SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Quantitative analysis of the IL-9R cell surface organization and molecular interactions in human T lymphoma cells

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1. INTRODUCTION

1.1 γ_c cytokines and their receptors in T cell function

The γc cytokine family, which includes the cytokines such as interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15, and IL-21, is named after the common γc (common γ) receptor subunit. Expression of the γ_c chain can be observed on most lymphoid-derived cells, including T cells. Consequently, cytokines belonging to the family are crucial in the development of these cell types, in the regulation of their homeostasis, activation and differentiation processes, and thus in the development of the coordinated functioning of the adaptive immune system.

From early development to the regulation of effector cell function, γc cytokines are essential for the formation and maintenance of normal T cell function at many levels. Each cytokine γ_c has a pleitotropic effect. Members of the family show some degree of redundancy in biological effects due to the use of the common receptor subunit and common signaling molecules. In addition to overlapping functions, they can also perform unique, often opposite functions even for the same cell type. Their defective functioning, which may be based on mutations/polymorphisms in genes encoding both cytokines and individual receptor chains and associated signaling elements, may be the basis for a number of immune dysfunctions, immunodeficiencies or malignancies. Thus, enhancing, inhibiting, or otherwise influencing the action of cytokines γ_c has therapeutic potential in the treatment of many diseases.

1.2 IL-9 and IL-2 receptors

IL-2 is the first identified member of the γ_c cytokine family. It can elicit its biological effects through both autocrine and paracrine mechanisms, having an effect on both IL-2-producing and other T cells in its vicinity. In addition to acting as a T cell growth factor in many cases, the presence of IL-2 is also critical in the process of activation-induced cell death (AICD) and plays an important role in regulating the function and homeostasis of various CD4+ and CD8+ T cell subpopulations. The cytokine IL-2 is also thought to play a central role in the regulation of other members of the cytokine family.

IL-2R is one of the heterotrimeric γ_c receptors: in addition to the γ_c chain and the cytokine-specific α subunit, the common β subunit - shared with the IL-15R - is also involved in the formation of the receptor structure. Previously we have shown in our institute that IL-2R is associated with MHC I and II glycoproteins on the surface of activated T lymphocytes and many human T lymphoma-derived cells. The lipid rafts were shown to have a crucial role in

the formation and maintenance of these clusters. The α-subunit of IL-15R was shown to be also enriched in these superclusters on cells expressing both receptor species.

The cytokine IL-9 was first identified as a helper T cell growth factor in mice with potential oncogenic activity. Nevertheless, the focus of IL-9 related research has long been on its role in the allergic immune response, based on its pleiotropic effect on mast cells. In recent years, its role in the development of T cell tumors has re-emerged, as demonstrated in both mouse model systems and human biopsies. The effect of IL-9 can be exerted directly on tumor cells or through the influence of factors regulating the development of lymphomas (eg Treg cells, tumor microenvironment); however, the exact mechanism is not yet clear. IL-9R is a heterodimeric member of the common γ_c cytokine receptor family, the expression of the IL-9R is observed primarily on T cell types that also express the heterotrimeric IL-2R complex (high affinity complex).

1.3 Investigation of the cell surface organization of membrane proteins

The plasma membrane plays an essential role in cell communication, the flow of matter and information, and their regulation. The organization/compartmentalization of membrane components plays a critical role in the coordination and execution of many physiological cellular functions, such as intra- and intercellular signaling.

One type of membrane domains created by compartmentalization can be observed on polarized cells, these are the so-called stable domains. These domains, which are on well-separated membrane regions with specialized function, can be characterized by a set of proteins and lipids as well as structure and size, defined by the function. The formation of membrane compartments can also be observed on non-polarized cells, however, the lifetime and size distribution of these structures cover a much wider range. In terms of their composition and/or structure, most of them are characterized by a certain degree of dynamism.

In case of the multi-subunit proteins, such as the interleukin-9 and -2 receptors I studied, the most basic level of organization is the direct molecular interaction of the subunits, and thus the formation of functional receptor complexes. The physical proximity (homo- and hetero-association) of the individual protein components plays a major role in the organization of membrane proteins on the nanometer distance scale, the so-called small molecular clusters (nanodomains) as well. Large-scale compartmentalization of membrane proteins in the range of several hundred nanometers (or even micrometers) has also been explored in many cases.

