**Mutations in the substrate binding site of human heat shock protein 70 (Hsp70) indicate specific interaction with HLA-DR outside the peptide binding groove**[a]

Running title: Mutation of peptide binding site in human Hsp70

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**Abstract**

Heat shock protein 70 (Hsp70):peptide complexes are involved in MHC class I and II-restricted antigen-presentation enabling enhanced activation of T cells. As shown previously, mammalian cytosolic Hsp70 (Hsc70) molecules interact specifically with HLA-DR molecules. This interaction might be of significance as Hsp70 molecules could transfer bound antigenic peptides in a ternary complex into the binding groove of HLA-DR molecules. The present study provides new insights into the distinct interaction of Hsp70 with HLA-DR molecules. Using a quantitative binding assay, it can be demonstrated that a point mutation of amino acids alanine 405 and valine 437 in the substrate binding domain leads to a reduced peptide binding compared to the wild type Hsp70 whereas HLA-DR binding remains unaffected. The removal of the C-terminal lid did neither alter the substrate binding capacity nor the Hsp70 binding characteristics to HLA-DR. Furthermore the truncated ATPase subunit of constitutively expressed Hsc70 revealed similar binding affinities to HLA-DR compared to the complete Hsc70. Thus it can be assumed that the Hsp70:HLA-DR interaction takes place outside the peptide binding groove and is attributed to the ATPase domain of HSP70 molecules. Hsp70-chaperoned peptides might thereby be directly transferred into the binding groove of HLA-DR, thus enabling enhanced presentation of the peptide on antigen-presenting cells leading to an improved proliferation of responding T cells as shown previously.

## Introduction

Heat shock proteins (HSP[b]) have been shown to play crucial roles in a variety of intracellular processes. In addition, important immunological functions in both, innate and adaptive immunity have been reported for most HSP molecules (1). HSP molecules may be released from cells by necrotic cell death, e. g. after infection, inflammation or in tumour cells (2, 3). The released HSP are able to induce cytokine secretion, expression of co-stimulatory molecules and to enhance antigen presentation in antigen-presenting cells (4, 5). Besides these innate HSP-effects, mainly members of the 70 and 90 kDa HSP families (HSP70[c] and HSP90[d]) additionally contribute to the adaptive immune response. These effects are attributed to the ability of HSP70 and HSP90 molecules to interact specifically with a wide variety of other peptides and proteins (6).

HSP70 molecules consist of a nucleotide binding domain with an adenosine triphosphatase (ATPase[e]) subunit and a substrate binding domain which is subdivided in the substrate binding pocket S and the c-terminal lid C. The substrate interaction is ATP-dependant causing a multitude of conformational changes of all subunits (7,8). Extracellular Hsp70:peptide complexes can be taken up into antigen-presenting cells by macropinocytosis or receptor-mediated endocytosis via specific receptors (reviewed in (9)). Former studies have shown that cluster of differentiation 91 (CD91[f]), along with other scavenger receptors, is supposed to be one of these specific receptors (10). The uptake of Hsp70:peptide complexes results subsequently in an enhanced presentation of Hsp70-chaperoned peptides via MHC class I and II, thus inducing improved antigen-specific cytotoxic T cell and $CD4^+$ T cell activation (11-15).

Whilst the HSP-enhanced cross-presentation of MHC class I-restricted epitopes is well investigated, the role in MHC class II-restricted antigen-presentation remains less clear. Nevertheless it becomes more and more obvious that peptides chaperoned by HSP, particularly of the 70 kDa family, are involved in MHC class II-restricted presentation (16). As a pre-condition for facilitated MHC class II presentation of Hsp70-chaperoned peptides, HSP:peptide complexes have to reach the MHC class II loading compartments to be correctly positioned for presentation of chaperoned peptides via MHC class II molecules. As Hsp70:peptide complexes could be detected after receptor-mediated uptake in MHC class II enriched compartments this important requirement is given (17). As a second mechanism, the antigen-presenting cells' own cytosolic HSP molecules may reach the MHC class II presentation pathway in complex with peptides from the cytosol via autophagic processes (18).

In a former study a direct and specific interaction between human Hsp70 and isolated intact HLA-DR molecules could be demonstrated (19). This interaction was increased at lower pH-values, whereas Hsp70-bound peptides were found to be released under these conditions. Additionally, Hsp70:HLA-DR interaction showed a higher affinity compared to normal Hsp70 substrate binding. These findings and the fact that the interaction of Hsp70 and HLA-DR in contrast to normal substrate binding was insensitive to nucleotide addition implicated that other structures than the substrate binding site of Hsp70 contribute to the specific binding of HLA-DR molecules.

