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Expansion and Proliferation of Skin-Homing T Cells in Atopic Dermatitis as Assessed at the Single Cell Level

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Key Words
CLA · CD7 · CD4+ T cell · DNA content · House dust mite antigen · Tetanus toxoid antigen

Abstract

Background: Sensitization to aeroallergens is a frequent event in patients with atopic dermatitis (AD). The role of allergen-specific T cells in the pathogenesis of AD is, however, not fully clear. A detailed immunological characterization of allergen-specific T cells able to migrate to inflamed skin might therefore be helpful in determining the relevance of allergen-specific T cells to clinical symptoms. ***Objective:*** To establish a simple assay to simultaneously monitor the expression of skin-related homing molecules and their proliferative response to an allergen on peripheral T cells. To evaluate whether this assay is able to identify AD patients in whom exposure to an allergen induces dermatitis. ***Methods:*** The expression of CLA, CD7 and CD4 was simultaneously measured by flow cytometry with the DNA content as a parameter of proliferation at the single cell level. House dust mite antigen (DerP) and tetanus toxoid (TT) were used as antigens. ***Results:*** CD4+ CLA+ CD7–, but not CLA– T cells from patients with AD proliferated and expanded following DerP stimulation. In contrast, TT stimulation resulted in proliferation of both, CLA+ and CLA– T cells from both, atopic and healthy donors. ***Conclusions:*** Single cell analysis revealed a predominant localization of allergen-reactive T cells within the CD7– CLA+ CD4+ T cell subset in AD. Thus, this simple assay not only confirms previously published results, but also extends our knowledge on the biology of CLA and CD7 cells in AD. Ultimately, this will be helpful in classifying those patients who might benefit from allergen avoidance therapies.

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Introduction

Atopic dermatitis (AD) is a multifactorial disease. One reason for exacerbation or chronicity of the disease might be the sensitization to common aeroallergens associated with the production of specific IgE and the development of allergen-specific, T helper 2 type (Th2)-skewed, skin-infiltrating T cells [1]. The discovery of receptors on lymphocytes enabling them to migrate into specific tissues has provided new insights into the process of homing [2]. The cutaneous lymphocyte antigen (CLA) has been described as a skin-specific homing receptor under both physiological [3] and pathological conditions [4]. Purified CLA+ CD45R0+ memory T cells from allergic donors with cutaneous, but no pulmonary symptoms, proliferated in vitro to the relevant allergen and produced IL-4 [5]

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and IL-13 [6], hallmarks of an allergen-specific Th2 response. The lack of CD7 expression has also been described to be associated with skin-infiltrating cells [7, 8] and a Th2 phenotype [9]. These studies suggest that T cells infiltrating the skin following allergen exposure in vivo are proliferating CLA+ and CD7– CD4+ T cells. We describe a single cell assay, which allows simultaneous determination of the expression of CD4, CD7 and CLA, and the corresponding DNA content as a marker of cell proliferation. Using this assay, we show that indeed CD7–CLA+ within unfractioned peripheral mononuclear cells (PBMCs) from AD patients proliferate and expand following house dust mite (DerP) antigen activation in vitro, whereas tetanus toxoid (TT), used as a control antigen, did not induce this selective activation. Thus, this simple assay not only confirms, but rather extends recent data and is therefore instrumental in investigating the role of allergens in homing of T cells in AD skin.

Materials and Methods

Patients and Cell Culture

Ten adult patients with AD were recruited to donate blood samples for the study. These patients had AD since childhood, were moderately to severely affected (mean SCORAD [10] 40.9 ± 16.6 SD), and had elevated serum IgE levels (mean 2,981 ± 2,469), specific IgE for DerP (mean 3.4 ± 0.7 RAST classes) and positive atopy patch tests to house dust mite antigen, which is an eczematous reaction after 24–72 h following epidermal application of the antigen. None of the patients were under systemic immunosuppressive treatment. The study was approved by the local ethics committee, with each patient giving written consent. Twelve healthy, age- and sex-matched volunteers served as controls.

