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Calcium Homeostasis Change in $CD4^+$ T Lymphocytes from Human Peripheral Blood during Differentiation *in vivo*

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Abstract—Resting naïve $CD4^+CD45R0^-CD45RA^+$ T cells are sensitive to ionomycin. In contrast, resting $CD4^+CD45RA^-CD45R0^+$ memory T cells show resistance to this Ca^{2+} ionophore. In the present study, the ability of activated T lymphocytes to respond to ionomycin during the transition from naïve precursors into memory T cells has been analyzed. Activated $CD4^+CD45RA^+CD45R0^+$ T cells are always present both in human peripheral blood (HPB) and in the ionomycin-resistant (IR) fraction. Therefore, some activated T cells are resistant toward the Ca^{2+} ionophore. CD69 molecules are markers of the very early stage of T cell activation. However, $CD4^+CD69^+$ T cells have never been found in the IR fraction. Thus, the majority of $CD4^+$ T lymphocytes at the early stage of activation are ionomycin-sensitive cells. The proportion of $CD4^+CD25^+$ T cells did not differ significantly in HPB and in the IR fraction. The presence of $CD4^+CD25^+$ T lymphocytes in the IR fraction reflects changes in the Ca^{2+}-signaling pathway at this differentiation step of activated cells. Depending on the expression level of CD25 molecules, the population of $CD4^+CD25^+$ cells is divided in T-regulatory ($CD25^{high}$) and proliferating ($CD25^{low}$) subpopulations. The action of ionomycin results in a decrease in the portion of the $CD4^+CD25^{low}$ T-cells, but it leads to an increase in the proportion of the $CD4^+CD25^{high}$ T lymphocytes. Consequently, greater portion of $CD4^+CD25^{high}$ T lymphocytes and smaller portion of $CD4^+CD25^{low}$ T cells are IR cells. Expression of HLA-DR molecules can be used as the marker for the late activation step. The IR fraction is significantly rich in $CD4^+$HLA-DR^+ T lymphocytes in comparison to the blood of the same donor. The link between different differentiation steps of $CD4^+$ T-lymphocytes and alterations in calcium ion homeostasis is discussed.

Key words: ionomycin, human peripheral blood, $CD4^+$ memory T cells

Human blood contains naïve T cells and memory T cells, which differ functionally and phenotypically [1]. All memory T cells occur as a result of differentiation of antigen-activated naïve precursors during normal development of the primary immune response *in vivo* [2]. In the case of human $CD4^+$ T lymphocytes, the isoforms of the CD45 molecule can be used to distinguish between naïve T cells and memory T cells. As a rule, $CD4^+CD45RA^-$-$CD45R0^+$ T lymphocytes are memory T cells and, respectively, $CD4^+CD45RA^+CD45R0^-$ are naïve T cells. Such classification is based exclusively on the ability of $CD4^+CD45RA^-CD45R0^+$ T cells, but not the naïve T lymphocytes, to respond intensively to repeated contact with the antigen *in vitro* [3, 4]. Rapid and intense response of memory T cells to specific antigen is their most important functional difference from the naïve precursors.

Resting $CD4^+$ memory T lymphocytes also differ from the naïve precursors in the system of intracellular signaling, resulting in resistance to the action of Ca^{2+} ionophores [5-8]. The ionomycin-resistant (IR) population of human $CD4^+$ T cells constitutes the main portion of the resting naïve $CD4^+CD45R0^-CD45RA^+$ T lymphocytes [8]. The majority of IR $CD4^+$ T cells are resting $CD4^+CD45RA^-CD45R0^+$ memory T lymphocytes. In this connection a quite logical question arises: at which differentiation stage of activated naïve $CD4^+$ T lymphocytes *in vivo* does the reconstruction of the system of Ca^{2+} homeostasis of the T cells leading to their resistance to ionomycin occur? To answer this question, populations of *in vivo* activated T lymphocytes from healthy donors at distinct differentiation stages have been analyzed for their resistance to low doses of ionomycin.

Abbreviations: HPB) human peripheral blood; IR) ionomycin-resistant cells; PE) phycoerythrin; HLA-DR) class II human major histocompatibility complex antigen; FITC) fluorescein-isothiocyanate; Cy5PE) cyanide 5–phycoerythrin complex; mAb) monoclonal antibodies; PBS) phosphate-buffered saline; FCS) fetal calf serum; Con A) concanavalin A; PHA) *Phaseolus vulgaris* agglutinin; PMA) phorbol 12-myristate 13-acetate.

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MATERIALS AND METHODS

Materials. Hanks medium, brefeldin A, fetal calf serum (FCS), phosphate-buffered saline (PBS), dimethylsulfoxide (DMSO), phorbol 12-myristate 13-acetate (PMA), concanavalin A (ConA), *Phaseolus vulgaris* agglutinin (PHA), and culture plastics were purchased from ICN (USA), ionomycin and heparin from Calbiochem (Switzerland), and Ficoll-Paque from Pharmacia (Sweden). All mAb used in this study were obtained from Immunotech (France).

Donors. Blood from donors whose immune status parameters determined with mAb combination for molecules CD3/CD19, CD3/CD4, CD3/CD8, CD45/CD14, (CD16 + CD56)/CD3, CD3/CD25, and CD3/HLA-DR corresponded to the data for normal healthy adults was used [9].

Isolation of cell population. Mononuclear fraction was isolated from donor's peripheral blood by centrifugation on Ficoll-Paque. Isolated cells were washed with PBS containing 1% FCS. Cells were incubated for 10 min with ionomycin at 37°C in Hanks medium containing 1% FCS. After incubation with ionomycin, cells were washed with PBS containing 5% FCS to remove the Ca^{2+} ionophore. Ionomycin-sensitive and IR T cells were separated on Ficoll-Paque (d = 1.077 g/cm^3). As a rule, ionomycin-sensitive cells did not retain their viability after treatment with the ionophore. IR cell fraction was harvested from the Ficoll-Paque surface and was washed twice with Hanks medium containing 1% FCS.

Flow cytometry. T-Cell phenotype was determined using mAb for the following cell surface markers CD4, CD25, CD26, CD29, CD38, CD45RA, CD45R0, CD62L, CD95, and HLA-DR labeled with fluorochromes cyanide 5–phycoerythrin complex (Cy5PE), phycoerythrin (PE), or fluorescein-isothiocyanate (FITC), respectively. Cells were analyzed using an EPICS ELITE flow cytometer (Coulter, USA). For correct exclusion from the analysis of all particles that did not correspond in size and granularity to viable lymphocytes, appropriate logical limitations were introduced in the histograms at low-angle and 90° light scatter. Mathematical analyses were performed using EXPO-32 software (Beckman-Coulter, USA). Each sample for analysis contained at least 10^4 cells.