Examining the arrangement of membrane proteins and their spatial relationship with other proteins, as well as exploring the factors that arrange and regulate them, can provide essential information for understanding the details of the processes that take place in the membrane. There are numbers of biochemical and biophysical methods that can be used to investigate this. The advantage of the latter (biophysical methods) is that they do can be performed on intact but at least quasi-intact cells. In addition, depending on the method chosen, the organization of proteins (and other membrane constituents) can be studied at different hierarchical levels, ranging from mapping molecular-level relationships to organization over a larger size range. In order to understand the complexity of processes that take place in the membrane, in most cases it is not enough to study only one hierarchical level, it is most expedient to study the structures formed by proteins and other membrane constituents at several levels simultaneously.

Among the biophysical methods for studying the organization of membrane constituents, the most obvious ones are the various microscopic methods. In these methods, the molecules to be examined are specifically labeled and then (one or more) images are recorded. Quantitative analysis of the images can be used to study the spatial distribution of the labeled molecule and, in the case of multiple labeled molecules, the relationship between the individual distributions.

Using confocal laser scanning microscopy (CLSM), the distribution of fluorescently labeled membrane constituents in the plasma membrane can be examined over a size range of a few hundred nanometers. When examining the spatial relationship of two membrane constituents, the primary question is whether they co-localize with each other, i.e., do they occur in the same region of the membrane? There are two basic ways to answer this question and to quantify the degree of colocalization: on the one hand, to determine the degree of correlation between their occurrences, and on the other hand, to determine their overlapping proportion (co-occurrence). In both cases, the quantitative parameter used to characterize the colocalization is determined by means of image pairs showing the distribution of the studied molecules, however, the values obtained in these ways characterize the spatial relationship of the molecules from different points of view.

A pivotal point in colocalization, especially correlation studies, is the separation of real colocalization (or even segregation) from randomly created “apparent” correlation (anti-correlation). It is difficult (practically impossible) to find a suitable biological control to check for randomness. To overcome this problem, Costes et al. introduced the so-called ‘confidence interval method’, originally developed to study the relationship between intracellular cell constituents. For a given cell, the random relationship of two molecules is modeled by mixing the pixels of the images detected from the distribution of the two molecules and the correlation

coefficient value is then calculated. By repeating the process many (several hundred) times, a confidence interval (usually 95%) for the random (uncorrelated) spatial relationship of the tested molecules can be determined, which reflects the expression conditions characteristic of the given cell. The value of the correlation coefficient calculated from the original pair of images, reflecting the actual spatial relationship of the two molecules, is compared to this confidence interval. If the value of the coefficient is outside the range, a true positive (or negative) correlation between the two molecules is likely. For the values in the interval, we can only say that there is no statistical evidence for the correlation (anti-correlation) of the two molecules, i.e. the effect of randomness cannot be ruled out.

While the confocal microscope provides information on the lateral distribution of the studied proteins in the ~100 nm range, the Förster fluorescence resonance energy transfer (FRET) method allows us to study the molecular association and direct physical proximity of the proteins (nanometer scale). Due to its relative simplicity, FRET efficiency determination from the fluorescence intensity change is the most common method for studying cell surface protein interactions. Intensity-based studies can be adapted to both flow cytometry and microscopy, taking advantage of the possibilities offered by the two instruments. The advantage of the intensity-based FRET method is that it can be applied to live samples, the FRET efficiency can be determined per cell, so the interaction of fluorescently labeled molecules can be examined per pixel and per cell. FRET efficiency can be calculated from the fluorescence intensity of the directly excited donor and acceptor and the sensitized (i.e. FRET-induced) emission of the acceptor. With this method, FRET efficiency can be determined per cell using either a flow cytometer or a confocal microscope. The basis of the accpbFRET method is that the fluorescence intensity of the donor decreases in the presence of acceptor due to FRET. If the acceptor is excited with high intensity light of the appropriate wavelength, it undergoes irreversible photochemical transformations that result in a change in its molecular structure, so it is no longer able to receive energy from the donor, i.e. donor fluorescence intensity increases depending on the FRET efficiency.

2. OBJECTIVES

Based on the previous results of our research group, IL-2R and IL-15R exerts their biological effect in the same membrane microdomains, in a similar molecular environment/common superclusters on human T lymphoma-derived cells. It is hypothesized that other cytokine receptors using the common γ_c chain, such as IL-9R, function in a similar environment, possibly in the same supercluster. This is supported by the fact that the IL-9Rα chain is primarily expressed on $CD25^+$ (IL-2Rα$^+$) cells, which also express the heterotrimeric IL-2R and where IL-2 plays an important regulatory role (Th2, Th9, Th17, Treg). In addition, IL-9Rα shows significant homology to the IL-2/15Rβ chain, which suggests the possibility of a similar protein-protein, and protein-lipid interactions.

