Impact of syndecan-4 on T cell-antigen presenting cell recognition and the immunological synapse

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To my Mum
Abstract

Syndecan-4 (SDC-4), a transmembrane heparan sulphate proteoglycan and a co-receptor of integrins for fibronectin, has been reported to modulate the adhesion of cells to a range of extracellular matrix ligands. In addition to the modulation of integrins, SDC-4 has been recently reported to modify the interaction of some growth factors with their receptors.

T cells are essential effectors of the adaptive immune response. When conjugating with antigen-presenting cells (APCs), T cells transform their shape to enable formation of a specialised morphological structure, called the immunological synapse (IS). The IS formation is associated with the polarisation of signalling and adhesive molecules towards the APC. The IS is formed and stabilized by similar adhesive forces and structures as in the motile fibroblasts - an extensively studied model of syndecan function.

In this thesis I have investigated the role of SDC-4 in T cell - APC recognition and the IS. First, I confirmed the tight regulation of SDC-4 expression in T cells during the process of activation. Flow cytometry and PCR data demonstrate increased presence of SDC-4 in stimulated human T cells compared to their resting counterparts, indicating involvement of SDC-4 in the processes of T cell activation. Transient transfection of exogenous SDC-4 into Jurkat T cells with low endogenous SDC-4 expression was used as a model to study the effect of increased SDC-4 expression on T cell function.

Using live cell imaging and advanced data image analysis I have quantitatively demonstrated that SDC-4 transfected Jurkat T cells are less likely to modify their shape during IS formation when compared to mock transfected Jurkat T cells. I also observed a delay in T cell antigenic response caused by SDC-4 over-expression. Moreover, I was able to visualise the exogenous SDC-4 in the IS using confocal microscopy and demonstrate the independence of this phenomenon on T cell receptor.

In summary, my observations indicate a regulatory role for SDC-4 in T cell adhesion causing delays in the IS formation and reduced transformation of T cell shape during conjugate formation.
Author declaration

All the work presented in this thesis is my own with the following exceptions:

- Definiens Software rulesets were designed or adopted by Dr Martin Spitaler
- Plasmids pEGFP-N1, pHA-rSDC-4-IRES-EGFP and pMEX-hSDC4 were kindly gifted by J. Couchman and Atsuko Yoneda
- Plasmid pHA-hSDC-4-IRES-EGFP was created by Dr Marek Cebeauer

Anna Maria Markiewicz........................................
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# Table of Contents

List of Figures .................................................................................................................. 10
List of supplementary movies ........................................................................................... 16
List of Tables ..................................................................................................................... 17
1 Introduction ..................................................................................................................... 20
  1.1 Proteoglycans ............................................................................................................. 21
  1.2 Glypicans ................................................................................................................... 21
  1.3 Syndecans .................................................................................................................. 22
    1.3.1 Basal expression of syndecans in eukaryotic cells .............................................. 24
    1.3.2 Regulation of syndecan expression ..................................................................... 24
    1.3.3 The domain structure of syndecans .................................................................... 25
      1.3.3.1 Extracellular domain and GAG chains ...................................................... 26
      1.3.3.2 Transmembrane domain (TM) .................................................................... 27
      1.3.3.3 Cytoplasmic domain .................................................................................... 28
    1.3.4 Syndecan-4 (SDC-4) .......................................................................................... 30
  1.4 B and T lymphocytes .................................................................................................. 33
    1.4.1 Types of T cells ................................................................................................... 35
      1.4.1.1 Cytotoxic T cells ......................................................................................... 35
      1.4.1.2 Helper T cells .............................................................................................. 35
    1.4.2 T cell receptor (TCR) ......................................................................................... 36
    1.4.3 T cell – APC interaction ..................................................................................... 38
    1.4.4 The immunological synapse ................................................................................ 40
      1.4.4.1 Stable IS ...................................................................................................... 41
    1.4.5 T cell activation .................................................................................................. 46
    1.4.6 Adhesion in T cell activation ............................................................................... 48
    1.4.7 SDC-4 expression in lymphocytes and other cells of the immune system .......... 50
      1.4.7.1 Inhibitory effect .......................................................................................... 50
    1.4.8 SDC-4 effect on maturation, motility and morphology ....................................... 52
  1.5 Agrin ............................................................................................................................ 54
## 1.6 Hypothesis and Aims

2.2.6.1 Hypothesis

2.2.6.2 Aims

## 2 Materials and methods

### 2.1 Materials and suppliers/sources

#### 2.1.1 General reagents and chemicals

#### 2.1.2 Molecular biology enzymes and reagents

#### 2.1.3 Molecular biology kits

#### 2.1.4 Tissue culture reagents and materials

#### 2.1.5 Western blotting equipment and materials

#### 2.1.6 Equipment

#### 2.1.7 Plasmids

#### 2.1.8 Bacterial strains

#### 2.1.9 Mammalian cell lines

#### 2.1.10 Buffers

#### 2.1.11 Antibodies and immunofluorescence reagents

### 2.2 Methods

#### 2.2.1 Antibody Production using BD CellTM MAb Medium in a CELLineTM Device

#### 2.2.1.1 Adaptation of hybridoma cells to low serum conditions

#### 2.2.1.2 Antibody Production

#### 2.2.1.3 Antibody Harvest

#### 2.2.1.4 Antibody purification

#### 2.2.1.5 OKT3 antibodies purification: Protein G-Sepharose 4 Fast Flow column

#### 2.2.2 Cell culture

#### 2.2.3 Frozen storage of cells

#### 2.2.4 Cell transfection

#### 2.2.4.1 Transfection of HEK293 cells

#### 2.2.4.2 Transfection of Jurkat T cells

#### 2.2.5 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

#### 2.2.5.1 Isolation of CD4+ T cells

#### 2.2.6 Molecular Biology Protocols

#### 2.2.6.1 DNA plasmid preparation
3 Results

3.1 Detection of endogenous SDC-4

3.1.1 Detection SDC-4 with 5G9 antibodies by flow cytometry

3.1.2 Detection of SDC-4 with RT-PCR

3.1.3 Summary

3.2 Detection of exogenous SDC-4 in Jurkat T cells

3.2.1 Jurkat T cell transfection optimisation

3.2.1.1 Microporator settings optimisation

3.2.1.2 Plasmid DNA concentration optimisation

3.3 Cellular localisation of SDC-4

3.3.1 Using 5G9 antibody

3.3.2 Using ab24511 antibody

3.3.3 Using HA tag antibody

3.3.4 Summary

3.4 Co-localisation of SDC-4 in human T cells

3.4.1 With TCR/CD3 complex

3.4.2 Proteins phosphorylated on tyrosine
3.4.3 Fibronectin ........................................................................................................................................ 113
3.4.4 Summary .......................................................................................................................................... 116
3.5 Conjugate formation analysis ............................................................................................................. 116
  3.5.1 Using dual-cell flow cytometry .................................................................................................... 116
  3.5.2 Using live cell imaging .................................................................................................................. 121
    3.5.2.1 Image analysis using Definiens ruleset .................................................................................. 121
  3.5.3 Summary .......................................................................................................................................... 132
3.6 T cell proximal signalling analysis ..................................................................................................... 133
  3.6.1 Immunoblotting ............................................................................................................................ 133
  3.6.2 Flow cytometry ............................................................................................................................. 135
  3.6.3 Summary .......................................................................................................................................... 136
4 General Summary and Discussion ......................................................................................................... 137
  4.1 Summary of the results ..................................................................................................................... 137
  4.2 Discussion and future perspectives ................................................................................................... 140
References .................................................................................................................................................. 144
List of Figures

**Figure 1.1.** Transmembrane proteoglycans. Four syndecans in the centre have heparan sulphate chains (blue) covalently attached to their N-terminal extracellular domain. In addition, proteoglycans frequently possess additional chondroitin sulphate chains (pink), e.g. syndecan-1 or CD44. Neuropilin-1 and betaglycan have dermatan sulphate chains instead (violet). Figure taken from Couchman et al. Annu. Rev. Cell Dev. Biol. 2010 [17].

**Figure 1.2.** General structure of syndecans. Extracellular domain contains attachment sites for HS (blue) and/or CS (green) chains. CS chains are attached only to murine SDCs.

**Figure 1.3.** An example of the chondroitin (top scheme), and heparan (lower scheme) glycosaminoglycan chain structures. All share a common stem sequence of four sugar residues; are highly sulfated and substituted on serine residues. These have an adjacent glycine residue on the carboxy-terminal side and usually lie within an area rich in acidic residues of the syndecan extracellular domain (modified from Couchman et al. Annu. Rev. Cell Dev. Biol. 2010 [17]).

**Figure 1.4.** The scheme and sequences of cytoplasmic domains of syndecans. The organisation of conserved (C1/C2) and variable (V) regions is shown with a summary of known interactions for each region.

**Figure 1.5.** Fibroblast cells plated on fibronectin (a) with well distinguishable FAs (arrowheads). Cells grown on the isolated integrin-only ligand (b) spread but fail to form FAs unless stimulated with an antibody against SDC-4 (c) or a syndecan-binding fragment of the fibronectin (d). Formation of FAs upon adhesion to a SDC-4 ligand can be blocked by disrupting the expression of SDC-4 (e) or protein kinase Cα (f). This highlights the specific role of SDC-4 in adhesion-contact formation and its immediate relationship with adhesion-driven signalling pathways. Images show fixed fibroblasts that have been immunofluorescently stained for the adhesion marker vinculin (green) and actin (red); scale bars represent 10 µm. Adopted from Morgan et al. Nat Rev Mol Cell Biol 2007 [4].

**Figure 1.6.** All lymphocytes originate in the bone marrow. T cells, in contrast to B cells, then mature in thymus. Cytotoxic T cells directly attack foreign cells. Helper T cells stimulate B cells by cytokines and other co-stimulatory signals (e.g. CD40-CD40L interaction). Plasma cells which release antibodies into the bloodstream are produced at the late stage of B cell development. Antibodies are soluble immunoglobulin receptors binding pathogens present in the blood. Some B cells differentiate into memory cells with the capacity to rapidly respond to invasions of pathogens in the future (figure adapted from www.medspace.com [70]).

**Figure 1.7.** Domain organisation of TCR chains. In colours are shown the variable regions Vα, Vβ (dark and light blue) and the constant regions Cα and Cβ (white and black).

**Figure 1.8.** TCR/CD3 complex. Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) are shown in black. In light blue and white, dark blue and black - T cell receptor αβ chains, respectively; light grey - δ chain; dark grey – γ chain; pale grey - ε chains; light/dark blue lines - ζ homodimer.

**Figure 1.9.** Phases of the interaction between T cell and APC. Figure taken from Fiedl et al. Nat. Rev. Immunol. 2005 [102].
Figure 1.10. The immunological synapse organisation. Examples of classical (a) and multifocal (b) immunological synapses (ISs) are presented. The fluorescence images are from experiments where CD4\(^+\) T cells were activated on supported planar bilayers containing fluorescently labelled pMHC and ICAM-1. TCR–pMHC and LFA-1–ICAM-1 interactions are green and red, respectively, in both representative diagrams and fluorescence images. Differential interference contrast (DIC) images are shown on the right to illustrate the shape of the cells. The scale bars represent 5 \(\mu\)m (modified from [114]). cSMAC (central supramolecular activation cluster) and pSMAC (peripheral supramolecular activation cluster) represent the classification of areas of the IS according to Kupfer and colleagues. Figure adopted from Thauland et al Immunology 2009. [114] ........................................................................................................................................44

Figure 1.11. Differences in TCR-pMHC crosslinking of the peptide-antigen and a superantigen driven event. ........................................................................................................................................47

Figure 2.1. Scheme of CELLine flask. Figure from www.BD........................................................................................................................................62

Figure 2.2. CELLine flask. Figure from........................................................................................................................................62

Figure 3.1. A. Basic principle of the flow cytometry; B. Cell analysis using specific markers. Figure from www.microporator.com [168, 169]........................................................................................................................................76

Figure 3.2. Dot plots illustrating the selection of stimulated T cells after flow cytometry analysis. The individual dot plots depict: the size (FSC) and granularity (SSC) of the cells (A), specific selection of T cells by gating for the CD4-positive cells (B) and the gating for the CD25-positive activated CD4 T cells (C). The histogram shows the SDC-4 expression of activated T cells, control corresponds to unstimulated T cells (D). The presented data are for the fourth day of PBMCs stimulation........................................78

Figure 3.3. Histograms of A surface and B total expression of SDC-4 in T cells stimulated (or not) for the indicated period of time – day 0 (ex vivo), day 1 and day 4. The PBMCs were stimulated with a mixture of superantigens and stained with antibodies against CD4 and CD25 for the identification of activated T cells and PE-labelled 5G9 antibody for SDC-4 detection. Control corresponds to cells unstimulated with 5G9-PE antibody on day 0. The histograms are representative of 10 experiments. 50000 cells were analysed per measurement. ........................................................................................................................................80

Figure 3.4. Surface and total expression of SDC-4 in stimulated primary human T cells. The PBMCs were stimulated with a mixture of superantigens and stained with anti-CD4_FITC antibodies against CD4 and anti-CD25-APC against CD25 for the identification of activated T cells. antiSDC-4-PE antibody (5G9-PE) were used to detect SDC-4. Control corresponds to cells not labelled with anti-SDC-4-PE antibodies. The graphs are representative of 10 experiments. 50000 cells were acquired per measurement. .............81

Figure 3.5. Histograms of A) surface and B) total expression of SDC-4 in Jurkat T cells stained with PE-labelled 5G9 (anti-SDC-4) antibody. Control corresponds to unstimulated cells. ........................................................................................................................................82

Figure 3.6. Histograms of total expression of SDC-4 in Jurkat T cells stained with antibody (against intracellular part of SDC-4) ab24511 (anti-SDC-4 rabbit polyclonal, Abcam) and with secondary anti-rabbit-Alexa555 antibodies. Control corresponds to the cells stained with secondary antibody only .....82

Figure 3.7. RT-PCR results for T cells day 0 (ex vivo), day 2 and day 4 of stimulated primary human T cell as well as HEK cells (negative control), MD-MBA-231 (positive control) and Jurkat T cells. GAPDH was used as a control of mRNA loading in the RT-PCR reaction ........................................................................................................84

Figure 3.8. Microporator main device (1) with microporator pipette (2), pipette station (3), tips (4) and the solution kit (5). Figure from www.microporator.com [168]. ........................................................................................................................................85
Figure 3.9. (A) Cuvette chamber with plate-type electrodes; and (B) pipette tip with needle-shaped electrode, as used in Microporation. Figure from www.microporator.com [168].

Figure 3.10. Jurkat T cells transfected with pEGFP-N1 plasmid (1µg/well) were analysed for the transfection efficiency using selected settings of the microporator. Flow cytometry was used for cell viability (FCS/SSC) analysis. Representative dot plots are shown for untransfected and selected conditions of microporation with the highest (80%) and the lowest (10%) viability of successfully transfected Jurkat T cells. Each dot in the graph corresponds to an individual cell. 10000 cells were analysed per sample.

Figure 3.11. Jurkat T cells transfected with pEGFP-N1 plasmid (1µg/well) were analysed for the transfection efficiency using selected settings of the microporator. Flow cytometry was used for the cellular EGFP expression analysis. Histogram showing the level of expression of EGFP for untransfected (black line) and selected the most (red line – 72%) and least (blue line) successfully transfected Jurkat T cells. Untransfected cells were treated similarly to all transfected samples except for the electric shock for the electroporation. The transfection efficiency was based on gating by marker M1, which measures all the positively transfected (EGFP-positive) cells. The highest transfection in this experiment corresponds to settings 1400V/30msec/1pulse and the lowest transfection of Jurkat T cells corresponds to settings 1450V/30msec/1pulse presented in Table 3.1.

Figure 3.12. (A) Representative dot plots with indicated viability (%) for Jurkat T cells, untransfected and transfected with pEGFP-N1 or pHA-SDC-4-IRES-EGFP plasmids using selected 1400V/30msec/1pulse condition of microporation. Each dot in the graph corresponds to an individual cell. 10000 cells were analysed per measurement. (B) Histogram showing the results achieved with optimised concentration of plasmids in order to attain as similar as possible level of expression of EGFP for both plasmids. Positive expression was determined based on the EGFP expression encoded by the plasmids (marker M1).

Figure 3.13. Histogram showing the level of expression of EGFP from the pHA-hSDC-4-IRES-EGFP and control pIRES2-EGFP plasmids in Jurkat T cells using microporator settings of 1400 V/10 msec/3 pulses, and concentration of 4µg/well pHA-hSDC-4-IRES-EGFP and 0.1µg/well pIRES2-EGFP.

Figure 3.14. Images of MD-MBA-231 (positive control) and HEK293 cells (negative control) stained with 5G9 antibody against the extracellular part of SDC-4 (cyan) followed by secondary Abs mouse anti-IgG2a Alexa 555 and with phalloidin Alexa 647 (for fibrillar actin; magenta). Brightfield (BF) shows the shape of cells. Example results of 20 conjugates analysed for each transfection.

Figure 3.15. Images of conjugates formed by Raji B cell and Jurkat T cells transfected with A) pHA-rSDC-4-IRES-EGFP or B) control pIRES2-EGFP plasmid and stained with 5G9 antibodies against the extracellular part of SDC-4 (cyan). Typical Lck localisation indicates the formation of productive IS (magenta). GFP indicates the transfection efficiency in Jurkat T cells. Brightfield (BF) shows the shape of cells.

Figure 3.16. Images of MD-MBA-231 (positive control) and HEK293 (negative control) cells labelled with ab24511 antibody against the intracellular part of SDC-4 followed by staining with secondary Ab anti-rabbit-Alexa555 (cyan)and with phalloidin-Alexa647 for fibrillar actin; (magenta staining). BF shows the cells in bright field.
Figure 3.17. HEK293 cells transfected with pHA-rSDC-4-IRES-EGFP plasmid to check the selectivity of ab24511 antibody for SDC-4 (B) (cyan). The phalloidin Alexa 647 staining of actin fibres (A)(magenta). Transfection efficiency control – EGFP (C), Margged image (D) of A and B. BF – cells in the bright field (E). Exo SDC-4 = exogenous SDC-4. ........................................................................................................ 103

Figure 3.18. Images of conjugates formed between: (A) Jurkat T cells transfected with pHA-rSDC-4-IRES-EGFP or (B) pIRES2-EGFP plasmid, and Raji B cells – stained with ab24511 antibody against the intracellular part of SDC-4. GFP serves to control for expression level. BF shows the cells in bright field. ......................................................................................................................... 104

Figure 3.19. CD3 downregulation in Jurkat T cells stably transfected with h-SDC-4. (A) gating for living cells; (B) gating for SDC-4 positive Jurkat T cells – the gating was done based on HA tag presence (detected with HA1.1 Abs and secondary anti murine IgG1-Alexa488; (C) histogram of CD3ε detection in stably and transiently transfected Jurkat T cells with pHA-hSDC-4-IRES-EGFP. The cells were stained with OKT3 Abs and anti-murine IgG2a-Alexa555. .................................................................................. 106

Figure 3.20. Images of Jurkat T cells transfected with (A) pHA-hSDC-4-IRES-EGFP or (B) pIRES2-EGFP plasmids and stained with the antibody against HA tag (HA.11) present on the N-terminus of SDC-4 and secondary anti-mIgG1-Alexa488 Ab. GFP images show the expression level. BF images show the cells in bright field. Representative image of 10 cells from 2 experiments is shown.............................................. 107

Figure 3.21. Representative images of HA-tagged hSDC-4 in conjugates formed between Raji B cells (APC) and Jurkat T cells transfected with A) pHA-hSDC4-IRES-EGFP and B) pIRES2-EGFP. GFP shows the expression level. BF shows the cells in bright field. Representative image of 20 conjugates from 3 experiments is shown. ........................................................................................................ 108

Figure 3.22. Representative image of one out of 20 conjugates formed by Jurkat T cells transfected with pHA-hSDC-4-IRES-EGFP and SAg-loaded Raji B cells, stained with antibodies against CD3ε chain (secondary Abs mlgG2a-Alexa555(magenta)) and HA tag for the SDC-4 localisation (secondary Abs mlgG1-Alexa633 (cyan). GFP (green) shows the level of transfection. Example result of 20 conjugates analysed from 3 different experiments. .................................................................................. 111

Figure 3.23. The images of the representative conjugate (1 out of 20 analysed) formed of Jurkat T cells transfected with pHA-hSDC-4-pIRES-EGFP and Raji B cells, and stained with 4G10 antibody against phosphorylated tyrosine(s)(secondary mlgG2a-Alexa555) (cyan) and HA tag for the SDC-4 localisation (secondary mlgG1-Alexa633) (magenta). GFP (green) shows the level of transfection. Example result of 20 conjugates analysed from 3 different experiments. .................................................................................. 112

Figure 3.24. Images of (A) MRC5 (positive control) and (B) MD-MBA-231 cells (negative control) stained with antibodies against fibronectin (cyan), (secondary anti-rabbit-Alexa555) and phalloidin- Alexa647 (fibrillar actin staining; magenta).................................................................................. 114

Figure 3.25. Representative images of the conjugate formed between a Jurkat T cell and a Raji B cell (APC) (preloaded with superantigen; SEE) labelled with the antiserum against fibronectin (secondary Ab anti-rabbit-Alexa555) (cyan) and Lck 3A5 (secondary anti-mlgG2b-Alexa633) (magenta). BF shows the cells in the bright field.................................................................................................................. 115

Figure 3.26. Dot-plots illustrating the procedure for flow cytometry data analysis: (A) Jurkat T cells (bottom right quarter), SAg-loaded Raji B cells (top left quarter) and conjugated Jurkat/Raji cells (top right quarter). (B) Quantitated numbers (% of total cells) for Jurkat T cells, Raji B cells and conjugates (indicated by red square) using FlowJo analytical software. .............................................................................. 117
Figure 3.27. Illustrative dot-plots showing A) a gating for unconjugated transfected Jurkat T cells (bottom right corner), Raji B cells (loaded with Sag) (top left corner) and untransfected Jurkat T cells (left bottom corner) as well as conjugated transfected Jurkat (either with SDC-4 or EGFP only)/Raji cells (top right corner); and for B) Jurkat T cells transfected with pHA-rSDC-4-IRES-EGFP and Raji B cells and C) Jurkat T cells transfected with pIRES2-EGFP and Raji B cells with quantitated number of conjugates (indicated in red). The quantisation was performed using FlowJo software. 50000 cells were acquired for each conditions.

Figure 3.28. Quantitation of the dual-cell flow cytometry analysis of the conjugates formed by: Jurkat T and Raji B cells (J/R); Jurkat T cells transfected with pIRES2-EGFP and Raji B cells (JEGFP/R) and Jurkat T cells transfected with pHA-rSDC-4-IRES-EGFP and Raji B cells (JSDC-4/R). SEE +/- corresponds to the presence or absence of SEE superantigen on the surface of Raji B cells. SD were calculated from 3 experiments.

Figure 3.29. Representative results of transient transfection efficiency for Jurkat T cells transfected with pHA-hSDC-4pIRES-EGFP or pIRES2-EGFP plasmids measured by flow cytometry. The cells were always tested on the day of each live cell imaging experiment.

Figure 3.30. Definiens software performs (A) analysis in five different layers of segmentation for each frame (created by Dr. M. Spitaler). Different levels of segmentation are illustrated here. The important information can be obtained at all levels of the segmentation; (B) scheme of ruleset map of the Definiens analysis for each frame of each movie.

Figure 3.31. Examples of the proximal and distal synapses determination in (A) Jurkat T cell forming a true IS and (B) Jurkat T cell just non-specifically touching Raji B cells (APC).

Figure 3.32. Illustrative graphs for (A) number of Jurkat T cells conjugating with Raji B cells and (B) the percentage of transfected Jurkat T cells conjugating with Raji B cells – an individual experiment is shown (see Figure 32 for a more global picture). Each frame was acquired every 10 seconds and the whole acquisition was taken over 30 minutes.

Figure 3.33. Graph summarising the average percentage of T cells forming IS with SEE-loaded Raji B cells. The values are per frame and x-axis indicates the frame number. Acquisition was set at 6 frames per minute. Red line corresponds to the cells transfected with the pHA-hSDC-4-IRES-EGFP (SDC-4) and blue line to the cells transfected with pRES2-EGFP (EGFP) plasmid. SDs were calculated from 40 datasets for every transfection.

