1 **Visualization of a continuum between heterogeneous IL-10-producing CD4 T cell sub-**

2 **sets and other T helper archetypes by pattern perception**

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23   **Methods**

24   **Mice**

25   NZBxW and C57BL/6 mice bred under specific-pathogen-free (SPF) conditions at the facility

26   of the Federal Institute for Risk Assessment, Berlin, Germany, as well as four-week-old

27   C57Bl6/J mice purchased from Janvier (France) were maintained at the experimental SPF

28   facility of the DRFZ. The analyzed mice were female (NZBxW), or male and female

29   (C57BL/6). All experiments were approved by the local authority (Landesamt für Gesundheit

30   und Soziales, Berlin, Germany; licenses T0187/01 and G0070/13). The disease was moni-

31   tored by weighing and proteinuria assessment in spontaneous urine (Uristix, Siemens).

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33   **Cell preparation and *ex vivo* activation**

34   The spleens were mashed through a 100-μm polyamide mesh to generate single-cell sus-

35   pensions. The erythrocytes were lysed with red blood cell lysis buffer Hybri-Max™ (Sigma-

36   Aldrich). After lysis, the splenocytes were resuspended in FACS buffer [phosphate buffer sa-

37   line (PBS) supplemented with 0.5% (w/v) bovine serum albumin (BSA)] and counted. The

38   cells were stimulated in complete RPMI medium with 10% fetal bovine serum for one hour

39   with 10 ng/ml phorbol 12-myristate 13- acetate (PMA) and 1 mg/ml ionomycin (Sigma) fol-

40   lowed by three additional hours with 5 mg/ml Brefeldin A (Sigma).

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42   **Flow cytometry**

43   After *ex vivo* stimulation, the cells were washed in PBS and stained with fixable LIVE/DEAD

44   aqua dye (Life Technologies). After washes in PBS, the cells were blocked for 10 min with

45   100 mg/ml of 2.4G2 antibodies at 4°C, and incubated with fluorescent antibodies against sur-

46   face markers in eBioscience Brilliant staining buffer (Life Technology) for 30 min at 4°C. After

47   washing in FACS buffer the cells were fixed with BD Cytofix™ (BD Biosciences) and incu-

48   bated with antibodies against cytokines in BD Cytoperm™ permeabilization buffer (BD Bio-

49   sciences). Antibody list and panels are detailed in Supplementary Table 1. The cells were

50   analyzed with a BD LSR Fortessa cytometer (BD Biosciences). The data were analyzed with

51   FlowJo (Treestar) and the CD44$^+$ CD4$^+$ T cells and IL-10$^+$ CD44$^+$ CD4$^+$ T cell population were

52   exported as FCS files for further analysis using the bin-approach PRI[4] (Baumgrass, DRFZ).

53   In PRI, the fluorescence intensities were transformed with inverse hyperbolic sine (arcsinh)

54   and a coefficient of 1 (Fig. 1B, d; Fig. 2A; Supporting Information Fig. S2A) or 5 (Fig. 2C),

55   and divided in 0.2 x 0.2 (Fig. 1B, d; Fig. 2) or 0.4 x 0.4 (Supporting Information Fig. S2A) bins

56   along the X and Y axis to draw bin plots. Different statistical values, including the frequency

57   (%) of cells positive for the z-parameter per bin, the mean signal intensity of all cells for the z-

58   parameter per bin (MSI), or the mean signal intensity of the cells positive for the z-parameter

59   (MSI+) per bin, are displayed with rainbow color gradients as a heat maps (low values are in

60   shades of blue, median values in yellow, and high values in red). Additional statistics are dis-

61   played in black, red, and green percentage numbers in each quadrant. Percentages in black

62   indicate the frequency of cells relative to total cells. The frequency of z$^+$ cells is given as rela-

63   tive to the cells inside the respective quadrants (red), or relative to total cells (green). All

64   events (cells) were included in the analysis but only bins containing > 5 cells are displayed,

65   balancing the impact of identifying comparatively rare subpopulations while retaining statisti-

66   cal robustness.

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68   **Statistics and graphic abstract**

69   Statistical analyses were performed in Graphpad Prism software [version 9.1.0 (211)]. The

70   proportion of cytokine-expressing cells between groups were compared by Mann-Whitney U

71   test. The graphical abstract was made in biorender.

