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

Assembly and function of interleukin receptors from the endoplasmic reticulum to the cell membrane

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The Examination takes place at the Discussion Room of Immunology Department, Faculty of Medicine, University of Debrecen, at 11:00 am, 15th of December, 2015.

Head of the **Defense Committee**:	László Csernoch, PhD, DSc
Reviewers:	Gábor Koncz, PhD
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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 13:00 pm, 14th of January, 2020.

1. INTRODUCTION

1.1. T lymphocytes

In adaptive immune responses two cell types of the immune system play main roles: T and B lymphocytes having the ability for recognition of various antigens. T lymphocytes originate from bone marrow stem cells, then mature in the thymus; γ_c chain of IL-2 receptor family and JAK3 kinase play an important role in their development. T cell differentiation proceeds on the periphery as well, where subpopulations having different effector functions are formed. Most resting T cells circulate in the blood, secondary lymphoid tissues and gut-associated lymphoid tissues. At least two signals are required for the activation, antigen-specific activation of naïve T cells and co-stimulatory signs together lead to expression of new genes for cell division and differentiation and enhanced level of receptors on the cell surface. Their fate depends on encounter with antigen presenting cell (APC) having MHC-peptide complex and on the co-stimulatory signal from their environment and the APC. These interactions determine the way of the cells: they can differentiate into effector cells by T cell receptor-mediated signal or become anergic cells or died via apoptosis.

1.2. IL-2 and IL-15 cytokines

Interleukin-2 belongs to the type I cytokine family. It contains four alpha helices, a glycoprotein with 15.5 kDa. It was identified in 1976 as a cytokine growth factor of bone-marrow-derived T lymphocytes. Its gene was cloned as the first one its cytokine family in 1983, the gene of its receptor one year later, and its crystal structure was solved in 1992. 18 years after IL-2, interleukin-15 was identified as a cytokine that could stimulate the proliferation of IL-2-depedent CTLL-2 T cells even in the presence of neutralizing anti-IL-2 antibody.

IL-2 is produced mostly by $CD4^+$ T lymphocytes after antigen stimulation. It is also expressed by $CD8^+$ T cells, NKT cells, activated dendritic cells and mast cells in smaller quantities, but not by macrophages. In addition to the action of IL-2 as an autocrine and paracrine factor, it has a pleiotropic effect on other cell types (e.g. increase the cytolitic activity of natural killer cells).

IL-15 is produced by monocytes, macrophages, dendritic cells, stroma cells and some epithelial cells. Activated monocytes and dendritic cells co-express IL-15 and alpha subunit of its receptor simultaneously. The cytokine binds to IL-15Rα in the endoplasmic reticulum then they are transported together to the cell surface allowing trans-presentation to T or NK progenitor cells.

While IL-15 is important for the survival and proliferation of conventional T cells, IL-2-mediated signals play essential role in the maintenance of the homeostasis of $CD4^+$ $CD25^+$ regulatory T cells (T_{reg}-s), so in maintenance of peripheral immune tolerance. Both cytokines stimulate the development and proliferation of T and NK cells, facilitate the proliferation and antibody production of B cells. IL-2 promotes activation induced cell death, while IL-15 inhibits this process. The latter one supports the survival of $CD8^+$ memory T cells, maintaining sustained, long-lasting immune response to pathogens.

1.3. Cytokine receptors: IL-2R and IL-15R

Both membrane receptors consist of three subunits. They use their β (CD122) and γ_c (CD132) subunits in common, which are required for signal transduction processes.They also have distinct, ligand-specific chains (IL-2Rα or CD25 and IL-15Rα or CD215), which are responsible for high affinity binding of the ligand. Based on their subunit composition, the receptors can form complexes with different ligand binding affinities in the cell membrane. IL-2Rα is expressed transiently after activation of TCR or binding of IL-2 to the other subunits. It can bind its ligand with low affinity ($K_d=10^{-8}$ M), without invoking signalling. The IL-2/15Rβγ_c heterodimer has intermediate ligand binding affinity to IL-2 or IL-15 ($K_d=10^{-9}$ M), while the heterotrimeric IL-2Rαβγ_c and IL-15Rαβγ_c have high affinity ($K_d=10^{-11}$ M). Heterodimers and heterotrimers can signal efficiently. In contrast to IL-2Rα, IL-15Rα alone can bind the cytokine with high affinity ($K_d=10^{-11}$ M). The β chain is shared by IL-2 and IL-15 only (IL-2/15Rβ), while the γ_c subunit is used by IL-4, IL-7, IL-9 and IL-21 receptors as well.

In IL-2R deficient humans different autoimmune disorders: haemolytic anaemia, inflammatory bowel disease are developed.

1.4. IL-2 transmembrane signaling

After receptor binding, IL-2 and also IL-15 – due to the shared signaling subunits – can activate many signal transduction pathways (Jak/STAT, PI-3K/Akt, Ras/Raf/MAPK). Heterodimerization of cytoplasmic domains of β and γ_c subunits leads to activation of Janus family tyrosine kinases (JAK1, JAK3); JAK1 associates with the β subunit and JAK3 with the γ_c constitutively. JAKs activate each other and phosphorylate the receptor chains. Phosphorylation mediates recruitment of STAT1, STAT3 and STAT5 transcription factors, from which the activation of STAT5 proteins is the most effective. STAT5A and STAT5B

molecules dock on the IL-2/15Rβ chain, become phosphorylated, dimerize and translocate to the nucleus, where they bind to their target genes which are required for T cell growth, differentiation and effector functions as well.

