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Human CD4$^+$ T Cells Are Predominantly Distributed among Six Phenotypically and Functionally Distinct Subsets[1]

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Human T cells are heterogeneous, varying in terms of their phenotype, functional capabilities, and history of Ag encounter. The derivation of a functionally relevant model for classifying CD4$^+$ T cells has been hampered by limitations on the numbers of parameters that may be measured using classical four-color flow cytometry. In this study we have taken advantage of the introduction of reagents for five-color flow cytometry to develop a detailed, functionally meaningful scheme for classifying human CD4$^+$ T cells. We show that CD4$^+$ T cells are predominantly distributed among six of eight possible compartments, identified by the expression of CCR7, CD45RA, and CD28. We demonstrate novel phenotypic and functional correlates that justify the choice of these three molecules to define CD4$^+$ T cell compartments. We note that CD4$^+$ T cells with different Ag specificities are distributed differently among the six described subsets. On the basis of these results, we propose a cross-sectional model for classification of peripheral CD4$^+$ T cells. Knowledge of where T cells lie on this model informs about their functional capacity and can reflect their history of Ag exposure. ***The Journal of Immunology,*** **2005, 175: 5765–5773.**

The peripheral pool of T cells includes cells with various histories of engagement with Ag and many different functional properties. After a first encounter with cognate Ag, naive T cells proliferate and acquire effector function. As infection is controlled, the majority of T cells mediating the primary immune response dies, and a small population remains to form the memory population (1–10). Where infections are persistent, then Ag-experienced T cells may be rechallenged recurrently (5–10).

Naive T cells are a relatively homogeneous population expressing a characteristic set of cell surface glycoproteins. The evolution of Ag-specific T cell responses is accompanied by changes in the expression of these cell surface molecules (5, 6, 8, 11–14). There has been interest in the idea that patterns of cell surface molecule expression, or phenotype, of cells may reflect their history of Ag exposure (10–12) or be indicative of functional capacity (15–22). Early work suggested that the expression of different CD45 isoforms distinguished between naive and memory cells (15, 16). The idea that naive T cells express CD45RA, but not CD45RO, has been upheld, although it has since become clear that Ag-experienced CD8$^+$ T cells can also have this phenotype (5–7, 11–14, 17, 18, 20–22). Analysis of the expression of other cell surface molecules, including the chemokine receptor CCR7 and the costimulatory molecules CD27 and CD28, has resulted in the development of three models of CD8$^+$ T cell memory, in which CD8$^+$ T cells are considered in different phenotypic compartments (12, 17, 18, 22).

The first model was based on the expression of CD45RA and CD27 (17). It allowed for the distinction of two major groups of Ag-experienced CD8$^+$ T cells with broadly different functional properties: CD45RA$^-$CD27$^+$ cells with the capacity to express IL-2, IL-4, TNF-α, and IFN-γ and CD45RA$^+$CD27$^-$ cells with the capacity to express TNF-α and IFN-γ, but not IL-2 or IL-4, and the ability to kill.

The second model focused on the expression of CD45RA and CCR7. Naive cells expressed both molecules. Ag-experienced cells were divided into central memory cells, expressing CCR7, but not CD45RA, and effector memory cells, which lacked the expression of CCR7. CCR7$^-$ cells were further subdivided according to the expression of CD45RA. Central memory cells were able to secrete IL-2 and home to lymph nodes, whereas effector memory cells secreted IFN-γ after short-term stimulation (18). The lineage relationship between central and effector memory populations has remained controversial. Although the original model suggested, on the basis of in vitro experiments, that the central memory population might serve as a pool from which effector memory cells derived, in vivo work in murine models has suggested that the central memory population itself derives from effector memory cells (23). Most recently, this type of model has been extended, with the CCR7$^-$CD45RA$^+$CD8$^+$ effector memory subset being further divided according to the expression of CD27 (22). Analysis of replicative history and mRNA for proteins involved in effector function suggested that the CCR7$^-$CD45RA$^+$CD27$^+$ population is intermediate between naive cells and CCR7$^-$CD45RA$^+$CD27$^-$ effector cells in terms of both the number of cell divisions experienced and the capacity to respond to Ag.

Finally, a third model has been suggested based on the expression of CD27 and CD28 (12). Although no correlates for function were proposed, it was noted that T cells with different specificities lay within different compartments in this model. Furthermore, progressive loss of first CD28 and then CD27 after Ag exposure in vitro lent support to the idea that analysis of the expression of these molecules allows one to define the positions of cells on a linear pathway of differentiation (24).

Analysis of CD4$^+$ T cell subsets has not been performed in equivalent detail. Data concerning CD4$^+$ T cells were presented in the context of the second model (18). CD4$^+$ T cells were originally considered as falling into only three compartments: naive

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CCR7$^+$CD45RA$^+$, central memory CCR7$^+$CD45RA$^-$, and effector memory CCR7$^-$CD45RA$^-$. Consistent with results from CD8$^+$ T cells, central memory cells appeared adapted for IL-2 production, whereas effector memory cells expressed IFN-γ and TNF-α. We and other groups have recently demonstrated the presence of CD4$^+$ T cells that are CCR7$^-$CD45RA$^+$ and respond to stimulation with preparations of EBV and CMV Ags (10, 25). It was subsequently reported that such CCR7$^-$CD45RA$^+$CD4$^+$ T cells have short telomeres, proliferate poorly in response to cytokines, and express high levels of CD57 (26). In parallel, studies have considered CD4$^+$ T cells as being distributed between subsets defined by the expression of CD27 and CD28 (10, 27, 28). Like CD45RA expression, the lack of CD28 expression has been correlated with high levels of expression of CD57. The extent to which subsets defined using CD27 and CD28 overlap those defined by CCR7 and CD45RA is not known. This necessarily limits the interpretation of experiments designed to investigate functional correlates for cell phenotype.

The development of reagents for five-color flow cytometry allows for a more detailed analysis of the CD4$^+$ T cell repertoire. The aim of this study was to define the major phenotypic subsets of human CD4$^+$ T cells and to draw detailed correlates between CD4$^+$ T cell phenotype and capacity for function. The results clearly reveal the presence of subsets of CD4$^+$ T cell subsets expressing CD45RA, but lacking the expression of CCR7. They define functional correlates for the expression of CD45RA by these CCR7$^-$ cells as well as for the expression of CD28 and CCR7. Overall, they support a model in which CD4$^+$ T cells may be considered as being predominantly distributed among six major compartments, which differ in terms of both the phenotypic and the functional properties of the cells that they contain.

