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T helper cells: a complete workflow for cell preparation, isolation, stimulation, polarization, and analysis

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Introduction

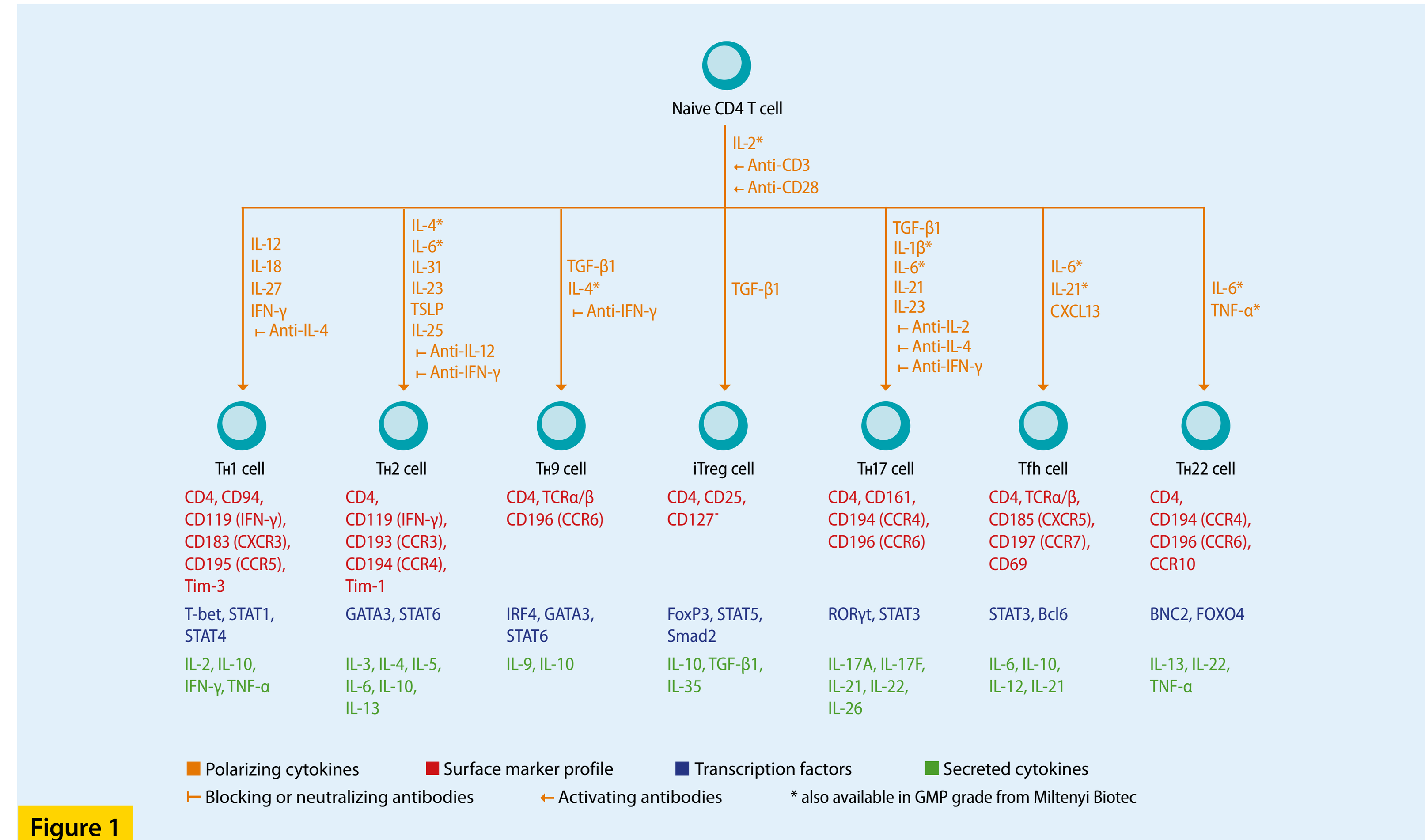

Figure 1
Polarization of naive CD4⁺ T cells into Tн cell subsets.

CD4⁺ T helper (Tн) cells play a central role in the adaptive immune system by controlling a variety of cellular responses, defending the host against pathogens and tumor development. Their cytokine secretion suppresses or stimulates immune responses and leads to antibody production by B cells, immunoglobulin class switch, and macrophage activation, for example.