1.5. IL-2Rα and Daclizumab

The ligand-specific alpha-chain of IL-2 receptor is a valuable target for immune therapy, since it is expressed among resting cells only by T_{reg} cells and $CD56^{hi}CD16^{lo}$ NK cells. At the same time there are abnormally elevated levels of IL-2Rα expression associated with an array of malignant and benign disorders, leukaemias and lymphomas, allograft rejection, autoimmune- and infectious diseases. A monoclonal antibody against IL-2Rα was described first in 1981, called anti-Tac (T cell activation antigen), which inhibits binding of IL-2 to IL-2Rα. The humanized version of anti-Tac, daclizumab (Zenapax, Roche), in 1997 was approved by FDA for prevention of renal allograft rejection. Daclizumab was the first humanized antibody targeting a cytokine receptor used in therapy. It is administered – although with limited success – in the treatment of T-cell-mediated autoimmune diseases (e.g. sclerosis multiplex, aplastic anaemia) or different types of neoplasia (adult T-cell leukaemia) as an inhibitor of T cell proliferation.

1.6. Adult T-cell leukaemia/lymphoma (ATL)

Adult T-cell leukaemia/lymphoma (ATL) is an aggressive T-cell malignancy caused by human T-cell lymphotropic virus I (HTLV-1) via infection of $CD4^+$ T cells. In peripherial blood an enhanced amount of $CD4^+$ T cells can be detectable with high CD25 expression. Based on its various clinical manifestations ATL is classified into four subtypes: acute, lymphomatous, chronic and smoldering. Whereas chronic and smoldering ATL are characterized by normal level of leukocytes and have a better prognosis, there is no curative therapy for acute ATL, associated with organ dysfunction and bad prognosis (4-10 month mean survival time). At early stage, autocrine growth period of leukemic $CD4^+$ T cells expressing of IL-2 and functional IL-2 receptors can be observed. Proliferation of the cells in this stage can be reduced by using anti-IL-2Rα (anti-Tac) antibody. Later, IL-2 production is abolished, but expression of CD25 on the cell surface is continuous. In several cases, this late stage is characterized by IL-2-independent growth and is associated with constitutively activated JAK-STAT signaling pathway.

1.7. Vesicular transport of the secreted and plasma membrane proteins

Secreted proteins – like interleukin-2 and -15 – go through the biosynthetic-secretory pathway from the place of synthesis to the plasma membrane. These proteins should have special signal sequences, which allow them to get from the ribosome (making synthesis) across the membrane of endoplasmic reticulum to its lumen. From here they flow to the cisternae of the Golgi apparatus, where some of them leave the Golgi after sorting in secretion vesicles and reach the plasma membrane and are secreted via exocytosis, while others get into the endosome/lysosome system.

Synthesis and trafficking of membrane proteins – like interleukin receptors – are similar to those of the secreted proteins with the difference that they never get to the lumen of ER with their full length. With special anchoring sequences proteins are attached to the ER membrane, from where they go to their final destination in a membrane-bound state with vesicular transport.

1.8. MHC proteins

On the short arm of the 6^{th} chromosome there is a complex, called major histocompatibility gene complex (MHC), which has the highest polymorphism in the human genome. HLA (human leukocyte locus A) antigens are coded by two main regions of classic or conventional MHC-genes and expressed in the plasma membrane as MHC I and MHC II proteins. They have outstanding importance in the immune system, mostly in the initiation of T-cell mediated immune responses. MHC I is expressed on almost all nucleated human cell, MHC II molecules are expressed constitutively only by professional antigen presenting cells, but they appear on some tumor cell membranes or can be induced by IFNγ. The main role of MHC I is presentation of the (peptide fragments of) endogenous antigens to $CD8^+$ T lymphocytes and it also takes part in the regulation of T cell signalling processes. MHC II on APCs presents exogenous antigens to T cell receptors (TCRs) on $CD4^+$ T lymphocytes. The two glycoproteins have different structures. MHC I is a heterodimer: a light chain (β_2-microglobulin molecule) binds to the polymorphic (α) heavy chain non-covalently. The β_2-microglobulin is required to form the conformation appropriate for peptide binding and for reaching the cell surface. MHC II consists of a polymorphic α- and a polymorphic β-chain.

1.9. Protein clusters in the cell membrane

Significant homoassociation of MHC I molecules as well as its associations with other cell surface receptors such as IL-2R, IL-15R, EGFR and MHC II have been reported. The homoassociations of IL-2Rα was also observed on some T cell lines. The β-β homodimer as a new form of functional IL-2 receptor was also reported to assemble spontaneously in the absence of γ_c subunit at the cell surface. Earlier our workgroup demonstrated that the four receptor subunits (IL-2Rα, IL-15Rα, IL-2/15Rβ and γ_c) could form heterotetrameric complexes in the absence of cytokine in the plasma membrane of T lymphoma cells, which were rearranged upon addition of relevant ligands. Clustering of receptors may influence their function and may have diagnostic value.

2. OBJECTIVES

In my PhD work I focused on the investigation of membrane proteins acting as key players in the regulation of the immune system; examination of the intracellular assembly of IL-2 receptors and disassembly of the IL-2R/MHC I protein cluster, might shed light on valuable information having clinical importance.

In cells producing IL-2 and also its receptor, e.g. in case of adult T-cell leukaemia, the effectiveness of blocking antibodies against cell surface receptors (and exogenous IL-2) may be explained by the fact that IL-2 receptors are pre-assembled after their synthesis and use endogenous IL-2 to initiate signal transduction before reaching the plasma membrane. Therefore we set the following goals:

- investigation of colocalization and molecular vicinity of the receptor subunits along the secretory pathway, from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane
- examination of IL-2-mediated signaling in the ER and the Golgi

Proteins and lipids involved in transmembrane signaling have a non-random distribution in the plasma membrane. Distributions might be modified upon changes of the expression level of proteins, which might also have functional consequences. MHC molecules and interleukin receptors form protein patterns at the cell surface of T lymphocytes, which are enriched in lipid rafts to enhance their signaling efficiency. Focusing on IL-2R/MHC I clusters we asked the following question:

- Are protein-protein interactions retained among the members of the supercluster if the expression of MHC I molecules decreased?