Materials and Methods

Blood samples and preparation

Peripheral blood was obtained from 14 healthy EBV- and CMV-seropositive donors. PBMCs were separated by centrifugation on a Lymphoprep gradient (Nycomed Pharma). The study was approved by the Oxford research ethics committee.

Five-color staining of ex vivo PBMC

Freshly isolated PBMCs were washed in a PBS buffer containing 0.16% BSA and 0.1% sodium azide (PBA).[3] Cells were incubated for 30 min on ice with saturating amounts of Abs, including anti-CD4-PE Cy7 (BD Pharmingen), anti-CD45RA-allophycocyanin (BD Pharmingen), anti-CD28-PE (BD Pharmingen), anti-CD27-FITC (BD Pharmingen), anti-CD45RO-FITC (BD Pharmingen), and anti-CD57-FITC (BD Pharmingen). Cells were stained for CCR7 by incubation with anti-human CCR7 mAb (BD Pharmingen), and binding was detected by subsequent staining with 0.25 μg of biotinylated goat anti-mouse IgG (DakoCytomation) and 0.15 μg of streptavidin-allophycocyanin Cy-7 (BD Pharmingen). Cells were then washed in cold PBA and resuspended in PBS containing 1% formaldehyde and 1% FCS. To detect the expression of perforin or granzyme A, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen) according to the manufacturer's instructions and then incubated on ice with either 0.25 μg of anti-perforin-FITC (Ansell) or 0.25 μg of anti-granzyme-A-FITC (BD Pharmingen) Abs diluted in Cytoperm buffer. After 60 min, cells were washed in cold PBA and resuspended in PBS containing 1% formaldehyde and 1% FCS. Data were collected on a CyAn flow cytometer and were analyzed using Summit software (DakoCytomation). Data were collected for between 500,000 and 1,000,000 events. Gates were set on live lymphocytes according to forward and side scatter characteristics.

Stimulation of PBMC

PBMCs were incubated for 18 h in RPMI 1640 supplemented with 10% FCS either alone or in the presence of an EBV-infected cell lysate, a CMV-infected cell lysate (both from East Coast Biologics), or *Staphylococcus* enterotoxin B (SEB; Sigma-Aldrich). Ags were used at a final concentration of 10 μg/ml, apart from SEB, which was used at a final concentration of 5 μg/ml. Brefeldin A was added after the first 2 h of incubation at a final concentration of 5 μg/ml. The incubation was performed in V-bottom tubes at 37°C in the presence of 5% CO_2. The EBV-infected cell lysate and CMV-endothelial cell lysate are reported by the manufacturer to be enriched for viral proteins and have been used successfully as tools to investigate CD4$^+$ T cell responses to EBV and CMV.

Four-color staining of stimulated PBMC

After 18 h of incubation, cells were washed in PBA and stained with saturating amounts of anti-CD4-PerCP (BD Pharmingen) and anti-CCR7 for 30 min on ice. Binding was detected by subsequent staining with 0.25 μg of biotinylated goat anti-mouse IgG, as described above, and 0.2 μg of streptavidin-PE (DakoCytomation). Cells were washed in PBA buffer, fixed and permeabilized with Cytofix/Cytoperm, and incubated with 3 μl of anti-IFN-γ-FITC (BD Pharmingen) and 0.1 μg anti-IL-2-allophycocyanin (BD Pharmingen) Abs diluted in Cytoperm buffer. After 60 min, cells were washed in cold PBA and resuspended in PBS containing 1% formaldehyde and 1% FCS. Data were collected and analyzed on a FACSCalibur flow cytometer (BD Biosciences). IgG1-FITC (DakoCytomation) and IgG2a-APC (BD Pharmingen) were used as isotype-matched negative control Abs. Five hundred thousand events were collected, and gates were set on live lymphocytes according to their forward and side scatter characteristics.

Five-color staining of stimulated PBMC

After 18 h of incubation, cells were washed in PBA and stained with saturating amounts of anti-CD45RA-FITC (BD Pharmingen), anti-CD28-PE, and anti-CD4-PE Cy7 for 30 min on ice. Cells were also stained for CCR7-allophycocyanin Cy-7 as described above. Cells were washed in PBA buffer, fixed and permeabilized with Cytofix/Cytoperm, and then incubated on ice with 0.1 μg of anti-IFN-γ-allophycocyanin (BD Pharmingen), anti-IL-2-allophycocyanin, or anti-TNF-α-allophycocyanin (BD Pharmingen) Abs diluted in Cytoperm buffer. After 60 min, cells were washed in cold PBS and resuspended in PBS containing 1% formaldehyde and 1% FCS. Data were collected and analyzed on a CyAn flow cytometer using Summit software. Isotype-negative control Abs included mouse IgG1-FITC and IgG2a-allophycocyanin. Two million events were collected, and gates were set on live lymphocytes according to forward and side scatter characteristics.

Cell sorting and proliferation assays

Freshly isolated PBMCs were stained for CD45RA (allophycocyanin) and CCR7 (allophycocyanin Cy7) as previously described in sterile conditions using filtered PBS with 1% FCS instead of PBA. Cells were then sorted using an operated MoFlow cell sorting cytometer (Becton Coulter). CFSE diluted in DMSO (Sigma-Aldrich) was added to the collected cells at a final concentration of 250 μM/10^7 PBMCs, and samples were incubated at 37°C for 12 min. Cells were then washed in 10 ml of cold RPMI 1640 and placed into culture with autologous adherent monocytes in 12-well plates in RPMI 1640 supplemented with 10% human serum and 5 μg/ml PHA (Sigma-Aldrich). IL-2 was not added to the cultures. Samples of cells were taken from the cultures between days 0 and 9 and were stained with Abs specific for CD45RA (allophycocyanin), CD28 (PE), and CD4 (PE Cy7). Samples were analyzed on a CyAn flow cytometer, and data were analyzed using Summit software as described above.

