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CD83 and GRASP55 interact in human dendritic cells

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ABSTRACT

CD83 is one of the best known surface markers for mature human dendritic cells (DCs). The full-length 45 kDa type-I membrane-bound form (mbCD83) is strongly glycosylated upon DCs maturation. As co-stimulatory properties of CD83 are attributed to mbCD83 surface expression is required for efficient T-cell stimulation by mature DCs. By yeast two-hybrid screening, we were able to identify GRASP55 as interaction partner of CD83. DCs maturation induces endogenous CD83 protein expression with simultaneous regulation of CD83 glycosylation, interaction and co-localization with GRASP55 and CD83 surface exposure. GRASP55 is especially known for its role in maintaining Golgi architecture, but also plays a role in Golgi transport of specific cargo proteins bearing a C-terminal valine residue. Here we additionally demonstrate that binding of CD83 and GRASP55 rely on the C-terminal TELV-motif of CD83. Mutation of this TELV-motif not only disrupted binding to GRASP55, but also altered the glycosylation pattern of CD83 and reduced its membrane expression. Here we show for the first time that GRASP55 interacts with CD83 shortly after induction of DC maturation and that this interaction plays a role in CD83 glycosylation as well as in surface expression of CD83 on DCs.

1. Introduction

CD83 is a strongly glycosylated type-I membrane protein expressed predominantly on human mature dendritic cells (mDCs), but also on thymic epithelial cells and subsets of activated B and T cells. During DC maturation CD83 is strongly upregulated and expressed as a homodimer on the cell's surface. Hereby, CD83 is the best known maturation marker for human DCs [1–3]. Two naturally occurring CD83 isoforms have been described; a soluble form (sCD83) that consists of the extracellular domain only and a full-length membrane-bound form (mbCD83) [4]. MbCD83 is vital for T cell development in the thymus, since complete CD83 knockout animals have strongly reduced $CD4^+$ T cell numbers in the periphery [5]. Moreover, *in vitro* studies revealed that it has co-stimulatory properties when expressed on human mDCs [6,7]. Interference with the nuclear export of CD83 mRNA in DCs, by blocking the CRM1 pathway, leads to a strongly reduced T cell stimulation [8]. Similar results were obtained using CD83-specific siRNA [7]. Interestingly, viruses also target CD83 on DCs thereby inducing specific immune evasion strategies. Infection of mDCs with HCMV or HSV-1 induces CD83 down-modulation leading to a reduced T cell stimulatory capacity [9,10].

Soluble CD83 exhibits strong immune suppressive properties demonstrated by the inhibition of DC-mediated T-cell stimulation *in vitro* [11]. Moreover, *in vivo* studies suggest sCD83 as an interesting therapeutic agent, reducing and even preventing clinical symptoms like paralysis in experimental autoimmune encephalomyelitis and significantly extending allograft acceptance upon skin, kidney, and heart transplantation [12–14]. Full-length CD83 consists of three functional domains: an extracellular domain containing a short signal peptide and an Ig-like V-set domain (144 amino acids [aa]), a single-span transmembrane domain (22 aa) and a short cytoplasmic tail (39 aa) [15].

CD83 expression is transcriptionally regulated by a tripartite promoter/enhancer complex via IRF and NFκB factors [16] and the mRNA is subsequently exported from the nucleus into the cytoplasm via the alternative CRM1/HuR-pathway [17]. However, regulation of CD83 protein expression, post-transcriptional processing, and transport within cellular organelles remained to be established.

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Golgi reassembly stacking protein 55 (GRASP55) was identified as integral Golgi structure protein required to maintain Golgi architecture [18]. Together with GRASP65 it plays essential roles in the stacking of Golgi cisternae through interaction with partner proteins Golgin-45 and GM130 [19,20]. In mammalian cells, interplay of GRASP55 and GRASP65 also regulates Golgi ribbon maintenance during interphase, Golgi fragmentation at the onset of mitosis, and cell polarization in migrating cells [21–23]. Furthermore, both proteins serve as essential transporters along conventional and unconventional secretory pathways [24,25].

Since protein interaction partners for CD83 were still completely unknown, we performed a yeast two-hybrid screening. Thereby we identified GRASP55 as potential interaction partner of CD83. Thus, the aim of this study was to confirm the observations from Y2H screening and to investigate which sequence domains of CD83 promote the interaction with GRASP55.

