Immunology 1993 **78** 237–243

The relation between T-cell expression of LFA-1 and immunological memory

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Accepted for publication 8 September 1992

SUMMARY

Antibodies against isotypes of the leucocyte common antigen (LCA, CD45) can be used to identify largely reciprocal subsets of human peripheral T cells, characterized by differential ability to respond to recall antigen *in vitro*. The transition from naive, unprimed T cells to memory cells capable of responding to recall stimulating has been associated with a switch in surface expression of CD45 from the CD45RA isotype to CD45RO. It has been proposed that this transition is accompanied by the co-ordinated up-regulation of a number of cell-surface molecules involved in cellular adhesion and/or activation, including the leucocyte function-associated antigens (LFA). In the present study we have examined the expression of LFA-1 on subsets of human peripheral T cells, and related it to the expression of markers of cellular activation and CD45 isotypes, and thus to immunological memory. Our results suggest that the intensity of LFA-1 expression on the surface membrane of human peripheral T cells is not tightly associated with maturation status as judged by LCA isotype expression, but rather reflects the degree of cellular activation. This conclusion is supported by data of T-cell function *in vitro*, showing similar antigen- and mitogen-induced proliferative responses in T-cell subsets characterized by low as well as high surface expression of LFA-1.

INTRODUCTION

Both $CD4^+$ and $CD8^+$ T cells can be subdivided into largely reciprocal subpopulations on the basis of their expression of isotypes of the leucocyte common antigen (LCA, CD45), and the differential expression of LCA isotypes has been demonstrated to be associated with memory formation.[1] T cells which express the CD45RA isotype are thus considered immunologically naive due to their inability to respond to recall antigens. Conversely, T cells which have been immunologically primed (memory cells), are characterized by expression of the isotype CD45RO.

In addition to the shift in LCA isotype expression, the transition from naive to memory cells is accompanied by the up-regulation of a number of cell-surface antigens known as the leucocyte function-associated antigens, which are involved in cellular adhesion.[1] Stimulation of T cells *in vitro* is thus accompanied by an increase in the expression of LFA-1 (CD11a/CD18), LFA-2 (CD2) and LFA-3 (CD58),[2] in addition to acquisition of the $CD45RA^-CD45RO^{hi}$ memory cell phenotype.[3] Furthermore, cord blood T cells are predominantly of the $CD45RA^{hi}CD45RO^-$ naive phenotype, and express only low levels of the LFA molecules.[2] Together, these data imply that $CD45RA^-CD45RO^+$ memory T cells are characterized by high expression of the LFA-1 molecule.[2] LFA-1 is universally expressed by human T cells, and the expression on unstimulated cells is bimodal, allowing the partition of these cells into an $LFA\text{-}1^{lo}$ and an $LFA\text{-}1^{hi}$ subset. In most donors, the majority of circulating T cells are $LFA\text{-}1^{lo}$. However, some healthy individuals appear to have a large proportion of $LFA\text{-}1^{hi}$ T cells without gross differences in other T-cell surface molecules (L. Hviid *et al.*, unpublished data), whereas individuals suffering from leucocyte adhesion deficiency (LAD) have T cells essentially devoid of LFA-1 due to a genetic defect.[4]

In the present study we have examined the relationship between T-cell surface expression of LFA-1, LCA isotypes, and markers of cellular activation in healthy donors with normal and high proportions of $LFA\text{-}1^{hi}$ T cells, as well as in one donor suffering from LAD.

Abbreviations: CDn, cluster determinant n; FBS, foetal bovine serum; FCM, flow cytometry; FITC, fluorescein isothiocyanate; FLn, fluorescence n; FSC, forward scatter signal; IL-2, interleukin-2; LAD, leucocyte adhesion deficiency; LFA, leucocyte function-associated antigen; mAb, monoclonal antibody; PBS, phosphate-buffered saline, PE, phycoerythrin; PerCP, peridinin chlorophyll protein; SSC, side scatter signal.

