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Expression of L-selectin (CD62L) discriminates Th1- and Th2-like cytokine-producing memory CD4$^+$ T cells

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SUMMARY

Human memory (CD45RO$^+$) CD4$^+$ T cells can be distinguished into two subpopulations on the basis of expression of the lymph node homing receptor, L-selectin (CD62L). In a prior study we showed that human L-selectin-positive memory T-helper (Th) cells promote the maturation of IgG- and IgA-producing cells by naive B cells. To further elucidate the contribution of memory CD4$^+$ T cells to B-cell differentiation, human memory CD4$^+$ T cells with or without L-selectin expression were evaluated for production of cytokines that participate in regulation of immunoglobulin production. It was found that L-selectin-positive human memory CD4$^+$ T cells produce mainly interleukin (IL)-4 and IL-5, whereas L-selectin-negative CD4$^+$ T cells produce mainly interferon-γ (IFN-γ). This profile of cytokine expression coincides with the profile that distinguishes Th1 and Th2 subsets. In contrast to the murine system, IL-10 production was similarly contributed by human L-selectin-positive and -negative memory CD4$^+$ T-cell subpopulations. These results suggest that the human L-selectin-negative and -positive subpopulations of human memory CD4$^+$ T cells contain Th1-like and Th2-like cytokine-producing cells, respectively.

INTRODUCTION

It is now widely accepted that the CD4$^+$ T-helper (Th) cells comprise functionally distinct subsets of Th1 and Th2 cells that are distinguished by patterns of cytokine production.[1] First identified by *in vitro* analyses of murine Th clones, accumulating evidence now exists for the existence of similar subsets *in vivo*, as a result of antigen (Ag)-mediated immune responses by naive CD4$^+$ T cells. Th1 cells secrete interferon (IFN)-γ, lymphotoxin and interleukin (IL)-2 and mediate delayed hypersensitivity, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 and promote immunoglobulin production by B cells. Another cell subset, termed Th0, has been shown to produce cytokines of both Th1 and Th2 types, and is thought to include obligatory precursors of Th1 and Th2 cells. Recent studies have shown that the development of Th subsets is regulated by cytokines, but the precise process by which naive CD4$^+$ T cells differentiate remains to be determined. Additionally, specific phenotypic markers for identification of possible precursors and effectors within circulating CD4$^+$ T cells have not been identified yet.

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Circulating human CD4$^+$ T cells contain both naive cells and cells previously primed with Ag, i.e. memory cells, which can be distinguished from each other on the basis of CD45RO expression.[2,3] Memory CD4$^+$ T cells provide more efficient help for immunoglobulin production by B cells than naive cells, and are good producers of the cytokine IL-4, IL-5, IL-10 or IFN-γ.[4–6] They can be distinguished into two subpopulations on the basis of expression of the lymph node homing receptor, L-selectin (CD62L).[7,8] In a previous study, we have demonstrated that Th-dependent generation of PWM-induced IgG- and IgA-producing cells is more effectively promoted by L-selectin-positive memory CD4$^+$ T cells than by L-selectin-negative cells, indicating that L-selectin-positive memory CD4$^+$ T cells play a role in isotype switching of naive B cells.[9] In the present study, we examined cytokine production by memory CD4$^+$ T cells that either do express or do not express L-selectin. The results suggest the existence in the circulation of Th1 and Th2 effectors that can be distinguished on the basis of differential expression of L-selectin.

MATERIALS AND METHODS

Reagents

Fluorescein isothiocyanate (FITC)-conjugated anti-CD45RO (UCHL1; Dako Japan, Kyoto, Japan), phycoerythrin (PE)-conjugated anti-CD62L (TQ-1; Coulter Immunology, Hialeah, FL) and peridium chlorophyll (Per-CP)-conjugated anti-CD4

(Leu-3a; Becton Dickinson Immunocytometry, San Jose, CA) monoclonal antibodies (mAbs) were utilized for isolation of CD4$^+$ T-cell subpopulations. Phorbol myristate acetate (PMA) (Sigma Chemical Co., St Louis, MO) and calcium ionophore A23187 (Behring Diagnostics, La Jolla, CA) were used for stimulation of CD4$^+$ T-cell subpopulations.