RESULTS

Activated CD4$^+$ T lymphocytes comprise an insignificant portion in the peripheral blood of healthy donors. This is the main methodological difficulty in the investigation of the differentiation of these cells. (Activated CD4$^+$ T lymphocytes usually constitute less than 10% of the total number of CD4$^+$ T lymphocytes in the peripheral blood of healthy donors [9].) Due to this fact, it is necessary to screen a large number of healthy donors and provide an immune status analysis for each of them.

Changes of calcium ion homeostasis in CD4$^+$ T lymphocytes were monitored based on their sensitivity to 2 μM ionomycin. Every CD4$^+$ T lymphocyte that retains its viability after exposure to ionomycin is regarded as an IR cell, whereas all CD4$^+$ T lymphocytes dying under these conditions are taken as sensitive to this Ca^{2+} ionophore [8].

A definitive phenotypical sign of differentiation of the human resting naïve CD4$^+$CD45R0$^-$CD45RA$^+$ T lymphocytes into resting memory T-cells is the appearance on the cell surface of CD45R0 molecules instead of the isoform CD45RA [3, 4]. This allows defining three subpopulations of human CD4$^+$ T lymphocytes: resting naïve CD45RA$^+$CD45R0$^-$ T cells, resting CD45RA$^-$-CD45R0$^+$ memory T cells, and activated CD45RA$^+$-CD45R0$^+$ T cells. (Mature CD4$^+$CD45RA$^-$CD45R0$^-$ T lymphocytes are not found in human peripheral blood (HPB) [4].) All activated CD4$^+$CD45RA$^+$CD45R0$^+$ T lymphocytes appear during antigen stimulation of naïve cells *in vivo*. In the present study, the definitions "CD45RA$^+$" and "CD45R0$^+$" mean all CD4$^+$ T cells labeled with mAb of the appropriate specificity. The results of alternative analyses of CD4$^+$ T lymphocytes using mAb to CD45RA and to CD45R0 molecules fully correspond to each other.

After adding 2 μM ionomycin to the HPB lymphocytes the proportion of naïve CD4$^+$CD45RA$^+$ T cells significantly decreases from 60-70% to 5-10% in comparison to the initial population of the same donor. Simultaneously, the fraction of CD4$^+$CD45R0$^+$ cells is enriched by 90% or more. We never observed complete disappearance of all CD4$^+$CD45RA$^+$ T-cells from the IR fraction in all conducted experiments (Fig. 1). The action of low doses of ionomycin on human CD4$^+$ T cells corresponds well with earlier published results [8]. Incomplete disappearance of CD4$^+$CD45RA$^+$ T cells from the IR fraction provides evidence that a portion of the activated CD4$^+$CD45RA$^+$CD45R0$^+$ T lymphocytes is resistant to ionomycin. Similar results were obtained for more than 70 donors. Thus, a change in calcium homeostasis in human CD4$^+$ T lymphocytes is observed during the antigen-dependent differentiation of memory T cells from activated naïve precursors.

The appearance of CD69 molecules is used as the main phenotypical sign of the earliest (first hours) activation stage of naïve CD4$^+$ T lymphocytes [10-12]. As a rule, in the blood of healthy donors it is not possible to detect more than 1-4% of CD4$^+$CD69$^+$ T cells (data not shown), and this corresponds well with data described in the literature [10-12]. However, even such a small portion of CD4$^+$CD69$^+$ T cells has never been observed among the lymphocytes from the IR fraction independently of their initial content in HPB. Similar results were obtained

