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Comprehensive Phenotyping of T Cells Using Flow Cytometry

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Abstract

The T cell compartment can form a powerful defense against extrinsic (e.g., pathogens) and intrinsic danger (e.g., malignant cells). At the same time, specific subsets of T cells control this process to keep the immune system in check and prevent autoimmunity. A wide variety in T cell functionalities exists, which is dependent on the differentiation and maturation state of the T cells. In this review, we report an overview for the identification of $CD4^+$ T-αβ cells (T-helper (Th)1, Th2, Th9, Th17, Th22, and $CD4^+$ regulatory T cells), $CD8^+$ T-αβ cells (cytotoxic T lymphocyte (Tc)1, Tc2, Tc9, Tc17, and $CD8^+$ regulatory T cells), and their additional effector memory status (naïve, stem cell memory, central memory, effector memory, and effector) using flow cytometry. These different subsets can be discriminated based on selective extracellular markers, in combination with intracellular transcription factor and/or cytokine stainings. Additionally, identification of very small subsets, including antigen-specific T cells, and important technical considerations of flow cytometry are discussed. Together, this overview can be used for comprehensive phenotyping of a T cell subset of interest. © 2019 International Society for Advancement of Cytometry

Key terms

T cell; phenotyping; flow cytometry; differentiation; T cell subset

T cells appear in different sorts and flavors, with distinct characteristics that can be visualized through flow cytometry. T cells originate from lymphoid progenitors and have the capacity to traffic from the bone marrow to the thymus. Here T-cell development takes place comprising T-cell receptor (TCR)-mediated selection and maturation into naive T cells (Fig. 1) (1,2). Lymphopoiesis starts with progenitor cells expressing CD2, CD5, CD7, and cytoplasmic CD3, which have the capacity to enter the cortex of the thymus (3,4). At this point, TCRs are formed after rearrangements of α, β, γ, and δ chains resulting in T cells with γδ-chains (T-γδ) and T cells with αβ-chains (T-αβ). These T-γδ and T-αβ cells can be characterized by flow cytometric gating for CD3 and the γδ-TCR or αβ-TCR, respectively (5–8). At this stage, NK-T cells originate from the CD3-expressing precursor T cells by expressing a specific α-chain (Vα14–Jα18) which pairs via a β-chain interaction with glycolipid-CD1d. Upon development NK-T cells acquire expression of the neural cell adhesion molecule (NCAM, CD56) (9). A CD1d tetramer together with CD56 antibody, can be used to study NK-T cells (10). Meanwhile, precursor T cells express CD3 at low levels on their surface and go through a rapid transition, where first CD8 and then CD4 expression is acquired. As expression of CD8 can occur prior to or after rearrangement of the TCRs, T-γδ cells can also separate from the αβ-path after upregulation of CD8, which results in approximately 30% the T-γδ cells being $CD8^+$ (8). The $CD4^+CD8^+$ double positive (DP) T-αβ cells continue their development by undergoing positive selection via interaction with either peptide:MHC class I complexes resulting in single expression of CD8 or peptide:MHC class II complexes resulting in single expression of CD4 (11). Thereafter, T-αβ migrate from the thymic