PBMCs were separated by Ficoll gradient centrifugation and 2×10^6 cells per milliliter were cultured in 24-well plates (Nunc, Wiesbaden, Germany) in RPMI-1640 + 5% FCS (Biochrom, Berlin, Germany). Cells were activated with an extract of house dust mite antigen containing predominantly DerP antigen (Bencard, Neuss, Germany), which is the starting material for the company's prick tests. The material was further purified by gel filtration over Sephadex G25 (PD-10; Pharmacia, Freiburg, Germany) and stored at –80°C at a concentration of 1 mg/ml in PBS. To stimulate PBMCs, we used 1 μg/ml. As a control, TT (kindly provided by Behringwerke, Marburg, Germany) was added at 21 Lf/ml.

Flow Cytometry

Freshly isolated cells and day 7 cultured cells were washed with PBS and stained with anti-CD4-TRI-Color (Caltag, Hamburg, Germany), anti-CD7-PE (Caltag) and anti-CLA-FITC (Pharmingen, Hamburg, Germany) antibodies. After staining, cells were washed and resuspended in a solution containing 50% PBS, 50% H_2O, 0.01% saponin (Sigma, Deisenhofen, Germany) and 1 μ*M* (4′,6′-diamidino-2-phenylindole-2-HCl) (DAPI; Sigma). Thirty minutes later, the cells were analyzed on a PASIII flow cytometer (Partec, Münster, Germany) equipped with a 488-nm argon laser and an HBO mercury arc lamp, able to measure 6 parameters simultaneously. To excite DAPI, UV light was filtered though a 10-nm band-pass filter at 365 nm. Emitted light was collected with a 20-nm band-pass filter at 460 nm. 50,000 cells per sample were collected in list mode and data were analyzed using WinList 3.0 (Verity Software, Topsham, Me., USA). For the determination of proliferating cells, the number of cells in the $S+G_2/M$ phase was calculated. To set gate definitions, regions were set on lymphocytes as identified by forward scatter versus side scatter, on CD4+ cells and on cells in the DNA histogram as defined by G_0/G_1, S (synthesis) and G_2/M (mitosis) phase. This procedure excluded debris, dead and apoptotic cells. These three regions were combined to define a gate by the logical operator AND. Further gates were defined based on CLA and CD7 staining resulting in four possible gates: CD7+CLA+, CD7+CLA–, CD7–CLA+, CD7–CLA–. The number of cells in the $S+G_2/M$ phase as the percentage of each of these four populations (set to 100%) is shown in the Results section and an example of the gating procedure is shown in figure 1. For measuring proliferation using ^{3}H-thymidine, cells were pulsed with 0.5 μCi (Amersham, Braunschweig) overnight, and radioactivity was counted in a beta counter (Beckman). All cultures were set up in triplicates.