2. Materials and methods

2.1. Yeast two-hybrid screening (Y2H)

Generation of the DC-specific cDNA library and the yeast two-hybrid screening were performed using Matchmaker Library Construction & Screening Kit (Clontech) according to the manufacturer's instructions (S1 A). In brief: immature DCs from two different donors were matured with LPS for 24 h. Two thirds of mDCs were further stimulated with H_2O_2 (100 μM) and sodium vanadate (0.035%) for 10 min, whereas the remaining third was left untreated. The "prey" was transformed into yeast strain AH109. The "bait", sequences coding for wildtype CD83 cytoplasmic tail (nucleotides 498–618), were transformed into yeast strain Y187. Selection was performed on high stringency yeast SD selection plates (Adenine$^-$/Histidine$^-$/Leucine$^-$/Tryptophan$^-$).

2.2. β-galactosidase assay

The β-galactosidase assay was performed using Z-buffer (60 mM Na_2HPO_4, 60 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, 0.02% BCIP/X-gal solution) and Whatman 50 filters (Amersham). Filters were pressed onto yeast plates, shock frozen for 20 sec in liquid nitrogen, thawed briefly and soaked with Z-buffer. After 4–8 h incubation at 37 °C blue color change was assessed.

2.3. Vectors used in Y2H and β-galactosidase assay

CD83 cytoplasmic domain was cloned into myc-tag containing pGBKT7 vector as "bait". Mutations converting TELV into TELS and GAAS, respectively, were introduced by PCR mutagenesis (performed by GeneArt). For "prey" vectors, purified cDNA was cloned into HA-tag containing pGADT7-Rec via the SMART III and CDS III anchor system (see S1 B and C). All cloned vectors were checked by DNA sequencing (Eurofins MWG Operon).

2.4. Vectors used for transfection experiments

CD8-stop (human CD8 lacking the cytoplasmic domain) and fusion constructs CD8/CD83 TELV, TELS, and GAAS (human CD8 extracellular and transmembrane domains fused to human CD83 cytoplasmic domain, either wildtype or mutated) were cloned into pEF-CX vector. GRASP55 was cloned into pEF-myc vector (see S2). CD83 full-length constructs, either wildtype or mutants, were cloned into pcDNA3.1 vector (Invitrogen). PIJ-GFP vector was used to assess transfection efficiency. PEF-CX, pEF-myc and pIJ-GFP vectors were kind gifts from A. S. Baur, Department of Dermatology, University Hospital Erlangen.

2.5. Generation of human monocyte-derived DCs (moDCs)

MoDCs were generated as described elsewhere [16]. Immature DCs were generated in presence of 800U granulocyte–macrophage colony stimulating factor (GM-CSF; Peptrotech) ml^{-1} and 250U interleukin-4 ml^{-1} (IL-4; Peprotech). Maturation of DCs was induced by addition of 0.1 ng/ml LPS (Sigma). Cells were routinely checked for their maturation status by flow cytometry. As indicated, cells were treated with 0.5 μg Tunicamycin ml^{-1} (Sigma). Isolation of PBMCs and generation of moDCs from leukapheresis products of healthy donors were approved by the local ethics committee.

2.6. Immunoprecipitation

DCs (12×10^6), 293T cells (1×10^6) or COS-7 cells (2×10^5) were lysed in 1 ml (200 μl for COS-7) lysis buffer (0.5% (v/v) NP-40, 137 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol and 50 mM Tris-HCl pH 8.0; 2 mM phenylmethylsulfonyl fluoride, 20 mM sodium fluoride, 2 mM sodium vanadate) for 1 h with 40C rotation at 4 °C. Cell debris was removed by centrifugation (10 min, 11,000 rpm). Supernatants were pre-cleared by a 1 h incubation with protein A-sepharose beads (CL-4B, GE Healthcare). Supernatants were added to protein A-sepharose beads together with 1 μg (0.6 μg for COS-7) of CD8- (H160; Santa Cruz Biotechnology), CD83- (HB15a; Beckmann Coulter) or IgG2b-specific antibody (isotype control; Biolegend) for overnight immunoprecipitation. Samples were washed three times with Brij-buffer (DCs; 1% Brij, 137 mM NaCl, 2 mM EDTA, 10% glycerol, and 50 mM Tris-HCl pH 8.0) or high salt buffer (293T and COS-7; 1% NP40, 450 mM NaCl, 2 mM EDTA, 10% glycerol, 50 mM Tris-HCl pH 8.0) and resuspended in 2x loading buffer containing 1 mM DTT, 10% SDS and 2.5% β-mercaptoethanol. Samples were denaturated for 10 min at 95 °C. To specifically immunoprecipitate mbCD83, cells were incubated for 2 h in 200 μl PBS containing 0.6 μg anti-CD83 antibody (HB15a; Beckmann Coulter). Cells were washed twice with PBS and lysed in 200 μl lysis buffer for 1 h. Cell debris was removed by centrifugation and supernatants were subjected to immunoprecipitation.