Figure 3.34. The analysis of asymmetry (A), border index (B), compactness (C), elliptic fit (D) roundness (E), border length (F), area (G), relative border of T cell to B cell (H) and the ratio between the border and area of the T cell (I). Frames 30, 60 and 180 from ~40 movies of Jurkat T cells transfected with pIRES2-EGFP or pHA-hSDC-4-IRES-GFP plasmids have been examined. Error bars were calculated as the error from the average value.
**Figure 3.35.** Conjugates between Jurkat T cells transfected with either pHA-SDC-4-IRES-EGFP (J_SDC4) or pIRES-EGFP (J_EGFP) plasmids and SEE-loaded Raji B cells were formed using varying incubation times (2, 8 and 20 minutes), lysed, subjected to 12% SDS-PAGE and immunoblotted. The membrane was probed with 4G10 Ab against proteins phosphorylated on tyrosines. Some bands (e.g. LAT, Lck, and ZAP-70) are labeled to indicate specific signalling molecules based on the experience from previous experiments; A.IMagee and M.Cecauer, personal communication). The membrane was then stripped and reprobed with Ab against α-Tubulin to control the loading of the samples on the gel. Example result is shown of three similar experiments.

**Figure 3.36.** The conjugates formed by Jurkat T cells overexpressing SDC-4 and SEE-loaded Raji B cells (SDC-4) and Jurkat T cells expressing only EGFP and SEE-loaded Raji B cells (EGFP). The cells were incubated for 2 (A) or 8 (B) minutes to form conjugates, fixed, permeabilised, washed and stained with 4G10 Ab against proteins phosphorylated on tyrosines (pY). Representative data are shown of 5 independent experiments.
List of supplementary movies

Movie 1. Live cell imaging data of conjugates formed between Jurkat T cells transfected with pIRES2-EGFP plasmid and Raji B cells.

Movie 2. Live cell imaging data of conjugates formed between Jurkat T cells transfected with pHA-hSDC-4-IRES2-EGFP plasmid and Raji B cells.

Movie 3. Live cell imaging data analysis of conjugates formed between Jurkat T cells transfected with pIRES2-EGFP plasmid and Raji B cells using Definiens software.

Movie 4. Live cell imaging data of conjugates formed between Jurkat T cells transfected with pHA-hSDC-4-IRES2-EGFP plasmid and Raji B cells using Definiens software.

Movie 5. Live cell imaging data analysis of conjugates formed between Jurkat T cells transfected with pIRES2-EGFP plasmid and Raji B cells (without brightfield) – example 1.

Movie 6. Live cell imaging data analysis of conjugates formed between Jurkat T cells transfected with pIRES2-EGFP plasmid and Raji B cells (without brightfield) – example 2.

Movie 7. Live cell imaging data of conjugates formed between Jurkat T cells transfected with pHA-hSDC-4-IRES2-EGFP plasmid and Raji B cells (without brightfield) – example 1.

Movie 8. Live cell imaging data of conjugates formed between Jurkat T cells transfected with pHA-hSDC-4-IRES2-EGFP plasmid and Raji B cells (without brightfield) – example 2.
List of Tables

Table 2.1. Antibodies used. WB = dilution for Western Blotting; IF = dilution used for Immunofluorescence; Mo and Po stand for Monoclonal and Polyclonal antibodies, respectively. D.A = does not apply.................................................................60

Table 3.1. The instrument settings optimisation tested for transfection of Jurkat T cells using pEGFP-N1 plasmid. Transfection efficiency and viability of the cells are shown for the indicated settings. The results presented in last two columns stand for transfection efficiency: No ≤20%........................................88

Table 3.2. The viability and transfection efficiency was determined for Jurkat T cells transfected with pEGFP-N1 plasmid using indicated microporator settings. First column corresponds to the individual setting (pulse voltage (V)/pulse width (msec)/pulse number). Second column represents the viability (%) / transfection efficiency (%) for every setting tested.................................................................91

Table 3.3. The viability and transfection efficiency was determined for Jurkat T cells transfected with the pEGFP-N1 plasmid (at concentration 1µg per well) using my previously established optimal settings- 1400V/30msec/1pulse and the ones suggested by Invitrogen (1400V/10msec/3pulses). I achieved the best results - shown in bold red.................................................................92

Table 3.4. The viability and transfection efficiency were determined for Jurkat T cells transfected with either pH4-rSDC-4-IRES-EGFP plasmid or pEGFP-N1 plasmid (both at a concentration of 1µg per well) using settings 1400V/30msec/1pulse.................................................................94

Table 3.5. The viability and transfection efficiency was determined for Jurkat T cells transfected with pH4-rSDC-4-IRES-EGFP or pEGFP-N1 plasmids using indicated concentrations and my previously determined optimal settings -1400V/30msec/1pulse. The data in bold red indicate the most comparable results of the transfection efficiency and viability for both plasmids. .................................................................95

Table 3.6. The viability and transfection efficiency was determined for Jurkat T cells transfected with pH4-rSDC-4-IRES-EGFP or pEGFP-N1 plasmid using concentrations optimised earlier in the project: 4µg/well of HA-rSDC-4-pIRES-EGFP and 0.1µg/well of pEGFP-N1; and Invitrogen-suggested and optimised settings of1400V/10msec/3pulses. .................................................................97

Table 3.7. Quantitation of Western Blotting bands (fold increase of signal and digital counts) for TCR signaling effector molecules ZAP70, Lck, LAT. Digital counts are presented also for the control of the loading α-Tubulin. Digital counts were calculated using ImageQuantTL software. Some bands are not detected for unconjugated Jurkat and Raji B cells - used as controls (-); half of the volume used for conjugated cells was loaded for unconjugated Jurkat and Raji controls.................................................................135
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abs</td>
<td>Antibodies</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Tissue Culture Collection</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BF</td>
<td>Bright field</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair(s)</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DC-HIL</td>
<td>Dendritic cell-associated heparan sulphate proteoglycan-dependent integrin ligand</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Cell Cultures</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>FAs</td>
<td>Focal adhesions</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney Cells</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>HSPGs</td>
<td>Heparan sulphate proteoglycans</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Inter-Cellular Adhesion Molecule 1</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IS</td>
<td>Immunological synapse</td>
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<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibition motif</td>
</tr>
<tr>
<td>LAT</td>
<td>Linker for activation of T cells</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>Lck</td>
<td>Lymphocyte-specific protein tyrosine kinase</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function-associated antigen 1</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph nodes</td>
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<tr>
<td>LysoPC</td>
<td>Lysophosphatidylcholine</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLR</td>
<td>Mixed lymphocyte reactions</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MTOC</td>
<td>Microtubule organizing centre</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffered saline Tween-20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PKCα</td>
<td>Protein kinase Cα</td>
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<tr>
<td>PGs</td>
<td>Proteoglycans</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
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<td>SAgS</td>
<td>Superantigens</td>
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<td>SB</td>
<td>Sample Buffer</td>
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<tr>
<td>SDCs</td>
<td>Syndecans</td>
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<tr>
<td>SDC-1 to -4</td>
<td>Syndecan-1 to -4</td>
</tr>
<tr>
<td>rSDC-4</td>
<td>rat Syndecan-4</td>
</tr>
<tr>
<td>hSDC-4</td>
<td>human Syndecan-4</td>
</tr>
<tr>
<td>SMAC</td>
<td>Supramolecular activation cluster</td>
</tr>
<tr>
<td>SOC</td>
<td>Super optimal catabolite repression media</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethlenediamine</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
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1 Introduction

Cells are the smallest functional units of living organisms. One of the abilities of cells is to adhere to each other and to the surrounding extracellular matrix (ECM) [1]. Such ability is essential for the existence of multicellular organisms. Moreover, adhesion provides physical support for cells, regulates their positioning and enables microenvironmental sensing. Aberrations in cell-to-cell or cell – ECM interactions and ECM organisation contribute widely to diseases, demonstrating the essential role of the adhesive forces and ECM in cellular function.

Many cell-surface receptors that mediate cell-to-cell and cell – ECM adhesions are members of two protein gene families — integrins [2] and syndecans [3]. Intriguingly, nearly all ECM molecules contain binding sites for both types of receptor [1] and there is substantial evidence that a full cellular adhesive function requires engagement of both types of receptors. Intracellularly, integrins and syndecans are required to generate a physical link with the cytoskeleton [4], for force transduction, spatial control of the assembly of the adhesion signalling complex (via adhesion contacts) [5] and regulation of the cytoskeletal dynamics [6].

For a long time, the studies in this field were focused on integrins. Integrins are a large family of surface receptors dominating the research of adhesion and cell motility, and with which several proteoglycans are known to associate [7]. One of these is syndecan-4 (SDC-4). Thus, observation of increased SDC-4 expression in activated compared to resting T cells brought us to the question: what is the function of this molecule in T cells during their activation? Is adhesion influenced by increased expression of SDC-4? As SDC-4 sits in the centre of my work, first, this family of small proteoglycans will be described in the following sections with a special attention towards SDC-4.
1.1 Proteoglycans

Proteoglycans (PGs) [8] can be classified into three main groups according to their localisation: secreted, surface associated and intracellular proteins. Each main group is further classified into subfamilies according to their gene homology, core protein properties, size and modular composition. Secreted PGs involve large aggregating PGs, named hyalectans, small leucine-rich PGs and basement membrane PGs. Cell-surface-associated PGs are divided into two main subfamilies (syndecans and glypicans), whereas serglycin is the only intracellular PG characterised to date [9, 10]. The wide molecular diversity of PGs derives from the multitude of possible combinations of protein cores, O-linked and N-linked oligosaccharides, and various types and numbers of glycosaminoglycan (GAG) chains. The specific structural characteristics of GAG types provide some of the structural basis for the multitude of their biological functions [11]. PGs may carry heparan sulfate (HS) and/or chondroitin sulfate (CS) or dermatan sulphate chains linked to their extracellular domain and were first described around 30 years ago [12].

Let me focus only on the cell-surface PGs, which include two major subfamilies: the syndecans [13-15], with four members cloned in mammals, and are type I transmembrane proteins mostly substituted with HS chains; and glypicans linked to the cell membrane by a glycosyl-phosphatidylinositol anchor, with six members cloned in mammals substituted with HS chains.

1.2 Glypicans

Glypicans show cell-type and developmental-stage-specific expression. They are involved in biological processes such as cell–ECM interactions and the control of cellular division, differentiation and morphogenesis [16]. At the level of signalling, they are involved in the regulation of pathways including fibroblast growth factor (FGF), hedgehog (Hh), bone morphogenic protein and insulin-like growth factor [16]. Therefore, depending on the biological context, glypicans can either stimulate or inhibit signalling activity. Notably, the HS chains are essential for the glypican-induced stimulation of FGF activity and partially required for the regulatory activity of glypicans in e.g. Hh and bone morphogenetic protein signalling [16].
1.3 Syndecans

Syndecans (SDCs) belong to a small group of transmembrane proteins which have GAG chains attached to the extracellular domain and belong to the family of PGs (Figure 1.1) [14, 15, 17].

Figure 1.1. Transmembrane proteoglycans. Four syndecans in the centre have heparan sulphate chains (blue) covalently attached to their N-terminal extracellular domain. In addition, proteoglycans frequently possess additional chondroitin sulphate chains (pink), e.g. syndecan-1 or CD44. Neuropilin-1 and betaglycan have dermatan sulphate chains instead (violet). Figure taken from Couchman et al. Annu. Rev. Cell Dev. Biol. 2010 [17].
Initially, a role for heparan sulphate proteoglycans (HSPGs) in adhesion was proposed [1]. Of note, this happened before the description of integrins. For two decades now it has been believed that focal adhesion (FA) formation during cell spreading on fibronectin depends on the engagement of an integrin and a cell-surface PG, specifically, α5β1 integrin and SDC-4 [18] (see SDC-4 section for more information).

Syndecans act as receptors for ECM proteins and for growth factors [15], engaging ligands through their large flexible GAG chains that make them ideal receptors for ligands that are dilute or distant from the membrane. The necessity of GAG chains is demonstrated by the fact that cellular responses to ligands such as fibronectin [19], fibroblast growth factor-2 (FGF2) and vascular endothelial growth factor (VEGF) are inhibited when the interactions of the sugar chains are compromised by addition of soluble competitors or disruption of the GAG biosynthetic machinery [19, 20].

Adhesion to the ECM regulates numerous signalling pathways [21]. Therefore, the ability of syndecans to be involved in the adhesion as well as in signalling processes brought the concept of syndecans as co-receptors.

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**Focal adhesions (FAs)**

Focal adhesions (FAs) are highly dynamic, multi-protein complexes, through which cells interact with ECM (e.g. via integrin receptors). These large assemblies, which can measure several micrometers in diameter, in general mediate interactions of cells with external surfaces, and are linked at their cytoplasmic side with actin bundles.
1.3.1 Basal expression of syndecans in eukaryotic cells

Syndecans are found in all mammalian cells. Whereas invertebrates express only a single syndecan, four genes (syndecans 1 to 4) encode syndecan proteins in vertebrates. Three out of four vertebrate syndecans (abbreviated SDC-1 to -4 subsequently) have a tissue-specific expression profile [3, 22].

- SDC-1 is expressed predominantly in epithelial cells and is thought to play an important role in maintaining epithelial phenotype and morphology. It is down-regulated in several types of carcinoma [23].
- SDC-2 was originally identified in the endothelium and lung fibroblasts, but is also highly expressed in mesenchymal tissues surrounding blood vessels, in organs such as kidney, lung, liver and stomach, and in cells forming cartilage and bones [13, 24].
- SDC-3 is enriched in neural tissues, but also in the developing musculoskeletal system. This syndecan has not been proven to be involved in interactions with the ECM [25].
- In contrast to the previous syndecans, SDC-4 is found in virtually every cell type [26], but its expression level differs between the cell types and the stage of their development.

Many cells express more than one type of syndecan, and they may possess other transmembrane proteoglycans as well.

1.3.2 Regulation of syndecan expression

In general, the expression profiles of all members of the syndecan family change in time and space (e.g. during tissue formation), but every mammalian cell express at least one syndecan type at the certain stage of its development [13]. Syndecan levels are tightly regulated as they are involved in tuning numerous signalling events. It has previously been shown that various growth factors play an important role in regulation of syndecan expression: e.g. tumour necrosis factor-α (TNF-α) upregulates SDC-2 and downregulates SDC-1 in endothelial cells [27]. Similarly, transforming growth factor–β2 (TGF-β2) upregulates SDC-4 and downregulates SDC-1 in epithelial cells [28] and FGF2 induces SDC-4 but not SDC-1 or SDC-2 expression in aortic smooth muscle cells [29]. Therefore, it is important to understand why expression levels of syndecans are tightly regulated by a number of factors depending on cell type. Interestingly, the same factors are responsible not only for a certain effect e.g. upregulation of one type of syndecan, but simultaneously for the downregulation of the other one in cells.
1.3.3 The domain structure of syndecans

Structurally, syndecans are composed of an extracellular domain (48 amino acids) containing several consensus sequences for three to five HS (and/or CS chains in mouse) (Figure 1.2). glycosaminoglycan (GAG) attachment; a single transmembrane domain (25 amino acids); and a C-terminal cytoplasmic domain (34 amino acids). The extracellular domain of syndecan interacts with a number of soluble and insoluble factors in the extracellular matrix (ECM). These interactions, mediated by both polysaccharide and protein components, activate syndecans to regulate transmembrane signaling events [30-32].

Figure 1.2. General structure of syndecans. Extracellular domain contains attachment sites for HS (blue) and/or CS (green) chains. CS chains are attached only to murine SDCs.
1.3.3.1 Extracellular domain and GAG chains

A wide array of extracellular ligands interact with heparin and HS [21] including growth factors, chemokines [33], lipid-regulating enzymes, ECM proteins [34], cell-cell adhesion molecules and blood-coagulation factors [35]. These molecules have one or more discrete domains that have heparin-binding characteristics and, in general, these have been demonstrated also to bind to the less sulphated HS, which forms distinct domains of GAG chains covalently attached to extracellular part of syndecans [21, 36, 37] (Figure 1.3).

**Figure 1.3.** An example of the chondroitin (top scheme), and heparan (lower scheme) glycosaminoglycan chain structures. All share a common stem sequence of four sugar residues; are highly sulfated and substituted on serine residues. These have an adjacent glycine residue on the carboxy-terminal side and usually lie within an area rich in acidic residues of the syndecan extracellular domain (modified from Couchman et al. Annu. Rev. Cell Dev. Biol. 2010 [17]).
The structural variation of HS and CS chains (Figure 1.3) results from a series of post-translational modifications delivered by a number of enzymes, such as N-acetylglucosamine transferase [38]. It has been previously assumed that all syndecan HS and CS chains are created equally, with no preference for a specific HS/CS sequence of the saccharides or their modifications on specific syndecans [39]. More recently, some studies demonstrated that, for example, murine SDC-1 core protein contains five potential GAG attachment sites, three for HS and two for CS. Also several SDC-4 cDNAs have been characterized for rat, human and chicken and although rat SDC-4 possesses three functional GAG attachment sites capable of bearing either CS or HS chains, chicken SDC-4 core protein has three potential sites for GAG attachment, modified with HS side chains, and SDC-4 isolated from human tissues has only HS chains. Besides, it is not clear how much exact sequence of saccharides and their modifications play a role in the function of PGs [26].

In addition to the interactions mediated via their HS and CS chains, the extracellular domain of SDC-4 polypeptide core can be directly involved in protein–protein interactions, e.g. midkine and pleiotrophin bind not only to the GAG chains [40], but also to the core protein of SDC-4 [36], supporting the possibility of cooperation between core protein and GAG chains. Also, previous studies have demonstrated that the amino acid sequence diversity of syndecan extracellular domains results in distinct cellular functions [13, 24].

1.3.3.2 Transmembrane domain (TM)

The syndecan transmembrane domain is composed of 25 hydrophobic amino acid residues and is responsible for dimerization/oligomerization of syndecan core proteins [41, 42]. These interactions are probably mediated through a strictly conserved GxxxD motif, which directly regulates homodimer formation of syndecans even in the presence of the strong anionic detergent sodium dodecyl sulfate (SDS), a property known as SDS-resistant dimerization [43]. The high concentration of this molecule in focal adhesions also favours this phenomenon [44]. Like other cell surface receptors, transmembrane domain-mediated non-covalent oligomerization appears to be crucial for the ability of syndecans to transduce signals from the ECM to the cytosol [41]. Multimerisation of SDC-4 has been detected using biochemical methods [45]. The function of such association is not clear but, for example in case of SDC-4, it has been shown to be required for activation of protein kinase C-α (PKCo) [45].
1.3.3.3 **Cytoplasmic domain**

The cytoplasmic domains of syndecans are relatively short, but contain three important regions: conserved regions 1 and 2 (C1 and C2) and a variable (V) region. The C1 domain, which is immediately adjacent to the inner leaflet of the cellular plasma membrane, is identical in all four mammalian syndecans (Figure 1.4) [26, 31].

<table>
<thead>
<tr>
<th></th>
<th>C1</th>
<th>V</th>
<th>C2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Interactions with ezrin,Scr/cortactin</td>
<td>Syndecan-specific interactions: PI4,5P2, PKCα and α-actinin (SDC-4)</td>
<td>Interactions with PDZ proteins (e.g., syntentin, synectin)</td>
</tr>
<tr>
<td>Syndecan1</td>
<td>RMKKKDEGSY</td>
<td>SLEEPKQANGGAYQK-PTKQE</td>
<td>EFYA</td>
</tr>
<tr>
<td>Syndecan2</td>
<td>RMKKKDEGSY</td>
<td>TLEEPKQA-SVTYQK-PTKQE</td>
<td>EFYA</td>
</tr>
<tr>
<td>Syndecan3</td>
<td>RMKKKDEGSY</td>
<td>DLGERK-PSSAAYQKAPTK—</td>
<td>EFYA</td>
</tr>
<tr>
<td>Syndecan4</td>
<td>RMKKKDEGSY</td>
<td>DLG-KK-P-I—YKKAPT—</td>
<td>EFYA</td>
</tr>
</tbody>
</table>

**Figure 1.4.** The scheme and sequences of cytoplasmic domains of syndecans. The organisation of conserved (C1/C2) and variable (V) regions is shown with a summary of known interactions for each region.
The cytoplasmic domain is thought to be involved in syndecan dimerisation [46] and in binding of several intracellular proteins, including ezrin, Src kinase, and cortactin [47-49]. The C-terminal conserved C2 domain contains a PDZ-binding motif and one tyrosine residue. Four PDZ domain–containing proteins have been identified to interact with syndecans: syntenin [50], synectin, synbindin and calcium/calmodulin-dependent serine protein kinase [51-55]. The V domain is highly heterogeneous among the four mammalian SDCs. This region has been studied most extensively for SDC-4 because of its unique features, which are described in the following chapter.

The interactions of cytoplasmic domains of transmembrane PGs, and SDCs in particular, with cytoskeleton are well established. However, the way how ligand interactions with HS chains transmit the appropriate signals through the core proteins is not yet clear. Similar to other receptors, SDCs can probably cluster in response to a ligand [45], although again, the molecular basis is unclear. Additionally, more recent developments show the importance of the ectodomain of the core protein in signal transduction [32]. There is growing evidence that the ectodomains may influence integrins, directly or indirectly, with further possibilities for affecting the ECM assembly or turnover [37]. As mentioned previously, all SDCs, apart from SDC-3, interact with ECM.

In summary, SDCs hold a distinct position within the group of PGs, as these molecules are placed at the centre of signal integration in the cell and have been named "tuners of transmembrane signalling" [24]. They are called like that because they possess the unique function to integrate signalling from solute ligands (e.g. growth factors) and ECM proteins with other cellular receptor systems such as integrins. Moreover, their intracellular domains and interacting proteins can regulate a number of signalling pathways.
1.3.4 Syndecan-4 (SDC-4)

As mentioned previously, it has been recognised that FA formation during cell spreading on fibronectin depends on the engagement of an integrin and a cell-surface proteoglycan, SDC-4 (Figure 1.5) [4]. The unique feature of SDC-4 here is that it can become incorporated into FAs together with integrins [18, 56]. So far, no other syndecan and proteoglycan has been proven to play a role in FA assembly. Studies from several groups have confirmed that SDC-4 enhances FA assembly, although the essential role of integrins is also well-established [44, 57, 58]. The interactions of fibronectin, SDC-4 and integrins involve a series of downstream signalling events, including activation of tyrosine kinases by the integrins. At the same time, SDC-4 cytoplasmic domain binds to membrane phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂), which initiates an intermolecular conformational change that provides a binding platform for PKCα [17, 59-61].
Figure 1.5. Fibroblast cells plated on fibronectin (a) with well distinguishable FAs (arrowheads). Cells grown on the isolated integrin-only ligand (b) spread but fail to form FAs unless stimulated with an antibody against SDC-4 (c) or a syndecan-binding fragment of the fibronectin (d). Formation of FAs upon adhesion to a SDC-4 ligand can be blocked by disrupting the expression of SDC-4 (e) or protein kinase Ca (f). This highlights the specific role of SDC-4 in adhesion-contact formation and its immediate relationship with adhesion-driven signalling pathways. Images show fixed fibroblasts that have been immunofluorescently stained for the adhesion marker vinculin (green) and actin (red); scale bars represent 10 µm. Adopted from Morgan et al. Nat Rev Mol Cell Biol 2007 [4].
SDC-4 certainly influences the cytoskeleton, since α-actinin can directly bind its cytoplasmic domain [62] and SDC-4 “knock-out” fibroblasts have been shown to display reduced organisation of actin with fewer microfilament bundles, perhaps indicative of a decreased tension exerted by the binding to the ECM [6, 19]. Such experiments support the function of the SDC-4 extracellular domain and its large GAG chains in cellular adhesion but the mechanisms involved are not yet fully understood [63].