Statistics

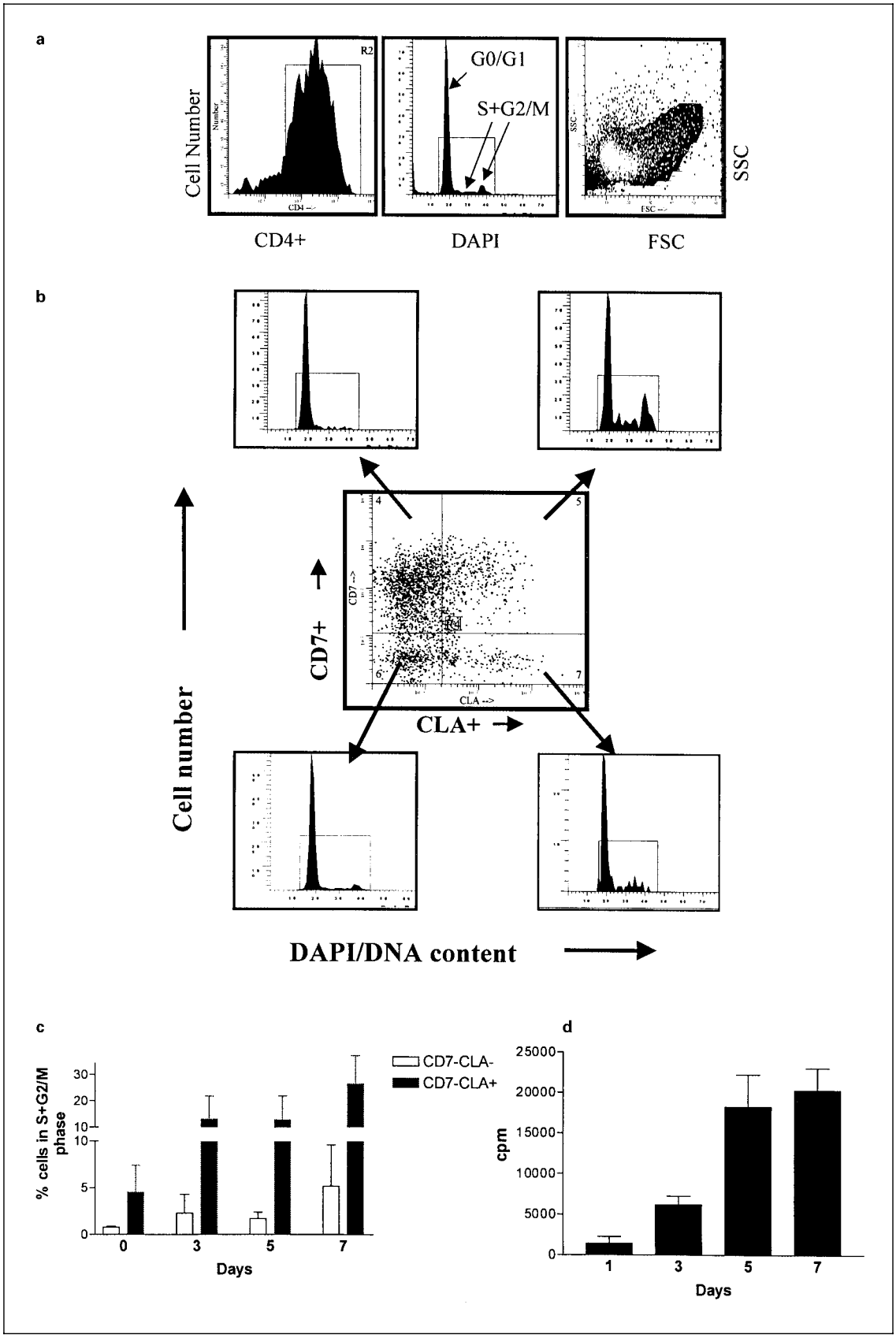
To verify the statistical significance of differences between healthy and atopic donors, the Mann-Whitney U test was performed (Prism 2.01, GraphPad Software). For analyzing paired values, the Wilcoxon test for matched pairs was used.

Results

Correlation of Cell Cycle Analysis with Cell Surface Marker Expression

A flow cytometer equipped with a UV HBO lamp and an argon laser allowed simultaneous excitation of four fluorescent dyes. This setting enabled us to measure the DNA content together with monoclonal antibodies directed at the cell surface molecules CD4, CD7 and CLA coupled to PE-Cy5, PE and FITC, respectively. Figure 1a shows an example of how regions were defined to subsequently gate on the four possible combinations between CD7 and CLA. The frequency of each of these subsets