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73   **Code availability**

74   The codes supporting the current study can be found at: https://github.com/InesHo/PRI-

75   demonstration and at the following links:

76   Source code 1

77   A notebook to show step by step how to create the bin plots (in HTML) is available for down-

78   load at:

79   https://cdn.elifesciences.org/articles/53226/elife-53226-code1-v2.zip

80   Source code 2

81   A notebook on how to create bin functions (in R) is available at:

82   https://cdn.elifesciences.org/articles/53226/elife-53226-code2-v2.zip

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84   **Author contributions**

85   Conception: RB

86   Methodology: AV, EM, FP, JR, JV, RB, SG, YH.

87   Investigation: AM, EM, JR, TH, TL, SG.

88   Writing&editing: AR, EM, JV, RB, TH.

89   Funding Acquisition, JV, RB.

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92   **Table S1.** Antibody list and panels

Target	Conjugate	Clone	Source / Catalog number	Concentration [μg/ml]	Panel
B220	Brilliant violet 785	RA3-6B2	Biolegend/103245	0.5	II, III
CD4	Pacific Blue	GK1.5	DRFZ	0.514	I
CD4	Alexa 700	GK1.5	DRFZ	0.963	II, III
CD8α	Brilliant violet 510	53-6.7	Biolegend / 100752	0.67	I
CD19	Brilliant violet 510	6D5	Biolegend / 115545	1	I
CD44	Pacific Blue	IM7	DRFZ	1.243	II, III
CD44	Brilliant violet 785	IM7	Biolegend / 103059	0.5	I
CD16/CD32	purified	2.4G2	DRFZ	28.45	
IFN-γ	PE/Cy7	XMG1.2	BD Biosciences / 557649	0.5	I, II
IL-2	Brilliant violet 510	JES6-5H4	Biolegend / 503833	0.5	II, III
IL-2	Brilliant violet 711	JES6-5H4	Biolegend / 503837	2	I
IL-10	APC	JES5-16E3	eBioscience / 17-7101-82	2	I, II, III
IL-21	PE	mhalx21	eBioscience / 12-7213-82	4	I
PD-1	PE	J43	eBioscience / 12-9985-83	1	II
PD-1	PerCP eFluor710	J43	eBioscience / 46-9985-82	1	III
PD-1	APC/Cy7	29F.1A12	Biolegend / 135234	1	I
TNF-α	FITC	MP6-XT22	DRFZ	0.65	I, II, III