On the other hand, there is an accumulating knowledge about the signalling events downstream of SDC-4 cytoplasmic tail, owing to the presence of well characterized PI(4,5)P₂ [64], PKCα and PDZ binding sites in the cytoplasmic domain of SDC-4 (see Figure 1.3). It suggests that interactions might vary according to the circumstances, allowing syndecans to act as environmental sensors [14, 65].
1.4 B and T lymphocytes

Human immunity consists of innate and adaptive systems. The innate immune system is capable of sensing viruses, bacteria, parasites and fungi through the expression of so-called pattern recognition receptors, which are expressed on the surface of macrophages, dendritic cells (DCs) and some other cells of the innate immune system [66, 67]. Adaptive immunity [68] is triggered when a pathogen breaks through the innate immune system and its sustained presence enables recognition of threshold levels of antigen. The cells of the adaptive immune system are special types of leukocytes called B and T lymphocytes, also called B and T cells. B and T cells are derived from pluripotent hematopoietic stem cells in the bone marrow [69] (Figure 1.6).

**Figure 1.6.** All lymphocytes originate in the bone marrow. T cells, in contrast to B cells, then mature in thymus. Cytotoxic T cells directly attack foreign cells. Helper T cells stimulate B cells by cytokines and other co-stimulatory signals (e.g. CD40-CD40L interaction). Plasma cells which release antibodies into the bloodstream are produced at the late stage of B cell development. Antibodies are soluble immunoglobulin receptors binding pathogens present in the blood. Some B cells differentiate into memory cells with the capacity to rapidly respond to invasions of pathogens in the future (figure adapted from www.medspace.com [70])
B cells are involved in the humoral immune response of higher organisms, whereas T cells are involved in cell-mediated immune response. Both B cells and T cells carry surface receptor molecules [67, 71] that specifically recognize “non-self” molecules called antigens (e.g. peptides, glycostructures, etc). T cells recognize exclusively peptide antigens (small fragments of proteins from the pathogen) which have been processed and presented in the combination with a “self” receptor called major histocompatibility complex (MHC) [72, 73]. This rather complicated process takes place in cells called antigen presenting cells (APCs). Nevertheless, MHC class I and II on the surface of APCs also present “self-antigens” which are peptides derived from the organism’s own proteins. Organisms have evolved mechanisms which ensure that self-peptides do not stimulate T cells [72, 74]. Unfortunately, there are situations when control systems do not function properly causing inappropriate immune responses. This may cause autoimmunity diseases or allergy [75].

Each individual T cell (and B cell) possesses T cell receptors (TCRs; BCRs for B cells) with specificity for the single antigen [72, 76, 77]. This means, for example, that a T cell can be activated only when its receptor binds to this antigen in a complex with the MHC class I or II of the other cell. TCRs, as well as BCRs, are polyclonal which means that each T cell (and B cell) has different specificity defined by their specific receptor [78]. Recognition of MHC:antigen complex is aided by co-receptors expressed on the surface of T cells: CD4 on helper and CD8 on cytotoxic T cells [79-81].

**Antibodies (Abs)**

Antibodies, also known as immunoglobulins, are globular proteins that are found in blood or other body fluids of vertebrates, and are used by the immune system to identify and neutralize foreign objects, such as bacteria and viruses. They are typically made of basic structural units—each with two large heavy chains and two smaller light chains. General structure of all antibodies is very similar, but a small region at the tip of the protein, known as antigen binding site, is a highly variable structure, allowing millions of antibodies binding to different and specific targets, known as antigens. This large diversity of antibodies allows the immune system to recognize an equally wide variety of antigens.
1.4.1 Types of T cells

1.4.1.1 Cytotoxic T cells

Cytotoxic T cells (CD8-positive; CD8+) are a sub-group of T cells that directly kill cells infected with viruses (or other pathogens), or are otherwise damaged or dysfunctional (e.g. tumour transformed) [82]. In general, they attack other cells carrying foreign or abnormal antigens on their surfaces. Their TCRs recognise MHC class I antigen complex. Cytotoxic T cell activation is tightly controlled and generally requires a very strong MHC/antigen activation signal, or additional activation signals provided by "helper" T cells (see below) [83].

After activation [84], cytotoxic T cells release cytotoxins, such as perforin, which form pores in the target cell's plasma membrane. Once the membrane of the attacked cell is perforated, osmosis drives extracellular ions and water to enter the target cell until it bursts causing its death. The other way of cytotoxic T cells to kill infected or aberrant cells employs the possibility to activate a suicide mechanism – called apoptosis – in the target cell. Killing of host cells by cytotoxic T cells is particularly important in the prevention of virus replication [85, 86].

1.4.1.2 Helper T cells

Helper T cells (CD4-positive; CD4+) have the capacity to regulate both innate and adaptive immune responses and help to determine which type(s) of immune response the body will generate for the eradication of particular pathogen(s) [87, 88]. These cells have no cytotoxic activity and do not kill infected cells or clear pathogens directly. Instead, helper T cells control the immune response by instructing the other cells, e.g. macrophages, to perform such tasks. TCRs of helper T cells recognize peptide antigen (derived from pathogens) bound to class II MHC molecules [77]. The MHC:antigen complex is also recognized by the helper cell's CD4 co-receptor with the associated intracellular Lck effector kinase which mediates efficient T cell activation [76]. The activation of naive helper T cells requires longer duration of the engagement with APC [85, 89]. The activation of a helper T cell causes the release of specific cytokines that influence the activity of many other cell types [90]. For example, cytokine signals produced by helper T cells enhance the microbicidal function of macrophages or the activity of cytotoxic T cells [89].
1.4.2 T cell receptor (TCR)

The T cell receptor or TCR is a molecule found exclusively in T lymphocytes [91]. The TCR is composed of two different polypeptide chains (subunits). In 95% of T cells, these are alpha (α) and beta (β) chains (Figure 1.7). The remaining 5% express gamma (γ) and delta (δ) chains. The TCR, which is a type I transmembrane glycoprotein, consists of two subunits which form a dimer. For the α chain, the variable region is called Vα and the constant region is called Cα; for the β chain these are called Vβ and Cβ, respectively [92].

The CD3 complex, which possesses four distinct chains (γ, δ and two ε) and a ζ2 complex (also called CD247 in mammals), is assembled together with the TCR heterodimer. Subunits of the CD3 complex contain characteristic sequence motifs for tyrosine phosphorylation in their intracellular tails, known as ITAMs (Immunoreceptor Tyrosine-based Activation Motifs) [93]. The TCR polypeptides themselves have very short cytoplasmic tails, and all intracellular signalling events are probably mediated exclusively through the CD3 subunits. The CD3 and ζ-chains, together with the TCR, form what is known as the TCR/CD3 complex (Figure 1.8) [93, 94].

![Figure 1.7. Domain organisation of TCR chains. In colours are shown the variable regions Vα, Vβ (dark and light blue) and the constant regions Cα and Cβ (white and black)](image)
Figure 1.8. TCR/CD3 complex. Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) are shown in black. In light blue and white, dark blue and black - T cell receptor αβ chains, respectively; light grey - δ chain; dark grey – γ chain; pale grey - ε chains; light/dark blue lines - ζ homodimer.

The essential function of the TCR complex is to recognize specific peptide antigen on MHC present on APC and elicit a distinct immune response [66]. Such recognition is enhanced by a tight contact formed between T cell and APC containing the unique antigen capable to cause T cell activation (details of the process in vivo described in following section). A large number of molecules are involved in the complex biochemical processes by which this activation occurs [92, 95-97] and of which only the main early and well documented events will be described in the following section.
1.4.3 T cell – APC interaction

An individual T cell–APC interaction consists of three phases: initiation of the contact by T cell (also known as ‘contact acquisition’); formation of an interaction plane (also called the immunological synapse (IS); see below); and, final phase, detachment of T cell which frequently continues to migrate (Figure 1.9) [77]. In vivo, the intercellular contacts are initiated by migrating T cells by their leading edge (showing similarities with the lamellipodium of motile fibroblasts) [98, 99] and are terminated by the re-initiation of the migration by the detachment from the APC [82, 100, 101].

**Figure 1.9.** Phases of the interaction between T cell and APC. Figure taken from Fiedl et al. *Nat. Rev. Immunol.* 2005 [102].
Three different physical states of the contact between T cells and APCs have been described (Figure 1.9), all of which have an impact on the contact duration, receptor engagement and downstream signalling.

First, well polarized, stable state results from the adhesion of T cell to APC after the recognition of cognate antigen [93, 98, 103]. Here, T cell rounds up and loses its migratory morphology, most notably, the posterior uropod is no longer detectable [104, 105]. Consequently, the lateral mobility of the cell is less than 1μm per minute, allowing receptor redistribution towards the cell–cell junction (Figure 1.9) [106].

Second, the slowly mobile state is prevalently observed in cell–cell clusters between T cells and APCs in three-dimensional tissue environments, such as lymph nodes [102]. To achieve this state, a T cell ceases migration and polarizes towards an APC, but ongoing cytoskeletal dynamics at the contact zone of both cells generate slow, non-directional lateral mobility of 1–2μm per minute. Therefore, with time, each cell changes position relative to the other, perhaps facilitating the receptor contact with the IS [71, 107].

Third, the migratory, or dynamic, junction results from the maintenance or re-initiation of the T-cell migration on the surface of APC [73]. Such migratory state is characterised by partial polarisation, but with a dynamic leading edge that generates up front in the direction of the movement, a roundish cell body and a uropod that is 'dragged' behind [73, 82]. Migratory contacts have velocities of 1–10μm per minute, which leads to a rapid position change of the T cell on the surface of APC, and consequently, these interactions are associated with a rapid detachment.

Although under numerous experimental conditions, one of these three states is usually dominant, the abovementioned status of the cell–cell interaction can convert during a single contact [73, 108]. Therefore, individual interactions can show sequential phases of the adhesion, slow lateral movement and dynamic crawling [109]. The type of interaction that occurs is determined by the initial maturation state of the T cell, the type of APC, the amount of antigen being presented by the APC and the location where the interaction takes place. The most important step is the contact time between the two cells when the IS is formed and the transmission of intracellular signals occurs [66].
1.4.4 The immunological synapse

Early studies of both helper and cytotoxic T lymphocytes interacting with APCs presenting antigens demonstrated that the microtubule organizing centre (MTOC) and the cytoskeleton of T cells are orientated towards the APCs [103, 110]. These cytoskeletal changes, resulting in changes in T cell morphology and flattening of T cell on the surface of the APC, occur immediately after the initial antigen recognition [72, 111]. Work from the Kupfer and Dustin laboratories further demonstrated that, in addition to the morphological changes, receptor–ligand complexes and signalling molecules involved in the adhesion and antigen recognition are organised into distinct domains or supramolecular clusters (see below) at the T cell–APC interface [104, 112]. This organisation is thought to help direct communication between two cells and has therefore been termed the ‘immunological synapse’ (IS), due to its functional and morphological similarities to the well-known synapse formed between connecting neurons [90, 106, 113, 114].
1.4.4.1 Stable IS

The stable IS (also known as the monocentric IS results from a polarised interaction between T cell and APC in the presence of cognate antigen [91]. In *vitro*, it frequently lasts 30 minutes or longer. It is defined by the recruitment and segregation of cell-surface receptors and adaptor proteins at the cell–cell junction, and was reported to be responsible for sustained transmission of the intracellular signals. The development of the IS comprises five phases [106, 115]:

1. cellular scanning, contact acquisition and adhesive arrest;
2. early IS assembly and signalling;
3. maturation and receptor segregation;
4. TCR internalisation;
5. IS dissolution.

Progression through all of these phases requires continuous pMHC–TCR interaction, sustained membrane-proximal signalling and an intact T-cell cytoskeleton [93, 99]. Although the processes that regulate the first four phases have been studied in detail, the processes that control the IS resolution and T-cell detachment are less well described [70].

1.4.4.1.1 Cellular scanning, contact acquisition and adhesive arrest

The first phase of the IS formation occurs when moving T cells recognise the immunogenic peptide–MHC complexes which activates lymphocytes function activation integrin – 1 (LFA-1) high affinity form for binding of intercellular adhesion molecule 1 (ICAM1), leading to a temporary arrest in migration, known as a ‘stop signal’ [77].

1.4.4.1.2 The IS assembly and signalling

The second phase in the ‘life’ of the IS is the essential phase for the optimal signalling and is therefore crucial for determining the outcome of the T-cell–APC interaction. Within first few seconds of contact, calcium signalling [66, 116] — followed by the recruitment of signalling molecules such as CD3ζ, Lck, ZAP70 and their phosphorylation — is observed [91]. In next step ZAP70 targets the membrane-anchored adaptor molecule LAT (linker for activation of T cells), which contains many ITAMs to which downstream effector molecules can bind [95].
A mature immunological synapse is formed at the cell–cell junction within 5–30 minutes of continuous T-cell–APC interaction [91, 106]. Maturation was defined by molecular segregation, which leads to a central zone containing subunits of the TCR/ CD3 complex, as well as associated PKCθ. This zone was named the central supramolecular activation cluster (cSMAC). It is surrounded by a ring-like zone with accumulated LFA1 and talin, which is known as the peripheral SMAC (pSMAC) and also as adhesive ring without which the IS formation is impossible [117] and a more distal zone rich in CD45, which is known as the distal SMAC (dSMAC) [104].

Although the organisation of ‘classical’ ISs has been the subject of intense study, non-classical ISs have been also observed under various conditions (Figure 1.10) [73, 114]. Mature IS has been mainly studied in T and B cells and T cells and planar bilyer interactions. It is less evident in T cells with DC interactions and in vivo, raising questions about its physiological significance. Also, experiments with conjugates fixed at various time-points and studies utilising time-lapse microscopy demonstrated that the IS formation is a dynamic process involving a variety number of intermediate profiles [112].

Imaging of the formation of the immunological synapse showed that the accumulation of antigen receptors in the centre of the synapse is preceded by microclustering of the antigen receptors in the periphery [118, 119]. Once formed, the microclusters are transported to the centre of the synapse by an actin-dependent process [118]. The synaptic microclusters appear to be the platforms for receptor activation and signal propagation. For example, microclusters recruit signalling molecules such as Src kinases and ZAP-70 [120]. They also exclude inhibitory phosphatases such as CD45 [94, 118]. However, many of the molecular mechanisms of antigen receptor activation inside these structures remain beyond the resolution of optical microscopy and could not be directly addressed by conventional imaging. Nevertheless, the results of the high-speed PALM imaging showed that TCRs on resting T cells were pre-clustered in small areas of about 70–140 nm in diameter called as ‘protein islands’ [96]. The islands were enriched in cholesterol and anchored by actin filaments. Antigen stimulation led to a more pronounced clustering of the TCR, with more TCRs present in the islands and multiple islands aggregating together. Taking into account the rapid movement of receptors seen in the single molecule studies, these results indicate that there is a dynamic partitioning of receptors into the islands in resting lymphocytes and that antigen-induced stability of the islands mediates immobilization of receptors and signalling molecules after activation [96, 121].
Specialized effector and downregulatory functions of the cSMAC are also suggested by its absence or modification according to cell-type. Prototypical cSMAC/pSMAC patterning, as originally described in T–B cell interfaces, is not commonly observed in T-cell interfaces with dendritic cells [122], and are absent from thymocyte interfaces [79]. Cytotoxic T cells appear to form a modified IS, in which the central region is divided between a TCR-rich cSMAC and a secretory domain for delivery of lytic granule [90].

TCR proximal signalling is thought to occur in TCR-rich submicrometer-scale plasma membrane domains which form almost instantaneously upon T cell contact with antigen-bearing surfaces in all T cell subtypes studied. These TCR microclusters form throughout the contact interface following TCR ligation [118], and after a brief stationary phase, translocate centripetally to assemble into the cSMAC. Signalling microdomains have recently emerged as a general organizing principle of spatially regulated signalling in lymphocytes and other cells [123, 124], leading to the suggestion that ligand-induced microclusters may constitute the basic signalling unit that initiates and organizes immune cell signalling [118, 120, 125].
**Figure 1.10.** The immunological synapse organisation. Examples of classical (a) and multifocal (b) immunological synapses (ISs) are presented. The fluorescence images are from experiments where CD4⁺ T cells were activated on supported planar bilayers containing fluorescently labelled pMHC and ICAM-1. TCR–pMHC and LFA-1–ICAM-1 interactions are green and red, respectively, in both representative diagrams and fluorescence images. Differential interference contrast (DIC) images are shown on the right to illustrate the shape of the cells. The scale bars represent 5 μm (modified from [114]). cSMAC (central supramolecular activation cluster) and pSMAC (peripheral supramolecular activation cluster) represent the classification of areas of the IS according to Kupfer and colleagues. Figure adopted from Thauland et al Immunology 2009. [114].

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1.4.4.1.3 TCR internalisation

In the later stage, during sustained signalling, the TCR is internalised to cytoplasmic vesicles in the cSMAC, thereby limiting its availability at the interaction plane [105]. This downregulation of the TCR is certainly important for the function, because it is predicted to contribute to the diminishment of the signalling [105], and, ultimately, to the last phase of the IS — contact resolution [95].

1.4.4.1.4 The IS resolution

The IS resolution occurs when the T cell and APC detach. In the most cases, the interactions are resolved by the re-onset of the migration of either cell. During detachment, portions of T-cell membrane (including the receptors they contain) can remain attached to (or fuse with) the APC (and vice versa), potentially causing focal zones of continued signalling [86]. It is unclear, however, whether the IS resolution is a prerequisite or a consequence of cell detachment [108]. At present, the mechanisms that have been proposed to elicit termination of the IS signalling and resolution of contact are based mainly on circumstantial evidence, and how these mechanisms would support the termination of the contact as a morphological process remains unclear.

It is important to note that the molecular events underlying the phases of stable IS formation and resolution also occur during the formation of other synaptic structures (Figure 1.10b), although the extent of TCR clustering and the identity of the recruited receptors can vary [76, 108, 119].
1.4.5 T cell activation

T cell activation results from signals delivered after the binding of ligands present on the surface of APCs to the TCR and co-stimulatory receptors on T cells [112, 126]. Moreover, the recognition of peptide antigens and the subsequent activation requires the physical interaction of T cells with APCs (e.g. LFA-1/ICAM-1 interactions). The migration and morphological reorganisation of T cell are essential for its activation [103]. The number of molecules (e.g. LFA-1, CD28, CD86) are responsible for the regulation of the cell’s contact [67, 119]. Adhesion of the T cell to the APC is crucial for the prolonged contact required for proper activation.

In addition to peptide antigens presented by MHC molecules, T cells can be activated with superantigens (SAgs) [127-129], a system used in our project. SAgs are disease-causing bacterial toxins [130] that are capable of interfering with the human immune system by crosslinking the TCR and MHC class II through the variable Vβ-domain of TCR (Figure 1.11) [92, 131, 132]. SAgs induce a massive release of cytokines and hence elicit a very strong immune response [131].
It has long been known that one reason for SAg potency is that the fraction of T cells that recognize and respond to particular SAgs (up to 20% of all T cells) is much larger than that which recognizes and responds to conventional antigens (about 0.001% of all T cells)[92]. In part, this is because, unlike peptide antigens, individual SAgs can bind either MHC II α or β chains [131, 133], or, in some instances, both. In addition, T cell recognition of SAg is linked to the expression of TCR β chain of a particular Vβ family [92, 132] and not to specific αβTCR clonotypes. Therefore, the number of potentially responsive T cells is much higher for given SAg than for an individual protein antigen.
More recently, crystallographic analysis has shown that SAgs do not interact with either the MHC or the TCR in the same way as conventional antigens [134]. Peptide antigens are folded into the antigen-binding cleft of MHC molecules, which are recognized in a head-to-head orientation by the TCR, which allows the co-receptors, CD4 or CD8, to bind to the membrane-proximal domain of the MHC molecule simultaneously [131]. In contrast, SAgs bind MHC II molecules outside of the peptide-binding groove and bridge the TCR to the MHC through an interaction with the external face of the TCR Vβ chain. Moreover, the co-receptor CD4 is not required for the recognition of SAgs, and in consequence, SAgs can stimulate both CD4+ and CD8+ T cells [92].

Despite the differences in the way peptides and SAgs drive MHC–TCR interaction, it has been widely assumed that stimulation of T cells by SAg mimics stimulation by antigen. SAgs have affinities for TCRs similar to those for pMHC, and their dissociation rates from TCRs are comparable to those measured for TCR-pMHC interaction [133]. Therefore, the stimulation of T cells with SAgs was assumed to resemble antigen stimulation of the TCR more closely than stimulation by means of cross-linking with antibodies directed against the subunits TCR/CD3 complex (usually strengthened by secondary antibody or immobilisation), as the latter have much higher affinity for TCR and slower dissociation rates. The most common mix of antibodies used for T cell activation are anti-CD3 and anti-CD28 antibodies against the ε subunit of the TCR complex and a co-receptor CD28 [135].

### 1.4.6 Adhesion in T cell activation

In the absence of antigen, T cells move rapidly within the lymph nodes (LNs), forming transient contacts with APCs that allow T cells to “scan” APCs for the presence of the cognate antigen [82]. Chemokine gradients present in the LN microenvironment may promote T cell scanning of APCs by activating LFA-1. Chemokine-stimulated increases in LFA-1 affinity are both extremely rapid and transient, characteristics that would support T cell scanning of APCs, but retain the specificity of the process. The scanning process by itself may promote integrin activation and facilitate more prolonged interactions between T cells and APCs [136]. The encounter of naïve CD4+ T cells with class II MHC-presenting self-peptide in the periphery promotes polarization of the TCR and signalling molecules, such as ZAP70, to the sites of T cell–APC contact [91]. Although LFA-1 distribution was not assessed in these studies, this tonic level of activation may polarise LFA-1 or shift the conformational equilibrium toward the higher affinity LFA-1 that facilitates T cell scanning [136, 137].
An external ring of LFA-1 surrounding a central TCR-rich region is a structural hallmark of a mature IS, which forms during the antigen-dependent T cell interactions with APCs [104, 112, 126] (as mentioned in section 2.3). Furthermore, studies using planar lipid bilayers have established that the IS can form when a T cell interacts with only pMHC complexes and ICAM-1 [112]. During the initial stages of T cell activation, LFA-1 is localized in the centre of the T cell–APC contact site while the TCR localizes primarily to the periphery [94, 112, 126]. Rapid clustering of LFA-1 coupled with its central localisation within the contact site suggests that LFA-1-mediated adhesion is needed to stabilize T cell–APC contact, resulting in a larger number of engaged TCRs and activation of signalling cascades required for T cell activation. This is supported by colocalisation of the TCR and activated tyrosine kinases, Lck and ZAP70, in the periphery of the IS [94, 126], which suggests that signalling cascades necessary for T cell activation are already initiated while LFA-1 and ICAM-1 are still clustered in the centre of the T cell–APC contact site.

T cell commitment to proliferation and effector function requires sustained T cell–APC interaction [84, 91, 138]. In the mature IS, the TCR moves to the centre of the T cell–APC contact site while LFA-1 translocates to more peripheral parts of the IS [91, 94, 139]. The kinetics of LFA-1 localisation to the IS correlate with the minimum amount of conjugation time necessary for T cell proliferation to occur [91, 139], suggesting that prolonged localisation of LFA-1 may facilitate molecular engagements at the T cell–APC contact site that result in signalling events necessary for complete T cell activation.

It is also important to note that factors such as the differentiation state of the T cell, the type of APC and the microenvironment where the T cell interacts with the APC may affect the initiation, maintenance, and functional consequences of IS formation. Memory T cells generally express higher levels of LFA-1 and other integrins than naïve T cells do, and thus memory T cells have enhanced basal and TCR-dependent integrin function [140]. These differences in integrin functions between naïve and memory T cells likely affect both the T cell scanning process and IS formation. Furthermore, effector T cells have elevated integrin activity when compared to resting memory T cells, and they likely interact with collagen and other ECM proteins in peripheral tissues.

Therefore, if integrins are involved in the process of IS formation and stabilisation, there is the possibility that proteoglycans may also be. Studies showing the expression profile of SDC-4 in T cells raised the idea to study the involvement of SDC-4 in T cell activation.
1.4.7 SDC-4 expression in lymphocytes and other cells of the immune system

SDC-4 has been a molecule of interest for a long time and much is known about it (section 1.4). On the other hand, its function and influence on cells of the immune system have got attention only recently and from very few groups.

