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Author: Paul, Petra
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Chapter 4

Studying MHC Class II Transport in Dendritic Cells

Paul P and Neefjes J

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Professional antigen presenting cells, such as dendritic cells, are effective in activating T lymphocytes due to their unique ability to present antigens in the context of both MHC class I and II molecules. After successful loading with antigenic peptides MHC class II molecules traffic from the late endosomal loading compartment to the plasma membrane to exert their function of presenting peptides to T helper lymphocytes. Various processes play a role in this event, which are only partly understood to date. The following protocols demonstrate a strategy of how to integrate high throughput datasets to select candidates possibly involved in MHC class II transport for in depth studies. A combination of proteomics, RNAinterference and biochemical experimentation can uncover novel pathways regulating transport processes in primary dendritic cells.

1. Introduction

Dendritic cells (DC) are professional antigen presenting cells (APC) that regulate the adaptive immune response by stimulating naïve T cells (see [1] for review). Expression of peptide-loaded MHC class II molecules at the cell surface is the result of tightly regulated transport processes from late endosomal compartments (called MIIC [2-4]), where antigen loading takes place. For a detailed description of the MHC class II pathway see [5, 6]. In DC MHC class II transport to the cell surface is enhanced when danger signals are encountered [7, 8]. Various intracellular proteins have been associated with this process, such as iNOS and caspases [9]. The ubiquitin ligase MARCH1 modifies MHC class II in human immature DC (imDC) [10, 11], clathrin and AP2 orchestrate endocytosis [12], and cystatins regulate cathepsin activities [13]. Despite these findings, many open questions remain when it comes to the control of MHC class II transport in DC. For example, which motors are involved in MIIC transport? Little is known about this. Myosin II has been reported to interact with invariant chain (Ii) to control MHC class II transport in B cells [14]. Another actin-based motor, myosin 1E, has been recently implicated to be responsible for the positioning of MHC class II-positive vesicles in DC [15]. The dynein motor is recruited to MIIC via the small GTPase Rab7 [16].

One way of identifying new players in the MHC class II transport route is to select candidates following an RNAinterference (RNAi) screen and other high throughput cell biological assays. Gene knockdown is achieved by introducing small interfering RNA molecules (siRNA) or short hairpin RNA (shRNA) which are provided as either genome-wide or specialized libraries (kinases, phosphatases, G-protein coupled receptors, etc.). The resulting candidates can then be tested in smaller scale in primary DC. RNAi screens can be performed by microscopy as well as flow cytometry when appropriate antibodies are available. For a comparison of the two methods see Table 1. For the RNAi screen described here, changes in cell surface expression of MHC class II detected by flow cytometry serves as a primary read-out. In general, a robust read-out is essential. Negative and positive controls are required. See [17] for a review on tips and tricks of RNAi screening. Secondary screens may subsequently be used to cluster candidates into phenotypically similar pathways, which is essential for creating new biology.

Primary immune cells are often unsuitable for screening. They are limited in number, difficult to manipulate and prone to differentiate. Cell lines can serve as substitutes. For a list of MHC class II-positive cell lines see Table 2. For any of these cell lines, functionality and transfection efficiency need to be determined and optimized before large scale screening activities. In some cases activation with cytokines is required for efficient antigen processing and presentation.

Following the primary screen, microarray or deep sequencing studies on APCs provide information whether genes that influence MHC class II surface levels in cell lines are expressed in primary cells. The model cell line may be used for further high throughput (HT) experimentation: (1) Quantitative RT-PCR (qRT-PCR) following silencing of genes identified in the primary screen reveals whether the transcription rate of the MHC locus is altered. (2) Immunofluorescence and confocal microscopy provide vital information on alterations of the intracellular MHC class II distribution. The large

Table 1 | Characteristics of Flow Cytometry versus Microscopy

	Flow Cytometry	Microscopy
Readout	Intracellular or PM	Mainly intracellular
Analysis	Simple	Complex, software programs needed
Quantity	Many data points (cells)	Few data points
Detail	Little insight	Precise localization, many features detected