Knowing these antecedents, the aim of my work is to place IL-9R on the map, i.e. to explore the spatial relationship of IL-2R/MHC with superclusters on human T lymphoma cells.

My objectives can be summarized in the following main points:

(1) the relationship of IL-9R to IL-2R / MHC superclusters, including the role of lipid rafts in developing the cell surface localization of IL-9R;

(2) assembly of the heterodimeric IL-9R, relationship between IL-9R and IL-2R;

(3) adaptation of the Costes confidence interval method in the correlation analysis of the spatial relationship of membrane proteins;

(4) adaptation of the confidence interval method in co-occurrence analysis of membrane proteins.

3. MATERIALS AND METHODS

3.1 Cell culture and cytokine treatment

Our experiments were performed on the following two human T lymphoma cell lines: **Kit225/IL9R**: HTLV-nonexpressing, cytokine-dependent human adult T lymphoma cell line with a helper/inducer phenotype, derived by stable transfection of IL-9Rα into Kit225 cells.
MT-2: is a HTLV-I–immortalized human adult T-cell leukemia cell line showing physiological expression of IL-2R and IL-9R. MT-2 cells are IL-9 producing cells.

Both cell lines exhibit constitutive expression of γ_c as well as IL-2Rα and β chains and are responsive to both IL-2 and IL-9. The MT-2 cell line also produces IL-9.
Cells were cultured in RPMI 1640 medium (SIGMA-Aldrich) supplemented with 10% FBS, penicillin, and streptomycin. We also added 30 U/ml human recombinant IL-2 (NCI, BRB Preclinical Repository) to the medium of Kit225/IL-9R cells every 48 h. The medium of Kit225/IL-9R cells contained 800 μg/ml G418 (Calbiochem®) to suppress the growth of wild type cells. In some experiments freshly harvested Kit225/IL-9R cells were washed twice in PBS and grown in IL-2-free medium for 48 h. Such cells were considered to be deprived of IL-2. Cells were then washed and incubated in fresh medium with IL-9 (0.25 μg/ml) at 37°C.

3.2 Labeling Cells with Fluorescent Markers

In our experiments, membrane proteins were labeled with fluorescent dye-conjugated monoclonal antibody and GM1 ganglioside was labeled with a fluorescently labeled cholera toxin B subunit (Molecular Probes). The antibodies were: MHC I light chain - L368, MHC I heavy chain – W6/32, MHC II – L243, IL-2Rα – anti-Tac, γ_c – TugH4, transzferrin receptor – MEM75 and the the two antibodies that bind to the IL-9Rα subunit were AH9R4 and AH9R7, which differ in the extent to which they inhibit the binding/action of the cytokine IL-9. According to the TS1H9RA3 proliferation assay, AH9R4 was a small inhibitor, while AH9R7 was a potent inhibitor. For this reason, in our studies where we studied the effect of IL-9 treatment, we used only the AH9R4 antibody.

3.3 Determination of protein expression

The expression levels of the tested membrane proteins were determined by flow cytometric analysis of the binding of monoclonal antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 546 dyes (FACSAria flow cytometer). The relative number of binding sites was calculated from the Mean Fluorescence Intensity (MFI) of the fluorescence intensity histogram of the cells corrected for autofluorescence and normalized to the labeling ratio of the antibody used. To determine the absolute number of available binding sites, MFI values were calibrated using microbeads containing a known number of dyes (QuantumTM Alexa Fluor 488 MESF kit).

3.4 Quantitative characterization of pairwise colocalization of membrane constituents

Membrane domain-level colocalization studies (on a distance scale of a few hundred nanometers) were performed with an Olympus FluoView 1000 confocal microscope. In the experiments, two or three cell surface constituents (membrane protein or GM1 ganglioside) were labeled with specific markers attached with spectrally separable spectral fluorophores.

Multi-channel images were recorded in optical slices in which the membrane slice was perpendicular (or nearly perpendicular) to the optical axis: in the layer close to the cover plate (bottom) and in the layer close to the slide (top). In this case, the smallest interference signal can be measured from parts outside the membrane area under study. The recorded 3 optical slices were combined into one image by projection after Gaussian filtering. To minimize the amount of overspill between the channels, the images are recorded in a "row-by-row sequential mode," which means that excitation with different lasers and detection of photons emitted by the excitation occur sequentially in each channel, row by row.

In the quantitative analysis of the spatial relationship of the membrane constituents, the cells were first manually circumscribed, and then the membrane and non-membrane "containing" pixels were segmented using the maximum entropy algorithm (Peter Nagy, MatLab plugin) and the threshold fluorescence intensity was determined. Subsequently, the program generated pixel blocks from adjacent pixels comparable to the lateral resolution of the microscope. Quantitative parameters characterizing colocalization were determined based on the intensities measurable in the pixel blocks.

