Ph.D.THESIS

IMPROVING THE FLOW CYTOMETRIC ENERGY TRANSFER METHOD WITH CELL-BY-CELL AUTOFLUORESCENCE CORRECTION: HOW TO INVESTIGATE THE ROLE OF DIFFERENT CD45 ISOFORMS IN T CELL SIGNALING

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I. INTRODUCTION

Protein-protein interactions are critical to most biological processes, extending from the formation of cellular macromolecular structures and enzymatic complexes to the regulation of signal transduction pathways. The cellular machinery is completely dependent on the exchange of signals, ions and nutrients between the extracellular environment and the interior of cells. Pre-existing and ligand-induced associations of membrane proteins are crucial to such processes.

CD45 protein tyrosine phosphatase is one of the most abundant glycoprotein expressed on immune cells. Although it has often attracted the attention of immunologists, the exact role of CD45, especially its different isoforms still remains unclear. It is presumed to play both positive and negative regulator in T cell signaling regulating the threshold of signal transduction.

In addition to functional assays, investigation of membrane topography is essential for determining the exact function of membrane proteins like CD45 tyrosin phosphatase. Flow cytometric fluorescence resonance energy transfer (FCET) method has been described for mapping the physical associations of various membrane molecules, and it provides quantitative measurements on thousands of individual cells, allowing convenient determination of the distribution of energy transfer efficiency in a large population with good statistics.

Contrarily to the relationship of CD45 and other molecules determined using invasive techniques, FCET provides an excellent approach to investigate the topography of cell surface proteins keeping the membrane intact, although due to the requirement for high expression of the molecule of interest its widespread application has been limited.

Possible role of CD45 in regulation of TCR signaling

CD45 has a large ectodomain with a receptor-like structure, although physiologically relevant ligand(s) remain to be identified. The CD45 tyrosine phosphatase regulates the threshold of T cell antigen receptor (TCR) signaling by modulating the actions of Src family $p56^{lck}$ and $p59^{fyn}$ kinases. The p56lck kinase associate with the CD4 and CD8 co-receptors and initiates TCR signaling cascades by phosphorylation of immuno-receptor tyrosine-based activation motifs (ITAMs) located in the TCR-ζ and CD3-ε chains.

Alternative splicing generates up to eight different CD45 isoforms of which five are expressed at significant level in T cells, and differential isoform expression is tightly controlled during thymic development and the activation of mature T cells. Naïve T cells express high molecular weight isoforms of CD45 including sequences encoded by exons A, B and C. Antigen-primed memory CD4+ T cells alter their CD45 isoform pattern and express predominantly a low molecular weight isoform lacking sequences encoded by the variable exons (CD45R0 or null isoform). Furthermore, CD45 splicing is regulated during T cell development; thymocytes express predominantly the CD45R0 isoform, whereas mature T cells express multiple isoforms. However, the molecular consequences of differential CD45 isoform expression for TCR signaling, if any, are not yet well understood.

Homoassociation of CD45 molecules also might exert influence on regulation of TCR signaling since it may lead to inhibition of its tyrosine phosphatase activity.

Fluorescence resonance energy transfer

Förster type resonance energy transfer is a physical process in which energy is transferred from an excited donor molecule to an acceptor molecule through long-range dipole-dipole interaction.

One of the most important factors influencing efficiency of energy transfer is the distance between donor- and acceptor-dye molecules. Energy transfer occurs in the 1-10 nm distance range with detectable efficiency, and these distances correlate well with macromolecular dimension, so the phenomenon can be used as a spectroscopic ruler in this range.

In the case of an ideal FRET dye-pair the emission spectra of the donor molecule overlaps with the absorption spectra of the acceptor. As a results the donor molecules become quenched, while the acceptor molecules become excited and can emit fluorescent light with their own quantum yield. This later process is called sensitized emission.