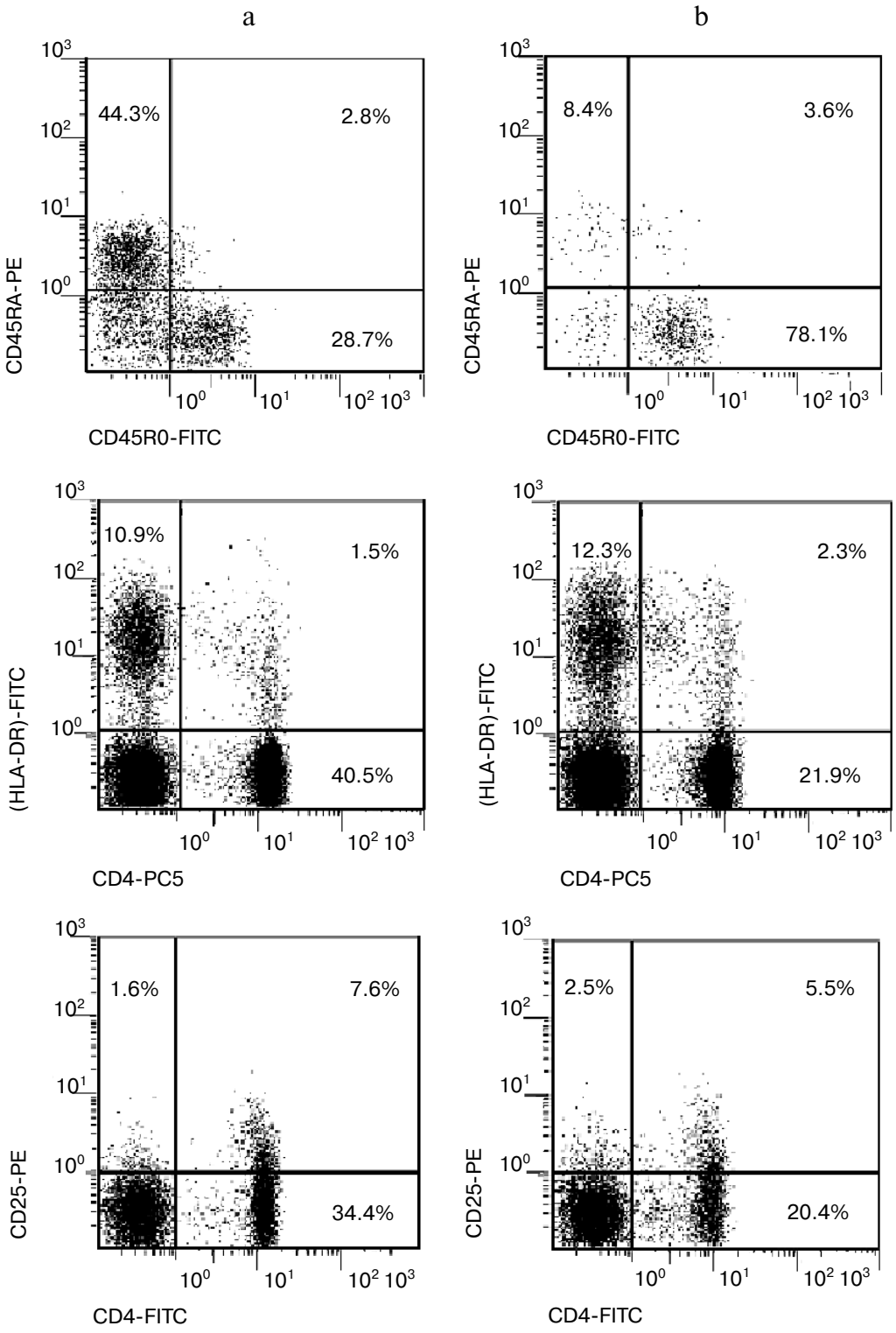

Fig. 1. Cell content analysis of human $CD4^+$ T-lymphocytes before (a) and after (b) exposure to ionomycin, using flow cytometry with three color staining. The abscissa axes show the logarithm of fluorescence intensity of FITC, the ordinate axes show the logarithm of fluorescence intensity of PE. Histograms show the expression of CD45RA, CD45R0, CD25, and HLA-DR molecules on the surface of $CD4^+$ T cells, stained with Cy5PE-labeled mAb to CD4. The data in the upper right corner of each quadrant represent mean value cell portion having given phenotype in the population. This figure shows one representative result of several (>10) independent experiments carried out on HPB of healthy donors.

for ten donors. It is most likely that all $CD4^+$-$CD45RA^+CD45R0^+CD69^+$ T lymphocytes are ionomycin-sensitive cells.

The appearance of CD25 molecules on the surface of $CD4^+$ T lymphocytes is usually considered as a specific sign of intensive antigen-specific proliferation of activated T-cells [13]. The absence of mature $CD4^+$-$CD69^+CD25^+$ T cells in the blood of healthy donors makes it possible to correctly distinguish a given activation stage of T cells from the previous one. Without counting in HPB the traces of $CD4^+CD45RA^+CD25^+$ "thymic emigrants", CD25 molecules are never found on resting $CD4^+CD45RA^+CD45R0^-$ T cells [13-15]. Most often, the action of ionomycin on $CD4^+$ T cells did not significantly change the portion of $CD4^+CD25^+$ T lymphocytes (Fig. 1). For several tested donors we did observe both a small decrease in the portion of this population in the IR fraction and a similar small increase in this portion. (It is hard to assume the existence of two healthy donors with absolutely equal content of activated T cells at each stage.) The results correspond well with the data described in the literature on the absence of changes in the binding pattern of interleukin-2 with its receptor after the interaction of T cells with low doses of ionomycin [16]. Most likely, there are in the HPB approximately equal portions of ionomycin-sensitive and IR $CD4^+CD25^+$ T cells at this stage of activation of $CD4^+$ T lymphocytes *in vivo*. These data suggest that the beginning of calcium ion homeostasis reconstruction occurs at this activation stage of human $CD4^+$ T lymphocytes *in vivo*.

The population of human $CD4^+CD25^+$ T cells is heterogeneous in its functional properties and phenotypical signs. It includes the population of proliferating $CD4^+CD45RA^+CD45R0^+CD25^{low}$ T cells and "regulatory" (TREG, T regulatory) $CD4^+CD45R0^+CD25^{high}$ T lymphocytes [13-15]. These cell populations are usually identified on the basis of cumulative quantitative analysis of expression of CD38, CD62L, CD95, and HLA-DR molecules [13-15]. The same were used for the lymphocyte analysis in HPB and IR fractions. In such case, the correct choice of the analysis area (Figs. 2 and 3) provides the most accurate description of human $CD4^+CD25^+$ T lymphocytes subpopulations. According to our results, phenotypical signs of T lymphocyte subpopulations $CD25^{high}$, $CD25^{low}$, and $CD25^-$ (Fig. 4) fully correspond to the existing criteria for these types of T cells [13-15].

Subpopulations of $CD4^+CD25^+$ T cells exhibit different sensitivity to ionomycin. In the IR fraction the proportion of $CD4^+CD25^{high}$ T cells is significantly increased in comparison to the full blood of the same donor, whereas the content of $CD4^+CD25^{low}$ T lymphocytes is decreased. Thus, the majority of $CD4^+CD25^{high}$ T lymphocytes are IR cells. Complete disappearance of $CD4^+CD25^{low}$ T lymphocytes in the IR fraction has never been observed. This suggests that also among the $CD4^+CD25^{low}$ T lymphocytes cells are present that differ in their ionomycin sensitivity. The change in the content of subpopulation of $CD4^+CD25^+$ T lymphocytes after ionomycin action was directly dependent on the initial proportion of T cells with given phenotype in the donor blood. However, the enrichment of the IR fraction with $CD4^+CD25^{high}$ T cells and simultaneous lowering of the proportion of $CD4^+CD25^{low}$ T lymphocytes was observed most frequently.

In comparison to the $CD4^+CD25^{high}$ T lymphocytes from HPB, the IR population (Fig. 3) was always enriched with cells showing high level of expression of HLA-DR, CD95, and CD38 molecules (increase in MEAN from 11.7 to 20.0, from 3.2 to 5.0, and from 2.7 to 3.4, respectively). Simultaneously, the IR $CD4^+CD25^{high}$ T lymphocytes are characterized by lower portion of $CD62L^+$ cells (Fig. 3) and significant decrease in the level of expression of these molecules (decrease of MEAN from 4.4 to 3.5). Hence, the portion of $CD4^+CD25^{high}$ T cells from HPB that do not exhibit the HLA-DR, CD95, and CD38 molecules but highly express the CD62L molecule is sensitive to ionomycin. Absolute values of proportions of all tested populations of $CD4^+CD25^{high}$ T cells were to a great extent dependent on their initial content in HPB of a particular donor, but qualitatively, a similar pattern of observed dependencies was observed in all (>10) cases. Compared to the $CD4^+CD25^{low}$ T cells from HPB, the IR fraction was enriched with cells showing levels of expression of $CD95^+$ molecules (increase in MEAN from 2.4 to 2.7) (Fig. 3). At the same time, the IR fraction of $CD4^+CD25^{low}$ T lymphocytes demonstrated significant decrease in the portion of $HLA\text{-}DR^+$ and $CD62L^+$ cells and lowering of the expression level of these molecules (decrease in MEAN from 14.9 to 11.9 and from 3.8 to 2.9, respectively). Among the IR $CD4^+CD25^{low}$ T lymphocytes, a decrease in $CD38^+$ cell portion (Fig. 3) occurred due to the lymphocytes showing low expression level of this marker (change of MEAN from 2.3 to 2.6).

