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11-color, 13-parameter flow cytometry: Identification of human naive T cells by phenotype, function, and T-cell receptor diversity

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Recent findings illustrate that diseases are often accompanied by changes in the numbers or function of 'fine' lymphocyte subsets, even if changes in the bulk lymphocyte populations are not evident. In some cases, such changes may provide powerful prognostic and diagnostic information. For example, Giorgi *et al.*[1] showed that the number of activated CD8 T cells ($CD38^+$/$HLA\text{-}DR^+$), but not the number of total CD8 T cells, was a better predictor of progression to AIDS than the commonly-used CD4 T-cell count. And, by accurately dividing T cells into naive and memory, we demonstrated that progression of HIV disease was accompanied by the loss of both CD4 and CD8 naive T cells[2,3]—a finding that could not be explained by then-popular models of cell loss by viral cytolysis.

These examples illustrate our increasing awareness of the complexity of the immune system. As many diseases are associated with expansions or reductions in major leukocyte subsets (for example, anemias, neutropenias, cancers and AIDS), it should come as no surprise that changes in representation (absolute number or percentage) of fine subsets of lymphocytes are also correlated with disease. Identifying such changes has been difficult because traditional three-color flow cytometric analyses can only resolve major lineages (B, T, natural killer, monocyte or neutrophil), or, at best, resolve a few subsets of any single lineage.

The findings that changes in fine subsets may be important in disease pathogenesis convinced us to develop a technology that could simultaneously measure many more distinct fluorescences from individual cells. This development effort required the concomitant development of new fluorescent dyes with suitable spectral properties, new hardware capable of measuring all of the fluorescences, and new software tools capable of analyzing, managing and reducing the large and complex data sets. Our current technology measures two scatter and 11 fluorescence parameters on each cell. We term this technology polychromatic flow cytometry (PFC). Although PFC is a derivative of flow cytometry, it has unique problems and potential artifacts that must be overcome[4]; the resulting data, however, provides a new view of the immune system.

Here we use the power of PFC to precisely define and characterize naive CD4 and CD8 T cells by phenotype, cytokine production and T-cell receptor diversity. Naive T cells are those that have been released by the thymus into circulation, but have not yet encountered their cognate antigens. Enumeration of naive T cells is critical to understanding, for example, potential immune reconstitution following antiviral therapy in highly-active anti-retroviral therapy[5–7] (HAART). For this reason, it is important to determine how accurately these cells can be identified by cell-surface immunophenotyping alone.

In the past, quantification of a single marker, such as expression of CD45RA or lack of expression of CD45RO, has been used to estimate naive cell counts. We and others previously demonstrated the necessity of including an additional marker such as CD62L or CD11a to better distinguish naive from memory[8,9]. Other phenotypic markers for naive cells include expression of CD27 and CD28, low or absent expression of activation markers (such as CD44 and HLA-DR), and low expression of FAS receptor[10–12] (CD95).

Naive T cells are known to differ functionally from memory T cells. After *in vitro* stimulation, they do not produce interferon-γ (IFN-γ), IL-4, or IL-5 and produce only low levels of IL-2 (ref. 13), and have different calcium mobilization[14] and proliferative responses[15]. In addition, the T-cell receptor diversity of the naive cell population is, by definition, the most diverse; memory subsets have a restricted repertoire as a consequence of antigenic selection. Based on these functional correlates, we used PFC to precisely define the phenotype of naive T cells, and quantify the limitation of two- or three-color analyses for enumerating naive T cells.

Multiple markers necessary for accurate naive cell identification

Analyzing the surface expression of multiple markers allowed us to determine that simultaneous quantification of multiple naive-associated phenotypic markers improves the accuracy of naive cell identification. Figure 1 shows an example of an experiment

Table 1 Ten and 11-color FACS staining combinations

	405 nm		488 nm excitation					595 nm excitation			
	CasBl	Cas Yel	FITC	PE	Cy5 PE	Cy5.5 PE	Cy7 PE	Alexa	APC	Cy5.5 APC	Cy7 APC
1[a]	CD45RA	CD3	CD28	CD49f	CD11a	CD27	CD4	CD62L	CD45RO	CD8	CD57
2[b]	CD45RA		CD11a	CD28	EMA	CD27	CD4	CD62L	IFN-γ	CD3	CD8
3[c]	CD45RA	CD3	Vβ	Vβ	CD11a	CD27	CD4	CD62L	CD28	CD8	CD57

[a]Staining combination used for Figure 1. This and similar combinations were used to generate the data in Fig. 2. [b]Staining combination used for Fig. 3. A cascade yellow reagent was not included. EMA, ethidium monoazide bromide, labels dead cells by intercalating into DNA and covalently binding to DNA when exposed to light. [c]Staining combination used for Fig. 4. For each blood sample, PBMC were stained with all these reagents except for FITC and PE. The stained cells were divided in aliquots and then individually stained with seven different FITC/PE Vβ reagent combinations (see Methods).