The flow cytometric fluorescence resonance energy transfer (FCET) can provide statistically very accurate information on the cell surface distribution of membrane proteins and conformational changes of biologically active molecules. This technique has been successfully applied to a wide range of biological systems, such as monitoring the association state of membrane proteins in immunologically competent cells and various tumor cells. Although FCET does provide

information on the lateral organization of molecules with high sensitivity, the technique is still not widely applied to cellular systems for two main reasons:

- Advanced dual-laser flow cytometric instruments with excitation wavelengths specific for the donor-acceptor pair, are required. Although the measurement of FRET-induced donor quenching does not need either dual-laser instruments or complicated evaluation, quenching cannot be used for cell-by-cell data analysis of FRET efficiency.
- In addition, both accuracy and reproducibility of FCET measurements are compromised if the ligands for the fluorescently labeled probes are expressed at low levels. In such cases the contribution of autofluorescence may be significant relative to the specific signal.

II. SPECIFIC AIMS

The major goal was to determine an assumable distinct regulator function of the various CD45 isoforms in T cell signaling. We had the following aims during our investigations:

- Can we detect physical association between CD45 and CD4/CD8 co-receptors in the intact cell membrane? If yes, is the heteroassociation pattern influenced by various isoforms of CD45?
- Does CD45 forms homodimers on an isoform-dependent way?
- What are the functional consequences of the differences in homo- and heteroassociation pattern of the distinct isoforms? Are various isoforms associated with specific differences in T cell signaling?
- Association pattern of molecules with low expression level (like expression of CD45 isoforms on transfected HPB-ALL T cells) determined with the traditional FCET method has low reliability, so development of a new, more sensitive technique with the ability of cell-by-cell correction for autofluorescence is required.
- As FRET applications are often limited by the lack of appropriate directly labeled monoclonal antibodies to serve as a donor/acceptor pair, the use of fluorophore-conjugated secondary antibodies or Fabs may overcome this problem. So we wanted to investigate whether the altered geometry of indirect labeling compared to direct labeling significantly affects the FRET efficiency values measured between intramolecular epitopes.

III. MATERIALS AND METHODS

Cells

EBV-transformed JY human B lymphoblast cells and the CD45 negative HPB-ALL T cells transfected with the CD45R0, CD45RBC and CD45RABC isoforms, and peripheral blood lymphocytes (PBL) from healthy donors gene-transduced with a single chain TCR:CD3ζ chimeric gene construct were used in our investigations.

Antibodies and conjugation of fluorophores

antigen	antibody	isotype
MHC I heavy chain	W6/32	IgG2a
MHC I β2-mikroglobulin	L368	IgG1
CD45	panCD45	IgG2a
TCR β chain	TCR Vβ1	IgG1
CD3	UCHT1	IgG1
CD3	OKT3	IgG2a
CD3	MEM	IgG2a
CD4	MEM	IgG2a
CD8	MEM	IgG2a

Cy3- and Cy5-conjugated affinity purified rabbit anti-mouse IgG2a (γ2a chain specific), rabbit anti-mouse IgG1 (γ1 chain specific) and Fab fragments of goat anti-mouse IgG (heavy and light chain specific) were used for indirect labeling.

Fab fragments were prepared as it was described earlier. Briefly, IgG mAbs were digested with activated papain. The enzyme activity was terminated by addition of iodoacetamide. The reaction mixture was passed through a Sephadex G-100 fine column and the collected Fab fractions were further separated from intact Ig by using a protein A sepharose column.

Antibodies or Fabs were labeled covalently with fluorescein izothiocyanate (FITC), tertamethyl rhodamin izothiocyanate (TRITC) or with the bifunctional succimidyl ester derivatives of sulfoindocyanine dyes (Cy3 and Cy5) as it was described earlier. Unreacted dye molecules were

removed by gel filtration through a Sephadex G-25 column. The dye-to-protein labeling ratio was determined by spectrophotometry and its value was around 3:1 in the case of whole IgGs and around 1:1 in the case of Fab fragments.

FRET labeling of cell surface molecules

Freshly harvested cells were washed twice in ice-cold PBS (pH 7.4). The cell pellet was suspended in 100 μl of PBS with 1% bovine serum albumin (BSA) (1 x 10^7 cells/ml) and incubated with mAbs at saturation concentration on ice in the dark.