Their crucial impact on immune responses and distinct role in the protection against disease make Tн cells a focus of many researchers studying immune regulation. The various Tн cell subsets can be differentiated from naive CD4⁺ T cells *in vitro* using specific combinations of polarizing cytokines (fig. 1).

Materials and methods

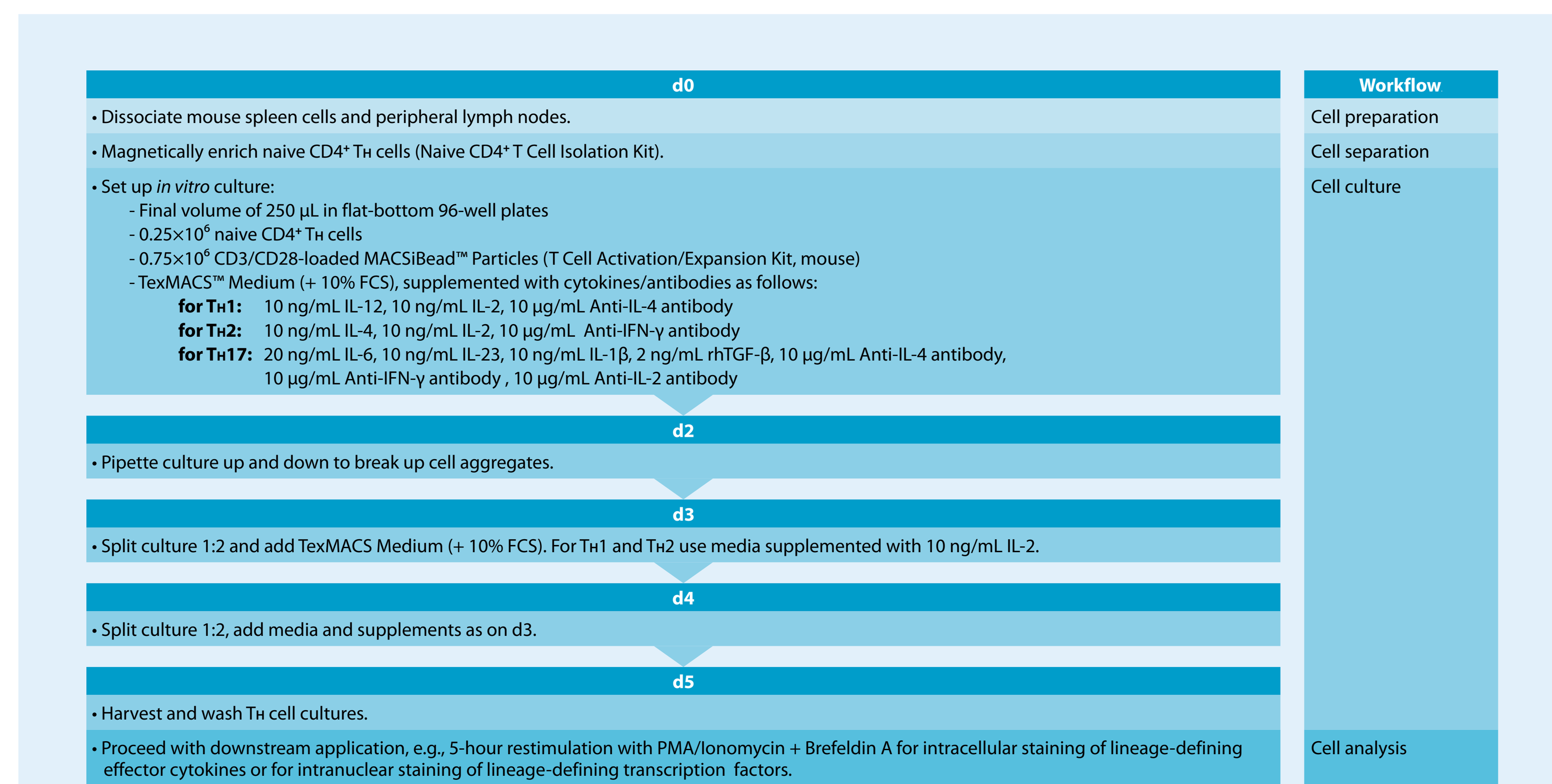

Figure 2
Workflow and protocol for *in vitro* mouse Tн cell differentiation

Results

1 Magnetic enrichment of naive CD4⁺ Tн cells

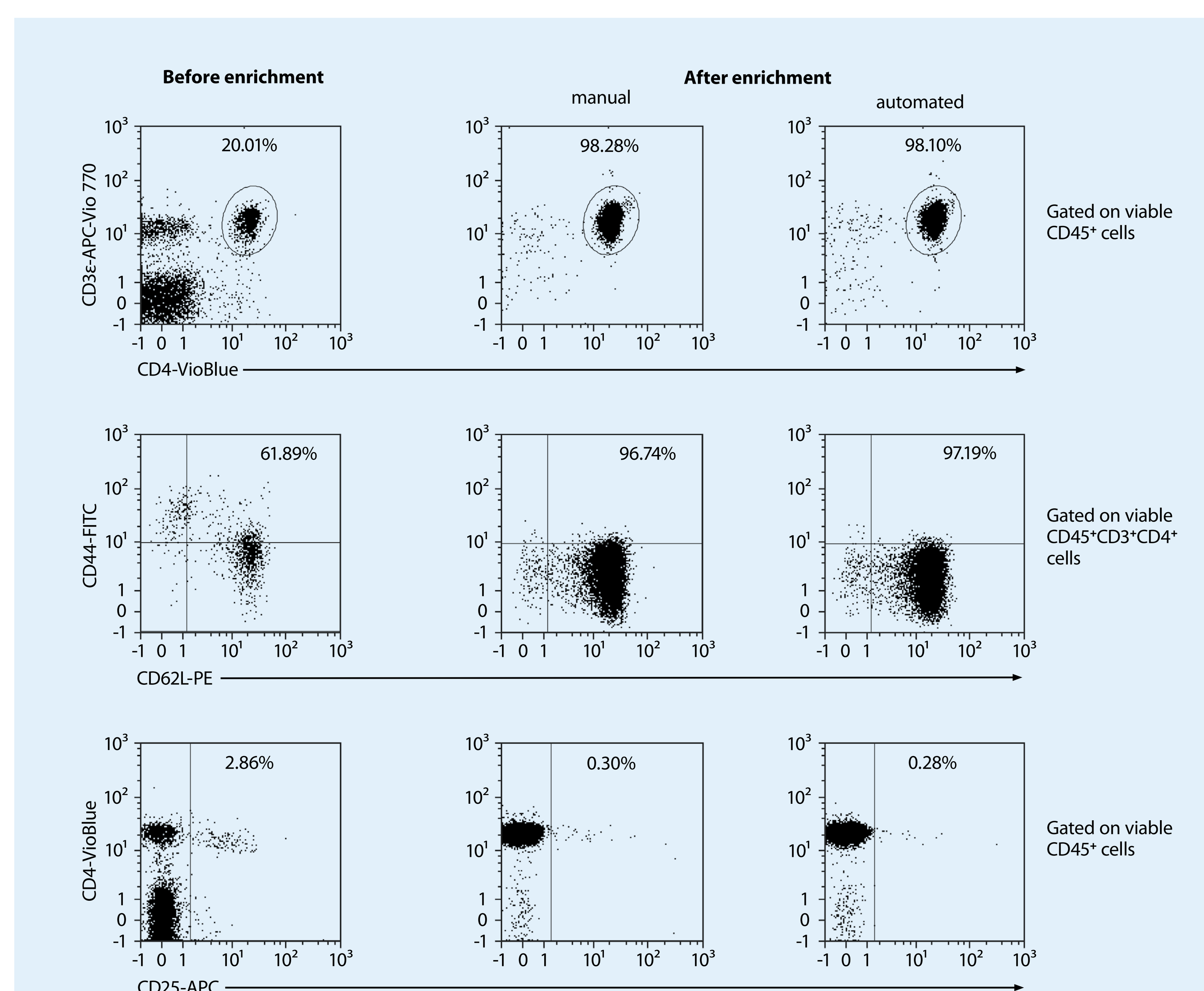

Figure 3
Example of magnetic enrichment of naive CD4⁺ Tн cells

Naive CD4⁺ T cells were isolated from a single-cell suspension, which was prepared from a 6-week-old BALB/c mouse spleen, using the Naive CD4⁺ T Cell Isolation Kit, mouse. Cell separation was performed either manually with MACS® Columns or automatically with the autoMACS® Pro Separator. Cells were fluorescently stained with CD45-VioGreen™, CD4-VioBlue®, CD62L-PE, CD3ε-APC-Vio® 770, CD44-FITC, and CD25-APC (all from Miltenyi Biotec) and analyzed by flow cytometry using the MACSQuant® Analyzer 10. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence, and a gate was set on CD45⁺ cells. The isolated naive (CD44$^{low/-}$CD62L⁺CD25⁻) CD4⁺ T cells with a purity of about 98% were used as starting material for *in vitro* polarization towards Tн1, Tн2, and Tн17 cells.