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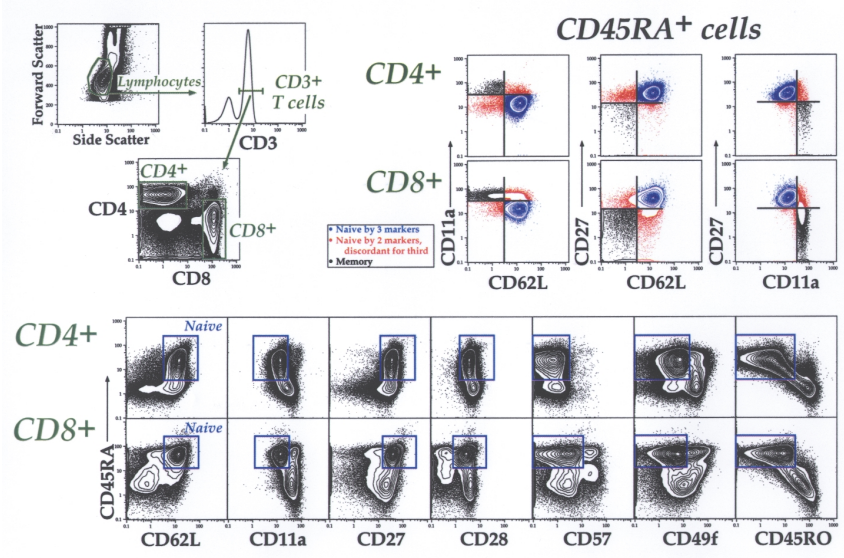

Fig. 1 13-parameter, 11-color PFC accurately defines naive T cells. Peripheral blood mononuclear cells from one donor were stained with one 11-color staining combination (Table 1) and analyzed by PFC. The upper left panels show the progressive gating to obtain CD4 and CD8 T cells. The lower panels show each of the naive-defining markers plotted against CD45RA, allowing two-marker definitions of naive cells (blue boxes). Among CD45RA-expressing cells, the expression of CD45RA is slightly higher for naive versus memory cells (median CD45RA fluorescence of $CD62L^+$ versus $CD62L^-$ cells was 20 versus 10 for CD4 T cells and 44 versus 31 for CD8 T cells). In the upper right panels, only cells expressing CD45RA are shown. For these cells, bivariate graphs of 2 other naive-defining markers are shown, allowing 3-marker definition of naive cells (blue). Memory cells (non-naive by both markers, although $CD45RA^+$) are shown in black. Discordant cells (naive by one marker and CD45RA, and non-naive by the other marker) are shown in red. Placement of the CD45RA gate is difficult and must be done separately for CD4 and CD8 T cells. For CD8 T cells, there is usually a clear separation between cells expressing high levels of CD45RA (which include naive cells) and the memory cells that express low levels of CD45RA, making the gate placement unambiguous. CD4 T cells express lower levels of CD45RA, and optimal placement of a CD45RA gate is more subjective. This makes the use of multiple markers to identify naive T cells (Fig. 2) even more important.

in which we stained peripheral blood mononuclear cells with monoclonal antibodies that recognize a series of surface markers known or suspected to be expressed differently on naive and memory T cells. The use of two markers to identify naive CD4 or CD8 T cells (that is, the limit of three-color flow cytometry) is also shown for comparison.

When a third marker is included to identify naive cells, a population of cells is found to be discordant, that is, naive based on two markers, but memory based on a third. Several examples of the use of three colors to define naive cells are shown in Fig. 1, where only cells classified as naive for one marker ($CD45RA^+$) are shown. Cells discordant in phenotype are evident in these graphs, especially among the CD8 T cells (for example, naive by CD62L and CD45RA, but memory by CD11a or CD27). As described below, we used functional studies to demonstrate that these discordant cells appear to be non-naive based on cytokine profile and Vβ repertoire.

