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Differential representations of memory T cell subsets are characteristic of polarized immunity in leprosy and atopic diseases

Dipendra K. Mitra[1,3], Stephen C. De Rosa[1], Andrew Luke[2], Arumugam Balamurugan[3], Binod K. Khaitan[4], James Tung[1], Narinder K. Mehra[3], Abba I. Terr[2], Anne O'Garra[5], Leonore A. Herzenberg[1], Leonard A. Herzenberg[1] and **Mario Roederer[6]**

[1]Department of Genetics and [2]Allergy Clinic, Immunology Division, Department of Medicine, Stanford University, Stanford, CA 94305, USA
[3]Histocompatibility & Immunogenetics Department and [4]Department of Dermatology, All India Institute of Medical Sciences, New Delhi 110029, India
[5]Department of Immunobiology, DNAX Research Institute, Palo Alto, CA 94304, USA
[6]Department of Stomatology, San Francisco, CA 94143-0422, USA

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Abstract

We identified functionally polarized subsets of CD4 memory T cells on the basis of the expression of CD11a, CD45RA and CD62L. Within the several phenotypically distinct subsets of CD4 memory cells are two that, upon stimulation, produce primarily IL-4 (MT_2, $CD45RA^-CD62L^+CD11a^{dim}$) or primarily IFN-γ ($MT_1$, $CD45RA^-CD62L^-CD11a^{bright}$). In addition, four other phenotypically distinct subsets of CD4 cells have unique cytokine profiles. To determine the clinical relevance of the representation of these cell types, we analyzed blood from patients with the chronic diseases leprosy and atopy. These diseases are characterized as immunologically polarized, since T cell responses in affected individuals are often strongly biased towards T_h1 (dominated by IFN-γ production) or T_h2 (IL-4 production). We show here that this polarization reflects homeostatic or differentiation mechanisms affecting the representation of the functionally distinct subsets of memory CD4 T cells, MT_1 and MT_2. Significantly, the representation of the MT_1 and MT_2 subsets differs dramatically between subjects with tuberculoid leprosy (a T_h1 disease), or lepromatous leprosy or atopic disease (T_h2 diseases). However, there was no difference in the cytokine profiles of these or any of the other finely resolved CD4 subsets, when compared between individuals across all disease states. Thus, it is the representation of these subsets in peripheral blood that is diagnostic of the polarized state of the immune system.

Introduction

It has recently become apparent that phenotypically-distinct subsets of memory CD4 T cells have distinct functional capacities. Indeed, selective expression of IFN-γ is associated with high CD11a expression in murine CD4 T cells (1) and selective expression of IL-4 is associated with CD62L expression in human CD4 T cells (2,3). More detailed definition of cytokine profiles of the many identifiable subsets of CD4 subsets has been nearly impossible because of the inability to simultaneously measure the expression of cytokine as well as the four or more surface molecules that uniquely identify functionally distinct subsets.

We have recently extended the capabilities of flow cytometric analysis to routinely perform eight-color analysis or higher (4). Using this technology, we can devote five colors to discriminate unique CD4 subsets while simultaneously measuring the production of cytokines such as IL-2, IL-4 and IFN-γ. In this report, we confirm the previous associations of biased cytokine production with phenotype and extend these analyses to demonstrate that four-color immunophenotyping identifies highly polarized (i.e. towards IL-4 or IFN-γ production) cell types. We also analyzed whether or not this phenotypic identification had clinical relevance using peripheral

Correspondence to: M. Roederer, Department of Stomatology, 521 Parnassus Ave C634C, San Francisco, CA 94143-0422, USA

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blood samples from humans with immunologically polarized disease.

Certain chronic human diseases can be characterized as immunologically polarized (5). For example, atopic disease manifestations to antigens such as pollen or mold spores include asthma, eczema and hay fever, and are characterized by allergen-specific IgE in the peripheral blood and activation of mast cells and eosinophils (6,7). Such responses have been shown to be associated with overproduction of IL-4, IL-5 and IL-13 , which are characteristic of the T_h2 subset of T lymphocytes (8–10).

Leprosy is a spectral disease associated with two polar forms. Tuberculoid leprosy (TT) is characterized by clinico-immunologically localized paucibacillary lesions and a T_h1-like cytokine profile (increased IFN-γ, decreased IL-4 and IL-5). Lepromatous leprosy (LL) exhibits diffuse, disseminated multibacillary lesions, with essentially no cell-mediated immunity against *Mycobacterium leprae* and a T_h2-like cytokine profile (11–16). Between these polar forms lie a number of other forms of the spectrum such as borderline tuberculoid (BT), borderline (BB) and borderline lepromatous (BL) showing a mixture of the distinctive characteristics found in each pole.

Characterizing the polarization of the immune response in chronic diseases such as leprosy can provide both diagnostic and mechanistic insights into the progression of the disease. However, identification and quantitation of cells mediating the polarized responses in such chronic diseases has proven quite difficult. Single-cell cytokine response assays to antigen are now becoming more common; for a majority of diseases, however, determining a functional T_h1 or T_h2 dominance requires cloning or selective expansion in the presence of antigen—a process usually requiring several weeks—or isolation of cells from the site of pathology during the peak of the immune response (9,14,17–20). Direct evaluation of the polarization in peripheral blood samples is preferable; however, even in patients with active chronic disease, there may be too few cells to readily define the cytokine profile of memory T_h cells stimulated with mitogen (21) or even with specific antigen (if the antigen is known).

In this report, we use multiparameter flow cytometry to study the cytokine profiles of discrete subsets of T cells identified on the basis of a combination of cell surface markers. We show that in chronic atopic diseases and lepromatous leprosy, or in tuberculoid leprosy, increased T_h2 or T_h1 responses (respectively) are manifested in blood as an increase in the representation of specific populations defined by these cell surface markers. Importantly, however, the functional profile of the individual cells within any given subset is the same across individuals in all disease states. Thus we show that the polarized response of the memory T cell compartment reflects the differential representation of distinct subsets rather than altered functionality of the cells in the compartments. These findings introduce a new potential for clinical monitoring of disease progression, particularly during therapeutic intervention such as allergic desensitization and/or vaccination protocols.