Results

Identification of subsets of CD4$^+$ T cells in healthy individuals

PBMC taken from healthy donors were stained for surface expression of CD4, CD45RA, CCR7, and CD28. A representative result is shown in Fig. 1, *A* and *B*. Analysis of the expression of CD45RA and CCR7 by CD4$^+$ T cells allowed us to discern four subsets (Fig. 1*A*). Naive cells are known to lie within the CCR7$^+$CD45RA$^+$ subset. The CCR7$^+$CD45RA$^-$ subset has previously been designated the central memory population, and the CCR7$^-$CD45RA$^-$ subset has been designated the effector memory population (18). We also documented a population of CCR7$^-$CD45RA$^+$CD4$^+$ T cells with a frequency ranging from 3.6–16.8% (mean, 10.1%) CD4$^+$ T cells. Costaining to detect CD28 demonstrated that both CCR7$^-$ subsets of CD4$^+$ T cells could be subdivided further (Fig. 1*B*). In contrast, few CCR7$^+$

[3] Abbreviations used in this paper: PBA, PBS buffer containing 0.16% BSA and 0.1% sodium azide; SEB, *Staphylococcus* enterotoxin B.

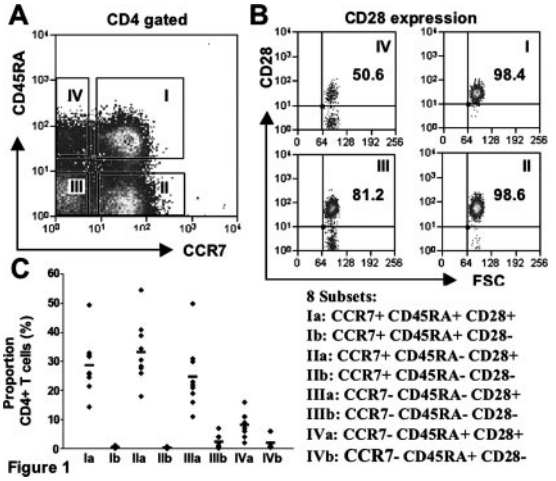

Figure 1

FIGURE 1. Expression of CD45RA, CCR7, and CD28 on $CD4^+$ T cells. PBMC were stained for the expression of CD4, CD45RA, CCR7, and CD28. *A*, Gates have been set on $CD4^+$ T cells, and staining for CD45RA and CCR7 is shown. *B*, Expression of CD28 by cells within the four subsets (I–IV) in *A*. The percentage of cells that express CD28 within each subset is shown. *C*, Distribution of $CD4^+$ T cells among eight subsets (Ia–IVb), defined by the expression of CD45RA, CCR7, and CD28. Data from nine healthy individuals are shown.

cells lacked expression of CD28. Thus, analysis of the expression of three cell surface receptors allows $CD4^+$ T cells to be categorized into six major compartments. We have designated these compartments as I–IV based on the expression of CCR7 and CD45RA and have appended the suffix, a or b, to denote whether cells are $CD28^+$ or $CD28^-$, respectively. The terminology has been chosen because it makes no a priori assumptions about functional capacity. Subset I corresponds to the previously defined naive population, subset II to the central memory population, and subset III to the effector memory population. We found that most $CD4^+$ T cells lie within compartments Ia, IIa, and IIIa, with some cells lying within compartments IIIb, IVa, and IVb. The distribution of $CD4^+$ T cells from nine healthy donors among these compartments is shown in Fig. 1*C*.

Phenotypic analysis of subsets of $CD4^+$ T cells

To further characterize the $CD4^+$ T cell subsets, their expression of other cell surface molecules, including those involved in costimulation (CD27) (29–31) and those putatively associated with Ag exposure (CD45 isoforms) (15, 16) and cell senescence (CD57) (32), was analyzed. In addition, intracellular expression of the cytolytic mediators, perforin and granzyme, was assessed. A representative example of the results is shown in Fig. 2. The use of five-color FACS analysis to simultaneously investigate the expression of four different receptors by $CD4^+$ T cells highlighted the heterogeneity of the $CD4^+$ T cell repertoire. Even within the $CCR7^+RA^+$ population (subset I), usually designated naive, some diversity existed, and the pattern of staining implied that not all cells within this population were Ag inexperienced. Subsets III and IV included several phenotypically distinct populations of cells, lending strong support to the idea that $CCR7^-CD4^+$ T cells should not be considered as a single compartment of effector memory cells.

Despite the diversity of the populations, clear phenotypic correlates emerged for lack of CD28 expression. The expression of the cytolytic molecules, perforin and granzyme, was strongly associated with the loss of CD28 expression. A high level of expression of CD57 was restricted to the $CD28^-$ population. Finally, virtually all $CD28^-CD4^+$ T cells also lacked the expression of CD27. Importantly, these observations held true whatever the level of expression of CD45RA and CCR7. In terms of the $CD28^+$ populations, it is also interesting to note the phenotypic similarity between compartments Ia and IVa.

Capacity of cells within different $CD4^+$ T cell subsets to express cytokines

We investigated the capacity of $CD4^+$ T cells within each subset to express IL-2, IFN-γ, and TNF-α. Freshly isolated PBMC from nine different healthy donors were incubated overnight with a polyclonal stimulus, SEB, in the presence of brefeldin A and then