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MATERIALS AND METHODS

Monoclonal antibodies

CD2: MT910 (DakoPatts, Glostrup, Denmark); CD3: SK7 (Leu-4; Becton-Dickinson, Mountain View, CA); CD4: SK3 (Leu-3a; Becton-Dickinson); CD8: SK1 (Leu-2a; Becton-Dick-

inson); CD11a: MHM24 (DakoPatts); CD14: TÜK4 (DakoPatts); CD16: GO22 (Leu-11b; Becton-Dickinson); CD18: MHM23 (DakoPatts); CD19: HD37 (DakoPatts); CD25: 2A3 (Becton-Dickinson); CD45RA: ALB11 (IOL2; ImmunoTech, Marseilles, France); CD45RO: UCHL1 (DakoPatts); CD54: 84H10 (ICAM; ImmunoTech); CD58: AICD58 (LFA-3; ImmunoTech). Most antibodies were used directly conjugated to either fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridinin chlorophyll protein (PerCP). Unconjugated antibodies were fluorochrome-labelled by FITC- or PE-conjugated $F(ab')_2$ fragments (DakoPatts). In each step, cells were labelled for 30 min in phosphate-buffered saline (PBS) + 3% foetal bovine serum (FBS), followed by washes in the same medium.

Donors and cells

Peripheral blood mononuclear cells (PBMC) from eight Danish healthy adults, six Sudanese healthy adults and one adult LAD patient were isolated by density centrifugation on Lymphoprep (Nyegaard, Oslo, Norway). Three of the PBMC samples from Danish healthy adults used for 1- and 2-colour flow cytometry (FCM) (see below) were used fresh, the remainder of the analyses were done using PBMC samples previously frozen by controlled-gradient freezing and stored in liquid N_2 until use.

Depletion of non-T cells by biomagnetic beads

PBMC were incubated either with a cocktail of CD14, CD16, and CD19 antibodies or with CD45RO antibody for 30 min, followed by a wash in the medium used for proliferation assays (see below), and incubation with anti-mouse IgG-coated biomagnetic beads (Dynal, Oslo, Norway) for 30 min. Antibody-coated monocytes ($CD14^+$), natural killer (NK) cells ($CD16^+$), B cells ($CD19^+$) or memory cells ($CD45RO^+$) were then removed by a powerful magnet (Dynal), and the uncoated $CD14^-CD16^-CD19^-$ or $CD45RO^-$ cells were washed once and processed for analysis.

Following depletion, the purity of depleted cells in six Danish and two Sudanese donors were [mean ± SD (95% confidence interval)] $CD2^+$: 88 ± 6% (83–93%) and $CD3^+$: 85 ± 6% (80–89%). For $CD4^+$ cells, the percentages were 58 ± 5% (53–64%) in Danish donors and 40 ± 1% in the Sudanese donors, and regarding $CD8^+$ cells the corresponding values were 25 ± 3% (22–29%) in the Danish and 46 ± 4% in the Sudanese donors. CD45RO-depleted cells contained 5 ± 1% (n = 2).

Flow cytometer equipment and phenotype analyses

Cell-sorting experiments and 1- and 2-colour flow cytometry (FCM) were done on a FACStar fluorescence-activated cell sorter (Becton-Dickinson), equipped with Consort 30 software. For 3-colour FCM, analyses were done on a FACScan instrument (Becton-Dickinson), equipped with Lysys II software. Data from a minimum of 10,000 cells, using a FSC live-gate to exclude cell debris, were collected for phenotype analysis.

Cell sorting of T cells by LFA-1 phenotype

PBMC samples for cell-sorting experiments were simultaneously labelled by FITC-CD11a, PE-CD14, PE-CD16, and PE-CD19. Samples were FSC-gated to exclude cell debris, FL2-gated to exclude $CD14^+$ monocytes, $CD16^+$ NK cells and $CD19^+$ B cells, and subsequently sorted on the basis of the FL1 signal into either LFA-1^{lo}, LFA-1^{hi} or LFA-1^{all} subsets. Following sorting, >95% of the sorted cells had the correct LFA-1 phenotype.