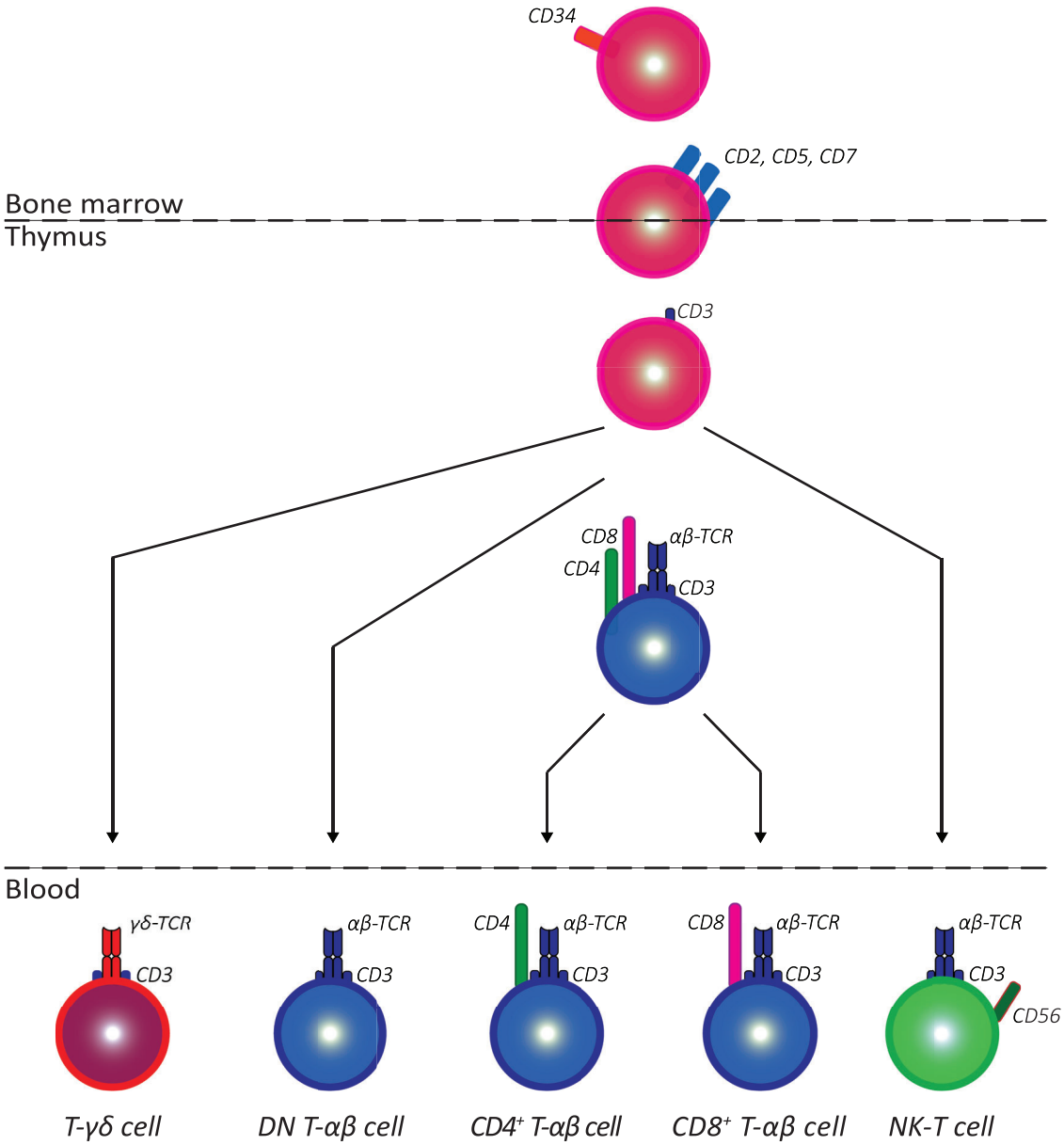

Figure 1. Discriminating T-αβ cells from their relatives. T-αβ cells arise from CD34$^+$ hematopoietic stem cells which acquire CD2, CD5, and CD7 expression during lymphopoiesis. After migration from the bone marrow to the thymus, CD3 is expressed and T-γδ cells and NK-T cells deviate from the T-αβ-path. T-γδ cells express the γδ-TCR and NK-T cells express a specific αβ-TCR which pairs with glycolipid-CD1d and they express CD56. The T-αβ cells gain both CD4 and CD8 and subsequent to positive and negative clonal selection, single positive CD4$^+$, and CD8$^+$ T-αβ cells are released into the blood. Double negative (DN) T-αβ cells are negative for both CD4 and CD8.

cortex to the medulla, where they undergo negative clonal selection to remove T cells that have a high affinity interaction for self-antigens. Finally, mature single-positive CD4$^+$ and CD8$^+$ T-αβ cells are released into the blood (12–15). A small percentage (~1%) of the peripheral blood T-αβ cells does not express CD4 nor CD8, and are known as double negative (DN) T-αβ cells (16–18).