2.7. Generation of whole cell lysates

Cells (2.5×10^5) were lysed in 10 μl lysis buffer for 1 h on ice. Cell debris was removed by centrifugation. Where indicated, lysates were treated with PNGaseF (NEB) following the manufacturer's instructions.

2.8. Immunoblotting

Proteins were separated by SDS-PAGE and subsequently transferred onto nitrocellulose filters (0.2 μm; Protran, Whatman). Membranes were incubated with primary antibodies against CD83, CD8 (F5 and H160, respectively; Santa Cruz Biotechnology), GRASP55 (ProteinTech Group), β-actin (Sigma) as well as against myc- and HA-tag (9E10 and HA.11, respectively; Hiss Diagnostics), followed by HRP-conjugated secondary antibodies (Cell Signaling Technology). Detection was performed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) on a high performance chemiluminescence film (GE Healthcare). Western Blots used for quantification of signal intensities were analyzed using Image Quant LAS4000 (GE Healthcare, Solingen). Quantification was carried out using Bio1D software (Peqlab).

2.9. Cell culture and transfection methods

293T and COS-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Lonza) supplemented with 10% (v/v) fetal calf serum (FCS; PAA) and 1% (v/v) penicillin/streptomycin/L-glutamine (PAA).

COS-7 cells were transfected using jetPEI (Polyplus transfection) according to the manufacturer's instructions. 293T cells were transfected using the calcium phosphate method. 293T cells (1×10^6) were seeded previous day into a 10 cm dish. 31.5 μl 2M $CaCl_2$ solution and 5 μg DNA were adjusted to a total volume of 250 μl by the addition of ddH_2O. 250 μl of 2x HBS buffer (280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM Hepes, pH 7.12) were added, samples mixed and incubated for 5 min at RT. Transfection solution was added drop-wise directly onto the cells. After 18 h cells were harvested and used for further experiments.

2.10. Immunofluorescence confocal microscopy

Immature DCs were allowed to adhere on poly-L-lysin (Sigma) coated glass cover slips, fixed with 3% paraformaldehyde and permeabilized using 1% Triton-X. Blocking was performed using 1% BSA in PBS. Antibodies used for immunofluorescence staining: anti-CD83 (HB15a; Beckmann Coulter; 1 μg/50 μl); anti-GRASP55 (ProteinTech Group; 1 μg/50 μl); Alexa488-, Alexa555-conjugated secondary antibodies (Invitrogen). DAPI (1 μg/ml) was used for nuclear staining. Confocal microscopy was performed using a Leica SP5 microscope (Leica Microsystems).

2.11. Statistics

Prism software was used for statistical analyses (GraphPad). One-way ANOVA with Bonferroni's Multiple Comparison *post hoc* test or students t-test was used as indicated to compare datasets.

2.12. Bioinformatics

CD83 protein sequences, glycosylation sites, disulfide bonds and protein-domains were identified using UniProt database. Protein interaction motifs were identified using Eukaryotic Linear Motif (ELM) database.

2.13. Flow cytometry

Cells were analyzed for CD83-surface expression (anti-CD83 (HB15e), BD Biosciences) using *FACS Scan* (Becton Dickinson) and results were analyzed using *CellQuest* software (BD Biosciences).

3. Results and discussion

3.1. GRASP55 interacts with CD83 in human moDCs

To elucidate potential binding partners of CD83, a Y2H screening was performed. The cytoplasmic CD83-domain was used as “bait” and screened using a cDNA library consisting of cDNA generated from LPS-matured and vanadate/H_2O_2-stimulated DCs. As a result, two GRASP55-specific cDNA fragments, encoding the potential CD83 binding partner, were identified. To confirm GRASP55 as binding partner of CD83, endogenous CD83 was immunoprecipitated from LPS-matured DCs and subsequently analyzed for their GRASP55 expression (Fig. 1A). GRASP55 was detectable in maturing DCs 1 h up to 24 h after LPS treatment, with the strongest signals at 4 h and 6 h. Samples of iDCs (0 h) as well as IgG showed no GRASP55-specific bands. The anti-CD83 immunoblot confirmed successful precipitation of CD83 (Fig. 1A, lower panel). Lysate controls showed equal expression of GRASP55 in immature and maturing DCs, whereas CD83 expression and glycosylation increased during DC maturation (Fig. 1B). The total length of 205 aa results in a calculated molecular weight of 22 kDa. Since CD83 is sequentially glycosylated during DC maturation, its molecular weight ranges between 28 kDa for the initially glycosylated “immature” form (iCD83), to 50 kDa for the terminally glycosylated “mature” form (mCD83) expressed on the surface of mDCs [15,26,27].