Fig. 1. Example of a 4-color analysis. PBMCs were stimulated with TT for 7 days, harvested and stained with mAbs and DAPI as described in the Materials and Methods section. a Regions which were combined to define a gate based on FSC/SSC properties, CD4 expression and DNA content containing $G_{0/1}$, S and G_2/M phase. b Typical analysis of the cell cycle for each gated subpopulation as defined by CD7 and CLA staining. The proliferative response of TT-activated PBMC is shown as mean ± SD of three experiments as measured by flow cytometric DNA analysis (c) and by ^{3}H-thymidine incorporation (d) as a parameter of time.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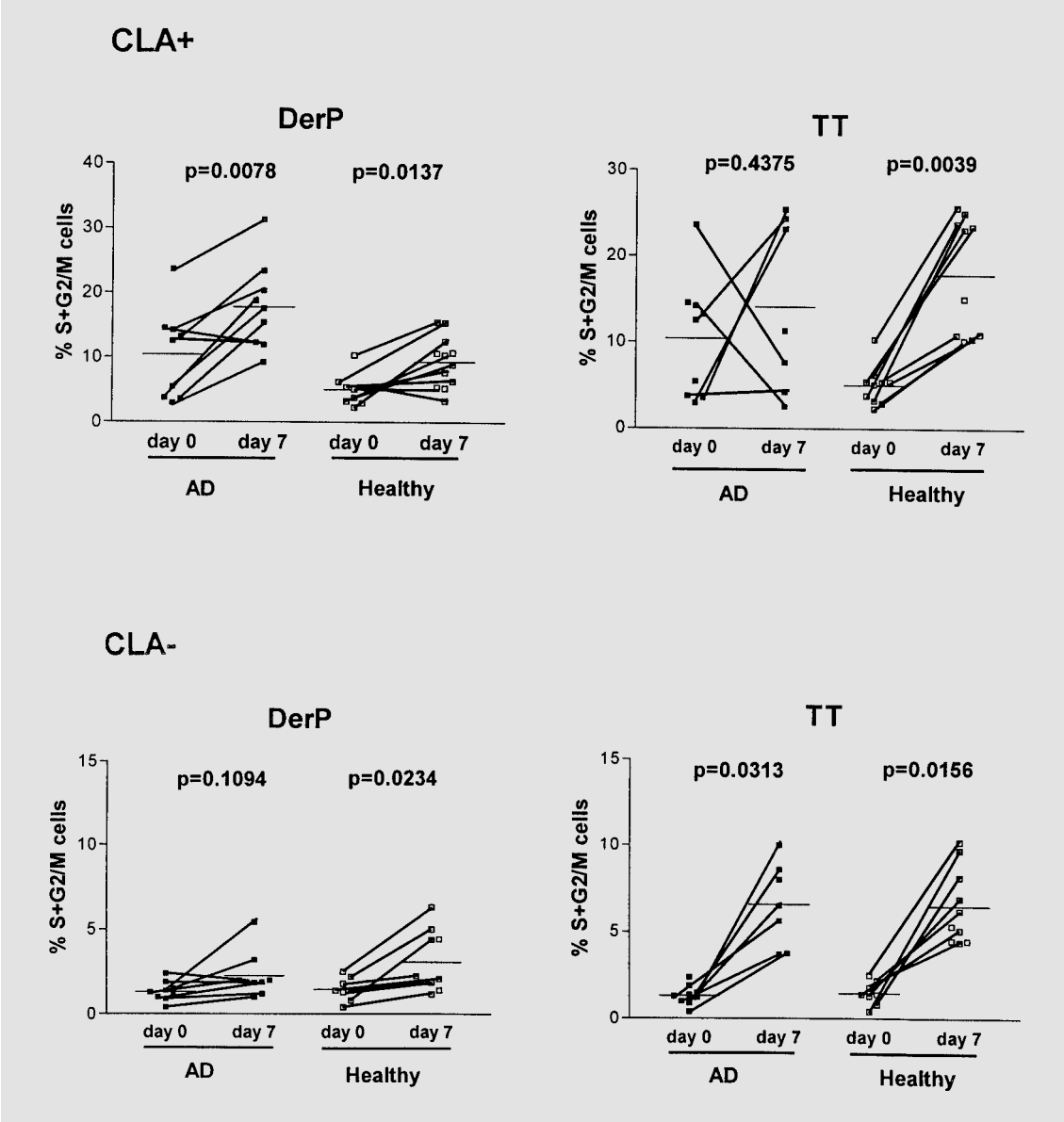