Mature T-αβ cell subsets can be further characterized by comprehensive phenotyping, keeping in mind that differentiation often shows gradual changes in expression patterns, and molecules previously described as subset specific (e.g., CD56), are also expressed by other immune cells (19). In this review we provide an overview of extracellular proteins, intracellular transcription factors, and effector molecules that can be used to characterize different populations of T-αβ cells.

T-αβ Cell Subsets Can Be Defined by Extracellular Proteins, Transcription Factors, and Cytokine Profiles

The most common strategy to segregate CD4$^+$ T-αβ helper (Th) cell subsets [i.e., Th1, Th2, Th9, Th17, Th22, and

Table 1. A comprehensive phenotype of cytokine-based subsets within $CD4^+$ and $CD8^+$ T-αβ cells

	CD4						CD8				
	TH1	TH2	TH9	TH17	TH22	TREG	TC1	TC2	TC9	TC17	TREG
Extracellular											
CCR10					+						
CD25 (IL-2Rα)						+					+
CD127 (IL-7Rα)						+/−					
CD152 (CTLA4)						+					+
CD161				+						+	
CD183 (CXCR3)	+						+				
CD194 (CCR4)		+		+	+			+		−	
CD196 (CCR6)	−	−		+	+				+	+	
CD294 (CRTH2)		+						+			
IL23R				+						+	
Intracellular											
AHR					+						
Foxp3						+					+
FOXO4					+						
GATA3		+						+			
IRF4		+	+	+			+		+	+	
PU.1			+								
RORγT				+						+	
T-bet	+						+			−	
Intracellular upon cell stimulation											
IFNγ	+	−		−	−		+	−			
IL2	+						+				
IL4	−	+		−	−	+	−	+/−			
IL5		+					−	+			
IL9		+	+	+	−				+		
IL10		+			−	+			+		+
IL13		+						+			
IL17				+	−					+	
IL21				+						+	
IL22				+	+						
TNF-α	+	+					+				
TGF-β						+					+
References	(20–22,24)	(20–23,30)	(30)	(8,21,22,28)	(21,25)	(22,26,31)	(29)	(23,29)	(29)	(27–29)	(29)

Boxes marked negative (–) indicate antigens, transcription factors or cytokines which are not expressed or produced by the corresponding subset. An empty box indicates that this is not used for the discrimination of a specific subset.

regulatory T cells (Tregs)] is based on their functionality. In the last decade, new research highlighted that this segregation can be applied to $CD8^+$ T-αβ cells as well, separating cytotoxic T lymphocytes (Tc)1, Tc2, Tc9, Tc17, and $CD8^+$ Tregs. The division is mainly based on extracellular markers, selective intracellular transcription profiles, and the cytokine production profile of the T cells. To define the various T-αβ subpopulations, a phenotype based on TCR-αβ, CD4, and CD8 staining should be combined with CD183 (CXCR3; Th1 and Tc1), CD194 (CCR4) and CD294 (CRTH2; both for Th2 and Tc2), CD196 (CCR6) or CD161 (KLRB1; both for Th17 and Tc17), CCR10 (Th22), and CD25 (IL-2Rα) and CD152 (CTLA4; both for $CD4^+$ and $CD8^+$ Tregs) (Table 1 and Fig. 2) (20–31). However, so far no distinct extracellular marker for Th9 and Tc9 cells has been identified. For better discrimination, subsequent intracellular transcription factor profiling can be performed. These include T-bet (Th/c1), GATA3 (Th/c2), PU.1 (Th9), RORγt (Th/c17), AHR, and/or FOXO4 (Th22) and FoxP3 ($CD4/CD8^+$ Tregs) (20–30). Selective transcription factors for Tc9 have not yet been found. Nevertheless, the best method to distinguish between the subsets is based on their cytokine secretion profile upon stimulation in the presence of a Golgi system inhibitor, like Brefeldin A or Monensin, to prevent cytokine release. Following subsequent fixation and permeabilization, cells can be stained for intracellular IFNγ and IL2 (Th1/Tc1), IL4 and IL5 (Th2/Tc2), IL9 (Th9/Tc9), IL10 ($CD4^+/CD8^+$ Treg), IL17 (Th17/Tc17), and IL22 (Th22) (20–30).