CD4$^+$ T-cell preparation

CD4$^+$ T-cell subpopulations were isolated by an electronic sorting using an EPICS Elite flowcytometer (Coulter Electronic, Inc., Hialeah, FL), as described.[10] In brief, peripheral blood mononuclear cells (PBMC) from healthy adult donors were separated from heparinized venous blood by Ficoll–Hypaque density gradient centrifugation, and partially depleted of adherent monocytes by incubation on plastic flasks. T cells were separated from non-adherent PBMC by rosette formation with 2-aminoethylisothiouronium bromide (Sigma Chemical Co., St. Louis, MO)-treated sheep red blood cells, followed by Ficoll–Hypaque gradient centrifugation as described.[11] E-positive T cells were simultaneously stained with Per-CP-conjugated anti-CD4, FITC-conjugated anti-CD45RO and PE-conjugated anti-CD62L mAbs. Based on the relation between CD45RO and CD62L expressed on CD4$^+$ T cells, three CD4$^+$ T-cell subpopulations, namely CD62L$^+$CD45RO$^-$, CD62L$^+$CD45RO$^+$ and CD62L$^-$CD45RO$^+$ cells, were isolated using three-colour fluorescence. Two cycles of sorting, performed to prevent contamination with other populations, resulted in isolation of populations that were more than 99% pure.

Stimulation of CD4$^+$ T-cell subpopulations

The culture medium consisted of RPMI-1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum, 25 mM HEPES, 5×10^{-5} M 2-mercaptoethanol, 0·3 mg/ml L-glutamine, 200 U/ml penicillin G and 10 μg/ml gentamicin. To assess cytokine mRNA induction following activation, CD4$^+$ T-cell subpopulations were stimulated with a combination of 10 ng/ml PMA and 250 ng/ml calcium ionophore at 5×10^5 cells/well in 48-well culture plates (Costar, Cambridge, MA) in a final volume of 500 μl. These cells were incubated for 24 hr at 37° in an incubator of 5% CO_2 in air, and harvested for RNA extraction described as below. For measurement of cytokines production, the cells were similarly stimulated with PMA plus calcium ionophore in duplicates at 2×10^5/well in 96-well flat-bottom microtitre plates (Corning Glass Works, Corning, NY) in a volume of 200 μl. The culture supernatants were obtained after 3 days of culture for enzyme-linked immunosorbent assay (ELISA).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for cytokine mRNA expression

RT-PCR was performed essentially as previously described.[4] Total RNA was extracted from stimulated CD4$^+$ T-cell subpopulations using a guanidium isothiocyanate/phenol-chloroform procedure. Total RNA from 5×10^5 cells was primed with oligo(dT)$^{12-18}$ (Pharmacia LKB Biotechnology, Bromma, Sweden) and the first-strand cDNA was synthesized with reverse transcription with RAV-2 (Takara-Shuzo Co., Ltd, Kyoto, Japan) in a final volume of 50 μl. PCR was performed using one-tenth of first-strand cDNA. To this was added 200 μM dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8·3), 1·5 mM $MgCl_2$, 0·001% gelatin, 2·5 U Taq polymerase (Perkin–Elmer/Cetus, Norwalk, CN) and 200 pmol of each sense/antisense primer in a total volume of 100 μl. Reactions were incubated in a Perkin–Elmer/Cetus DNA thermal cycler for 25 to 40 cycles. The primers used for IL-2, IL-4, IL-5 and IFN-γ were described previously.[4] Additional primers included those for L-10 (5′-ATGCCCCAAGCTGAGAACCAAGACCCA; 3′-TCTCAAGGGGCTGGGTCAGCTATCCCA) and β-actin (5′-TACATGGCTGGGGTGTTGAA; 3′-AAGAGAGGCA-TCCTCACCCT). An aliquot (10 μl) of the amplified products was subjected to 1·3% agarose gel electrophoresis, transferred to a nylon membrane and hybridized with each cDNA probe labelled with digoxigenin (Boehringer Mannheim Biochemicals, Indianapolis, IN). The immuno-enzymic detection of hybridized DNA was carried out according to the recommendations of the manufacturer.