For indirect staining cells were incubated with non-conjugated mAbs and after washing the cells twice in PBS, they were further incubated with Cy3- or Cy5-conjugated polyclonal rabbit anti-mouse antibodies against the IgG γ1 or γ2a chain.

In case of indirect staining with Fab fragments the following labeling sequence was applied: (*i.*) non-conjugated W6/32 mAb; (*ii.*) Cy3-conjugated Fab fragments of goat anti-mouse IgG (*iii.*) unlabeled Fab fragments of polyclonal goat anti-mouse antibodies in order to block the free binding sites remaining on W6/32 mAbs (*iv.*) L368 mAb (*v.*) Cy5-conjugated Fab fragments of goat anti-mouse IgG.

Flow cytometric energy transfer measurements

FRET measurements were carried out using a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Jose, CA) to determine FRET efficiency between FITC- and Rhodamine-conjugated mAb. In brief, the donor fluorescence was excited with the 488 nm line of an argon ion laser, and fluorescence emission of the donor was detected at 530±30 nm. Donor fluorescence of the double-labeled samples was compared to that of samples where the acceptor antibody was replaced by non-labeled antibody to compensate for any competition between the donor and acceptor antibodies. FRET efficiency was calculated from the fractional decrease of the donor fluorescence in the presence of the acceptor.

A Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) was used to measure energy transfer efficiency between Cy3- and Cy5-labeled molecules. Four fluorescence intensities were measured. Three of them were excited at 488 nm and detected at 530±15 nm, 585±21 nm and above 670 nm, respectively, while the fourth fluorescence intensity

was excited at 635 nm and detected at 661±8 nm. Energy transfer efficiency (E) was calculated from the four fluorescence intensities on a cell-by-cell basis.

Immunoprecipitation and immunoblotting

Stimulated cells were treated with lysis buffer, nuclei and cellular debris were removed by centrifugation. For analysis of whole cell lysates by immunoblotting, samples were boiled with Laemmli's buffer. For TCR-ζ immunoprecipitation the lysates were pre-cleared by rotating packed Protein-G Sepharose beads. Pre-cleared lysates were incubated with immunoprecipitating antibody. Immune complexes were recovered by incubation with packed protein G-Sepharose beads. For CD4 immunoprecipitates, cells were coated with CD4 mAb. For *in vitro* kinase assay, immune complexes were washed once in kinase buffer. After 10 min incubation with kinase buffer (dithiotreitol, ATP and [γ-^{32}P]ATP) the reaction stopped by the addition of Laemmli's buffer and boiling. Quantification of immunoblots and autoradiographs was carried out using a phosphorimager (Fuji FLA3000).

IV. RESULTS

Cell-by-cell correction for autofluorescence in FCET measurements

In order to improve the applicability of the FCET method in very low signal-to-noise systems, we applied the red shifted donor/acceptor pair Cy3 and Cy5. In addition, we supplemented the FCET method with a mathematical treatment allowing cell-by-cell correction for autofluorescence. Since individual cells have different autofluorescence intensities, subtraction of the mean autofluorescence intensity of non-labeled cells from each cell in the labeled sample, as used in the conventional FCET method, may falsify the specific fluorescence values. Two biological systems were tested using our modified technique:

1. The expression level of the R0 isoform of the CD45 tyrosine phosphatase on a transfected HPB-ALL T cell line was too low to determine the extent of homodimerization of these molecules accurately by the traditional FCET method (signal:autofluorescence ratio is 1:1). Cell-by-cell autofluorescence correction by the modified FCET method resulted in a more reliable measurement of the energy transfer histogram: the improved method yielded a shift to the positive range and a lower standard error of mean (mean of the frequency distribution histogram = (4.9± 2.6)%), as compared to the result of the traditional way of background correction, i.e. the use of constant autofluorescence values (mean = (-0.1± 7.6)%).