3. MATERIALS AND METHODS

3.1. Cell culture, gene silencing

Assembly of IL-2/15 receptor subunits was examined in a human cervix carcinoma (HeLa) cell line, cultured in RPMI 1640 medium completed with 10% (v/v) foetal bovine serum (FBS), L-glutamine and gentamycin. In case of experiments for intracellular signaling, human ATL cell lines, ED40515(+) wild type and its IL-2 transfected version, ED/IL-2 were used. For growing of wild type ED cells, 100 U/ml human recombinant IL-2 was added to the culturing medium.

FT7.10, $CD4^+$ human T lymphoma cells, stably expressing IL-15Rα (produced from Kit 225 K6 wild type cell line), were used for investigation of protein clusters in the plasma membrane. 500 pM human recombinant IL-2 was added to the culturing medium every 48 hours. Selection of IL-15Rα positive cells was achieved by addition of 0.8 mg/ml G418.

Gene silencing of FT7.10 cells was carried out with siRNA sequences against the light chain of MHC I, using AMAXA Nucleofector II electroporator according to the manufacturer's protocol. Non-electroporated samples or transfected with irrelevant (anti-GFP or negative control) siRNAs were used for controls. Viability of the cells was checked by fluorescein-diacetate/propidium-iodide staining 48 hrs after the electroporation. Absolute amount of MHC I, MHC II, IL-2Rα and IL-15Rα molecules per cell were determined with Dako QIFIKIT fluorescent beads, based on the protocol recommended by manufacturer.

3.2. Cloning, Transient transfection

Human IL-2Rα and IL-15Rα cDNAs were cloned in EcoRI/BamHI sites of pEGFP-N1, pmCherry-N1 (expression vector) plasmids downstream of the EGFP or mCherry gene. The signal peptide of bovine preprolactin was used in BglII/EcoRI sites of the vectors upstream of the inserted cDNA.

IL-2Rβ-YFP and γc-YFP plasmids, containing the human genes for IL-2Rβ and γ_c were kindly gifted by Thierry Rose (Institute Pasteur, France). Fluorescent proteins were changed to EGFP or mCherry using standard PCR. dsDNA fragments were cleaved and subcloned in AgeI/NotI sites. In some experiments we used a truncated version of the IL-2/15Rβ chain (β-C7), which after deletion of 279 amino acids from the C-terminus, has a 7 aa long cytoplasmic tail.

The plasmids encoding TagBFP targeted to the cytoplasmic surface of the ER (ER marker) or Golgi (Golgi marker) were created by Prof. Péter Várnai (Semmelweis University).

Transient transfection of HeLa cells in a 8-well chambered cover slip was achieved with FuGene HD transfection reagent, according to the manufacturer's protocol. HeLa cells were plated 24 hrs before transfection (10^4 cells/well/300 µl culturing medium). 24 hrs after transfection, before the measurement, culturing medium (containing the transfection mix) was replaced to Leibovitz's L-15 „imaging" medium.

3.3. Retroviral transduction

HEK293T cells serving as a packaging cell line (maintained in Dulbecco's Modified Eagle's Medium) were transfected with the retroviral transfer vector pBMN-Z-IN encoding the cDNA of the appropriate insert (the IL-2Rα, IL-2/15Rβ or γ_c receptor subunit subcloned in ORF frame 1 of the vector) and helper plasmids: VSVG and PAX2 by using the calcium phosphate transfection method. 25 µM chloroquine was added to the cells to aid transfection. Supernatants containing retrovirus were collected 48 hours after transfection, filtered with a 0.45 µm syringe filter, and used for infecting HeLa cells. 10 µg/ml polybrene was added to increase the infection efficiency, which was checked 48-72 h later using immunofluorescence labeling and confocal microscopy.

3.4. Immunofluorescent labeling

HeLa-IL-2Rα, HeLa-β and HeLa-γ_c cell lines – created by viral transduction – without any fluorescent protein tag, were visualized by direct immunofluorescent labeling. Cells were plated 24 h before labeling in eight-well chambered cover slip. After washing in ice-cold Hanks' balanced salt solution (HBSS), cells were incubated with 50 µg/ml fluorescent mAbs for 30 min on ice, then washed twice in ice-cold HBSS and fixed with 2% formaldehyde/PBS. The following mouse monoclonal antibodies were used: AlexaFluor546-conjugated anti-Tac for IL-2Rα against the cytokine-binding epitope, Mikβ3 for IL-2/15Rβ, TUGh4 for γ_c.

For labeling of membrane proteins of FT7.10 cells, the following mouse monoclonal antibodies were used: W6/32 for MHC I heavy chain, L368 for β2-microglobulin, anti-Tac for IL-2Rα, anti-FLAG-M2 or 7A4-24 for IL-15Rα, MEM-75 for transferrin receptor and

MEM102 for GPI-anchored CD48 protein. Antibodies were conjugated with succinimidyl esters of Alexa Fluor 488, 546 or 647 dyes.

