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

Investigation of Interleukin-15 trans-presentation

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The PhD Defense takes place at the Life Science Building, F.008-009 lecture hall, Faculty of Medicine, University of Debrecen, at 15:00, 26th of January, 2023.

1. Introduction

1.1 The role of lymphocytes in the immune system

Lymphocytes play a prominent role in both natural and adaptive immunity. They are made up of natural killer (NK) cells, B cells and T cells, which are produced in the bone marrow. As part of the natural immunity, NK cells recognize tumour or virus-infected cells and destroy them by lysis or by inducing apoptosis. After forming in the bone marrow, B cells also continue to develop there. Their primary role is antibody production and antigen presentation as part of adaptive immunity. T cells develop in the thymus and are essential components in the regulation of immune processes.

1.2 IL-2 and IL-15 cytokines

There are many groups and subgroups of cytokines, and in general they include proteins involved in the immune response and signal transduction. Interleukin-2 and -15 (IL-2 and IL-15), of relevance to this thesis, belong to the family of four α-helix bundle cytokines. The IL-2 cytokine was first described by Robert Gallo's group, which led to the identification of the first retrovirus (HTLV) in humans. The IL-15 cytokine was discovered almost simultaneously by Thomas A. Waldmann's and Kenneth H. Grabstein's research groups. The IL-2 and IL-15 receptors (IL-2R, IL-15R) are heterotrimeric in structure, containing cytokine-specific α subunits that bind their specific ligands with different affinities. IL-15Rα (CD215) binds IL-15 with higher affinity (Kd: 50 pM) than IL-2Rα (CD25) binds IL-2 (Kd: 10 nM). Both receptors use the IL-2/IL-15Rβ subunit (CD122) and the γ_c (CD132) chain (also found in IL-4, IL-7, IL-9 and IL-21 receptors). Both IL-15 and IL-2 stimulate T-cell proliferation, induce the production of cytotoxic lymphocytes (CTL) and stimulate the expansion of natural killer (NK) cells. However, IL-2 and IL-15 may play different roles in many adaptive immune responses. In contrast to IL-2, IL-15 inhibits IL-2-induced cell death (AICD), does not activate functional T_{reg} cells, and does not cause capillary leak syndrome, as has been shown in

animal and clinical studies. IL-15 promotes maintenance of $CD44^{hi}CD8^{+}$ effector/memory T cells and survival of virus-specific memory $CD8^{+}$ T cells. Furthermore, IL-15 is critical for the development of the tissue memory phenotype $CD103^{+}CD28^{+}CD8^{+}$ T cells.

1.3 IL-15 trans-presentation

Dubois et al. demonstrated that, unlike the cytokine IL-2, IL-15 ensures T cell survival for a longer period after culturing cells in medium devoid of both cytokines. They also showed that IL-15 on the cell surface is dependent on the expression of IL-15Rα. Furthermore, endosomal recycling requires the presence of the IL-15Rα/IL-15 complex. This type of IL-15 recycling is not dependent on β subunit expression but is significantly dependent on the presence of IL-15Rα. Their most significant observation was that the IL-15Rα/IL-15 complex stimulated proliferation of adjacent cells, a process they termed IL-15 trans-presentation (TP). The IL-15Rα/IL-15 complex on the surface of activated monocytes induced trans-proliferation of $\beta\gamma_c$-expressing neighbouring cells. This unique ligand delivery mode is enabled by the high ligand-binding affinity of IL-15Rα, which allows a stable IL-15Rα/IL-15 complex to assemble intracellularly and thus be delivered to the cell surface. T-cells expressing only IL-2/15Rβ and γ_c subunits bind IL-15 with lower affinity (Kd: 1 nM) and since soluble IL-15 is not present at such high concentrations in vivo, these cells require an alternative, directed ligand targeting method. Therefore, IL-15 is primarily presented to these cells by IL-15Rα. One way of presentation is by proteolytic cleavage of the IL15Rα/IL-15 complex in the extracellular compartment, resulting in a soluble IL-15Rα/IL-15 (sIL-15Rα/IL-15) complex, thereby inducing IL-15 signalling on responding cells. Another IL-15 trans-presentation method described is trans-endocytosis, whereby an immunological synapse is formed between the presenting and reacting cells, where IL-15Rα on the surface of the presenting cell presents the IL-15 cytokine to the IL-2/15R$\beta\gamma_c$ heterodimer on the responding cell. The entire IL-15R$\alpha\beta\gamma_c$/IL-15 TP complex,

including part of the presenting cell's membrane, is then internalized by the responding cell.

