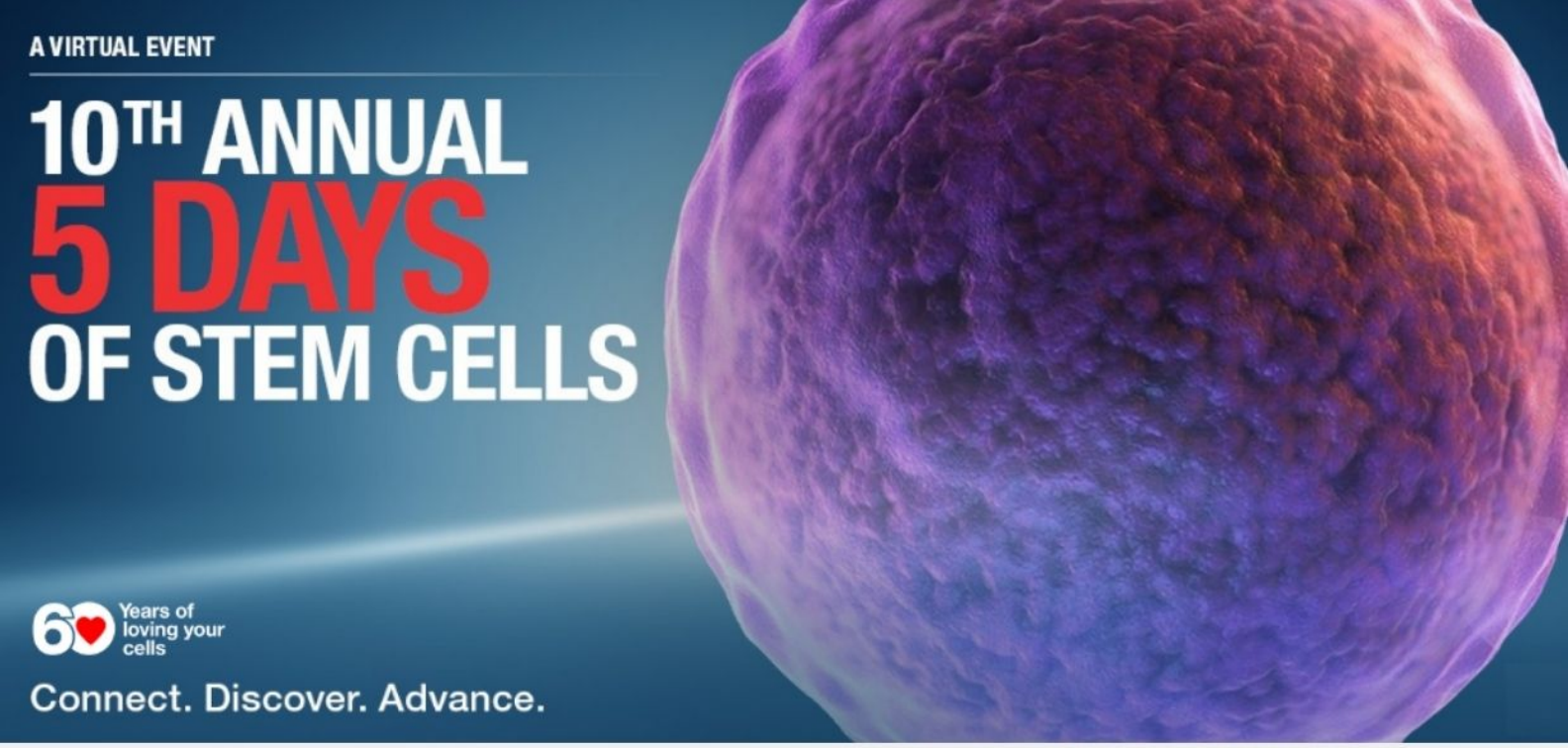

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Notes and Insights

Bin-based visualization of cytokine-co-expression patterns of IL-10-producing CD4 T cell subsets

Recently, the dogma representing effector CD4 T cell (Teff) diversity as discrete subsets has been challenged by unsupervised analyses of single-cell RNA sequences. In particular, two studies from Cano-Gamez *et al.* and Kiner *et al.* showed that *in vivo* differentiated Teffs do not cluster into discrete populations but rather form a transcriptional continuum following either a gradient of "effectorness" [1] or a temporal gradient reflecting the kinetics of response to infections [2].