Methods

Patient samples

Fourteen patients (non-smokers, seven female) with either allergic rhinitis or asthma seen at Stanford University Allergy Clinic between February and April 1997 were selected for this study. All had at least four positive wheal and flare epicutaneous skin prick test reactions for inhalant allergens including tree, grass, weed pollens and dust mite. None of the patients received systemic corticosteroid therapy. Control subjects were selected during the same time period and had no history of atopic manifestations and completely negative skin tests. All subjects gave informed consent. In addition, peripheral blood mononuclear cells (PBMC) from 11 TT leprosy and five LL leprosy patients from the Leprosy Clinic of the All India Institute of Medical Sciences, India, were obtained for this study. Patients were diagnosed by clinical and bacteriological criteria, and clinic-immunologically classified as polar TT or LL leprosy according to the Ridley and Jopling classification (22). All TT patients had well-demarcated single paucibacillary lesions that were histopathologically confirmed; all LL patients had multiple disseminated asymmetrical lesions. Patients were initially diagnosed within 4–6 weeks before collecting PBMC and none had received any form of corticosteroid since anti-leprosy therapy (dapsone and rifampicin) was initiated at diagnosis. All subjects gave informed consent.

To evaluate the effect of the long shipping period on the samples from India, we collected blood samples from control subjects at Stanford at the same time as the collection in India; these were left on the bench top until all samples were co-processed. In addition, control samples were also collected from the same endemic area and from the same ethnic group in India, and shipped with the TT and LL samples. Both of these sets of controls were indistinguishable (once EMA^+ dead cells were excluded) in our assays from the fresh samples obtained from the Stanford control group and were therefore grouped together as a single control group.

Samples of whole blood were shipped by courier from India on two occasions. In one instance, shipment required 120 h; these five TT leprosy samples showed poor functional responses and were not included in the analysis of Fig. 5(C) (however, these samples showed normal phenotypic properties indistinguishable from the other samples and were included in the phenotyping analyses). In the other case, shipment was accomplished in 60 h and full functional analysis was performed.

A majority of the control and atopy samples and all but five TT leprosy samples were coded; sample preparation and data analysis were concluded before the disease status was revealed. At least one control sample was nearly always included whenever leprosy or atopy samples were analyzed.

Preparation of cells

Cells were isolated from heparinized blood by Ficoll-Hypaque centrifugation, washed 3 times with RPMI 1640 (Gibco/BRL) and resuspended in medium at 1×10^6/ml for further processing. For stimulations or culture, cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bioproducts, Calabasas, CA), L-glutamine (290 μg/ml), penicillin (100 U/ml) and streptomycin (70 μg/ml) in a 37°C CO_2 incubator.

Stimulation and immunofluorescence staining

CD4 is rapidly internalized after stimulation of protein kinase C [phorbol myristate acetate (PMA)]; CD62L is proteolytically

removed from the cell surface by metalloproteinases (MMP) after even mild stimulation. To overcome these problems, we pre-stain cells with fluorescently conjugated antibodies to these two molecules. Thus, when CD4 is internalized, the fluorochrome is internalized with it; $CD4^+$ T cells can be easily identified and discriminated from $CD8^+$ or $CD4^-CD8^-$ T cells. The inclusion of monensin to cause accumulation of cytokines in the Golgi also prevents degradation of the internalized fluorochromes. Identification of $CD62L^+$ cells is accomplished by the inclusion of a specific inhibitor of MMP, KB-R8301 (generous gift of K. Okumura), which, in combination with the presence of the anti-CD62L Ig, significantly inhibits the down-modulation of CD62L (23). Pre-staining with antibodies did not affect the responsiveness of the cells (24).

All antibodies were either obtained from DNAX (Palo Alto, CA) or PharMingen (San Diego, CA) and conjugated in our laboratory. PBMC (2×10^6) were pre-stained in 100 μl of culture medium at room temperature with Cy7–phycoerythrin (PE) –CD4 and Texas Red–CD62L for 15 min and then washed twice with room temperature culture medium. PBMC were then cultured in 1 ml medium in a 24-well plate. The following were added to the cultures for stimulation: PMA (1 ng/ml), ionomycin (2 μM), monensin (Calbiochem, CA; 2 μM) and KB-8301 (1 μM). After 6 h, which we determined to be optimal for simultaneous detection of IL-2, IL-4 and IFN-γ (25), cells were washed twice with PBS supplemented with 1% BSA and 0.1% sodium azide (PBS/BSA/Azide). Cells were then surface stained with ethidium monoazide bromide (EMA), CD8, CD11a and CD45RA for 15 min at room temperature, and washed in PBS twice. Cells were fixed in 2% formaldehyde for 20 min at room temperature in the dark and then washed twice with PBS/BSA/Azide. For permeabilization, cells were resuspended in 150 μl 0.5% saponin (in PBS/BSA/Azide) and incubated at room temperature for 10 min in the dark. Cells were pelleted and resuspended gently in 25 μl of 0.5% saponin buffer containing optimal amounts of fluorochrome-conjugated anti-cytokine mAb. Cells were further incubated for 30 min at room temperature in the dark; then washed twice in 0.5% saponin buffer, washed 3 times in PBS/BSA/Azide and finally resuspended in 200 μl PBS/BSA/Azide at 4°C. FACS analysis was done within 12 h; $1–3 \times 10^5$ events were collected for analysis. Eight-color FACS analysis was performed as described (4).

In most experiments, we stained with 5 μg/ml EMA (Molecular Probes, Eugene, OR) simultaneously with the antibody stains, to exclude dead cells which can non-specifically bind antibodies. After washing and before fixation, cells were exposed to fluorescent light for 10 min and then washed. EMA diffuses into dead cells and intercalates into DNA; upon exposure to fluorescent light it is photo-cross-linked and is covalently bound.

The discrimination between $CD11a^{high}$ and $CD11a^{low}$ cells is often difficult to determine in the $CD45RA^-$ population. We adopted the following procedure to set the gate position objectively. The CD11a histogram for the total lymphocyte population was examined. This histogram is always bimodal, segregating into $CD11a^{high}$ and $CD11a^{low}$ populations. The CD11a gate was set to the histogram minimum between these two peaks and applied to the $CD45RA^-$ population as described in the text.

RNA quantitation

Quantitative PCR was performed as previously described (26), with minor modifications. For CD3 stimulation, we used Leu4 in the presence of P815 cells (no co-stimulation), or P815 cells stably transfected with human CD80 or CD86 (kind gift of L. Lanier) (27) at final ratio of 5:1 T cells:P815.