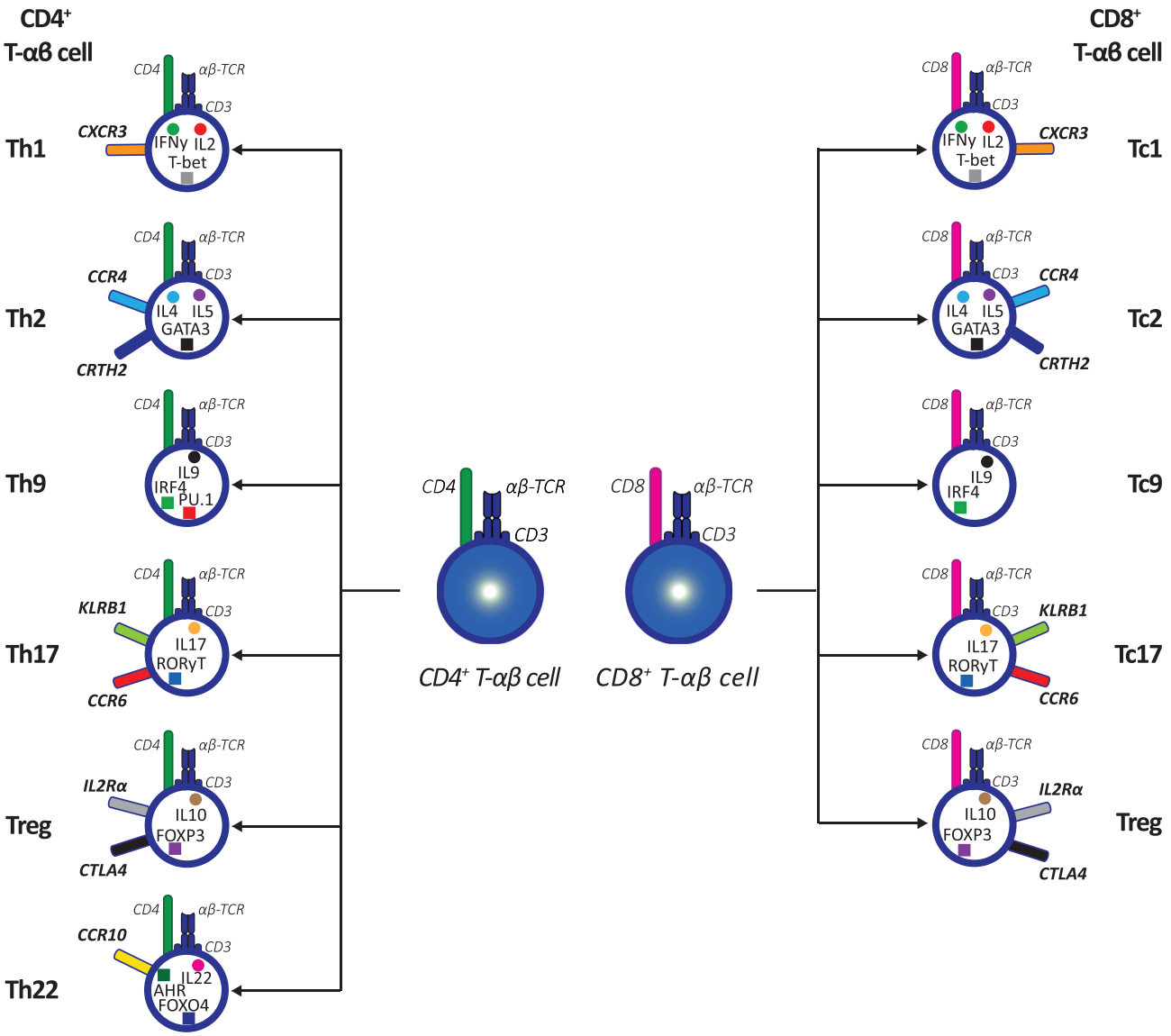

Figure 2. Identification of CD4$^+$ and CD8$^+$ T-αβ subsets. A combination of both surface and intracytoplasmic stainings for cytokines and transcription factors should be used for the correct identification of CD4$^+$ T-αβ helper (Th) cell subsets Th1, Th2, Th9, Th17, Th22, and Treg, and CD8+ cytotoxic T-αβ (Tc) cell subsets Tc1, Tc2, Tc9, Tc17, and CD8$^+$ Tregs. Discriminative surface proteins are visualized on the cell surface, cytokines intracellular as circles and transcription factors intracellular as squares.