Fig. 2. Proliferation of CD4+CD7–CLA+ and CLA– T cell following antigen activation. Cell cycle analysis was performed on resting (day 0) and DerP- and TT-activated PBMCs cultured for 7 days from healthy and atopic (AD) donors. Data show analyses for CD4+CD7– T cells expressing or not expressing CLA. Thus, 10% of CLA+ cells being in the S+G_2/M phase means that 10% of all CD4+CD7–CLA+ T cells are in the cell cycle. p values were obtained after performing paired statistical analyses for the proliferative responses between day 0 and day 7. Note that data from day 0 lacking a corresponding value from day 7 (and vice versa) are also shown. The means are shown as horizontal lines.

could be calculated as the percentage of 100% CD4+ cells. In addition, the percentage of cells in the S + G_2/M phase from each of the four subsets was recorded (fig. 1b). To determine the optimal time point of measurement following in vitro stimulation of the cells, a kinetic study was performed. As shown in figure 1c, the proliferative response of both the CD4+CD7–CLA– and CD4+CD7–CLA+ cells following TT stimulation was highest after 7 days. Thus, the proliferative response measured based on DNA content correlated well with a ^{3}H-thymidine incor-

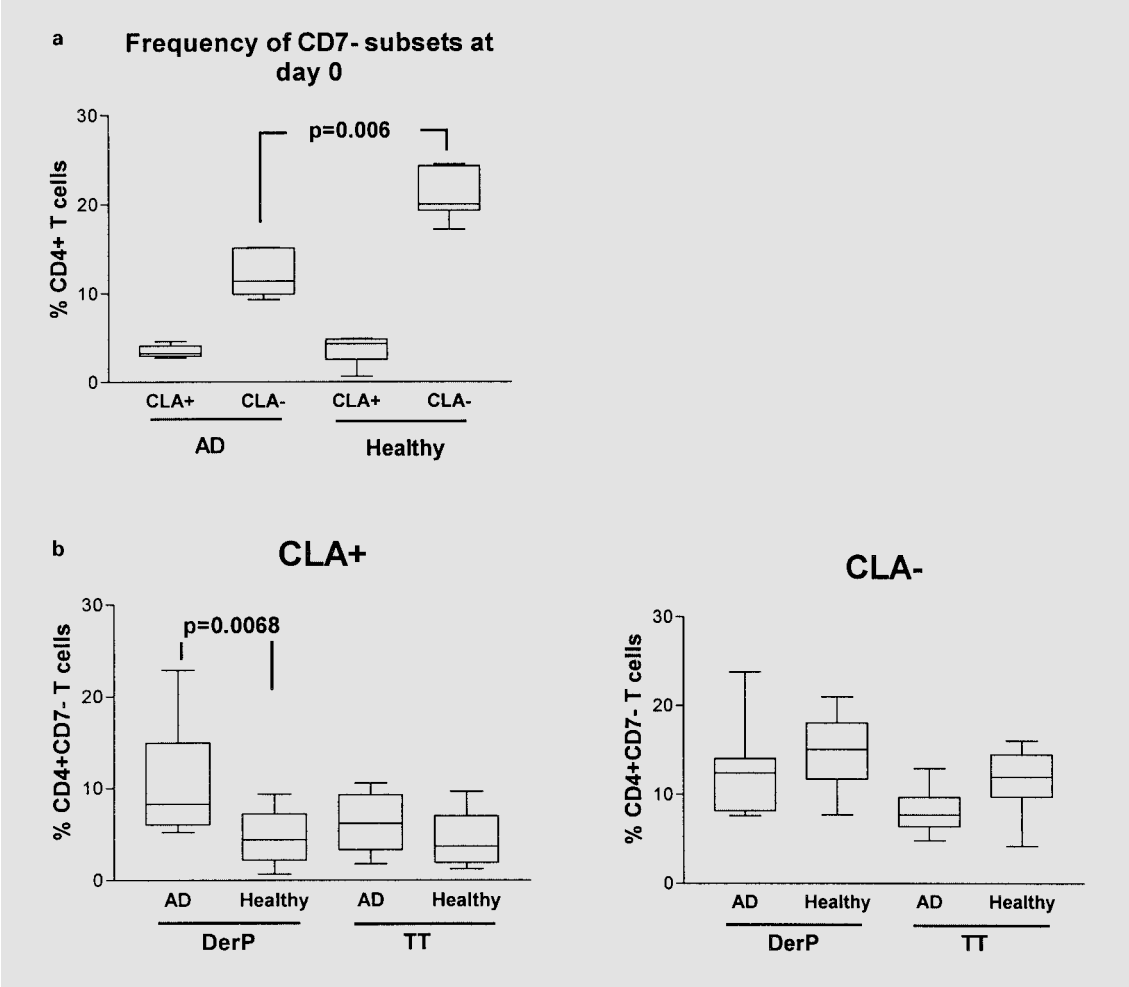

Fig. 3. Relative frequencies of CLA+ and CLA– cells within the CD4+CD7– T cell subset. a The percentage of CD4+CD7– T cells expressing or not expressing CLA on day 0 (resting cells) is shown for healthy and atopic donors (AD) as box and whiskers plots (mean and 25th and 75th percentiles, plus the very exteme outliers at the top and bottom). b The relative distribution of CLA+ and CLA– CD4+-CD7– T cells is shown for cultured T cells (day 7) following DerP and TT activation.

poration assay following TT stimulation (fig. 1d). In addition, DerP-activated PBMC also showed the highest frequencies of proliferating cells after 7 days of activation (data not shown).

Analysis of Proliferating CLA+ and CLA– CD4+CD7– T Cells in AD Patients and Healthy Controls following DerP and TT Stimulation

Cell cycle analysis of CLA+ and CLA– CD4+CD7– T cells activated with DerP or TT in vitro for 7 days demonstrated proliferative activity compared to resting cells (fig. 2). Although CLA+ T cells from atopic subjects tended to have a higher frequency of mitotically active resting cells (day 0) as compared to healthy donors, the difference was statistically not significant. When the proliferative activity of DerP-activated CLA+ T cells was compared to resting cells by means of paired statistical analysis, a significant increase in proliferation was observed for both, atopic and healthy subjects. However, comparison of the frequency of proliferating DerP-activated CLA+ cells at day 7 between healthy and atopic subjects revealed a clear statistically significant difference ($p = 0.0045$), indicating that atopic CLA+ T cells proliferated more vigorously than healthy control T cells. CLA+ T cells from healthy donors activated with TT proliferated substantially more than resting T cells, in contrast to