Table 2 | Possible Cell Lines for an MHC Class II-related RNAi Screen

Endogenous	Type	ATCC	Reference
MUTZ-3	Human		[25]
KG-1	Human	CCL-246	[26]
THP-1	Adherent, macrophage-like after stimulation with phorbol ester	TIB-202	[27]
LCL	Human Epstein Barr virus-transformed		Transformation protocol [28]
MelJuSo	Adherent human melanoma cell line		[29]
Ectopic	**Manipulation**	**ATCC**	**Reference**
RAW264.7	Adherent murine monocyte/macrophage cell line transfected with MHC class II	TIB-71	[30]
Fibroblasts	Transfected with MHC class II and Ii		[31]
HeLa	Transfected with CIITA	CCL-2	[32]

amount of images generated can be analyzed and clustered based on similarity using open software programs such as Cell Profiler and CP Analyst [18, 19]. To select candidates for in-depth follow-up studies, stringent criteria must be set. When addressing the question of what regulates MHC class II transport in DC, the following key points need to be considered. Silencing a gene that is a positive regulator of transport might result in reduced MHC class II levels at the plasma membrane. Knocking down a negative regulator, on the other hand, will result in higher cell surface expression levels. If this regulator is involved in the maturation-induced outward transport of MHC class II, its expression level might be lower in mature (m)DC compared to imDC, information that can be deduced from the gene expression data set. Finally, the intracellular distribution of MHC class II might be altered after silencing. For an example of HT data set integration and candidate selection strategy see [15].

The protocols described below are dedicated at how candidates selected from HT data sets can be validated and investigated in DC. First, the effects of silencing the candidate genes observed in the screen need to be confirmed in the primary immune cell. Lentiviral transduction is an efficient tool for the introduction of shRNA into human monocytes which can then be differentiated into imDC. In principle, such viral particles can also be used to introduce overexpression constructs. Once candidates that alter the transport of MHC class II are confirmed and changes of MHC class II gene transcription are excluded, the search for interaction partners/effectors can begin. Different methods can be applied for this purpose depending on which type of protein is selected. Yeast Two Hybrid (Y2H) technologies have been proven to be useful when looking for effectors of small GTPases. In our experience, for effector molecules of other protein classes (e.g. scaffold proteins) GST pulldown might be a more suitable method [20]. Kinases in general exceed very short lived interactions, which are difficult to detect in either assay. Any interaction identified using these methods needs to be confirmed by alternative methods, e.g. coimmunoprecipitation. All these techniques and considerations are required to build new pathways from high content screening data.

An RNAi screen only helps identifying potential candidate genes controlling a biological phenomenon. To understand the cell biology at a high content level, secondary HT screens can be applied. Subsequently, these are integrated with other datasets such as those from microarray, yeast two-hybrid and proteomics. When properly done, new pathways can be generated allowing definition of the control of MHC class II transport in primary DC.

2. Materials

2.1 Lentiviral Transduction of DC

- Human monocytes isolated from peripheral blood
- Cell line for lentivirus production (e.g. human 293T cells)
- Media and supplements: Cellgro medium (Cellgenix) or other medium for serumfree cultivation of dendritic cells, DMEM (GIBCO), fetal calf serum (FCS, Greiner), Phosphate-Buffered Saline (PBS, dissolve 1 tablet in 500 ml of distilled water, GIBCO) pH 7.4, trypsine-EDTA (GIBCO), IL-4 (Cellgenix), GM-CSF (Cellgenix), maturation trigger such as lipopolysaccharide LPS (Invivogen) and IFN-γ (Immukine,

Boehringer Ingelheim)
- Transfection reagent: Fugene 6 (Roche) or alike
- Plasmids: packaging constructs [e.g. pMDLg/pRRE, pRSV-Rev and pCMV-VSV-G [21]], control vectors (e.g. pLKO.1empty, pLKO.1shEGFP, pLKO.1shLuciferase, pLKO.1shSCRAMBLE), lentiviral plasmid containing shRNA of choice (pLKO.1 vectors from Open Biosystems, Thermo Scientific, usually 4-5 different sequences per gene)
- 0.45 μm filter units (Millipore)
- Polybrene (Millipore) dissolved in PBS
- Ultracentrifugation tubes (Beckman Coulter)
- Ultracentrifuge (Beckman Coulter Rotor SW28)

2.2 Flow Cytometry

- Wash Buffer: PBS containing 2% FCS
- Antibodies: PE anti-human HLA-DR (L243), FITC anti-human CD14, FITC anti-human CD83, PE anti-human CD80, APC anti-human CD86, APC anti-human CD40, APC anti-human DC-SIGN (all from BD Biosciences), diluted to 1-10 μg/ml in Wash Buffer
- FACS Calibur flow cytometer (BD Biosciences)