We compared the accuracy of naive cell identification achieved using from 1 to 4 naive-associated markers in combination, relative to those cells identified as naive using all five markers—the most stringent identification we used (Fig. 2). In agreement with previous studies, the use of any single marker (for example, CD45RA) is poor for identifying naive cells. Adding a second marker greatly improves the specificity for naive cells (97–98% for CD4 cells, 87–93% for CD8 cells); a third marker further improves the specificity (99% for CD4 cells, 96% for CD8 cells).

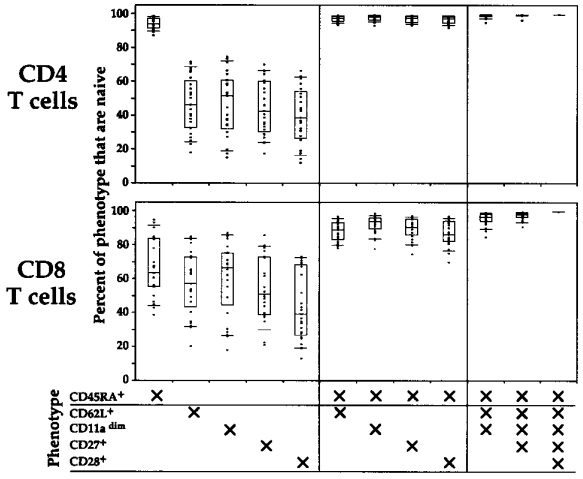

Fig. 2 The use of multiple markers increases the accuracy of naive cell determinations. Peripheral blood mononuclear cells derived from 5 adults were stained to identify naive CD4 and CD8 T cells with 5 different sets of staining combinations, each of which includes the 6 naive-defining reagents listed. The gating method used to define the CD4 and CD8 overall and naive T cells is shown in Fig. 1. The frequency of cells identified as naive by all 5 markers (last column) is used as the reference for the most pure naive population. The first set of columns shows the percentage of the cells that are naive relative to this reference when only one naive marker is used. The next set of columns shows this accuracy of naive determination when two naive markers are used. Similarly, the accuracy using 3 or 4 markers is also shown. The box plots show the median, interquartile range (box), and 10^{th} and 90^{th} percentiles for measurements from all samples.

Discordant cells are non-naive by functional analysis

As memory T cells produce large amounts of IFN-γ, and naive T cells produce little if any of this cytokine upon stimulation, we used the *in vitro* production of IFN-γ to determine if the naive subset as identified by two surface markers includes contaminating cells that are functionally behaving as memory cells. The intracellular production of IFN-γ was measured using minor modifications of a flow cytometric assay for cytokine production[13].

Among the cells identified as naive by two markers, there are cells that produce IFN-γ and are likely not naive (Fig. 3). Indeed, these IFN-γ–producing cells are excluded from the subset of cells defined as naive when additional surface markers are used (right panel). Therefore, cells discordant for naive phenotypic markers

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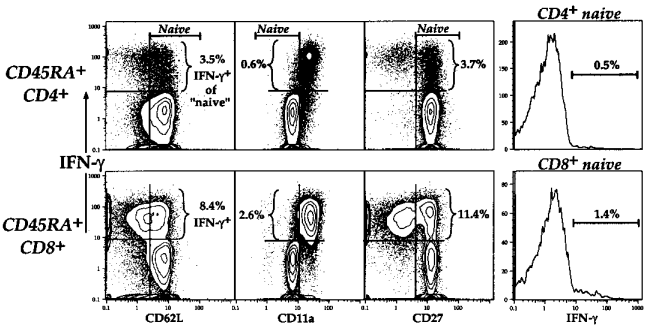

Fig. 3 Cells discordant for naive markers express IFN-γ upon stimulation. Stimulated peripheral blood mononuclear cells were stained for surface markers and for intracellular IFN-γ (ref. 13). CD4+ and CD8+ T cells that express CD45RA, gated as in Fig. 1, are shown in the 6 panels on the left. These show IFN-γ expression in cells identified as naive based on the expression of the marker on the x-axis (that is, $CD62L^+$, $CD11a^{dim}$, $CD27^+$) in combination with CD45RA. The percentage of these cells that are IFN-γ^+ is indicated. The two panels on the right show the expression of IFN-γ in cells identified as naive by all four markers ($CD45RA^+CD62L^+CD11a^{dim}CD27^+$). The use of CD62L to define naive cells after stimulation can sometimes be difficult to interpret, because stimulation leads to activation of a specific protease that normally hydrolyzes CD62L off the cell surface. Although we include a metalloproteinase inhibitor during the *in vitro* stimulation to inhibit this proteolysis[17,18], the resolution of CD62L positive versus negative cells is adversely affected. Note that the combination of CD11a with CD45RA works particularly well to exclude IFN-γ^+ cells.

have a cytokine profile consistent with memory cells, in contrast to the cells with the proper expression of all naive markers.