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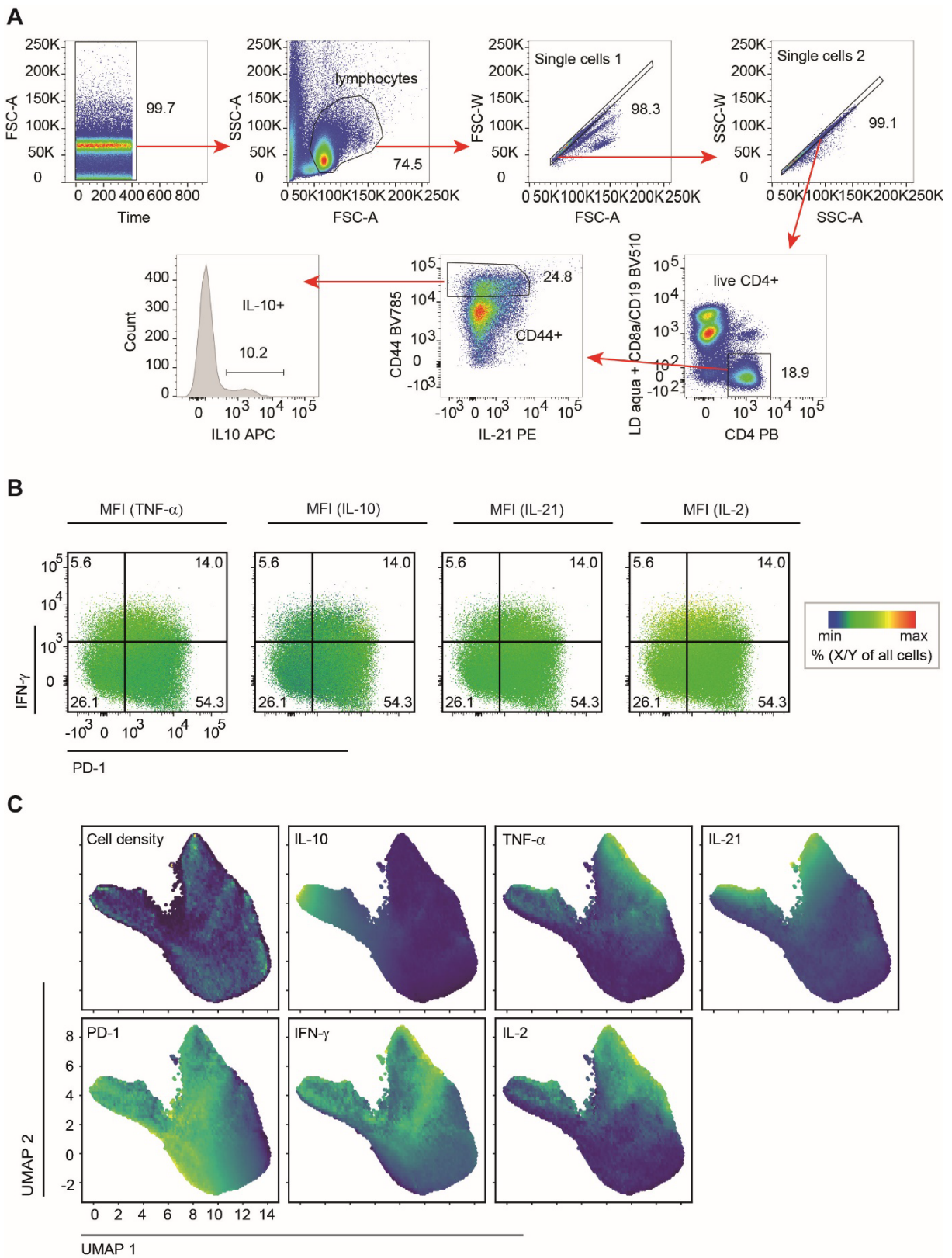
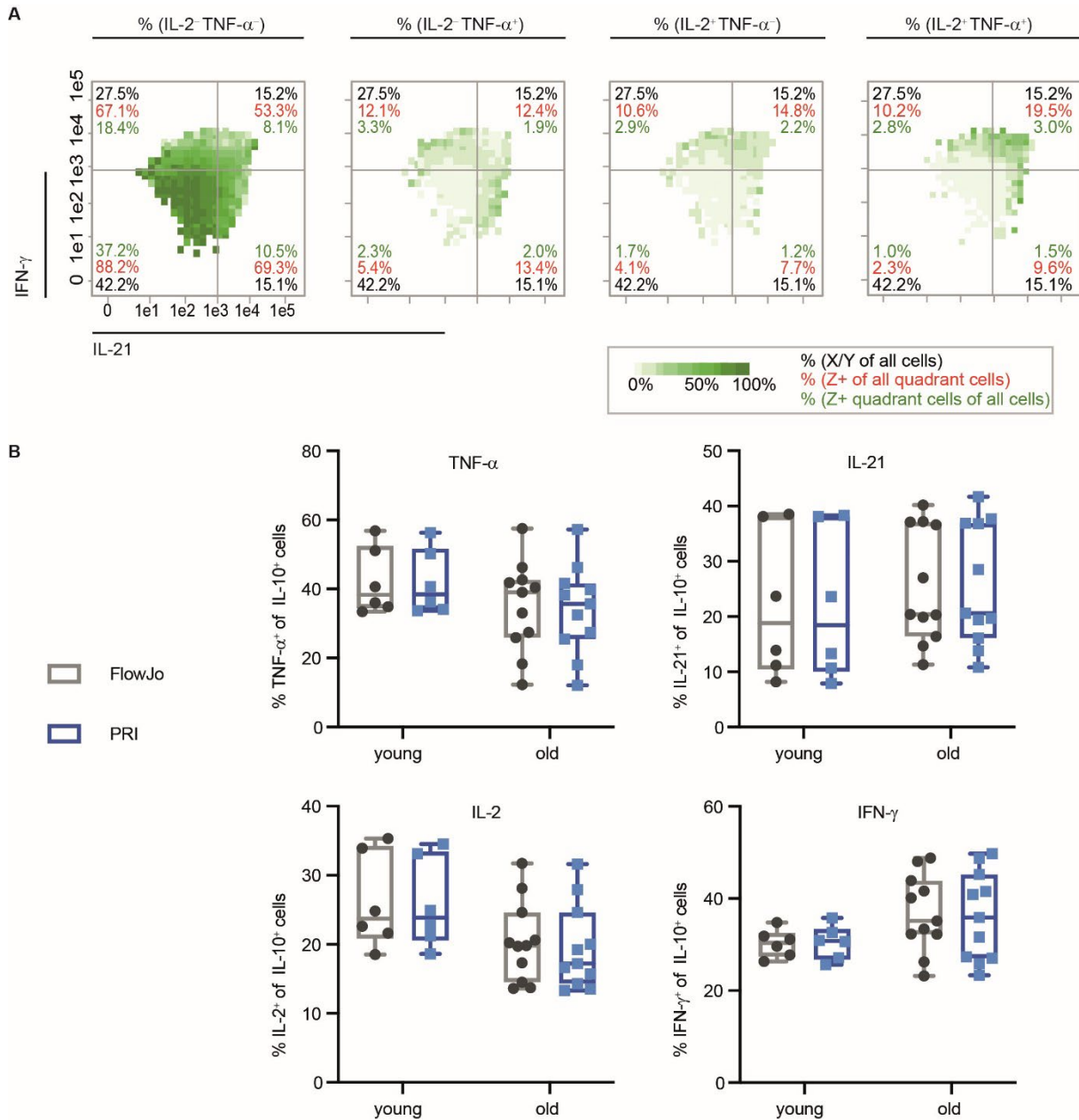## Figures