1.4. IL-2 and IL-15 signaling

Since both IL-2R and IL-15R use the same β and γ_c subunits responsible for signal transduction, their signalling uses the same three main pathways: JAK/STAT, Ras/Raf/MAPK and PI-3K/AKT/mTOR. Activation of the JAK/STAT pathway can lead to proliferation, differentiation, migration or apoptosis. Its signal transduction uses a relatively simple mechanism. The subunits bind constitutively tyrosine kinases JAK1/3 (β subunit) and JAK3 (γc subunit). Upon ligand binding, two (or more) JAKs come into proximity, trans-phosphorylate each other and their receptors, and can bind STAT1, STAT3 or STAT5, which are also phosphorylated by JAK1/3. The phosphorylated STAT dimerizes or tetramerizes and is transported by importin α5- and Ran-dependent transport into the nucleus where it binds to specific regulatory regions to activate or inhibit its target genes. The Ras/Raf/MAPK pathway is one of the best characterised signalling pathways in cell biology, as it is involved in several critical processes (e.g., cell cycle regulation, integrin signalling, cell migration, angiogenesis). The PI-3K/AKT/mTOR pathway has functions similar to the two signalling pathways already described (proliferation, angiogenesis, apoptosis). PI3K, also activated by JAK, performs PIP_2 to PIP_3 conversion. PIP_3, as a secondary messenger, binds PDK-1 to the cell membrane and the most important protein of this signalling pathway, AKT. AKT has several target proteins through which it can regulate many cellular functions.

1.5 Antigen presentation

Antigen presentation (AP) is a well-studied immunological process that is essential for T cell activation. Antigen-presenting cells (APCs) such as B cells, macrophages and dendritic cells are the link between natural and adaptive immunity. They are able to internalise antigens of exogenous origin, which are processed and then bound to major histocompatibility (MHC) molecules and delivered to the cell surface where they are

presented to the T cell receptor/CD3 (TCR/CD3) complex. During the TCR-MHC II interaction, CD4, the co-receptor of the TCR, also binds to the MHC II receptor, further strengthening the intercellular protein complex. In $CD4^+$ T cells, secondary signals are provided by additional protein-protein interactions: CD28 is a receptor on the T cell that binds to CD80 or CD86 on the presenting cell. Expression of additional proteins such as ICOS, 4-1BB and OX40 on T cells and their ligands on APC are also essential for T cell activation. These co-stimulants are only available when the presenting cell encounters and appropriately presents a pathogen. Additional protein-protein interactions such as CD40/CD40L and LFA-1/ICAM-1 further stabilize the immunological synapse (IS). The structure of the IS can be divided into three parts forming concentric rings composed of different segregated protein clusters. This supramolecular activation cluster (SMAC) can be divided into three domains: central SMAC (c-SMAC), peripheral SMAC (p-SMAC) and distal SMAC (d-SMAC). Previous studies have provided insights into the protein composition of these three regions. CD4/CD8 has been shown to be enriched in the c-SMAC. The leukocyte functional antigen-1 (LFA-1) and the cytoskeletal protein talin are concentrated in the p-SMAC, while CD43 and CD45 localize in the d-SMAC. Our institute and other research groups have also shown that various ion channels, predominantly the voltage-dependent potassium channel Kv1.3, are also enriched in the IS by the adaptor protein PSD-95 during the early stages of T cell activation.

2. Objective

Since both IL-15 TP and AP contribute to T cell activation and are based on similar cell-cell interactions, the question arises whether these two processes influence each other or are independent. There are components in immunological synapses, namely inhibitory receptors of the KIR family, expressed by NK cells, that negatively regulate the effect of IL-15 TP between B and NK cells. However, the possible effects of AP on IL-15 TP have not yet been investigated. The experiments described in this thesis study the relationship between IL-15 TP and AP using biophysical methods. The B-cell - T-cell model system used here expresses the proteins required for AP and IL-15 TP, and both "trans" (intercellular) and "cis" (occurring on the same cell) interactions have been investigated. Our aim was to provide the first direct biophysical evidence for the assembly of IL-15R subunits expressed by the two cells during IL-15 TP and to determine whether the receptors involved in AP and IL-15 TP translocate to the immune synapse in a common complex or independently. We also sought to answer whether the signalling efficiency of IL-15 is affected by AP and vice versa.

In the second part of the thesis, we investigated the homodimerization of γ_c subunits. In 2008, Pillet et al. described that the IL-2/15Rβ subunit is homodimerized in the membrane of COS-7 cells, in the absence of ligand or even without the presence of the γ_c or IL-2Rα and IL-15Rα subunits . Our group has shown by FRET experiments that the γ_c subunit behaves similarly, with assembly already occurring intracellularly in HeLa cells. To answer further questions on protein dynamics, we performed single-plane illumination microscopy-based fluorescence cross-correlation (SPIM-FCCS) measurements on the γ_c subunit, which we first applied to the study of membrane proteins.