2.3 Immunofluorescence

- Cover glasses or μ-Slide 18-well plates (IBIDI)
- Coating: Fibronectin (Invitrogen) diluted to 20 μg/ml in PBS
- Fixation: PBS with 3,75% formaldehyde (free from acid, Merck)
- Permeabilization: PBS with 0.1% Triton X-100 (Sigma)
- Blocking: 0.5% bovine serum albumin (BSA, Sigma) in PBS
- Primary Antibody (AB): mouse anti-human CD63 (or any other late endosomal marker) and rabbit anti-HLA-DR [2] diluted in blocking buffer
- Fluorophore-conjugated Secondary Antibody (Invitrogen): diluted in blocking buffer, e.g. goat anti-mouse IgG Alexa 488
- HOECHST 33342 nuclear dye (Invitrogen) diluted to 2 μg/ml in secondary AB solution
- Phalloidin-Alexa568 (Molecular Probes) diluted to 0.4 U/ml in secondary AB solution
- Mounting medium: Vectashield (Vector Laboratories) or 80% glycerol (Merck) in PBS
- AOBS confocal microscope (Leica)

2.4 qRT-PCR

- Roche mRNA Capture Kit (Roche)
- Transcriptor High Fidelity cDNA Synthesis Sample Kit (Roche)
- Lightcycler 480 SYBR Green 1 Master (Roche)
- PCR-grade, RNAse-free water: DEPC-treated water (Invitrogen)
- Plates (Lightcycler 480 Multiwell 96, Roche)
- 10x primer solution: 3,3 μM of both forward and reverse primer in PCR-grade water; 18S rRNA reference primers: Forward 5'-CGGCTACCACATCCAAGGAA-3', Reverse 5'-GCTGGAATTACCGCGGCT-3'; HLA-DR α-chain primers: Forward 5'-CATGGGCTATCAAAGAAGAAC-3', Reverse 5'-CTTGAGCCTCAAAGCTGGC-3'
- PCR machine (Peltier Thermal Cycler, MJ Research)
- Light Cycler 480 Detection System (Roche)

2.5 Sample Preparation for Y2H and Glutathione-S-transferase(GST) Pulldown

- cDNA clone of gene of interest (e.g. IMAGE clone)
- Y2H suitable vector: e.g. pGBT9 (**http://www.dkfz.de/gpcf/y2h.html**)
- Cells of choice for GST pulldown, e.g. monocyte-derived imDC or mDC, peripheral blood mononuclear cells (PBMC)
- Lysis buffer: 50 mM NaCl (can be up to 500 mM to reduce unspecific binding), 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 0.8% NP-40 and protease inhibitors (EDTA-free, Roche Diagnostics) in water
- Wash buffer: same as lysis buffer, but only 0.08% NP-40 and without protease inhibitors
- Recombinant GST-tagged protein of choice plus free GST for control purposes
- Glutathione Sepharose beads 4G (GE Healthcare)

2.6. Coimmunoprecipitation

- Cell line to be transfected with proteins identified to interact (e.g. MelJuSo or 293T) or primary immune cell type to study interaction of endogenous proteins
- Expression vectors encoding tagged proteins of interest (possible tags: GFP, HA, myc etc.)
- NP-40 lysis and wash buffers: see 2.5
- Antibodies: anti-GFP, anti-HA, anti-myc or antibodies raised against the proteins of interest
- Protein G Sepharose beads 4 Fast Flow (GE Healthcare)

2.7 SDS-PAGE and Western Blot

- 2x Sample Buffer: 4 ml distilled water (dH_2O), 10 ml 0.5 M Tris-HCl pH 6.8, 8 ml glycerol, 16 ml 10% SDS, few flakes of Bromphenol blue, store aliquots at -20°C, add 400 μl of β-mercaptoethanol per 3,8 ml of sample buffer before use.
- Resolving gel buffer: 1.5 M Tris-HCl pH 8.8, store