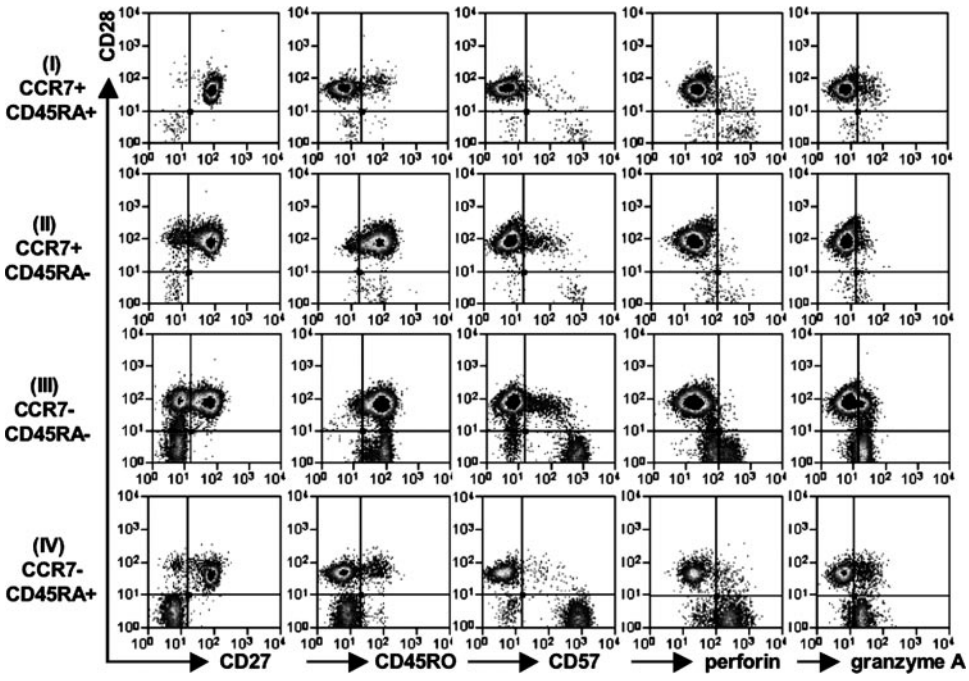

FIGURE 2. Expression of CD27, CD45RO, CD57, perforin, and granzyme A by $CD4^+$ T cells. PBMC were stained for the expression of CD4, CD45RA, CCR7, CD28 and CD27, CD45RO, CD57, perforin, or granzyme A. Gates have been set on $CD4^+$ T cells and then on cells within each of subsets I–IV.

stained for the expression of CD4, CD45RA, CCR7, and CD28 and for the presence of intracellular cytokines. The protocol induced the expression of IL-2, IFN-γ, and TNF-α by CD4$^+$ T cells. A representative result is shown in Fig. 3*A*, and a summary of all results is shown in Fig. 3*B*. Again, it proved possible to draw some clear correlates from the results.

The CCR7$^+$ subsets (subsets I and II) had limited capacity for cytokine expression. Cells within the CCR7$^+$CD45RA$^-$ subset (subset II) were more likely to express IL-2 than IFN-γ, in keeping with previous reports. The proportion of cells expressing TNF-α was similar to that expressing IL-2 and was greater than that expressing IFN-γ.

The CCR7$^-$ subsets showed good capacity for cytokine expression, although important differences in the functional capacity of the different subsets were evident. The expression of IL-2 was prominent among the CCR7$^-$ cells, but was predominantly a feature of CD28$^+$ T cells. CCR7$^-$ cells that expressed CD28 were much more likely to produce this cytokine than CCR7$^-$ cells that did not express CD28 (mean, 28.2 vs 6.7%; $p < 0.00002$). Overall, IL-2 expression was found most commonly within cells of subset IIIa.

The expression of IFN-γ was virtually entirely limited to cells that lacked the expression of CCR7 (subsets III and IV). CCR7$^-$ cells that did not express CD45RA (subset III) were much more likely to express IFN-γ than those that expressed CD45RA (subset IV; mean, 17.7 vs 5.6%; $p < 0.01$). There was a trend toward higher IFN-γ expression by CD28$^-$ compared with CD28$^+$ cells within the CCR7$^-$ subsets, but this did not reach statistical significance. Overall, IFN-γ expression was found most commonly within cells of subset IIIb.

The capacity to express TNF-α was also highest in cells that lacked the expression of CCR7 (subsets III and IV). The expression of CD28 by CCR7$^-$ cells correlated with an increased capacity to express TNF-α, with a mean of 36% of CD28$^+$ cells expressing TNF-α and a mean of 21.6% of CD28$^-$ cells expressing this cytokine ($p < 0.005$). Overall, TNF-α expression was found most commonly within cells of subset IIIa.

The five-color phenotypic analysis illustrated in Fig. 2 revealed that CD28$^+$ T cells within subsets Ia and IVa had very similar phenotypic properties, with most differing only in terms of the expression of CCR7. Analysis of the results presented in Fig. 3, however, shows that these two subsets of CD28$^+$ cells are clearly different in their capacity to express cytokines, particularly IL-2 and TNF-α. The capacity of cells in subset IVa to express IL-2 and TNF-α, but little IFN-γ, suggests that their properties are intermediate between those of subsets Ia and IIIa.

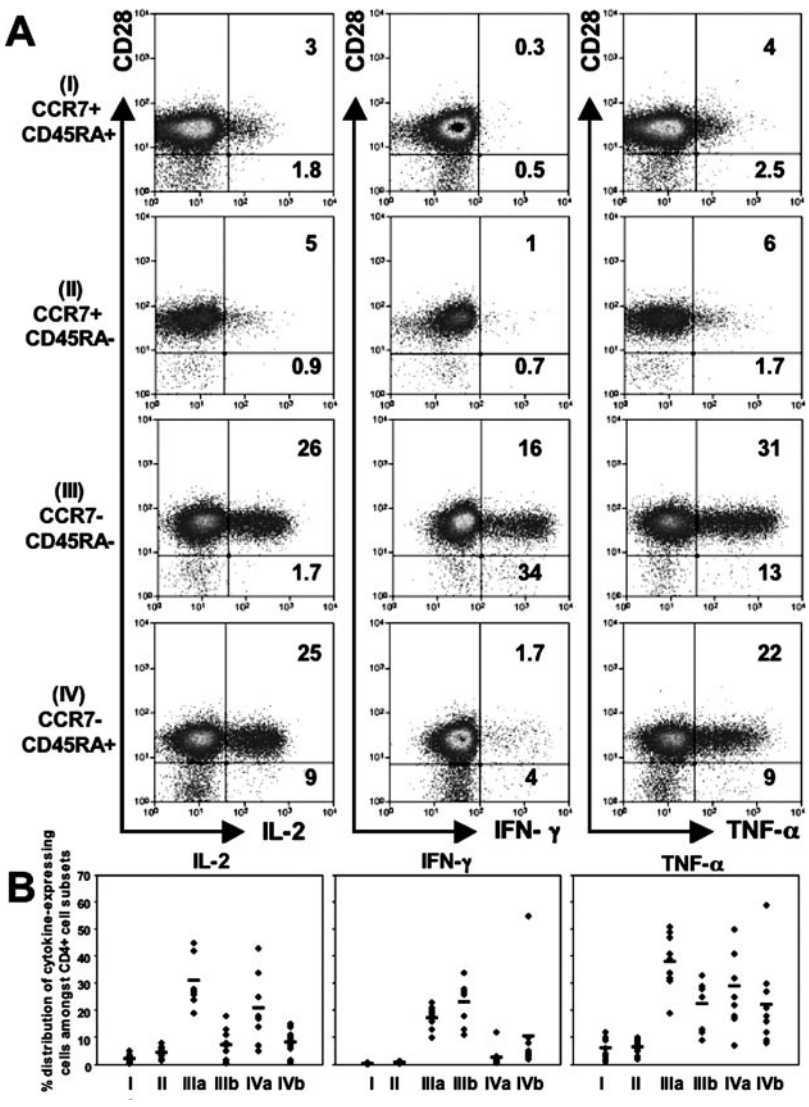

FIGURE 3. Expression of IL-2, IFN-γ, and TNF-α by CD4$^+$ T cells after polyclonal stimulation. PBMC were stimulated with SEB and then stained for the expression of CD4, CD45RA, CCR7, CD28, and cytokines. *A*, A representative example of the results is shown. Gates have been set on CD4$^+$ T cells and then on cells within each of subsets I–IV. The percentage of cells within each subset, I–IVb, that express each cytokine is shown. *B*, A summary of results from experiments performed on nine donors, showing the expression of IL-2, IFN-γ, and TNF-α by cells within subsets I–IVb after polyclonal stimulation.