Both, Y2H as well as co-immunoprecipitation experiments suggest an interaction between CD83 and GRASP55. To elucidate whether CD83 and GRASP55 co-localize in primary DCs *in vivo*, maturing DCs were subjected to immunofluorescence staining and analyzed using confocal microscopy (Fig. 1C). Immature DCs were treated for 1 h and 2 h with LPS and afterwards stained for GRASP55 (Fig. 1C, green) and CD83 (Fig. 1C, red). Nuclei were stained with DAPI (Fig. 1C, blue). GRASP55 and CD83 co-localize in DCs after 1 h and 2 h LPS treatment. Therefore, these data support the hypothesis that CD83 is indeed transported through the Golgi of maturing DCs by the rather uncommon interaction with GRASP55. Since myeloid DCs isolated from peripheral blood also express CD83 we hypothesize that also in these cells CD83 and GRASP55 will colocalize [28].

3.2. GRASP55 binds CD83 cytoplasmic domain via the TELV-motif

Next, computational *in silico* analyses were performed for an initial validation of the potential interaction between GRASP55 and CD83. Using ELM database, a highly conserved C-terminal valine-bearing motif (TELV) in the cytoplasmic domain of CD83 was identified as a potential binding site for PDZ domains (Fig. 2A). GRASP55 contains two PDZ domains, which mediate homodimerization required to form Golgi structures. These PDZ domains were shown to engage in interactions with membrane proteins bearing C-terminal valine motifs like transforming growth factor α (TGF-α), members of the p24 cargo receptor family and CD8α, suggesting the CD83 TELV-motif, as a GRASP55 binding site [15,18,29,30].

To determine the role of this TELV-motif, several CD83 mutants were generated and their binding to GRASP55 was analyzed in yeast (Fig. 2B). Therefore, “bait” vectors encoding either the wildtype CD83 cytoplasmic sequence (Fig. 2B, sample 2) or a mutated TELS (sample 3) and GAAS (sample 4) variant or without insert (sample 5) instead of the TELV-motif were screened against the “prey” vector containing the GRASP55 cDNA. Binding was monitored using the lacZ reporter gene and the β-galactosidase screening assay. This test revealed a positive interaction signal for the wildtype cytoplasmic CD83-domain, but no interaction for the mutant variants (Fig. 2B). Thus, the single mutation of the C-terminal valine residue in the TELS construct was sufficient to disrupt the CD83/GRASP55 interaction in yeast. These observations are in line with observations made for TGF-α and CD8α, in which the C-terminal valine residue functions as transport signal. Mutation of this residue results in a strong delay in protein trafficking to the plasma membrane or to protein mislocalization [30–32]. In the case of CD8α, mutation of the terminal valine residue resulted in a ~4-fold decrease in the transport kinetics and impaired accumulation of CD8α in the intermediate compartment [33].

To further confirm the CD83/GRASP55 interaction, co-immunoprecipitation analyses were performed. Therefore, 293T cells were transfected with expression vectors coding for GFP, GRASP55 (GRASP55-myc), CD8stop or CD8/CD83 fusion proteins including the wildtype CD83 cytoplasmic domain (CD8/CD83 TELV) or the mutated variants (CD8/CD83 TELS and CD8/CD83 GAAS) (Fig. 2C). Immunoprecipitation was performed using a myc-specific antibody and co-precipitated CD8/CD83 fusion proteins were detected by subsequent immunoblot analyses with a