at 4°C
- Stacking gel buffer: 0.5 M Tris-HCl pH 6.8, store at 4°C
- 10% sodium dodecylsulphate (SDS) in dH2O
- Acrylamide/Bis-acrylamide: extremely toxic and carcinogenic! 40% (ratio 37.5:1, Biorad)
- TEMED (N,N,N,N-Tetramethylethylenediamine, Sigma)
- Initiator: 10% ammonium persulphate solution (APS) in dH2O, prepare just prior to use
- n-Butanol, water saturated: add dH2O, shake, let phases separate, use upper layer
- Electrophoresis buffer: 10x TGS (Biorad), final: 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3, dilute to 1x with dH2O
- Molecular weight standard: Page Ruler prestained protein ladder (Thermo Scientific)
- Gel fixative: 40% methanol and 10% acetic acid in dH2O
- Silver stain kit: SilverQuest (Invitrogen)
- Transfer buffer: 10x TG (Biorad), final: 25 mM Tris, 192 mM glycine, pH 8.3, dilute to 1x with dH2O and add 20% methanol
- PVDF membrane (Immobilon-P, Millipore)
- Blotting paper: Whatman (GE Healthcare)
- PBS/T: 0.1% Tween-20 (Sigma) in PBS
- Blocking buffer: 5% skim milk powder in PBS/T
- Primary AB: diluted in 1% blocking buffer
- Secondary AB: e.g. swine anti-rabbit IgG horse radish peroxidase-conjugated (Dako), diluted 1:5 000 in 1% blocking buffer
- Detection: Amersham ECL Detection Reagents (GE Healthcare)
- Biorad Mini Protean and Transfer System

3. Methods

3.1 Lentivirus Production

- 293T cells are grown in 150 cm^2 flasks in DMEM supplemented with 10% FCS.
- Cells are washed with PBS, trypsinized and seeded at 3.5 x 106 per 10 cm tissue culture dish. Per lentiviral shRNA construct four dishes are required.
- After 24 h cells are transfected. The three packaging constructs are mixed 1:1:1. The ratio of this packaging trio to shRNA construct (pLKO.1) is 1:1. To 1.4 ml of serum-free DMEM (room temperature) add 56 µl of Fugene 6. In a separate tube (see Note 1) mix 7 µg (see Note 2) of packaging vectors with 7 µg of pLKO.1shRNA. Combine Fugene/DMEM and DNA, incubate for 30 min at room temperature. Replace the medium in the 10 cm dishes with 12.6 ml of DMEM/FCS. Add DNA/Fugene dropwise.
- After 24 h change medium to 8 ml Cellgro. The cells should be 50% confluent. Handle them

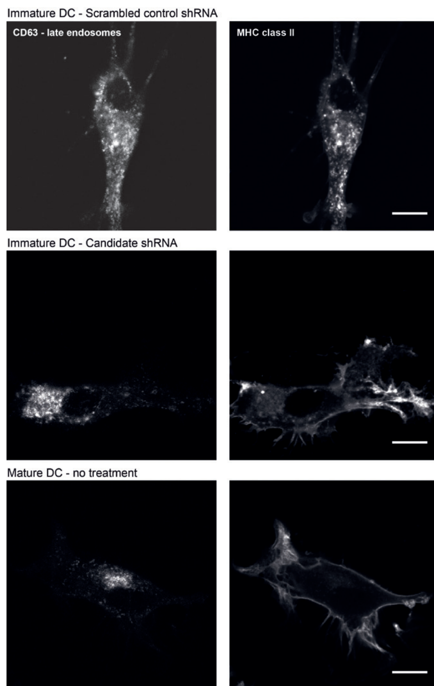

Figure 1 | Redistribution of MHC Class II after Candidate Gene Silencing
While MHC class II colocalises with the late endosomal marker CD63 on intracellular vesicles in immature DC (imDC, upper panel), most of MHC class II resides at the plasma membrane (PM) in mature DC (mDC, lower panel). When factors negatively influencing this transport process are silenced MHC class II molecules are redistributed to the PM, which resembles the phenotype of an mDC, while other maturation markers remain negative as is typical for an imDC (determined by flow cytometry). DC are fixed and stained with antibodies against CD63 and MHC class II. Fluorophore-conjugated secondary antibodies are used to visualize the localization of the two molecules by confocal microscopy. Bar = 10 µm.

carefully as they detach very easily.

- After another 24 h harvest the supernatant. Filter through a 0.45 µm filter. Concentrate the viral supernatant 100x by ultracentrifugation at 20.000 x g at room temperature for 2 h. Resuspend the viral pellet in 360 µl Cellgro (see Note 3) and snap freeze in liquid nitrogen. Store at -80 (see Note 4). The viral suspension obtained is enough to perform transduction of at least three different monocyte donors.
- Add another 7 ml of Cellgro to the 293T cells. A second harvest can be performed the day after.