3. Materials and methods

3.1 Cell culture

Raji B cells and Jurkat T cells used for IL-15 trans-presentation assays were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS and GlutaMAX. Homodimerization of γ_c subunits was examined in HeLa cell line, also cultured in the medium described above. HEK293T cells used for retroviral transduction were maintained in DMEM medium with the previously mentioned supplements. All cells were cultured in 5% CO_2 humidified atmosphere at 37 °C. Raji and Jurkat cells stably expressing IL-15 receptor subunits as EGFP-IL-15Rα, mCherry-IL-15Rα, IL-15Rα and IL-2/15Rβ (hereafter referred to as Raji-EGFP-IL-15Rα, Raji-mCherry-IL-15Rα, Raji-IL-15Rα and Jurkat-IL-2/15Rβ) were selected by geneticin.

3.2. Plasmids

The cDNA of IL-15Rα and IL-2Rα was cloned into the MCS (multiple cloning site) of the expression vectors pEGFP-N1, pmCherry-N1 and pmCherry-C3. The bovine preprolactin signal sequence was inserted upstream of the subunit gene for the C-terminally tagged subunits and upstream of the fluorescent protein gene for the N-terminally tagged subunits. In the ORF (open reading frame) of the pBMN-Z-IN retroviral transfer vectors, the gene of the respective tagged or untagged subunit (EGFP-IL-15Rα, mCherry-IL-15Rα, IL-15Rα, IL-2/15Rβ) was inserted into the LacZ site using BamHI and SalI restriction cleavage enzymes.

3.3. Transient transfection

Plasmids encoding mCherry-IL-2Rα and IL-2Rα-mCherry were introduced into Raji cells by transient transfection (electroporation). Transfection was performed using an Amaxa Nucleofector II device using the manufacturer's recommended protocol. HeLa cells were transfected with FuGene HD transfection reagent.

3.4. Retroviral transduction

The required plasmids (retroviral transfer vector, VSVG envelope and psPAX2 packaging) were delivered into HEK293T cells used as packaging cells. The filtered supernatant containing retrovirus was added to Raji or Jurkat cells with an additional 10 µg/ml polybrene and incubated for 48 h, after which the cells were immunofluorescently labelled to verify the efficiency of transduction.

3.5. Immunological synapse training

IL-15 (100 nM) and/or SEE superantigen (1 µg in 50 µl RPMI 1640 medium) was added to 10^5 (for microscopic measurement) or 6×10^5 (for flow cytometric measurement) Raji cells and incubated at 37 °C for 20 min. After washing in RPMI 1640 medium, the Raji cells were mixed with Jurkat cells (10^5 or 6×10^5), incubated at 37 °C for 20 min after centrifugation at 200×g for 1 min, and then placed on ice for immunofluorescence labelling.

3.6. Immunofluorescence labelling

Raji and Jurkat cells were labelled with a mouse monoclonal antibody conjugated to Alexa Fluor 488 or Alexa Fluor 546 to detect receptors and receptor subunits. Anti-Tac for IL-2Rα, Mikβ3 for IL-2/15Rβ, OKT3 for CD3 and L243 for MHC II (HLA DR) were used for labelling. Cells were washed once in PBS before labelling and incubated on ice for 30 min with 50 µg/ml fluorescent mAb. After labelling, cells were washed twice and fixed with 2% formaldehyde and placed in an 8-well microscopy sample chamber.

3.7 Intensity-based FRET measurement by confocal microscopy

The association of receptor subunits (proximity in the 2-10 nm range) was determined by Förster resonance energy transfer (FRET) microscopy on a pixel-by-pixel basis using an LSM 880 confocal microscope. Alexa Fluor 488 was excited with an Ar-ion laser at 488 nm and mCherry at 543 nm with a HeNe laser. The signals were recorded in 3 channels: I_1 - donor (excitation: 488 nm, emission: 500-560 nm), I_2 - transfer

(excitation: 488 nm, emission: 590-700 nm) and I_3 - acceptor (excitation: 543 nm, emission: 590-700 nm).

FRET efficiencies were calculated pixel by pixel for regions with pixel intensity values above a threshold in both channels 1 and 3 (dynamic threshold was set to 10% of the maximum pixel intensity for each image). After thresholding, ROIs were further manually narrowed based on the transmission image to include only synaptic regions. The FRET efficiencies obtained for the selected pixels were averaged to obtain a value representative of a given synapse. The acceptor/donor ratio (N_A/N_D) affects the FRET efficiency, so this was also determined.