We also determined the T-cell receptor Vβ repertoire to characterize the naive, memory and discordant cells. Naive T cells have, by definition, the broadest Vβ repertoire. In contrast, memory T cells often have a repertoire that is restricted to varying degrees due to clonal selection by antigen. Using PFC, we quantified the usage of 21 Vβ genes among the CD4 and CD8 T cells in the naive and memory subsets. On average, these 21 Vβ genes account for 60% of the total T-cell repertoire.

The Vβ distributions for memory T-cell subsets are skewed compared to naive (Fig. 4). Moreover, the phenotypically discordant naive T cells have skewed distributions, indicating that these cells are comprised of relatively few clones presumably selected by antigenic stimulation. This unequivocally defines the phenotypically discordant cells as memory.

Discussion

A major unanswered question in immunology concerns the extent of the complexity and heterogeneity of the cells of the immune system. A full understanding of this complexity includes identifying and characterizing the most basic units—the fundamental subsets that cannot be subdivided further and that contain cells that are functionally and phenotypically homogeneous. Achieving this goal requires the ability to measure a sufficient number of parameters for each cell to resolve the underlying heterogeneities. We developed the PFC technology with this goal in mind; our current technology is capable of measuring two scattered-light and 11 fluorescence measurements simultaneously.

The question we addressed here is the extent to which the current methods of identifying naive cells are subject to artifacts due to the inclusion of memory T cells within the designated subset. Our data show that measuring a minimum of three differentiation markers is required to consistently enumerate naive T cells with better than 95% accuracy. Moreover, the use of only two markers, the most common method of identifying naive cell populations, defines a population that is significantly contaminated by memory cells. Although in general this contamination with memory cells is relatively small for healthy individuals (2–3% for CD4 cells, 7–13% for CD8 cells), it can be significantly greater in the context of acute or chronic infection. Furthermore, this level of contamination can introduce significant artifacts in functional studies. For example, a 10% contamination of memory CD8 T cells can lead to a significant production of effector cytokines in what was supposedly a pure naive T-cell population. Undoubtedly, this contamination has been the source of the confusion regarding the naive phenotype, as well as explaining the apparent reversion of memory to naive phenotypes described in the literature.

The need to accurately enumerate naive T cells is particularly important when the response of these cells to disease state and therapeutic intervention is clinically relevant. For example, in HIV disease, both CD4 and CD8 naive T cells are known to be lost as the disease progresses. In the current era of HAART, the return of naive cells needs to be accurately monitored as a measure of immune reconstitution. This is especially important given that many HIV-infected patients have very low numbers of naive T cells before treatment, and that although some increase is observed following HAART, this increase is relatively small. The accuracy of the naive cell measurement could strongly affect the assessment of whether or not the treatment results in immune reconstitution.

Methods

Peripheral blood was drawn from volunteers and peripheral blood mononuclear cells were obtained by density centrifugation. Purified antibodies to the cell-surface markers listed in Table 1, as well as to IFN-γ, were obtained from PharMingen (La Jolla, California) and conjugated to the indicated fluorochomes in our laboratory using standard protocols (http://drmr.com/abcon). Fluorescein isothiocyanate (FITC), Alexa-594, Cascade Blue and Cascade Yellow were obtained from Molecular Probes (Eugene, Oregon). Phycoerythrin (PE) and antigen-presenting cells APC were obtained from ProZyme (San Leandro, California). Cy5, Cy5.5, and Cy7 were obtained from Amersham Life Science (Pittsburgh, Pennsylvania). For staining of 21 Vβ alleles a prototype TCR Vβ kit was obtained from Antje Necker (Immunotech, Marseille, France). Approximately $10–20 \times 10^6$ peripheral blood mononuclear cells were stained for 15 min with nine reagents (not including the FITC and PE anti-Vβ), then divided in 7 aliquots and stained for 15 min with the FITC/PE Vβ sets. Data were collected on a modified FACStarPlus (Becton Dickinson, San Jose, California) connected to MoFlo electronics (Cytomation, Fort Collins, Colorado). Data were collected by FACS-Desk software and compensated, analyzed, and presented using FlowJo (Tree Star, San Carlos, California).