Data analysis

Compensation for spectral overlap as well as all gating, statistical analysis and graphical presentation of flow cytometric data was performed using FlowJo (Tree Star, San Carlos, CA). Statistical comparison and presentation of clinical data was performed using JMP (SAS Institute, Cary, NC). Comparisons of distributions were performed by the non-parametric two-sample Wilcoxon rank test.

Results

Cytokine profiles of T cell subsets

In healthy adults, roughly half of the mature T cells in blood are naive T cells, which arise from positive and negative selection in the thymus but have not yet encountered their cognate antigenic peptide. Naive T cells are identified by the expression of CD45RA and CD62L, low-level expression of CD11a, and the lack of expression of activation antigens (28,29). When stimulated by antigen presentation, they proliferate rapidly and produce IL2; however, they do not produce effector cytokines such as IL-4, IL-5, IL-10 or IFN-γ.

The remaining peripheral T cells are a heterogeneous population of memory T cells that have undergone proliferative expansion in response to antigen, and have differentiated to phenotypically and functionally distinct subsets. Three subsets in peripheral blood of healthy subjects are identified by the expression of a combination of T cell lineage markers, CD4 and CD8, and the CD45RA and CD62L differentiation markers (Fig. 1). One of these subsets, $CD45RA^-CD62L^+$, includes the only cells that can provide effective help for B cells (2). A second subset, $CD45RA^-CD62L^-$, is equally well represented on average. Cells expressing CD45RA but not CD62L are relatively rare in healthy people (averaging 5% of CD4 T cells); however, this population can be significantly expanded in pathogenic states such as HIV infection (30).

Each of these subsets has a unique cytokine profile after stimulation with PMA and ionomycin (Fig. 1). The cytokine profiles, identified by FACS analysis after stimulation with PMA and ionomycin, are essentially identical to those seen in purified populations stimulated with other mitogens including phytohemagglutinin, anti-CD3 and anti-CD3 with co-stimulation, and assessed by quantitative PCR (Fig. 1). As indicated above, naive ($CD45RA^+CD62L^+$) T cells produce IL-2 but little IL-4 or IFN-γ. The $CD45RA^+CD62L^-$ T cells, in contrast, have a differentiated effector cytokine profile. Furthermore, although like naive cells they do not express CD45R0, they do express high levels of CD11a, and often express CD38 and CD57 (data not shown), supporting the designation as a memory/effector subset. Finally, consistent with previous findings showing that $CD45RA^-CD62L^+$ provide B cell help for antibody production and isotype switching (2), cells in this subset are the main producers of IL-4.

In these experiments, we used EMA staining to discriminate cells which died during the stimulation from live cells. Dead cells will bind some (but not all) antibody reagents non-specifically, leading to potentially artefactual results. As shown in Fig. 1(a), for example, most apparently CD4, CD8 double-positive cells are dead cells that non-specifically bind antibodies to CD4, CD8, and IL-4 (but not IFN-γ). Without the ability to exclude dead cells, the IL-4-producing cells in the CD4 compartment would be significantly over-reported. For our experiments, the average fraction of dead cells after PMA/ionomycin stimulation was 6% (CD4), 8% (CD8), 16% ($CD4^-$ $CD8^-$) and 88% ($CD4^+CD8^+$).

Identification of functionally polarized CD4 subsets

By further subdividing the two $CD45RA^-$ memory cell subsets on the basis of expression of CD11a, we noted that IFN-γ was associated with high CD11a expression in these cells, in keeping with recent results from the mouse (1). This combination of CD62L with CD11a allows the identification of four subsets of $CD4^+CD45RA^-$ cells with unique cytokine profiles (Fig. 2).

The $CD11a^{bright}CD62L^-$ subset, which we designate MT_1, is the most 'T_h1-like' in that it has the highest ratio of IFN-γ-producing to IL4-producing cells (i.e. it is the most IFN-γ-biased). Similarly, the $CD11a^{dim}CD62L^+$ (MT_2) subset is the most 'T_h2-like', in that it has the highest ratio of IL4 producers to IFN-γ producers (it is the most IL4-biased). The $CD11a^{bright}CD62L^+$ (MT_0) subset is 'T_h0-like'; finally, few cells in the $CD11a^{dim}CD62L^-$ (MT_n) subset make either IL-4 or IFN-γ. Interestingly, this functional division is also seen in the CD8 subsets (although the relative proportion of IL-4-producing subsets is lower than in the CD4 lineage; furthermore, IL-4 production by CD8 cells is highly donor dependent). Thus, the CD4 and CD8 lineages have differentiated phenotypes with similar cytokine profiles, consistent with studies showing that differentiation of T_c1 and T_c2 can be achieved using the same signals that drive T_h1 and T_h2 development (31).

While the apparent differences in IL-4 production between different CD4 subsets is small in absolute magnitude, it is highly statistically significant. The sensitivity of the FACS assay for IL-4 production is much lower than that for IFN-γ and may underestimate the proportion of IL-4-producing cells. In Table 1, we computed the fraction of each of the six CD4 subsets that produce IL-4 or IFN-γ and, for each subset, the ratio of cells producing these cytokines. Note that naive T cells and MT_1 T cells, which do not make IL-4 (Fig. 1B), show about 1–2% positive cells by FACS: this value can be viewed as the background for the FACS IL-4 assay, making the relatively small increment in IL-4-producing cells in, for example, the MT_2 subset, substantially larger by comparison. Even without taking this background into account, MT_2 cells have nearly 4 times as many IL-4-producing cells as MT_1 in all disease states we examined [average ratio of $IL\text{-}4^+$ cells in MT_2 to MT_1 is 3.7 (IQR 1.3–5.4) for individuals in all disease states; this value is different from 1.0 with $p < 10^{-4}$].

Clinical correlates of functionally polarized subsets

Since atopic disease or TT leprosy are disorders previously characterized as T_h2- or T_h1-dominated, respectively, we used the phenotypic analyses described above to examine differences in peripheral blood T cell subsets of subjects with these diseases. Because the precise antigen(s) dominating the immune responses in these individuals is unknown, it was necessary to use a generic stimulus (i.e. PMA/ionomycin) to determine the functional state of the composite immune system. As shown in Fig. 1, the analysis of cytokine profile using the flow cytometric assay (with PMA/ionomycin stimulation) is essentially the same as using more physiological stimuli such as CD3/CD28 and is most likely representative of the profile obtained for any given fine T cell subset after antigen stimulation.