3.8 FLIM-FRET measurement with confocal microscopy

FRET was also determined by the decrease in fluorescence lifetime of donor molecules using a picosecond pulsed laser excitation with a repetition rate of 20-40 MHz. The FRET efficiency can be determined by the following equation:

$$E = 1 - \frac{\tau_{DA}}{\tau_D} \qquad (1)$$

Where τ_{DA} and τ_D are the amplitude-weighted average fluorescence lifetimes of the donor in the presence and absence of an acceptor, respectively, but in the same environment.

3.9 Quantification of receptor translocation

The extent of receptor translocation was evaluated from confocal images by calculating the ratio of the average pixel intensity within the immunological synapse to the average pixel intensity of the whole cell membrane using the MATLAB program as follows:

$$\langle I_{IS,norm} \rangle = \frac{\sum I_{IS} / N_{IS}}{\sum I_{tot} / N_{tot}} \qquad (2)$$

where I_{IS} is the intensity of a given pixel in the IS, N_{IS} is the number of pixels in the IS, I_{tot} is the intensity of pixels in any region of the cell membrane (including the IS) and N_{tot} is the number of pixels from the membrane.

3.10 Flow cytometric measurement of phosphorylated STAT5 and CD3ζ in Jurkat cells

Prior to the formation of immunological synapses, Jurkat cells were labelled with Alexa Fluor 546-W6/32 mAb to distinguish them from Raji cells. After IS formation, cells were separated by trypsin digestion at 37 °C for 5 min. Cells were then labelled with Alexa Fluor 647-anti pSTAT5 mAb or IgG1 κ isotype control mAb. Jurkat cells were scored based on Alexa Fluor 546 positive and EGFP negative (from Raji-EGFP-IL-15Rα) signal; pSTAT5 levels were determined based on Alexa Fluor 647 signal.

To determine the extent of TCR-induced signal transduction, flow cytometry experiments were performed to measure pSTAT5 and pCD3ζ levels. Cells were labelled with anti-pCD3ζ mAb with allophycocyanin label or IgG2b κ isotype control. For evaluation, T cells and T cell - B cell conjugates were determined for cells with a positive signal of Alexa Fluor 546-W6/32, and p-CD3ζ levels were determined from the intensity of allophycocyanin.

3.11 Determination of the co-mobility of γ_c subunits by single-plane illumination microscopy-based fluorescence cross-correlation spectroscopy (SPIM-FCCS)

The co-mobility of receptor subunits was determined by SPIM-FCCS measurements, also known as imaging FCCS. This method is capable of detecting stable co-diffusion of fluorescent particles within the observation time window (diffusion time of particles in the detection volume). The measurements were performed on a custom-built SPIM instrument. To study the homodimerization of γ_c subunits, γ_c-EGFP + γ_c-mCherry co-transfection was used. From the combined beams, a cylindrical lens with a thickness of approximately 1.3 µm formed a light plane that illuminated the cells. Fluorescence

from the sample was collected using a long working distance water-immersion objective with the optical axis perpendicular to the excitation light plane. The collected fluorescence was split into green (500-550 nm) and red (>593 nm) components.

From the fluorescence images, the autocorrelation functions of each channel and the cross-correlation function of the two channels were calculated for each pixel using the QuickFit 3 software. The diffusion coefficients and the ratio of green-red dimer fractions were obtained from the fitting parameters. For model-independent determination of the fraction of molecules in the green-red complex, i.e., interacting proteins, the amplitude of the relative cross-correlation function (*RCCF*) was also calculated for the selected pixels:

$$RCCF(x,y) = \frac{\sum_{i=1}^{n} G_{CCF(x,y)}(\tau_i)}{\sum_{i=1}^{n} G_{ACF(x,y)}(\tau_i)} \qquad (3)$$

where $G_{CCF,(x,y)}$ and $G_{ACF,(x,y)}$ are the values of the cross-correlation and autocorrelation functions in time τ_i and pixel coordinates x,y, respectively. In the computation, n is the number of correlation channels (τ_i values) at the beginning of the correlation curves used for averaging (n = 5 was used). The dynamic range of the *RCCF* ratio is defined by the values of the negative control (co-expressed EGFP and mCherry) and the positive control (EGFP-mCherry fusion protein).

3.12. Statistical analysis

Student's t-tests were performed using GraphPad Prism 8 software. Differences were considered significant at $p<0.05$.