Characterization of Effector Memory Differentiation

T-αβ cell subsets can be further characterized based on their effector memory differentiation status. Either via a linear or partly progressive model, stem cell memory (T_{SCM}), central memory (T_{CM}), effector memory (T_{EM}), and effector T cells (T_{EFF}) develop from the naive (T_N) repertoire upon activation (32–37). Effective T cell activation requires interaction of the TCR-CD3 complex with the peptide:MHC complex on antigen-presenting cells, co-stimulatory signaling and cytokine support. Every effector/memory subset has unique characteristics which provide them with differential migratory capacity, longevity, and functionality. While CD197$^+$CD62L$^+$ T_N, T_{SCM}, and T_{CM} home to lymphoid tissues in search of their specific antigen being presented by antigen-presenting cells, CD197$^-$CD62L$^-$ T_{EM} and T_{EFF} patrol peripheral tissues and blood and efficiently produce effector molecules upon antigen encounter (36–38). Notably, early memory T cells (T_{SCM} and T_{CM}) are known for their longevity and high proliferative capacity, which gradually decreases upon differentiation (36). Although the expression of various extracellular markers changes during activation and differentiation (Table 2), one can already discriminate the different CD4$^+$ and CD8$^+$ effector memory T cell subsets using a small set of markers, including CD45RA or CD45RO in combination with CD197 (CCR7) and CD95 (Fig. 3A). Additionally, often the

Table 2. A comprehensive phenotype of memory differentiation subsets within $CD4^+$ and $CD8^+$ T-αβ cells

	CD4				CD8				
	T_N	T_{SCM}	T_{CM}	$T_{EM/EFF}$	T_N	T_{SCM}	T_{CM}	$T_{EM/EFF}$	REFERENCES
Extracellular									
CD11a (LFA-1)	−/+	+	+	+	−	+	+	++	(44,45)
CD27	+++	+++	++	+/−	+++	+++	++	+/−	(40,44,46–51)
CD28	++	+++	+++	+/−	++	+++	+++	+/−	(40,44,46–48)
CD38	−	+	+	+	+++	++	++	+	(37,44,52)
CD45RA	+	+	−	−	+	+	−	+/−	(46–51,53,54)
CD45RO	−	−	+	+/−	−	−	+	+/−	(44,46–49,51,53,54)
CD57	−*	−*	+	++	−*	−*	+	++	(40)
CD58 (LFA-3)	−	+	++	++	−	+	++	++	(44,48,49)
CD62L (L-selectin)	+++	++	++	−	+++	+++	++	−	(38,44,46–49)
CD69	−*	−*	+	+	−*	−*	+	+	(38,55)
CD95 (FAS)	−	++	+++	++	−	++	+++	++	(37,44,46–51)
CD122 (IL-2Rβ)	−	+	+	++	−	+	+	++	(37,44,46–49)
CD127 (IL-7Rα)	++	++	++	+/−	+	++	++	+	(44,47,53,56)
CD130	+++	++	++	+	+++	++	++	+	(37)
CD134 (OX40)	+*	+*	+++	++	+*	+*	++	++	(40)
CD183 (CXCR3)	−	+	+	++	−	++	++	+/−	(37,38,44,48,50,57,58)
CD184 (CXCR4)	+++	++	++	+	+	+++	+++	++	(38,44)
CD195 (CCR5)	−	+	+	+++	−	+	++	+++	(37,50)
CD197 (CCR7)	+++	+++	++	−	+++	+++	++	−	(38,44,46–51)
CD218a (IL-18Rα)	−	+	++	+++	−	++	++	+++	(37)
CD244 (2B4)	−*	−*	−	+	−*	−*	−	+	(40)
CD278 (ICOS)	+*	+*	++	++	+*	+*	++	+	(40)
HLA-DR	−	+	+	++	−	+	+	++	(37,38)
Intracellular									
ACTN1					+++	++	+	−	(44)
EOMES	−	+	+	++	−	+	++	+++	(24,44,46)
FOXO1					+	+	+	−	(59)
FOXP1	+++	++	++	+	+++	++	++	+	(32,44,46)
ID2					+	+	++	+++	(46)
ID3					+++	+++	++	+	(46)
IL6ST					+++	+	+	−	(44)
LASS6					+++	++	+	−	(44)
LEF1	+++	++	+	−	+++	++	+	−	(32,44,46)
KLF7					+++	++	+	−	(46)
PRDM1	−	+	++	+++	−	+	++	+++	(32,44,46)
PRF1	−	−			−	−	+	+++	(44,50)
TAF4B					+++	++	+	−	(44)
TBX21	−	+	+	+++	−	+	+	+++	(32,44,46)
TCF7	+++	+++	++	+	+++	+++	++	+	(32,46)
Zeb2	−	+	++	+++	−	+	++	+++	(32,46)
Intracellular upon stimulation									
IFNγ	−	+	++	+++	−	+	++	+++	(38,44,46,47,50,58)
IL-2	−/+	+++	+++	+	−/+	+++	++	+/−	(38,46,47,58)
TNFα	−	−/+	++	+++	−	+	++	+++	(46,47,50)
GZMA	−	+		++	−	+	+	+++	44,50,60,61
GZMB	−	+/−		++	−	−*	−*	++	(50,60–62)
Perforin				++	−	+*	+*	+++	(60,61)