The present study investigates the binding characteristics of human purified HLA-DR with recombinantly expressed Hsp70 molecules with either a mutation in the substrate binding cavity or an Hsp70 molecule, lacking the C-terminus which leads to a constitutively open

conformation enabling a fast binding and release of the substrate. Investigating the binding affinity of mutated bacterial Hsp70 homologue DnaK Mayer et al. (20) proved that the amino acids methionin at position 404 and alanine at position 429 are essential for substrate binding. A mutation of valine at position 437 to phenylalanine caused a steric effect completely disabling enzyme-substrate interaction. Methionin at position 404 was proven to be responsible for enclosing the peptide backbone thus influencing the substrate release. The C-terminal subdomain is an α-helical lid covering and opening the substrate binding domain in an ATP-dependant manner. In the absence of ATP, open and closed conformation are in equilibrium, while presence of ATP causes an open conformation and an increase in substrate binding and release rates with lowered substrate affinity (7). Considering these results respective mutations in the substrate binding cavity of Hsp70 for alanine 405 to glycine (mutant A405G) and valine 437 to glycine (mutant V437G) were performed. To investigate the role of Hsp70's C-terminus, a C-fragment shortened Hsp70 molecule (mutant AS-fragment) was produced. Binding assays of the recombinantly expressed Hsp70 molecules were performed with peptides (tetanus toxin $TT_{947-966}$ and hemagglutinin $HA_{307-319}$) and human HLA-DR*04:02. To further investigate the physiological effects of the mutated Hsp70 molecules on the Hsp70-enhanced MHC class II dependant antigen presentation *in vitro*, T cell proliferation assays were performed.

The present studies provide evidence that the HLA-DR binding can be likely referred to the ATPase subunit of Hsp70 and is independent of the substrate binding cavity. Thus Hsp70-chaperoned peptides might be transferred into the binding groove of HLA-DR in a ternary complex, resulting in facilitated antigen presentation and improved proliferation of responding T-cells as demonstrated.

## Materials and Methods

*Antibodies and Proteins*

The monoclonal antibodies to human stress-inducible Hsp70 (ADI-SPA-810) as well as recombinant human stress-inducible Hsp70 (ADI-ESP-555), bovine constitutive Hsc70 (ADI-SPP-751) and ATPase fragment of bovine Hsc70 (ADI-SPP-752) were purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA). Affinity-purified HLA-DR molecules were isolated from HLA-DR homozygous EBV-transformed B-lymhoblastoid cell lines. Cell culture, purification and labelling of HLA-DR molecules was performed as previously described (18, 21).

*Construction of mutant plasmids and expression of proteins*

A pEQ plasmid containing human hsp70-gene (Gene ID: 3303), especially designed for expression in *E. coli* was purchased from Qiagen (Hilden, Germany). Mutagenesis was performed using the Stratagene QuikChange® II XL site-directed mutagenesis kit (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's protocol with the primers 3'-tgggtctggaaacCGGaggtggtgttatgac-5' and 3'-gtcataacaccacctCCGgtttccagaccca-5', causing a mutation of amino acid alanine 405 to glycine (A405G) and the primers 3'-ccagatttttaccACCggctccgataatcagccgg-5' and 3'-ccggctgattatcggagccggTGGtaaaaatctgg-5'causing a mutation of amino acid valine 437 to glycine (V437G). A C-terminal shortened protein, containing only substrate binding domain and ATPase-domain (amino acids 1-1632, AS fragment) of Hsp70 could be designed using primer pair 3'-ataaataCTCGAGcagggcatttttgcgctaacacgttcacgc-5' and 3'-taatattaGGATCCatgaaacaggcaaaagcagcagcaattgg-5' containing a *BamH*I and *Xho*I-restriction site. After amplification in a PCR reaction, DNA was digested, purified using PCR purification kit (Qiagen, Hilden, Germany) and ligated into a pET-28a(+) vector (Merck

KGaA, Darmstadt, Germany). All plasmids were sequenced prior to expression (Agowa, Berlin, Germany).

All proteins (wild type Hsp70, A405G, V437G and AS-fragment) were expressed in *E. coli* BL21 at 22°C and their expression was induced with 0.6 mM Isopropyl β-D-1-thiogalactopyranoside (Peqlab, Erlangen, Germany). Cell pellets from a 200 mL culture were lysed by using BugBuster (Merck) according to the manufacturer's protocol and purified using Ni-nitrilotriacetic acid columns. Fractions containing the protein were pooled and dialysed using Amicon Ultra-15 centrifugal filter units with ultracell-50 membranes (Merck). Proteins were stored at 4°C and used in all experiments for no longer than 7 days.