3.2 Silencing Candidate Genes in DC

Human monocytes are transduced with lentivirus carrying shRNA constructs targeting the selected candidate genes followed by differentiation into imDC. Several control vectors should be used. An average of their phenotype serves as a reference point. The experiment should be performed in several donors as a big difference between individuals is usually observed when working with primary cells. General effects can be easily separated from MHC class II-specific ones by including additional cell surface markers such as MHC class I (for effects on the entire MHC locus), CD63 (for effects on recycling between PM and late endosomes) and transferrin receptor (for recycling in early endosomes).

- Thaw frozen monocytes. Wash 2x with Cellgro. Plate cells at 1 x 10^6 in 12-well plate in 900 µl Cellgro supplemented with IL-4 (800 U/ml final concentration), GM-CSF (1000 U/ml) and Polybrene (4 µg/ml). Add 100 µl of 100x viral supernatant.
- After 24 h remove 400 µl of medium and refresh with 1.4 ml of Cellgro/IL-4/GM-CSF.
- On day 5 mature a well of untreated imDC with LPS (2.5 µg/ml) and IFN-γ (1000 U/ml) to use as an mDC control.
- After 24 h determine the cell surface levels of MHC class II and other maturation markers by flow cytometry and the intracellular MHC class II distribution by immunofluorescence. To ensure that imDC were successfully generated monitor the reduction of CD14 and the increase of DC-SIGN cell surface levels compared to monocytes by flow cytometry. Furthermore, the cells are subjected to qRT-PCR to establish the level of knockdown and to rule out any effects on MHC class II gene transcription.

3.3 Phenotyping DC by Flow Cytometry

DC are semi-adherent and can be detached by repetitive pipetting and flushing the well.

- Approx. 50% of cells remain after differentiation from monocytes to imDC. Transfer approx. 100 000 DC per staining to a round-bottom 96-well plate. Spin cells down and discard supernatant. Wash cells one time with buffer and spin down.
- Add 20 µl of antibody cocktail (e.g. FITC-CD83, PE-MHCII, APC-CD40) and incubate for 30 min on ice in the dark. Wash cells with buffer followed by centrifugation.
- Resuspend pellet in 100 µl of wash buffer. Measure the cell surface expression of the respective markers using a plate reader attached to the flow cytometer.

3.4 Intracellular MHC Class II Distribution

In imDC MHC class II mainly resides in late endosomes where it colocalizes with CD63. When exposed to danger signals DC undergo striking morphological changes one of which is a redistribution of MHC class II to the plasma membrane. To determine whether any of the silenced candidate genes is responsible for maintaining MHC class II's endosomal location the colocalisation between CD63 and MHC class II is being determined (Figure 1).

- Coat µ-slides for 30 min at 37°C with fibronectin and rinse with PBS.
- Add 30 µl DC suspension and incubate in a wet chamber at 37°C for 6-7 h. All the following steps are carried out at room temperature.
- Fix cells with 3.75% formaldehyde for 15 min and wash by immersing the slide in a chamber filled with PBS. Slides may be stored for several days in PBS at 4°C.
- Permeabilize the cells with Triton-X for 10 min and wash with PBS.
- Unspecific binding is prevented by blocking with 0.5% BSA solution for 45 min.
- Add the primary antibody solution and incubate for 60 min. 15 µl of solution are enough to cover the entire surface area of one well.
- Wash the slides 3x for 5 min by placing the PBS-filled chamber on a shaking platform.
- Add the secondary antibody solution containing HOECHST as a nuclear dye and phalloidin to stain the actin cytoskeleton and incubate for 30 min in the dark.
- Wash the slides again 3x for 5 min and fill each well with 80% glycerol. The slides can be stored at 4°C for a few days until analysis with a confocal microscope. When using individual cover glasses perform all washing steps in

multi-well plates and mount the glasses in the end using a drop of mounting medium on a glass slide. Remove excess mounting medium by pressing the glass slide on tissue.
- Analyze the MHC class II distribution in DC by confocal microscopy.

3.2 Determination of mRNA Levels in DC by qRT-PCR

This method is used to determine the knockdown efficiency on one hand and to rule out any effects on transcriptional regulation of MHC class II on the other hand (see Note 5).