No discrimination between T_N and T_{SCM} cells. Empty boxes are not specifically described for the indicated subset.

cell adhesion molecule and homing receptor L-selectin (CD62L, Fig. 2B), co-stimulatory molecules CD27 and CD28, and cytokine receptors IL-2Rβ (CD122) and IL-7Rα (CD127) are included in effector memory differentiation analyses. Upon stimulation, besides expression of co-stimulatory markers, activated T-αβ cells can also start to express co-inhibitory receptors, including CTLA-4, PD-1, TIGIT, and TIM-3 (39,40). While this prevents sustained T cell activation via a natural feedback loop, it can also hamper immune responses in patients with chronic viral infections or cancer (41).

The analysis of all the described subsets should be always preceded by a proper gating strategy which includes the exclusion of doublets using a single cell gate (42) (Fig. 3C), dead cells with a life/dead stain (Fig. 3D), and, when necessary, autofluorescent cells using an empty fluorescence channel. This is especially important when small (low frequent) populations including T_{SCM} and antigen-specific T cells are analyzed. Antigen-specific $CD8^+$ T-αβ cells can be visualized using single or double (combinatorial encoding) tetramer or pentamer stainings (Fig. 3E). Tetramers and pentamers consist of respectively 4 or 5 biotinylated peptide–MHC complexes linked to a fluorescent label. The use of double tetramer staining with two different fluorescent labels eliminates the chance of false positive results, thereby increasing the analysis specificity. Combinatorial staining with 6 different fluorochromes results in 15 unique color combinations, and makes it possible to identify up to 15 different epitopes with a high specificity (43). Additionally, in particular when performing deep multiparametric phenotypes, also Fluorescence Minus One (FMO) controls should be taken into consideration. An FMO control includes cells stained with all fluorochromes of the panel except one, and can be used to verify the cut-off point of background fluorescence and thereby the positive population (Fig. 3F) after optimum concentrations have been established via an antibody titration assay. Finally, single stains on the cells of interest should be performed to set the appropriate compensations followed by a check of compensation settings when using the complete panel. It is essential to synchronize flow cytometer settings over time and also harmonize your analyzers to acquire identical data on different machines. The right settings, together with accurate gating and a complete flow cytometry panel can be used for comprehensive phenotyping of your T-cell subset of interest.