atopic CLA+. CLA– T cells also proliferated following activation with DerP or TT: however, the increment of proliferation compared with resting cells was less or even nonsignificant compared with the activity of CLA+ T cells. Thus, DerP stimulation preferentially activated the proliferation of CLA+ T cells from atopic donors. In contrast, TT activation promoted the proliferation of both CLA+ and CLA– T cells, predominantly in healthy donors.

DerP-Activated CLA + T Cells Expand following Proliferation

To analyze whether the selective proliferation of a T cell subpopulation would result in an outgrowth of this subset, the relative distribution of CLA+ and CLA– CD4+CD7– T cells was determined in resting (fig. 3a) and in activated cells (fig. 3b). While the frequency of resting CLA+ T cells did not differ between atopic and healthy donors, the proportion of resting CLA– T cells was significantly decreased in atopic subjects. DerP-mediated proliferation of CLA+ T cells in AD patients was accompanied by an enhanced number of CLA+ T cells after 7 days of in vitro culture (fig. 3b). Although TT activation induced a strong proliferative response in both CLA+ and CLA– T cells from healthy subjects, this did not result in an altered relative frequency of CLA+ or CLA– T cells. Thus, while DerP preferentially activates the proliferation and expansion of CLA+ T cells from AD patients TT induces proliferation of both CLA+ and CLA– T cells from healthy subjects, but does not determine a significant change in their relative distribution.

Discussion

In this report we describe the monitoring of proliferating CD4+ T cells subpopulations within cultures of blood mononuclear cells following in vitro stimulation with antigen. A newly established approach to measure the DNA content simultaneously with 3 cell surface markers allowed not only to determine the relative frequency of CD4+ T cell subsets, but also to quantify their mitotic activity, based on the proportion of cells in the DNA synthesis (S) and G_2/mitosis (G_2/M) phase. The results show that a cell cycle analysis of each gated CD4+ subpopulation could be accurately delineated. This is mainly due to a well-resolved detection of the G_0/G_1 peak following DAPI staining [11], which usually is superior to other DNA intercalating fluorescent dyes.