We found no differences in the cytokine profiles of the phenotypically defined subsets (MT_n, MT_0, MT_1 or MT_2) in atopy or TT leprosy compared to controls (Fig. 3). However, we found that atopic subjects have a significant increase in the *numbers* of MT_2 cells ($CD62L^+CD11a^{dim}$: IL4 biased) and a significant decrease in MT_1 cells ($CD62L^-CD11a^{bright}$: IFN-γ biased) in the peripheral blood (Fig. 4). Conversely, subjects with TT leprosy have a significant decrease in MT_2 cells and a significant increase in MT_1 cells. The number of $IL4^+IFN\text{-}\gamma^+$ (double-producing) cells within all subsets and in all subjects tested was exactly predicted by the proportions of single-positive T cells (data analysis not shown).

Unlike the case for CD4 T cells, we found no statistically significant differences in the representations or cytokine profiles of the fine subsets of CD8 T cells between individuals with either TT or LL leprosy, atopy or no disease. This is not inconsistent with our study design, selecting subjects with known functional polarization in the CD4 compartment (i.e. T_h1- or T_h2-biased diseases).

Note that enumeration of these subsets can be performed using commercially available four-color flow cytometers by measuring CD4, CD11a, CD45RA and CD62L. The eight-color analyses we performed was required to demonstrate the correlation between phenotype (i.e. surface markers) and function (i.e. intracellular cytokine production).

The statistical summary in Figs 5 and 6 further demonstrates that changes in the cytokine profiles of individual subsets, or in the representation of the MT_n and MT_0 subsets, do not occur in atopic diseases or polar leprosy—either tuberculoid or lepromatous (Fig. 5C). In contrast, there is an inverse effect on the numbers of MT_1 and MT_2 cells in these polarized diseases (Fig. 5A), i.e. MT_1 is preferentially increased in TT leprosy, a T_h1 disease, whereas MT_2 is preferentially increased in atopic disorders or LL, both considered as T_h2 diseases. Figure 6 shows the significant difference in the CD4 memory representations of these individuals. Whether this remodeling occurred prior to disease (and possibly contributed to the clinical course of disease) or as a result of disease remains to be determined. In any case, the relative representations of the functionally distinct immune subsets can be taken to be an index of the degree of polarization of the peripheral immune system (Fig. 5B).

Discussion

Studies presented here show that the dominance of the T_h1 and T_h2 response pattern by peripheral blood T cells from patients with atopic disease or polar leprosy is manifested as a change in T cell subset representation rather than in the