3.5. Determination of FRET efficiency by confocal microscopy

Molecular associations of the receptor chains at the 2-10 nm level were assessed by FRET microscopy on a pixel-by-pixel basis using Zeiss LSM 880 or Leica TCS SP5 II confocal microscopes. For excitation of EGFP the 488-nm line of an Ar ion laser and for mCherry a 543-nm (Zeiss) or 594-nm (Leica) HeNe laser were used. Signals were detected in 3 channels: donor (ex: 488 nm, em: 500-550 nm), transfer (ex: 488 nm, em: 604-687 nm) and acceptor (ex: 543 or 594 nm, em: 604-687 nm). Spectral crosstalk factors were calculated from cells singly transfected with EGFP (as FRET donor) or mCherry (acceptor), respectively. Background correction in each channel was achieved by subtracting average autofluorescence intensities measured from non-transfected cells. The α factor relating the signal intensities in the donor and transfer channels arising from equal amounts of excited donor and acceptor proteins was assessed by using cells transfected with the EGFP-mCherry fusion protein (expressing the two fluorescent proteins at a 1:1 ratio) The FRET efficiency (E) was calculated for all samples co-expressing donor- and acceptor-tagged proteins.

Target sequences of Sac1 (ER marker) or giantin (Golgi marker) tagged with TagBFP (excited at 405 nm) were used to identify these cellular organelles. By selecting pixels where TagBFP fluorescence was above a threshold, FRET data were evaluated in an organelle specific manner. Data analysis was carried out using the RiFRET plugin written for the FiJi software.

3.6. Determination of FRET efficiency by flow cytometry

Doubly labeled cells - with mAbs tagged with donor (Alexa Fluor 546) and acceptor (Alexa Fluor 647) dyes – were used for the measurements, which was carried out on a FACSAria III instrument. Three fluorescence intensities (in the donor, FRET and acceptor channels) were detected from each cell. The following excitation wavelengths and detection bands were used: 561/595±25, 561/>635 and 633/>635 nm. Spectral overspill factors and α factor relating the detection efficiencies of the donor and the acceptor were determined from samples singly labeled with donor or acceptor antibodies. Dead cells were excluded from the analysis based on side scatter vs. forward scatter dot plots. FRET data were analyzed with a software written in-house, REFLEX.

3.7. Golgi isolation from ATL cells

Golgi compartment was isolated from ED40515(+) wild type cells (after 48 hrs IL-2-starving) and from ED/IL-2 cells by ultracentrifugation, using Golgi Isolation Kit based on the protocol recommended by the manufacturer (Sigma-Aldrich).

3.8. Western blot from isolated Golgi fraction

Cells were dissolved in protein lysis buffer containing protease inhibitor and phosphatase inhibitor at 4°C and their protein concentration was determined by Bradford assay. Protein concentration was determined by Bradford assay. Lysates containing 2 mg/mL protein were mixed with equal volumes of denaturation buffer and incubated at 99°C for 10 minutes. Protein (20 μg) was electrophoresed on 8% SDS–polyacrylamide gels and electroblotted onto a PVDF membrane. Antibodies against IL-2 signaling elements, ER, Golgi and for detection of possible contamination (lysosome/late endosome, recycling endosome, plasma membrane) were applied, bands were visualized by ECL Kit (Advansta).

3.9. Statistical analysis

Student's *t* tests with Holm-Sidak correction for multiple comparision were performed with the GraphPad Prism software.

4. RESULTS

4.1. Intracellular preassembly of IL-2/15 receptors

4.1.1. Confocal microscopy shows that cotransfected receptor chains are partially colocalized in the ER and the Golgi

A plausible explanation for the evasion of IL-2-producing cells from antagonistic antibody block targeting cell surface receptors (and exogenous IL-2) would be that IL-2 receptors could already preassemble and signal before reaching the cell surface using endogenous IL-2. Therefore, we set out to investigate the colocalization and molecular vicinity of receptor subunits along the secretory pathway, in the ER and the Golgi. We gained a first insight into the extent of colocalization between coexpressed receptor chains at ~200-300 nm resolution by confocal microscopy in different compartments of living HeLa cells. The extent of co-localization was assessed by the Pearson's correlation coefficient, *C*, between the green and red pixel intensities in the two images. The maximal value of this parameter is 1 for perfectly overlapping distributions, it is 0 for independent ones and negative for mutually exclusive ones. As a positive control, I used the EGFP-mCherry fusion protein, for which the signals in the green and red channels were proportional resulting a homogenous yellow merged image and a high average *C* value of 0.93. As a negative control, the pixels of confocal images were randomized yielding an average *C* value of zero. TagBFP-labeled ER/Golgi resident proteins were used as markers to visualize these organelles and study colocalization of receptor chains in an organelle specific manner. The colocalization of the γ_c-EGFP and IL-2/15Rβ-mCherry chains was only partial in the ER and the Golgi; besides areas where both chains were present at similar concentrations, there were also regions where one or the other protein dominated. The partial colocalization is reflected by the lower values of *C* (0.55 and 0.5 in the ER/Golgi) as compared to the positive control. For IL-2Rα-EGFP and β-C7-mCherry, the C-terminally truncated version of IL-2/15Rβ, the correlation was high in the ER and the Golgi ($C \approx 0.7$), but even higher in the plasma membrane ($C=0.83$). For IL-15Rα-EGFP and β-C7-mCherry the tendency was similar: partial colocalization in the ER and the Golgi ($C=0.62$ and 0.67) and more extensive in the plasma membrane ($C=0.83$). The distinct EGFP-IL-2Rα and mCherry-IL-15Rα subunits had intermediate *C* values in the ER/Golgi (0.54, 0.48) and a very high value in the plasma membrane (0.89). The colocalization of co-transfected γ_c-EGFP and γ_c-mCherry in the ER/Golgi was also partial ($C=0.5$ and 0.68).

4.1.2 Control samples for confocal microscopic FRET measurements

I used confocal microscopic FRET measurements to assess the intracellular pre-assembly of IL-2/15 receptor subunits at the molecular level in an organelle-specific manner. The positive control, the EGFP-mCherry fusion protein resulted an average FRET efficiency of E=24.5% ± 2.3% (mean ± s. d.).