The major question to be answered by this approach was whether such a simple single cell assay would allow the identification of a candidate peripheral T cell subset, preferentially responding to an allergen. We therefore focussed on CD4+ CD7– T cells, which represent the fraction containing skin-infiltrating T cells [7, 12]. Finally, CLA was chosen based on the finding that skin-infiltrating lymphocytes preferentially express CLA [3] and that CLA+ T cells expand following in vitro activation with a dermatitis-relevant allergen [13, 14]. A combination of CD7 and CLA for the analysis of allergen-responsive T cells has not been previously analyzed.

Cell cycle analysis of freshly isolated CLA+ T cells from both healthy and atopic subjects showed that these cells were preactivated in vivo as compared to CLA– T cells, since a substantial portion of the cells were already in the S + G_2/M phase of the cell cycle. Atopic donors tended to have more proliferating CLA+ cells, which is in concordance with previous reports showing higher HLA-DR and/or CD25 expression on, as well as higher spontaneous proliferation of, freshly isolated CLA+ cells [6, 15, 16].

Following in vitro activation with DerP, CLA+ T cells from AD patients, but not CLA– T cells, responded by increasing their mitotic activity. Consequently, the selective proliferation of atopic CLA+ T cells was associated with a higher relative frequency of CLA+ T cells after 7 days of culture. Also T cells from healthy donors reacted to DerP stimulation, but less vigorously than atopic T cells and without any preference regarding CLA expression. Therefore, the relative distribution of CLA+ and CLA– T cells in healthy donors after 7 days of DerP activation was not changed, due to the fact that both subsets proliferated similarly. TT activation induced similar proliferative responses in CLA+ and CLA– T cells, irrespective of CD7 expression (data not shown). Thus, as expected, no change in relative frequencies of these T cell subsets was observed despite proliferation of all cells. Altogether, our data, obtained at the single cell level, confirm previous reports and suggest that CLA+ T cells from AD patients respond to a clinically relevant allergen [5, 6] and expand [13, 14]. In addition to previous reports, our single cell analysis, however, clearly demonstrates that the selective induction of proliferation in AD patients following DerP activation was restricted to those CLA+ T cells which lack CD7 expression (data not shown). It is not clear why the absence of CD7 seems to favor the infiltration of T cells into the skin. CD7 is thought to be involved in signal transduction [17] and in adhesion [18]; further investigation, however, is hampered by the fact that the

natural ligand is still unknown. The loss of CD7 has been associated with a maturational stage of proliferating cells [19] and thus, as CLA, determines a subset of circulating mature T cells. The data presented here show that the frequency of CD7-cells was lower in AD patients, which has not been described so far [7, 12]. Since the absence of CD7 on CD4+ cells has been shown to be associated with skin-homing abilities [8, 12], our data might indicate that preactivation in vivo might have resulted in an enhanced migration of these cells into the inflamed skin of our patients. However, the reduction of CD7– cells was only evident within the CLA–, but not within the CLA+ subset. This was unexpected since cells migrating into inflamed skin are expected to express CLA. A recent study, however, demonstrated that in acute inflammatory reactions, skin-infiltrating T cells are not necessarily CLA+, but are comprised of different subsets [20].

In conclusion, a newly established assay to monitor the expression of three cell surface molecules and the corresponding DNA content has enabled us to demonstrate that AD patients allergic to DerP and having positive atopy patch tests, show higher frequencies and enhanced proliferation of CD7–CLA+CD4+ T cells compared with healthy controls. Importantly, these changes were not observed using TT as an antigen. This simple assay identifies allergen-reactive T cells within the CD7–CLA+ T cell subset at the single cell level. Because of its simplicity, further use of such an assay may be helpful as an objective parameter in double-blind, placebo-controlled allergen challenges in AD patients.

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