Appearance of HLA-DR molecules on the $CD4^+$ T lymphocytes is thought to be a sign of late activation stage of these cells [17, 18]. The existence of $CD4^+$-$CD25^+HLA\text{-}DR^+$ T cells in HPB does not permit complete separation of this stage of activation of T lymphocytes from the previous one. Most $CD4^+HLA\text{-}DR^+$ T cells from HPB usually have the phenotype $CD4^+$-$CD45RA^-CD45R0^+$. The results correspond well with those described in the literature [17, 18]. The IR fraction always contained a higher portion of $CD4^+HLA\text{-}DR^+$ T cells than HPB of the same donor. Comparable results were obtained for more than 20 donors. Most probably, *in vivo* in the late stages of activation during normal development of primary immune response the main portion of these human $CD4^+$ T lymphocytes change qualitatively their pattern of intracellular Ca^{2+}-regulation and become resistant to low doses of ionomycin.

The marker of the very late *in vivo* activation stage of human T lymphocytes is CD29 (VLA, very late antigen)

molecule. It is frequently used as a supplemental marker of resting $CD4^+CD45RA^-CD45R0^+$ memory T-cells [19-21]. The IR fraction contains significantly more $CD4^+CD45R0^+CD29^+$ T cells than does the respective population of the same donor (Fig. 4). At the same time, a significant portion (approximately almost a half) of $CD4^+CD45RA^-CD45R0^+$ T lymphocytes from the IR fraction lacks this antigen. There are currently no experimental results described in the literature on the interdependency between CD29 and CD45R0 molecules. The degree of enrichment of the IR fraction with $CD4^+$-$CD45R0^+CD29^+$ T lymphocytes depends on the initial proportion of the cells with this phenotype in the HPB; however, in all tested samples the CD29 molecules were expressed on a smaller portion of IR $CD4^+CD45RA^-$-$CD45R0^+$ T cells. Usually, only in the blood of chronic infection patients the $CD4^+CD45R0^-CD29^+$ T cells are observed in detectable quantities [19]. Comparable results were obtained for the lymphocytes from more than ten donors. Most probably, at the late activation stage the greater portion of $CD4^+CD45R0^+CD29^+$ T lymphocytes consists of IR cells, and, possibly, some por-