Despite a technologic burst allowing for multiparametric protein expression analysis at single-cell level by flow or mass cytometry [3], gradients of protein expression remain hardly tangible because conventional analyses use successive gating that creates discrete cell subsets. Other methods, using dimensional reduction and clustering like t-SNE or UMAP, require down-sampling and render quantitative comparisons problematic. Here, we tested whether the $CD4^+$ T cell continuum described at the transcriptomic level also existed at the protein level by analyzing flow cytometric data with our new semi-continuous bin-based algorithm for "pattern recognition of immune cells" (PRI) [4]. We took the example of the $CD44^+CD4^+$ T memory cells (Tmems) expressing IL-10 and those expressing other cytokines, such as IFN-γ-producing T helper type 1 cells (Th1), IL-21-producing T follicular helper cells (Tfh), or $TNF\text{-}\alpha^+IFN\text{-}\gamma^+IL\text{-}2^+IL\text{-}21^+$ lupus-associated T super helper cells (Tsh) [4], in mouse autoimmunity and aging.

First, we compared IL-10 expression in Tmems relatively to PD-1 and IFN-γ by conventional two-parameter analysis (Fig. 1A; Supporting Information Fig. S1A) and three-parameter bin-plot analysis with PRI (Fig. 1B). Despite lower frequencies of IL-10-producing cells (Fig. 1C) in young wild-type C57BL/6 and pre-lupus NZBxNZW F1 (NZBxW) mice, the IL-10 pattern was conserved independently of age or health status. This pattern characterized by largely overlapping IFN-γ and IL-10 patterns (60% of co-expressers), albeit $IL\text{-}10^+IFN\text{-}\gamma^+$ cells produced low IFN-γ level (Fig. 1A, B). The $IL\text{-}10^+$ cells mainly clustered in the $PD\text{-}1^{+/high}/IFN\text{-}\gamma^+$ area, with minute proportions in the $IFN\text{-}\gamma^{high}$ and $PD\text{-}1^-$ areas (Fig. 1B,D). Further analysis with PRI, allowing a more comprehensive analysis of IL-10 level in all (mean signal intensity [MSI]) and $IL\text{-}10^+$ cells (positive mean signal intensity [MSI+]) than the "color mapping of dots" in FlowJo software (Supporting Information Fig. S1B), revealed a gradient of IL10 expression originating from the $PD\text{-}1^{-/low}$ area and culminating in the $PD\text{-}1^{high}$ area (Fig. 1D). The positive correlation between IL-10 and PD-1 expression was most striking in sick NZBxW mice but was also visible in old C57BL/6 mice.

Next, comparing IL-10, TNF-α, IL-2, IL-21, and IFN-γ expression in relation with PD-1 and IFN-γ level showed distinct but partially overlapping patterns that formed a cloud gathering multiple cytokines producers in the $PD\text{-}1^{-/low}IFN\text{-}\gamma^+$ area (Fig. 2A). In particular, the $PD\text{-}1^+IFN\text{-}\gamma^+$ quadrant contained overlapping $IL\text{-}21^+$ and $IL\text{-}10^+$ areas. TNF-α and IL-2 displayed similar patterns, modestly overlapping IL-10 pattern. We explored the actual combinatorial expression of IL-10 with the other cytokines by 4-parameter bin-plot analysis (quadru-plots) (Supporting Information Fig. S2A). The largest $IL\text{-}10^+$ subset (37.23%) produced exclusively IL-10 (Fig. 2B), while a third of the $IL\text{-}10^+$ cells co-expressed an additional cytokine (Fig. 2B and Supporting Information Fig. S2B). Most frequent double-expressers produced IFN-γ (18.44%) and IL-21 (10.47%), whereas $IL\text{-}10^+IL\text{-}2^+$ and $IL10^+TNF\text{-}\alpha^+$ cells were scarce. Triple-expressers were mainly $IL\text{-}10^+IFN\text{-}\gamma^+IL\text{-}21^+$ cells (8.1%). Finally, in double-, triple-, quadruple- and quintuple-producers, the highest IL-10 intensities were associated with IL-21 and IFN-γ expression (Fig. 2B, vignettes with yellow and orange bins), whereas the lowest intensities correlated with IL-2 and TNF-α expression. UMAP approach confirmed little co-expression of IL-10 with TNF-α and IL-2 (Supporting Information Fig. S1C). Further analysis revealed populations of high TNF-α and/or IL-2 producers in the $IL\text{-}21^{high}$ areas, excluded from the $IL\text{-}10^+$ quadrants and likely representing Tsh cells. This distribution confirmed negative correlations between IL-10 and TNF-α or IL-2 expression (Fig. 2C).