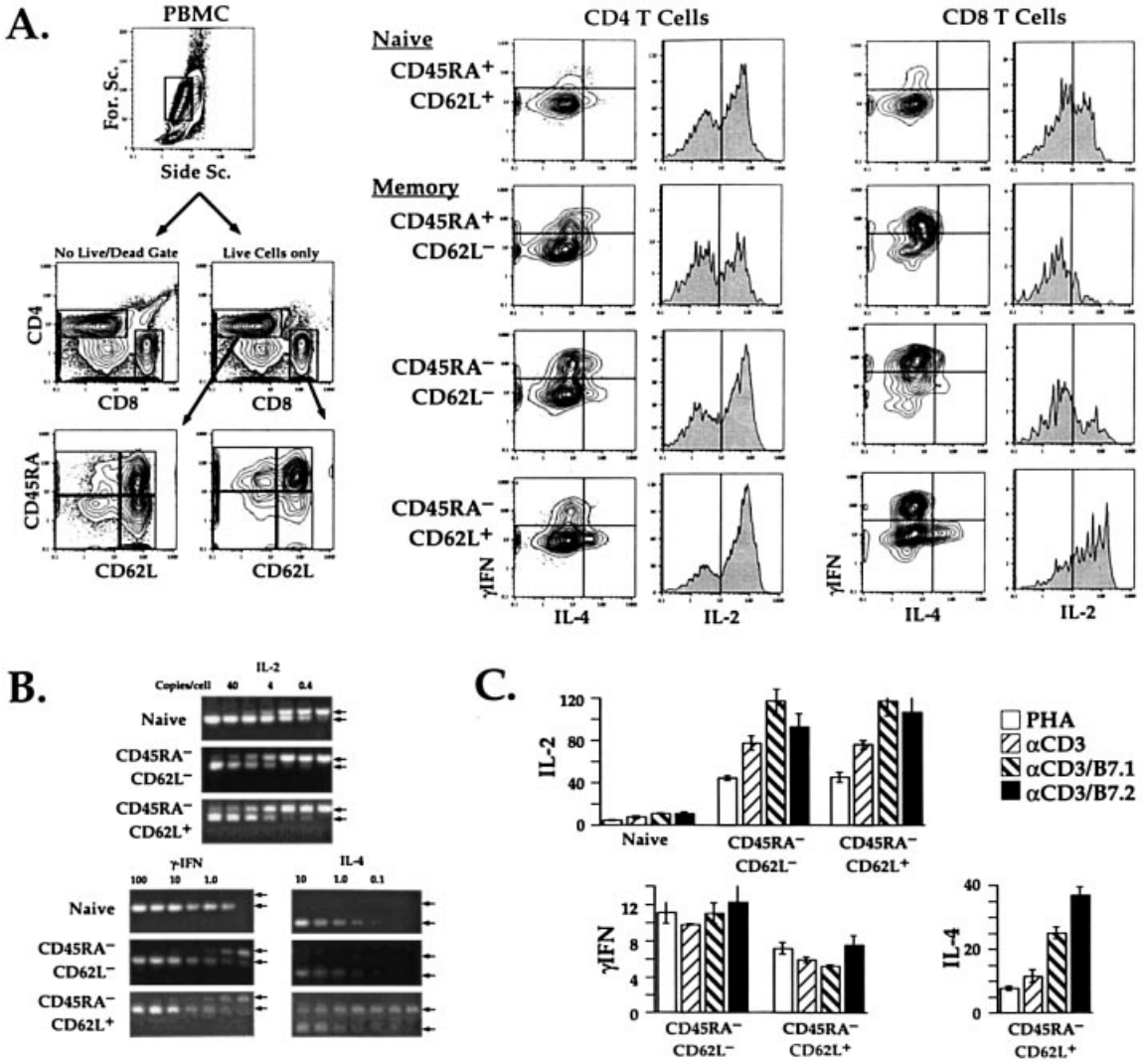

Fig. 1. T cell subsets have unique cytokine profiles. (A) Flow cytometric determination of cytokine profiles. Eight-color, 10-parameter FACS(4) was used to determine the cytokine profile of fine T cell subsets stimulated with PMA/ionomycin. The left panels show progressive gating of the data: by scatter for lymphocytes, by CD4 or CD8 for lineage and then by CD45RA and CD62L to distinguish naive ($CD45RA^+CD62L^+$) cells from three types of memory cells. EMA fluorescence could be used to exclude dead cells, which non-specifically bind some antibodies including anti-CD4 and anti-IL4 (in general, <5% of CD4 and up to 20% of CD8 became EMA^+ after stimulation with PMA and ionomycin). On the right, the distribution of expression for IL-4 versus IFN-γ and for IL-2 are shown for each of the differentiation stages of CD4 or CD8 T cells. Note that the apparent division of the 'negative' populations into a main population and one on the axis is a mathematical and visual artefact of the data compensation process. All events in the first decade of fluorescence are essentially equivalent regardless of their position within that decade. (B) Quantitative PCR determination of cytokine profiles. CD4 subsets were sorted to >98% purity by FACS, based on the expression of CD4, CD45RA and CD62L. In most healthy individuals, $CD45RA^+CD62L^-$ CD4 cells are too rare to sort enough cells for quantitative PCR analysis. Sorted naive and memory cells were stimulated either with PHA, anti-CD3 or anti-CD3 with co-stimulation provided by B7-1 or B7-2. Shown are example amplifications of mRNA with dilutions of specific cytokine competitor RNA for the three cell types stimulated with anti-CD3+B7-1. No IL-4 production could be detected in the naive or $CD45RA^-CD62L^-$ cells. Arrows point at the expected locations of the cell-derived amplicon (wild-type length, upper band) and artificially constructed competitor-derived amplicon (lower band) for each cytokine message. (C) Densitometric scanning of the gels such as shown in (B) was used to quantitate the absolute number of mRNA molecules for each cytokine; results are presented as cytokine mRNA molecules per cell. Error bars represent ± SD for results from four different experiments on T cells sorted from four different individuals. Unstimulated T cells were also analyzed, but showed no detectable cytokine message. Naive T cells have ~10-fold less message per cell than do memory T cells; however, this amount is easily detectable and is still several orders of magnitude above the signal for unstimulated T cell.

proportion of cytokine-producing cells within any given subset (Table 1 and Fig. 5). These findings extend the initial observation made by Field *et al.* (32), who found that the $CD45RA^-CD62L^+$ subset (MT_2+MT_0) was expanded in a single atopic individual. In essence, we show that simultaneous measurement of CD4, CD11a, CD45RA and CD62L allows enumeration of the MT_1 (IFN-γ-biased) and MT_2 (IL4-biased) subsets, the ratio of which reflects the balance of cytokine production characteristic of the chronic disease state.