Lymphocyte proliferation assays

All proliferation assays were done in triplicate in round-bottomed 96-well plates (Nunc, Roskilde, Denmark), using RPMI-1640, supplemented with 58·4 μg/ml L-glutamine, 20 IU/ml penicillin and 20 μg/ml streptomycin (all Gibco, Paisley, U.K.) as culture medium. Cultures were either unstimulated or stimulated by 47 μg/ml phytohaemagglutinin (PHA; Difco, Detroit, MI), 12 μg/ml purified protein derivative of tuberculin (PPD; State Serum Institute, Copenhagen, Denmark), or 10 μg/ml tetanus toxoid (TT; State Serum Institute), and contained 5×10^4 sorted cells, supplemented with 5×10^4 irradiated (2400 rads) autologous PBMC as feeder cells in 170 μl medium. Cells were cultured at 37° in a humidified atmosphere containing 5% CO_2 for 3 days (PHA-stimulated cultures) or 7 days (antigen-stimulated cultures). Cultures were pulsed by 20 μl/well [^{3}H]thymidine for the last 24 hr of incubation, and subsequently harvested onto glassfibre filters. Incorporation of radiolabel was determined by liquid scintillation spectrometry.

Statistical analyses and data presentation

Differences within donor groups were tested by *t*-tests of the differences (paired data). Results of FCM analyses are expressed as means ± SD, and the 95% confidence intervals are also given for n > 5. Approximation of data to the normal distribution was verified by probability plots. Minitab 8 software (CLECOM Ltd, Birmingham, U.K.) was used for all statistical analyses. Results of proliferation experiments are given as median incorporation of triplicate wells.

RESULTS

Expression of CD11a on T-cell subpopulations

The surface expression of LFA-1 on various T-cell subsets is summarized in Table 1. T cells from all healthy donors expressed the CD11a antigen, which constitutes the α-chain of the LFA-1 molecule; $CD4^+$ cells generally containing relatively less LFA-1^{hi} cells than $CD8^+$ cells.

The Sudanese donors had considerably less cells of the LFA-1^{lo} phenotype (and hence more of the LFA-1^{hi} phenotype) than Danish donors, partially due to the lower CD4:CD8 ratio in the Sudanese donors. However, the observed differences within $CD4^+$ cells and $CD8^+$ cells can not be explained this way. T cells from the LAD donor had no detectable LFA-1 expression (data not shown).

Expression of activation markers on LFA-1^{lo} and LFA-1^{hi} cells

To characterize the LFA-1^{lo} and LFA-1^{hi} subsets further, cells from six Danish donors were examined for expression of various markers of cellular activation by 2-colour FCM. Cells were depleted of $CD14^+$, $CD16^+$ and $CD19^+$ cells prior to FCM analysis. The expression of CD2, CD3, CD4 and CD8 were similar in LFA-1^{lo} and LFA-1^{hi} cells, when compensating for the slightly larger size (FSC signal) of LFA-1^{hi} cells compared to LFA-1^{lo} cells (data not shown). As expected, the expression of CD18, constituting the β-chain of the LFA-1 molecule, very closely parallelled CD11a expression in both LFA-1 subsets

Table 1. Expression of LFA-1 (CD11a) (given as the percentages having the LFA-1^{hi} phenotype) in various subsets of T cells

	Subset			
Donor group	CD2$^+$	CD3$^+$	CD4$^+$	CD8$^+$
Danish, healthy (n = 6)	21* ± 6 (14–28)	15 ± 6 (9–22)	14 ± 7 (6–21)	31 ± 8 (23–39)
Sudanese, healthy†	52 ± 11	45 ± 12 (33–57)	32 ± 11	65 ± 4

* Mean ± SD (95% confidence interval).
† n = 6 (CD3$^+$ data) or n = 2 (rest).