Three kinds of negative controls were applied. First, I used cotransfected EGFP and mCherry, which were distributed evenly in the whole cell yielding E=0.9% ± 1.7%. Second, I coexpressed N-terminally tagged EGFP-IL-2Rα with mCherry-GPI, a glycosylphosphatidylinositol-anchored protein, in which case the donor and the acceptor are both at the extracellular side of the plasma membrane, resulting E=0.4% ± 1.1% in the ER and 1.5% ± 0.9% in the plasma membrane. This control allowed us to assess the extent of random FRET that may occur between two unrelated membrane components expressed in lipid rafts at similar concentrations as those of the receptor chains in our experiments. The low E values indicate that random FRET is not a concern at the expression levels used. Third, I applied C-terminally labeled IL-2Rα-EGFP coexpressed with mCherry-GPI, resulting E=1.1% ± 0.6% in the cytosol (no ER/Golgi marker was used) and 0.4% ± 0.9% in the plasma membrane. In this case IL-2Rα is tagged at the cytoplasmic, whereas GPI at the extracellular side, the donor and the acceptor being separated by a distance at least as large as the thickness of the lipid bilayer (5-10 nm). This control showed that for a sample where the donor-acceptor distance is outside the range of FRET, the resultant FRET efficiency is practically zero, thus proving that spectral crosstalk factors are determined accurately making our FRET calculations accurate. Importantly, mean FRET efficiencies were ≤1.6% for each negative control.

4.1.3. The IL-2/15Rβ chain assembles with γ_c in the ER and the Golgi

After determining the FRET efficiencies of the control samples, I studied the assembly of the intermediate affinity IL-2/15Rβ—γ_c heterodimer. FRET measurements were carried out first with cells cotransfected with γ_c-EGFP and IL-2/15Rβ-mCherry. FRET efficiencies calculated in pixels where the ER or Golgi marker was present resulted 5.5% ± 2.7% and 4.6% ± 2.5% (average ± s. d.), respectively. Using donor and acceptor labeling in reverse order (IL-2/15Rβ-EGFP and γ_c-mCherry) resulted in similar FRET efficiencies, 5.5% ± 2.4% (ER) and 3.7% ± 2.2% (Golgi). All these values are significantly higher than that of the negative control (EGFP+mCherry) indicating that the IL-2/15Rβ and γ_c chains are at least partially pre-

assembled in these organelles. The fluorescence intensity from coexpressed γ_c-EGFP and IL-2/15Rβ-mCherry subunits at the cell surface was hardly discernible from the autofluorescence intensity; therefore, I did not measure FRET in the plasma membrane. Among the IL-2/15R subunits IL-2/15Rβ has the longest cytoplasmic tail, 200 amino acids longer than the γ_c chain. To bring the C-termini of the two receptor chains closer together, I also used a C-terminally truncated version of the β chain (β-C7) having a cytoplasmic tail of only 7 aa. Contrary to our expectations, this modification did not influence the FRET efficiency to a significant extent; its value was E=4.4% ± 1.5% in the ER and 4.1% ± 2.0% in the Golgi.

4.1.4. IL-2/15Rβ subunit assembles partially with IL-2Rα or IL-15Rα along the secretory pathway before full assembly at the cell surface

Next, I assessed the association of the IL-2/15Rβ chain with the cytokine specific IL-2Rα and IL-15Rα chains. I compared N- or C-terminally labeled subunit pairs and examined the effects of shortening the β subunit as well as the presence of unlabeled γ_c chains. FRET efficiencies measured between N-terminally tagged subunits, EGFP-IL-2/15Rβ and mCherry-IL-2Rα (5.5% ± 1.3% in the ER, 5.0% ± 1.7% in the Golgi) or mCherry-IL-15Rα (6.9% ± 2.4% in the ER, 6.2% ± 2.3% in the Golgi) were significantly higher than those for the negative control. These results suggest that each α chain is at least partially pre-assembled with the β chain prior to reaching the cell surface. I received smaller FRET efficiencies between C-terminally tagged IL-2Rα-EGFP and IL-2/15Rβ-mCherry (4.3% ± 1.1% in the ER, 2.3% ± 0.9% in the Golgi) than between the N-terminally labelled counterparts, which is probably due to a larger distance between the C termini of these subunits. Therefore, I also measured FRET between the C-terminally truncated β-C7-mCherry and the IL-2Rα-EGFP (5.0% ± 1.7% in the ER, 4.7% ± 2.0% in the Golgi) or IL-15Rα-EGFP (4.9% ± 1.6% in the ER, 4.5% ± 1.3% in the Golgi) yielding higher FRET efficiencies than achieved with the full length β chains. When β-C7-mCherry was coexpressed with IL-2Rα-EGFP or IL-15Rα-EGFP, all receptor subunits were highly expressed in the plasma membrane, where they resulted much higher FRET efficiencies (21.6% ± 5.4% for IL-2Rα and 21.6% ± 5.1% for IL-15Rα) than in the ER or the Golgi. The high FRET efficiencies measured in the plasma membrane imply that full assembly of these receptor chains ensues only at their final destination, i.e., pre-assembly along the secretory pathway is partial.

In order to examine whether γ_c, the third element of the high affinity $\alpha\beta\gamma_c$ receptor heterotrimers influences the assembly of the α- and β-chains, I also used a cell line stably

expressing non-tagged, dark γ_c. The presence of γ_c did not significantly influence the interaction between the IL-15Rα and β-C7 subunits.