4. Results

4.1. Direct evidence for the assembly of the IL-15 trans-presentation complex

In our experiments, the presenting cell was a Raji B cell expressing a labelled (mCherry or EGFP at the N-terminal) or unlabelled IL-15Rα subunit, while the target cell was a Jurkat T cell expressing an untagged IL-2/15Rβ subunit (endogenously expressing the γc subunit). We checked the expression of receptors or receptor subunits involved in IL-15 TP or AP on different cells. Our measurements showed that MHC II had the highest expression level on Raji B cells followed by IL-15Rα, which was comparable to the expression of CD3 on Jurkat T cells. These were approximately 3× higher than that of the IL-2/15Rβ subunit. The γ_c subunit has two orders of magnitude lower expression than IL-2/15Rβ. To directly observe the assembly of the IL-15Rα/IL-15 - IL-2/15Rβ complex at the molecular level and to study the effect of AP on IL-15 TP, we performed ratiometric FRET experiments. FRET efficiency was evaluated on a pixel-by–pixel basis. IL-15 treatment alone resulted in increased FRET efficiencies between IL-2/15Rβ labeled with A488-Mikβ3 mAb and mCherry-IL-15Rα, indicating assembly of IL-15 receptor subunits and the ligand forming a complex in the IS. Dual treatment with IL-15 and SEE superantigen further increased the FRET efficiency. The low FRET value in the treatment with SEE alone indicated that in the absence of IL-15, there was no significant association between the subunits tested; random FRET may occur due to the high N_A/N_D acceptor-donor molecular ratios.

4.2 CD3 and MHC II assemble only in the presence of antigen

To verify whether AP is established in our model system, we measured the association between CD3 and MHC II in the IS by FLIM-FRET, for which CD3 was labelled with Alexa488-OKT3 and MHC II with Alexa546-L243 mAb. When we treated Raji cells with IL-15 alone, there was no significant FRET between the two proteins despite a high N_A/N_D ratio. However, with SEE treatment, FRET efficiency was significantly increased,

although the N_A/N_D then dropped to half of that with IL-15 treatment. The dual treatment had no additional effect compared to treatment with SEE alone, suggesting that the antigen alone is necessary and sufficient for effective AP.

4.3 No significant cis-interaction between IL-2/15Rβ and CD3

The question may arise as to whether proteins translocating to the IS on the T cell move together or independently. Wpossible found no interaction between the IL-2/15Rβ subunit and CD3 in either AP or IL-15 TP. When measured at the IS, neither IL-15- nor double-treated samples resulted in significant FRET increases. The slightly elevated FREt efficiency upon SEE treatment is likely due to the increased N_A/N_D ratio, which is higher than that observed in other samples. Based on these results, we can conclude that there is no significant association between IL-2/15Rβ and CD3 on either solitary or IS-forming cells.

4.4 The strong cis-interaction between IL-15Rα and MHC II is further enhanced by the SEE antigen

Our finding that AP enhanced the association of IL-15Rα and IL-2/15Rβ subunits raises the question whether IL-15Rα and MHC II also interact with each other on B cells. We investigated the cis-interaction between IL15Rα and MHC II in IS by FLIM-FRET experiments. We first measured FRET on lone B cells and observed an increased FRET efficiency, suggesting that these molecules form a complex in the absence of ligand. We then measured FRET in the IS. The IL-15- and double-treated samples showed similar results; meanwhile, the SEE-only treated sample yielded a significantly higher value, compared to the two formal treatment. We also calculated the efficiency of FRET outside the synaptic region of the cell membrane. The results showed a similar trend as observed in the IS. These results indicate that IL15Rα and MHC II are present in a common complex on both solitary and IS-forming cells.

4.5 Receptor translocation into the IS

The enrichment of proteins involved in AP and IL-15 TP in IS was quantified using the $\langle I_{IS,norm} \rangle$ parameter, which gives the average intensity per pixel of the molecule of interest in the IS compared to the average intensity measured in the cell membrane as a whole. IL-15Rα shows a constant, slight enrichment in the IS for all three treatments. In contrast to IL-15Rα, the IL-2/15Rβ subunit was only enriched in the IS when IL-15 was presented by IL-15Rα: in the case of IL-15 TP, the number of IL-2/15Rβ subunits in the IS was significantly increased. MHC II behaved similarly to the IL-15Rα subunit, showing low but nearly constant enrichment for all treatments. CD3, like IL2/15Rβ, translocated to the synaptic region only when MHC II presented its own ligand (SEE antigen). In this case, the enrichment was significant but lower than that of the IL-2/15Rβ subunit.