It is important to note that the most important conclusion of

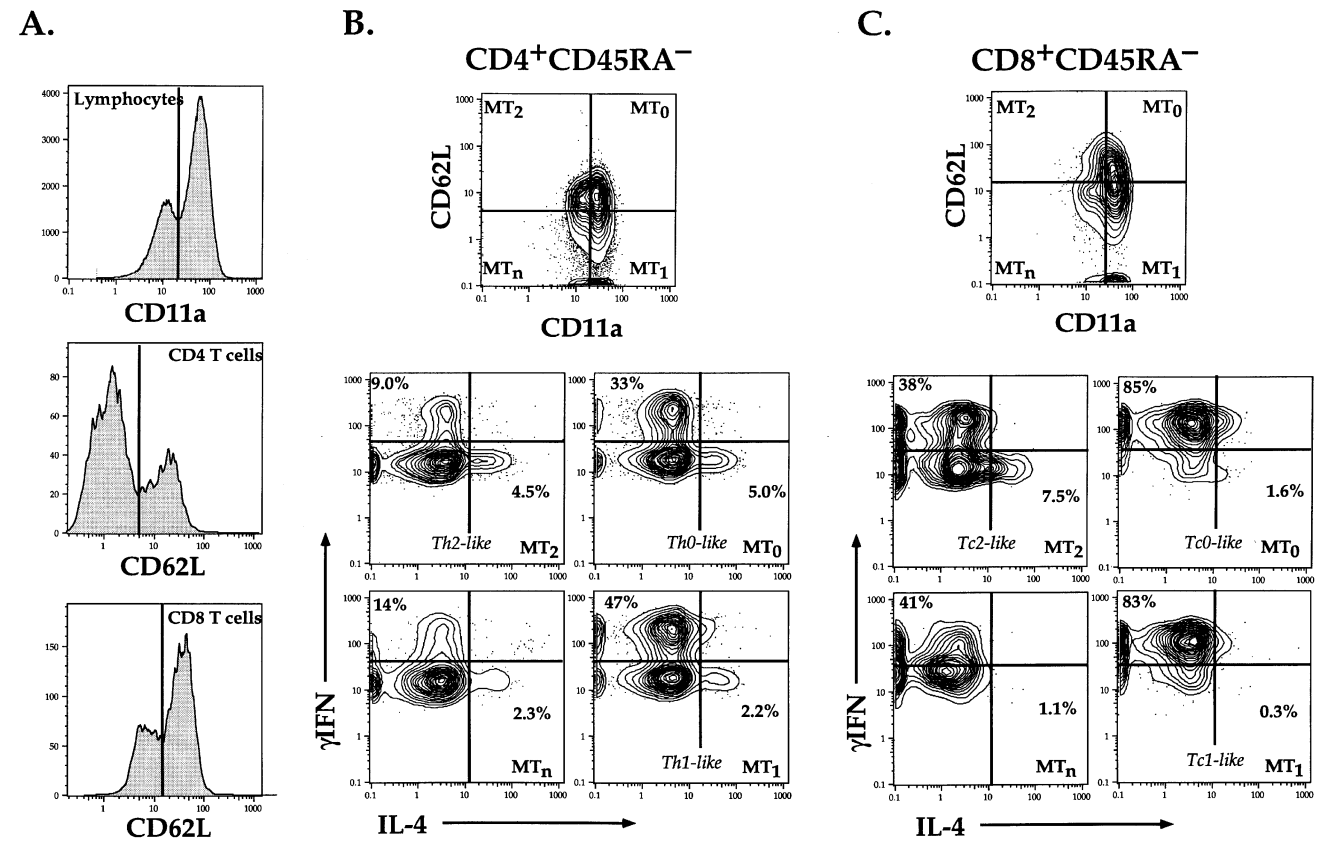

Fig. 2. Identification of functionally polarized memory T cell subsets. Cells were stained similarly to those in Fig. 1, with the addition of antibody to CD11a. (A) The gate used for CD11a is determined by the midpoint between the positive and negative peaks found for lymphocytes; that for CD62L by the midpoint for CD4 or CD8 T cells (graphs from different individuals in different experiments are shown). The precise location of the gate depends primarily on which fluorophore was conjugated to the antibody in any given experiment, since the background levels for some fluorophores (such as Cy7–allophycocyanin and Cy7–PE) are much higher than others. (B and C) The top two panels show the profile of CD62L versus CD11a for CD4 (B) or CD8 (C) cells that are CD45RA$^-$. Based on CD11a and CD62L staining as shown in (A), we divide CD45RA$^-$ cells into four subsets: CD11a^{dim}CD62L$^-$ (MT_n), CD11a^{bright} CD62L$^+$ (MT_0), CD11a^{bright}CD62L$^-$ (MT_1) and CD11a^{dim}CD62L$^+$ (MT_2). The IL-4 versus IFN-γ profile for each of these four cell types is shown. The numbers refer to the percent of cells within the subset expressing IL-4 or IFN-γ. While there is apparently a small difference in (for example) the IL-4 production in different subsets, this difference is highly significant statistically (see Table 1), and, given the functional analysis in Fig. 1, biologically.

our report, i.e. that polarized disease states have characteristic alterations in subsets of memory CD4 T cells, does not depend on the functional measurements (intracellular cytokine staining) that we performed. Four-color immunophenotyping is sufficient to quantitate the degree of functional polarization in the immune system.

Previously identification of T_h1 and T_h2 functional dominance in a number of diseases including leprosy, leishmaniasis, schistosomiasis and atopic manifestations (5,7,33–36) could only be achieved by selective expansion of antigen-specific effector clones or by visualization of effector T cells at the site of inflammation (19). In contrast, we show that this dominance can be rapidly identified by flow cytometry on fresh blood samples. This can be performed by simultaneous measurement of CD4, CD45RA, CD62L and CD11a—on newer, four-color flow cytometers; it does not require a stimulation assay nor an intracellular staining assay. Thus, the methodology we describe could facilitate clinical monitoring of changes in chronic disease that may lead to pathology or immunosuppression as a result of imbalance of T_h1 and T_h2 responses (5). Furthermore, this approach may be especially valuable for monitoring changes in cytokine profiles during immunotherapy in allergic disease or drug therapy of other diseases such as leprosy, leishamaniasis or even in vaccination protocols.

A number of studies have attempted to define phenotypic markers for T_h1 or T_h2 cells. Preferential IL-12Rβ2 mRNA expression in T_h1 cells was observed in mice and in humans (37,38). Selective expression of receptors for P-selectin and E-selectin was noted for T_h2 and T_h1 cells respectively, and hypothesized to mediate the recruitment of these cells to the site of inflammation (39). On the other hand, CD62L (L-selectin), necessary for homing to lymphoid tissues, is preferentially expressed on IL-4-producing T cells (Fig. 2) (3,32). Our findings extend these observations by showing that enumeration of clinically relevant polarized T cell subsets requires the measurement of CD11a, CD62L as well as CD45RA on CD4 T cells.

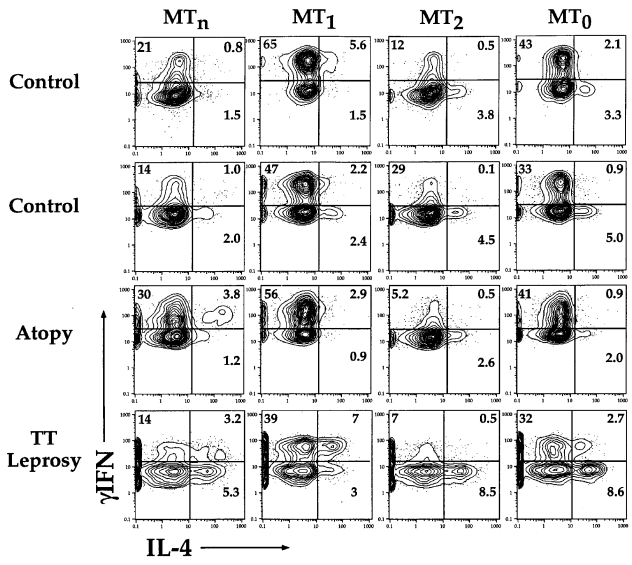

Fig. 3. Cytokine profiles of T cell subsets are unchanged in pathogenic states. The cytokine profiles for CD45RA$^-$ T cell subsets are shown for two healthy controls, one atopic and one TT leprosy patient. While there is individual variation in the fraction of each subset that will produce IL-4 or IFN-γ, the overall pattern remains constant across all individuals. There is no statistically significant difference in the cytokine profiles for individuals such as these (Fig. 5C). The position of the quadrant distinguishing cytokine-positive from -negative cells was chosen based on the profiles found for the parallel unstimulated cultures.