4.1.5. IL-2Rα and IL-15Rα form a common cluster

Earlier our workgroup has shown that IL-2Rα and IL-15Rα were coexpressed in a common complex on T cells, which probably plays a role in efficient sharing of the signaling β and γ_c subunits. We were interested whether the two α chains are also pre-assembled along the secretory pathway. The distinct α chains, N-terminally tagged with EGFP or mCherry, displayed a positive FRET efficiency suggesting that they could form heterodimers in the ER and the Golgi (ER: E=4.8% ± 1.4%; Golgi: 3.8% ± 2.2%). In the plasma membrane a higher FRET efficiency, E=9.5% ± 2.5% was measured; thus, the intracellular assembly is probably partial. To test whether the presence of the IL-2/15Rβ subunit influences the interaction of the two distinct α subunits, we used a HeLa cell line stably transfected with non-tagged IL-2/15Rβ. There was a slight decrease of FRET efficiency at each cellular organelle suggesting that there is a competition between the interaction of the two α chains and their heteroassociation with IL-2/15Rβ.

4.1.6. Control experiments for investigation of overlapping of cell organelles

In the colocalization and FRET experiments TagBFP-tagged ER or Golgi marker were used and the analysis was restricted to pixels where this marker was present. I determine apparent colocalization between ER (BFP-Sac1) or Golgi (BFP-Giantin) and recycling endosomes (Rab11-mCherry) or lysosomes (Lysotracker Red) by targeting these organelles with fluorescent markers and calculating the Pearson's correlation coefficients between their distributions.

Low correlation coefficients suggest that although there is some overlap between the distributions, confocal microscopy using organelle markers could sufficiently discern the ER/Golgi from recycling endosomes and lysosomes.

4.1.7. Phosphorylation of IL-2 signaling elements in the Golgi/ER of ATL cells

To assess the signaling competence of preassembled IL-2R in the ER/Golgi, we isolated Golgi from our ATL cell lines and investigated phosphorylation of signaling elements of the

IL-2 pathway by Western blot. Jak1 binds and phosphorylates the β chain, whereas Jak3, binding constitutively, phosphorylates the γ_c chain. pJak1, pJak3 and phosphotyrosine bands around the expected molecular mass of γ_c (65-70 kDa) chains were detected from the isolated Golgi fraction of IL-2-expressing ED/IL-2 cells; the signals from all three phosphorylated components were weaker for IL-2 non-expressing, IL-2-starved ED40515(+) wild type cells. The isolated Golgi fractions were GM130 positive (Golgi marker), but also contained calreticulin, characteristic for the ER. No lysosome/late endosome marker (LAMP1), recycling endosome marker (Rab11) or plasma membrane marker (MDR1 having a half-life of >100 hours) were found in the isolates. Thus, the Golgi fraction does not contain a significant fraction of plasma membrane contamination.

4.2. Partial disassembly of MHCI-IL2R protein clusters at the cell surface

4.2.1. Expression of IL-2/15 receptors and MHC glycoproteins before and after MHC I gene silencing

IL-2R, IL-15R, MHC I and MHC II form superclusters in lipid rafts of Kit 225 FT7.10 T lymphoma cells. To define the role of MHC I, the most abundant element in the organization of these clusters, we knocked down MHC I expression by anti-β2m siRNA. Because of the high abundance of MHC I it was essential to maximize silencing efficiency. MHC I expression at the cell surface decreased with increasing siRNA concentration reaching a maximal effect at 100 μg/ml where expression was reduced to 5-10%. KD was specific: expression levels of IL-2Rα, IL-15Rα or MHC II did not change. Irrelevant siRNA (anti-GFP or anti-erbB1) used as control caused no variation in the expression of MHC I, IL-2Rα or IL-15Rα.

4.1.2. Partial disassembly of MHC I/IL-2Rα/IL-15Rα clusters upon MHC I KD, decrease of the extent of homo- and heteroassociations of MHC I

To dissect interactions between individual protein species at the nanometer scale, we applied Förster resonance energy transfer (FRET). Homo- and heteroassociations of IL-2R and IL-15R subunits, MHC I and II glycoproteins were analyzed on a cell-by-cell basis using flow cytometry. As a negative control, the FRET efficiency between coated-pit-located transferrin receptors and lipid-raft-located GPI-anchored CD48 proteins was measured, yielding E=3±1%. As a positive control, we measured FRET between the light and heavy chains of MHC I, which decreased from 52±1% to 28±1% upon MHC I KD. Here, the FRET process is only partially

intramolecular: FRET can also occur between the light and heavy chains of distinct MHC I molecules residing next to each other in an aggregate. Homoclustering of MHC I heavy chains diminished significantly upon MHC I knockdown: the FRET efficiency between heavy chains dropped from 35±1% to 11±1%. Here, MHC I was targeted using donor- and acceptor-tagged antibodies specific for the heavy chain of intact MHC I containing both β2m and the heavy chain.

The heteroassociation of donor-labeled IL-2Rα and acceptor-labeled MHC I also decreased significantly upon MHC I KD, from 47±1% to 9±1%. FRET between IL-15Rα and MHC I decreased similarly, from 44±1% to 7±1%. This is not surprising because the acceptor-to-donor ratio decreased, thereby the probability that a given donor-labeled protein had an acceptor-labeled partner within FRET-range (2-10 nm), diminished. The heteroassociation of IL-15Rα with IL-2Rα did not change significantly, suggesting that interleukin receptor assembly was not influenced by interactions with MHC I.

