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Short Communication

Proximity ligation assay combined with flow cytometry is a powerful tool for the detection of cytokine receptor dimerization

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ABSTRACT

Many cytokine receptors are cell surface proteins that promiscuously combine to form active signalling homo- or heterodimers. Thus, receptor chain dimerization can be viewed as a direct measure of a high probability of intracellular signalling by specific cytokines. Proximity ligation assay (PLA) is an antibody-based method for selective and highly sensitive detection of protein interactions by microscopy. As proof of concept, the aim of this study was to combine antibodies towards interleukin 7 receptor alpha (IL-7Rα) and the common gamma chain (γc) with PLA and flow cytometry to enable the detection of IL-7 receptor heterodimers. The presence of IL-7 receptor heterodimers on the surface of the HPB-ALL T cell line was detected by PLA and microscopy with a resolution of one complex per cell. Optimisation of the PLA reaction on cell suspensions identified buffer effects with critical importance for the flow cytometric outcome. In addition, blocking, fixation and incubation conditions were optimised to prevent unspecific antibody binding. PLA combined with flow cytometry very sensitively detected receptor heterodimers on the cell surface. Thus, the method is a powerful tool for the investigation of cytokine receptor dimerization.

1. Introduction

Intense studies of cytokines and cytokine receptors during the last decade have led to a better understanding of their role in the pathogenesis of inflammatory diseases. Many cytokine receptors are cell-surface proteins that combine to form active signalling homo- or heterodimers. The identification of the cell types influenced by a specific cytokine is challenged by the fact that receptor chains are promiscuous in their dimer associations. Thus, measurements of cell-surface expression of one or more receptor chains by standard immunofluorescence and flow cytometry cannot provide information on the dimerization status and hence receptor activity.

This study focused on the IL-7 receptor heterodimer because of the specific antibody requirements for the assay. IL-7 is a leading differentiation and survival factor for memory T cells [1,2] believed to play a central role in sustaining inflammation, e.g. in rheumatoid arthritis [3]. IL-7 is a member of the IL-2 γ_c family of interleukins, which also includes IL-2, IL-4, IL-9, IL-15 and IL-21. The receptor chains of the family are promiscuous and redundancy in signalling has been demonstrated, e.g. the loss of IL-7 signalling in γ_c knockout mice can be partly restored by TSLP administration [4]. It is believed that IL-7 binding leads to signalling through an active receptor heterodimer formed by the γ_c and the IL-7Rα chain [5].

PLA is an antibody and rolling circle amplification (RCA) based method for the specific detection of protein interactions by microscopy of primary cells [6]. A PLA signal from an IL-7 receptor heterodimer can thus be viewed as a direct measure of a high probability of activity leading to downstream effects. As proof of concept, the aim of this study was to combine specific antibodies towards IL-7Rα and the γ_c with PLA and flow cytometry to enable the detection of heterodimeric IL-7 receptors with high sensitivity.

2. Materials and methods

2.1. HPB-ALL

The human T cell line HPB-ALL (The German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) established from an acute lymphoblastic leukaemia [7] was cultured in

Abbreviations: APC, allophycocyanin; CFSE, carboxy-fluorescein diacetate succinimidyl ester; DAPI, 4′,6-diamidino-2-phenylindole; EGFR, epidermal growth factor receptor; IL-7Rα, interleukin 7 receptor alpha; IL, interleukin; MFI, median fluorescence intensity; PE, phycoerythrin; PLA, proximity ligation assay; RCA, rolling circle amplification; TSLP, thymic stromal lymphopoietin; γ_c, common gamma chain.

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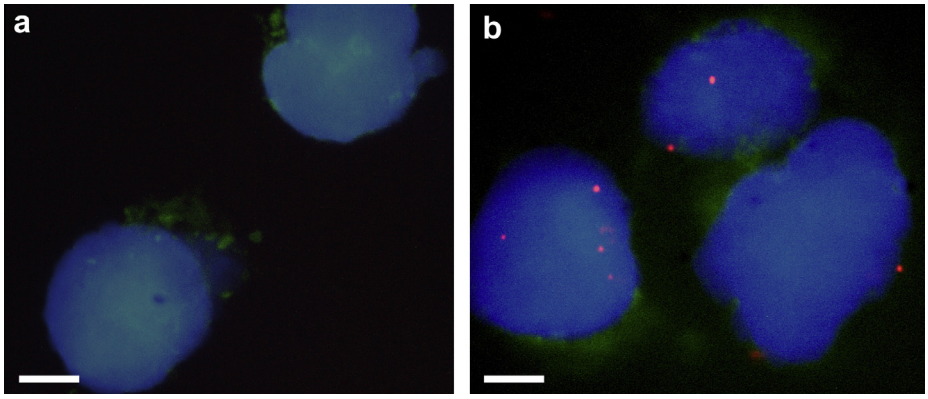

Fig. 1. *Visualisation of IL-7 receptor heterodimers on HPB-ALL.* The PLA signal from each IL-7Rα and γ_c heterodimer was visualised as defined red spots on the cell surface by fluorescence microscopy. (a) Technical negative control with only MINUS probe, (b) PLA with PLUS and MINUS probes. Cell nuclei are blue by DAPI staining and cell cytoplasm is green by CFSE staining.