Pearson's correlation analysis

In the case of the image pair recording the distribution of the two membrane constituents, the Pearson correlation coefficient was determined, then the pixel blocks of one member of the image pair were randomly rearranged ("shuffled"). The value of the correlation coefficient between the shuffled and the other unchanged image was determined. By repeating this process 500 times, we can obtain the distribution of the correlation coefficient in the case when the spatial locations of the signals measured in each channel (i.e., the membrane constituents examined) are actually independent of each other (C = 0). The distribution can be used to determine the 95% confidence interval for each image pair (i.e., each cell tested) by which it can be determined that the correlation coefficient obtained for the original image pair (i.e., the actual cell being tested) is an actual correlation (anti-correlation). Using the confidence interval for a given cell, we can define three categories: "true positive", "true negative", and "zero". If the Pearson correlation coefficient obtained for a given cell falls into the first two categories, then an actual correlation ("true positive") or anti-correlation (i.e., segregation of the two membrane constituents, "true negative" category) can be assumed between the spatial localization of the two membrane constituents.

Determination of the overlapping proportion of membrane constituents:

The coefficient for the quantitative characterization of the overlapping proportion of membrane constituents (co-occurrence analysis) was determined using both intensity and area-based approaches. Here, we also applied the confidence interval method introduced for correlation coefficients: we determined the confidence interval for the value of the overlapping ratio in the case where the distribution of the two molecules is random relative to each other, and then the actual value was compared to this interval. Overlap of the two molecules on a given cell was considered proven if the value of the coefficient was higher than the upper limit of the confidence interval.

3.5 Fluorescence resonance energy transfer (FRET) studies

Performance of ratio-based FRET (ratiometric FRET, rFRET) measurements:

rFRET measurements were performed on a Zeiss LSM 880 confocal microscope. Three fluorescence intensities were detected in each field of view, corresponding to the donor, FRET, and acceptor channels. The images were evaluated using the DipImage application running under Matlab: to segment the membrane and non-membrane "containing" pixels, we used the

"manually seeded watershed" algorithm and then determined the parameters needed to calculate the FRET using the rFRET plugin. Single-labeled samples, i.e., donor-only and acceptor-labeled samples, were used to calculate correction factors and relative fluorescence efficiencies. Finally, FRET efficiency was calculated on double-labeled cells (together with donor and acceptor dye) per cell.

Acceptor Photobleaching FRET (accpbFRET) assays: AccpbFRET assays were performed with an Olympus FluoView 1000 confocal microscope. During the measurements, a single-slice, two-channel image of the donor and acceptor intensity distribution of the double-labeled cells (carrying both donor and acceptor fluorophores) was recorded at low excitation laser intensity. Subsequently, donor excitation was turned off, and several scans were performed at maximum power with the laser used to excite the acceptor. Using repeated (3×) scans, the intensity of the acceptor was reduced to virtually zero (photobleaching). Another two-channel image was then taken at low laser intensity and the change in donor intensity was examined. During the evaluation of the images, we reduced the background noise by lowpass filtering. The FRET efficiency per pixel was determined with the ImageJ AccPbFRET module, which allowed for the necessary corrections (e.g., unwanted burnout of the donor, possible slippage of sequential images, etc.). The result of the evaluation is a color-coded FRET map and a histogram of the FRET efficiency distribution per pixel for the given cell. The mean FRET efficiency for a given protein pair was determined by averaging the median values of the FRET histograms per cell.

3.6 Statistical calculations

Two-sample t-test and ANOVA were used to compare the coefficients used to quantitatively characterize the colocalization, while the distribution of rFRET values was examined using the Kolmogorov-Smirnov test.

4. RESULTS

4.1 Membrane domain-level relationship between IL-9Rα and IL-2R/MHC superclusters: Pearson's correlation analysis

The membrane domain-level relationship of IL-9Rα and IL-2R/MHC superclusters was characterized by quantitative analysis of confocal microscopic images using Pearson's correlation coefficient. In the course of the analysis, we successfully adapted the confidence interval method previously used by Costes et al. To study the spatial relationship of intracellular cell constituents, which can be used to distinguish between random and actual colocalization between two membrane constituents. The applicability of the method was validated using positive and negative biological controls (MHC I light and heavy chain - positive control, transferrin receptor and GM1 ganglioside - negative control).