All published data confirm ubiquitous and, at the same time, tightly regulated expression of SDC-4 in all cells, including T cells. Ariizumi and co-workers demonstrated increased expression of SDC-4 in activated mouse [141, 142] and human T cells [143] in comparison to their resting (naïve) counterparts. On the other hand, SDC-4 is constitutively expressed at high levels in B cells and monocytes [144]. An increase in SDC-4 expression has been reported after lipopolysaccharide (LPS) stimulation of macrophages and DCs [145, 146].

1.4.7.1 Inhibitory effect

T lymphocyte activation is regulated by stimulatory, co-stimulatory and inhibitory signals transduced together with those induced by the binding of TCRs to their corresponding ligands on APCs [80]. Stimulatory receptors tend to be present constitutively even on resting T cells, whereas inhibitory receptors require primary activation for their expression [147]. Akiyoshi and co-workers characterised a novel inhibitory pathway in T cells, which is transduced by the interaction of the highly glycosylated APC surface molecule dendritic cell-associated heparan sulphate proteoglycan-dependent integrin ligand (DC-HIL) and SDC-4 as its exclusive receptor [141, 143, 148, 149]. DC-HIL specifically recognizes a particular structure of heparan sulphate specific for SDC-4 [141, 150]. This binding strongly attenuates the response to anti-CD3 antibodies, which can activate murine T cells. In T cells, it also causes a block in the production of proinflammatory cytokines and in their proliferation. These data suggest that DC-HIL/SDC-4 interaction can inhibit the T cell activation process in vivo [141].
The inhibitory role of SDC-4 in T cells was also studied by Espel's group [142]. They examined whether anti-SDC-4 antibodies could hinder in vitro T cell alloreactivity requiring cell-cell interactions. They looked at the stimulatory response of cells in a mixed lymphocyte reaction (MLR). The cells were incubated in the presence of an anti-SDC-4 Abs which partially inhibited proliferation; control cells proliferated normally. The combination of anti-SDC-2 and anti-SDC-4 Abs did not inhibit proliferation further. These findings support the evidence that SDC-4 may have an inhibitory function during the activation of primary T lymphocytes (human as well as murine).

The abovementioned MLR stimulation in presence of anti-SDC-4 Abs suggested that SDC-4 plays an important role during T cell stimulation. Further studies showed that the inhibition of T cell proliferation by cross-linkage of SDC-4 was independent of the accessory cells [142]. The same effect of proliferation inhibition was observed when purified T cells (from unseparated peripheral blood mononuclear cells (PBMCs)) were cultured on anti-CD3 plus anti-SDC-4-coated plates. This means that extensive cross-linking of SDC-4 on T cells leads to the inhibition of T cell proliferation stimulated with anti-CD3 antibodies. This suggests that cross-linked SDC-4 Abs have higher inhibitory capacity on T cells than soluble Abs (MLR above). Moreover, cross-linking of SDC-4 also inhibited anti-CD3 Ab-induced TNFα production by CD4 T cells in a dose-dependent manner. These results show that SDC-4 has an inhibitory function in human T cells, in agreement with what has been suggested for SDC-4 in mouse and human T cells by Ariizumi and colleagues [141, 143].

**Mixed lymphocyte reaction (MLR)**

Peripheral blood lymphocytes from two individuals are mixed together in tissue culture for several days. Lymphocytes from incompatible individuals stimulate each other (alloreactive reaction) to proliferate significantly whereas those from MHC compatible individuals do not.
1.4.8 SDC-4 effect on maturation, motility and morphology

Interesting studies emphasizing the function of SDC-4 in cells of the immune system have been performed by the Buhlingen group. They demonstrated that SDC-4 is upregulated in human monocyte-derived dendritic cells (DCs) during their maturation. Increased presence of SDC-4 influenced the motility of DCs to lymphatic tissues [151].

Maturation and motility of DCs, following contact with an antigen, are crucial steps of the immune response [152], as well as of maintenance of tolerance. Both processes are important steps of the antigen-specific adaptive immune response during which mature DCs present the processed antigens to T cells in secondary lymphatic tissues. DCs stay in tissues from where they migrate into lymph nodes. There they contact number of T cells, what is regulated by actin driven processes.

Averbeck and co-workers investigated the effects of lysophosphatidylcholine (LysoPC) on DC motility. In addition, they investigated the effect of LysoPC on the involvement of PKCδ phosphorylation-dependent regulation of DC motility by SDC-4 and PKCα [153]. LysoPC is generated during the apoptosis of cells and acts as a “find-and-eat-me” signal thought to prevent autoimmunity [146]. This molecule also upregulates the surface expression of MHC and co-stimulatory molecules, e.g. CD86 in DCs, but has no effect on the expression of SDC-4. Furthermore, LysoPC-induced SDC-4 phosphorylation leads to a decrease in adhesion, podosomes formation and mobility of DCs.

Buehligen and co-workers also showed that LysoPC inhibits the adhesion of DCs to fibronectin and their motility by decreasing podosomes formation. What seems to be important for my project is that DC podosomes formation and motility are both regulated by SDC-4. Moreover, both processes are controlled by PKCδ-dependent phosphorylation of SDC-4 and were inhibited in LysoPC-matured DCs. Thus, these DCs are defective in adhesion and migration that might delay migration of DCs to lymphoid organs and thus prevent autoimmunity. Buehligen and co-workers found that LysoPC stimulation decreases motility and adhesion in immature and mature DCs, which suggests that the adhesive events are independent of maturation. They have also previously shown that a shift from intracellular to cell surface-associated SDC-4 occurs during the maturation of DC [146, 151].
Yamashita and co-workers demonstrated another effect of SDC-4 on the morphology of immune system cells. Activated murine B lymphocytes, when seeded on a SDC-4 antibody-coated surface, exhibited dramatic morphological changes such as the formation of well-distinguishable and large fillopodial extensions. In consequence, SDC-4 was proposed to increase the cell size [154].

In summary, SDC-4 regulatory effect on DC’s adhesion and their motility, and B cells morphology, together with changes in SDC-4 expression in T cells, brought us to the idea that SDC-4 may influence the T cell activation process when recognising the antigen on the surface of APCs.

The general recapitulation of the published knowledge on SDC-4 function and characteristics in mammalian cells is as follows:

- It is present in FAs and influences junction assembles between cells [114]
- SDC-4 in coordination with fibronectin [20, 65, 155] and integrins [4, 5] is involved in a number of downstream regulation processes including activation of tyrosine kinases
- SDC-4 highly affects cytoskeleton organisation [62, 156], and it may possible be its effect in IS as actin organisation is highly reorganised during IS formation
- SDC-4 is a binding partner for many proteins, e.g. PDZ [52], but may influence indirectly other receptors e.g. TCRs [88, 90]
- The association of SDC-4’s extracellular core protein domain and other cell surface molecules may mediate cell-cell adhesion or potentially function as a shed or matrix-embedded adhesive ligand - adhesion is important for T cell activation.

Recent publications of a few groups which already described expression changes in T cells made us more interested in the interaction or correlation between the molecule and these cells [142, 143, 148, 150].
1.5 Agrin

Interestingly, another heparan sulphate proteoglycan agrin has recently also been implicated to function as a co-stimulatory signal at the immunological synapse [157].

Agrin is a heparan sulphate proteoglycan with a large core protein backbone that includes a number of distinct structural domains. There are extensive O- and N-linked glycosylations at the amino-terminal half of the protein with the addition of heparan sulphate GAG at the O-linked carbohydrate moieties [158]. The transcript of the Agrin gene can be differentially spliced to produce different isoforms of the protein, which determine its localization and function. Alternative splicing at the amino terminus produces either a type II transmembrane protein (TM-Agrin) or a secreted protein (SS-Agrin) [159, 160].

Expression of Agrin in T cells has been documented by Western blotting, and PCR. Initially, Khan and colleagues [157] reported its expression in murine thymocytes and splenocytes, and demonstrated that following T cell activation it is post-translationally modified. Co-stimulation with anti-CD3 and anti-Agrin antibodies augmented T cell proliferation, particularly when low concentrations of the anti-CD3 antibody were used. Furthermore, purified Agrin from activated, but not resting, T cells when added to the culture medium of non-activated T cells induced clustering of lipid rafts [161] and of the TCR [157]. Also, Agrin was localized at the IS during antigen presentation [157, 162]. It has been reported that agrin lowers the threshold for antigen-induced lymphocyte proliferation in vitro by recruiting lipid rafts that contain complexes of CD3 and T-cell receptors, CD4 or CD8, and Lck [157]. It functions on lymphocytes at picomolar concentrations and within minutes. As mention earlier, Agrin is present in resting, as well as activated, T cells, but interestingly only deglycosylated form isolated from activated T cells induces TCR aggregation and recruitment of lipid rafts [163] as observed at the immunological synapse [164]. Given that agrin's function in T cells is regulated by glycosylation and that agrin aggregates lipid rafts, its deglycosylation might induce cytoskeletal reorganization in T cells. So, on T-cell activation, the deglycosylation of agrin could eventually allow its dissociation from the CD44–agrin complex and the delivery of the accompanying lipid rafts to the immunological synapse [164]. Based on these results, it was proposed that T cell activation induces an as yet unknown modification of Agrin that endows it with higher aggregating activity, and furthermore redistributes the protein to the site of the IS where it may facilitate antigen presentation [164].
1.6 Hypothesis and Aims

1.6.1 Hypothesis

This project is aimed to add another perspective to the ongoing research studying factors influencing the formation and stability of the immunological synapse (IS) formed between T cells and APC. More specifically, the poorly understood involvement of proteoglycans in this process. I particularly studied one member of this protein superfamily, SDC-4 with its extensive glycosaminoglycan moieties exposed to the extracellular space surrounding the cells (glycocalyx). SDC-4 has been found previously to influence the contact with neighbouring cells. Specifically, I hypothesised that SDC-4 would play a role in T cell-APC interaction by affecting IS formation and/or stability, and designed experiments to test this.

1.6.2 Aims

My aims to test the hypothesis were:

- To confirm the expression profile of SDC4 in naïve and activated human peripheral T cells as well as the Jurkat T cell line using available methods. SDC-4 is expressed in T cells at low level, but it is the only known syndecan which was reported to be upregulated by T cell activation [141-143]
- To localise SDC4 in activated T cells and study its co-localisation with the T cell receptor and proximal signalling molecules using immunofluorescence.
- To test the possibility of involvement of fibronectin, an extracellular ligand of SDC-4, in IS formation and stabilisation using immunofluorescence.
- To quantitate the impact of SDC4 on conjugates formation using flow cytometry.
- To study the dynamics of the IS in the presence or absence of increased level of SDC-4 expression employing live cell imaging and quantitative image analysis.
## 2 Materials and methods

### 2.1 Materials and suppliers/sources

#### 2.1.1 General reagents and chemicals

<table>
<thead>
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<th>Supplier</th>
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#### 2.1.2 Molecular biology enzymes and reagents

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<tr>
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<td>Roche</td>
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<td>Lipofectamine 2000</td>
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<td>Luria-Bertani (LB) broth</td>
<td>Sigma</td>
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<td>Needles</td>
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RNase A  
OligodT  
Protein G beads  
SDS-PAGE Protein all blue ladder  
S.O.C. medium  
Taq DNA polymerase  

Qiagen  
Invitrogen  
Amersham  
Biorad  
Invitrogen  
Qiagen  

2.1.3 Molecular biology kits

Qiagen endofree plasmid maxi kit  
Taq PCR kit  
Dynabeads® Untouched™Human CD4 T Cells separation kit  

Qiagen  
Invitrogen  
Invitrogen  

2.1.4 Tissue culture reagents and materials

Coverslips  
Cryotubes  
BD Cytopern/Cytofix™  
Dimethyl sulfoxide (DMSO)  
Dulbecco’s Modified Eagle Medium (DMEM)  
Foetal Calf Serum (FCS)  
OptiMEM®  
Penicillin  
Kanamycin  
Phosphate Buffered Saline (PBS) (without calcium and magnesium)  
RPMI 1640 medium  
Sodium pyruvate  
General microscopy slides  
Tissue culture and microbiology plastic ware: NUNC, BD Biosciences, Falcon, FisherScientific  
Trypsin-EDTA  
WILCo-dishes  

VWR, UK  
Nunc  
BD  
Sigma  
PAA  
PAA  
Invitrogen  
PAA  
Sigma  
PAA  
VWR  
Intracel, UK  

2.1.5 Western blotting equipment and materials

PROTRAN® Nitrocellulose transfer membrane  
SDS-PAGE gel tanks  
Western Semi-Dry Blotting Machine  
3MM paper  
Amersham™ ECL Western Blotting Detection System  

Whatman International Ltd, UK  
BioRad  
Hoeffer, Germany  
Whatman International Ltd, UK  
GE Healthcare
2.1.6 Equipment

Centrifuge tubes
Falcon ref.352052 BD Biosciences
Eppendorf 5810 centrifuge
Eppendorf, Germany
Eppendorf 5415R centrifuge
Eppendorf, Germany
FACS Calibur
BD Biosciences, UK
FACS BD LSR II
BD Biosciences, UK
Eppendorf 5415R centrifuge
Eppendorf, Germany
Storm 820 gel drier
Pharmacia Biotech
Sorvall RT-7
Sorvall, UK
Sorvall RC-5B centrifuge
Sorvall, UK
Sorvall RC-5B centrifuge
Sorvall, UK
MWG, DNA thermal cycler
MWG, Germany
MWG, DNA thermal cycler
MWG, Germany
ETC Enzyme Imager
GE Healthcare, UK
Micro Centaur microcentrifuge
Scotlab, UK
Tissue culture CO₂ incubator
Nuaire

2.1.7 Plasmids

pEGFP-N1
gift from J. Couchman
pHA-rSDC-4-IRES-EGFP
gift from J. Couchman
pIRES2-EGFP
Clontech
pMEX-hSDC4
gift from J. Couchman
pHA-hSDC-4-IRES-EGFP
created in the group of A.I. Magee

2.1.8 Bacterial strains

XL10 Ultracompetant cells
Tet' (mcrA)183 (mcrCB-hsdSMR-mrr)173 endA1
supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB lacF Δ(ZM15 Tn10 (Tet') Amy Cam')]a
Stratagene

DH5α subcloning efficient cells
F- φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 thi-1
 gyrA96 relA1 λ-
Invitrogen

2.1.9 Mammalian cell lines

Jurkat T cells
gift from J. Burkhardt lab
HEK293A cells
ATCC
MD-MBA-231 cells
ECACC
MRC5 cells
ECACC
Raji B cells
gift from J. Burkhardt lab
OKT3 hybridoma
ATCC
2.1.10 Buffers

Cell Lysis Buffer: 1% Octylglucoside (OG) in TNEPF + 1mM DTT, (150mM NaCl, 1.5mM MgCl$_2$, 50mM NaF, 1mM Na$_3$VO$_4$)

5X Electrophoresis Running Buffer: 0.6M Tris Base, 0.5M Glycine, 0.05% (w/v) SDS, distilled water to 1 litre and pH 8.3.

DNA loading Buffer: 6mM EDTA, 300mM NaOH, 18% Ficoll (in water), 0.15% Bromocresol Green, 0.25% Xylene Cyanol

Fixation Buffer: 4% paraformaldehyde + 2% sucrose in 25ml PBS, 400µl 1M NaOH, 200µl 1M HCl

SDS-PAGE Sample Buffer (SB): 100 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 15% glycerol

5X Transfer Buffer: 25mM Tris, 0.05% SDS, 20% (v/v) Methanol, made to 1000ml with distilled H$_2$O.

RIPA Buffer: 50mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 0.5% Triton X100, 0.5% Sodium deoxycholate (need to pH with NaOH), 0.1% (w/v) SDS (optional) plus mini complete/EDTA cocktail of inhibitors.

PBS-T: 0.05% (v/v) Tween-20 in 1X PBS

SDS-PAGE Gel Buffers stocks:
Lower Tris Buffer pH 6.8: 1M Tris,
Upper Tris Buffer pH 8.6: 1M Tris,

WB Blocking Buffer: 5% (w/v) non-fat dried milk powder in PBS-T and filtered through 5MM blotting paper
### 2.1.11 Antibodies and immunofluorescence reagents

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Raised In</th>
<th>Type</th>
<th>Concentration Used</th>
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<td>Mouse</td>
<td>Mo IgG1</td>
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<td>Covance, USA</td>
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<tr>
<td>Anti-CD25 APC</td>
<td>Mouse</td>
<td>Mo</td>
<td>1:200</td>
<td>BD Biosciences, USA</td>
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<tr>
<td>Anti-CD4 FITC</td>
<td>Mouse</td>
<td>Mo</td>
<td>1:200</td>
<td>BD Biosciences, USA</td>
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<tr>
<td>Anti-Lck</td>
<td>Rabbit</td>
<td>Po 2166</td>
<td>1:100</td>
<td>Generated in our group</td>
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<td>Anti-Lck, 3A5</td>
<td>Mouse</td>
<td>Mo</td>
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<td>Invitrogen, UK</td>
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<td>Alexa Fluo®488</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alexa Fluo®647</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Anti-Mouse IgG2a conjugated to:</td>
<td>Goat</td>
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<td>Alexa Fluo®647</td>
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<td>Alexa Fluo®647</td>
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<tr>
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<tr>
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<td>Mo, IgG2b</td>
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<td>Millipore</td>
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<td>Anti-rSDC-4, 5G9</td>
<td>Mouse</td>
<td>Mo</td>
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<td>SantaCruz Biotech., USA</td>
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<tr>
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<td>Mouse</td>
<td>Mo</td>
<td>1:50 (IF)</td>
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<td>Rabbit</td>
<td>Po</td>
<td>1:200 (IF)</td>
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<td>Lab creation</td>
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<tr>
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<td>Mouse</td>
<td>Mo</td>
<td>1:200</td>
<td>AbCam</td>
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<tr>
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<td>D.A.</td>
<td></td>
<td>1:200</td>
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Table 2.1. Antibodies used. WB = dilution for Western Blotting; IF = dilution used for Immunofluorescence; Mo and Po stand for Monoclonal and Polyclonal antibodies, respectively. D.A = does not apply.
2.2 Methods

2.2.1 Antibody Production using BD CellTM MAb Medium in a CELLineTM Device

The CELLine™ is a novel cell culture system based on nutrient-permeable membrane technology. In general, cells are maintained in a small (15 ml) cultivation chamber (cell compartment) that is separated from a one litre nutrient supply compartment by a semi-permeable membrane. Nutrients and other small molecules pass across the semi-permeable cellulose acetate membrane into the cell chamber. Cell-secreted products (e.g. antibodies with Mr~150kDa) with a molecular weight greater than 10kDa are retained in the cell compartment of the device. The moulded silicone membrane on the bottom of the device allows oxygen to reach the cells from underneath. The cells settle upon the silicone membrane at the bottom of the cell compartment, which provides direct access to oxygen and carbon dioxide gases that rapidly diffuse across the membrane. This approach leads to high cell concentrations within the small volume of medium in the cell compartment (Figure. 2.1 and 2.2).

The CELLine flask can generate an average yield of 30-50mg of antibody every two-four weeks from cells adapted to low serum-containing medium.
Figure 2.1. Scheme of CELLine flask. Figure from www.BD

Figure 2.2. CELLine flask. Figure from
2.2.1.1 Adaptation of hybridoma cells to low serum conditions.

The use of a CELLine system requires that cells adapt to low serum culture medium. BD Cell Serum-Free MAb Medium is a synthetic medium that does not require high serum supplementation. In general, OKT3 hybridoma cells were grown in DMEM +10% foetal calf serum (FCS) for two weeks after bringing to culture. The cells were seeded into an adaptation culture vessel using a 3:1 mixture of BD Cell MAb Serum Free Medium to the pre-adaptation DMEM medium formulation. Then the cells were maintained for a week and split in the mixture when necessary.

Slow continuous increase of BD Cell MAb Serum Free Medium content was performed over 6 weeks. After this period cells were ready to grow in 100% BD Cell MAb Serum Free Medium containing 1.25% immunoglobulin (Ig)-low Serum. Importantly, a high cell density should be maintained throughout the process for successful adaptation.

2.2.1.2 Antibody Production

An adaptation procedure was followed to prepare cells for the use of the CELLine system. The nutrient and cell compartment medium was prewarmed to 37°C to prevent condensation and to eliminate significant temperature fluctuations inside the CELLine device.

First, the nutrient compartment was prewet with 25ml of the medium to avoid damage of the membrane. Then the cells were resuspended in 15ml of BD Cell Serum-Free Medium, containing 1.25% Ig-low serum, at 2x10⁶/ml and loaded to the cell compartment. The nutrient compartment was filled with one litre of serum free BD Cell MAb Medium.

2.2.1.3 Antibody Harvest

On day seven, cells with antibody-containing medium were collected from the cell compartment. The sample was centrifuged for 5min at 200xg. Antibody-containing supernatant was collected to a separate tube, 0.1% sodium azide was added as preservative and it was stored at +4°C. The CELLine device was reloaded with the cells resuspended in 15ml of BD Cell MAb medium containing 1.25% low-Ig serum.
2.2.1.4 Antibody purification

For purification of antibodies isolation I used two kinds of affinity chromatography media. Protein A- and Protein G-Sepharoses have different IgG binding specificities, depending on the origin of the IgG. Compared to protein A, protein G binds more strongly to mono and polyclonal IgG, for example from cow or sheep. Furthermore, unlike protein A, protein G binds monoclonal rat IgG to which the OKT3 belongs. In that case, Protein A-Sepharose was applied for purification of the control antibodies (036 and 037 rabbit antisera) and Protein G-Sepharose for OKT3 IgG2a antibodies.

2.2.1.5 OKT3 antibodies purification: Protein G-Sepharose 4 Fast Flow column.

Prior to the affinity purification of OKT3 the supernatant was centrifuged (1000xg for 10minutes) and diluted with the equal volume of Binding buffer. A 5ml Protein G-Sepharose column was prepared according to the manufacturer’s instruction. The column was equilibrated with 5 bed volumes of Binding buffer and the sample, diluted 1:1 in Binding buffer, applied to the column. Any large impurities were eliminated by filtration of sample through a 0.45μm syringe filter. The column was washed with 5 volume of Binding buffer. In the next step the antibodies were eluted with Elution buffer and 500μl samples were collected into 1.5ml tubes, containing 100μl of Neutralizing buffer. The column was regenerated with Binding buffer.

Materials:
Binding and washing buffer: 20mM Sodium Phosphate, pH 7.0
Elution buffer: 0.1M glycine-HCl, pH 2.4
Neutralizing buffer: 1M Tris-HCl, pH 9.0
2.2.2 Cell culture

All procedures were carried out in a laminar flow class 2 tissue culture hood. Jurkat T cells and Raji B cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FCS at 37°C in the atmosphere of 5% CO₂. Cells were grown in tissue culture flasks until the adequate density (2-10 × 10⁶ cells/ml) was reached. Cells were counted regularly using a haemocytometer.

MRC5. HEK293A and MD-MBA-231 cells were cultured in DMEM medium supplemented with 10% FCS, at 37°C in the atmosphere of 5% CO₂. Cells were grown in flasks and multi-well dishes until 80% confluence was reached; they were then washed with PBS, treated with Trypsin-EDTA for 2 minutes to detach the cells, and then resuspended in growth medium and incubated as described above.

2.2.3 Frozen storage of cells

For long term storage of cells, these were harvested from a confluent 25cm² T-flask and taken up in 3 ml of ice-cold “Freezing medium” (FCS containing 10% (v/v) DMSO). The suspension was aliquoted into two cryotubes, placed in a neoLab-Freezing box Cryo-Safe (-1°C, 120 mm Ø, 86 mm high) to allow slow decrease of the temperature and incubated for 24 hours at -80°C freezer. Then the tubes were stored in liquid N2.
2.2.4 Cell transfection

2.2.4.1 Transfection of HEK293 cells

Lipofectamine 2000 transfection reagent (Invitrogen) was used for transfection of pHA-rSDC-4-IRES-EGFP and pIRES2-EGFP plasmids into HEK293A cells. Transfection was performed on autoclaved coverslips in 24-well dishes. A day before the transfection, $2 \times 10^5$ cells per well were seeded in the antibiotic-free serum-containing medium in a 24-well dish. On the day of transfection, the medium was replaced with fresh medium followed by the addition of the transfection mixture. For each well, the transfection mixture was created by mixing 2.5µg of DNA construct diluted in 35µl of OptiMEM® medium and 1.2µl of Lipofectamine™ 2000 diluted in 35µl of OptiMEM® I Medium. Both solutions were incubated for 5 minutes at room temperature, then the diluted DNA was combined with the diluted Lipofectamine™ 2000 and incubated at room temperature for another 20 minutes to allow DNA-Lipofectamine™ 2000 complexes, also called transfection mixture, formation. The transfection mixture was added directly to each well containing cells and mixed gently by rocking the dish back and forth. Samples were incubated for 4 hours at 37°C in a CO₂ incubator. Then the medium was changed and the cells were used for the immunofluorescence 24 hours post-transfection.