4.5. Signal transduction is successfully triggered by IL-15 TP, unaffected by AP

To study whether IL-15 TP can indeed induce signal transduction in the target cell and to determine whether AP had an effect on it, we measured the amount of phosphorylated STAT5 in lone Jurkat cells and Jurkat cells exposed to differently treated Raji cells. We found that solitary Jurkat cells already had a positive base pSTAT5 level, which was slightly elevated by SEE treatment. Interaction with IL-15-treated Raji cells greatly increased pSTAT5 levels, but there was no significant increase with additional SEE treatment. For the latter two treatments, we detected a responsive and a non-responsive population and fitted a two-component lognormal function to the histograms. For comparison of pSTAT5 levels, we used the values of the responding populations corrected for the baseline pSTAT5 levels of lone Jurkat cells. These results indicate that IL-15 TP alone can initiate signal transduction and that AP has no significant effect on this process.

4.6 Effect of IL-15 TP on T cell signalling

The possible effect of IL-15 TP on AP-induced T cell signalling was investigated by flow cytometry and Western blotting by measuring the phosphorylation of the CD3ζ chain of the TCR/CD3 complex. We first measured phosphorylated CD3ζ levels in Jurkat cells exposed to differently treated Raji cells (SEE, IL-15, SEE+IL-15) by flow cytometry. Relative to the isotype control signal, a non-zero pCD3ζ level was already detectable in lone Jurkat cells and in samples where only IL-15 TP occurred. AP alone (SEE treatment) significantly increased pCD3ζ levels. The signal from the dual (SEE + IL-15) treatment was slightly lower than from SEE treatment alone, although the difference was not statistically significant.

We also examined CD3ζ phosphorylation at the cell population level by Western blot. These experiments showed a similar trend, with the strongest pCD3ζ bands in the SEE treated Raji+Jurkat samples. The intensity of the bands confirmed that simultaneous IL-15 TP, although not statistically significant, reduces the efficiency of AP-induced TCR signalling. However, the same trend was always observed in all five replicate experiments.

4.7 SPIM-FCCS measurements demonstrate stable co-diffusion of γ_c homodimer complexes.

Using fluorescence cross-correlation spectroscopy, we can demonstrate the co-diffusion of associated molecules when they form a stable complex. The analysis of the auto- and cross-correlation functions (ACF, CCF) of the fluorescence intensity over time reveals the mobility of the molecules and the fraction of molecules that form a complex. SPIM-FCCS was used to study the homoassociation of γ_c chains.

Cells co-transfected with separate EGFP and separate mCherry encoding plasmids were used as a negative control. To detect homo-oligomerization of γ_c chains, we measured γ_c-EGFP and γ_c-mCherry in co-transfected cells. The measurements were performed on the inner membranes located in the cytoplasm, which were clearly

distinguishable from the plasma membrane. To estimate the extent of dimerization, the model-independent value of the $RCCF_g$ ratio was calculated from the average of the first five measurement points of the CCF cross-correlation function and the green channel ACF_g correlation function. The negative control soluble proteins were evenly distributed throughout the cytoplasm, while γ_c chains were restricted to smaller areas. The $RCCF_g$ value of the γ_c-EGFP + γ_c-mCherry sample was significantly higher than that of the negative control, thus these results indicate that at least some of the intracellular γ_c subunits are in dimeric (or oligomeric) form. From the fitting of the correlation curves, the concentration of each molecular population can be obtained. The green-red dimer fraction $c_{gr}/(c_g+c_r+c_{gr})$ can be calculated from the fitted ACF and CCF curves. The γ_c-EGFP + γ_c-mCherry sample resulted in a significantly higher dimer fraction compared to the negative control.

Finally, the particle mobility was determined from the ACF and CCF functions. ACFs of all samples and CCFs of controls were fitted with a two-component diffusion model. The average values of the fast and slow diffusion coefficients from the ACFs were similar for both controls and γ_c-EGFP + γ_c-mCherry samples. However, the fraction of the slow component of the γ_c-γ_c dimer is much larger than that of the soluble controls, indicating slower diffusion of γ_c subunits in cytoplasmic membrane systems. Our SPIM-FCCS measurements confirmed the existence of γ_c dimers or oligomers in the cytoplasm and demonstrated their slow co-diffusion.

5. Discussion

IL-15 is a pleiotropic cytokine that stimulates a variety of cell types and functions, but also plays a pathogenic role in several organ-specific immune diseases. The question arises as to why among the γ_c cytokines only IL-15Rα/IL-15 employs the TP mechanism. IL-15 TP develops in cases where IL-15 is required in the context of cell-cell contact. The IL-15 TP interaction plays an important role in the formation of $CD^{44hi}CD8^+$ memory T cells, which are the main functional components of immunological memory.