2 Analysis of lineage-specific transcription factors

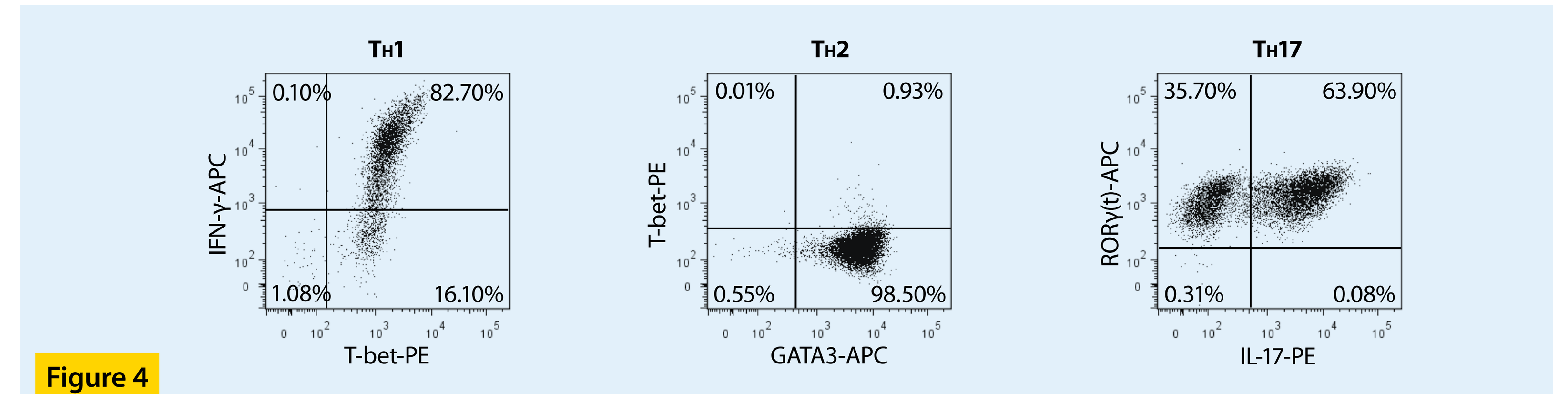

Figure 4
Analysis of lineage-specific transcription factors in Tн1, Tн2, and Tн17 cells

Differentiation into the Tн cell subsets was accomplished using the T Cell Activation/Expansion Kit, TexMACS™ Medium, recombinant cytokines, and functional-grade antibodies (all from Miltenyi Biotec). On day 5 of the *in vitro* culture, the differentiation into the various Tн cell subsets was examined by analyzing the expression of the lineage-specific transcription factors T-bet, GATA3, and RORγ(t). To this end, cells were intranuclearly stained with the respective fluorochrome-conjugated antibodies and the FoxP3 Staining Kit (all from Miltenyi Biotec) and analyzed by flow cytometry.