Distribution of cytokine-expressing, virus-specific CD4$^+$ T cells among phenotypically defined subsets

In additional experiments we examined the cytokine profiles and phenotypes of populations of Ag-specific CD4$^+$ T cells. PBMC freshly isolated from seropositive donors were incubated for 18 h with either an EBV-infected B cell lysate or a CMV-infected endothelial cell lysate. Cells were stained for the expression of CD4, CCR7, CD45RA, and CD28 and for the presence of intracellular IL-2, IFN-γ, or TNF-α. Experiments were performed using blood from eight different donors. Results from a representative donor are shown in Fig. 4, *A* and *B*, and a summary of results is presented in Fig. 4, *C* and *D*.

In response to the EBV Ag preparation, a mean of 0.15% of CD4$^+$ T cells responded by expressing IFN-γ, and 0.16% responded by expressing TNF-α. A smaller number (0.09% of CD4$^+$ T cells) responded by expressing IL-2 (Fig. 4, *A* and *C*). A higher number of CD4$^+$ T cells responded to CMV, but the overall pattern of cytokine production was similar, with a mean of 0.37% cells expressing IFN-γ, 0.5% expressing TNF-α, and 0.1% expressing IL-2 (Fig. 4, *B* and *C*).

Although some cells responsive to EBV were present throughout the CD4$^+$ T cell compartments, they were highly enriched in the CCR7$^-$RA$^-$CD28$^+$ subset (subset IIIa; Fig. 4, *A* and *D*).

CD4$^+$ T cells that reacted to CMV Ags by expressing IL-2 were also enriched in the CCR7$^-$RA$^-$CD28$^+$ subset (subset IIIa). However, in contrast with T cells specific for EBV, T cells specific for CMV that responded to Ag by expressing IFN-γ or TNF-α were also commonly found in the CCR7$^-$RA$^-$CD28$^-$ subset (subset IIIb), with clear populations detectable in the CCR7$^-$RA$^+$CD28$^-$ subset (subset IVb; Fig. 4, *B* and *D*).

The difference in phenotypes between the EBV- and CMV-reactive CD4$^+$ T cells was consistent among the various donors; this supports the idea that CD4$^+$ T cells with different specificities tend to be distributed differently between phenotypic compartments. The data were consistent with the results presented above showing that IL-2 expression was predominantly limited to CD28$^+$ T cells, but was not limited to CCR7$^+$ cells, whereas the expression of IFN-γ and TNF-α occurred in CD28$^+$ or CD28$^-$ cells, but was usually associated with a CCR7$^-$ phenotype.

Correlates among the expressions of CCR7, IL-2, and IFN-γ

Our results bring into question the idea that CCR7 expression by CD45RA$^-$ cells identifies a subset of cells that is adapted for IL-2 expression; in response to both superantigen and specific Ag, we have found that IL-2 was more commonly expressed by a population of CCR7$^-$CD28$^+$CD4$^+$ T cells (subsets IIIa and IVa). To investigate the association between CCR7 expression and capacity to express IL-2 and IFN-γ further, in the context of Ag-specific responses, we stimulated PBMC from EBV- and CMV-seropositive donors with the preparations of EBV or CMV Ags and stained