- Wash 50 000 DC 2x with cold PBS (see Note 6) and lyse in 50 µl lysis buffer (see Note 7).
- Add 4 µl of biotinylated oligo dT primer and anneal for 5 min at 37°C. Transfer lysate to a streptavidin-coated tube and incubate for another 5 min at 37°C (see Note 8).
- Add 200 µl of wash buffer and discard. Repeat washing step 3x. Leave the liquid of the last wash in the tube while preparing the cDNA synthesis master mix.
- Per reaction you will need: 6 µl 5x Reaction Buffer, 20 µl RNAse-free water, 0.5 µl RNAse Inhibitor, 2 µl nucleotides (dNTPs), 1 µl DTT, and 0.5 µl Reverse Transcriptase.
- Discard wash buffer completely. When pipetting away the liquid avoid touching the walls of the tube as this might destroy the layer of streptavidin. Add 30 µl of master mix.
- Reverse transcribe the captured mRNA into cDNA with a PCR program of 30 min 50°C and 5 min 85°C. Stop at 99°C. At such high temperature the bond between biotin and streptavidin is broken and the cDNA can be removed.
- Quickly remove the 30 µl of liquid and replace them with 50 µl of PCR-grade water. Incubate for 3 min at 99°C. Remove the liquid and combine it with the previous 30 µl. The cDNA can be stored at -20°C.
- For the qRT-PCR reaction 96-well plates are used. Per well add 5 µl of SYBR Green, 1 µl of 10x primer solution (3.3 µM each) and 1.5 µl of water. Add 2.5 µl of cDNA. Seal the plate and spin it to collect all fluid at the bottom of each well.
- Insert the plate in the LightCycler and start the run, which will take about 1 h 20 min.
- Analyze using the comparative CT method (ΔΔCT). Relate the results to the 18S rRNA values and normalize it to the average of control shRNA-treated cells.

3.3 Identification of Interaction Partners/ Effectors by Y2H

- The Y2H analysis can be outsourced (e.g. **http://www.dkfz.de/gpcf/y2h.html**). Alternatively, a protocol on how to perform Y2H can be found here [22].
- To prepare the sample for Y2H clone the cDNA of your gene of interest into a Y2H-combatible vector.
- Choose an appropriate cDNA library to screen (e.g. lymph node). Choose a cell type/tissue where your gene of interest is expressed under normal conditions. Information about tissue distribution can be found at **http://biogps.org** [23].

3.4 Identification of interaction partners/ effectors by GST pulldown

- To prepare a protein for GST pulldown clone the cDNA of your gene of interest into a GST expression vector. Produce the recombinant GST-tagged protein as well as free GST in e.g. *Escherichia coli* and purify them.
- Wash 120 µl of glutathione beads 3x in lysis buffer. Spin at 500 x g for 3 min. Remove liquid carefully (see Note 9).
- Couple 50 µg of recombinant protein dissolved in lysis buffer to the beads for 1 h. All subsequent incubation steps are performed at 4°C on a spinning wheel. Wash beads 3x in lysis buffer (see Note 10).
- Lyse 200 x 106 PBMC [cell number may vary depending on (1) the expression level of the protein of interest and (2) the availability of the cell type you want to use] in 750 µl lysis buffer for 30 min. Centrifuge for 10 min at maximum speed.
- Incubate beads with the supernatant of lysed cells for 1 h or overnight. Wash 1x in lysis buffer and 3x in wash buffer (see Note 10).
- Remove all liquid (see Note 9). Add 25 or 50 µl of 1x sample buffer (prepare by mixing equal volumes of water and 2x sample buffer), boil for 5 min at 100°C, centrifuge at max. speed and load supernatant on one or two gels, respectively.

3.5 SDS-PAGE and Mass Spectrometry

- Wash glass plates, clean with 70% ethanol and dry.
- Prepare two 1 mm 12% resolving gels by mixing 4.35 ml water with 2.5 ml resolving gel buffer, 0.1 ml 10% SDS solution and 3 ml acrylamide/bis-acrylamide solution. Start the polymerization by adding 50 µl of 10% APS solution and 5 µl of TEMED. Pour the gel leaving space for the

stacking gel. Overlay with n-butanol.