Quantitation of cytokines in culture supernatants

Production of IL-2, IL-4, IFN-γ and IL-10 was measured by using commercial ELISA kits (Medgenix Diagnostics, Brussels, Belgium). IL-5 were quantitated by using a sandwich ELISA as described elsewhere.[12]

Statistical analysis

Group comparisons were performed by the Student's *t*-test.

RESULTS

Three-colour immunofluorescent analysis of CD4$^+$ T cells in adult blood showed that the vast majority of naive (CD45RO$^-$) CD4$^+$ T cells express L-selectin (CD26L), whereas a small but sizable fraction (about 25%) of memory (CD45RO$^+$) CD4$^+$ T cells lack CD62L expression (Fig. 1). Thus, on the basis of CD62L surface expression, two subpopulations of memory CD4$^+$ T cells can be identified, a large CD62L$^+$CD45RO$^+$ subset and a small CD62L$^-$CD45RO$^+$ subset. Naive (CD45RO$^-$) CD4$^+$ T cells, CD62L$^+$ and CD62L$^-$ memory (CD45RO$^+$) CD4$^+$ T cells isolated from adult donors by an electronic sorting were evaluated for production of the cytokines IL-2, IL-4, IL-5, IL-10 and IFN-γ. It was previously shown that naive CD4$^+$ T cells produce only IL-2, whereas memory CD4$^+$ T cells are good producers of IL-4, IL-5, IL-10 and IFN-γ.[4–6] We analysed whether the two subpopulations of memory CD4$^+$ T cells may differ in their ability to produce IL-4, IL-5, IL-10 and IFN-γ. For this purpose, isolated CD4$^+$ T-cell populations were stimulated by a combination of PMA and calcium ionophore, and levels of secreted cytokines were assessed by the ELISA (Fig. 2). All CD4$^+$ T-cell subpopulations produced substantial levels of IL-2 in response to stimulation with PMA plus calcium ionophore. As expected, variable levels of IL-4, IL-5, IL-10 and IFN-γ were detected in the culture supernatants of both memory CD4$^+$ T-cell subsets, but not in the culture supernatants of naive CD4$^+$ T cells. Importantly, the levels of IL-4 and IL-5 detected in culture supernatants of CD62L$^+$ memory CD4$^+$ T cells were significantly higher than those detected in culture supernatants of CD62L$^-$ memory CD4$^+$ T cells. In addition, IFN-γ production was prominent in cultures of CD62L$^-$ memory CD4$^+$ T cells, but modest in cultures of CD62L$^+$ memory CD4$^+$ T cells. Although CD62L$^+$ memory CD4$^+$ T cells showed somewhat higher amounts of IL-10 compared with CD62L$^-$ memory CD4$^+$ T cells, this difference was not statistically significant.