Table 2. Expression (percentage positive) of the activation markers IL-2 receptor (CD25), ICAM-1 (CD54) and LFA-3 (CD58) on non-T-cell depleted PBMC from six healthy Danish donors

Cell subset	IL-2 receptor (CD25)	ICAM-1 (CD54)	LFA-3 (CD58)
LFA-1^{lo} cells	5* ± 3 (2–9)	3 ± 2 (0–5)	19 ± 9 (10–28)
LFA-1^{hi} cells	39† ± 17 (18–60)	40† ± 15 (24–56)	92† ± 7 (83–100)

* Mean ± SD (95% confidence interval).
† Difference between corresponding LFA-1^{lo} and LFA-1^{hi} cell subsets significant at 0·01 level (CD25), 0·002 level (CD54) or 0·0001 level (CD58).

(data not shown). A small proportion of the cells were expressing detectable levels of IL-2 receptor (CD25), ICAM-1 (CD54) or LFA-3 (CD58). Compared to the LFA-1^{lo} cells, LFA-1^{hi} cells were markedly enriched for all these markers, suggesting that LFA-1^{hi} cells enclose *in vivo* activated cells. The differences in expression of activation markers between LFA-1^{lo} and LFA-1^{hi} cells were highly significant (Table 2).

The above results clearly suggested a higher level of activation in LFA-1^{hi} T cells compared to LFA-1^{lo} cells. However, as the cells analysed were prepared by depletion of non-T cells, remaining CD2$^-$ and/or CD3$^-$ cells might compromise the findings. To exclude this possibility, and to extend the analysis to CD4$^+$ and CD8$^+$ cells, T cells from two further Danish donors were examined in a similar way by 3-colour FCM. In these experiments the cells were not depleted by magnetic beads, but were labelled by PerCP-conjugated antibody to either CD3, CD4 or CD8, in addition to the labelling used in the 2-colour FCM experiments. Cells were gated by FSC as above, and furthermore FL3-gated to include only CD3$^+$, CD4$^+$ or CD8$^+$ cells. The cell subsets were thus directly identified. The results were essentially identical to those described above, showing clear correlation between expression of LFA-1 and the activation markers (data not shown).

The relation between T-cell expression of LCA isotypes and LFA-1

T-cell expression of CD45RA and CD45RO is very variable, ranging from entirely negative to highly positive. The expression of these isotypes is negatively correlated, and expression ranges from CD45RA^{hi}CD45RO$^-$, over CD45RA^{lo}CD45RO^{lo}, to CD45RA$^-$CD45RO^{hi} (Fig. 1). The T-cell expression of LCA isotypes in the LAD patient was similar to that in healthy donors, despite the absence of LFA-1 on cells from this donor (data not shown).

T-cell enriched PBMC from six Danish and two Sudanese donors were analysed by 2-colour FCM for expression of LFA-1 in relation to the expression of LCA isotypes. PBMC from these donors were divided into either CD45RA$^-$, CD45RA^{lo}, CD45RA^{hi}, CD45RO$^-$, CD45RO^{lo} or CD45RO^{hi} subsets, with subsequent analysis of LFA-1 expression in each subset. In CD45RA^{hi}, CD45RA^{lo}, CD45RO$^-$ and CD45RO^{lo} cells, LFA-1 expression was clearly bimodal. In contrast, no clear LFA-1 bimodality was evident in CD45RA$^-$ and CD45RO^{hi} cells, and

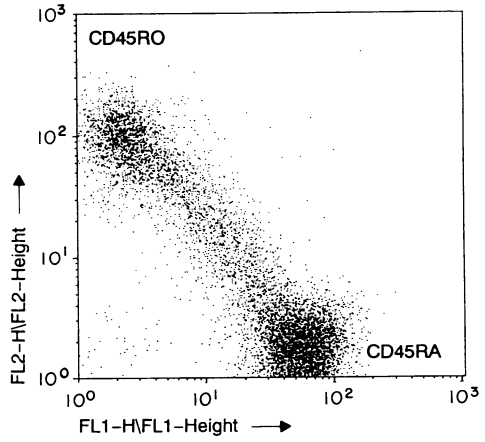

Figure 1. T-cell surface expression of the LCA isotypes CD45RA and CD45RO.