2. Biological samples may contain different subpopulations of cells with respect to the association between surface molecules of interest. Discrimination between such subpopulations is essential but used to be difficult with the conventional FCET dye pairs (fluorescein and rhodamine) due to the high variance of the energy transfer distribution. Our new FCET technique is capable of a much more accurate distinction between subpopulations of cells with different energy transfer efficiencies. FCET studies with primary human T lymphocytes transduced with single chain chimeric TCR chains show that the membrane topology of an exogeneous TCR molecule (i.e. scTCR:CD3ζ) differs from that of full length, non-modified TCR chains. The majority of transduced T lymphocytes do express the scTCR:CD3ζ chain (i.e., cells positive for Vα12 and Vβ1), but the existence of a subpopulation of T lymphocytes, albeit a small subpopulation, which express an endogenous TCR chain recognized by the anti TCR antibody (i.e., a Vβ1 chain) may hamper the intended FRET studies significantly. In this particular example the energy transfer

histogram had a bimodal rather than a unimodal frequency distribution when the endogenous TCR β chain appeared to be present in a fraction of the cells.

Geometry of labeling scheme influences FRET efficiency values

When staining the heavy and the light chain (β2m) of HLA class I molecules with W6/32 and L368 mAbs, respectively, the direct or indirect labeling procedure differ slightly with respect to the distance between donor and acceptor fluorophores, as a consequence of the different sizes of the used antibody complexes. The alterations in distance between fluorophores markedly affect energy transfer measurements. Labeling of both HLA epitopes with Cy3- and Cy5-conjugated secondary Fab fragments led to lower energy transfer efficiencies (15%) compared to direct labeling with fluorophore-conjugated primary mAbs or Fab fragments of these mAbs (around 30-28%). When cells were labeled indirectly with fluorophore-conjugated polyclonal whole IgG instead of Fab fragments, an even lower FRET efficiency (~8%) was observed. Combining indirect labeling with Fab fragments on the donor side *with* direct labeling on the acceptor side caused an intermediate value of energy transfer efficiency (24%). Interestingly, combining direct labeling on either side and indirect labeling *with* whole IgG on the other side provides similar FRET values as indirect staining on both sides (i.e. ~9%).

Association pattern of different CD45 isoforms on HPB-ALL cells

Our flow cytometric energy transfer results indicated that there was substantial association between CD45R0 and CD4 and CD8, respectively, but not between CD45RBC or CD45RABC with CD4 or CD8. The energy transfer values for CD45R0-CD4/CD8 association were found to be in the range 7.2-12.2%, demonstrating that a pool of CD45R0 molecules are within 10nm at the cell-surface of a pool of CD4 or CD8 molecules.

To investigate homodimerization of various CD45 isoforms, FRET analysis using the FACS was carried out on the different sub-clones labeled with a 50:50 mixture of Cy3- and Cy5-CD45 Fab fragments. The three CD45 isoforms under investigation, only CD45R0 was found as homodimers, generating energy transfer efficiency values of 11.0±4.4 based on four independent experiments, in contrast to 1.2±0.4 and 1.8±1.4 for the $CD45RBC^+$ and $CD45RABC^+$ sub-clones, respectively.

Investigation of TCR signaling

CD4-associated $p56^{lck}$ kinase activity was, on average, two-fold higher in the $CD45R0^{+}$ than in the $CD45RBC^{+}$ sub-clone. Consistent with this finding, basal protein tyrosine phosphorylation levels were consistently higher in the $CD45R0^{+}$ than in the $CD45RBC^{+}$ cells.

Immunoprecipitation of the TCR-ζ chain followed by immunoblotting for phosphotyrosine showed that upon CD3-CD4 co-ligation, generation of the TCR-ζ p21 and p23 phosphoisomers was greatly amplified in the $CD45R0^{+}$ compared to the $CD45RBC^{+}$ sub-clone. Recruitment of ZAP-70 was also increased, as was tyrosine phosphorylation of the kinase.

V. DISCUSSION

Our investigation was aimed to find a possible regulator function for CD45 isoforms in TCR signaling by determining the association patterns of cell surface molecules and by analyzing signaling processes in $CD45R0^+$, $CD45RBC^+$ and $CD45RABC^+$ HPB-ALL T cell lines. As we could not use the classical FCET method on cell surface molecules with low expression level, improvement of flow cytometric energy transfer method was aimed too. Our results are the follows:

(1) Application of red emitting fluorophores Cy3 and Cy5 in comparison with traditionally used fluorescein-rhodamin pair significantly reduced autofluorescence. We introduced a fourth fluorescent parameter in order to detect the autofluorescence independently, which allowed us to develop a new mathematical algorithm for cell-by-cell correction of autofluorescence increasing both accuracy and reproducibility of FCET method.