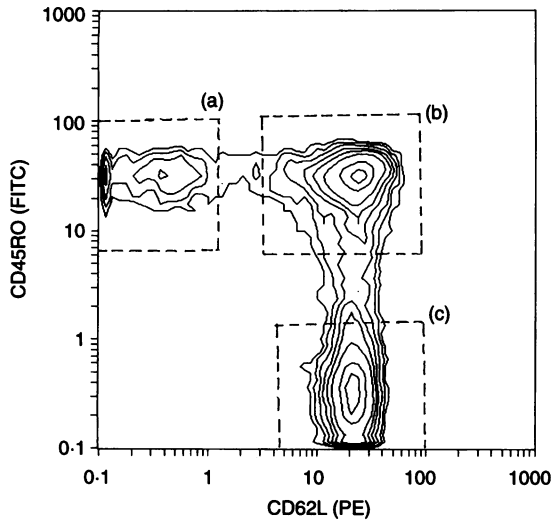

Figure 1. Three-colour immunofluorescent analysis of CD45RO and L-selectin (CD62L) expressed on $CD4^+$ T cells. E-rosetting T cells from adult peripheral blood were simultaneously stained with Per-CP-conjugated anti-CD4, FITC-conjugated anti-CD45RO and PE-conjugated anti-CD62L mAbs. Two-colour profiles of CD45RO (FITC) and CD62L (PE) expression on $CD4^+$ T cells, gated on red fluorescence, were obtained using an EPICS Elite flowcytometer. As outlined by the dashed lines, three subpopulations of $CD4^+$ T cells were isolated by this method; (a) $CD65L^-$ memory ($CD45RO^+$) cells, (b) $CD62L^+$ memory ($CD45RO^+$) cells and (c) naive ($CD62L^+CD45RO^-$) cells.

Measurement of cytokines produced revealed that IL-4 and IL-5 were preferentially produced by $CD62L^+$ memory $CD4^+$ T cells, whereas IFN-γ production was a selective production of $CD62L^-$ memory $CD4^+$ T cells. In light of the current view that Th cells can be distinguished into Th1 and Th2 subsets based upon patterns of cytokines produced,[1] our findings suggested that $CD62L^-$ and $CD62L^+$ subpopulations of memory $CD4^+$ T cells are distinguishable based upon production of Th1-like and Th2-like cytokines, respectively. To assess this further, we studied cytokine mRNA expression in stimulated $CD4^+$ T cells by RT-PCR. The results of these experiments are depicted in Fig. 3. All $CD4^+$ T-cell subpopulations expressed similar levels of IL-2 mRNA in response to PMA plus calcium ionophore. In contrast, we found that mRNA expression of IL-4 and IL-5 was much higher in $CD62L^+$ memory $CD4^+$ T cells than in $CD62L^-$ memory $CD4^+$ T cells. In addition, IFN-γ mRNA expression was evident in $CD62L^-$ memory $CD4^+$ T cells, but barely detectable in $CD62L^+$ memory $CD4^+$ T cells. Also, IL-10 mRNA expression was similar in subpopulations of memory $CD4^+$ T cells.

DISCUSSION

Final differentiation of B cells into immunoglobulin-producing cells is believed to require a cognate interaction with activated T cells and the presence of T-cell-derived cytokines. Recent identification of the CD40 B-cell Ag and its ligand (CD40L) expressed on activated T cells has supported the importance of a B–T cell interaction for immunoglobulin production by B