4.1.3. Dependence of FRET efficiencies on the expression levels of donor- and acceptor-tagged proteins

FRET efficiencies as a function of expression levels of the interacting proteins was also analyzed. Cells were classified into three classes according to the expression (low, medium, high) of the donor-tagged proteins. For the heteroassociation of donor-tagged IL-15Rα or IL-2Rα with acceptor-tagged MHC I, there is a clear tendency of increasing FRET efficiency versus increasing N_A/N_D acceptor/donor molecular ratio, and faster saturation of the graph for higher donor expressors, just as expected for association equilibria.

In the case of homoassociation of MHC I, FRET efficiency increases with increasing expression level of MHC I. Selected cells had about equal numbers of donor- and acceptor-tagged MHC I molecules: N_A/N_D was in the range of 0.9-1.1.

5. DISCUSSION

5.1. Intracellular preassembly of IL-2/15 receptors

IL-2 and -15 receptor subunits can form various homomeric and heteromeric complexes in the plasma membrane having different ligand binding affinities. Of these, the $\beta\gamma_c$ intermediate affinity heterodimers (Kd~10^{-9} M ligand binding affinity) and the high affinity $\alpha\beta\gamma_c$ heterotrimers (Kd~$^{10\text{-}11}$ M) are capable of efficient signaling. Earlier our workgroup has shown that IL-2 and -15 receptors are preassembled in the plasma membrane even in the absence of ligand, and their conformation changes upon ligand binding and also demonstrated that the two receptor kinds form a common complex including the shared β and γ_c chains and the cytokine specific IL-2Rα and IL-15Rα chains. It is an intriguing question whether the newly synthesized constituents of multicomponent membrane receptors find each other only in the plasma membrane, or they arrive there in a preassembled form. If the receptor subunits can already associate along the secretory pathway, they may potentially signal even before reaching the plasma membrane. This would define a new way of autocrine signaling in cells that produce the receptors and their ligands as well.

In addition to the endocrine and paracrine action, autocrine mechanism has also been described in case of growth hormone receptor (GHR) belonging to the same cytokine receptor superfamily. According to the model, growth hormone in the ER binds to its receptor immediately after synthesis facilitating the maturation of the receptor. Exogenous growth hormone cannot bind to the hormone-receptor complex at the cell surface. Receptor dimers in the ER cannot initiate signaling, activation of GHR occur only when it is in or past the Golgi.

Autocrine signaling has importance in disorders as well, contributing to self-sufficiency of tumor cells, facilitating key functions of growth, survival and invasion. The function of major angiogenic factor, VEGF (vascular endothelial growth factor), is not be restricted to endothelial cells, it can influence the function of tumor cells as well. Presence of specific VEGF receptors on carcinoma cells implies that such cells have the potential to respond to VEGF in either an autocrine or paracrine manner. In addition to its survival functions, VEGF autocrine signaling also contributes to tumor progression by inducing CXC4 chemokine receptor expression.

We demonstrated the presence of pJak1 and pJak3 as well as a phosphotyrosine signal around the expected molecular mass of the γ_c chain (which is phosphorylated by Jak3) on Western blots from the isolated Golgi fraction of ED/IL-2 cells. In the Golgi fraction of IL-2-dependent wild type cells after 48 h IL-2 withdrawal these phosphoproteins were not detectable.

These findings can be explained by formation of efficient ligand-receptor signaling complexes in the intracellular space before receptors reached the membrane.

Production and intracellular binding of the IL-2 ligand to its receptor in T cells involved in these immune reactions may reduce the efficiency of antibody therapies targeting receptors expressed in the plasma membrane only but having no access to them in intracellular organelles.

To test the hypothesis that receptors could preassemble before reaching the plasma membrane thereby creating the possibility of intracellular signaling, I studied the interaction of the IL-2 and -15 receptor chains in the ER and the Golgi. FRET microscopy is a well-established method to report on molecular interactions. Earlier the colleagues of our department used this technique to study the interaction between IL-2/9/15 receptor subunits. Here, we used fluorescent ER and Golgi markers allowing us to study protein-protein interactions in living cells in an organelle-specific manner. The lateral and axial resolution of the confocal microscope is ~200 nm and ~500 nm, respectively; therefore, an area identified as positive e.g. for the ER marker contains not only the membrane and lumen of the ER but also the cytoplasmic space between neighbouring cisternae.

My FRET data gained from HeLa cells expressing fluorescent protein-tagged IL-2/15 receptor chains revealed the pairwise association of the β + γ_c, β + IL-2Rα as well as β + IL-15Rα subunits in the ER and the Golgi. I also found that the distinct α chains of IL-2R and IL-15R were associated with each other in these organelles, in line with our previous findings in T cell plasma membranes. These observations imply that following their synthesis, the subunits of IL-2R and also IL-15R start their assembly along the secretory pathway, which may be a general phenomenon for different receptors. The very low FRET efficiency detected between EGFP-IL-2Rα and mCherry-GPI used as a negative control proved that accidental colocalization of unrelated proteins expressed at similar levels as those of the labeled receptor chains could not result in a significant random-FRET in the membrane or the ER.

The FRET efficiencies characterizing the interactions between the β and α chains or the IL-2Rα and IL-15Rα subunits were lower in the ER/Golgi than in the plasma membrane. Such a discrepancy may result from the following scenarios. On the one hand, the conformation of the receptor chains at different cellular localizations may differ, leading to different donor-acceptor distances and thus different FRET efficiencies within a single D-A pair. On the other hand, in the ER/Golgi there may be a mixture of assembled receptor complexes and monomers, while this equilibrium is shifted toward a higher ratio of assembled complexes in the plasma membrane. Overlay images of the fluorescence intensity distributions of coexpressed receptor

chains showed that their relative concentration ratios were different at various regions of the ER or the Golgi. After their synthesis by distinct ribosomes at different parts of the ER, receptor chains start to mix in the endomembrane systems by diffusion; however, their mixing is obviously incomplete. Besides areas where both receptor chains are present at similar concentrations, there are also regions where one of the two receptor chains is dominantly present. In contrast, in the plasma membrane the mixing of the receptor chains at the few-hundred-nanometer scale is more perfect. The values of the Pearson's correlation coefficient, a measure of colocalization at this distance scale, were higher in the plasma membrane than at the ER/Golgi for all studied pairs of receptor chains.