Altogether, this analysis revealed a continuum between IL-10-producing Tmems and cells with cytokine profiles reminiscent of Th1, Tfh and Tsh cells, with all possible cytokine combinations at single-cell level. The consistency of the cytokine patterns in mice of different age and health status suggests that these may represent conserved programs of CD4 T cell differentiation that vary quantitatively depending on the physiological context. Progressive accumulation of multifunctional Tmems evokes the "effectorness" gradient evidenced by single-cell transcriptomics [1]. Negative correlations between IL-10 and IFN-γ level, and TNF-α and IL-2 expression suggest a repression of these cytokines in IL-10-producing cells. Given the positive correlation between IL-10 and PD-1 expression, this down-regulation could involve PD-1 [5]. Supporting these hypotheses, IL-10 blockade increases TNF-α and IFN-γ production *in vitro*, and lupus manifestations in mice [6, 7].

The best characterized $IL\text{-}10^+IFN\text{-}\gamma^+$ cells are $Foxp3^-$ Type 1 regulatory $CD4^+$ T cells with clear suppressive functions [8].

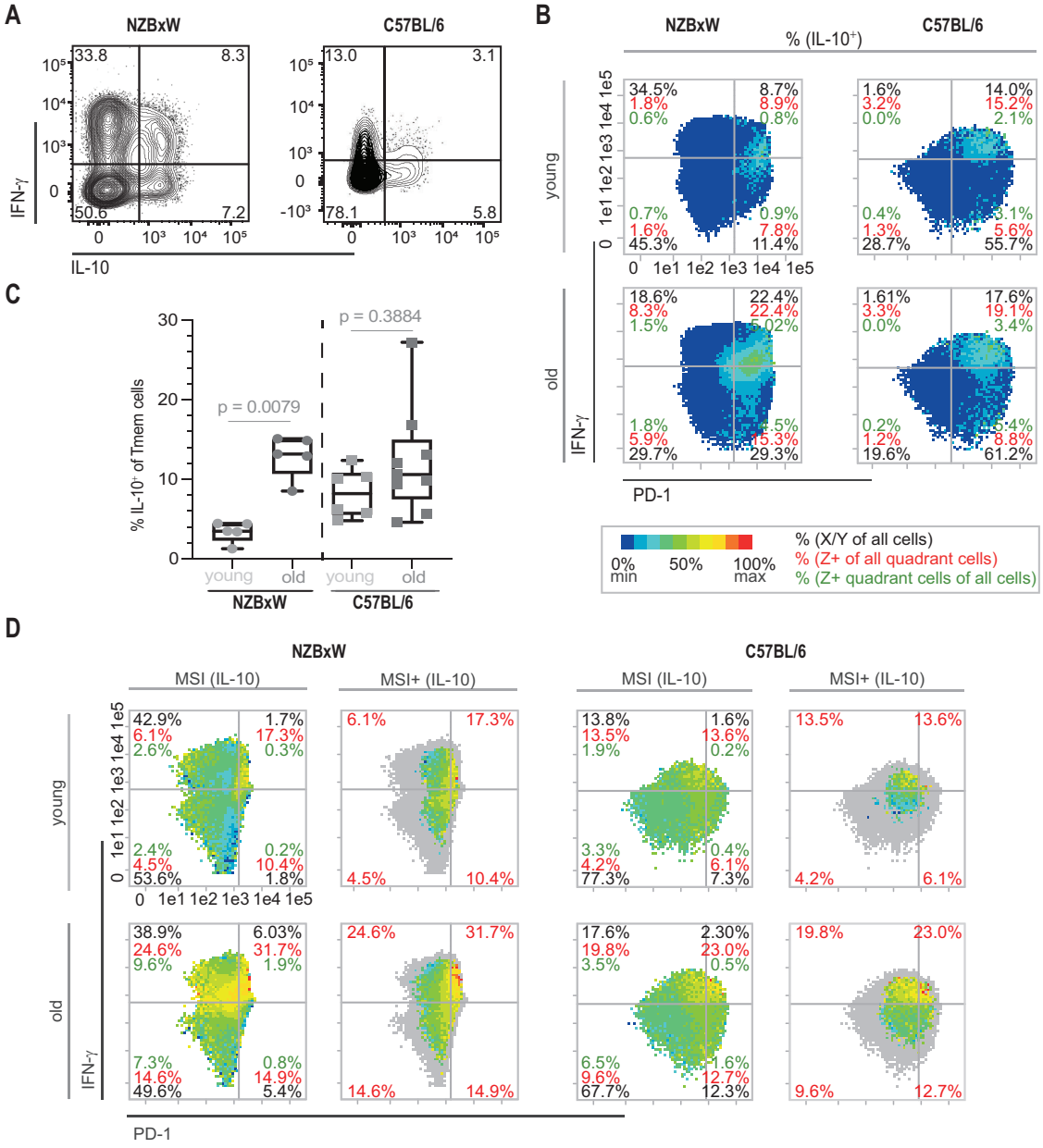

Figure 1. IL-10 co-expression with IFN-γ and PD-1 in Tmems from C57BL/6 and NZBxW lupus mice. Flow cytometric analysis of *ex vivo* restimulated splenocytes from C57BL/6 or NZBxW mice (antibody panels: Supporting Information Table S1). (A) Representative contour plots showing IL-10 and IFN-γ co-expression in Tmems from a diseased NZBxW and a two-year-old C57BL/6 mouse (Panel