2.2.4.2 Transfection of Jurkat T cells

First, the appropriate number of wells in a 24-well dish were filled with 0.5 ml of complete medium and pre-conditioned in the 5% CO₂ incubator (37°C) for at least 15 minutes. Transfections of Jurkat T cells using pHA-rSDC-4-IRES EGFP, pHA-hSDC-4-IRES-EGFP and pIRES2-EGFP plasmids were performed using the electroporation technique called microporation (see section 3.2.1 for details). For microporation, were used per well of the 24-well dish: 3µg of pHA-rSDC-4-IRES-EGFP DNA, 4µg of pHA-hSDC-4-IRES-EGFP DNA or 0.1µg of pIRES2-EGFP DNA in 10 µl of Buffer R supplied by the manufacturer (Invitrogen). The cells were first centrifuged at 800 rpm for 2min at room temperature, washed with PBS and resuspended in Solution R buffer by gentle pipetting to obtain a single cell suspension. The DNA was added and mixed by tapping of the tube with a finger. Such mixture was directly applied for the microporation in the supplied electroporation tip. Similarly, $2 \times 10^6$ cells, ten times larger amount of the DNA plasmid and 6-well dish containing 2 ml of pre-conditioned medium were used for the microporation of Jurkat T cells in the 100 µl electroporation tip.
The Microporator settings were optimised for 1400V, 10msec, 3pulses, independent of the electroporation sample size (see section 3.2.1.1 for details).

2.2.5 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

10% sodium citrate venous blood was obtained from a donor, diluted 1:1 with serum-free RPMI 1640 medium and gently layered on Ficoll-Paque™ PLUS in the ratio 1:2 (Ficoll:blood). After centrifugation at 1000rpm (no brakes) at room temperature for 30 minutes, the white, cloudy interface between the bottom Ficoll layer and upper RPMI layer was transferred to a new tube. Cells were washed twice with PBS. Samples were centrifuged after each wash at 800rpm at RT for 10 minutes. Pellets were resuspended in serum-containing RPMI-1640 medium, transferred to a 96-well plate and either left inactivated or a mix of superantigens (1µg/ml Staphylococcal enterotoxin SEE, 2µg/ml SEA, 2µg/ml SEB) in RPMI-1640 supplemented with 10% FCS was added to activate them. The cells were cultured at 37°C in the atmosphere of 5% CO₂ for a week and flow cytometry analysis was performed each day.

2.2.5.1 Isolation of CD4⁺ T cells

The PBMCs were cultured for 4 days and T cells were isolated every day by negative selection using the Invitrogen Dynabeads® Untouched™ Human CD4⁺ T Cell Isolation Kit. 1x10⁷ cells were incubated with 100µl of Isolation Buffer (PBS + 2%FCS and 2mM EDTA) containing 20µl of heat activated FCS and 20µl of Antibody Mix for 20min at 4°C, then washed with 2ml of Isolation Buffer and centrifuged at 300xg for 8 min at 4°C. The Isolation Buffer and pre-washed Depletion MyOne Dynabeads were added to the washed cells and incubated for another 15 min at 24°C with gentle tilting and rotation. The beads were removed with a magnet. The supernatant was transferred to the new tube and cells were centrifuged for 10min at 300rpm. RNA extractions were performed from selected T cells followed by RT-PCR.
2.2.6 Molecular Biology Protocols

A standard PCR reaction contained the following: 5µl of 10X PCR buffer, 2.5mM dNTPs, 10pmol of each primer (Forward and Reverse), 1 unit of Taq DNA polymerase (Invitrogen), 1-5µl of DNA (~500ng), made to 50µl with sterile water. For reactions with more than 2 samples a master mix was prepared.

2.2.6.1 DNA plasmid preparation

2.2.6.1.1 Transformation of bacteria

50 μl DH5α subcloning efficient cells were thawed on ice for 5 min, mixed with 2 μl plasmid DNA and incubated on ice for 30 minutes. The mixture was heat-shocked at 42°C for 45 seconds and placed on ice for another 2 minutes. 950 μl of the pre-heated SOC medium (Invitrogen) was added to the cells which were then incubated at 37°C for 1 hour with orbital shaking applied. Finally, 200 μl of the culture were plated on selective LB-agar Petri dishes (ø 9 cm) and placed at 37°C overnight.

2.2.6.1.2 Plasmid purification

Plasmids pHA-rSDC-4-IRES-EGFP, pHA-hSDC-4-IRES-EGFP and pIRES2-EGFP were purified using the EndoFree Plasmid Maxi kit (Qiagen Cat.No.12362). DNA concentration and purity were determined by measuring absorbance at 260 nm and 280 nm employing a Nanodrop ND-1000 spectrophotometer. Purity was assessed by the A260/A280 ratio where ratios of 1.8-2.0 were considered free of contaminants. The plasmids were sent to MWG Eurofins (Germany) for sequencing.

2.2.6.2 RNA isolation and Reverse-Transcription PCR

Total RNA was extracted from the isolated T cells using the Trizol® Reagent. Approximately 1 x 10⁷ of PBS-washed T cells were used for each individual extraction and the spin protocol for the RNA extraction was followed as provided by the manufacturer. Quantitation of RNA quality and concentration was carried out using 260nm absorbance analysis in a spectrophotometer and the RNA integrity was visualised on a 1% agarose gel.
Then the SuperScript® first strand for RT-PCR was used and the manufacturer’s protocol was followed. Finally, the RT-PCR reaction consisted of 2.5µl of cDNA, 5µl of 10X Buffer, 1.5µl of MgCl₂, 1µl of dNTPs (10mM), 0.5µl primer (25pmol mix) and sterile water to a total of 50µl. The cycles used were as for PCR reported in section 2.2.3.1. Primers were used as for PCR reactions. PCR for the human SDC-4 sequence was carried out with 2µl of cDNA and the following primers F (Forward) – CGAGAGACTGAGGTCATCGAC, R (Reverse) – GCGGTAGAACTCATTGGTG (product of 453bp). Amplification was performed using the following protocol: 94°C – 2 minutes, 94°C – 50 seconds, 53°C – 50 seconds, 72°C – 6 minutes, repeated for 35 cycles. PCR was also carried out with GAPDH with 2µl of the RT-PCR reaction. These GAPDH primers were used as a control for the RT reaction and possible genomic contamination when used in the absence of an RT enzyme. The product amplification was analysed by 1% agarose gel electrophoresis (with 5µl ethidium bromide (10mg/ml) per 50ml of gel) and a 1kb or more suitable ladder.

2.2.6.3 SDS-PAGE

Protein samples were separated by molecular weight using a Tris/glycine SDS-polyacrylamide gel electrophoresis (SDS-PAGE) system. The SDS binds to the proteins creating an uniform negative charge and the Electrode buffer permits the movement of current and thus the movement of charged proteins down the gel. Homemade gels were prepared using a Biorad mini gel system. 10-15% gels composed of running gel (250 mM Tris pH 8.8, 10-15% acrylamide-bisacrylamide mix (37.5:1) (BIORAD), 0.2% APS, 0.1% SDS, 0.082% TEMED) and 4% stacking gel (158 mM Tris pH 6.8, 0.3% APS, 0.1% SDS, 0.15% TEMED). For each layer of the gels to set, a layer of propan-2-ol was poured on top and left at room temperature for 30 minutes. Gels were cast between two glass plates (BioRad system) which were held together with clamps. Plastic combs were used to create the sample wells. After setting of the gels, the apparatus was assembled and the chambers filled with running buffer. All protein samples were prepared in NuPage LDS sample buffer (Invitrogen) with 12.5 mM DTT added. All samples were incubated at 95°C for 5 minutes prior to loading,
### 2.2.6.4 Western Blotting

Proteins were transferred onto cellulose membranes (pre-soaked in transfer buffer) using a semi-dry transfer system. Transfer buffer used was electrode buffer (25 mM Tris (pH 8.3), 192 mM glycine and 1% SDS). Transfer was run for 1 h 30 min at 1 mA/cm² for home-made 0.75 mm gels. After transfer, membranes were blocked in 5% filtered non-fat milk for 1 h at room temperature or overnight at 4°C. Details of primary antibodies used are shown in Table 2.1.11. All antibody incubations were done for 1 h at room temperature. Secondary goat anti-mouse and goat anti-rabbit HRP-coupled antibodies (Southern Biotech) were used at 1:20,000 dilution. Washes after all antibody incubations were done in PBS-T 4-5×5 min. Bands were detected using ECL Plus Western blotting reagent (GE Healthcare) and light detection at 530 nm using an Ettan DIGE Imager (GE Healthcare).

### 2.2.6.5 Proximal signalling analysis in conjugates

Conjugates were prepared the same way as in section 1.2.6.3.1, but varying time of incubation at 37°C was applied: 2, 4 and 8 minutes. Conjugates were prepared with Raji B cells pre-loaded with SEE or not (for control). After the incubation and brief spin, 100µl of Cell lysis buffer (pervanadate and DTT were added at the latest possible point) were added to samples and these were left on ice for 40 minutes (lysis). The insoluble material was removed by a short centrifugation step of 3000g for 3 minutes at 4°C. 70µl from each sample were moved to a new Eppendorf tube, 30µl of 5x sample buffer (SB) buffer were added to each tube, the samples were boiled for 3min at 100°C and then equal volumes of samples were loaded to SDS-PAGE. The levels of Phosphotyrosine and α-Tubulin were then determined using Western blotting with specific antibodies.
2.2.7 Microscopy

2.2.7.1 Preparation of Fixation solution

4% paraformaldehyde (PFA) was prepared by adding 1g of PFA and 0.5g sucrose in 25ml of PBS and heated at 65°C for 5 minutes, then 400µl of 1M NaOH were added and heated at 65°C for 10 minutes. Finally, approximately 250µl of 1M HCl were added to adjust pH to 7.2.

2.2.7.2 PLL-coated coverslip preparation

The coverslips were washed with Piranha solution (30:70 (v/v) 30% H₂O₂ stock:95-98% H₂SO₄ stock). The solution was prepared by gently mixing the H₂SO₄ with H₂O₂ and the coverslips were left in the solution for 15 minutes. Then the solution was removed and the coverslips were washed 10x with high quality water. Later, a 0.01% poly-L-lysine solution (0.1% stock) was added for 1h at RT, then removed and the coverslips dried at 65°C for 30 minutes. The coverslips were washed twice with PBS and water just before use.

2.2.7.3 Preparation of coverslips with fixed cells

Adherent HEK293A cells were cultured on coverslips in 24-well dishes overnight at 37°C with 5% CO₂ atmosphere after being sparsely plated. Cells were then treated with 4% PFA to fix the cells (500µl/well), then the fixation solution was aspirated and then the cells were washed twice with PBS+0.1M ammonium chloride and once with PBS only.

2.2.7.3.1 Preparation of conjugates formed between Jurkat T cells and Raji B cells

Raji B cells were counted and the appropriate volume was transferred to 15ml tubes, and pelleted for 5min at 1000rpm. Then the pellet was resuspended at 1×10⁶ cells per ml in RPMI. SEE was added to a final concentration of 1µg/ml and the tube was incubated with the lid loose for 1h at 37°C, in a CO₂ incubator. Meanwhile, the Jurkat T cells were counted (the same number of cells as for Raji cells), pelleted cells were washed with serum-free RPMI-1640 and resuspended in serum-free RPMI at the same concentration as Raji B cells.
At the end of the SEE incubation, the Raji cells were washed with serum-free RPMI and resuspended to $1 \times 10^6$ cells/ml. Then 200µl of Raji B cells, pre-loaded (or not) with SEE, were added to 200µl of Jurkat T cells in 5ml centrifugation tubes (Falcon) spun at RT for 1min at 100xg and incubated without disturbing the pellet in a 37°C water bath for 5min. Then, 200µl of the supernatant from the top of the sample was taken off using a tipped pipette, then the pellet was broken up by pipetting up-down gently with a cut p20 pipette tip and a 50µl sample was transferred onto a 12mm round coverslip. The coverslips placed in a 24-well dish were floated on a 37°C water bath for 2min, then fixed with 500µl Fixation solution (containing 4% PFA) for 20min at RT.

### 2.2.7.4 Immunofluorescence

After fixation, cells were washed twice with PBS + 0.1M ammonium chloride and once with PBS only. Then, 0.2% TritonX100 in PBS was added to samples and left for 8 minutes at RT to permeabilise the cells. The liquid was then aspirated and the cells were washed 3x with PBS, blocked with 5% BSA + 0.2% fish skin gelatine for 1h at RT and, after aspirating the liquid, the primary antibody was added to the sample at an appropriate concentration (as previously optimised) in 5% BSA/PBS and left for 1 hour at RT. After washing 10x by dipping the coverslip in a beaker containing PBS and once for 5min on a drop of PBS, the secondary antibody with an appropriate fluorophore was added at 1:200 in 5% BSA/PBS for 30 minutes at RT. Cells were then washed in a beaker with PBS as for primary antibody and twice on a drop of PBS. Finally, the sample was desalted by dipping in water and left to air dry for 5min. Coverslips were mounted onto slides using Prolong® Gold antifade reagent.

### 2.2.7.5 Imaging

For confocal imaging, the cells were imaged on an inverted laser scanning confocal fluorescence microscope (Zeiss LSM 510) using a 63X 1.2NA oil-immersion objective). For dual labelling experiments laser line 543nm (20%) was used for Alexa555 and filter BP560-615 was used to select the detection wavelength; laser line 633nm (20%) was used for Alexa633 and filter LP650 to select the detection wavelength. Finally, the images were analysed with ZEN 2008 software by Carl Zeiss.
2.2.7.6 Live cell imaging

On the day of the experiment, Raji B cells (APCs) were labelled with Orange cell tracker (2µl per 1ml of resuspended cells; 1x10⁶ cells per ml of serum-free RPMI-1640) for 30 min at 37°C, washed, resuspended in RPMI-1640 containing 10%FCS and loaded with SEE (1µg/ml) for 1h at 37°C. Afterwards, the cells were washed once with serum-free RPMI-1640 and resuspended to 0.5x10⁶ cells per ml of serum–free and Phenol Red-free RPMI-1640. 200µl of Raji B cells were applied on the microscopy glass-bottomed MatTek dish (cat. P35G-0-20-C, 35mm, 20mm glass diameter). To reduce the mobility of the Raji B cells for imaging, they were left to settle and attach to the glass bottom of the dish for 2 minutes. Then, the medium was removed and 300 µl of Jurkat T cells (0.5 x 10⁶ cells per ml of Phenol Red-free RPMI-1640 containing 0.5% FCS) were added, followed by the immediate acquisition of the events. One frame every 10 sec was taken over a period of 30 minutes. The images were taken using an inverted epi-fluorescence microscope Zeiss Axiovert 200M with a X20, PlanApo objective, Zeiss. Orange cell tracker labelled B cells were detected with 555 laser and transfected Jurkat T cells with 488 laser.

In next step the results were analysed with Definiens® software (Germany). All details and explanation of the steps are presented in Results - section 3.5.2.1.

2.2.8 Flow Cytometry analysis

2.2.8.1 Conjugates formation

The conjugates were prepared the same way as described in section 2.2.7.3.1. The cells were fixed with Fix/Perm Kit (BD Biosciences) according to the manufacturer’s recommendations or left unfixed. Unfixed cells were kept at 37°C till the measurement was performed using the LSRII flow cytometer.

2.2.8.2 SDC-4 detection in T cells

PBMC were selected from blood and either left unactivated or activated with a mix of superantigens (1µg/ml Staphylococcus SEE, 2µg/ml SEA, 2µg/ml SEB). The mixtures were cultured in RPMI-1640 supplemented with 10% FCS at 37°C in the atmosphere of 5% CO₂/95% air over a week. For each day the 96well plate was prepared with 20 wells containing 200µl of PBMCs suspension (1x10⁶/ml).
Every day one plate with the cells were first stained with primary conjugated antibodies to detect CD4+, CD25+. For detection of CD4+ T cells I used antiCD4–FITC conjugated Abs, for CD25+ T cells the antiCD25-APC conjugated Abs were used in concentration 10µg/ml. The cells were stained for 30min (10µl/well was used). Then the cells were washed twice with 100 µl PBS (3min spun, 500rpm) and either immediately stained for SDC-4 (for surface staining) or permabilized with BD cytofix/cytoperm buffer (10µl per well) and then stained (for total staining) For SDC-4 detection the SDC-4-PE Abs were used in conc. 2µg per 1×10⁶ cells. The cells were stained for 30min and washed twice with PBS as previously. Measurement was done using a FACS Calibur flow cytometer. In each measurement 50000 cells were acquired. All the data were analysed using FlowJo flow cytometry software.

2.2.8.3 Proximal signalling analysis in conjugates

Conjugates were prepared the same way as in section 2.2.7.3.1 but varying the time of incubation at 37°C, 2, 4 and 8 minutes. Conjugates were prepared with Raji B cells pre-loaded with SEE or not (for control). After the incubation and brief spin, 100µl of Cytoperm/Cytofix solution was added, incubated for 20min at 8°C, washed with TBS and stained with 4G10 Abs against proteins phosphorylated on tyrosine for 30min at RT. Then the cells were washed twice with TBS and secondary antibodies (anti mouse IgG2a Alexa555, Invitrogen) were applied for 30 min. After that the cells were washed once again and the measurement was taken.
3 Results

3.1 Detection of endogenous SDC-4

SDC-4 has been reported to be expressed by resting and activated human T cells at different levels [142]. I verified the expression of SDC-4 in these cells as well as in Jurkat T cells, which I planned to use as model cells. Flow cytometry using commercial antibodies which were reported in similar studies [142, 165, 166] was employed for these experiments.

In flow cytometry, the analysis is performed by passing hundreds of fluorescently-labelled cells per second through a laser beam and capturing the light that emerges from each cell as it passes through (Figure 3.1). As a cell passes through the laser beam, it refracts or scatters the light at all angles. Forward scatter counts (FSC) represent the amount of light that is scattered in the forward direction and is roughly proportional to the size of the cell. Light scattering measured from the side (SSC) is caused by the granularity and structural complexity inside the cell. The side-scattered light is focused through a lens system and collected by a detector usually located 90 degrees from the laser’s path. The fluorescent light emitted by labelled cells travels along the same path as the SSC signal and is directed through a series of filters and mirrors, so that particular wavelength ranges are delivered to the appropriate detectors [167]. The obtained data can be plotted on two-dimensional dot plots or simple histograms and processed by analytical flow cytometry software (e.g. FlowJo, CellQuest, WinMDI). Regions on these plots can be sequentially separated, based on specific criteria, by creating a series of subset extractions, termed "gates." The gating is frequently done based on the levels of specific markers (e.g. anti-CD4 or anti-CD25 antibodies for T cells) which are fluorescently labelled and specifically bind cells.
Figure 3.1. A. Basic principle of the flow cytometry; B. Cell analysis using specific markers. Figure from www.microporator.com [168, 169].
3.1.1 Detection SDC-4 with 5G9 antibodies by flow cytometry

First, I examined the expression of SDC-4 in resting (naïve) and activated primary human T cells over a period of 5 days using flow cytometry and 5G9, an anti-human SDC-4 antibody (Santa Cruz Biotechnologies, Santa Cruz, USA), the most frequently used tool for SDC-4 expression analysis found in the literature [142, 143, 165, 166]. A multicellular population of peripheral blood mononuclear cells (PBMCs) was used for T cell activation and expression profile experiments. For activation, PBMCs and a mixture of superantigens (SEA, SEB, SEE) were incubated in a 96-well U-bottom plate to best mimic the physiological situation where T cells are crowded in a limited space of the lymph nodes.

In order to distinguish T cells within the mixed population, PBMCs were stained with fluorescently-labelled anti-CD4 (FITC-conjugated), a marker of T cell subpopulation involved in humoral immune response, and anti-CD25 (APC-conjugated) for the selection of activated T cells. The expression of SDC-4 was detected using the 5G9 anti-SDC-4 antibody (also directly PE-conjugated Figure 3.2)

**CD4 and CD25**

*CD4* is a co-receptor of T cell receptor (TCR) and it helps TCR during the activation of T cell following an interaction with APC. It directly interacts with MHC class II molecules on the surface of the APCs via its extracellular domain.

*CD25* (IL2Ra chain) expression is upregulated during the activation of T cell after the contact with APC presenting antigens or superantigens (see for more details Introduction).
Figure 3.2. Dot plots illustrating the selection of stimulated T cells after flow cytometry analysis. The individual dot plots depict: the size (FSC) and granularity (SSC) of the cells (A), specific selection of T cells by gating for the CD4-positive cells (B) and the gating for the CD25-positive activated CD4 T cells (C). The histogram shows the SDC-4 expression of activated T cells, control corresponds to unstained activated T cells (D). The presented data are for the fourth day of PBMCs stimulation.
Staining for SDC-4 was performed before or after fixation/permeabilisation of cells to acquire data for the surface as well as the total (including intracellular and surface) expression, respectively (Figure 3.2). I followed the expression profile of SDC-4 for stimulated T cells over 5 days (Figure 3.3). The results indicate the increase of the SDC-4 expression level in course of time. In general, a higher SDC-4 expression was detected in activated T cells compared to their resting counterparts (ex vivo). At the surface protein level, SDC-4 expression was only slightly increased over the tested period (Figure 3.4 A) but the total expression increased more dramatically and reached the highest level approximately on day four (Figure 3.4 B).
Figure 3.3. Histograms of A surface and B total expression of SDC-4 in T cells stimulated (or not) for the indicated period of time – day 0 (ex vivo), day 1 and day 4. The PBMCs were stimulated with a mixture of superantigens and stained with antibodies against CD4 and CD25 for the identification of activated T cells and PE-labelled 5G9 antibody for SDC-4 detection. Control corresponds to cells unstained with 5G9-PE antibody on day 0. The histograms are representative of 10 experiments. 50000 cells were analysed per measurement.
Figure 3.4. Surface and total expression of SDC-4 in stimulated primary human T cells. The PBMCs were stimulated with a mixture of superantigens and stained with anti-CD4 FITC antibodies against CD4 and anti-CD25-APC against CD25 for the identification of activated T cells. antiSDC-4-PE antibody (5G9-PE) were used to detect SDC-4. Control corresponds to cells not labelled with anti-SDC-4-PE antibodies. The graphs are representative of 10 experiments. 50000 cells were acquired per measurement.

The data presented on Figure 3.4 represent one of 10 experiments. Due to variation in results between the experiments I am not able to conclude on which day syndecans expression is the highest. It has been mentioned by Ariizumi group that they also noticed differences in SDC-4 expression levels and time of the highest expression in the cells coming from different donors [141]. The reason for that can be that some donors already has quite a high level of activated T cells (e.g. after having cold).
The expression level of SDC-4 was also examined in Jurkat T cells using 5G9 and ab24511 (rabbit anti-hSDC4 –intracellular domain) antibodies and flow cytometry since these cells were used as model T cells in my project. Results presented in Figure 3.5 and 3.6 indicate very low surface but detectable intracellular expression of SDC-4 in Jurkat T cells.

**Figure 3.5.** Histograms of A) surface and B) total expression of SDC-4 in Jurkat T cells stained with PE-labelled 5G9 (anti-SDC-4) antibody. Control corresponds to unstained cells.

**Figure 3.6.** Histograms of total expression of SDC-4 in Jurkat T cells stained with antibody (against intracellular part of SDC-4) ab24511 (anti-SDC-4 rabbit polyclonal, Abcam) and with secondary anti-rabbit-Alexa555 antibodies. Control corresponds to the cells stained with secondary antibody only.
3.1.2 Detection of SDC-4 with RT-PCR

To check presence of SDC-4 I also performed RT-PCR. The PBMCs were isolated from blood and incubated with a mixture of superantigens. On days 0, 2 and 4 the samples were collected, the T cells were isolated, RNA prepared and RT-PCR was performed on day 4. The quality of RNA was checked on agarose gel and concentration was measured on Nanodrop.