The Kit225 / IL-9R cell line is a version of the Kit225 cell line stably transfected with the IL-9Rα subunit. It has been previously shown that IL-2R and MHC proteins enrich significantly in common membrane regions on Kit225 cells: thus, we first checked whether transfection modified the colocalization of IL-2R and MHC glycoproteins. Our results confirmed a large, non-random overlap of both MHC I and MHC II proteins with the IL-2Rα subunit on Kit225/IL-9R cells. Similar results were obtained for previously unexamined MT-2 cells constitutively expressing the α-subunit of IL-9R.

Qualitative analysis of CLSM images already suggested a significant overlap of the other members of the IL-9Rα and IL-2R/MHC superclusters in both cell lines examined. Pearson's correlation analysis of the images confirmed this hypothesis: a significant percentage of cells fell into the "true positive" correlation category for both IL-9Rα and IL-2Rα and the spatial relationship between IL-9Rα and each MHC glycoprotein. Similar results were obtained for IL-9Rα and GM1-containing lipid rafts. However, correlation analysis also revealed that in a significant proportion of cells, IL-9Rα and the other membrane constituents examined occur (primarily) in distinct (segregated) membrane regions.

4.2 Molecular interaction between IL-9Rα and IL-2R/MHC superclusters

In the following, we investigated whether there was a "closer" molecular-level interaction between IL-9R and IL-2R/MHC clusters in addition to the domain-level relationship using the FRET method based on photobleaching. As expected, positive and negative biological control showed a high positive FRET efficiency between MHC I glycoprotein light and heavy chains for both cell lines, while a low mean FRET value close to zero for the negative control (GM1 ganglioside and transferrin receptor) was detected.

Between IL-2Rα and MHC I and between IL-2Rα and MHC II proteins, positive FRET efficiencies were detected in both cell lines, significantly higher than the negative control, i.e., the MHC glycoproteins and IL-2R were in the molecular proximity of each other. Significantly positive FRET efficiency was also detected between IL-9Rα and both types of MHC protein, as well as IL-9Rα and IL-2Rα.

4.3 Membrane domain-level relationship between IL-9R and IL-2R: Manders co-occurrence analysis

In order to get a more accurate assumption of the relationship between IL-9Rα and IL-2Rα, and to see if the overlapping and segregated membrane regions detected by the correlation analysis could occur on the same cell, the correlation analysis of the confocal microscopic images was supplemented with the Manders co-occurrence analysis. With this method, the overlapping proportion of membrane constituents can be determined.

In addition to determining the coefficients characterizing the degree of overlap, for the cells for which I have previously presented the result of Pearson's correlation analysis, we also performed new experiments where both types of analysis were performed.

Similarly to the Pearson analysis, the confidence interval for the random distribution was determined for each cell for the Manders coefficients, however, due to the different nature of the information carried by the coefficient value, a one-way comparison was performed in this case. The values of the obtained coefficients for each examined cell fell towards the confidence interval, i.e. the degree of overlap was significantly higher in all cases than the value for co-occurrence generated only by chance.

As expected, for cells in the "true positive" correlation category, both the IL-9 and IL-2 receptor α subunits showed significant overlap with the other receptor α subunit. For IL-9Rα,

the overlap ratio was comparable to the values obtained for the overlapping ratio of the light (β2m) and heavy chain (HC) MHC I glycoprotein used as a positive biological control ($M_{\beta 2m}$ = 0,70 ± 0,22 and M_{HC} 0,83 ± 0,13). Accordingly, the vast majority of IL-9Rα is located in the same membrane region as the IL-2Rα subunit. In the case of IL-2Rα, the overlap is slightly smaller, approx. 50%.

For cells in the "true negative" category, we still observed a much lower, but at the same time - compared to random generation - significant overlap between the receptor subunits of the two receptors, i.e., the segregation of the two proteins was incomplete. This is also supported by the fact that the two α subunits are significantly higher than the values obtained for the transferrin receptor and GM1 ganglioside used as a negative biological control.

4.4 Effect of cytokine milieu on membrane domain-level association of IL-9R and IL-2R

Next, we examined whether IL-9 binding affects the membrane domain-level relationship between the two interleukin receptors. MT-2 cells themselves produce the cytokine IL-9, so these experiments were performed on Kit225/IL-9R cells. When culturing Kit225/IL-9R cells, IL-2 must be added to the medium at regular intervals. Since there is a possibility that the effect of IL-9 may be influenced by IL-2 remaining on the cell, I used cells that were cultured in IL-2-free medium for 48 hours after passage ("starved" cells) to study the effect of IL-9.

Based on quantitative analysis of CLSM images, both common and segregated membrane areas can be found on "starved" cells. Neither the value of Pearson's correlation coefficients nor the percentage distribution of cells in each correlation category differed significantly between "starved" and cells cultured in the presence of IL-2 (hereafter "normal").