3 Analysis of lineage-specific effector cytokine expression

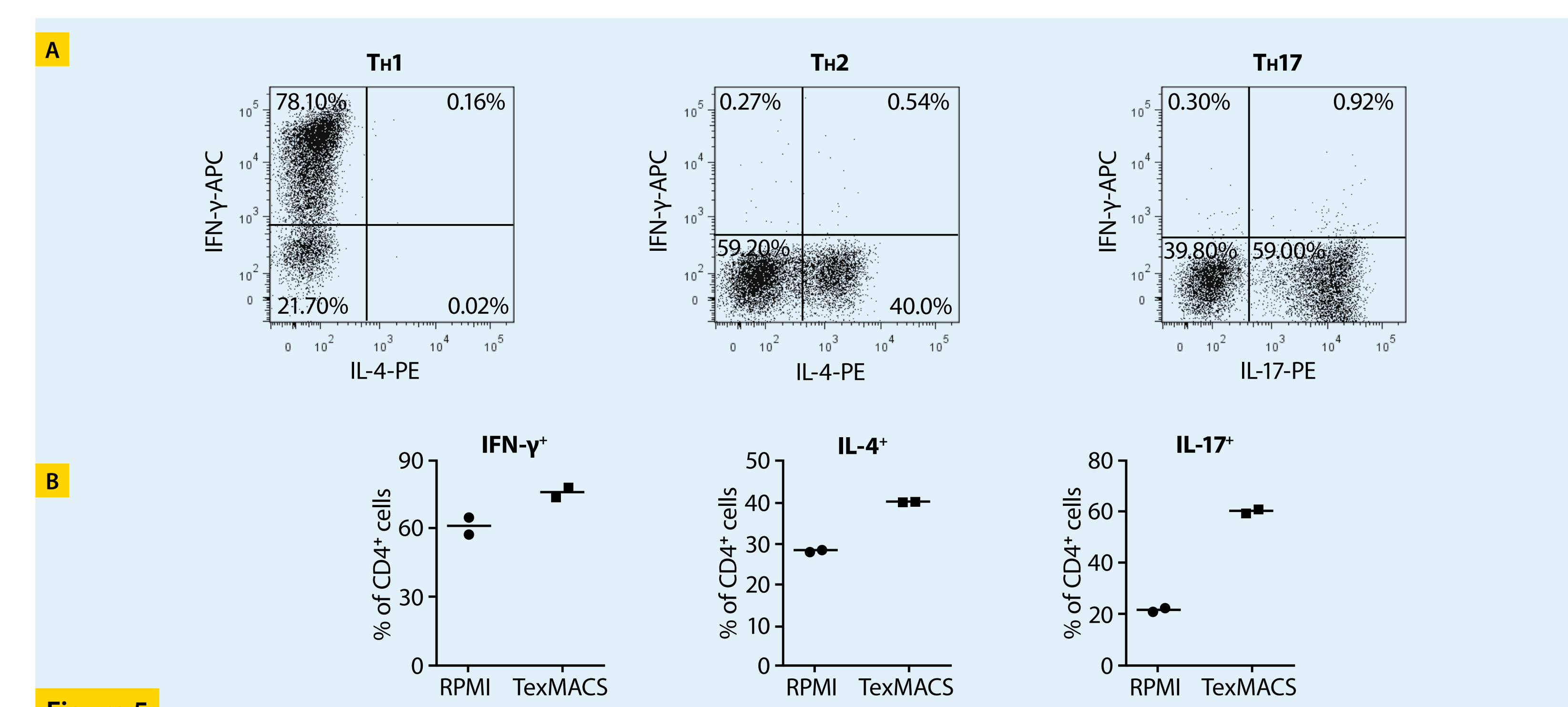

Figure 5
Analysis of lineage-specific effector cytokine expression

To determine the effector function of T cells after polarization, the capacity of the cells to produce lineage-specific effector cytokines (see overview in fig.1) was analyzed by flow cytometry on day 5. To this end, cells were restimulated with PMA/Ionomycin for 5 hours and stained intracellularly for the detection of IFN-γ, IL-4, and IL-17, using the respective fluorochrome-conjugated antibodies in combination with the Inside Stain Kit (all from Miltenyi Biotec). In line with the expected characteristic cytokine expression profiles, the Tн1 culture showed a high percentage of IFN-γ–producing, but no IL-4–producing cells. The Tн2 culture featured a high frequency of IL-4–, but no IFN-γ–expressing cells, whereas the Tн17 culture was characterized by a high level of IL-17–producing cells and the absence of IFN-γ–producing cells (A). TexMACS Medium was developed specifically for T cell activation and expansion. *In vitro* Tн cell differentiation in the presence of TexMACS Medium led to a higher expression level of the characteristic effector cytokines in the various Tн subsets compared to RPMI 1640 (B).