The results in Figure 3.7 show that there is SDC-4 weakly expressed in primary T cells on day 4. Robust SDC-4 expression was detected in Jurkat and positive control MB-MDA-231 breast carcinoma cells. GAPDH was used as a positive control transcript for successful RT-PCR amplification; however, GAPDH mRNA was also undetectable on day 0 and was barely detectable on day 2. I did not detect SDC-4 on day 0 and 2 so I cannot unequivocally confirm the increase of SDC-4 level in activated T cells using this technique. Lack of results on days 0 and 2 may be due to too low amount of DNA used or lack of my experience with the technique. However, because the ex vivo T cells are quiescent and have low metabolic activity it is probable that GAPDH, an enzyme involved in glycolysis and energy metabolism, is genuinely expressed at low levels and is only subsequently induced upon cell activation, becoming detectable by day 4. This makes it very difficult, and perhaps impossible, to choose an appropriate control transcript, as most genes will be downregulated in quiescent cells.
3.1.3 Summary

The expression level of endogenous SDC-4 in human T cells is reported to be low and to increase following activation. Similarly, Jurkat T cells (my model cells) have low endogenous surface SDC-4 expression, which makes them more suitable for my model.

Studying why SDC-4 is increased over time in activated T cells can bring some new information about IS formation and its stability. Unfortunately, a model system is needed for experiments studying the effect of increased SDC-4 expression – Jurkat T cells in our case - as the level of endogenous expression of T cells is too low.

Figure 3.7. RT-PCR results for T cells day 0 (ex vivo), day 2 and day 4 of stimulated primary human T cell as well as HEK cells (negative control), MD-MBA-231 (positive control) and Jurkat T cells. GAPDH was used as a control of mRNA loading in the RT-PCR reaction.
3.2 Detection of exogenous SDC-4 in Jurkat T cells

As shown in previous section the surface SDC-4 levels are very low in tested cells. Therefore, exogenous expression in a model system, Jurkat T cells, has been applied for further studies focused on the SDC-4 impact on the IS formation and stability.

3.2.1 Jurkat T cell transfection optimisation

Optimalisation of the transfection was essential step as numerous protocols for Jurkat T cell plasmid DNA transfection were reported in published works and on-line but only a handful were found to work outside the laboratory which presented the technique. Of those, two main systems were successfully used: viral transduction and electroporation. Since the first one requires cloning of genes into specialised vectors (plasmids) and work with viruses, I decided to focus on electroporation. Nucleofection, which refers to the electroporation technique established by AMAXA (version 2; currently Lonza, Germany), was previously successfully applied for DNA delivery to Jurkat T cells in our laboratory. However, the viability of transfected cells was low (~ 20-30%; M. Cebecauer, personal communication) even though 50-70% transfection efficiency could be achieved. Therefore, I decided to focus on a novel electroporation technology called microporation (Figure 3.8.)

![Microporation setup](image)

**Figure 3.8.** Microporator main device (1) with microporator pipette (2), pipette station (3), tips (4) and the solution kit (5). Figure from [www.microporator.com](http://www.microporator.com) [168].
Microporation is a proprietary electroporation technology developed by NanoEnTek (South Korea), now commercially available from Life Technologies/Invitrogen under the commercial name Neon™. The majority of electroporation techniques employ plate-type electrodes and cuvette style chambers for the electric shock application. Microporation is unique in using a pipette tip with a needle-shaped electrode as an electroporation space (Figure 3.9.)

![Figure 3.9](image)

**Figure 3.9.** (A) Cuvette chamber with plate-type electrodes; and (B) pipette tip with needle-shaped electrode, as used in Microporation. Figure from www.microporator.com [168].

The advantage of using microporation with the tip-shaped electrode is in the generation of a more uniform electric field which minimises heat generation, metal ion dissolution, pH variation and oxidation, all of which are harmful events to the cell (probably causing reduced viability of the nucleofected cells using AMAXA system). Improved transfection efficiency and cell survival rate are benefits which made this technique my choice.
3.2.1.1 Microporator settings optimisation

First, transfection efficiency and viability of the cells were tested for a number of settings recommended by the manufacturer, using the control pEGFP-N1 plasmid at a concentration of 1µg per well (Table 3.1). The voltage, pulse width and pulse number can be varied. Flow cytometry was used for the viability and transfection efficiency analysis (see below for details of the examination).
<table>
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<th>Pulse voltage (V)</th>
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*Table 3.1.* The instrument settings optimisation tested for transfection of Jurkat T cells using pEGFP-N1 plasmid. Transfection efficiency and viability of the cells are shown for the indicated settings. The results presented in last two columns stand for transfection efficiency: No ≤20%, 20%<Low ≤ 50%, High > 50% and viability: Poor ≤ 50% and Good > 50%.
In my study, the viability of the cells was analysed using SSC and FSC. Healthy, viable cells were gated first (Figure 3.10). In the next step, the transfected cells were analysed and compared with untransfected ones. The same gating was applied for all samples. Correct gating is based on the type of cells and experience.

**Figure 3.10.** Jurkat T cells transfected with pEGFP-N1 plasmid (1µg/well) were analysed for the transfection efficiency using selected settings of the microporator. Flow cytometry was used for cell viability (FCS/SSC) analysis. Representative dot plots are shown for untransfected and selected conditions of microporation with the highest (80%) and the lowest (10%) viability of successfully transfected Jurkat T cells. Each dot in the graph corresponds to an individual cell. 10000 cells were analysed per sample.
The transfection efficiency was measured by the fluorescent light detector and the data are presented in the form of a histogram. The efficiency of the transfection (percentage of successfully transfected Jurkat T cells) was achieved by range gating, which means that a subset of the cells, which has higher fluorescence emission than untransfected cells, are counted as positive counts (transfected) (Figure 3.11).

**Figure 3.11.** Jurkat T cells transfected with pEGFP-N1 plasmid (1µg/well) were analysed for the transfection efficiency using selected settings of the microporator. Flow cytometry was used for the cellular EGFP expression analysis. Histogram showing the level of expression of EGFP for untransfected (black line) and selected the most (red line – 72%) and least (blue line) successfully transfected Jurkat T cells. Untransfected cells were treated similarly to all transfected samples except for the electric shock for the electroporation. The transfection efficiency was based on gating by marker M1, which measures all the positively transfected (EGFP-positive) cells. The highest transfection in this experiment corresponds to settings 1400V/30msec/1pulse and the lowest transfection of Jurkat T cells corresponds to settings 1450V/30msec/1pulse presented in Table 3.1.
In summary, Figure 3.10 shows representative dot plots of the untransfected and exemplars of the most and least successfully transfected Jurkat T cells. Each dot in the graph corresponds to an individual cell. These representative results indicate that the transfection settings highly influence the viability of the cells. Figure 3.11 shows the histogram of the EGFP level of expression for the untransfected and selected most and least successfully transfected Jurkat T cells. Untransfected cells were treated similarly to all other (transfected) samples except for the electric shock for electroporation. In general, all microporator settings resulted in a reduced viability but at different levels of transfection efficiency.

The data presented in Table 3.2 summarise the results for settings selected in Table 3.1 (wells number #2, 15 and 21) and their more subtle modifications (+/- 50V of the applied pulse voltage). The results expressed in red bold again indicate the highest viability and transfection efficiency - setting 1400V/30msec/1pulse; the lowest viability and transfection – setting 1450V/30msec/1pulse are select in blue bold. The results acquired for untransfected cells are shown for comparison.

<table>
<thead>
<tr>
<th>Settings (V/msec/pulses)</th>
<th>EGFP viability (%) / transfection efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>untransfected</td>
<td>82/1</td>
</tr>
<tr>
<td>1400/20/1</td>
<td>29/45</td>
</tr>
<tr>
<td>1450/20/1</td>
<td>46/56</td>
</tr>
<tr>
<td>1500/20/1</td>
<td>54/68</td>
</tr>
<tr>
<td>1350/30/1</td>
<td>44/66</td>
</tr>
<tr>
<td><strong>1400/30/1</strong></td>
<td><strong>46/72</strong></td>
</tr>
<tr>
<td><strong>1450/30/1</strong></td>
<td><strong>17/38</strong></td>
</tr>
<tr>
<td>1350/20/2</td>
<td>41/68</td>
</tr>
<tr>
<td>1400/20/2</td>
<td>39/66</td>
</tr>
<tr>
<td>1450/20/2</td>
<td>31/63</td>
</tr>
</tbody>
</table>

**Table 3.2.** The viability and transfection efficiency was determined for Jurkat T cells transfected with pEGFP-N1 plasmid using indicated microporator settings. First column corresponds to the individual setting (pulse voltage (V)/pulse width (msec)/pulse number). Second column represents the viability (%) / transfection efficiency (%) for every setting tested.
By fine tuning of the instrument settings I managed to optimise the conditions for 1400V-pulse voltage, 30msec-pulse width, 1 pulse which was my choice at this stage of the work as I achieved results of ~70% transfection efficiency and a approximately 50% viable Jurkat T cells using these values for microporation.

At a later stage of the project, Invitrogen acquired the system from the microporator inventor, performed intense evaluation of the technique and suggested some critical improvements for customers, and have now launched the technology to the world-wide market under the name, "Neon™ Transfection System". I therefore decided to set up a new optimisation experiment using settings based on my previous experience and compared these to the values recommended by Invitrogen researchers. A fine tuning (increase and decrease in voltage by 25V) was employed during this optimisation step. Again, the EGFP encoding plasmid was employed for the transfection optimisation. Results are presented in Table 3.3.

<table>
<thead>
<tr>
<th>Settings (V/msec/pulses)</th>
<th>EGFP viability (%) / transfection efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>93/2</td>
</tr>
<tr>
<td>1375/30/1</td>
<td>90/40</td>
</tr>
<tr>
<td><strong>1400/30/1</strong></td>
<td><strong>90/55</strong></td>
</tr>
<tr>
<td>1425/30/1</td>
<td>84/40</td>
</tr>
<tr>
<td>1300/10/3</td>
<td>94/21</td>
</tr>
<tr>
<td>1325/10/3</td>
<td>94/21</td>
</tr>
<tr>
<td>1350/10/3</td>
<td>94/24</td>
</tr>
<tr>
<td>1375/10/3</td>
<td>95/37</td>
</tr>
<tr>
<td><strong>1400/10/3</strong></td>
<td><strong>95/68</strong></td>
</tr>
<tr>
<td>1425/10/3</td>
<td>94/35</td>
</tr>
</tbody>
</table>

*Table 3.3. The viability and transfection efficiency was determined for Jurkat T cells transfected with the pEGFP-N1 plasmid (at concentration 1µg per well) using my previously established optimal settings-1400V/30msec/1pulse and the ones suggested by Invitrogen (1400V/10msec/3pulses). I achieved the best results - shown in bold red.*

Applying the Invitrogen-suggested settings (1400V/10msec/3 pulses), a better viability (95%) and good transfection efficiency (68%) was achieved. Therefore, these settings were applied for the following experiments.
3.2.1.2 Plasmid DNA concentration optimisation

3.2.1.2.1 EGFP- and rat SDC-4-encoding plasmids

In addition to the instrument settings, DNA plasmid concentration is a sensitive parameter influencing the transfection efficiency and cell viability after electroporation. I used pEGFP-N1 as a standard testing DNA but used a plasmid encoding HA-tagged rat (r) SDC-4 in the concentration set up analysis due to uneven behaviour of different plasmids in such technique. The EGFP expression level was again used for the transfection efficiency determination since the pHA-rSDC-4-IRES-EGFP plasmid encodes enhanced green fluorescent protein (EGFP) downstream of the internal ribosomal entry site (IRES). This EGFP fluorescence was also used for the regular testing of the transfection efficiency using flow cytometry or the identification of transfected Jurkat T cells in microscopy.

In next step, I optimised the concentration for each plasmid separately to make the transfection efficiency and viability for both plasmids as similar as possible. I compared the transfection efficiency of pHA-rSDC-4-IRES-EGFP plasmid with our standard pEGFP-N1 plasmid in Jurkat T cells. As shown in the Table 3.4, the EGFP expression efficiency is significantly higher for the control pEGFP-N1 plasmid compared to pHA-rSDC-4-IRES-EGFP. The transfection efficiency was optimised based on the EGFP expression, which was higher in case of pEGFP-N1 plasmid (~4700 base pairs), possibly as it is smaller than pHA-rSDC-4-IRES-EGFP (~6300 bp) plasmid.

**Internal ribosomal entry site (IRES)**

An internal ribosome entry site (IRES), is a nucleotide sequence that allows for translation initiation in the middle of a messenger RNA (mRNA) sequence as a part of the complex process of protein synthesis.
Table 3.4. The viability and transfection efficiency were determined for Jurkat T cells transfected with either pHA-rSDC-4-IRES-EGFP plasmid or pEGFP-N1 plasmid (both at a concentration of 1µg per well) using settings 1400V/30msec/1pulse.

Later, Jurkat T cells were transfected either with pHA-rSDC-4-IRES-EGFP plasmid or pEGFP-N1 plasmid using two different concentrations based on previous results. Applied microporator settings were 1400V/30msec/1pulse since these were the selected best conditions at this stage of the project.

Table 3.5 shows the results for Jurkat T cells transfected either with pHA-rSDC-4-IRES-EGFP plasmid in concentrations 1µg/well and 4µg/well, or pEGFP-N1 used in concentrations: 0.1µg/well and 0.05µg/well. Using the same settings of the microporator for both plasmids results in very different expression efficiency, therefore the pEGFP-N1 plasmid concentration was lowered in order to get similar transfection efficiency for both plasmids.
Table 3.5. The viability and transfection efficiency was determined for Jurkat T cells transfected with pHA-rSDC-4-IRES-EGFP or pEGFP-N1 plasmids using indicated concentrations and my previously determined optimal settings 1400V/30msec/1pulse. The data in bold red indicate the most comparable results of the transfection efficiency and viability for both plasmids.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>pHA-rSDC-4-IRES-EGFP viability (%) / transfection (%)</th>
<th>pEGFP-N1 viability (%) / transfection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>88/1</td>
<td>87/1</td>
</tr>
<tr>
<td>1 µg/well</td>
<td>31/38</td>
<td></td>
</tr>
<tr>
<td>4 µg/well</td>
<td>40/42</td>
<td></td>
</tr>
<tr>
<td>0.10 µg/well</td>
<td>48/32</td>
<td></td>
</tr>
<tr>
<td>0.05 µg/well</td>
<td>44/20</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.12 presents the viability (A) dot plots and transfection efficiency (B) histogram for the results achieved using optimised concentration (Table 3.5) of plasmids and the previously optimized microporator settings 1400V/30msec/1pulse.
Figure 3.12. (A) Representative dot plots with indicated viability (%) for Jurkat T cells, untransfected and transfected with pEGFP-N1 or pHA-SDC-4-IRES-EGFP plasmids using selected 1400V/30msec/1pulse condition of microporation. Each dot in the graph corresponds to an individual cell. 10000 cells were analysed per measurement. (B) Histogram showing the results achieved with optimised concentration of plasmids in order to attain as similar as possible level of expression of EGFP for both plasmids. Positive expression was determined based on the EGFP expression encoded by the plasmids (marker M1).
Plasmid concentrations were optimised for 1400V/30msec/1pulse since these were the selected best settings at this stage of the project. The most similar expression efficiency was achieved using 4 µg/well of pHA-rSDC-4-IRES-EGFP and 0.1µg/well of pEGFP-N1 plasmid and the above mentioned microporator settings.

After getting better results of the viability and transfection with pEGFP-N1 plasmid for the Invitrogen suggested settings (Section 3.2.1.1), the previously optimised plasmid concentrations were tested using the settings of 1400V/10msec/3pulses. The results are presented in Table 3.6.

<table>
<thead>
<tr>
<th>Settings</th>
<th>pHA-rSDC-4-IRES-EGFP viability (%)/ transfection (%)</th>
<th>pEGFP-N1 viability (%)/ transfection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1400/10/3</td>
<td>81/63</td>
<td>79/78</td>
</tr>
</tbody>
</table>

Table 3.6. The viability and transfection efficiency was determined for Jurkat T cells transfected with pHA-rSDC-4-IRES-EGFP or pEGFP-N1 plasmid using concentrations optimised earlier in the project: 4µg/well of HA-rSDC-4-IRES-EGFP and 0.1µg/well of pEGFP-N1; and Invitrogen-suggested and optimised settings of 1400V/10msec/3pulses.

3.2.1.2.2 The plasmid encoding human SDC-4

In a later stage of the project, I received a plasmid encoding HA-tagged human (h) SDC-4 in the pMEXneo vector (pMEXneo-HA-hSDC-4). This vector does not encode EGFP. Therefore, anti-HA tag antibody (HA.11 from Covance) was used for the immunofluorescent detection of the exogenous protein in transfected Jurkat T cells. As for the rSDC-4-encoding plasmid, DNA concentration optimisation of the transfection efficiency was performed for pMEXneo-HA-hSDC-4 plasmid (data not shown).
I required EGFP expression driven from the same plasmid for the subsequent experiments. Therefore, the insert (DNA fragment encoding HA-tagged hSDC-4) was subcloned into the pIRES-EGFP vector (kindly performed by Dr. Marek Cebecauer). This plasmid was used in the functional studies (such as live cell imaging) as I was more interested in the effect of hSDC-4 in human T cells. I was, therefore, able to base the transfection efficiency control on the EGFP expression. Also, at that point of the project I changed control plasmid from pEGFP-N1 to pIRES2-EGFP to apply more similar vector for the functional assays. Both of these plasmids encode the same protein EGFP, but pIRES2-EGFP is expressing from the IRES, the same way as the plasmids encoding human (and rat) SDC-4. I used Invitrogen-suggested settings of 1400V/10msec/3 pulses and the concentration of 4µg/µl of pHA-hSDC-4-IRES-EGFP and 0.1µg/µl of pIRES2-EGFP. I got similar expression and viability levels using these conditions (presented in Figure 3.13). These conditions were used for all following experiments.

Figure 3.13. Histogram showing the level of expression of EGFP from the pHA-hSDC-4-IRES-EGFP and control pIRES2-EGFP plasmids in Jurkat T cells using microporator settings of 1400V/10 msec/3 pulses, and concentration of 4µg/well pHA-hSDC-4-IRES-EGFP and 0.1µg/well pIRES2-EGFP.
3.3 Cellular localisation of SDC-4

3.3.1 Using 5G9 antibody

The next step of my study was to determine the spatial localisation of SDC-4 in T cells. First, I used 5G9 antibody (Santa Cruz Biotechnologies, Santa Cruz, USA) recognising an epitope in the extracellular part of SDC-4 known from the literature [148, 166]. I tested the antibody on cells which are known to express high levels of SDC-4 – human breast cancer cells MD-MBA-231 (positive control), and on cells which were reported to express minimal levels of SDC-4 - human embryonic kidney 293 cells (HEK293; negative control) (Figure 3.14).

**Figure 3.14.** Images of MD-MBA-231 (positive control) and HEK293 cells (negative control) stained with 5G9 antibody against the extracellular part of SDC-4 (cyan) followed by secondary Abs mouse anti-IgG2a Alexa 555 and with phalloidin Alexa 647 (for fibrillar actin; magenta). Brightfield (BF) shows the shape of cells.
Intense spot-like signals were observed on images of positive control cells (MD-MBA-231), which can correspond to the accumulation of SDC-4 in focal adhesions in agreement with previously published data [4]. The dramatic difference between positive and negative control cells (Figure 3.14) indicated that this antibody can be applied for the immunofluorescence studies performed on conjugates formed between Jurkat T and Raji B cells (functioning here as APCs) (Figure 3.15). Conjugation takes place after mixing Raji B cells (loaded in advance with superantigens) with Jurkat T cells and incubation at 37°C for 5 minutes.
Figure 3.15. Images of conjugates formed by Raji B cell and Jurkat T cells transfected with A) pHA-rSDC-4- IRES-EGFP or B) control pIRES2-EGFP plasmid and stained with 5G9 antibodies against the extracellular part of SDC-4, followed by secondary Abs mouse anti-IgG2a Alexa633 (cyan). Typical Lck localisation (cells stained with anti-Lck abs, followed by secondary mouse anti-IgG2aAbs) indicates the formation of productive IS (magenta). GFP indicates the transfection efficiency in Jurkat T cells. Brightfield (BF) shows the shape of cells. Example results of 20 conjugates analysed for each transfection.
The observed results do not detect any presence of SDC-4 in the conjugating cells. One possible reason why there was no signal detected is because the 5G9 antibody was not sensitive enough for this technique.

### 3.3.2 Using ab24511 antibody

The ab24511, a commercially (Abcam, Cambridge, UK) available rabbit polyclonal antibody, was generated against a peptide derived from the intracellular part of human SDC-4. Again, I tested ab24511 first on cells which were reported to express, or not, high levels of SDC-4, MD-MBA-231 and HEK293 respectively (Figure 3.16). My results exhibited similar distribution of SDC-4 in positive cells (MD-MBA-231) to the one which was previously observed using 5G9 antibody, but with reduced background. Negative control indicates the specificity of the staining. In order to better test the specificity of the ab24511 antibody, I decided to transflect HEK293 with HA-rSDC-4-encoding plasmid (Figure 3.17).

![Images of MD-MBA-231 (positive control) and HEK293 (negative control) cells labelled with ab24511 antibody against the intracellular part of SDC-4 followed by staining with secondary Ab anti-rabbit-Alexa555 (cyan) and with phalloidin-Alexa647 for fibrillar actin; (magenta staining). BF shows the cells in bright field.](image)

**Figure 3.16.** Images of MD-MBA-231 (positive control) and HEK293 (negative control) cells labelled with ab24511 antibody against the intracellular part of SDC-4 followed by staining with secondary Ab anti-rabbit-Alexa555 (cyan) and with phalloidin-Alexa647 for fibrillar actin; (magenta staining). BF shows the cells in bright field.
Figure 3.17. HEK293 cells transfected with pHA-rSDC-4-IRES-EGFP plasmid to check the selectivity of ab24511 antibody for SDC-4 (followed by secondary anti-rabbit-Alexa 555 antibodies) (B) (cyan). The phalloidin Alexa 647 staining of actin fibres (A)(magenta). Transfection efficiency control – EGFP (C), Mergegd image (D) of A and B. BF – cells in the bright field (E). Exo SDC-4 = exogenous SDC-4.

The results suggest high specificity of the ab24511 antibody for SDC-4 since the intense signal was detected only in the cells transfected with the pHA-rSDC-4-IRES-EGFP plasmid. These cells were determined based on the GFP fluorescence, which was expressed from the IRES of the plasmid.

Next, these antibodies were used for the analysis of the SDC-4 localisation in conjugates formed between Jurkat T cells, transfected with the pHA-rSDC-4-IRES-EGFP or pIRES2-EGFP plasmids, and APC (Raji cells) (Figure 3.18).
Figure 3.18. Images of conjugates formed between: (A) Jurkat T cells transfected with pHA-rSDC-4-IRES-EGFP or (B) pIRES2-EGFP plasmid, and Raji B cells – stained with ab24511 antibody against the intracellular part of SDC-4 and secondary anti-rabbit-Alexa 555 antibodies. GFP serves to control for expression level. BF shows the cells in bright field.

Non-homogeneous fluorescence signal (spots) for SDC-4 staining in tested cells shows similar pattern to the results which were achieved for the positive control represented by MD-MBA-231 cells (Figure 3.14 and 3.16) or HEK293 cells transfected with pHA-rSDC-4-IRES-EGFP (Figure 3.17). More intense signal for SDC-4 was observed for Jurkat T cells transfected with pHA-rSDC-4-IRES-EGFP (Figure 3.18A) in comparison to Jurkat T cells transfected with pIRES2-EGFP (Figure 3.18B). The strong accumulation of SDC-4 in the pHA-rSDC-4-pIRES-EGFP-transfected T cell indicates the presence of SDC-4 in Jurkat T cells. Unfortunately, there was strong signal detected in Raji cells conjugating with the transfected and untransfected Jurkat T cells. The high level of endogenous SDC-4 expression in Raji cells made it impossible to specifically localise SDC-4 in Jurkat-Raji conjugates. Therefore, I looked for another strategy of exogenous SDC-4 localisation in Jurkat T cells.
3.3.3 Using HA tag antibody

I took the advantage of the presence of a short peptide tag in the constructs encoding SDC-4 (e.g. pHA-rSDC-4-IRES-EGFP) and employed antibody against HA tag (HA.11, Covance) for the localisation of the exogenous SDC-4 in Jurkat T cells) (Figure 3.20). The addition of the HA tag to the protein of interest allows the detection of SDC-4 with an antibody against the HA sequence using immunofluorescence methods.