In terms of its tendency, $M_{IL\text{-}9R\alpha}$ was found to be lower and $M_{IL\text{-}2R\alpha}$ higher in the fasted cells in both the "true positive" and "true negative" correlation categories for both dye pairs. The significant difference ($p < 0.05$) obtained with the two-sample t-test was $M_{IL\text{-}9R\alpha}$ for the Alexa Fluor 546-AH9R4 + Alexa Fluor 647-antiTac dye pair and $M_{IL\text{-}9R\alpha}$ for the Alexa Fluor 546-AH9R4 + Alexa Fluor 488-antiTac dye pair. It was observed when comparing 2Rα coefficients. For cells in the "0" category, there was no significant difference between "starved" and "normal" cells.

We then examined the effect of IL-9 binding on the spatial relationship of the two proteins. Starved cells were subjected to 20 min of IL-9 treatment (in our preliminary studies, maximal STAT1 activation was observed after 20 min of stimulation). IL-9 binding did not

cause a significant difference in either the value of the Pearson correlation coefficient, the distribution of cells between each correlation category, or the overlapping proportion of the two subunits. Thus, at the membrane domain level, IL-9 binding did not affect the spatial relationship between the α-subunits of IL-9R and IL-2R.

4.5 Effect of IL-9 on the molecular interaction of γ_c, IL-9Rα, and IL-2Rα

In the following, we examined whether IL-9 binding does have any influence on the molecular interaction of the γ_c chain and the α subunits and on the molecular interaction of the two α subunits. Similar to the protocol used in the domain-level relationship study, the effect of IL-9 binding was studied on 20-minute stimulated, "starved" Kit225/IL-9R cells. FRET efficiency was determined by the ratio-based FRET (rFRET) method, per cell, based on the intensities detected in images taken in the donor, acceptor, and FRET channels, respectively. We chose this method because, based on literature data, it proved to be statistically most reliable when examining the interactions of low-expression proteins (e.g., γ_c chain) compared to other microscopic FRET techniques (e.g., accpbFRET).

Based on our results, the mean frequency histograms of FRET efficiency were ~18% for the γ_c - IL-9Rα pair, ~17% for the IL-9Rα - IL-2Rα pair, and ~25% for the γc - IL-2Rα pair, which refers to the molecular interaction of the tested proteins. IL-9 binding did not cause a significant change in the FRET efficiency between elements of the heterodimeric IL-9R complex. In contrast, the FRET frequency histograms characterizing the relationship between IL-9Rα – IL-2Rα and γc – IL-2Rα were significantly shifted upon IL-9 treatment ($p < 0.007$, Kolmogorov-Smirnov test): the mean of the histograms was was significantly increased for the IL-9Rα – IL-2Rα pair, while it was significantly decreased for the γc – IL-2Rα pair after IL-9 binding.

5. DISCUSSION

The aim of my work was to map the cell surface organization of the interleukin-9 receptor on cells of human T lymphoma origin. IL-9R is a heterodimeric member of the common γ_c cytokine receptor family, the expression of which is observed primarily on T cell that also express a high-affinity heterotrimeric IL-2R complex. Earlier in our institute it was shown that the IL-2R, which is another member of the γ_c cytokine family, forms common superclusters with MHC I and II glycoproteins on the surface of activated T lymphocytes and many human T lymphoma-derived cells. In the formation and maintenance of these superclusters lipid rafts play a prominent role. The α-subunit of IL-15R is also enriched in these superclusters on cells expressing both receptor species. An important issue in our studies is the exploration of the spatial relationship between the IL-9 receptor and IL-2R/MHC superclusters based on the above, which we studied at several hierarchical levels.

The spatial relationship between IL-9R and IL-2R/MHC-containing lipid rafts was explored on a distance scale of a few hundred nanometers by Pearson correlation and Manders co-occurrence analysis of confocal microscopic images. In the Pearson analysis, we successfully adapted the confidence interval method previously introduced by Costes et al. To investigate the spatial correlation of intracellular cell constituents. With this method we have the possibility to categorize the correlation coefficient determined for the given cell (below, towards or just falling within the confidence interval), so that the actual correlation (anti-correlation) can be recognized by eliminating potential distortion factors (eg expression differences, etc.). The confidence interval method was also used in the analysis of the overlapping proportion of membrane proteins (co-occurrence) with the appropriate modification: here the overlap was considered statistically justified in case of colocalization coefficient higher than the upper limit of the interval.