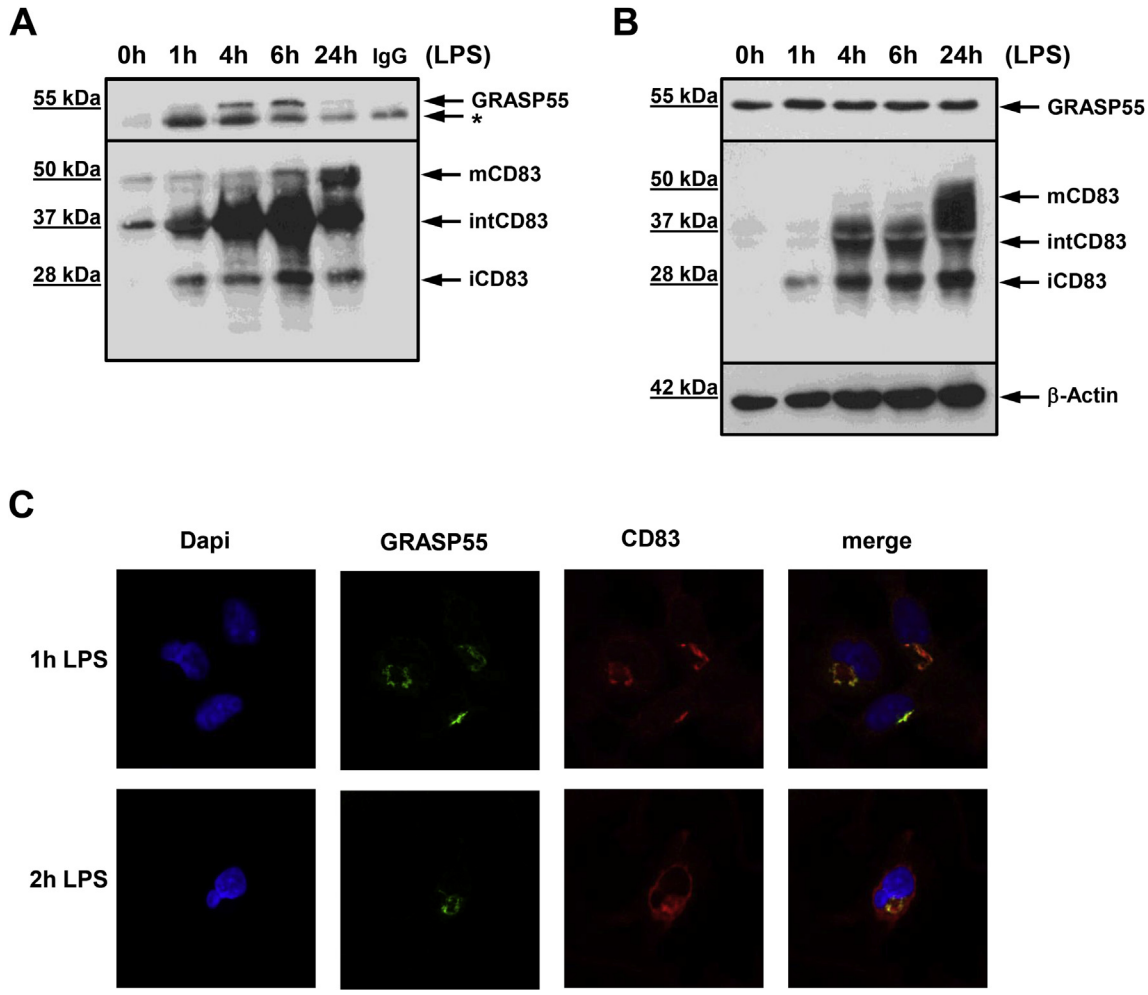

Fig. 1. GRASP55 interacts with CD83 in mDCs. A. Cell lysates obtained from iDCs or 1 h, 4 h, 6 h and 24 h LPS-matured DCs were subjected to immunoprecipitation using an anti-CD83 antibody or the according IgG isotype control (24 h matured DCs) as indicated. Immunoprecipitated proteins were analyzed by immunoblotting using anti-GRASP55 and anti-CD83 antibodies as indicated. *: unspecific band. B. Lysate controls of A. C. Immunofluorescence analyses of LPS-matured DCs. Immature DCs were treated with LPS for 1–2 h. Cells were fixed and stained for GRASP55 (green) and CD83 (red) as well as DAPI (blue). Cells were analyzed using confocal immunofluorescence microscopy.

CD8-specific antibody (Fig. 2C). GRASP55 efficiently co-immunoprecipitated the CD8/CD83 TELV fusion protein, while only very weak signals were obtained with the CD8/CD83 TELS and CD8/CD83 GAAS mutants. Comparable expression of GRASP55-myc and the CD8/CD83 fusion constructs was assessed by lysate controls (Fig. 2D). Thus, using CD8/CD83 fusion proteins we could confirm the TELV-dependent interaction of the cytoplasmic CD83 domain with GRASP55.

3.3. Glycosylation is necessary for efficient CD83 surface expression

Staab and coworkers showed that CD83 is exclusively N-linked glycosylated [27]. Indeed, after PNGase F as well as Tunicamycin treatment only the 22 kDa uCD83 form was detectable (Fig. 3A). Furthermore, cells treated with Tunicamycin displayed a significantly reduced surface expression of CD83 24 h after LPS maturation compared to untreated controls (Fig. 3B). These data lead to the hypothesis that proper glycosylation of CD83 concurred with efficient CD83 membrane expression. As CD83 surface expression correlates with the capacity of mDCs to induce potent T cell responses [7,11] we concluded that proper glycosylation of CD83 is crucial for the biological function of mDCs.