*SDS-PAGE*

All recombinantly expressed proteins were analysed for size and purity by Coomassie staining in a gel electrophoretic separation. Protein concentration was determined by a BCA Protein Assay (Pierce, Rockford, IL, USA) according to manufacturer's protocol. 200 mL preparations yielded an average amount of 10-12 mg protein total. Protein preparations were mixed with NuPAGE® LDS Sample buffer (Invitrogen, Karlsruhe, Germany). 1 µg of each purified protein was loaded per lane and size-separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) in Tris-HEPES-SDS 4-20% protein gels (Pierce). Coomassie staining was performed using Imperial Stain (Pierce) according to manufacturer's protocol.

*Peptides*

Tetanus toxin $TT_{947\text{-}966}$[g] (FNNFTVSFWLRVPKVSASHL) and influenza hemagglutinin $HA_{307–319}$[h] (PKYVKQNTLKLAT) peptide synthesis was performed on a modified SyRo

multiple peptide synthesizer (MultiSynTech, Witten, Germany) as described previously (19). Biotinylated peptides were specifically labelled at the N-terminus using succinimidyl-6-(biotinamido)hexanoate prior to deprotection and cleavage. Identity and purity of peptides were verified by reversed phase HPLC and electrospray ionization-mass spectrometry (>90 %).

*ELISA anti-Hsp70 monoclonal antibody*

To detect different binding affinities of anti-Hsp70 monoclonal antibody to either the wild type Hsp70 or the mutated molecules, an indirect ELISA was performed. Wells of 96 well FluoroNunc™ Maxisorp microtiter plates (Thermo, Rochester, NY, USA) were precoated overnight at 4°C with Hsp70 molecules (20 µg/mL in PBS). After blocking with blocking reagent (Roti®-block, Carl Roth), proteins were incubated with anti-Hsp70 monoclonal antibody (2 µg/mL) for 1h at 25°C. Wells were extensively washed with PBST (Hank's PBS, PAA, with 0.1% Tween 20, Carl Roth) and treated with 0.02 µg/mL horse radish peroxidase conjugated anti-mouse-IgG monoclonal antibody (0.02 µg/mL, BML-SA-204, Enzo) for another hour at room temperature. Antibody binding was quantified by using TMB substrate (TMB substrate kit, Pierce) according to manufacturer's protocol. Absorbance was read out at 450 nm in a microplate reader (Milenia Kinetic Analyzer, DPC, Los Angeles, CA, USA).

*Hsp70:peptide-binding assays*

Peptide and protein binding were assayed via dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA$^{i}$) (22) using the same conditions as described previously (19). For complex formation Hsp70 molecules (10 µg/mL) and biotinylated peptide (0.01 to 40 µg/mL) were incubated overnight at 37°C in phosphate buffer pH 7.0. 2 mM ATP (Sigma) were added to this assay as indicated to investigate the impact of the ATPase subunit on substrate

binding. 96 well FluoroNunc™ Maxisorp® (Nunc, Wiesbaden, Germany) plates were coated overnight at 4°C with 5 µg/mL anti-Hsp70 mAb (ADI-SPA-810, clone C92F3A-5). Subsequently plates were blocked with blocking reagent Roti®-block (Carl Roth) for 1h. Plates were washed with DELFIA washing buffer (PerkinElmer, Boston, MA, USA), the HSP:peptide mixture was transferred to the microtiter plate and incubated for 1h at room temperature. After another washing step, the biotinylated peptides were incubated with europium-labelled streptavidin in DELFIA assay buffer (PerkinElmer) for 1h. After adding DELFIA enhancement solution fluorescence was detected by time resolved fluorescence measurement at 615 nm with a Viktor 1420 multilabel (PerkinElmer).

*Hsp70:HLA-DR protein-binding assays*

To determine the affinity of Hsp70 to HLA-DR, wild type Hsp70 and its mutated analogues were precoated overnight at 4°C to microtiter plates (20 µg/mL in PBS). After blocking with blocking reagent Roti®-block, the proteins were incubated for 1h with biotinylated HLA-DR*04:02 molecules at a concentration of 6 µg/mL PBS in a total volume of 50 µL/well. The bound HLA-DR molecules were detected in a DELFIA fluorescence assay as described above. To determine the interaction of the ATPase fragment with HLA-DR molecules microtiter plates were precoated with 5 µg/mL Hsp70, Hsc70, ATPase fragment of Hsc70 (Hsc70-ATPf) or human serum albumin (HSA). Binding of 10 nM biotin-labelled purified HLA-DRB1*0401 was analysed in 150 mM phosphate buffer pH 5 (0.05 % Tween-20) and the resulting fluorescence was measured in a DELFIA assay as described above.