Pearson analysis of confocal microscopic images revealed a “true positive” correlation between IL-9Rα and IL-2Rα/MHC-containing lipid domains (lipid rafts) in a significant proportion of cells in both human T lymphoma cell types examined. Complementing our studies with FRET measurements, we demonstrated the molecular proximity of the elements of IL-9Rα and IL-2R / MHC clusters, i.e., IL-9R is an additional component of these protein patterns.

Comparing our results with previous data on IL-2 and IL-15 receptors, it is likely that the organization of γ_c cytokine receptors is driven by a general regulatory principle in the plasma membrane of activated or transformed T cells in which both lipid rafts and MHC

glycoproteins play a key role. The exact function of the protein clusters formed in this way is still to be clarified, but their role in the formation of functional receptor complexes, thus in the induction of signaling processes and in the regulation of their efficiency, is probable. The presence of MHC I glycoproteins can, among other things, influence the dynamic properties of γ_c receptors and, through this, indirectly interfere with their signaling processes. Based on previous FCS, STED and FRET data, the IL-2R, IL-15Rα and MHC I proteins enriched in the lipid rafts of Kit225/FT7.10 cells, which are Kit225/IL-9R cells "related" cells and form smaller size, varied composition protein aggregates within the given membrane region. Some of these units are mobile, others have limited mobility. Decreasing cell surface MHC I expression significantly increased the mobility of IL-2Rα/IL-15Rα-containing protein aggregates without modifying the geometry of the IL-2Rα/IL-15Rα relationship.

Based on Pearson's correlation analysis of CLSM images, IL-9Rα and IL-2R/MHC superclusters occur not only in common membrane regions (lipid rafts) but also in segregated membrane areas, as seen in the correlation coefficients of significant proportion of the cells. Based on visual analysis of CLSM images, the presence of segregated domains was not limited to the higher number of membrane constituents.

It is important to note that our results are independent of the type of cell tested: similar results were obtained for transfected IL-9R expressing Kit225/IL-9R and constitutively expressing IL-9Rα MT-2 cells.

In order to obtain a more comprehensive picture of the spatial relationship between IL-9Rα and IL-2Rα subunits, the information obtained by correlation analysis was supplemented by the study of co-occurrence. After one of our aims was to study the cytokine million effect, these assays were performed on Kit225/IL-9R cells. Between IL-9Rα and IL-2Rα we demonstrated a minimal but significant overlap on all cells regardless of the correlation category. All this supports the co-occurrence of distinct and overlapping domains on the same cell. Our rFRET studies provided further evidence: the one-mode frequency distribution of FRET efficiencies determined between IL-9Rα and IL-2Rα per cell, as well as the significantly positive value of FRET efficiencies in the vast majority of cells.

The overlapping proportion of IL-9Rα with the IL-2Rα subunit was decreased by IL-2 deprivation (starvation), suggesting that IL-2 may play a regulatory role in the cell surface organization of IL-9R, thereby regulating the signaling efficiency of the receptor complex. IL-9 treatment did not cause a significant change in either the spatial correlation or the overlapping proportion of each α subunit. Based on previous NSOM and STED studies, the average radius of IL-2Rα domains on Kit225/FT7.10 cells is ~300-400 nm. According to our own results, even

if IL-9 binding causes some rearrangement, the extent is not sufficient to detectably alter the spatial relationship between the two receptor species in the few 100-nanometer size ranges.

The assembly of the heterodimeric IL-9R and the effect of IL-9 binding on the structure/conformation of the IL-9R complex and its molecular interaction with the IL-2Rα subunit were also studied in starved Kit225/IL-9R cells by the rFRET method. We have shown that IL-9Rα and γ_c subunits are in close molecular proximity to each other in the absence of IL-9, so that heterodimeric IL-9R complexes are present, at least in part, pre-assembled on Kit225/IL-9R cells. Previous antibody-induced fluorescent co-patching experiments have yielded similar results on human cells.

In contrast to the results of the aforementioned co-patching studies, we did not observe a significant change in the detectable FRET efficiency between γ_c and IL-9Rα upon IL-9 binding. In contrast, the distribution of FRET efficiencies detected for γ_c/IL-9Rα and IL-2Rα/IL-9Rα pairs was significantly shifted, suggesting a change in the internal structure (conformation and/or composition) of IL-9/2R clusters. The discrepancy between the co-patching results and our own FRET data is only apparent: an increase in the number of IL-9R$\alpha\gamma_c$ heterodimers does not necessarily mean that there is a detectable change in the mean distance between the two subunits at the FRET level (at least for specific epitopes).