- After polymerization is complete pour away the butanol and rinse the gel with water. Absorb residual water using blotting paper.
- Prepare 4% stacking gel mixing 3.22 ml water with 1.25 ml stacking gel buffer and 50 µl 10% SDS solution and 0.5 ml acrylamide/bis-acrylamide solution. Start the polymerization by adding 25 µl of 10% APS solution and 5 µl of TEMED. Pour the liquid on top of the resolving gel and insert the comb.
- When polymerization is complete, assemble the gel unit, remove the combs, fill the unit with 1x electrophoresis buffer and flush each sample well using a long slender tip or syringe with needle.
- Load samples. Fill empty wells with 1x sample buffer. Include a weight marker in one lane.
- Close the unit and connect it to a power supply. Set the power supply to constant voltage and run at 90 V. When the sample front has passed the stacking gel the voltage may be increased to 110 V.
- When the SDS-PAGE run is finished, remove the gel and fix it for at least 20 min before proceeding to the silver staining.
- Perform mass spectrometric analysis to determine the proteins that have interacted with your protein of choice, but not with GST alone (see Note 11).

3.6 Confirmation of Interaction by Coimmunoprecipitation

Interactions identified by Y2H or GST pulldown need to be confirmed. If antibodies are available it is better to perform the coimmunoprecipitation (CoIP) with endogenous proteins in e.g. imDC or mDC. If such antibodies are not available ectopic expression of the proteins of interest in a cell line may be applied. By tagging the proteins with two different tags interactions can be studied.

- For CoIP either isolate the cell type of choice to confirm the interaction or transfect a cell line with plasmids encoding tagged versions of the two putative interaction partners. Use e.g. Fugene 6 for the transfection following the steps described under 3.1.
- Wash 50 µl of protein G beads 3x in lysis buffer. Spin at 500 x g for 3 min. Remove liquid carefully (see Note 9).
- Couple 5 µl (the amount might be reduced to as little as 1 µl depending on the strength of the antibody) of antibody (against interaction partner one or against the tag of interaction partner one) diluted in lysis buffer to the beads for 1 h. All subsequent incubation steps are performed at 4°C on a spinning wheel. Wash beads 3x (see Note 10).
- Lyse 5 x 106 transiently transfected cells (more primary cells are necessary when detecting endogenous proteins) in 750 µl lysis buffer for 30 min. Centrifuge for 10 min at maximum speed.
- Mix 50 µl of supernatant with an equal volume of 2x sample buffer, boil for 5 min at 100°C, centrifuge and store at -20°C. Load on a gel as a total lysate control.
- Incubate beads with the remaining supernatant of lysed cells for 1 h or overnight. Wash 1x in lysis buffer and 3x in wash buffer (see Note 10).
- Remove all liquid (see Note 9). Add 25 of 1x sample buffer (prepare by mixing equal volumes of water and 2x sample buffer), boil for 5 min at 100°C, centrifuge and load supernatant on a gel.
- Perform SDS-PAGE as described under 3.5.
- Prepare pieces of blotting paper and PVDF membrane slightly larger than the gel and soak the membrane in 100% methanol for 2-3 min.
- Fill a tray with 1x transfer buffer. Submerge the transfer cassette. Stack a foam pad and three pieces of blotting paper on the dark plastic side of the cassette. Remove the gel from the electrophoresis chamber, rinse with water, and place onto the filter paper. Place the membrane on the gel (avoid the introduction of bubbles. Add three pieces of blotting paper prewet in transfer buffer. Remove any bubbles in between the different layers by rolling a serological pipette over the stack. Place wet foam pad on top and close the cassette.
- Insert the cassette into the transfer tank. Black side of the cassette facing the black side of the tank. Double check that the membrane is placed between the gel and the anode (+). Proteins will migrate towards the anode. If inserted the opposite way proteins will be lost in the buffer.
- Place an ice block in the transfer tank and fill the tank with 1x transfer buffer. Add a magnetic stirrer.
- Place tank on a stirrer platform, close lid and perform transfer at constant current of 150 mA for 1.5 h. Alternatively, the transfer can be performed at constant 20 mA in the cold room overnight.
- Remove the PVDF membrane from the transfer unit (see Note 12). The prestained molecular weight marker should be clearly visible.
- Block the membrane in 10 ml of milk solution for 1 h at room temperature (RT) rocking on a shaking platform. Wash the membrane 3x with PBS/T for 5 min.