At another level, IL-15 TP is essential for the survival and proliferation of natural killer (NK) cells. It was observed that NK cells did not develop in mice lacking either one of the three subunits of IL-15R or IL-15. In this case, IL-15Rα/IL-15 is also expressed on the presenting cells and formed IL-15 TP with NK cells expressing $\beta\gamma_c$ subunits.

As a third function, suggested by Jabri and Abadie, IL-15 acts as a danger signal to regulate tissue resident T cells and tissue damage. IL-15 has a very broad expression compared to other γ_c cytokines and can be expressed by hematopoietic and other cell types. IL-15Rα/IL-15 can be induced in these diverse tissues by cellular infection and sterile inflammation as well as in autoimmune diseases. IL-15-induced effector cytotoxic cells act locally only when an active tissue "emergency signal" is in progress, regulated by either an infectious or a non-infectious stress signal. Although this is valuable to the host in some cellular infections, it also underlies autoimmune diseases such as gluten sensitivity, type 1 diabetes, patchy hair loss, inflammatory bowel disease and sarcoidosis.

In the light of the role of IL-15 TP described above, it was an interesting question whether IL-15 TP could occur autonomously between an APC and a T cell or only as an AP chaperone. Both IL-15 TP and AP are critically dependent on ligand-driven intercellular protein-protein interactions, and in a T-cell model system we have previously demonstrated the interaction between the two presenting proteins, IL-15Rα and MHC II. Here, we found direct evidence for the generation of IL-15 TP by

examining the association of IL-2/15Rβ and IL-15Rα subunits in both the absence and presence of AP.

The IL-15R heterotrimeric receptor assembled only when IL-15Rα presents the bound IL-15 to the IL-2/15Rβγc complex. During IL-15 TP, each IL-15R subunit was partially translocated to the IS, but, probably due to differences in expression levels, the enrichment of the lower expressed IL-2/15Rβ was found to be more significant than that of IL-15Rα and only occurred when IL-15 was present. To monitor the efficiency of IL-15 signalling, we determined the level of STAT5 phosphorylation in T cells. The results showed that IL-15 TP alone induces the JAK/STAT pathway and that this process is independent of AP. Jurkat cells express IL-2, which may trigger JAK/STAT signalling in an autocrine or intracrine manner, which may explain the elevated basal STAT5 phosphorylation in untreated lone T cells. An interesting phenomenon is that when AP occurred concomitantly with IL-15 TP, the association between IL-2/15Rβ and IL-15Rα subunits was increased; this tighter association could be explained by the stronger IS due to AP and/or the previously described co-association of MHC II and IL-15Rα, which we have also demonstrated in this model system. Our experiments demonstrated the association of MHC II and IL-15Rα on the Raji B cells we used; these molecules translocated together to the synaptic area when IL-15 or antigen was present. In addition, we found that when MHC II bound the SEE superantigen, FRET efficiency between IL-15Rα and MHC II increased not only in the synaptic region but also outside the IS and on solitary B cells.

Similar to IL-15 TP, we also provided direct evidence for the assembly of the antigen-presenting complex by intercellular FRET measurements between CD3 and MHC II. As expected, association of these molecules only occurred when B cells were treated with SEE antigen. We also examined JAK/STAT signalling during AP and found that phosphorylation of STAT5 was low but significantly increased compared to baseline phosphorylation levels. This is caused by an overlap in IL-15 and TCR signalling, with

STAT5 being phosphorylated by JAK1 or JAK3 in IL-15, whereas in TCR signalling it is carried out by the lymphocyte-specific tyrosine kinase Lck. In contrast, SEE-induced phosphorylation of the CD3ζ subunit (via Lck) is moderately reduced in the concomitant presence of IL-15 TP. It is conceivable that the association of IL-15Rα with the $\beta\gamma_c$ heterodimer may inhibit the interaction between IL-15Rα-associated MHC II and the TCR, or possibly intracellularly limit the availability of the CD3ζ phosphorylation site; however, further research is needed to elucidate this phenomenon.

In our model system, similar to IL-15 TP participants, the proteins involved in AP had different expression levels. Therefore, not surprisingly, CD3 with lower expression levels showed a higher translocation to the IS than MHC II. Similar to the IL-2/15Rβ subunit, CD3 was only enriched in the synaptic region when MHC II antigen was presented. Interestingly, the potassium channel Kv1.3, crucial for T cell activation, was also shown to co-localize with CD3, as well as to get enriched in the IS.