Figure S1. Gating strategy and common three-parametric and multi-parametric visualizations of cytometry data.

99   **(A)** Representative plots showing the gating strategy of mouse splenocytes used to analyze Tmem

100 (live CD44+CD4 T cells) and IL-10+Tmem cells (gate on histogram). The red arrows show the succes-

101 sive gating leading to one gate to the next plot. For PRI analysis, the total the Tmem [Fig. 1 (B), (D);

102 Fig. 2(A), (C)], or IL-10+Tmem [Fig. 2(B)], populations were extracted in a separate FCS files.

103 **(B)** FlowJo's color maps showing MFI of different cytokines corresponding to the representative sam-

104 ple analyzed in Fig. 1b by bin-plotting. The rainbow color gradient represents different mean fluores-

105 cence intensity (MFI) of individual cells overlaid on dot plots displaying PD-1 (X axis) and IFN-γ (Y

106 axis) expression.

107 **(C)** Representative UMAP (Uniform Manifold Approximation and Projection) analysis of the Tmem

108 cells from a representative wild-type C57BL/6 mouse. Prior to UMAP analysis, the data underwent

109 logicle transform and robust scaling. The color gradient represents different MFI of individual bins.

110

A

B

Figure S2. Cytokine co-expression of IL-10 producing cells.

(A) Combinatorial analysis of cytokine expression of IL-10 producers by four-parametric bin-plots. The plotted IL-10 producers were obtained by concatenating equal numbers of IL-10-producing Tmem cells from three different two-year old C57BL/6 mice analyzed in a single experiment. The cells were binned according to the semi-continuous intensities of IL-21 (X axis) and IFN-γ (Y axis) expression. The frequency pattern of the cells expressing the four possible combination of TNF-α and IL-2, i.e., TNF-α^- IL-2$^-$, TNF-α^+ IL-2$^-$, TNF-α^- IL-2$^+$, and TNF-α^+ IL-2$^+$ (Z axis, shades of green gradient). The black percentages represent the proportion of cells in each quadrant according to IL-21 and IFN-γ expression. The green percentages indicate the proportion of cells with the Z phenotype among the total population of IL-10$^+$ cells and were used as source data to generate the pie chart in Fig. 2b.

123   **(B)** Box and minimum to maximum whiskers graphs summarizing the medians of the percentages of

124   total IL-10$^+$ Tmem cells co-expressing TNF-α, IL-21, IL-2, and IL-21 in young (6–8 months) or old (24

125   months) C57BL/6 mice, as calculated with FlowJo (grey) or bin-plotting (blue). Data from four inde-

126   pendent experiments involving 2–3 mice/group. Two-group comparisons were statistically assessed

127   by non-parametric Mann-Whitney U test. No significant differences between groups of different ages

128   or analyses were detected (P values > 0.05).