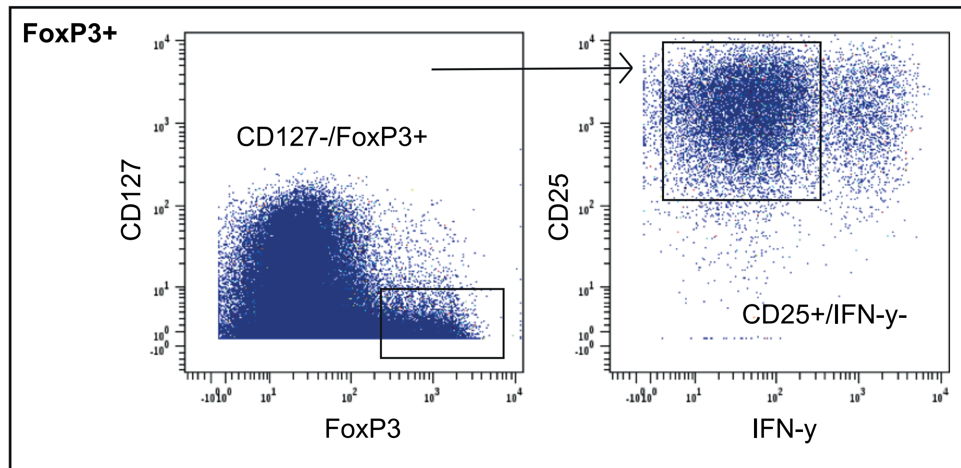


Figure S6. Different T cell subsets were identified for further visualisation in biaxial viSNE plots (shown in figure 3A).

Channel	Target
141	CD3
142	CD19
143	CD56
144	CD11b
145	CD4
147	CD20
148	IL-4
149	CCR4
150	CD62L
151	CD123
152	TNF-a
153	CD45RA
154	CD45
156	IL-6
158	IL-2
159	CD154
160	Tbet
162	CD69
164	IL-17
165	IFN-y
166	CD33
166	CD34
166	CD15
167	GATA3
168	CD8
169	CD25
169	CD25
170	IL-10
171	FoxP3
171	FoxP3
171	FoxP3
172	CD38
174	HLA-DR
175	CD279
176	CD127

Table S1. Antibody panel for mass cytometry staining
protocol with cell surface markers, cytokines and transcription factors.