3.4. Mutation of the CD83 TELV-motif impairs its glycosylation and surface expression

These findings together with the known functions of GRASP55 in cargo transport and Golgi structure [18,21,25] suggested a link between TELV-dependent CD83/GRASP55 interaction and CD83 glycosylation as well as membrane expression. To assess the functional relevance of the CD83 TELV-motif for glycosylation and cell surface expression, COS-7 cells were transfected with vectors coding for wildtype full length CD83 or mutated TELS or GAAS variants (Fig. 4A). In whole cell lysates of CD83 of the TELS and GAAS mutants we observed comparatively increased levels of iCD83 (28 kDa) compared to wildtype CD83 (Fig. 4A) indicating that mutant variants express lower levels of mCD83. In addition, when only mbCD83 was immunoprecipitated CD83 wildtype transfected cells showed significantly higher amounts of CD83 on their cell surface compared to cells transfected with CD83 TELS- and GAAS-mutations (Fig. 4B). Furthermore, flow cytometric analyses supported these data since significantly decreased CD83 surface expression levels were observed when CD83 mutants were compared with CD83 wildtype (Fig. 4C). To confirm these observations we quantified the signal intensities of mCD83 in

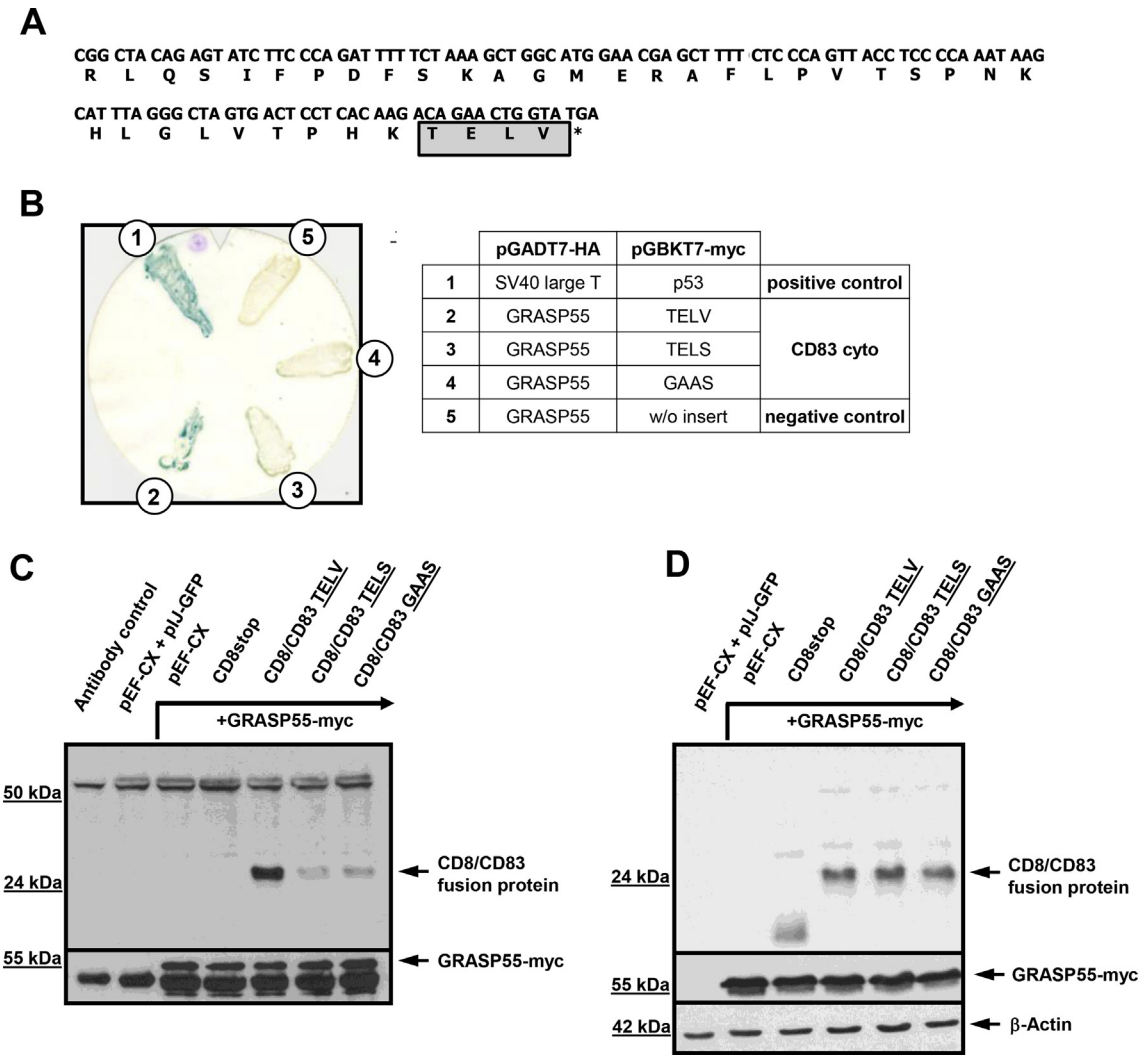

Fig. 2. GRASP55 binds the CD83 cytoplasmic domain via the TELV-motif. A. Amino acid sequence of the CD83 cytoplasmic tail with the predicted TELV-motif B. Reanalysis of CD83-TELV/-TELS/-GAAS variants and GRASP55 interaction in yeast using the lacZ reporter gene. C. Cell lysates obtained from transfected 293T cells were immunoprecipitated with an anti-myc antibody and subsequently analyzed by immunoblotting: lanes 1 and 2 show controls without lysates containing only Sepharose-A beads and the anti-myc antibody to assess unspecific/Ig heavy chain signals (lane 1) and the transfection/negative control with lysate from cells transfected with pIJ-GFP control vector in combination with empty pEF-CX vector backbone (lane 2). Lanes 3 and 4 depict the immunoprecipitation controls with lysates from cells transfected with the GRASP55-myc vector in combination with either pEF-CX empty vector backbone or CD8stop. Lanes 5–7 contain cell lysates from cells co-transfected with GRASP55-myc vector and the CD8/CD83 fusion proteins, either as wildtype variant (TELV; lane 5) or mutated variants (TELS; lane 6 and GAAS; lane 7). SDS-PAGE was followed by immunoblot analyses using an anti-CD8 antibody and subsequently an anti-myc antibody as control for GRASP55-myc expression. D. Lysate controls of C.