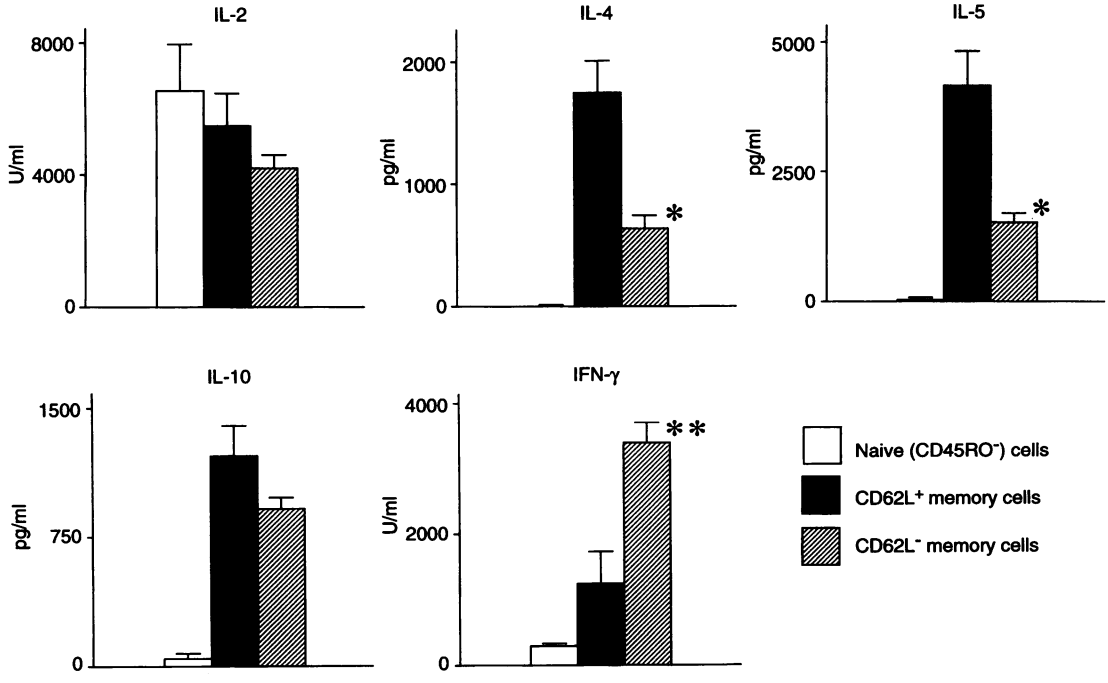

Figure 2. Cytokine production by $CD4^+$ T-cell subpopulations. Isolated naive $CD4^+$ T cells and memory $CD4^+$ T cells with or without L-selectin (CD62L) expression were stimulated with a combination of PMA and calcium ionophore for 3 days. Cytokine levels in culture supernatants were assessed by ELISA. Data represent the means (± SEM) of seven different experiments. * $P < 0{\cdot}01$, ** $P < 0{\cdot}002$, statistical significance of comparisons between $CD62L^+$ and $CD62L^-$ memory $CD4^+$ T cells.

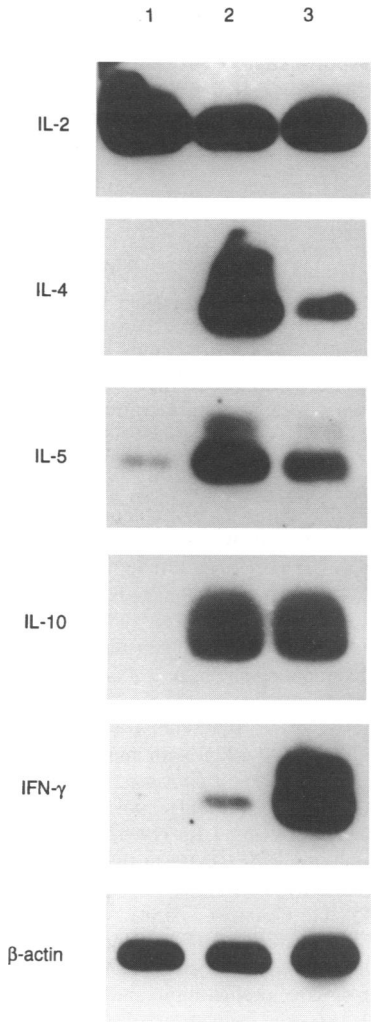

Figure 3. Cytokine mRNA expression in CD4$^+$ T-cell subpopulations. Naive (lane 1), CD62L$^+$ memory (lane 2) and CD62L$^-$ memory (lane 3) CD4$^+$ T cells were stimulated with PMA plus calcium ionophore for 24 hr. mRNA expression of cytokine and β-actin genes in stimulated cells was evaluated by RT-PCR, followed by Southern hybridization with digoxigenin-labelled corresponding cDNA probes.

cells.[13] This notion was further supported by the finding that defects involving the CD40L gene are responsible for X-linked hyper-IgM syndrome, resulting in the failure of B cells to switch from IgM to IgG and IgA production.[14,15] In a previous report, we have described that, both in newborns and adults, generation of IgG- and IgA-producing cells (in addition to IgM production) by naive (IgD$^+$) B cells, is more effectively promoted by L-selectin-positive memory CD4$^+$ T cells than by the L-selectin-negative cells. These findings indicated the crucial role played by L-selectin-positive memory CD4$^+$ T cells in isotype switching of naive B cells.[9] Although CD40L expression appears to be defective in neonatal naive T cells, it was shown that CD40L mRNA and protein are expressed at the same levels on both naive and memory CD4$^+$ T cells from adult individuals after activation.[16] It is therefore unlikely that CD40L may be expressed preferentially on subsets of memory CD4$^+$ T cells on the basis of L-selectin expression. Furthermore, studies have indicated that T-cell-derived cytokines, including IL-4, IL-5 and IL-10, are necessary for induction of immunoglobulin production by B cells through the CD40/CD40L system.[17–19] These findings suggested that Th-derived cytokines, rather than a physical contact with T cells, may play a key role in isotype switching of naive B cells.