In the light of the literature, our results can be interpreted as follows: IL-9R, together with the IL-2 receptor (and presumably other proteins such as MHC I and II glycoproteins), forms loosely structured, rearrangable protein clusters in the lipid rafts of human T lymphoma cells These serve as a "hot spot" (signaling center) to allow rapid initiation of signaling processes after cytokine binding. Cytokine binding results in a functional conformation of the receptor complexes and an increase in the number of assembled receptor complexes, thereby regulating the efficiency of signal transduction. Regardless of the correlation category of IL-9R and IL-2R, the common membrane domains observed on all cells can serve as a kind of "niche" for receptor complexes. By concentrating IL-9/2R subunits in a given membrane region, they can regulate the rate of formation of functional receptor complexes and provide a platform for IL-2 and IL-9 to efficiently share the γ_c subunit.

There is increasing evidence that the level of the γ_c chain plays a key role in determining the signaling efficiency of cytokines γ_c (threshold cytokine concentration, signaling strength). The colocalization of IL-2R and IL-9R (or other γ_c cytokine receptors) presents an opportunity to regulate the available γ_c level that does not require actual modification of the expression level (e.g., through downregulation or synthesis of a new protein), thereby making it faster and more efficient. IL-2 binding may reduce the number of γ_c chains available to other cytokines by

removing common γ_c chains and related signaling elements (at least a portion). Depending on the actual cytokine milieu appearing in the microenvironment of the cell, this mechanism allows fine-tuning of the signal strength induced by IL-9 (and other γ_c cytokines). (This is supported by our STAT1 activation studies, in which the efficacy of IL-9-induced STAT1 activation was attenuated in a concentration-dependent manner by pretreatment with IL-2.) Of course, this mechanism may work not only for the regulatory role of IL-2 but for any two (or more) γ_c cytokine. Thus e.g. it has recently been shown that the efficiency of IL-2 signaling is inversely correlated with the expression of IL-7Rα on Treg cells, which is underpinned by the preassociation of IL-7Rα and γ_c chains.

Based on the results presented in my dissertation, the colocalization of γ_c cytokine receptors and IL-2R is a general phenomenon, the formation of which is independent of the composition of the given receptor complex (heterodimer or heterotrimer). In addition to allowing the economical and efficient sharing of γ_c with significantly lower expression levels compared to cytokine-specific α subunits between different receptor species, this arrangement may help to play the putative regulatory role of IL-2 in the system of γ_c cytokines. Furthermore, our observations support that the study of the organization of membrane proteins at different hierarchical levels requires the combined use of available biophysical methods as well as several complementary evaluation methods to obtain a reliable picture of membrane protein distribution, which may contribute to a better understanding of signaling processes.

6. SUMMARY

In my PhD work, I studied the cell surface assembly of the interleukin-9 receptor, which plays an important role in the regulation of the immune system, and its spatial relationship with IL-2R/MHC supercluster elements at several hierarchical levels in human T lymphoma cells.

Our FRET and CLSM measurements demonstrated that protein patterns previously generated on other T cells with IL-2R and MHC glycoproteins were found on both MT-2 and Kit225/IL-9R cells. IL-9R is expressed, at least in part, in lipid rafts shared with IL-2R/MHC clusters, in the molecular proximity of IL-2R and MHC proteins, i.e., IL-9R is an additional component of these clusters. Comparing our previous data on IL-2 and IL-15, it is likely that the organization of γ_c cytokine receptors is driven by a general regulatory principle in the plasma membrane of activated or transformed T cells in which both lipid rafts and MHC glycoproteins play a key role.

Pearson's correlation analysis of CLSM images revealed that in addition to common domains, IL-9R and IL-2R/MHC clusters may also occur in segregated membrane regions, even on the same cell. The ratio of overlapping and non-overlapping membrane areas can vary from cell to cell, to the extent that can be seen in the value of the correlation coefficient. This is also supported by the co-occurrence method, which complements the correlation analysis, by which we demonstrated a minimal but significant degree of overlap between IL-9Rα and IL-2Rα on all cells, regardless of the correlation category.

On Kit225/IL-9R cells, FRET measurements revealed the molecular proximity of the common γ_c subunit and IL-9Rα even in the absence of IL-9. After a short period of IL-9 treatment, changes in the internal structure (conformation and/or composition) of the IL-9/2R cluster were detected. According to our hypothesis, on the surface of the studied human T lymphoma cells, the loosely structured clusters formed by IL-9R and IL-2R complexes (as well as other proteins, such as MHC I and II glycoproteins) promote efficient signaling after ligand binding. On the other hand, they allow efficient sharing of common γ_c receptor subunits and thus fine-tuning the evoked response.

7. PUBLICATIONS

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