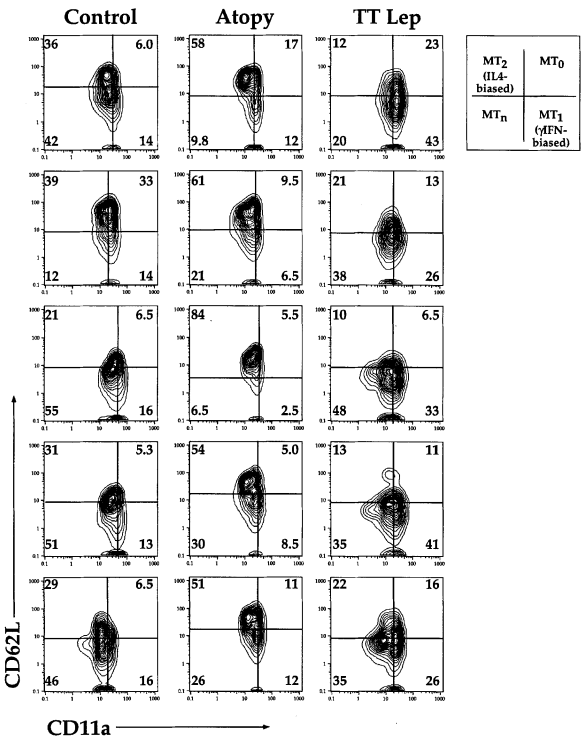

Fig. 4. Representation of CD45RA$^-$ T cells in leprosy or atopy. Five representative examples of T cell profiles of healthy, atopic or TT leprosy individuals are shown. Samples were gated for CD4$^+$CD45RA$^-$ lymphocytes as shown in Fig. 1(A). The bottom-most panel for the control group is a control sample obtained from India, representing the same ethnic group and endemic area as the leprosy samples. Different combinations of fluorochrome–antibody conjugations were used in some of the experiments shown here; hence, the absolute fluorescence between different individuals is not comparable. The relative position of the CD62L gate was based on negatively versus positively stained cells; the relative position of the CD11a was chosen to split the two major peaks that are always easily discerned in the total lymphocyte population.

Table 1. IL-4 and IFN-γ expression in CD4 T cell subsets

CD4 cell type	Percent of subset expressing		IFN-γ/IL-4
	IFN-γ	IL-4	
MT_1	54 (39–71)	2.0 (0.9–2.8)	29 (14–53)
MT_2	11 (7.3–16)	4.4 (2.6–6.8)	2.8 (1.6–5.1)
Naive	5.0 (2.6–9.4)	12.0 (0.5–1.8)	5.5 (2.2–13)
MT_n	19 (12–28)	2.7 (1.9–4.6)	11.5 (4.8–21)
MT_0	39 (30–53)	4.0 (2.2–6.8)	9.3 (4.7–20)
CD45RA$^+$CD62L$^-$	34 (22–53)	4.2 (1.9–6.4)	10.3 (5.3–18)

Values are the median (interquartile range) for 35 individuals from all disease states. There is no significant difference, between disease states, when comparing cytokine profiles for any given subset (see Fig. 5C). The ratio of IFN-γ to IL-4 production for MT_1 cells is different from that for MT_2 cells with a probability $P < 10^{-11}$.

The differential expression of the selectins or selectin receptors may be related to the selective recruitment of cytokine-polarized effector T cells at the pathologic sites. However, many factors may influence the selective differentiation and expansion of T_h1 and T_h2 cells, including the antigen-presenting cell, hormones, adjuvant, dose of antigen, co-stimulatory molecules, affinity of the immunogenic peptide for MHC, regulatory cytokines and the genetic background of the individual. Among these, cytokines have been shown most consistently to be an important driving factor (40–43). The altered levels of defined subsets such as those we have demonstrated here may also represent antigen-driven or cytokine-driven expansion. Alternatively, or in addition, such changes may be influenced by the genetic background of the individual as well as environmental factors. In any case, the representation of these subsets is highly stable over a period of several years in any given individual, implying a strong homeostatic control mechanism.

It is important to note that the measurements we report here are based on the cells found in the peripheral blood. As such, it is expected that these measurements may not accurately reflect the T cells in either secondary lymphoid tissue or, perhaps more importantly, at the site of the lesion itself. Preliminary analysis of cells isolated from biopsies of leprosy patients confirm that the local effector cells show a similar phenotypic bias as we found for the peripheral blood: in lesions from T_h1 disease (TT leprosy), T cells expressed high levels of CD11a and CCR5; in lesions from T_h2 disease (LL leprosy), T cells expressed low levels of CD11a and high levels of CCR3 (D. K. Mitra and N. K. Mehra, unpublished). Thus, the imbalance that we measure in the peripheral blood may herald a much larger imbalance in the antigen-specific-immune response carried out at the lesion itself.