relation to the total CD83 signal intensity (Fig. 4D; for quantification strategy see S3). The amount of mCD83 was decreased in CD83 TELS- and CD83 GAAS- mutants when compared to the CD83 TELV-construct, supporting the hypothesis that the mutant variants generate lower levels mCD83. Furthermore, immunefluorescence staining of transfected COS-7 cells indicate less CD83 membrane expression of the TELS and GAAS variants compared to CD83 wildtype (see S4). In conclusion these data strongly suggest that the TELV-motif is necessary for interaction of CD83 with GRASP55 and therefore for proper glycosylation and membrane expression of CD83. These observations are in line with data of Xiang and coworkers which showed that binding to GRASP55 slowed down protein trafficking to ensure complete glycosylation in the Golgi apparatus [34].

Only a few proteins, including TNF-α were so far identified as cargos of GRASP55-containing complexes [30,32]. Both, CD83 and TNF-α transcription are regulated by a multipartite and IRF/NF-κB-dependent promoter complex and in addition nuclear mRNA export is regulated by the CRM1 pathway [16,35,36]. Furthermore, blocking CRM1-mediated nuclear export not only inhibits CD83 expression but also TNF-α expression [18,19]. This special requisite for CD83 glycosylation emphasizes not only its need for strict regulation, but also its potential regulatory relationship to cytokines such as TGF-α.

In summary, this work provides for the first time data regarding the intracellular trafficking and glycosylation of CD83. Y2H screening and co-immunoprecipitation from transfected 293T cells showed a specific interaction between GRASP55 and CD83. In addition, mutation of the cytoplasmic TELV-motif impairs CD83 glycosylation and subsequently CD83 cell surface expression, which might be essential for its biological function. Thus, the relevance of the C-terminal TELV-motif for both, the interaction of CD83 with GRASP55 and the glycosylation-dependent surface expression of CD83, strongly supports a functional role of CD83/GRASP55 interaction in DC maturation and their immune stimulating capacities.