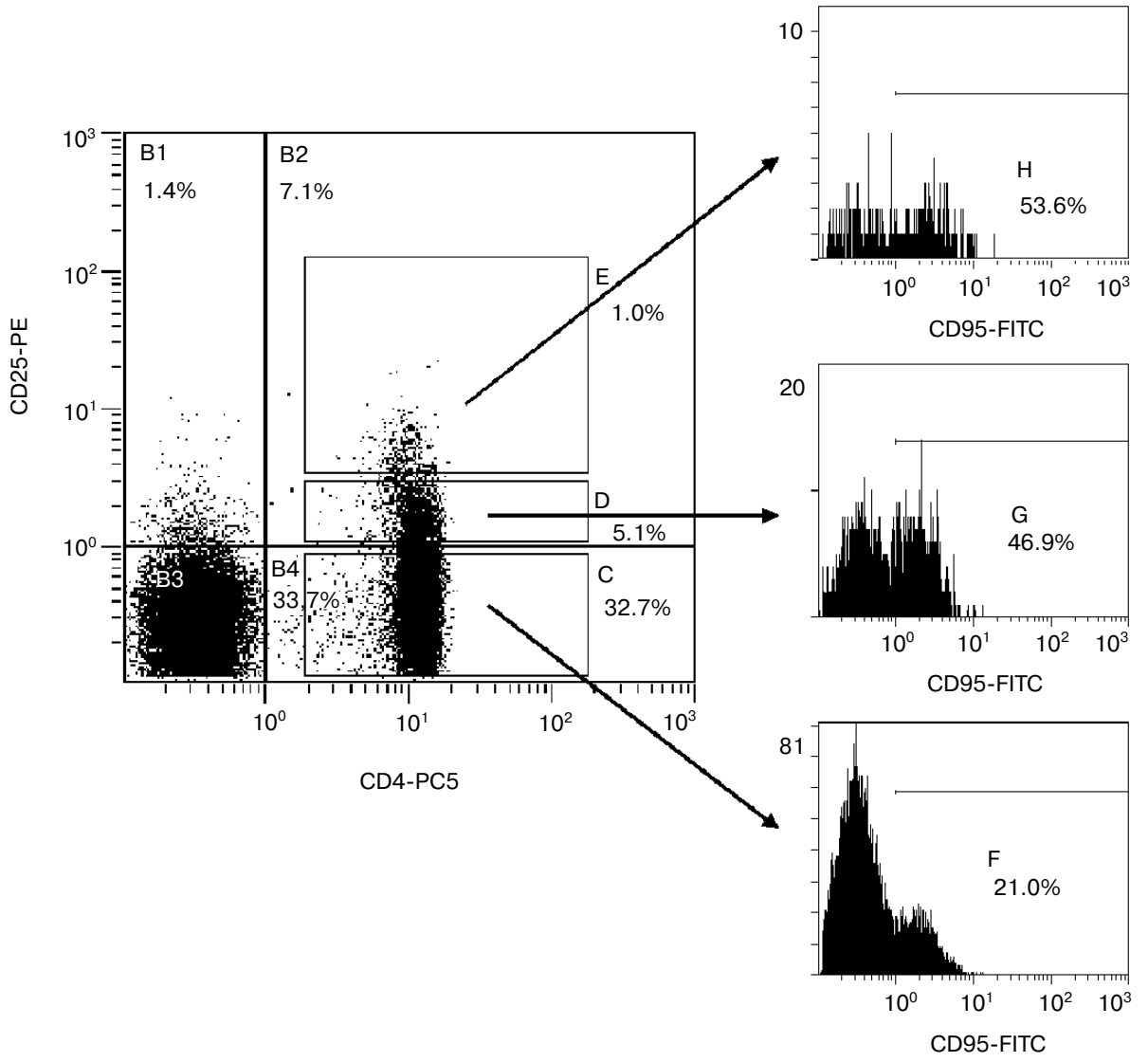

Fig. 2. Algorithm used for flow cytometry analysis of subpopulation of human $CD4^+CD25^+$ T-lymphocytes. The histogram shows the chosen zones of analysis of $CD4^+$ T-cells with high level of expression of CD25 molecules ($CD25^{high}$), zone "E"; $CD4^+$ T-cells with low level of expression of CD25 molecules ($CD25^{low}$), zone "D"; and $CD4^+CD25^-$ T cells, zone "C". Arrows from each zone of analysis show single-parameter histograms of distribution of $CD95^+$ molecules (as an example) on the $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, and $CD4^+CD25^-$ T-lymphocytes, respectively. The numbers on histograms "H", "G", and "F" show the portion of $CD95^+$ cells in the respective subpopulation. The distribution of CD38, CD62L, and HLA-DR molecules on the surface of $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, and $CD4^+CD25^-$ T-cells has similarly been evaluated using same areas of analysis. Ionomycin-resistant $CD4^+$ T-lymphocytes were analyzed using the same method.

tion of $CD4^+CD45R0^+CD29^-$ T lymphocytes is ionomycin sensitive. The reason for this phenomenon remains obscure.

Appearance of $CD4^+CD26^+$ T lymphocytes is usually regarded as a sign of specific activation of T cells, although it remains unknown at which particular stage this marker occurs. Normally, almost a quarter of $CD4^+$ T cells from HPB express CD26 molecules [22]. In the IR fraction the portion of $CD4^+CD26^+$ T lymphocytes is always significantly higher than in the HPB of the same donor, and it usually represents more than half of the respective population (Fig. 4). Comparable results were obtained for five donors. Most likely, a significant portion of human $CD4^+CD26^+CD45RA^-CD45R0^+$ T lymphocytes is IR.

Thus, the resistance to low doses of ionomycin and changes in calcium ion homeostasis in $CD4^+$ T cells appears at very late differentiation stages of *in vivo* activated human $CD4^+$ T lymphocytes. To the full extent this property belongs to the long-lived resting human $CD4^+CD45RA^-CD45R0^+$ memory T lymphocytes.

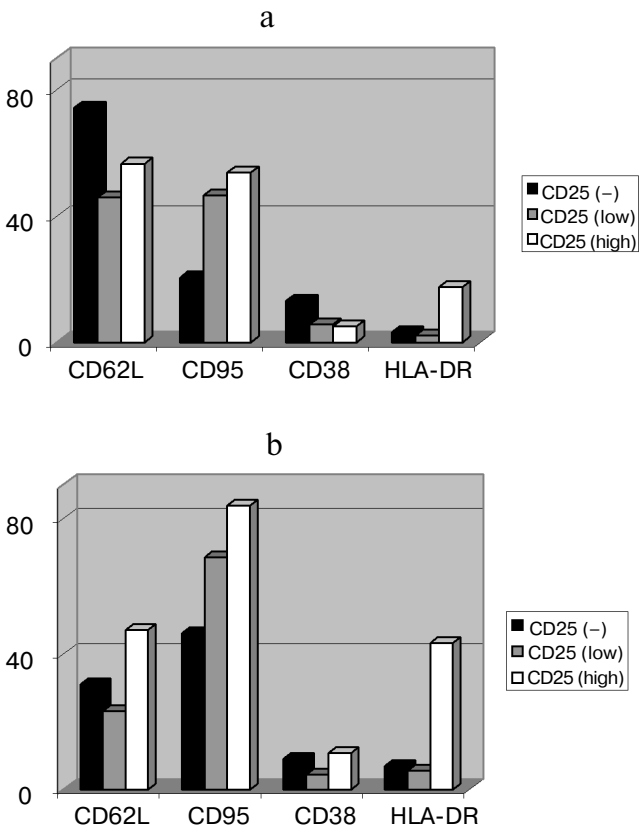

Fig. 3. Subpopulation analysis of human $CD4^+$ T-lymphocytes before (a) and after (b) cell exposure to ionomycin, using flow cytometry with three color staining and the developed algorithm (see Fig. 2). The ordinate axes show the meaning of cell portion in the appropriate population. This figure shows one representative result of several (>10) independent experiments carried out on HPB of healthy donors.

DISCUSSION

The cell cycle of T cells in the body can be subdivided into two phases. A foreign antigen independent differentiation stage of T cells results in the appearance in the bloodstream of mature naïve T lymphocytes, each of them being able to respond only to its "own" antigen. During primary response, antigen-stimulated T cells undergo further differentiation. Here we present a study of *in vivo* calcium ion homeostasis changes in $CD4^+$ T lymphocytes during the primary immune response. The complete answer to this question is fully dependent on the correct description of the phenotype of $CD4^+$ T lymphocytes at each stages of activation and differentiation of memory T cells from naïve precursors. Unfortunately, not all stages of activation and differentiation of $CD4^+$ T lymphocytes have been described equally completely.

As can be expected from a system working by the principle of clonal selection, each antigen activates only a very small part of the total population of T lymphocytes [23]. To fulfill their functions, naïve T lymphocytes must proliferate (since only several clones containing a small number of cells respond to antigen, it is necessary to multiply to develop an intensive response). For this reason, most studies on the mechanisms of T lymphocyte activation have been conducted *in vitro* using mitogenic lectins (ConA and PHA), mixtures of PMA with Ca^{2+} ionophores (A23187 or ionomycin), or mAb to complex CD3–TKP [24-26]. Each step of specific T cell activation occurs *in vivo* in a particular microenvironment, which is hardly possible to reconstruct in experimental conditions. (If even the presence of erythrocytes in the population significantly changes the intensity of T lymphocytes response to the stimulation with mAb against CD3 [27], then the degree of "participation" of different types of immunocompetent cells in the development of T lymphocyte response to polyclonal stimuli is difficult to describe reliably.) Correspondence of the mechanisms depicted *in vitro* in model systems with the reactions proceeding in the organism *in vivo* is widely discussed; however, everybody understands how risky it is to expect full identity of the processes that are switched on *in vivo* in cells by polyclonal stimuli and antigens.