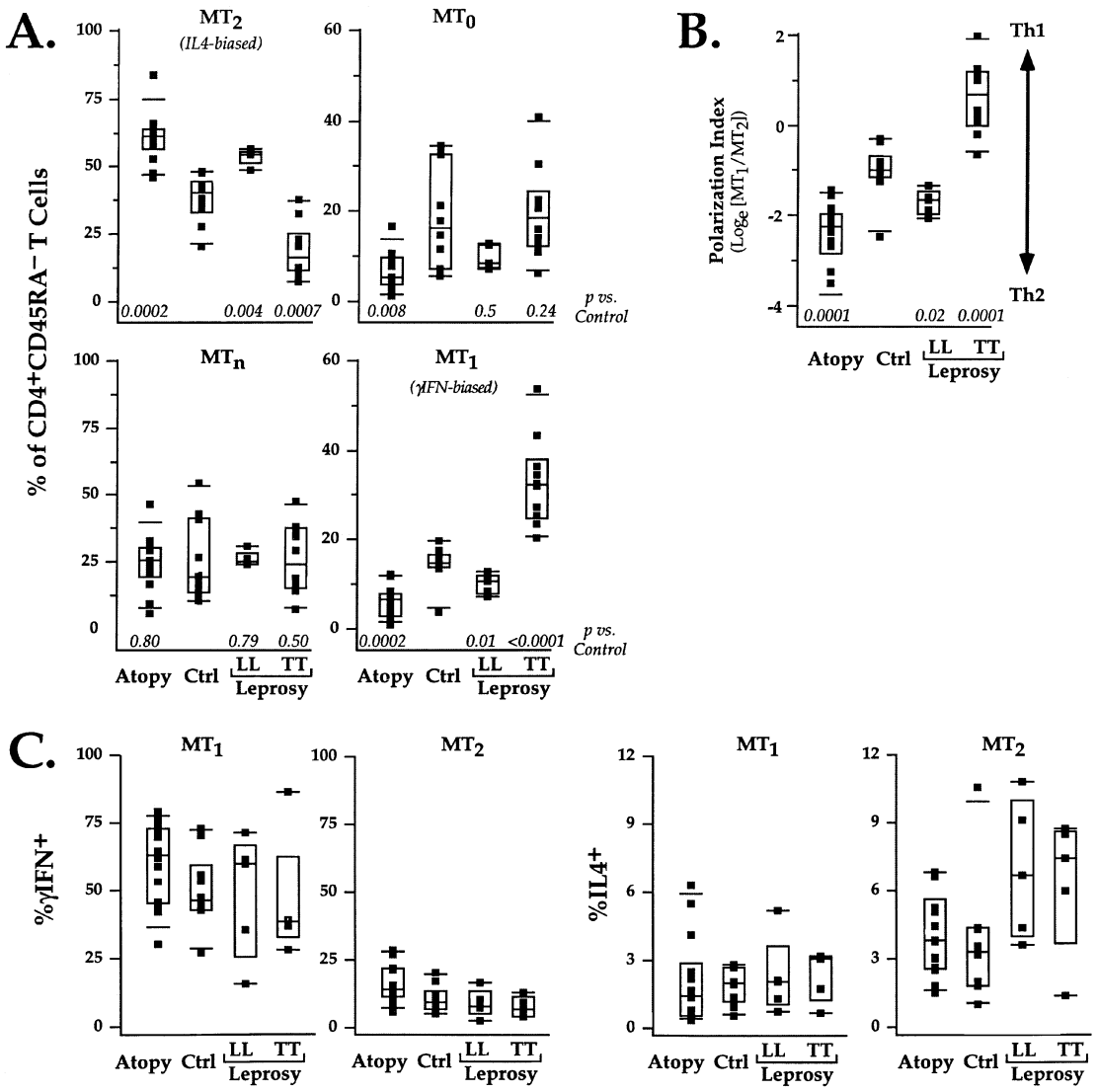

Fig. 5. Representation of functionally polarized T cell subsets in individuals with atopy or polar leprosy. (A) The fraction of $CD4^+CD45RA^-$ T cells that fall into each of the four subtypes shown in Fig. 2 was measured for 14 atopic patients, 12 healthy non-atopic controls, 11 TT and five LL leprosy patients. Wilcoxon rank comparisons of each patient group to control ('Ctrl') were performed; numbers in italic indicate the *p* value. (B) For each patient, the logarithm of the ratio of MT_1 to MT_2 cells was computed ['Polarization index' (PI)]; higher values indicated a higher proportion of MT_1 cells (IFN-γ biased). (C) Despite a change in the representation of the MT_1 and MT_2 cells between patient groups, the functional profiles of these subsets remain unaltered. The proportion of each cell type expressing IL-4 or IFN-γ is shown; none of the distributions are significantly different from the control group. (A–C) To assess reproducibility, four subjects were analyzed 2–3 times over a period of 6 months. The coefficient of variation for any of the measurements shown here was between 6 and 30%.

A subset imbalance and the resulting cytokine imbalance may dictate resistance or susceptibility to pathogenic organism, or susceptibility to normally non-pathogenic stimuli such as allergens or self-antigens, or, in the case of leprosy, affect the clinical course of disease (i.e. TT versus LL). Recent data supports the hypothesis that the pre-existing balance of these cells can affect the course of disease. For example, in a mouse model, animals predisposed to IL-4 production developed less delayed-type hypersensitivity and were protected from T_h1-mediated autoimmune inflammatory diseases (44). The hypothesis is also supported by recent clinical investigations in humans (45,46) showing inverse associations between tuberculin reactivity (T_h1) and atopic disorders, and between multiple sclerosis (T_h1 like) and atopy.

These findings raise the question as to whether, upon exposure to *M. leprae*, individuals with pre-existing high levels of MT_1 (IFN-γ-biased) cells are more likely to develop a T_h1 response and thus tuberculoid leprosy, whereas those with high levels of MT_2 (IL-4-biased) are more likely to develop a T_h2 response leading to the LL form. Our preliminary studies on a small number of untreated individuals with LL leprosy confirm that they have elevated numbers of MT_2 cells and decreased numbers of MT_1 cells—similar to that found in individuals with atopic diseases. However, we cannot rule out

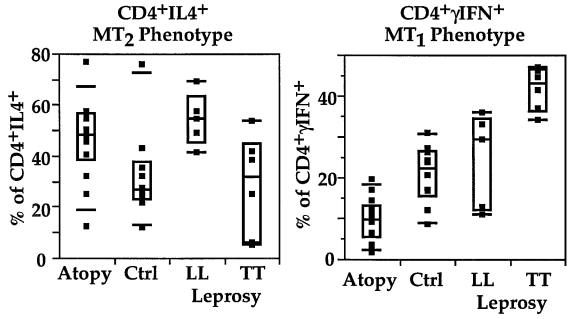

Fig. 6. The increased IL-4 or IFN-γ expression in CD4 T cells in individuals with atopy or polar leprosy is explained by a change in representation of MT_1 or MT_2 cells. For each individual, the fraction of IL-4-expressing CD4 cells belonging to the MT_2 subset (left) or the fraction of IFN-γ expressing CD4 cells belonging to the MT_1 subset (right) is shown. These data show that the change in cytokine expression that accompanies chronic diseases is due to a change in the representation of the phenotypically defined memory subsets. Since the cytokine profile of any given subset has not changed (Table 1 and Fig. 5C), the polarization of the peripheral immune system is solely explained by homeostatic or differentiation mechanisms leading to altered cell counts, rather than a functional change at the cell level.

that the imbalance in subset representation is a consequence of other factors that determine the T_h1/T_h2 polarization after exposure to the causative agents. In any event, studies presented here provide a method for testing this hypothesis, and for determining the relationship between subset representation and the progress and nature of chronic diseases characterized by polarized immune responses.

Finally, we find no evidence that there is any alteration in the functional capacity of any given T cell in chronic diseases—rather, the functionality of a T cell from such an individual can be predicted by examining the functionality of a T cell from the same subset in a healthy individual. Rather, our data show that the functional polarization of the immune system is most likely due to homeostatic and/or differentiation mechanisms that regulate the number of these functionally distinct T cell subsets.

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Abbreviations

EMA	ethidium monoazide bromide
LL	lepromatous leprosy
MMP	metalloproteinases
PBMC	peripheral blood mononuclear cell
PE	phycoerythrin
PHA	phytohemagglutinin
PMA	phorbol myristate acetate
TT	tuberculoid leprosy

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