RPMI 1640 with 10% (v/v) FCS (Invitrogen, Paisley, UK), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine at 37 °C with 5% CO_2 and 75–85% humidity. HPB-ALL was mycoplasma negative by PCR.

2.2. Antibodies and proximity probes

For optimisation of antibody concentration and incubation conditions before PLA experiments mouse PE-conjugated anti-human IL-7Rα (hIL-7R-M21, BD Pharmingen, San Jose, CA) and rat APC-conjugated anti-human γ_c (tUGh4, Biolegend, San Diego, CA) antibodies were titrated by flow cytometry. Unspecific binding was not observed using PE-conjugated mouse IgG1 (DAK-GO1, DAKO, Glostrup, Denmark) and APC-conjugated rat IgG2b (RTK4530, Biolegend) isotype controls. Antibody binding before and after blocking and fixation was compared because cells must be fixed to prevent dimer dissociation during PLA and identical results were obtained (data not shown).

To obtain the proximity probes, unconjugated anti-IL-7Rα antibody (hIL-7R-M21) was concentrated and desalted by filtration in a 100 kDa Amicon Ultra centrifugal unit (Millipore, Billerica, MA) according to the manufacturer's guidelines. Concentrated and purified anti-IL-7Rα antibody and unconjugated anti-γ_c antibody (tUGh4) were conjugated to DNA oligonucleotides for RCA using Probemaker PLUS and MINUS kits (Olink Biosciences, Uppsala, Sweden), respectively, following the manufacturer's guidelines. In the PLA experiments described below, a technical negative control with only one of the probes (The anti-γ_c MINUS probe) was included.

2.3. IL-7 receptor PLA on cytospin preparations for microscopy

Cell cytoplasm was stained using CellTrace CFSE Cell Proliferation kit (Invitrogen). HPB-ALL cultures were centrifuged and adjusted to 10^6 cells/ml in PBS with 0.1% (v/v) FCS and incubated with 0.5 μM CFSE for 10 min at 37 °C. Ice-cold growth media was added to increase the volume fivefold followed by two washes in ice-cold PBS with 0.5% BSA and fixation with ice-cold 0.9% (v/v) formaldehyde for 10 min on ice. Cells were washed and adjusted to 10^7 cells/ml in PBS with 0.5% BSA. Suspensions were spun onto Superfrost PLUS slides (Menzel-Gläser, Braunschweig, Germany) using Shandon cytofunnels (Thermo Scientific Shandon, Leicestershire, UK). Blocking and PLA reactions were performed in 40 μl open droplets and slides were washed in TBS with 0.05% (v/v) Tween 20 between reaction steps. Blocking buffer (PBS with 1% (v/v) BSA and 5 μg/ml sonicated salmon sperm DNA (GE Healthcare, Uppsala, Sweden) was added and slides were incubated in a humidity chamber overnight at 4 °C. Blocking buffer was tapped off the slides and proximity probes diluted in proximity probe dilution buffer from the Probemaker kit were added and incubated for 25 min at room temperature. PLA was performed using Orange Detection Reagent (Olink Biosciences). Reagents and enzymes were diluted according to manufacturers' guidelines and ligation and RCA were performed at 37 °C for 30 and 100 min, respectively. After RCA, slides were washed twice in TBS and mounted with anti-fade and DAPI. Fluorescence was visualised using the 64× oil immersion lens on a Zeiss LSM 710 (Carl Zeiss MicroImaging, Jena, Germany) confocal microscope and the 100× oil immersion lens on an AX70 microscope (Olympus, Hamburg, Germany).

2.4. IL-7 receptor PLA on HPB-ALL suspensions for flow cytometry

HPB-ALL culture was washed in PBS with 0.5% (v/v) BSA and adjusted to 10^6 cells/ml in PBS with 0.5% (v/v) BSA. Cell viability was evaluated by staining a part of the cells with LIVE/DEAD near-IR (Invitrogen) according to the manufacturers' guidelines. Cells were fixed with ice cold 0.9% (v/v) formaldehyde on ice for 10 min. After one wash in PBS with 0.5% (v/v) BSA the concentration was adjusted to 5×10^6 cells/ml in blocking buffer and 100 μl cell suspension was added to 5 ml polypropylene vials (Sarstedt, Nümbrecht, Germany) followed by incubation overnight at 4 °C. Proximity probes were added (10 μg/ml PLUS probe and 2.5 μg/ml MINUS) and incubated for 25 min at RT followed by two washes in TBS with 0.05% (v/v) Tween 20, TBS or TBS with 0.5% (v/v) HSA. PLA was performed using Far Red Detection Reagent (Olink Biosciences). Reagents and enzymes were diluted according to manufacturers' guidelines and 80 μl was added to relevant 5 ml polypropylene vials. Ligation and RCA were performed at 37 °C for 30 and 100 min, respectively, each followed by two washes in TBS with 0.05% (v/v) Tween 20, TBS or TBS with 0.5% (v/v) HSA. After the last centrifugation, cells were suspended in TBS.

Flow cytometry was performed the same day using a FC 500 flow cytometer (Beckman Coulter). Following acquisition, samples were analysed using FlowJo software (TreeStar Inc., Ashland, OR). In all cases IL-7 was not added to the cultures and three or more experiments were carried out.

3. Results

3.1. Visualisation of IL-7Rα and γ_c dimerization by PLA

HPB-ALL was used because the cell line expresses IL-7Rα and proliferates in response to IL-7 [8].

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