The accepted phenotype of naïve human $CD4^+$ T cells may be described as $CD45RA^+CD45R0^-CD25^-$-$CD29^-CD62L^+CD69^-CD95^-HLA\text{-}DR^-$. (In the given formula, only those differentiation clusters are mentioned that have been analyzed in the present study.) Most of the resting naïve $CD4^+CD45RA^+CD45R0^-$ T lymphocytes, as well as the thymic T-cells, are sensitive to the action of Ca^{2+} ionophores [8, 28]. A large increase in $[Ca^{2+}]_i$ always occurs most sharply in the first minutes of T lymphocytes stimulation and reaches a maximum in 5-10 min [7, 24-

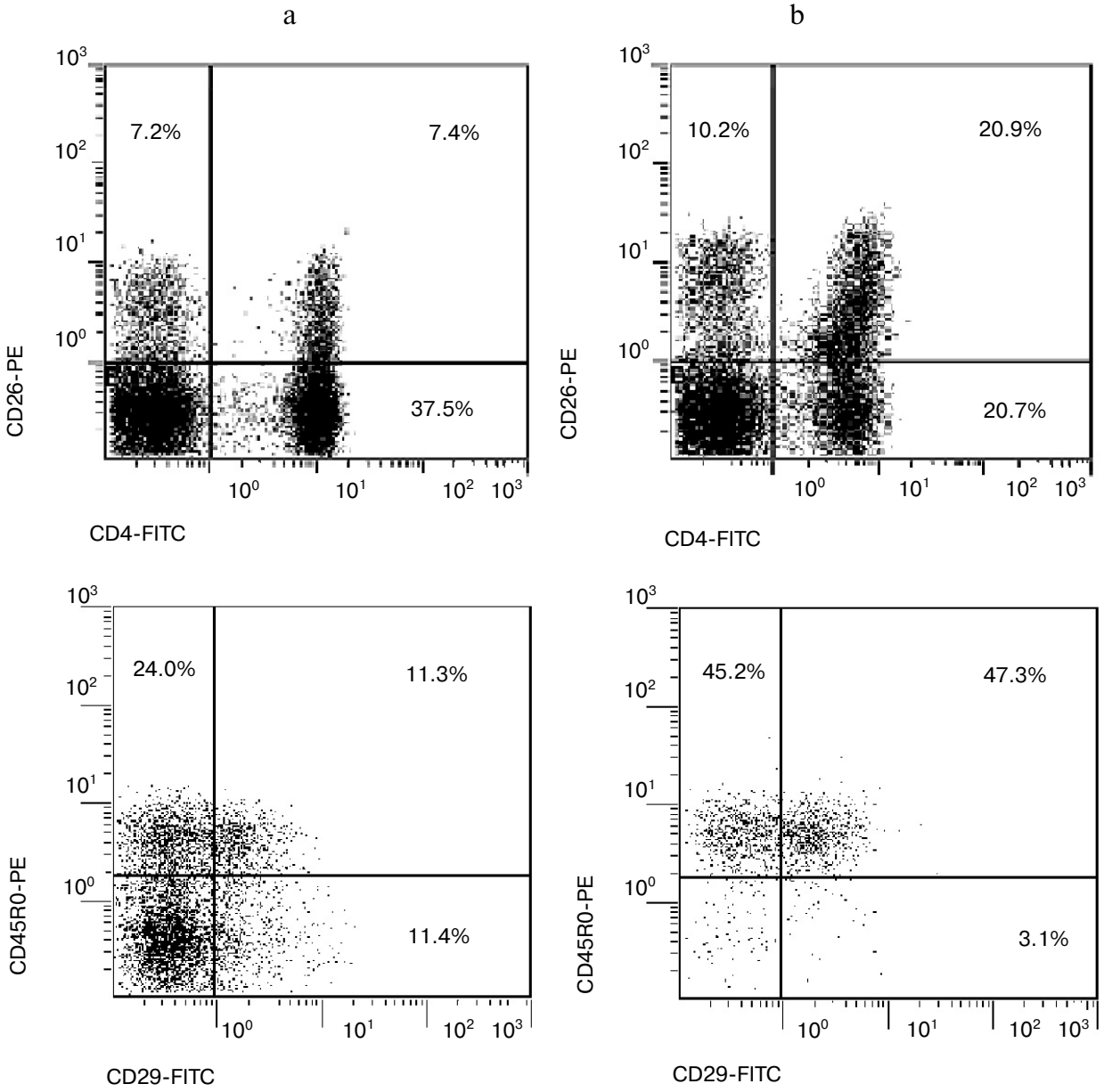

Fig. 4. Cell content analysis of human $CD4^+$ T-lymphocytes before (a) and after (b) exposure to ionomycin, using flow cytometry with three color staining. The abscissa axes show the logarithm of fluorescence intensity of FITC, the ordinate axes show the logarithm of fluorescence intensity of PE. Histograms show the expression of CD45R0, CD26, and CD29 molecules on the surface of $CD4^+$ T-cells, stained with Cy5PE-labeled mAb to CD4. The data in the upper right corner of each quadrant represent mean value cell portion having given phenotype in the population. This figure shows one representative result of several (>10) independent experiments carried out on HPB of healthy donors.

26, 29]. Therefore, sensitivity to the presence of calcium ions in the environment as well as to ionomycin is an obligate condition for induction of activation stage in naïve T lymphocytes.

The sensitivity of early activation steps (from the moment of the beginning of polyclonal stimulation until the appearance of CD25 molecules on the surface of T lymphocytes) to the presence of calcium ions in the surrounding medium as well as their dependency on the amplitude of $[Ca^{2+}]_i$ increase in response to stimuli is accepted by most researchers [24-26, 30, 31]. The duration of this activation step is estimated to last from 8 to 12 h. The same time needed for the exposition of CD69 molecules [10-12]. The appearance of CD69 molecules on the activated T cells is fully blocked by EGTA [32]. Among the lymphocytes in the IR fraction $CD4^+CD45R0^-CD45RA^+CD69^+$ T cells have never been observed. Probably, most human $CD4^+CD45RA^+$-

$CD45R0^{low}CD69^+$ T lymphocytes are ionomycin sensitive cells. Perhaps, prior to the exposition of CD25 molecules on the activated $CD4^+CD45R0^-CD45RA^+$ T lymphocytes, these cells proceed through several steps of initial activation that are not accompanied by qualitative changes in sensitivity to ionomycin. Hence, for the initiation of activation the ionomycin sensitivity of T cells correlates with their highest dependency on Ca^{2+} concentration in the medium. Since calcium ions are always present in the organism, resistance to ionomycin is simply impossible to occur at this stage of activation.