(2) The significantly increased sensitivity of our new FCET method incorporating cell-by-cell correction of autofluorescence allowed the detection of small subpopulations with different energy transfer values.

(3) The limit of applicability of the newly developed FCET method was tested on various immunfluorescent labeling schemes. Labeling the heavy and light chains of HLA class I molecules with different direct and indirect protocols we found that intramolecular energy transfer efficiency was significantly affected by the complexity of the applied antibodies. Whereas staining directly both donor and acceptor molecules resulted the highest energy transfer values, using secondary antibodies caused a decrease in FRET efficiency.

(4) We represented the first report that CD45R0 isoform preferentially forms homodimers and the first time that CD4/CD8-CD45R0 associations have been described using non-invasive FRET technology.

(5) In accordance with preferential associations we showed the increased CD4-associated $p56^{lck}$ basal kinase activity and enhanced TCR signal transduction intensity of $CD45R0^+$ cells. Homoassociation of CD45 might lead to the inhibition of its phosphatase activity, but the dominant effect of CD45R0 in the present experimental context was to associate with CD4/CD8 and to amplify TCR signal transduction coupling by generation a more active pool of CD4-associated $p56^{lck}$ molecules.

VI. UTULIZATION OF RESULTS

Our results contribute to a better understanding of TCR signaling due to emphasizing the correlation between TCR signaling and the presence of CD45 isoform-specific protein complexes. These findings represent the first report that CD45R0 preferentially forms homodimers and the first time that CD4/CD8-CD45R0 associations have been described using non-invasive FRET technique, which may explain the regulation of CD45 protein tyrosine phosphatase and thereby that of TCR signaling.

Our modified FCET method allows energy transfer efficiency to be determined on cellular systems with a very low expression level of the molecules. Furthermore the lower variance of FCET distributions enables a much more accurate discrimination of subpopulations having distinct FRET efficiencies. The practical advantage of the new method is that it can easily be implemented on a commercially available dual-laser benchtop flow cytometer without the need for hardware modifications.

VII. THE THESIS IS BASED ON THE FOLLOWING PUBLICATIONS:

1. **Sebestyén Z**., Nagy P., Horváth G., Vámosi G., Debets R., Gratama J.W., Alexander D.R., Szöllősi J.: Long wavelength fluorophores and cell-by-cell correction for autofluorescence significantly improves the accuracy of flow cytometric energy transfer measurements on a dual-laser benchtop flow cytometer. *Cytometry* 2002, 48:124-35. **IF: 2,557**

2. Dornan S., **Sebestyén Z**., Gamble J., Nagy P., Bodnár A., Alldridge L., Doe S., Holmes N., Goff L.K., Beverley P., Szöllősi J., Alexander D.R.: Differential association of CD45 isoforms with CD4 and CD8 regulates the actions of specific pools of $p56^{lck}$ tyrosine kinase in T cell antigen receptor signal transduction. *J. Biol. Chem.* 2002, 277: 1912-1918.
IF: 7,368

OTHER PUBLICATIONS:

1. Nagy P., Vereb G.**, Sebestyén Z.,** Horváth G., Lockett S.J., Damjanovich S., Park J.W., Jovin T.M., Szöllősi J: Lipid rafts and the local density of ErbB proteins influence the biological role of homo- and heteroassociations of ErbB2. *J. Cell Scien.* 2002 *(accepted)*
IF: 5,996

2. Szöllősi J., Nagy P., **Sebestyén Z**., Damjanovich S., Park J.W., Mátyus L.: Application of fluorescence resonance energy transfer for mapping biological membranes. *Rev. Mol. Biotech.* 2002, 82: 251-26

3. Szöllősi J., **Sebestyén Z.**, Nagy P.: Molecular superstructures in biological membranes (Molecular and Cellular Biology: from plant to human, *pp*:184-194, EMBO lecture course, 2000, Debrecen)