II). (B) Three-dimensional bin-plots showing IL-10$^+$ Tmems frequency (color-coded, Z-axis) according to semi-continuous PD-1 (X-axis) and IFN-γ (Y-axis) level. Each plot represents a concatenated sample made of Tmems from three different mice of same age, strain, and disease score (Panel II). The quadrants delineate the positive thresholds for PD-1 and IFN-γ. (C) Splenic IL-10$^+$ Tmem proportion (mean±SEM) in different groups (Panel II). Two-group comparisons by Mann-Whitney test (1–3 independent experiments involving 3–5 mice/group). (D) Bin-plots from individual young and old NZBxW (Panel III) or C57BL/6 (Panel II) mice revealing IL-10 intensity pattern in Tmems (rainbow color-coded intensities) according to PD-1 and IFN-γ levels. The bin-plots depict the mean signal intensity of all cells/bin (MSI) or IL-10$^+$ cells/bin (MSI+)

However, IL-10 has been linked to lupus progression [6], while IFN-γ-receptor signaling reduces disease development [9]. Thus, IFN-γ and IL-10 may synergize to achieve higher anti-inflammatory functions. Conversely, IL-21/IL-10 association may promote autoantibody production and aggravate autoimmunity [10]. Therefore, IL-10 could be protective or pathogenic, depending on its association with other cytokines at single-cell level. Further, the amount of cytokines per cell may be significant to disambiguate the biological activity of IL-10-producing Tmems. PRI, integrating the combinatorial and level of protein expression, may help extracting clinically relevant quantitative data.