One of the key steps in the activation process is assumed to be the appearance of $CD4^+CD25^+$ T cells [13-15]. The influence of calcium ion removal from the medium by EGTA on the expression of CD25 molecules by T cells was strongly dependent on the moment of addition of the chelator [33]. Usually, the earlier the activation step chosen for the removal of calcium ions from the cell incubation medium, the higher the effect it realized on the expression of CD25 molecules [25, 31]. Perhaps, *de novo* induction of CD25 molecule synthesis absolutely requires calcium ions [16, 32]. The expression of CD25 molecules on activated T cells that have already accomplished the induction step is much less dependent on EGTA [34, 35]. For very late activation stages, no effect of EGTA on $CD25^+$ T cells or binding pattern of IL-2 with its receptor has been noted [16, 34, 35]. This suggests significant decrease in the dependency on surrounding calcium ion concentration of this and, probably, subsequent T cell activation steps.

The portion of $CD4^+CD25^+$ T cell subset in the IR fraction differs insignificantly from that in the blood of the same donor. This suggests that IR T cells appear having rebuilt their calcium ion homeostasis exactly at this *in vivo* activation stage of $CD4^+$ T lymphocytes. There are proliferating and regulatory $CD4^+$ T lymphocytes in the population of $CD4^+CD25^+$ T cells from HPB [13-15]. It remains unknown whether there are mutual interconversions between $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ populations. Subpopulations of these cells demonstrate different sensitivity to Ca^{2+} ionophore. Most $CD4^+CD25^{high}$ T lymphocytes are IR cells. Compared to the $CD4^+$-$CD25^{high}$ T lymphocytes from HPB, the IR fraction is always enriched in $HLA\text{-}DR^+$, $CD95^+$, and $CD38^+$ cells, but at the same time, it contains fewer $CD62L^+$ cells. Compared to the $CD4^+CD25^{low}$ T cells from HPB, the IR population is enriched with $CD95^+$ having high expression levels for this molecule. We observed a significant decrease in the portion of $HLA\text{-}DR^+$ and $CD62L^+$ cells in the IR fraction of $CD4^+CD25^{low}$ T lymphocytes. This suggests that T cells with altered calcium ion homeostasis occur at the stage of intensive proliferation of activated $CD4^+$ T lymphocytes, but represent a smaller portion of this population. In the population of regulatory $CD4^+CD25^{high}$ T lymphocytes, the portion of IR cells is significantly higher. There is currently no evidence on the dependency of these populations on the presence of calcium ions in the medium.

Expression of HLA-DR is an accepted sign of the late activation stage of $CD4^+$ T lymphocytes [17, 18]. There have been no special studies on the role of calcium ions in the expression of the HLA-DR molecule; however, minimal involvement of calmodulin-dependent processes (cAMP- or cGMP-dependent protein kinases) in the expression has been noted [36]. The portion of IR $CD4^+HLA\text{-}DR^+$ T cells was always higher than in the blood of the same donor. This suggests that at the late stages of *in vivo* activation most activated $CD4^+$ T cells change qualitatively their pattern of intracellular calcium ion regulation and become resistant to ionomycin.

The molecules of CD29 are frequently used as an additional marker for resting $CD4^+CD45RA^-CD45R0^+$ memory T cells [19-21]. There is no clear evidence on the dependency of its expression on the activation stage of $CD4^+$ T lymphocytes. The role of calcium ions in the expression induction is not known as well. The IR fraction is significantly enriched with $CD4^+CD45R0^+CD29^+$ T cells in comparison to the initial population in the donor's blood. However, the majority of IR $CD4^+CD45RA^-CD45R0^+$ T cells lacks this antigen. Perhaps, at this relatively late activation stage of $CD4^+$ T lymphocytes, there is a complete change of isoforms CD45RA to CD45R0. Probably, at this activation stage most $CD4^+CD29^+$ T lymphocytes become ionomycin resistant. There are currently essentially no studies on the comparative analysis of functional properties of $CD45R0^+CD29^-$ and $CD45R0^+CD29^+$ $CD4^+$ T cells.

An additional marker for the resting human $CD4^+CD45RA^-CD45R0^+$ memory T lymphocytes is $CD28^+$ cells [3, 4, 8, 9]. The action of ionomycin on the HPB lymphocytes is accompanied by significant enrichment of the population with $CD4^+CD45RA^-$-$CD45R0^+CD28^+CD29^+CD62L^{low}$ T cells. Different ionomycin sensitivity of $CD62L^{high}$ and $CD62L^{low}$ T lymphocytes [8] correlates well with the known classification of human $CD4^+$ T cell populations according to their migration pathways in the body [37]. There is clear evidence on different sensitivity for apoptosis of $CD28^-$ and $CD28^+$ cells for human $CD4^+$ T lymphocytes: the $CD4^+CD95^{high}CD28^-$ cells are thought to be the most committed for apoptosis [38]. According to the modern classification, phenotypically human IR $CD4^+$ T cells are most compatible with the memory T-cell population of the "central nucleus" [39]. Within this population of memory $CD4^+$ T-lymphocytes resting human T cells are represented that have undergone complete antigen-dependent differentiation during primary immune response and possess limited ability to migrate to highly endothelial venules.

Limited ability to respond to mitogenic doses of Con A has been shown for human IR T cells [40]. This corresponds well with the fact that the ionomycin sensitive

fraction of murine T cells contains the majority of spleen and lymphatic T lymphocytes responsive to Con A [7]. The inability of Con A to influence the level of $[Ca^{2+}]_i$ in IR T cells in the spleen of mice supports this evidence [9]. The age-related accumulation of memory T cells in the body and simultaneous decrease in the Con A-responsive cell fraction provides evidence on the inability of Con A to stimulate the memory T cells [41, 42].

IR T cells were described earlier for spleen and lymphatic nodes of mice [5-7]. Using laboratory animals gives a rare possibility to conduct experiments with the adoptive transfer technology: the isolated and characterized immune cell populations from donors and as a control, intact cells, are transferred to the naïve syngene recipients, where the functional properties of transferred cells are tested *in vivo*. (Clearly, it is not possible to get similar results on humans.) On the appropriate models only the isolated IR splenic T cells from healed or vaccinated mice have been able to transfer adoptively to the naïve recipients the resistance to consecutive inoculation with *Mycobacterium tuberculosis* and *Neisseria meningitidis* [43-45]. These data provide direct evidence on the ability of IR T cells to perform the main functions of the memory T lymphocytes, including recognition of infectious antigens as well as rapid and enhanced *in vivo* response leading to an effective recipient defense against the pathogen. Most of the antigen-specific murine T lymphocytes from spleen and lymphatic nodes are also located in the IR fraction of T cells [7].