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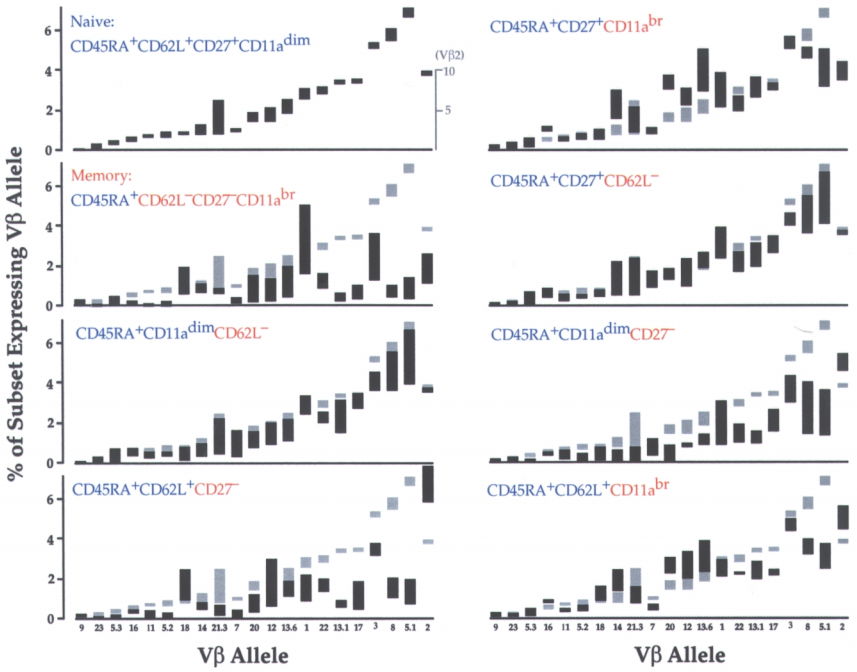

Fig. 4 CD8 T cells discordant for naive markers display a restricted Vβ repertoire and are therefore memory cells. Data are shown for 1 adult analyzed 4 times over a two-week period. Reagents detecting 21 Vβ alleles (see Methods and Table 1) were used in combination with a set of 11-color staining reagents (Table 1) chosen to allow identification of naive and memory CD8 T cells. The percentage of cells in each subset that express the indicated Vβ allele is graphed for each subset; bars indicate the range for the four measurements. (The vertical scale is uniform except for Vβ2, the most frequent allele; see axis scaling on the right of the top-left graph). The Vβ profile for naive cells identified by the complete phenotype $CD3^+CD8^+CD4^-CD11a^{dim}CD62L^+CD27^+CD45RA^+$ is shown on the top left. Immediately below, the phenotype for CD8 T cells in the memory subset that is $CD45RA^+$ but non-naive for the other 3. The other 6 graphs show data for $CD45RA^+$, discordant naive T cells (that is, naive by two markers but non-naive by a third). The profile for the naive T cells is overlayed on each graph for comparison. These patterns, showing restricted repertoires in discordant subsets compared to naive T cells, were also observed for CD4 T-cell subsets, and were observed in 3 of 3 other donors (data not shown).

Using PFC, we can identify at least 75 phenotypically distinct subsets of lymphocytes in human peripheral blood (Fig. 1; examples of CD4 and CD8 T-cell subsets). Each of these subsets has a unique functional profile, and likely has a unique role in the immune system and disease pathogenesis. Indeed, we were able to show that the number of cells of functionally-polarized (Th1-like or Th2-like) CD4 memory subsets was highly correlated with disease states such as atopy, or tuberculoid or lepromatous leprosy[13]. These findings indicated that the functional polarization of the immune system (described for the bulk lymphocyte population) is due to a change in the representation of functionally distinct subsets—and that enumeration of those cells may be clinically useful.

The highly multiparametric nature of PFC also allowed us to perform the most thorough analysis of unmodified antigen-reactive T cells from cancer patients to date. These cells are typically extremely rare, for example, 1 in 10^5 peripheral blood mononuclear cells, making bulk functional assays impossible. The paucity of these cells renders detailed three- or four-color immunophenotyping (using multiple distinct staining combinations) similarly difficult or impossible. With ten-color analysis, however, we were able to identify that tumor-reactive T cells in patients with metastatic melanoma fell into two distinct phenotypic categories, and to show that these cells were functionally anergic *ex vivo*, perhaps explaining their inability to control tumor growth *in vivo*[16].

These studies exemplify how PFC, while revealing the rich heterogeneity and complexity of the peripheral immune system, can actually simplify our understanding of immunopathogenesis. Polychromatic flow cytometry gives us the ability to focus only on the relevant cell populations and ignore the majority of cells that may play no role in the disease.

Acknowledgments

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