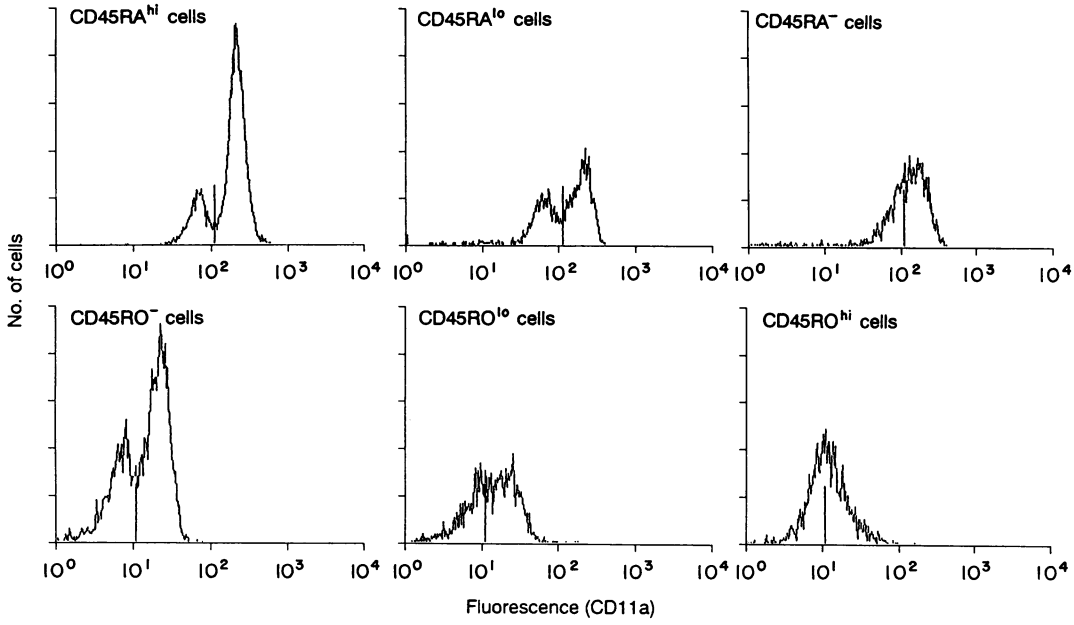

Figure 2. Expression of LFA-1 (CD11a) in $CD45RA^{hi}$, $CD45RA^{lo}$ and $CD45RA^{-}$ cells (upper panels), and in $CD45RO^{-}$, $CD45RO^{lo}$ and $CD45RO^{hi}$ cells (lower panels) enriched for T cells by depletion of non-T cells by biomagnetic beads. Results from a healthy Sudanese donor are shown.

the level of LFA-1 expression in these latter subsets was between that of $LFA\text{-}1^{lo}$ and $LFA\text{-}1^{hi}$ cells from the former four subsets (Fig. 2).

In additional 3-colour FCM analyses of two Danish donors, the above findings were confirmed, when examining either directly identified $CD2^{+}$ (data not shown) or $CD3^{+}$ cells (Fig. 3, left). In addition, we found most $CD4^{+}$ cells to have either low or intermediate LFA-1 expression, the former being predominantly $CD45RA^{hi}CD45RO^{-}$, the latter $CD45RA^{-}CD45RO^{hi}$ (Fig. 3, centre). $CD8^{+}$ cells with high LFA-1 expression were mostly $CD45RA^{hi}CD45RO^{-}$ (Fig. 3, right). Finally, whereas $CD45RA^{lo}CD45RO^{lo}$ $CD4^{+}$ cells were mostly $LFA\text{-}1^{lo}$, the corresponding $CD8^{+}$ cells were primarly $LFA\text{-}1^{hi}$ (Fig. 3, centre and right).