Human IR T cells retain their ability to respond to the mixture of PMA and ionomycin [40]. For the isolated human $CD4^+CD45R0^+$ memory T cells, it was also shown that there is a significantly weaker dependency of proliferation and IL-2 production on the calcium ion

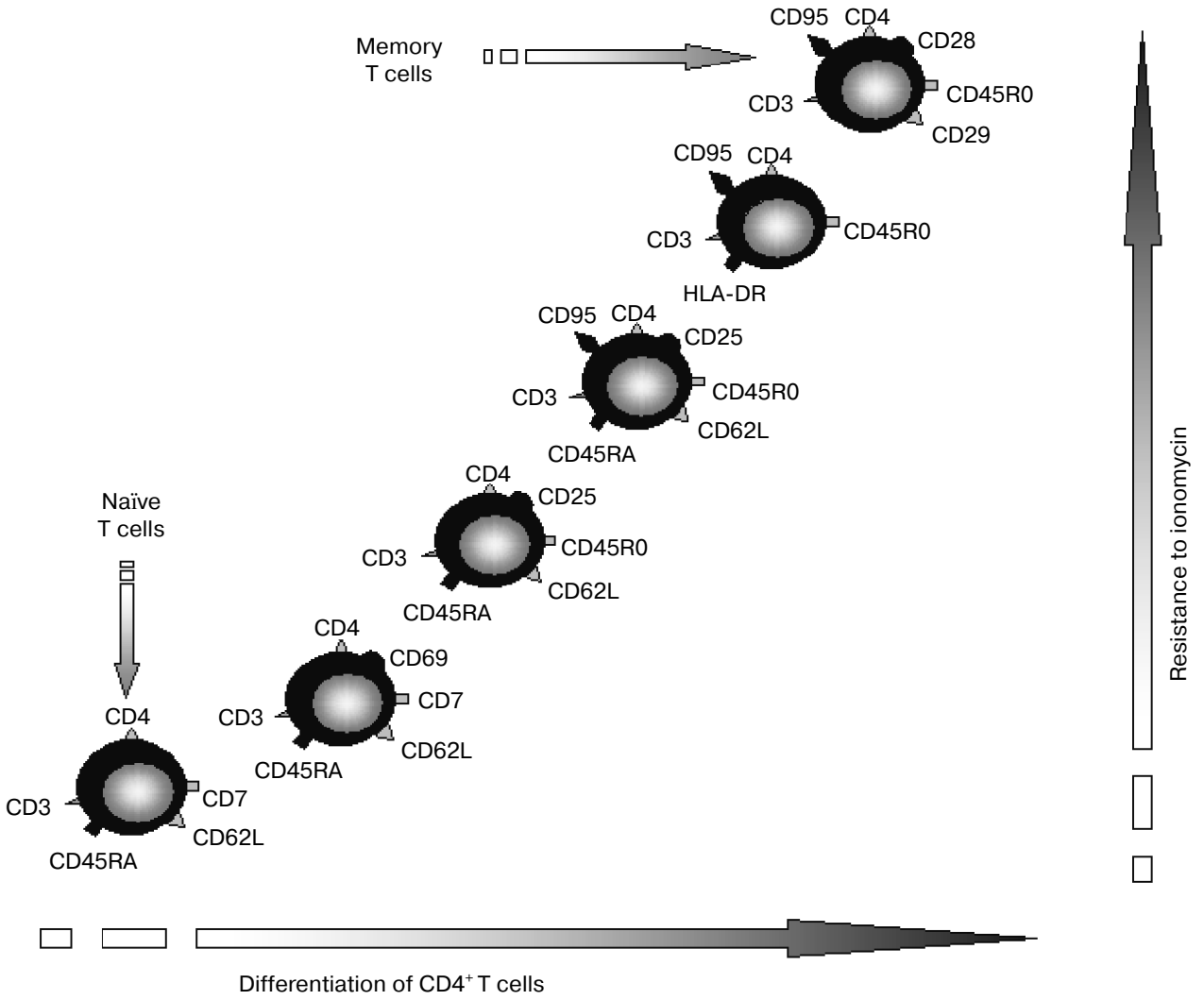

Fig. 5. Schematic diagram showing the appearance of the resistance to ionomycin of human $CD4^+$ memory T lymphocyte during antigen-dependent differentiation from naïve precursors by the development of primary immune response *in vivo*.

concentration in the medium on stimulation with mAb to CD3, compared to the naïve $CD4^+$ T cells from the same donor [46]. Therefore, the resistance to ionomycin of the memory T cells "does not prohibit" them from performing the most important functions of the memory T cells both *in vivo* and *in vitro*.

The experimental data are summarized in a scheme (Fig. 5) showing the changes in calcium ion homeostasis in $CD4^+$ T lymphocytes during their activation and differentiation *in vivo* parallel to the dependency of activation stage on the presence of calcium ions in the medium. This scheme is hypothetical, since for $CD4^+$ T cells not all steps of activation and differentiation *in vivo* are described equally comprehensively. Thus the discussion of the results might lead to conclusions on the sequence of particular activation stages of T cell activation that could become controversial after some time.

Calcium ions are always present in the body. They are needed for many biochemical reactions in the cell. During *in vivo* differentiation of activated T lymphocytes, the role of calcium ions changes constantly. All the resting naïve $CD4^+CD45RA^+CD45R0^-$ T cells are sensitive to low doses of ionomycin. Induction of the activation of naïve T lymphocytes always leads to an increase in $[Ca^{2+}]_i$. At this stage of T cell activation, they retain their sensitivity to ionomycin, since alternatively, their response to stimulation would be virtually impossible. Late stages of activation are less depending on the presence of calcium ions, and a portion of the activated cells becomes resistant to ionomycin. The resting $CD4^+CD45RA^-CD45R0^+$ memory T cells are completely resistant to ionomycin; however, this does not prevent them from performing their physiological functions related to the recognition of infectious antigen as well as rapid and enhanced response to their action *in vivo* leading to an effective recipient defense against the pathogen.

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