*T-cell purification and culture*

A tetanus-preimmunised donor with the HLA-DR haplotype HLA-DRB1*11 was chosen for T-cell proliferation experiments. HLA-DR typing was performed by oligonucleotide typing

(Institute for Clinical and Experimental Transfusion Medicine, Tuebingen). $CD4^+$ T cell purification and irradiation treatment of antigen presenting cells (APC) was performed as described previously (11). All cells were cultured in very low endotoxin-RPMI 1640 medium (Biochrom AG, Berlin, Germany) supplemented with 10% heat inactivated pooled human serum, 2 mM L-Glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 10 mM HEPES buffer (all Biochrom AG, Berlin, Germany).

*Staining and Proliferation*

CFSE staining of $CD4^+$ T cells was performed using CellTrace™ Cell Proliferation Kit (Invitrogen Ltd., Paisley, UK). Cells were incubated with 10 µM CFSE at room temperature for 6 minutes. Staining was quenched by adding 5-fold amount of cold pure fetal calf serum (FCS, Biochrom AG, Berlin) and washing in culture medium. Irradiated antigen presenting cells were incubated with either protein:peptide complexes (0.1 µg/mL Hsp70 molecules/0.01 µg/mL $TT_{947-966}$), or protein or peptide alone, respectively. 2 x $10^5$ stained $CD4^+$ T cells were co-cultured with 5 x $10^4$ irradiated and preincubated APC at 37°C for 7 days. $CD4^+$ T cells were labelled with anti-CD4-PerCP fluorescent dye (BD Biosciences) for 10 minutes at 4°C. Washed cells were analysed on a FACSCalibur flow cytometer (BD Biosciences).

*Statistical Analysis*

All resulting data was subjected to analyses using GraphPad Prism software version 5.04 for Windows (GraphPad Software, San Diego, CA, USA). To determine significant differences (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$) between all test groups and control groups ANOVA analysis of variance and Bonferroni's post test were carried out. Outliers were detected and eliminated due to Grubb's outlier test.

**Gene bank accession number:**

**3303 (M11717)** HSPA1A Heat shock 70kDa protein 1A (*Homo sapiens*)

## Results

*Mutation of the substrate binding cavity of Hsp70*

As a binding of Hsp70 to HLA-DR independent of the substrate binding is expected, a mutation in the substrate binding cavity supposedly causing a reduction of substrate binding was performed on His-tagged HSP70A1A-DNA. Mutation was carried out using the Stratagene QuikChange® Mutagenesis Kit XL-II (Agilent Technologies). Substrate binding amino acids in the binding cavity, alanine 405 and valine 437 (23), were replaced by glycine. The resulting mutated proteins were called A405G and V437G, respectively. To narrow down the possible binding site of HLA-DR to Hsp70 a C-terminal shortened protein, consisting of amino acids 1-1632 only, was expressed. This truncated protein, further called AS-fragment, lacks the C-terminal lid but expresses the ATP-binding domain as well as the substrate binding domain. The lack of the C-terminal lid results in an ATP-independent all-open conformation of the protein thus enhancing binding and dissociation of peptides.

To ensure a high purity and correct expression of both wild type Hsp70 and mutated Hsp70 proteins, pooled and washed elution fractions were separated on an SDS-PAGE and stained with Coomassie. Figure 1 demonstrates protein bands at a size of approximately 70 kDa for wild type Hsp70 A1A and mutated A405G and V437G. C-shortened AS-fragment with a calculated size of 55 kDa shows a protein band at a size of about 60 kDa.

*Anti-Hsp70 mAb binding to mutated Hsp70 molecules is not affected*

Prior to Hsp70-peptide fluorescence assays it was analysed whether the mutation of the substrate binding site of Hsp70 affects the binding affinity of mAb anti-Hsp70 to Hsp70 molecules. Figure 2 displays the results of an indirect ELISA with anti-Hsp70 mAb (ADI-SPA810) and wild type Hsp70 A1A, mutated A405G and V437G as well as C-shortened AS-

fragment. All tested Hsp70 molecules showed no significantly different ($p>0.05$) binding affinity to anti-Hsp70 mAb.