We may speculate about further possible reasons for this discrepancy. Another possibility is that the lipid environment in the ER/Golgi may differ from that in the plasma membrane, which may also affect interactions between the receptor chains. Colleagues of our department have shown earlier that the cholesterol content of the plasma membrane, probably due to its contribution to the integrity of lipid rafts, plays an important role in the distribution and function of IL-2R in Kit225 T lymphoma cells. Cholesterol depletion significantly reduced the efficiency of IL-2 signaling and made the originally patchy, clustered distribution of IL-2R more diffuse in the membrane. It also reduced interactions between IL-2R subunits and MHC I and II glycoproteins coexpressed in lipid rafts.

Based on my FRET data, combined with the Western blot results indicated signaling activity in the Golgi of IL-2-producing ATL cells, we put forward the possibility of a new autocrine signaling mechanism that utilizes intracellular IL-2 binding to preassembled receptors along the secretory pathway. This mechanism has clinical implications regarding antibody therapies targeting receptors in the plasma membrane.

5.2. Partial disassembly of MHCI-IL2R protein clusters at the cell surface

Membrane proteins form dynamic clusters at different hierarchical levels, which may be regulated by several factors. These include protein-protein interactions between transmembrane helices or extra/intracellular domains, the lipid composition of the membrane, the cytoskeleton, or crosslinking of glycoproteins by galectin lattices. In T cells, disruption of lipid rafts by cholesterol depletion resulted in blurring of IL-2R clusters and impairment of IL-2 signaling.

In the thesis there is a detailed view of the effects of knocking down a highly expressed member of a membrane protein cluster on the lateral organization of the remaining elements of the cluster. FRET measurements gave insight into protein-protein interactions at the nanometer scale. Associations involving MHC I diminished upon its KD as indicated by the decrease of FRET efficiencies. Importantly, not only the homoassociation of MHC I diminished, but also the heterotypic interactions between IL-2Rα/IL-15Rα and MHC I became less abundant as expected. On the other hand, the interaction between IL-2R/IL-15R subunits did not change significantly, suggesting that the interactions holding these subunits together were not critically dependent on the presence of MHC I. Previously our research group found that IL-2/15 receptors were associated with lipid rafts even in the absence of specific ligands, whereas other results suggested that the migration of IL-2 receptors to rafts was induced by ligand binding.

Lipid raft domains confine IL-2Rα/IL-15Rα and MHC glycoproteins in superclusters, in which smaller interacting tight aggregates are formed as identified by FRET. In conclusion, MHC I knockdown significantly influenced cell surface supramolecular patterns. Changes of clustering properties induced by changes of MHC I expression level may modulate signaling properties of receptor complexes associated with MHC I. Our data demonstrate that modifying the expression of one element of a cluster alter protein-protein associations within the cluster.

6. SUMMARY

The membrane receptors (consisting of three subunits) of the interleukin-2 and -15 cytokines play essential roles in the regulation of the immune system and are important in several lymphomas and autoimmune disorders. I studied two aspects of these receptors: (1) assembly of their subunits intracellularly, and (2) disassembly of their superclusters formed with MHC molecules at the cell surface. Our results can be summarized as follows:

In the ER and the Golgi of live HeLa cells we demonstrated partial heteroassociation between β and γ_c subunits, β and IL-2Rα or IL-15Rα subunits, between the two ligand-specific α-chains, moreover homoassociation between γ_c-chains. The presence of neither IL-2Rα nor β influenced this homoassociation significantly. There is a competition between the interaction of the two α chains and their heteroassociation with the β subunit. We showed that colocalization and assembly of the ILR subunits are more complete in the plasma membrane than in these intracellular organelles. Our measurements suggest that the intracellular domain of the β chain has a strongly folded conformation. We identified pJAK1 and pJAK3 kinases in the Golgi fraction of IL-2-producing human ATL cells indicating efficient signaling induced by intracellularly produced cytokine.

Our results imply the formation of efficient ligand-receptor signaling complexes intracellularly; IL-2/15 receptors start to assemble in the ER/Golgi, before they reach the plasma membrane and signal, utilizing the IL-2 in IL-2-producing adult T-cell leukemia/lymphoma cells. This finding raises the possibility of a new intracellular autocrine signaling mechanism, which explains resistance to antiproliferative daclizumab (anti-IL-2Rα) therapies targeting receptors at the cell surface.

MHC I forms superclusters with several membrane receptors including IL-2/15R at the cell surface. Silencing of MHC I (minimalized upon gene silencing) influences not only its homoassociations, but also its heteroassociations with the IL-2/15 receptors. Although interactions holding IL-2/15 receptor chains together, are independent from presence of MHC I, alteration of clustering properties might affect the signaling efficiency of receptor complexes associated with MHC I molecules.

7. PUBLICATIONS

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List of publications related to the dissertation

1. **Volkó, J.**, Kenesei, Á., Zhang, M., Várnai, P., Mocsár, G., Petrus, M. N., Jambrovics, K., Balajthy, Z., Müller, G., Dóczy-Bodnár, A., Tóth, K., Waldmann, T. A., Vámosi, G.: IL-2 receptors preassemble and signal in the ER/Golgi causing resistance to antiproliferative anti-IL-2R[alfa] therapies.
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   * These authors contributed equally to this work.
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IF: 3.711

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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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