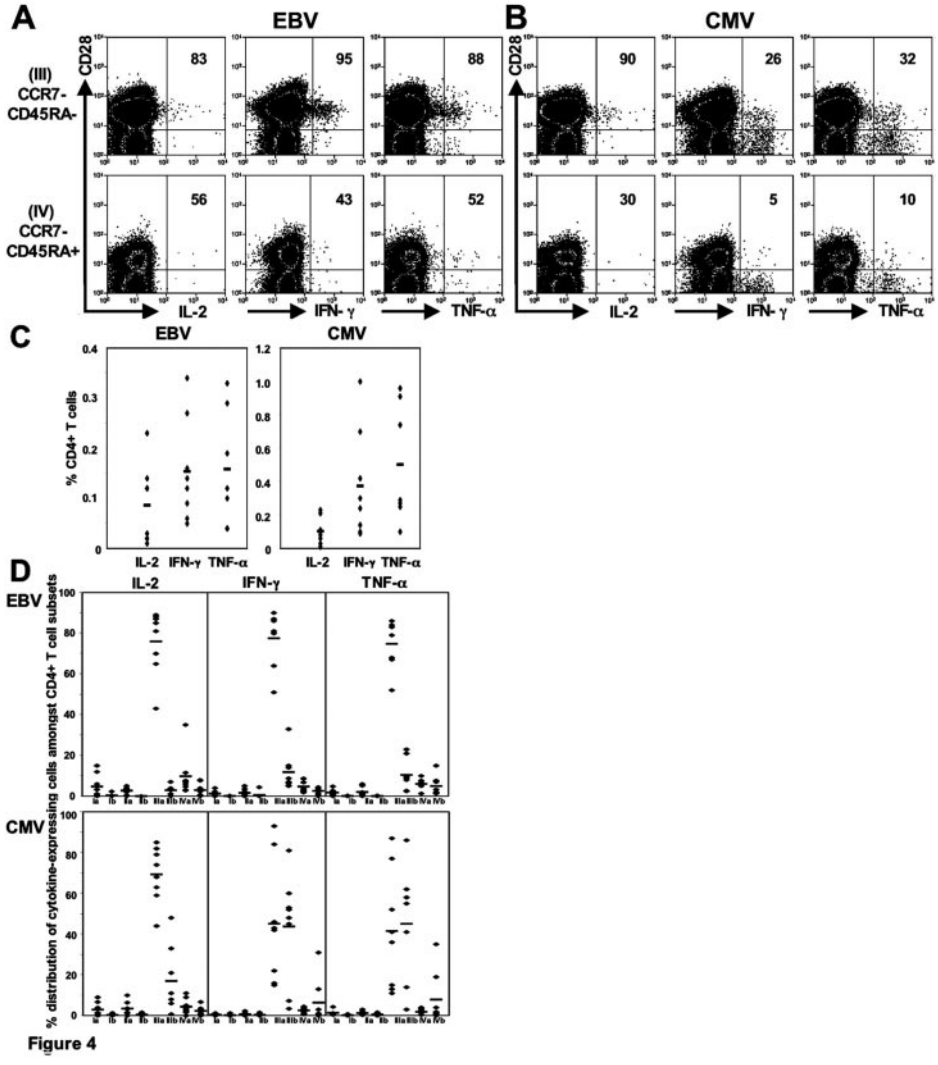

Figure 4

FIGURE 4. The expression of IL-2, IFN-γ, and TNF-α by CD4$^+$ T cells after stimulation with preparations of EBV or CMV Ags. PBMC from EBV- and CMV-seropositive donors were stimulated with preparations of EBV or CMV Ags and then stained for the expression of CD4, CD45RA, CCR7, CD28, and cytokines. *A*, A representative result showing cytokine expression after stimulation with EBV Ags. *B*, A representative result showing cytokine expression after stimulation with CMV Ags. Gates have been set on CD4$^+$ T cells and then on cells within each of subsets I–IV. The expression of IL-2, IFN-γ, and TNF-α is shown on the *x*-axis. The percent expression of CD28 by cells that respond to the stimulus by cytokine expression is shown for subsets III and IV. Smaller numbers of CD4$^+$ T cells within compartments I and II responded (FACS profiles not shown, but all data summarized in *C* and *D*). *C*, Summary of results from experiments performed using blood from eight different donors, showing the percentage of CD4$^+$ T cells expressing IL-2, IFN-γ, or TNF-α after stimulation with EBV or CMV Ags. *D*, The distribution of IL-2-, IFN-γ-, and TNF-α-expressing cells of these donors among subsets Ia–IVb after stimulation with EBV or CMV Ags.

for the expression of CD4, CCR7, IL-2, and IFN-γ. In line with a previous report, we were able to detect cells that responded by expressing IL-2 alone, both IL-2 and IFN-γ, and IFN-γ alone (33). Results from one experiment are shown in Fig. 5*A*, with gates set on cells that respond to the EBV or CMV Ag preparations by producing IL-2 or IFN-γ, and staining for the second cytokine and CCR7 shown. Cells that expressed IL-2, but not IFN-γ, were most likely to express CCR7. In contrast, cells that expressed both IL-2 and IFN-γ or IFN-γ, but not IL-2, were usually CCR7$^-$. This pattern held true for responses to EBV and CMV, and results were consistent when experiments were performed using blood from 10 donors who were EBV seropositive and eight donors who were CMV seropositive (Fig. 5*B*). Overall, a mean of 68% of CD4$^+$ T cells that responded by secreting IL-2 alone expressed CCR7 compared with a mean of 24% of cells that produced both IL-2 and IFN-γ ($p < 0.005$) and a mean of 22% of cells that produced IFN-γ alone ($p < 0.005$). The results provide additional support for the concept that the loss of expression of CCR7 correlates more closely with the gain of capacity to express IFN-γ than with the lack of capacity to express IL-2.

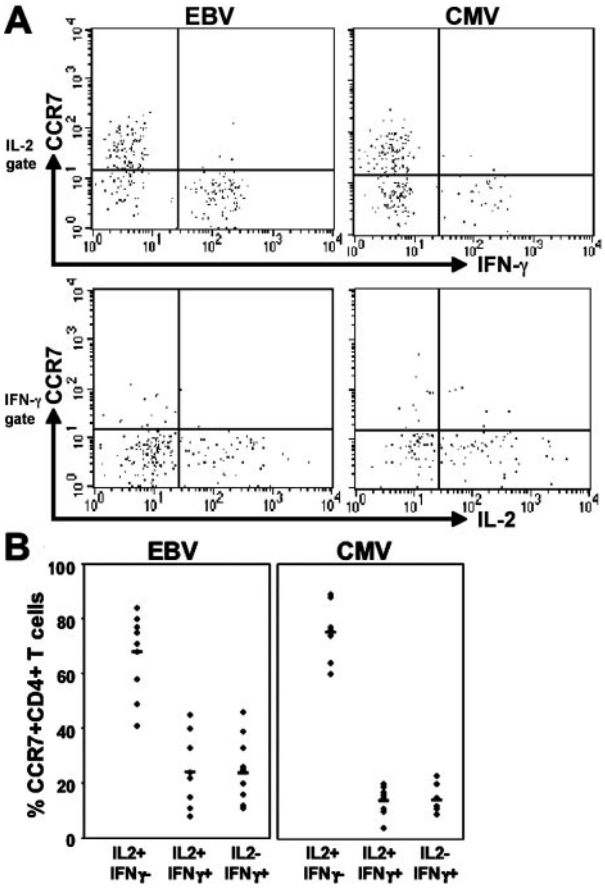

FIGURE 5. The expression of CCR7 by CD4$^+$ T cells that secrete IL-2 or/and IFN-γ. PBMC from EBV- and CMV-seropositive donors were stimulated with preparations of EBV or CMV Ags and then stained for the expression of CD4, CCR7, IL-2, and IFN-γ. *A*, Results from a representative individual. Gates have been set on CD4$^+$ T cells that respond by expressing IL-2 (*upper panel*) or IFN-γ (*lower panel*), and plots show the expression of the second cytokine vs CCR7. *B*, Summary showing results from 10 samples of PBMC stimulated with EBV Ags and eight samples stimulated with CMV Ags. The *y*-axis shows the percent CCR7 expression by CD4$^+$ T cells that respond by secreting IL-2 only, IL-2 and IFN-γ, or IFN-γ only.