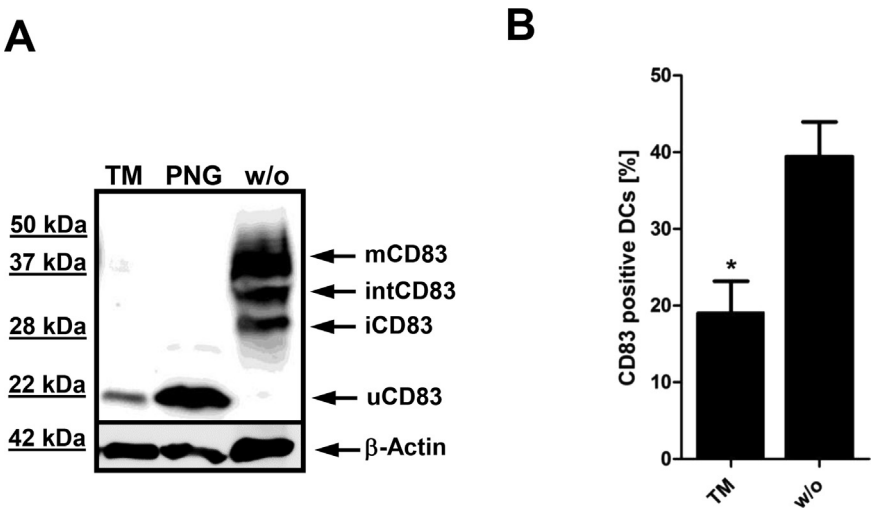

Fig. 3. Glycosylation is necessary for proper CD83 surface expression A. Immunoblot analyses of 24 h LPS-matured mDCs in the presence or absence of Tunicamycin (TM). The second lane shows a lysate control treated with PNGaseF (PNG) and the last lane an untreated lysate control. B. CD83 surface expression on 24 h LPS-matured DCs in the presence or absence of TM. Experiments were performed four times with cells of different healthy donors. *P < 0.05, student's t-test.

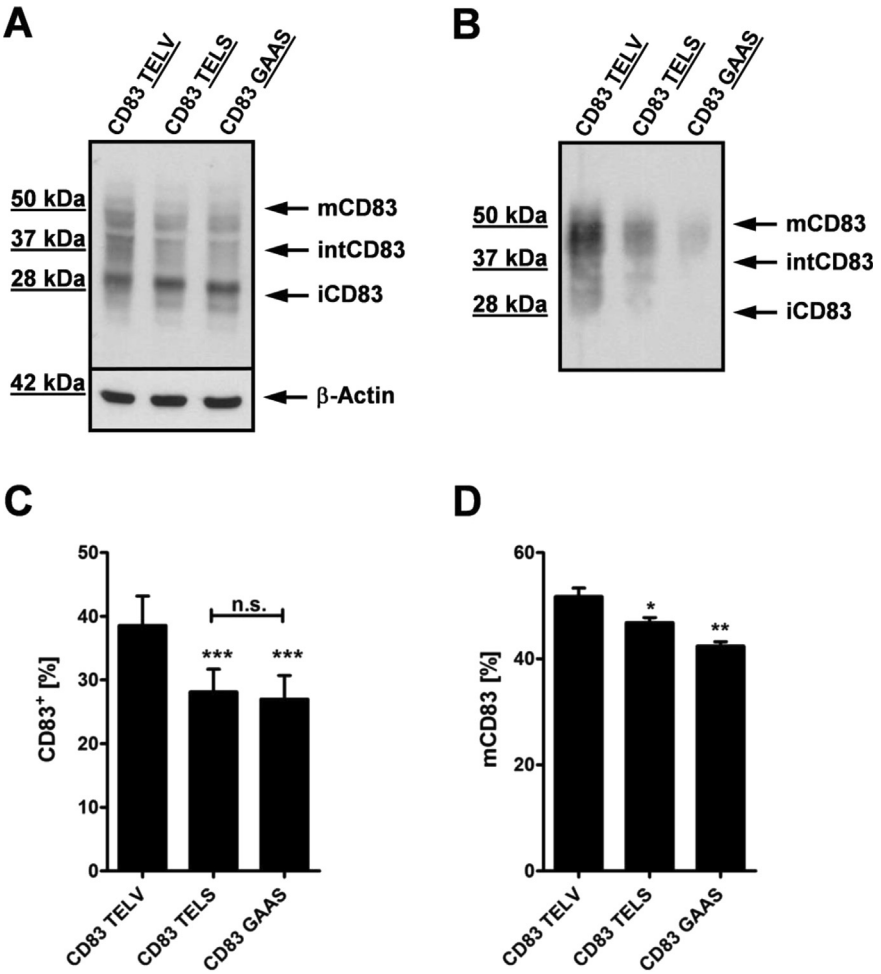

Fig. 4. Mutation of CD83 TELV-motif impairs its glycosylation and surface expression. COS-7 cells were transfected with CD83 (full length) expression constructs, encoding either the wildtype cytoplasmic tail (TELV) or the mutated tails (TELS and GAAS). A. Western blot analyses of whole cell lysates. B. IP of mbCD83. Cells were incubated with a CD83-specific antibody before lysis and immunoprecipitated protein was analyzed for CD83 expression using immunoblotting. C. CD83 surface expression on COS-7 cells (n = 4). D. Quantification signal intensities of A. Depicted is the percentage of mCD83 proportionately to the total CD83 signal intensity (n = 5). ***P < 0.001, **P < 0.01, *P < 0.05; one-way ANOVA with Bonferroni's Multiple Comparison *post hoc* test; mean ± s.e.m.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.057.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.057.

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