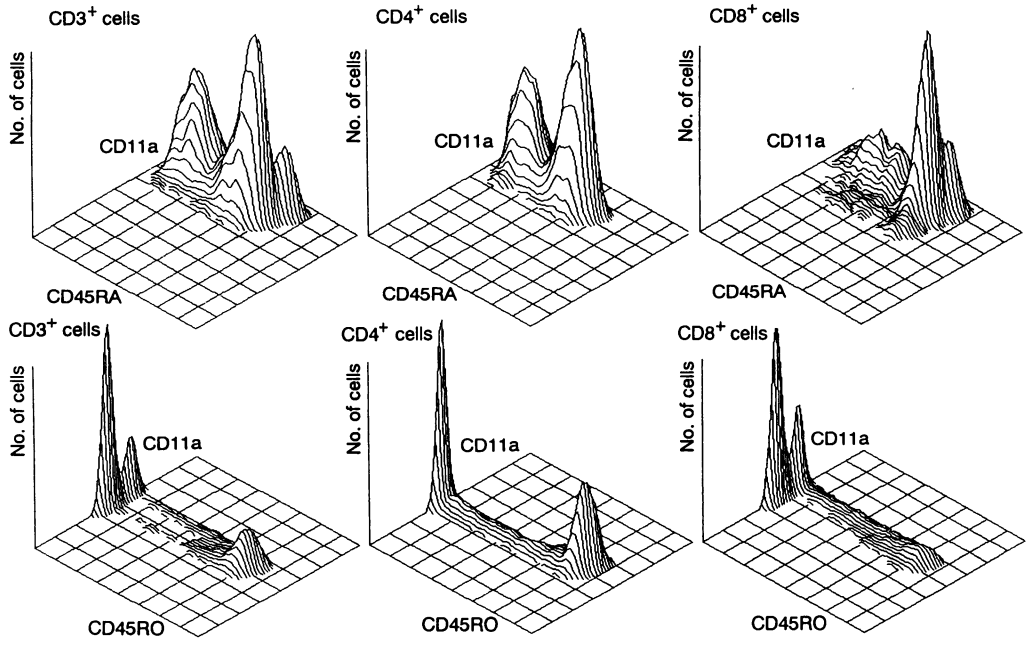

Figure 3. Expression of LFA-1 (CD11a) versus CD45RA (upper panels) or CD45RO (lower panels) in $CD3^{+}$, $CD4^{+}$ and $CD8^{+}$ T cells from a healthy Danish donor.

Table 3. Lympho-proliferation induced by mitogen or antigens

Stimulus	Exp.	[^{3}H]thymidine incorporation (median c.p.m. $\times 10^{-3}$)				
		LFA-1^{lo}	LFA-1^{hi}	LFA-1^{all}	CD45RO$^-$	CD45^{all}
PHA (3 days)	1	39·5	26·4	47·2	ND	ND
	2	29·1	ND	22·6	ND	ND
	3	18·1	14·5	44·9	47·0	53·9
	4	26·3	ND	15·5	27·0	34·5
PPD (7 days)	1	11·0	32·2	14·8	ND	ND
	2	19·7	ND	15·4	ND	ND
	3	19·5	8·6	17·9	0·4	15·9
	4	11·8	ND	11·9	0·1	17·2
TT (7 days)	1	17·0	10·4	11·7	ND	ND
	2	0·1	ND	0·2	ND	ND
	3	0·1	0·3	2·3	0·1	3·1
	4	1·9	ND	0·1	0·1	1·3

Triplicate wells of 5×10^4 cells sorted according to LFA-1 or CD45 phenotype (supplemented with 5×10^4 irradiated autologous PBMC) were either left unstimulated or stimulated with either PHA, PPD or TT. Cells were pulsed by [^{3}H]thymidine for the last 24 hr of incubation. Incorporation in unstimulated cultures was always less than 1·6 c.p.m $\times 10^{-3}$.