Our results suggest that IL-15 TP between APCs and T cells may not only occur in association with AP but may take place independently. Thus, we can consider IL-15 trans-presentation as an autonomous, self-contained process that contributes to the survival of memory T cells when these cells interact with IL-15-presenting cells during their life cycle.

IL-15 TP and AP are autonomous processes that can occur alone or simultaneously. When one of the ligands (IL-15 or antigen) is present, the corresponding receptors on B and T cells translocate to the synapse and establish a cell-cell interaction by forming an intercellular protein complex. IL-15Rα and MHC II on the B cell are in constant association with each other; thus, when the ligand-binding protein translocates to the IS, the other protein follows it and also accumulates there. In contrast, IL-2/15Rβ and CD3 on the T cell do not interact with each other; therefore, only the protein whose ligand is presented by the B cell is translocated into the synapse, the other protein gets

enriched only if its ligand is also present in the IS. IL-15 TP alone is able to induce signalling in the T cell, and although the association of IL-2/15Rβ and IL-15Rα is enhanced by AP, the efficiency of IL-15 TP-induced JAK/STAT signalling is not affected. In addition to the heteromeric structure of IL-15R, homomeric complexes have also been shown: the IL-2/15Rβ subunit can form a dimer in the cell membrane, but its function is not yet clear, and the γ_c subunit has been shown to form trimers in vitro. Our SPIM-FCCS measurements have shown stable association and co-diffusion of γ_c complexes in the intracellular membrane system. Our measurements also demonstrated that γ_c subunits are mobile, similar to Golgi-residue proteins, which have been shown to undergo anomalous diffusion by FRAP and FCS experiments. Our data suggest that γ_c chains have a fast and a slow diffusion subpopulation, but that the dimer/oligomer form belonged entirely to the slow population. The diffusion coefficients of these complexes were similar to those measured for IL-2Rα and IL-15Rα subunits in the plasma membrane, suggesting intracellular membrane diffusion. The diffusion coefficient of the fast fraction of the monomer γ_c was two orders of magnitude higher, similar to that of the soluble controls: the EGFP, mCherry and EGFP-mCherry fusion proteins. We can speculate that some of the γ_c chains may exist in a non-membrane bound form, e.g., due to misfolding, the receptor chain may be complexed with a chaperone protein while being transported into the proteasomes.

6. Summary

Interleukin-15 plays a key role in the long-term maintenance of T cells and immunological memory. IL-15 acts primarily via trans-presentation (TP), whereby an antigen-presenting cell (APC) expressing IL-15Rα subunit binding IL-15 presents its ligand to the $\beta\gamma_c$ receptor heterodimer on a neighbouring T/NK cell. Antigen presentation (AP), the initial step in T cell activation, is also based on APC-T cell interaction. The question arises whether AP influences IL-15 TP or whether they are independent processes. In our Raji B-cell - Jurkat T-cell model system, we studied inter/intracellular protein interactions during the formation of IL-15 TP and AP receptor complexes using Förster resonance energy transfer measurements. We observed enrichment of IL-15Rα and IL-2/15Rβ at the synapse and positive FRET efficiency when Raji cells were pretreated with IL-15 provided direct biophysical evidence for IL-15 TP. IL-15Rα and MHC II formed a complex and translocated to the immunological synapse in the presence of either ligand. IL-15 TP initiated STAT5 phosphorylation in Jurkat cells, which was not affected by AP. Our results demonstrate that in our model system IL-15 TP and AP can occur independently with AP having no significant effect on IL-15 signalling during TP. Thus, IL-15 TP can be considered an autonomous, antigen-independent process.

In addition to heteromeric complexes, IL-15 receptor subunits can also appear as homomeric complexes on the cell surface. Using SPIM-FCCS measurements, we successfully detected the existence of such homomeric complexes for the γ_c subunit. These data also provide evidence that homomeric complexes are formed intracellularly and exhibit co-localization within cytoplasmic membrane compartments during and after protein synthesis.

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List of publications related to the dissertation

1. **Kenesei, Á.**, Volkó, J., Szalóki, N., Mocsár, G., Jambrovics, K., Balajthy, Z., Dóczy-Bodnár, A., Tóth, K. Á., Waldmann, T. A., Vámosi, G.: IL-15 Trans-Presentation Is an Autonomous, Antigen-Independent Process.
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List of other publications

3. Borbásné Sebestyén, V., Nagy, É., Mocsár, G., Volkó, J., Szilágyi, O., **Kenesei, Á.**, Panyi, G., Tóth, K. Á., Hajdu, P., Vámosi, G.: Role of C-Terminal Domain and Membrane Potential in the Mobility of Kv1.3 Channels in Immune Synapse Forming T Cells.
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