Proliferative capacity of subsets of CD4$^+$ T cells

Having examined the phenotypic properties and cytokine-expressing capacities of subsets of CD4$^+$ T cells, we investigated the proliferative potentials of these subsets. Freshly isolated PBMCs were stained for CCR7 and CD45RA and then cell sorted into the four described subsets: subset I, CCR7$^+$RA$^+$; subset II, CCR7$^+$RA$^-$; subset III, CCR7$^-$RA$^-$; and subset IV, CCR7$^-$RA$^+$. After sorting, the cells were stained with CFSE, polyclonally stimulated with PHA, and placed into culture with autologous adherent monocytes. Samples were taken on days 3, 5, and 9 and stained for the expression of CD4, CD45RA, and CD28. Gates were set on the CD4$^+$ T cells, and proliferation was examined in each subset by measuring changes in CFSE fluorescence intensity. Experiments were performed using blood from three different donors. Fig. 6 illustrates the results from a representative experiment.

The CCR7$^+$CD45RA$^+$ subset (subset I) was slow to start division, and by day 5, many cells had only undergone one or two divisions. In contrast, the CCR7$^+$CD45RA$^-$ subset (subset II) had good proliferative capacity, with cells proliferating on day 3 and showing evidence of up to six divisions by day 5. The four different CCR7$^-$CD4$^+$ T cell subsets (subsets IIIa, IIIb, IVa, and IVb) varied in their proliferative capacities. CD28$^-$ cells, regardless of the expression of CD45RA, did not proliferate well in response to PHA (subsets IIIb and IVb). This is best seen at the day 5 point. Both CCR7$^-$CD28$^+$ subsets had proliferative capacity. However, of these two subsets, CD45RA$^+$ cells performed best (subset IVa), at least in this in vitro assay system. As the cells within this subset proliferated, the expression of CD45RA was down-regulated (data not shown).

Discussion

The possibility that T cells may be classified into meaningful subsets on the basis of the expression of surface proteins is attractive. Markers for T cells with the ability to express particular cytokines, to home to certain sites, or to proliferate will be useful when designing vaccines or T cell therapies for infectious and malignant

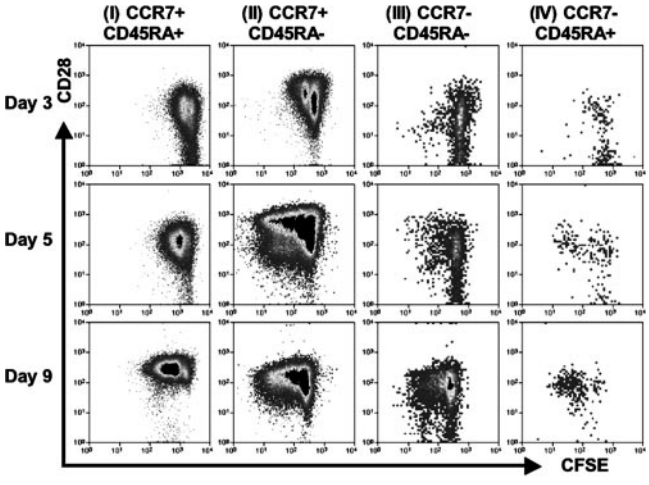

FIGURE 6. Proliferation of CD4$^+$ T cells after stimulation with PHA. CD4$^+$ T cells from PBMC were sorted into subsets I–IV, labeled with CFSE, and stimulated in vitro with PHA in the presence of autologous monocytes. Cultures were sampled on days 3, 5, and 9, and cells were stained for the expression of CD4 and CD28. Gates have been set on CD4$^+$ lymphocytes, and staining for CD28 is shown against CFSE intensity. Results are representative of experiments performed using blood from three different donors.

disease (34, 35). Several different models for classifying $CD8^+$ T cells have been suggested, and evidence is accumulating to justify their use (12, 17, 18, 22). The complexity of the $CD4^+$ T cell repertoire is less well understood. Technical advances in multicolor flow cytometry have allowed us to analyze this in detail. The work performed in this study has revealed that the $CD4^+$ T cell pool is very heterogeneous. Although the results show that T cells with many different phenotypes circulate in healthy individuals, they make a case for considering $CD4^+$ T cells as being distributed among major subsets, defined by the expression of CCR7, CD45RA, and CD28. The majority, although not all, of $CD4^+$ T cells fall within six of the possible eight subsets defined by these three molecules. In contrast to most previous studies, but in keeping with our previous work (10) and two other recent publications (25, 26), we have found that populations of $CCR7^-CD4^+$ T cells expressing CD45RA are present within peripheral blood, and these cells make up two of the six subsets. We provide a justification for use of the model we propose in terms of the functional correlates of expression of each of the three molecules chosen to define the subsets.

The inclusion of CCR7 in the description of subsets is justified on the basis of a very clear correlation with cytokine expression. $CD4^+$ T cells that are capable of expressing IL-2, but not IFN-γ, are most likely to express CCR7. We stress that the loss of CCR7 expression does not imply that cells have lost the capacity to express IL-2. Indeed, in the context of the two Ag-specific responses that we have studied, the majority of IL-2 production is from cells that lack the expression of CCR7. The lack of expression of CCR7 correlates well with their enhanced capacity to express both IFN-γ and TNF-α. One would predict that the in vivo consequence of the observed correlates would be that cells that circulate to lymphoid organs may express IL-2, but will not express IFN-γ; cells that home to peripheral sites may express both IL-2 and IFN-γ.

The expression of CD28 was not considered in the original subclassification of $CD4^+$ T cells proposed by Sallusto et al. (18), recently modified by Harari et al. (26). Populations of $CD28^-CD4^+$ T cells have been noted in patients who have recovered from primary CMV infection (36), patients with rheumatoid arthritis (37, 38), and patients with unstable angina (39, 40) and have been referred to as cytotoxic $CD4^+$ T cells (28). Their role in protective immunity has been unclear, although it has been suggested that they may be terminally differentiated cells, generated as a consequence of persistent immune stimulation, and/or that they may play a role in the response to organisms that establish intracellular infection of HLA-class II-expressing cells by killing such cells. In the context of recovery from CMV infection, $CD28^-CD4^+$ T cells were found to be granzyme B^+, consistent with a putative role as killer cells, and to lack the expression of CD27 and CD45RA (36). In the rheumatoid arthritis patients, the cells were noted to express CD45RO, CD57, and killer Ig-like receptors (37, 38). We found some $CD28^-CD4^+$ T cells in all our donors, particularly among the $CCR7^-$ cell populations. Of relevance, all donors selected were seropositive for CMV. Our experiments demonstrate strong correlates between the expression of CD28 and phenotypic and functional properties of $CD4^+$ T cells. The lack of expression of CD28 is associated with the expression of CD57, perforin, and granzyme A. Furthermore, it is associated with a much reduced capacity to express IL-2 and a diminished capacity to produce TNF-α. Finally, the lack of expression of CD28 identifies $CD4^+$ T cells that proliferate poorly in response to PHA in vitro.