We found that there were marked differences in the patterns of IL-4, IL-5, IL-10 and IFN-γ production between memory CD4$^+$ T cells, with and without L-selectin surface expression. Although these cytokines were produced by both memory CD4$^+$ T-cell subpopulations, not naive CD4$^+$ T cells, it was found that L-selectin-positive memory CD4$^+$ T cells produced higher levels of IL-4 and IL-5 than their counterparts, whereas IFN-γ production was predominant in the latter subpopulation. The notion that Th clones should be distinguished into Th and Th2 subsets based on the types of cytokines was suggested by a number of investigations on murine and human Th clones.[1] The consensus is that IFN-γ is predominantly secreted by Th1 cells, but IL-4, IL-5 and IL-10 are predominantly produced by Th2 cells. With the exception of IL-10 production, our findings raised the possibility that cytokine production profiles in L-selectin-negative and -positive memory CD4$^+$ T cells might correspond to those of Th1 and Th2 subsets. This was further confirmed by analysis of cytokine mRNA expression.

With regard to IL-10 production, murine Th2 clones have been shown to produce IL-10 selectively.[20] In the present study with human cells, IL-10 production was found in both L-selectin-positive and -negative memory CD4$^+$ T cells. Evaluation of IL-10 mRNA by RT-PCR also indicated that IL-10 mRNA was similarly expressed in both L-selectin-negative and -positive memory CD4$^+$ T cells. Consistent with our findings, it was reported that IL-10 was measurable in supernatants of Th1 as well as Th2 clones, but IL-10 mRNA expression was somewhat increased in Th2 clones.[21] It is possible that regulation of IL-10 production by Th1 and Th2 subsets may be different in human and mouse, and this issue should be further investigated.

Although the distinction between Th1 and Th2 subsets was derived from many *in vitro* studies of established Th clones, it could be that naive Th cells are driven by antigenic stimuli in local lymphoid tissues to differentiate into effectors of a specific Th subset, subsequently migrate into the blood and constitute a proportion of circulating Ag-primed or memory-like Th cells. Studies on human CD4$^+$ Th clones have shown that Th2 clones exhibit more efficient helper activity for immunoglobulin production by B cells than Th1 clones.[22] These data supported the idea that circulating memory CD4$^+$ T cells with and without L-selectin contain Th1-like and Th2-like cytokine-producing cells. L-selectin is involved in cell migration from the blood into peripheral lymphoid tissues or sites of infection and inflammation.[23] Thus, it is natural that memory CD4$^+$ T cells with Th2-like function express L-selectin in the circulation, permitting cells to actively participate in regulation of Ag-mediated B-cell responses in local lymphoid tissues. In contrast, L-selectin-negative memory CD4$^+$ T cells, which migrate freely in non-lymphoid spaces, appear to exert the Th1-like function. Generally, L-selectin expression is lost after lymphocyte activation, but can be re-expressed upon return to the resting state.[23] Unlike human memory CD4$^+$ T cells, the vast majority of murine memory CD4$^+$ T cells have been shown not to

express L-selectin.[24] However, such differences remain to be further investigated.

To our knowledge, this work is the first demonstration that memory $CD4^+$ T cells in the blood can discriminate into Th1-like and Th2-like cells on the basis of the surface-marker L-selectin, although the antigen CD30 or CD60 has been previously shown to identify Th clones producing Th2 cytokines.[25,26] An alteration in the Th1/Th2 response has been implicated in the pathogenesis of parasitic infections, atopic diseases, HIV infection and autoimmune diseases.[27] L-selectin-based examination of memory $CD4^+$ T cells could lead to a better understanding of the pathological significance of Th1 and Th2 subsets.

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