ND, not done.

LFA-1 expression in relation to memory cell function

The results of phenotypic analyses reported above implied that if resting T cells were divided into LFA-1^{lo} and LFA-1^{hi} cells, both these subsets would contain memory cells able to proliferate *in vitro* in response to stimulation by recall antigens. To test this hypothesis, we simultaneously labelled PBMC from four Danish donors by PE-CD14, PE-CD16, PE-CD19 and FITC-CD11a antibodies, and sorted them into CD14$^-$CD16$^-$CD19$^-$CD11a^{lo} and CD14$^-$CD16$^-$CD19$^-$CD11a^{hi} cells, respectively. The cell sorting was done preserving the sterility of the cells, using a FACStar cell-sorting instrument. A third population, labelled as above but sorted to obtain CD14$^-$CD16$^-$CD19$^-$ cells irrespective of LFA-1 expression, was obtained as control cells (LFA-1^{all}). Sorted cells were subsequently tested in a proliferation assay using irradiated autologous PBMC to compensate for the depletion of antigen-presenting cells inflicted by the sorting procedure. As summarized in Table 3, cells from either subset were able to respond to both mitogenic and antigenic stimulation. Despite the inhibitory effects of CD11a antibodies on T-cell proliferation, the distinct proliferation seen in both LFA-1^{lo} and LFA-1^{hi} cells suggest that functionally defined memory cells reside in each of these.

In the same donors, depletion of CD45RO$^+$ cells resulted in abrogation of responsiveness to recall the antigens PPD and TT, thus confirming the correspondence between LCA-phenotype and memory cell function (Table 3).

DISCUSSION

Resting T cells can be subdivided into largely reciprocal subsets by their expression of isotypes of the leucocyte common antigen (LCA, CD45). CD45RA^{hi}CD45RO$^-$ CD4$^+$ T cell are functionally characterized by their inability to respond to stimulation by recall antigens *in vitro*, and this subset is generally considered to comprise immunologically unprimed, or naive, T lymphocytes. The complementary CD45RA$^-$CD45RO^{hi} subset, composed of cells which are highly responsive to recall antigens *in vitro*, is regarded as containing previously primed, or memory, cells (reviewed in ref 1). The maturational status of the intermediate subset with concurrent low-intensity expression of both the above isoforms is presently unclear.[5] In CD8$^+$ T cells, a similar concordance between phenotypic and functional characteristics appears to exist.[6] Following cellular activation, CD45RO is acquired while CD45RA is gradually lost.[3,5] The leucotype function-associated antigen LFA-1 (CD11a/CD18) is playing a central role in cellular adhesion,[7] and is widely distributed on human leucocytes, including all peripheral T cells. The expression of LFA-1 on resting human T cells is bimodal, with variable numbers of T cells having either low (LFA-1^{lo}) or high (LFA-1^{hi}) expression of this molecule. Following activation *in vitro*, essentially all T cells acquire the LFA-1^{hi} phenotype.[8] In addition to this up-regulation of LFA-1, the expression of a number of activation markers (e.g. IL-2 receptor, ICAM-1 and LFA-3) is increased.[2,5] Based on the above findings, and on the fact that cord blood leucocytes are predominantly CD45RA$^+$,[9] and have low expression of LFA-1 and other markers mentioned above, it has been proposed that LFA-1 constitutes a marker of immunological memory.[2]