4 Identification of human Tн cell subsets *ex vivo*

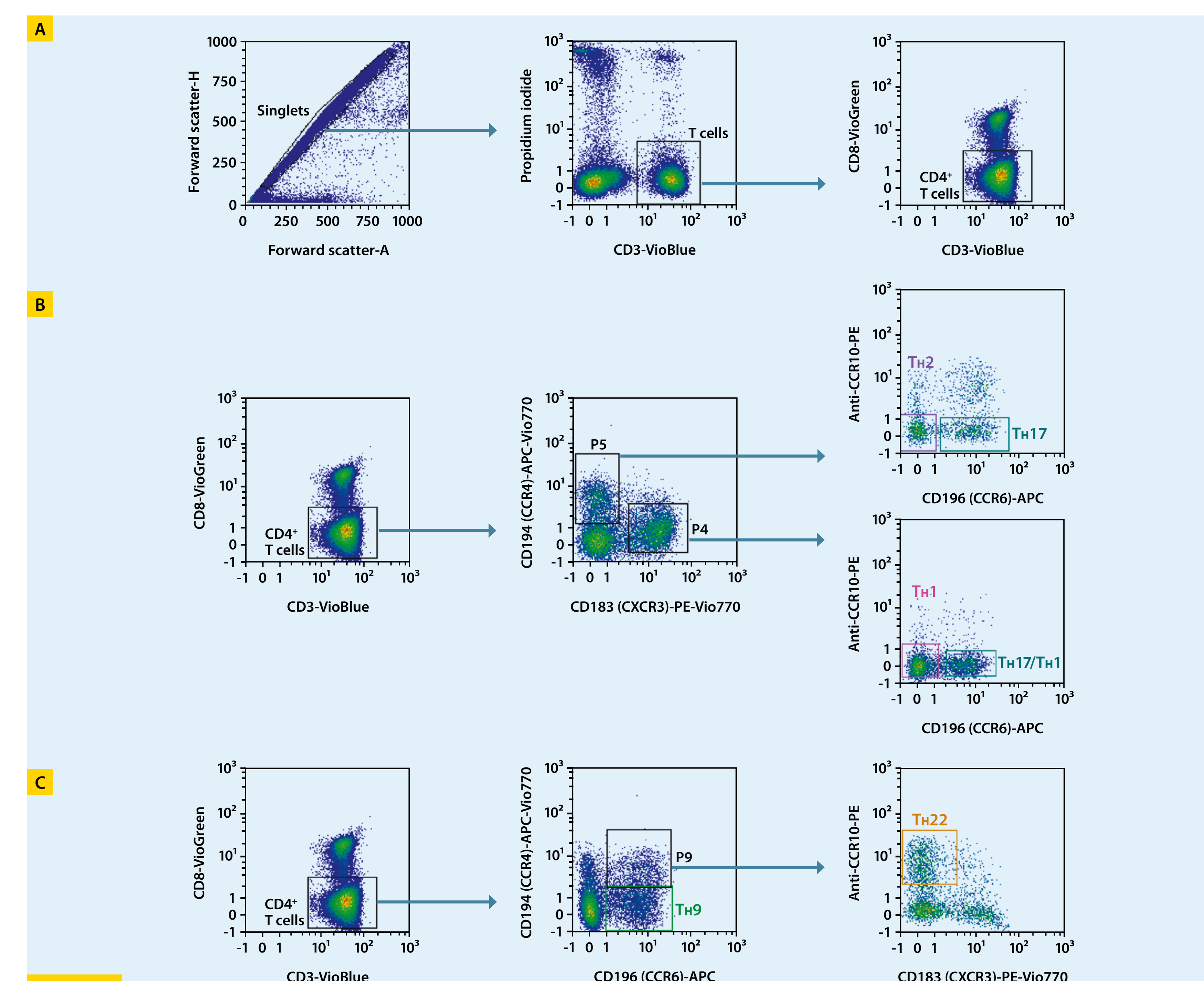

Figure 6
Example of flow cytometry analysis of Tн1, Tн2, Tн9, Tн17, and Tн22 lineage-specific chemokine receptor profiles in a single blood sample. (A) Singlets were identified by FSC-A vs. FSC-H blots (A, left). After gating on live T cells (PI⁻CD3⁺; A, middle), CD4⁺ Tн cells were identified by excluding CD8⁺ Tc cells (A, right). (B) The CD4⁺ cells (B, left) were further analyzed for CXCR3 and CCR4 expression (B, middle). Tн2 and Tн1 cells could then be distinguished from Tн17 cells by CCR6/CCR10 analysis (B, right). (C) The CD4⁺ cells (C, left) were also analyzed for CCR6 and CCR4 expression to identify Tн9 cells (C, middle). Tн22 cells were then detected by CXCR3/ CCR10 analysis (C, right).

The polarized human Tн cell subsets (Tн1, Tн2, Tн9, Tн17, Tн22) were simultaneously identified in a single sample of human whole blood. A panel of fluorochrome-conjugated antibodies recognizing the typical chemokine receptors on the cell surface was used for multicolor flow cytometry. An established analysis template providing the gating strategy enabled the automated characterization of Tн cell subsets on the MACSQuant Analyzer 10 (from Miltenyi Biotec).

Conclusion

We show a complete workflow for reliable and efficient mouse Tн cell differentiation, starting with single-cell preparation, followed by isolation of naive CD4⁺ T cells and *in vitro* activation and differentiation, through to comprehensive cell analysis. The reagents, instruments, and protocols are proven tools for research on Tн cell subset development and function.

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