- Add the primary antibody solution (against interaction partner two or tag of interaction partner two) and incubate for at least one hour at RT or overnight at 4°C. Wash the membrane 3x with PBS/T for 5 min.
- Add the secondary antibody solution and incubate for 1 h at RT. Wash the membrane 3x with PBS/T for 10 min.
- Prepare the ECL reagents by mixing equal volumes of the two components. Remove the membrane from the washing solution. Remove excess buffer by blotting one corner of the membrane on tissue. Place membrane on clear plastic foil, cover with ECL mix and wrap in foil. Ensure complete coverage of the membrane by striking the liquid from one side to the other repeatedly.
- After 1 min remove excess liquid and wrap membrane in clean foil. Place membrane in a film cassette.
- In a dark room expose X-ray film to the chemiluminescence signals and develop afterwards. The exposure time needs to be adjusted to the signal strength.

3.7 Colocalisation Studies on putative Interaction Partners using Immunofluorescence Microscopy

Colocalisation observed by immunofluorescence (IF) represents an alternative method to support protein interaction data.

- Prepare primary cells as described under 3.4 or transiently transfected cells as described under 3.6.
- Determine the localization of the two putatively interacting proteins by staining them with antibodies directly or indirectly (anti-tag). See 3.4 for procedure.
- To place these interactions in context with MHC class II transport stain also for MHC class II or for the compartments involved in MHC class II antigen presentation (e.g. CD63 for late endosomes). Phalloidin is a stain for the actin cytoskeleton and will help to determine whether transport occurs along actin filaments. Furthermore, the microtubule network may be visualized using anti-tubulin antibodies.

3.8 Conclusion

An RNAi screen often results in a list of genes that are somehow affecting a biological process. To classify these 'hits' HT follow-up screens are needed. When all these individual datasets are integrated and stringent criteria are set a list of candidates emerges that might play a role in e.g. the control of MHC class II transport. To study and confirm these candidates experiments in primary immune cells (DC) need to be performed. Silencing can be achieved by introduction of shRNA constructs using lentivirus transduction. Changes in cell surface levels of MHC class II and its intracellular distribution are measured. To extend the findings of individual new players to whole pathways interaction partners and effectors can be identified using Y2H and proteomic approaches. All new findings require further confirmation applying alternative methods. Including appropriate controls ensures that the new findings are MHC class II-specific and do not affect any general transport or protein secretion pathway.

4. Notes

1. All tubes should be made from polyethylene to limit the rate of liposomes sticking to the tube's wall.
2. In order to monitor the transfection efficiency 0.5 µg of pEGFPC1 (Clontech) may be added to the packaging vector mixture. The amount of pLKO.1shRNA construct should be reduced to 6.5 µg respectively. More than 90% of 293T cells should be GFP-positive after 48 h.
3. Add Cellgro to pellet and leave it standing for a few minutes before pipetting up and down gently (try not to introduce bubbles).
4. To be able to set a certain mode of infection (MOI) the viral titer of your lentivirus preparation needs to be determined as transducing units per ml (TU/ml). Produce GFP-expressing lentiviral particles by introducing the pLKO.1TURBOGFP construct. Infect monocytes with decreasing volume of virus supernatant and determine the amount of GFP-positive colonies by microscopy after 48 h. Multiply this number with the dilution factor.
5. A useful program to design primers against your gene of interest for qRT-PCR is Perlprimer [24]. Adjust the settings according to what is being recommended with the SYBR Green you are using. BLAST the primer sequences to ensure they only recognize one gene.
6. To ensure RNAse-free working conditions wipe surfaces and tools with 70% ethanol. Use filter tips and RNAse-free tubes and reagents. Avoid working in a laminar flow hood as the flow of air will favor contamination with RNAses.
7. The solution is very viscous. One freeze-thaw cycle at -80°C helps to complete the lysis.
8. When working with the SA-coated tubes avoid the introduction of bubbles and scratching the wall of the tubes while pipetting!
9. Use a needle of diameter 0.4 mm. Liquid can be

entirely removed without loss of beads.

10. Transfer beads to a fresh tube during the last washing step. This will help eliminate proteins that unspecifically stick to the tube's wall.
11. Contamination of gels with keratine can cause problems during mass spectrometry. To ensure keratine-free conditions pre-cast gels can be used.
12. Use forceps to handle the membrane. The transfer of the prestained marker to the membrane gives an indication of the quality of protein transfer. To get an overview of protein transfer across the entire membrane stain with Ponceau S for a few minutes. Destain by rinsing with excess of water.

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