Later in the project, I acquired the human (h) HA-SDC-4 protein-encoding plasmid (pMEXneo-HA-hSDC-4) (a kind gift from Prof. John Couchman’s lab, University of Copenhagen) and I successfully generated Jurkat T cells stably transfected with human pHA-SDC-4. For detection of the positively transfected cells I used Abs against HA tag (Figure 3.19) and Flow cytometry technique first. Stable over-expression of SDC-4 unfortunately resulted in the down-regulation of the surface TCRs so these cells were not suitable for my subsequent purposes. No change in TCR surface expression was observed in Jurkat T cells transiently transfected with plasmid encoding human SDC-4 (Figure 3.19C).
Figure 3.19. CD3 downregulation in Jurkat T cells stably transfected with h-SDC-4. (A) gating for living cells; (B) gating for SDC-4 positive Jurkat T cells – the gating was done based on HA tag presence (detected with HA1.1 Abs and secondary anti murine IgG1-Alexa488; (C) histogram of CD3 detection in stably and transiently transfected Jurkat T cells with pHA-hSDC-4-IRES-EGFP. The cells were stained with OKT3 Abs and anti-murine IgG2a-Alexa555. Control histogram for transiently transfected Jurkat stained with secondary Abs only overlapped the histogram result for stably transfected stained Jurkat T cell.
In next step the insert (DNA fragment encoding HA-hSDC-4) was subcloned into pIRES-EGFP vector (kindly performed by Marek Cebecauer). Thus, in the next step, I transfected Jurkat T cells with pHA-hSDC-4-IRES-EGFP and pIRES2-EGFP plasmids, and stained the cells with antibody against HA tag for immunofluorescence microscopy (Figure 3.20). These results for the first time demonstrate exogenous SDC-4 localisation in (Jurkat) T cells. SDC-4 was accumulated in the plasma membrane and also in vesicles in the cell. Also, SDC-4 was not distributed equally throughout the whole plasma membrane, but rather accumulated in certain areas of this structure.

**Figure 3.20.** Images of Jurkat T cells transfected with (A) pHA-hSDC-4-IRES-EGFP or (B) pIRES2-EGFP plasmids and stained with the antibody against HA tag (HA.11) present on the N-terminus of SDC-4 and secondary anti-mlgG1-Alexa633 Ab. GFP images show the expression level. BF images show the cells in bright field. Representative image of 10 cells from 2 experiments is shown.
I also employed the antibody against the HA tag to localise SDC-4 in the conjugates formed between transfected Jurkat T cells and Raji B cells (APC) (Figure 3.21A and B). The results display relatively intense and specific signal for HA tag in the conjugates formed between Jurkat T cells transfected with pHA-hSDC-4-IRES-EGFP plasmid and Raji cells loaded with SEE. Only a very weak signal for HA tag was detected in Jurkat T cells transfected with pIRES2-EGFP plasmid conjugated with Raji B cells loaded with SEE. These data demonstrated the accumulation of SDC-4 in perinuclear membranous area of the cell and near the contact site (the IS) of the conjugating cells. This is in good agreement with the results achieved when using ab24511. Thus, the immunofluorescence microscopy allowed me to confirm the specificity of the antibodies and the correlation of the HA tag and SDC-4 antibody signals within Jurkat T cells. Such results also allowed me to use these antibodies for the co-localisation studies (the following section).

Figure 3.21. Representative images of HA-tagged hSDC-4 in conjugates formed between Raji B cells (APC) and Jurkat T cells transfected with A) pHA-hSDC4-IRES-EGFP and B) pIRES2-EGFP and stained with the antibody against HA tag (HA.11) present on the N-terminus of SDC-4 and secondary anti-mlgG1-Alexa633 Ab. GFP shows the expression level. BF shows the cells in bright field. Representative image of 20 conjugates from 3 experiments is shown.
3.3.4 Summary

In this section I presented the approaches used to demonstrate SDC-4 localisation in unconjugated and conjugated Jurkat T cells. The data were acquired using untransfected cells, as well as those transfected with rat or human SDC-4 fused to HA tag and cloned into pIRES-EGFP plasmid. The lack of good direct antibodies against SDC-4 made it more difficult to determine the exact localisation of the native protein.

The first antibody tested – 5G9 from Santa Cruz Biotechnologies, Ltd. - exhibited poor specificity. The other antibody tested – ab24511 from AbCam, Ltd. – gave specific results but, unfortunately, intense signal for endogenous SDC-4 expressed in Raji B cells (functioning as APC in my assays) was also detected. This disabled the spatial detection of endogenous SDC-4 in T cells, the cells of my primary interest. In my last approach, the antibody against HA tag, Covance, Ltd. (genetically fused to the N-terminus of rat and human SDC-4) allowed me to demonstrate more precisely the localisation of SDC-4 within unconjugated and conjugated transfected Jurkat T cells.

No conclusive SDC-4 localisation, but rather highly varying pattern, has been detected in conjugates formed between Jurkat T and superantigen-loaded Raji B cells (APCs). SDC-4 in transfected T cells was detected in the plasma membrane, intracellular vesicles, uropod or perinuclear membraneous recycling compartment as presented in my work.
3.4 Co-localisation of SDC-4 in human T cells

After studies on SDC-4 localisation I hypothesised that the presence of SDC-4 may not be random and may have an impact on conjugates formation, as well as cellular function and stability of the IS. In the next step I tried to determine the localisation of SDC-4 in relation to the receptors and signalling molecules which are either known to interact with the SDC-4 molecule, such as fibronectin, or potentially are involved in T cell activation (TCR/CD3 complex and proteins phosphorylated on tyrosine(s) - results shown in next section).
3.4.1 With TCR/CD3 complex

The results from the localisation of SDC-4 show different accumulation of this molecule in T cells. The prevalent pattern was the confinement to the perinuclear recycling membranous compartment, which is situated just below the contact side of the T cell and APC in the productive synapses. TCR/CD3 complex and Lck kinase were shown previously to accumulate in the IS but also in this compartment during or after the activation of T cells [93]. Therefore, I first tested the co-localisation of the SDC-4 with TCR/CD3 receptors.

I transfected the T cells with pHA-hSDC-4-IRES-EGFP plasmid and formed conjugates with Raji B cells pre-loaded with superantigen. Then I performed immunostaining with OKT3 antibody against CD3ε chain (description in the Introduction section) and HA.11 against HA tag to localise SDC-4. Results are presented in Figure 3.22. The results showed no co-localisation for SDC-4 and TCR/CD3 complex.

Figure 3.22. Representative image of one out of 20 conjugates formed by Jurkat T cells transfected with pHA-hSDC-4-IRES-EGFP and SAg-loaded Raji B cells, stained with antibodies against CD3ε chain (secondary Abs mlgG2a-Alexa555(magenta)) and HA tag for the SDC-4 localisation (secondary Abs mlgG1-Alexa633 (cyan). GFP (green) shows the level of transfection. Example result of 20 conjugates analysed from 3 different experiments.
3.4.2 Proteins phosphorylated on tyrosine

In next step, I checked the co-localisation of SDC-4 with signal coming from the proteins phosphorylated on tyrosine(s). Tyrosine phosphorylation is one of the earliest events in the T cell activation and is involved in the initiation the signalling cascades. Jurkat T cells transfected with pHA-hSDC-4-IRES-EGFP plasmid formed conjugates with Raji B cells and were stained with 4G10 (Upstate Biotechnology) antibody against phosphorylated tyrosine (pY) and HA.11 antibody against HA tag to localise SDC-4. Results are presented in Figure 3.23. They showed little (probably accidental) or no co-localisation for SDC-4 and proteins phosphorylated on tyrosine(s).

Figure 3.23. The images of the representative conjugate (1 out of 20 analysed) formed of Jurkat T cells transfected with pHA-hSDC-4-pIRES-EGFP and Raji B cells, and stained with 4G10 antibody against phosphorylated tyrosine(s)(secondary mlG2a-Alexa555) (cyan) and HA tag for the SDC-4 localisation (secondary mlG1-Alexa633) (magenta). GFP (green) shows the level of transfection. Example result of 20 conjugates analysed from 3 different experiments.
3.4.3 Fibronectin

In addition to the molecules involved in T cell activation I tested whether SDC-4 can interact or co-localise with its well documented binding partner, fibronectin. Since no data were available about fibronectin and T cells, I was interested in the presence of this extracellular matrix molecule in the IS or at the surface of T cells and APCs. I first checked the specificity of the rabbit antiserum against fibronectin (kind gift from Prof. John Couchman). I used MRC5 human skin fibroblast cells as a positive control and MD-MBA-231 cells as a negative control for the immunofluorescence studies of the fibronectin (Figure 3.24). The typical fibrillar fibronectin staining localised at attachment sides of the actin fibres (stained with phalloidin) to the surface of the coverslip. The difference in signal between positive and negative samples confirmed the specificity of the antiserum against fibronectin.
Figure 3.24. Images of (A) MRC5 (positive control) and (B) MD-MBA-231 cells (negative control) stained with antibodies against fibronectin (cyan), (secondary anti-rabbit-Alexa555) and phalloidin- Alexa647 (fibrillar actin staining; magenta).
After confirming that the antibodies are specific to fibronectin, I investigated the presence of fibronectin in conjugates formed between Jurkat T cells and Raji B cells (APC) (Figure 3.25). Immunofluorescence results demonstrated that there is no detectable fibronectin present in the IS or at the surface of Jurkat T and Raji B cells, despite the cells being grown in fibronectin-rich serum.

**Figure 3.25.** Representative images of the conjugate formed between a Jurkat T cell and a Raji B cell (APC) (preloaded with superantigen; SEE) labelled with the antiserum against fibronectin (secondary antibodies anti-rabbit-Alexa555) (cyan) and Lck 3A5 (secondary anti-mIgG2b-Alexa633) (magenta). BF shows the cells in the bright field.
3.4.4 Summary

The results showed no co-localisation between SDC-4 and TCR/CD3 complex or proteins phosphorylated tyrosine(s) in conjugated Jurkat T cells and Raji B cells. Such results do not support SDC-4 having a direct role in the TCR signalling, but rather it may play a role in the regulation of T cell properties (most probably adhesion), similar to its function demonstrated in motile fibroblasts. I did not detect any presence of fibronectin, the known ligand of SDC-4, in the IS or at the surface of T cells and APCs. Nevertheless, I observed anecdotally that Jurkat T cells transfected with SDC-4 appeared to be less likely to form conjugates with Raji B cells loaded with superantigen). Thus, in the next step, I decided to quantitate the number of conjugates formed by Jurkat T cells transfected with either pHA-rSDC-4-IRES-EGFP or pIRES2-EGFP and Raji B cells pre-loaded or not with superantigen.

3.5 Conjugate formation analysis

3.5.1 Using dual-cell flow cytometry

The dual-cell flow cytometry technique was employed for a quantitative analysis of T cells forming conjugates with antigen-presenting cells (APCs). Flow cytometry application for this kind of analysis is rarely used as it requires expensive equipment and careful control of conditions.

Using two cell trackers with different fluorescence properties I demonstrated the formation of conjugates between Jurkat T and Raji B cells (APCs) (Figure 3.26). In this experiment I labelled Jurkat T cells with Green cell tracker (excitation-492nm/emission-517nm) and Raji B cells with Orange cell tracker (excitation-541nm/emission-565nm). Raji cells were loaded with SEE or not, then Jurkat and Raji cells were mixed, allowed to interact by mild centrifugation for 1 min, conjugated by incubation for 5 min at 37°C and immediately analysed using flow cytometry (details in Materials and Methods section). The cells were kept at approximately 37°C throughout the whole measurement. The results were quantitated using flow cytometry analytical software FlowJo (Tree Star, North America). (Figure 3.26).
Using dual-cell flow cytometry, I successfully demonstrated the formation of conjugates between Jurkat T and SAg-loaded Raji B cells (APCs). I assumed that the number of T-B conjugates reflects the frequency of synapse formation between T cells and APCs under the tested conditions. On the other hand, very similar numbers of conjugates formed in the presence and absence of superantigen showed that conjugates are independently formed and are probably more likely to be formed by the cells which were briefly centrifuged (in order to help conjugates formation – see Discussion for more details).

Since I aimed to study the influence of SDC-4 on the T cell activation process, in the next step, Jurkat T cells were transiently transfected with pHA-rSDC-4-IRES-EGFP (label: Jurkat SDC-4 T cells) or pIRES2-EGFP (label: Jurkat EGFP T cells) plasmids, Raji B cells were labelled with Orange cell tracker and the experiment was repeated using the same conditions as for the untransfected cells. Here, the GFP fluorescence encoded into the plasmid was used instead of Green cell tracker, used in set-up experiments, for the identification of Jurkat T cells - Figure 3.27).
The experiment was repeated three times and the quantitation is presented in Figure 3.28. I observed only a minor decrease in number of conjugates formed by Jurkat T cells transiently transfected to overexpress SDC-4 compared to T cells transfected with EGFP only. Interestingly, slight decrease in conjugates formation was observed in the presence, but also in the absence, of the superantigen on the APCs.

Moreover, the number of conjugates formed between transfected Jurkat T cells and Raji B cells was lower in comparison to the conjugates formed by untransfected T cells and Raji B cells. The possible explanation for this phenomenon is that this may be a side effect of the microporation which, similar to the other electroporation techniques, influences the cell membranes.
Figure 3.27. Illustrative dot-plots showing A) a gating for unconjugated transfected Jurkat T cells (bottom right corner), Raji B cells (loaded with Sag) (top left corner) and untransfected Jurkat T cells (left bottom corner) as well as conjugated transfected Jurkat (either with SDC-4 or EGFP only)/Raji cells (top right corner); and for B) Jurkat T cells transfected with pHA-rSDC-4-IRES-EGFP and Raji B cells and C) Jurkat T cells transfected with pIRES2-EGFP and Raji B cells with quantitated number of conjugates (indicated in red). The quantisation was performed using FlowJo software. 50000 cells were acquired for each conditions.
Figure 3.28. Quantitation of the dual-cell flow cytometry analysis of the conjugates formed by: Jurkat T and Raji B cells (J/R); Jurkat T cells transfected with pIRES2-EGFP and Raji B cells (JEGFP/R) and Jurkat T cells transfected with pHA-rSDC-4-IRES-EGFP and Raji B cells (JSDC-4/R). SEE +/- corresponds to the presence or absence of SEE superantigen on the surface of Raji B cells. SD were calculated from 3 experiments.

The dual-cell flow cytometry technique is highly dependent on the conditions during the experiment. I discovered that a small change in temperature during the experiment dramatically influenced the results (see relatively high SDs). Moreover, this technique does not allow the differentiation between poorly conjugated Jurkat and Raji cells, compared to those forming the mature synapse (see the following section). Therefore, live cell imaging was employed to better characterise the effect of SDC-4 on the T cell activation.
3.5.2 Using live cell imaging

In my last approach, I employed live cell microscopy to image the cells when forming conjugates. The advantage of this technique is that it enables to partially mimic the physiological conditions which happen during the immune response. Moreover, as conjugate formation between the T cells and APCs is a fast process, with this approach I was able to monitor the first tens of seconds and minutes after the contact between the cells. None of the previously used techniques in this project was able to follow the shape changes of the T cell. Also, the analysis of the data let me quantitate a number of cells and conjugates to increase the probability to detect a more subtle effect of SDC-4 on conjugates formation and/or morphology of T cells.

3.5.2.1 Image analysis using Definiens ruleset

The day before each experiment Jurkat T cells were transiently transfected with pHAb-hSDC-4-IRES-EGFP or pIRES2-EGFP (used here as a control – mock transfection) using the microporator and the conditions optimised before (see section ‘SDC-4 detection’ for details). The transfection efficiency was regularly tested by flow cytometry. Representative results are presented in Figure 3.29

![Image](image.png)

**Figure 3.29.** Representative results of transient transfection efficiency for Jurkat T cells transfected with pHAb-hSDC-4pIRES-EGFP or pIRES2-EGFP plasmids measured by flow cytometry. The cells were always tested on the day of each live cell imaging experiment.
On the day of the experiment, Raji B cells (APCs), labelled with Orange cell tracker for their easy identification, were loaded with superantigen (SEE) for 1h at 37°C, washed and applied onto the microscopy glass-bottom MatTek dish as described in Materials and Methods, (section 2.2.7.6). To reduce the mobility of the APCs for imaging, they were loaded in a serum-free medium and left for 2 minutes to settle the cells down at the bottom of the dish. Then, the medium was removed and the T cells were added, followed by the immediate acquisition of the events on a CCD camera of the wide-field microscope.

Overall, we performed over 50 experiments, which correspond to the 50 individual days when transfection was completed (the day before) and cells (including various conditions and DNA constructs) were tested. The first 15 experiments were performed to optimise the conditions of the experiments, as there was no literature describing this kind of approach available. After testing different microscopy dishes, microscope settings, optimising the number of cells applied on the dish, and acquisition time and frequency, I found satisfactory conditions. Total acquisition time was 30 minutes with each frame collected every 10 seconds (Movies 1 and 2; in the attached DVDs).

**Movie 1.** Live cell imaging data of conjugates formed between Jurkat T cells transfected with pIRES2-EGFP plasmid and Raji B cells.

**Movie 2.** Live cell imaging data of conjugates formed between Jurkat T cells transfected with pHA-hSDC-4-IRES2-EGFP plasmid and Raji B cells.

In both movies-Orange cells- Raji B cells are stained with Orange cell tracer; Green cells-Jurkat T cells are transfected with pIRES2-EGFP (movie 1) or pHA-hSDC-4-EGFP (movie 2). Grey cells –untransfected Jurkat T cells.

Each day, 8 to 12 datasets were acquired (4 to 6 for Jurkat T cells transfected with SDC-4-encoding plasmid and equal number for EGFP only plasmid). This gave me over 500 datasets in total for the analysis.
In next step, the results were analysed using Definiens® software (Germany), which allowed us to quantitate the number of individual cells (T and B cells separately), as well as the conjugates formed. Moreover, the shape (morphology) analysis was enabled after advanced tuning of the analytical approach which was facilitated by the increased number of datasets acquired and analysed using its various versions. (Movies 3 and 4; in the attached DVDs presents the Definiens analysis of Movies 1 and 2)

**Movie 3.** Live cell imaging Definiens data analysis of conjugates formed between Jurkat T cells transfected with pIRES2-EGFP plasmid and Raji B cells. Raw data presented at Movie 1.

**Movie 4.** Live cell imaging Definiens data analysis of conjugates formed between Jurkat T cells transfected with pHA-hSDC-4-IRES2-EGFP plasmid and Raji B cells. Raw data presented at Movie 2.

In both movies-Blue cells- Raji B cells are stained with Orange cell tracer; Red cells-Jurkat T cells are transfected with pIRES2-EGFP (movie 3) or pHA-hSDC-4-IRES32-EGFP (movie 4). Yellow cells –Jurkat T cells detected as the ones forming synapses.

The ruleset (Attachment 1) was kindly created and provided by Dr. Martin Spitaler (the manager of the FILM Imaging Facility of the Imperial College London), but I was involved in the tuning process by applying the ruleset in progress on the representative datasets and during discussions about their improvement.

Analysis using Definiens® software was performed in five different layers of segmentation (Figure 3.30 A) and as a final output yielded the number of individual Raji B cells (APCs), number of T cells present and the number of T cells forming synapses per frame. Each layer corresponds to different parameter which let to detect T cell, detect B cells, detect distal and proximal synapses and finally the conjugated T cells forming IS. The final analysis (shown at movies 3 and 4) is compensation of all layers analysis performed according to designed ruleset (figure 3.30 B).
A.
Figure 3.30. Definiens software performs (A) analysis in five different layers of segmentation for each frame (created by Dr. M. Spitaler). Different levels of segmentation are illustrated here. The important information can be obtained at all levels of the segmentation; (B) scheme of ruleset map of the Definiens analysis for each frame of each movie.
The optimisation of the ruleset for Definiens software was a long and complicated process, and different approaches must be tested. Originally, the relative border of the T cell to Raji cell was the only condition required, but it generated a number of false positive signals for cells which were just overlapping each other and not forming real synapses (conjugates). To solve this problem, Martin Spitaler brought the idea to define the proximal and distal synapses and apply the ratio between these two values as another condition for the IS analysis, as the proximal synapse should be always larger than the distal one in the proper IS. The ‘proximal’ and ‘distal’ synapses are defined based on the relative distance from the Raji cell (Figure 3.31). At the end, the detection of T cells forming synapses was based on two major conditions which need to be met. First of all, there has to be a large contact area relative to the total T cell border between neighbouring T and B cells (within a range of 10-15%) and the area of the proximal synapse has to be ~25-30% larger than of the distal one.

Figure 3.31. Examples of the proximal and distal synapses determination in (A) Jurkat T cell forming a true IS and (B) Jurkat T cell just non-specifically touching Raji B cells (APC).
Time-lapse results from the individual imaging experiments are presented on the graph plot in terms of absolute (Figure 3.32A) and relative numbers (Figure 3.32B). Unfortunately, the number of Jurkat T cells expressing EGFP from the pIRES2-EGFP plasmid was always higher than for those transfected with pHA-hSDC-4-IRE5-EGFP plasmid. Therefore, instead of the absolute number of Jurkat T cells conjugating with Raji B cells, I used relative number of the cells, i.e. percentage of transfected T cells forming conjugates with the APCs, to describe quantitatively the capacity of Jurkat T cells to form the IS.

Figure 3.32. Illustrative graphs for (A) number of Jurkat T cells conjugating with Raji B cells and (B) the percentage of transfected Jurkat T cells conjugating with Raji B cells – an individual experiment is shown (see Figure 32 for a more global picture). Each frame was acquired every 10 seconds and the whole acquisition was taken over 30 minutes.
The quantitative analysis was performed for all (fifteen) individual experiments (different transfections; experimental days) which, in total, resulted in 40 datasets for each, control EGFP only and SDC-4-transfected, cells. The average time-lapse values for all the data are presented on the graph in Figure 3.33. Large error bars and little difference between individual values of global conjugates formation per frame do not support any difference in the capacity of Jurkat T cells to form conjugates after the transfection either with pHA-SDC-4-IRES-EGFP or pIRES2-EGFP plasmids.

**Figure 3.33.** Graph summarising the average percentage of T cells forming IS with SEE-loaded Raji B cells. The values are per frame and x-axis indicates the frame number. Acquisition was set at 6 frames per minute. Red line corresponds to the cells transfected with the pHA-hSDC-4-IRES-EGFP (SDC-4) and blue line to the cells transfected with pRES2-EGFP (EGFP) plasmid. SDs were calculated from 40 datasets for every transfection.
At first, I was disappointed by the fact I was unable to quantitate a difference between two sets of cells, a difference which had appeared evident by visual observation. Nevertheless, I hypothesised that more detailed analysis of the movies might reveal a difference in the shape of T cells forming IS, when transfected with the two different plasmids. In my next attempt I focused on the frame-by-frame digital image analysis of factors such as: asymmetry, border index, compactness, elliptic fit, roundness, border length, area, relative border of T cell to B cell and the ratio of the border of the T cell to its area. Movies 6 to 8 (in the attached DVDs) show examples of data acquired. The BF is turned off to better present the shape changes.

**Movie 5.** Live cell imaging data analysis of conjugates formed between Jurkat T cells transfected with pIRES2-EGFP plasmid and Raji B cells – example 1.

**Movie 6.** Live cell imaging data analysis of conjugates formed between Jurkat T cells transfected with pIRES2-EGFP plasmid and Raji B cells – example 2.