The expression of RA and RO isoforms of CD45 on T cells was originally thought to distinguish naive from Ag-experienced cells (15, 16). That naive T cells express CD45RA continues to be an accepted truth. However, the expression of CD45RA on some Ag-experienced $CD8^+$ T cells has been noted (5–7, 11–14, 17, 18, 20–22). Its relevance to function has been controversial. It has been argued that CD45RA-expressing, Ag-experienced $CD8^+$ T cells have good functional capacity and are relatively resistant to apoptosis (20, 41, 42). In keeping with our previous work, we found that CD45RA is expressed on significant numbers of circulating $CCR7^-CD4^+$ T cells. Importantly we found that CD45RA expression by $CCR7^-CD4^+$ T cells had correlates in terms of cytokine expression and was associated with a significantly reduced capacity to express IFN-γ. Other properties of the $CD45RA^+$ cells varied according to CD28 expression, and we found that the subpopulation of cells that also expressed CD28 (subset IVa) had good proliferative capacity.

Overall, our work suggests that cells within subset IIIa have the best capacity to produce a broad range of cytokines, including IL-2 and TNF-α, and can also proliferate in response to stimulation. Interestingly, this is where we found EBV-specific $CD4^+$ T cells to accumulate. CMV-specific $CD4^+$ T cells were also found within subset IIIa, but additionally, many were found within subset IIIb, where the cells would be less likely to express IL-2 or proliferate rapidly, but would be more likely to express cytolytic mediators. The accumulation of T cells with different specificities within the different compartments may reflect adaptations that allow for optimal control of the different pathogens.

It is of interest that we were able to detect virus-specific $CD4^+$ T cells, albeit relatively few, in compartment IVb when an assay to detect either IFN-γ or TNF-α by stimulated T cells was used. Overall, we have found that only a small proportion of cells present within subset IVb expresses these cytokines after stimulation. We would therefore only expect to detect a small proportion of the virus-specific $CD4^+$ T cells present within this compartment with these methods. This raises the possibility that there may be relatively large numbers of virus-specific $CD4^+$ T cells within subset IVb. The observation that the overall numbers of $CD28^-CD4^+$ T cells within the circulation increases after CMV infection is consistent with this (36). Additional studies using reagents such as MHC/peptide multimers to detect the physical presence of CMV-specific $CD4^+$ T cells are required to investigate this in more depth.

Despite the different approaches, the conclusions of our study of the $CD4^+$ T cell repertoire in many ways parallel the results of interesting recent work published by Rufer et al. (22), in which they propose that the $CD8^+$ T cell population may be considered as being distributed among six major compartments. This group chose to use CD27 as well as CCR7 and CD45RA to define the $CD8^+$ T cell compartments, whereas we chose CD28 instead of CD27.

A diagrammatic, cross-sectional representation of the described subsets of $CD4^+$ T cells is shown in Fig. 7. We have not investigated the lineage relationship among the different subsets, although we have noted that CD45RA expression is lost from $CCR7^-$ cells as they proliferate (data not shown). Thus, cells move from IVa to IIIa and from IVb to IIIb. However, the relevance of changes in phenotype seen in vitro experiments to those happening after Ag stimulation in vivo is unclear. If one were to assume that changes in the expression of individual molecules occur sequentially, then it would be possible to draw hypothetical pathways for T cell differentiation from our model. If two or more molecules were to change in their expression pattern simultaneously, then the cell would effectively take two or more steps along the differentiation pathway. It is theoretically possible that cells might move in either direction along the pathway; therefore, for completeness, bidirectional movement is shown on the diagram. Previous work has, for example, shown that after exposure to IL-12, $CD4^+$ T cells

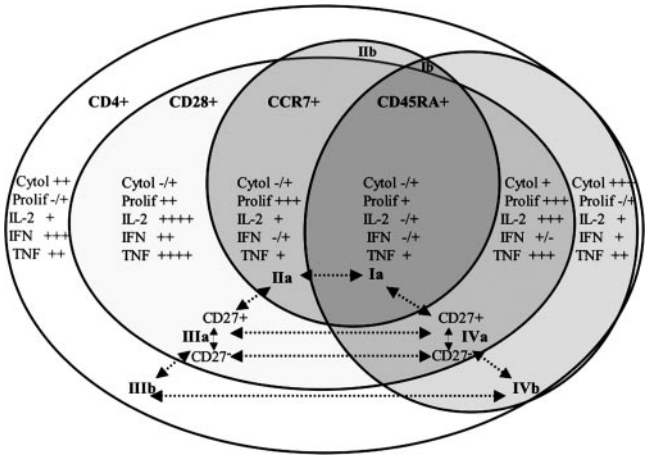

FIGURE 7. A cross-sectional model of the peripheral $CD4^+$ T cell repertoire. Overlapping populations of cells expressing CCR7, CD28, and CD45RA are represented within the pool of peripheral $CD4^+$ T cells. The six major subsets of $CD4^+$ T cells, Ia, IIa, IIIa, IIIb, IVa, and IVb are shown, as are the two very minor subsets, Ib and IIb. The functional profiles of cells within the subsets are given. Cytol, presence of cytolytic mediators; prolif, proliferative capacity in response to PHA; IL-2, IFN, and TNF, capacity to express IL-2, IFN-γ, and TNF-α in response to polyclonal stimulation. This is a cross-sectional representation; theoretically possible lineage relationships between the compartments are shown by dotted arrows. The diagram does not reflect the actual sizes of the compartments.

may re-express CD28 (43). The resulting pathways, illustrated in the model, provide a framework hypothesis for the differentiation of $CD4^+$ T cells. Of interest, this hypothesis suggests that cells might differentiate from subset I, via the newly described subset IVa, to subset IIIa. In support of this, during primary CMV infection, CMV-specific $CD4^+$ T cells have been noted to be $CD62L^-$, but $CD45RA^+$; such cells showed variability in terms of CD27 expression (8). The details of this model need to be tested in future studies of the evolution of $CD4^+$ T cell responses in vivo to understand how $CD4^+$ T cells with different types of functional capacity are generated and maintained.

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Disclosures

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