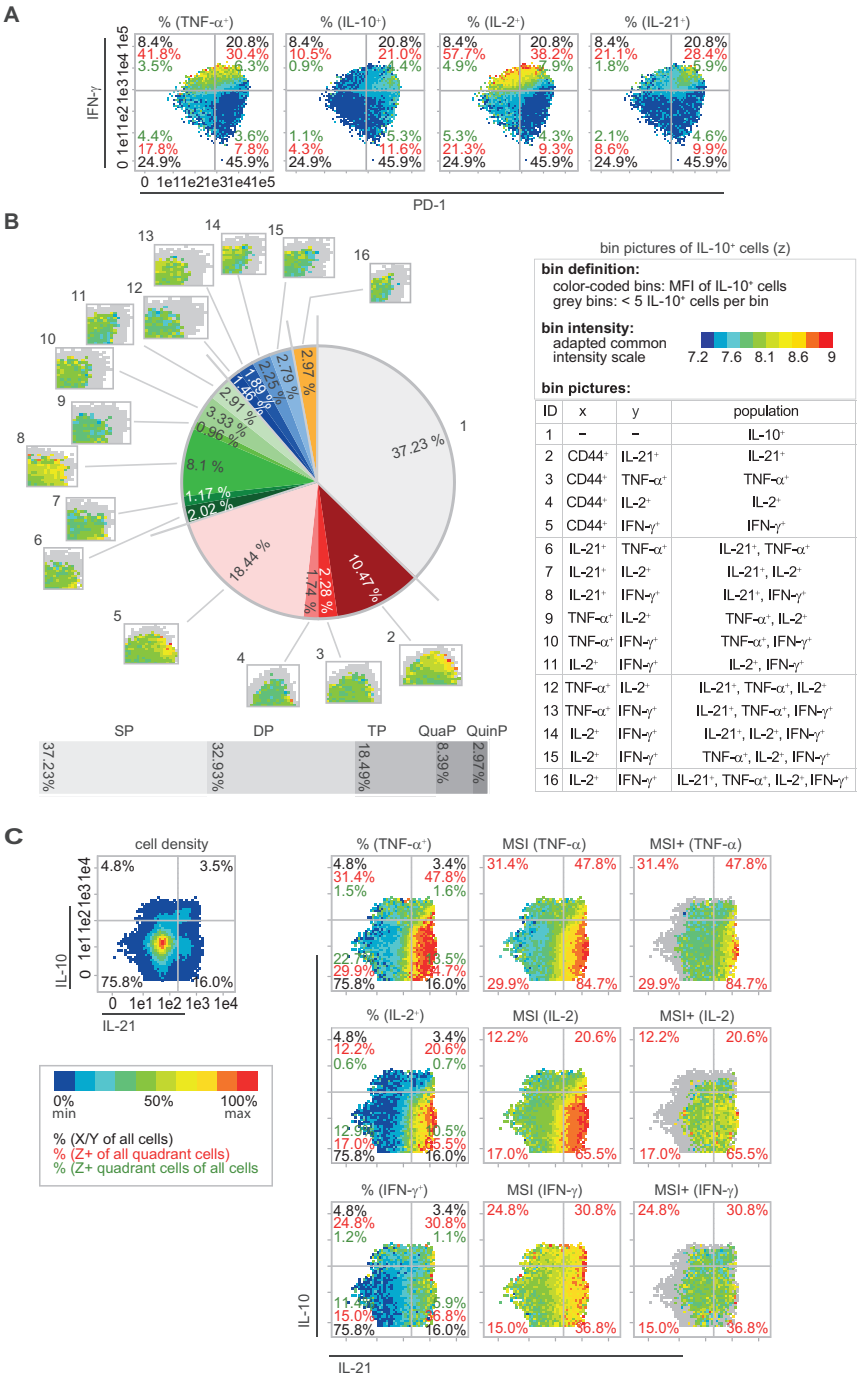

Figure 2. IL-10 co-expression with multiple cytokines. Flow cytometric analysis of *ex vivo* stimulated C57BL/6 splenocytes (Panel I: Supporting Table S1). (A) IL-10, TNF-α, IL-2, and IL-21 patterns (color-coded frequencies, Z-axis) in Tmems, according to semi-continuous PD-1 (X-axis) and IFN-γ (Y-axis) level. Representative data from a two-year-old C57BL/6 mouse (n = 10, four independent experiments). (B) Pie chart and 100%-stack bar: relative proportions of single(SP)-, double(DP)-, triple(TP)-, quadruple(QuaP)-, and quintuple(QuinP)-cytokine producers in a representative concatenated sample made of equal proportions of IL-10$^+$ Tmems from three mice from a single experiment. Frequencies were analyzed in three- and four-parametric bin-plots (Supporting Information Fig. S2). The numbered vignettes associated to the sectors are quadrants from three-parametric MSI+ bin-plots drawn with the X- and Y-axis indicated in the table and displaying IL-10 signal intensity of IL10$^+$ cells (Z-axis). (C) Representative bin patterns of cytokine expression according to IL-10 and IL-21 expression. The Tmems from a two-year-old mouse were analyzed for cell density, frequencies (%), mean signal intensity (MSI), and mean signal intensity of the Z$^+$ cells (MSI+)

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