During studies of expression of T-cell surface molecules on PBMC from healthy donors of a malaria-endemic area of Sudan we have observed many donors with an unusually high proportion of LFA-1^{hi} cells without concomitant obvious differences in the other cellular markers mentioned. We have since confirmed this finding in donors from a variety of geographically wide-

spread locations in Africa (L. Hviid *et al.*, unpublished data). Furthermore, clinical episodes of *Plasmodium falciparum* malaria appear to be associated with transient depletion of LFA-1^{hi} T cells from the peripheral circulation without prominent changes in other T-cell surface markers.[10] These data are not easily reconciled with a tight association between LFA-1 expression and immunological memory. We thus decided to examine the expression of LFA-1 on resting T cells in more detail, and to clarify the relation between LFA-1 expression and immunological memory. The expression of LFA-1 on T cells and T-cell subpopulations in the Danish donors corresponded well to data previously published.[11,12] Whether the difference in LFA-1 expression between CD4$^+$ and CD8$^+$ cells reflects a constitutive feature of these cells as has been suggested,[11] or indicates a larger proportion of marginally activated circulating cells in the CD8$^+$ than in the CD4$^+$ T-cell subset is at present unclear. In the Sudanese donors, a considerably larger fraction of circulating T cells was of the LFA-1^{hi} phenotype compared to previously reported data. We are currently investigating the background of this difference.

In the absence of *in vitro* stimulation, most circulating T cells are either negative or have only very low expression of cellular markers of activation such as IL-2 receptor, ICAM-1 and LFA-3, although appreciable amounts of these markers are present on some cells. The expression of these markers and of LFA-1 has been reported to be co-ordinated,[2,13] a finding which is supported by the present study. It can thus be assumed that LFA-1^{hi} T cells represent cells marginally activated *in vivo*.

Although high LFA-1 expression has been described as being characteristic of memory T cells,[1,2] the data presented here clearly demonstrate that LFA-1^{hi} T cells can be found even in CD45RA^{hi}CD45RO$^-$ T cells. Indeed, in one of the Sudanese donors examined, the majority of these cells were LFA-1^{hi} (Fig. 2). Furthermore, it appears that CD45RA$^-$CD45RO^{hi} cells generally have intermediate LFA-1 surface expression, without clear LFA-1 bimodality. In this context it may be of interest to note that LFA-1 has been reported to be physically associated with CD45RA but not with CD45RO.[14] In the LAD patient examined, typical expression of CD45 isotypes was found, demonstrating that acquisition of the CD45RO isotype can occur in the absence of detectable LFA-1 expression. Finally, in T cells sorted into LFA-1^{lo} and LFA-1^{hi} cells, substantial antigen-induced proliferation was observed in each subset, suggesting the presence of immunologically primed T cells in both.

In conclusion, we feel that the present data advise a modification of the concept that acquisition of immunological memory is characterized by sustained high expression of the LFA-1 antigen. Increased expression of LFA-1 may rather indicate a recent stimulatory event, quite possibly due to non-specific, cross-reactive stimulation incurred *in vivo*.[15] The intermediate LFA-1 expression on the CD45RA$^-$CD45RO^{hi} cells might indicate that regulation of LFA-1 expression is bidirectional, and that the apparently relatively slow transition from naive to memory phenotype is only completed following some reduction in LFA-1 expression following the stimulatory event, which is likely to have taken place outside the peripheral circulation. Finally, if CD45 isotype expression is a reliable marker of T-cell memory function, as it certainly appears from the literature, even the transition from naive to memory cells may not be entirely unidirectional, as CD45RO$^+$ T cells may revert to the CD45RA$^+$ phenotype.[16] Thus, T-cell expression of markers of cellular activation, adhesion and maturation appears to be dynamic, with a substantial and complex flux of cells between subpopulations defined according to expression of such markers.

ACKNOWLEDGMENTS

Gitte Pedersen and Jette Dalsten are thanked for excellent technical assistance. Ole Borregaard is thanked for cell samples from the LAD patient. Riad A. Bayoumi and James B. Jensen are thanked for invaluable support of our work in Sudan, and the continuing support of the inhabitants of Daraweesh village, Eastern State, Sudan is gratefully acknowledged. This study received financial support from the Danish International Development Agency (DANIDA) (grant 104. Dan.8/357), the Danish Medical Research Council (grant 12-7311), and NIH, Bethesda, MD (grant AI 16312). L. Hviid holds a senior research fellowship at University of Copenhagen.

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