**Movie 7.** Live cell imaging data of conjugates formed between Jurkat T cells transfected with pHA-hSDC-4-IRES2-EGFP plasmid and Raji B cells – example 1.

**Movie 8.** Live cell imaging data of conjugates formed between Jurkat T cells transfected with pHA-hSDC-4-IRES2-EGFP plasmid and Raji B cells – example 2.

In both movies-orange cells- Raji B cells are stained with Orange cell tracer; Green cells-Jurkat T cells are transfected with pIRES2-EGFP (movies 5 and 6) or pHA-hSDC-4-IRES-EGFP (movies 7 and 8). Brightfield was turned off to better show the shape changes of the T cells. The movies represent two different experiments for two different transfection with each plasmid.

For the advanced analysis, we selected only frames 30, 60 and 180, corresponding to 5, 10 and 20 minutes from the beginning of the movie (the addition of T cells to the Raji B cells), and performed the test over ~40 movies for each, Jurkat T cells transfected with pIRES2-GFP and pHA-hSDC-4-IRES-GFP plasmids. The data presented in Figure 3.34 do not examine T cells which have not been identified as forming conjugates. Analysis only of T cells forming conjugates with Raji B cells should be valid since the percentage of conjugating T cells was similar for samples expressing EGFP only and those expressing SDC-4 (Figure 3.32).
Figure 3.34. The analysis of asymmetry (A), border index (B), compactness (C), elliptic fit (D) roundness (E), border length (F), area (G), relative border of T cell to B cell (H) and the ratio between the border and area of the T cell (I). Frames 30, 60 and 180 from ~40 movies of Jurkat T cells transfected with pIRES2-EGFP or pHA-hSDC-4-IRES-GFP plasmids have been examined. Error bars were calculated as the error from the average value.
The T cell morphology analysis was performed with automatically calculated by software parameters like asymmetry etc. The results confirmed the asymmetric character of Jurkat T cells transfected with pIRES-EGFP (EGFP only control), but less pronounced asymmetry for Jurkat T cells transfected with pHA-hSDC-4-IRES-EGFP (SDC-4) (Figure 3.34). Compactness, relative border of the T cell to B cell and the ratio between border and area of the T cells are the parameters which show the biggest differences between control samples and those transfected with SDC-4, but only at frame 60. At the beginning (frame 30) and end of the analysis (frame 180) these parameters are almost the same. This is in agreement with the visual observations that after the first big engulfing step (big increase in compactness and roundness for EGFP at frames 60), in the next step cells forming functional IS with APC acquire a more round shape again. Similarly, the relative border of T cell to B cell and the ratio of the T cell border to its area are lower for the SDC4-transfected cells, which confirms the observation that SDC-4 over-expressing Jurkat T cells do not intensely engulf B cells, but still form conjugates. Such data also indicate that the presence of SDC-4 on the surface of T cells allows contact formation between T cells and APC but somehow reduces their capacity to expand the contact area (see Discussion for more details).

<table>
<thead>
<tr>
<th>Asymmetry, Border index, Compactness, Elliptic fit, and Roundness of the object</th>
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<tr>
<td><strong>Asymmetry</strong> - describes the relative length of an object, compared to a regular polygon. An ellipse is approximated around the object, which can be expressed by the ratio of the length of its minor and major axes.</td>
</tr>
<tr>
<td><strong>Border index</strong> - describes how jagged an image object is; the smallest rectangle enclosing the image is created and the index is calculated as the ratio between the border lengths of the image object and the smallest enclosing rectangle.</td>
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<tr>
<td><strong>Compactness</strong> - describes how compact an image object is and is a product of the length and the width, divided by the number of pixels.</td>
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<tr>
<td><strong>Elliptic fit</strong> - describes how well an image object fits into an ellipse of similar size and proportions.</td>
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<tr>
<td><strong>Roundness</strong> - describes how similar the object is to an ellipse and is calculated by the difference of the enclosing ellipse and the enclosed one.</td>
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Roundness and Elliptic fit of SDC-4-overexpressing T cells show only minor differences between the T cells expressing SDC-4-EGFP and EGFP only.

Parameters like Asymmetry, Border index, Border line and Area of the T cell are generally higher for pIRES-EGFP transfected T cells, but show almost the same alteration over the time.

### 3.5.3 Summary

Live cell imaging application presented above is a powerful technique which can bring a lot of information and some explanations to the current knowledge of the IS formation. Such an approach for the analysis of the IS formation brings the possibility of acquiring large datasets and makes the technique highly reliable. The Achilles’ heel of this technique is the data analysis. I did not get a conclusive answer about the stability or speed of the IS formation by global image analysis. On the other hand, the changes in the shape of the IS for Jurkat T cells expressing EGFP only are significantly more pronounced than those for SDC-4-overexpressing Jurkat T cells. Moreover the effect can be analysed in big number of cells in this approach.
3.6 T cell proximal signalling analysis

A slight delay in the conjugates formation has been observed for Jurkat T cells transfected with pHA-hSDC-4-IRES-EGFP plasmid compared to their pIRES-EGFP plasmid transfected counterparts (see Figures 3.32 and 3.33). I, therefore, experimentally verified if there are any differences in proximal signalling events using biochemical (immunoblotting) and cell biology (flow cytometry) assays. I was unable to detect these signalling events (e.g. calcium mobilisation) using our live cell imaging system mainly because during the first two or three minutes of imaging, the period when such signalling events are most active, the acquisition was disturbed by the landing of T cells and the flow of the medium.

3.6.1 Immunoblotting

Immunoblotting assay was performed on conjugates formed between Jurkat T cells transfected with pHA-hSDC-4-IRES-EGFP or pIRES-EGFP plasmid and Raji B cells. T cells were transfected a day before the experiment, washed and mixed with Raji B cells pre-loaded with SEE. The mix was briefly spun, incubated at 37°C for 2, 8 and 20 minutes and lysed with Cell lysis buffer. Then the samples were subjected to 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane, which was probed with 4G10 Ab against proteins phosphorylated on tyrosine and HRP-conjugated anti-mouse secondary antibody. Then the Abs were stripped and the membrane was reprobed with Ab against α-tubulin to control for equal sample loading. The signal was analysed and processed using ImageQuantTL software. Results presented in Figure 3.35 and Table 3.7 show that very early signalling events produce weaker phosphotyrosine signal for the conjugates formed by Jurkat T cells transfected to overexpress SDC-4 (and SEE pre-loaded Raji B cells) in comparison to the conjugates formed by Jurkat T cells transfected to express EGFP only. The most dramatic difference was observed for bands which should represent phosphorylated LAT, Src-family kinases (Lck) and ZAP-70 kinase (Table 3.7).
Figure 3.35. Conjugates between Jurkat T cells transfected with either pHA-SDC-4-IRES-EGFP (J_SDC4) or pIRES-EGFP (J_EGFP) plasmids and SEE-loaded Raji B cells were formed using varying incubation times (2, 8 and 20 minutes), lysed, subjected to 12% SDS-PAGE and immunoblotted. The membrane was probed with 4G10 Ab against proteins phosphorylated on tyrosines. Some bands (e.g. LAT, Lck, and ZAP-70) are labeled to indicate specific signalling molecules based on the experience from previous experiments; A.I.Magee and M.Cebecauer, personal communication). The membrane was then stripped and reprobed with Ab against α-Tubulin to control the loading of the samples on the gel. Example result is shown of three similar experiments.
Table 3.7. Quantitation of Western Blotting bands (fold increase of signal and digital counts) for TCR signaling effector molecules ZAP70, Lck, LAT. Digital counts are presented also for the control of the loading α-Tubulin. Digital counts were calculated using ImageQuantTL software. Some bands are not detected for unconjugated Jurkat and Raji B cells - used as controls (-); half of the volume used for conjugated cells was loaded for unconjugated Jurkat and Raji controls.

3.6.2 Flow cytometry

The proximal signalling events were also investigated using flow cytometry and 4G10 Ab against proteins phosphorylated on tyrosines. The results (Figure 3.36) are in good agreement with the immunoblotting data described in the previous section, i.e. the early signalling events were delayed in conjugates formed by Jurkat T cells transfected with SDC-4-encoding plasmid and SEE-loaded Raji B cells in comparison to the conjugates formed by Jurkat T cells expressing only EGFP. There was no detectable phosphotyrosine signal in conjugates formed by Jurkat T cells overexpressing SDC-4 after 2 min of incubation, but the signal became stronger after 8 min of the incubation with Raji B cells pre-loaded with the superantigen.
Figure 3.36. The conjugates formed by Jurkat T cells overexpressing SDC-4 and SEE-loaded Raji B cells (SDC-4) and Jurkat T cells expressing only EGFP and SEE-loaded Raji B cells (EGFP). The cells were incubated for 2 (A) or 8 (B) minutes to form conjugates, fixed, permeabilised, washed and stained with 4G10 Ab against proteins phosphorylated on tyrosines (pY). Representative data are shown of 5 independent experiments.

3.6.3 Summary

T cell proximal signalling analysis using immunoblotting and flow cytometry techniques demonstrates that early signalling events in the IS formed between SDC-4-transfected Jurkat T cells and SEE-loaded Raji B cells are delayed in comparison with Jurkat T cells expressing EGFP alone. The differences are noticeable especially for 2min timepoint, interestingly for the 8min timepoint the values are going up and stay high even for 20min timepoint, which in case for control conjugates goes down. It surely proofs the delay in signalling.
4 General Summary and Discussion

The human immune system has been intensely studied over many years but a number of questions stay unanswered. A hot topic related to the function of T lymphocytes, one of the key cells of the immune system [68], is a better understanding of the properties and functions of the IS [66, 88, 105, 106, 108, 113, 170] formed between T cells and APCs. The structure and general function of the IS are well described [75, 104, 106], but new results are opening new questions and new techniques make it possible to study the IS in more details. That means, many facets of the IS structure and function require further experimental studies. Moreover, there are many aspects of the IS which can be studied. For example, one can focus on its formation, stability [108], or signalling events generated by the engagement of TCR [76, 97, 118]. On the other hand, a number of unpredicted proteins which may appear in the IS only temporarily, and their function, could turn out to be crucial for the proper in vivo function of the IS formed between T cells and APCs.

4.1 Summary of the results

I hypothesised that one such protein can be SDC-4. Its tightly regulated expression level in human naive and activated T cells was published recently [142, 143] and I was able to confirm this observation (see section ‘SDC-4 detection’). SDC-4 expression is upregulated by the activation of T cells. What does this mean? Can SDC-4 help to organise the IS or, in opposite, it can inhibit the IS formation and regulate the detachment of the T cell from APC after the essential recognition of the antigen period is over and not required anymore, i.e. be involved in down-regulating the T cell-APC interaction?

Low levels of endogenous SDC-4 expression in human naive T cells and the sensitivity of these primary cells to handling, as well as their small size for imaging, made it difficult to monitor SDC-4 localisation and function in these cells. Therefore, I had to find another model cell for my studies. The cells of choice were human Jurkat T cells, derived from an acute T cell leukaemia. The level of their endogenous SDC-4 expression is very low, so they are more similar to the resting T cells. In order to see the effect of increased SDC-4 expression I decided to transfec these cells with exogenous SDC-4. Transfection of Jurkat T cells is an inefficient process when using plasmids.
Viral vectors were successfully applied for the transfection of these cells by other groups. In consultation with my supervisors, I decided to avoid work with these complex genetic tools and focused on optimisation of the settings for the microporator (a novel version of electroporation). A careful and time-consuming optimisation process, including determination of the best concentration for individual plasmids, resulted in satisfactory results. During this process, I discovered that longer than 24h culture of the cells after transfection lowered their viability. In addition, I successfully generated Jurkat T cells stably transfected with human SDC-4, but this approach unfortunately resulted in the down-regulation of the surface TCRs so these cells were not suitable for my purpose. No change in TCR surface expression was observed in Jurkat T cells transiently transfected with plasmid encoding human SDC-4 (see 3.3.3 section in Results). Possible explanation for that may be that as SDC-4 expression increases over the time, the activity of PKCα becomes deregulated [61] which can cause the down-regulation of the TCR in stably transfected Jurkat T cells [171].

Jurkat T and Raji B cells forming conjugates in the presence or absence of antigen were selected as an experimental system for the functional assays. As an antigen, SEE superantigen was used for the pre-loading of Raji B cells. Efficient formation of conjugates and of the stable IS was observed for untransfected and EGFP-transfected Jurkat T cells in the presence of antigen-loaded Raji B cells. The advantage of the system is that it mimics well the native situation of T cells searching for antigen presented by APCs (e.g. dendritic cells).

SDC-4 is intensely studied in migrating fibroblast [19] but little is known about its function in T cells and especially, according to my knowledge, in the formation and stability of the IS [172]. Therefore, I have focused on the process of T cell conjugation with the APC employing a number of imaging techniques. I investigated the localisation and effect of SDC-4 in the IS formed by Jurkat T cells transfected with plasmids encoding SDC-4 and Raji B cells (APC) presenting (or not) the antigen. I managed to detect a low level of exogenous SDC-4 in Jurkat T cells as well as in conjugates formed by these cells with Raji B cells by confocal microscopy (section ‘Localisation of SDC-4’). Rather random localisation of SDC-4 in the membranes of individual (unconjugated) Jurkat T cells was observed, but also of those forming conjugates with Raji B cells. I also tested the co-localisation of the proteoglycan with its well-described ligand (fibronectin) and proteins of the TCR/CD3 complex, as well as effector proteins phosphorylated on tyrosine (section ‘Co-localisation of SDC-4 with receptors). I did not find any specific co-localisation with these examined proteins.
SDC-4 was previously demonstrated to regulate the interaction of eukaryotic cells with the ECM and neighbouring cells via adhesion [173, 174]. Therefore, my primary interest became the quantitation of conjugates formed by Jurkat T cells transfected to overexpress SDC-4 with APCs, in comparison to Jurkat T cells expressing EGFP only. Such experiments should provide information on the role of SDC-4 in this process. Using the EGFP expressing Jurkat T cells as a control allowed me to eliminate the effect of the transfection in data analysis as it was involved in both cases.

The first technique used for conjugates quantitation was flow cytometry as I assumed that the number of T-B conjugates reflects the frequency of synapse formation between T cells and APCs under the tested conditions. The results presented in the section 3.5.1 show a certain decrease in the number of conjugates formed by SDC-4 Jurkat T cells and Raji B cells pre-loaded with the superantigen. Nevertheless, the technique was extremely sensitive to the experimental conditions, such as the sample temperature throughout all steps of the preparation.

Later, live cell imaging was applied for more advanced analysis of T cell conjugation with antigen-bearing APCs. The advantage of this technique is that one can 'see' all steps of the interaction between the cells and observe the conjugates over a longer period of time (section Live cell imaging) to investigate their stability and morphology. The results achieved using live cell imaging of transfected Jurkat T cells and Raji B cells indicate that Jurkat T cells overexpressing SDC-4 form conjugates slightly less frequently than those transfected with EGFP but there is no significant difference in the overall numbers of formed conjugates. These data correlate with those achieved using flow cytometry and point to the fact that the global analysis of conjugates cannot uncover differences in the IS formation in the presence or absence of the increased SDC-4 expression.

Such differences were well observable by eye and I therefore decided to take advantage of single cell image data analysis of individual cell morphology which may influence T cells activation [108]. A dissimilarity was confirmed in shapes of Jurkat T cells transfected with SDC-4-encoding plasmid but forming conjugates with Raji B cells and those transfected with EGFP-only plasmid. Due to the time constraints I was unable to find the optimal way of the shape analysis yet, but factors such as asymmetry, roundness and relative border of T cell to B cell well document my visual observations. The data indicate that the engulfment of Raji B cells by SDC-4 overexpressing Jurkat T cells is smaller than Jurkat T cells expressing only EGFP.
Also, the contact site between conjugating cells is much smaller for proteoglycan-expressing Jurkat T cells than for controls.

The downside of the live cell imaging mentioned above is that the images taken during the first 2-3 minutes of the conjugates formation are ‘disturbed’ by the landing cells and the detection (image data analysis) of cells is more difficult. I observed reduced formation of conjugates by Jurkat T cells overexpressing SDC-4 compared to those expressing EGFP only. Keeping in mind the abovementioned drawback of live cell imaging I tested the signalling in conjugating Jurkat T cells using Western blotting. I observed significant delay in the signalling events (herein represented by phosphorylation of effector proteins on tyrosine) in SDC-4 overexpressing Jurkat T cells compared to those expressing EGFP only. The data were also confirmed using flow cytometry to detect the signal for individual cells.

**4.2 Discussion and future perspectives**

T lymphocyte activation is regulated by stimulatory and inhibitory signals transduced simultaneously or after the binding of TCR to its corresponding ligands on APCs [97, 147, 150]. Stimulatory receptors tend to be present constitutively on the surface of resting T cells, whereas inhibitory receptors require activation for their expression or localisation to the IS [175]. Syndecans, similar to other accessory molecules, can contribute positively or negatively to the antigenic stimulation of T cells by antigen. Recent results indicate an inhibitory role for SDC-4 in human and mouse T cells [143, 150]. The novel ligand for SDC-4 (DC-HIL expressed by human monocytes and dendritic cells) promoted its inhibitory function [143, 149], but published results did not analyse the effect of SDC-4 on T cell morphology and early activation events. Ariizumi et al noticed negative effect on distal signalling effects (such as IL-2 production)[143], but I did not observe any overall dramatic effect of SDC-4 overexpression on proximal signalling in Jurkat T cells. The only detectable difference was the delay in signalling. Also, I am not aware of DC-HIL expression in B cells, so I do not expect the effect of this interaction on the morphology of T cells described in my work but this needs to be tested. I decided to investigate the impact of SDC-4 on IS formation. First, I did not manage to prove a systematic global effect, such as complete block of the IS formation, but the shape changes indicate that SDC-4 influences the IS formation or, at least, adhesive forces involved in these morphological changes.
The fact is that syndecans cannot be considered in isolation for any system studied. For example, syndecans and integrins collaborate extensively in mediating cell adhesion [1] to a broad range of ECM molecules and can combine synergistically to regulate FA-complex formation [176], cell spreading and directional migration [19, 65]. In addition, some growth factors and their high-affinity receptors can influence cell-matrix relationship based on the modulation of syndecan-integrin network [4, 32].

Given the propensity of the syndecans to homodimerise and multimerise [45] in response to ligand engagement [148], it seems likely that the SDC-4 extracellular domain might regulate the assembly of other multi-molecular cell surface signalling or adhesion complexes because of GAG chains presence [21]. Looking from the T cell side, molecular structures and biochemical events involved in T cell activation are taking place in the supramolecular structures formed at the plasma membrane and have been increasingly well-described [76]. Nevertheless, scientists using biophysical and imaging approaches naturally tend to focus on the events directly related to the TCR engagement and proximal signalling using simplified experimental approaches, such as stimulatory supported planar bilayers containing just two ligands (pMHC and ICAM-1) or antibody stimulation [73, 105]. Such approaches may miss the complex character of T cell activation, in which molecules such as SDC-4 may play a regulatory role. My observations expand the knowledge on the factors regulating T cell morphology and adhesion during its activation.

Recent publications of Ariizumi group [177] indicates that because SDC-4, as mentioned before the coinhibitor for DC-HIL, lacks immunoreceptor tyrosine-based inhibitory motifs (ITIMs), through which most coinhibitory receptors regulate T-cell responses that recruits protein tyrosine phosphatases to mediate inhibitory function. Therefore, they posited that SDC-4 links with a protein tyrosine phosphatases in an ITIM-independent manner. They showed that SDC-4 associates constitutively with the intracellular protein syntenin [50], but not with the receptor-like protein tyrosine phosphatase CD148 on human CD4+ T cells. Binding to DC-HIL allowed SDC-4 to assemble with CD148 through the help of syntenin as a bridge, and this process upregulated the activity of CD148, which is required for SDC-4 to mediate DC-HIL’s inhibitory function [177]. It may also explain my observation, that there is no colocalisation of SDC-4 with TCRs, as SDC-4 may be excluded from the IS and be accumulated there at the resolution point, when inhibitory molecules like CD148 are brought [178].
In summary, SDC-4 is a complex molecule, is highly expressed only in activated T cells, and its interactions with other ligands (e.g. DC-HIL) influence T cell activation (as shown by Ariizumi group) [141, 143, 177]. Its overexpression in T cells also influences the IS formation (presented in my thesis). I have not been able to describe the detailed mechanism behind the effect of SDC-4 on T cell conjugation with APCs.

Therefore, there are still numerous issues regarding SDC-4 and T cells to study. My results showing the shape differences between Jurkat T cells transfected with pHA-hSDC-4-IRES-EGFP and pIRES-EGFP plasmids which form conjugates with Raji B cells (APCs) (loaded with SEE) suggest limited contact between these cells - less engulfment of Raji B cell by the T cell. Such impact may have consequences in delayed search for pMHC because of the presence of big GAGs attached [63], or, in later phases to reduce the contact and help the detachment of T cell from the APC. I was actually able to observe delayed proximal signalling in my model system.

Due to time constraints I was unable to perform all experiments one would think of after seeing the impact of SDC-4 on the morphology of IS. For example, it would be interesting to test the effect on the IS formation using SDC-4 lacking HS chains (e.g. by applying heparinise for their removal, or the use of a mutated - unglycosylated – gene of SDC-4 for the transfection ). In addition, my results indicate some effect of SDC-4 on early signalling events. SDC-4 can stabilize and activate the activity of PKC-α [61, 171] with possible influence of TCRs function. Therefore, in future, these data should be expanded in a more detailed study using a variety of phospho-specific Abs to get more precise information on which specific signals have been delayed or whether the initiation of TCR signalling on its own was affected.

Actin cytoskeleton is a driving force of the attachment to and engulfment of T cells on APC [179]. From the studies performed using motile fibroblast, it is also known that SDC-4 can influence actin cytoskeleton [62, 173]. Therefore, the possible explanation of my observation regarding shape changes in Jurkat T cells during conjugates formation may be due to the SDC-4 regulatory role for the actin cytoskeleton [62]. It would be interesting to check the actin and SDC-4 co-localisation and the impact on actin-driven forces using our model system. Indeed, in collaboration with the group of Robert Endres (CISBIC, Imperial College London) we are analysing our live cell imaging data with respect to the forces generated during the conjugates formation.
It may be possible that SDC-4 present on the surface, but not interacting with fibronectin and generating the ECM-driven signal, can influence the adhesion between two interacting cells [62]. There is possibility that SDC-4, in this way, interacts with integrins on the membrane of the same cell *in cis* (e.g. the plasma membrane of T cell in our case), and modulates intracellular events such as integrin-actin bundling and cytoskeletal changes normally stimulated by cell-to-cell adhesion [1]. Such interaction can make the conjugate formation more difficult, especially during stages of the contact. Thus, it would be interesting to investigate the localisation of integrins (not just LFA-1) in the IS and their co-localisation with SDC-4, as well as the effect of the SDC-4 over-expression on their function.

It is important to note that all my results were achieved using CD4+ T cells or the model Jurkat T cells (mimicking early stage of CD4+ T cell development) and cannot be simply implicated for cytotoxic (CD8+) T cells without further testing. Also, no other SDCs have been described in T cells so far.

Although SDC-4 was initially identified as a co-receptor of integrins, its expression in other tissues, including T cells, implies a more widespread role. Results from studies in T cells suggest that SDC-4 function is linked to TCR signalling and cell activation. Despite these initial findings, there are big gaps in our knowledge regarding the function of SDC-4 in T cells and the immune system in general, at both the molecular level and at the level of the whole organism. At the molecular level important areas of investigation, although by no means the only ones, are: to understand the nature of SDC-4 modification induced early on during T cell activation; to explore the role of SDC-4 in cell-cell adhesion during antigen presentation; and to identify the receptor(s) that mediate the effects of SDC-4 in immune cells. At the organism level, vital information will be generated by: studying how T cells and the immune system in general develop in the absence of SDC-4 expression; assessing changes in immune responses of viable SDC-4-/- mice compared to wild type; and studying whether higher expression of SDC-4 in T cells in engineered mice predisposes to T cell hyperactivity and autoimmunity. Many tools are already available to study the biology of SDC-4 and we anticipate that these issues will be addressed in the near future by immunologists with different areas of expertise. These studies may well prove that SDC-4 has a critical role in the immune system.
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