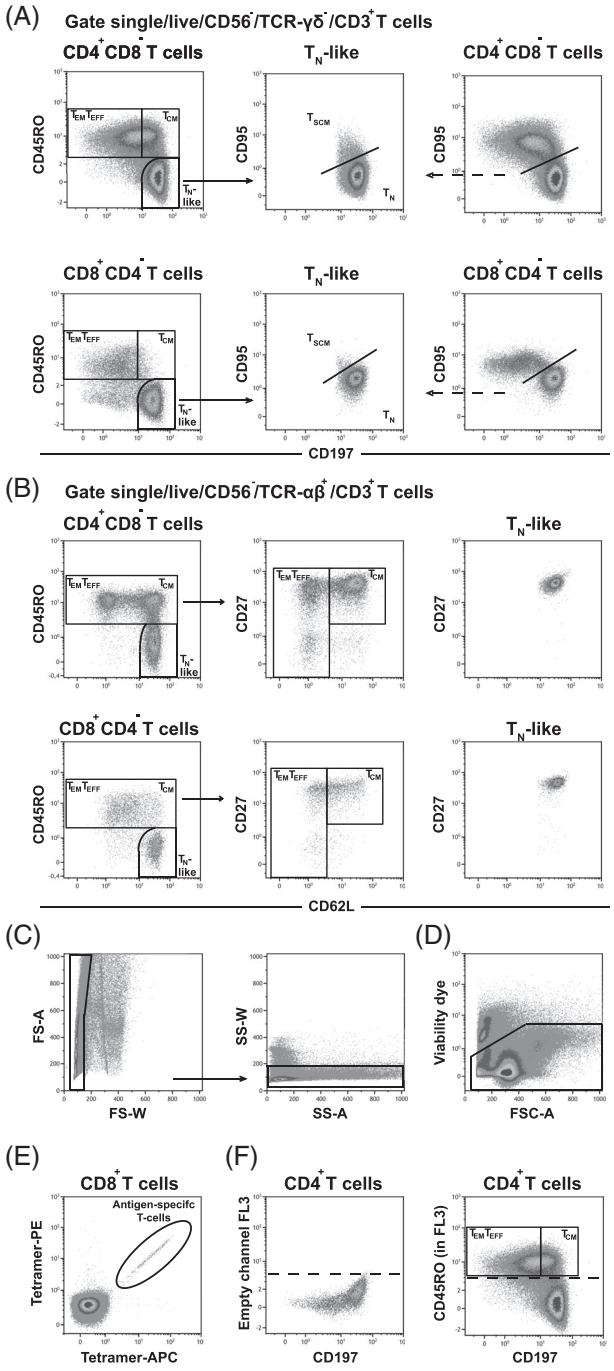

Figure 3. Gating for flow cytometry. Peripheral blood mononuclear cells were resuspended in PBS containing 0.5% bovine serum albumin followed by extracellular staining for 30 min at 4°C in the dark. (A) Gating strategy to discriminate $CD4^+$ and $CD8^+$ T cell memory populations within single, live, $CD56^-TCR^-\gamma\delta^-CD3^+$ cells. TN-like cells are $CD45RO^-CCR7^+$, TCM cells are $CD45RO^+CCR7^+$ and TEM/EFF cells are $CD45RO^+CCR7^-$. The discrimination between TN and TSCM within TN-like was based on the CD197-differentiation pattern of total $CD4^+$ or $CD8^+$ T cells (– ->). (B) Gating strategy to discriminate $CD4^+$ and $CD8^+$ T cell memory populations with CD45RO, CD62L, and CD27 within single, live, $CD56^-TCR^-\alpha\beta^+CD3^+$cells. TN-like cells are $CD45RO^-CD62L^+CD27^+$, TCM cells are $CD45RO^+CD26L^+CD27^+$ and TEM/EFF cells are $CD45RO^+CD62L^-CD27^{+/-}$. (C) Discrimination of single cells by plotting forward scatter width (FS-W) against forward scatter area (FS-A) as well as plotting side scatter width (SS-W) against side scatter area (SS-A). Events deviating from the linear cell population are called doublets. (D) Live cells can be evaluated by gating on viability dye-negative cells. (E) Antigen-specific $CD8^+$ T cells are analyzed by using antigen-specific tetramers, labeled with two different fluorescent labels. (F) Fluorescence minus one (FMO) control for CD45RO expression within FL3 contains all antibodies except CD45RO-ECD. This is used to determine the position of $CD45RO^+$ cells

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