*Substrate binding of mutated A405G and V437G is reduced*

Binding capacity to $HA_{307-319}$ and $TT_{947-966}$ was tested for all Hsp70 molecules. For that purpose, anti-Hsp70 mAb was precoated to microtiter plates. Preincubated Hsp70:peptide complexes or Hsp70 molecules alone were added and incubated for one hour. Peptide binding was evaluated in a DELFIA. As displayed in figure 3 substrate binding of biotinylated $TT_{947-966}$ was significantly reduced for mutated A405G and V437G ($p<0.001$) but not for AS-fragment ($p>0.05$) compared to substrate binding of wild type Hsp70 A1A. Addition of 2 mM ATP during preincubation of Hsp70 molecules and peptide could significantly reduce the substrate binding of all Hsp70 molecules ($p<0.001$). A similar outcome of the binding assay could be observed for biotinylated $HA_{307-319}$ showing reduced binding capacity for A405G and V437G compared to wild type A1A. Binding capacity of AS-fragment to $HA_{307-319}$ was not reduced but even increased. Additionally Hsp70:$TT_{947-966}$ binding affinity was analysed in saturation experiments. Figure 4 shows titration curves for A1A, A405G, V437G and AS with increasing amounts of $TT_{947-966}$. Wild type Hsp70 A1A and AS-fragment revealed similar titration curves whereas titration curves for mutant A405G and V437G where significantly different from wild type Hsp70 A1A ($p<0.001$).

*HLA-DR binding is not affected by mutation of the substrate binding site*

To investigate the impact of the mutation of the substrate binding site on the binding affinity of Hsp70 to HLA-DR molecules, an indirect ELISA was performed. Therefore, Hsp70 molecules were precoated to microtiter plates and subsequently incubated with biotinylated HLA-DRB1*0402. HLA-DR binding was detected in a DELFIA fluorescence assay. Figure 5

demonstrates equal HLA-DR binding characteristics to both wild type Hsp70 and mutated Hsp70 molecules ($p>0.05$). These experiments indicate an interaction of HLA-DR and Hsp70 independent of conformational changes in the substrate binding cavity.

*Specific Hsp70-binding to HLA-DR molecules is mediated by the ATPase subunit of Hsp70*

So far, the HLA-DR:Hsp70 interaction outside of the substrate binding domain of Hsp70 could be attributed as the HLA-DR binding is not influenced by a lack in substrate binding or a split-off of the C-terminal lid. With a quantitative binding assay (DELFIA) a specific interaction between human Hsp70 and Hsc70 molecules and different allelic variants of purified and recombinant HLA-DR molecules could be demonstrated (19). This specific interaction was demonstrated for both, the constitutively expressed (Hsc70) and the stress-inducible (Hsp70) form of cytosolic Hsp70 molecules. The interaction was saturated and could be competed by unlabelled molecules. $K_D$ values for the Hsp70:HLA-DR interaction were calculated from the experiments and were found to be in the low nM range (e.g. 26.6 nM for the interaction of Hsp70 with HLA-DRB1*0401). The interaction was not sensitive to the addition of ATP. Together with the findings demonstrated above, it can be assumed that regions other than the peptide-binding domain of the Hsp70 molecules are involved in the Hsp70:HLA-DR interaction. In order to clarify which subunit of the Hsp70 molecule mediates the HLA-DR binding, binding experiments using both the N-terminal ATPase fragment of Hsc70 protein and complete Hsc70 were performed. Figure 7 shows the interaction of purified HLA-DRB1*0401 to Hsp70, Hsc70 and the ATPase-subunit of Hsc70 (Hsc70-ATPf). Similar results were obtained with different other allelic variants of purified HLA-DR molecules (HLA-DRB1*0801 and HLA-DRB1*0402, data not shown). In accordance with our previous findings, a slightly higher HLA-DR affinity was found for Hsp70 as compared to Hsc70. Interestingly, a significant differences ($p>0.05$) in signal

strength between complete Hsc70 and the truncated Hsc70 ATPase-fragment lacking the peptide-binding domain with any of the allelic HLA-DR variants could not be detected. This finding suggests that the interaction with HLA-DR is mediated by the ATPase subunit of Hsp70.

*Mutation of the substrate binding site influences the Hsp70:peptide-mediated MHC class II dependant antigen presentation*

As shown previously (11), peptide-complexation to Hsp70 enhances the proliferation of human $CD4^+$ T cells compared to antigenic peptide alone. As demonstrated in this study, mutations of recombinantly expressed Hsp70 in the substrate binding domain reduces peptide binding, but does not affect the adhesion of HLA-DR. The influence of these mutations on the Hsp70-facilitated antigen presentation of the antigenic tetanus peptide $TT_{947-966}$ was therefore further investigated. As the recombinantly expressed Hsp70 molecules are of bacterial origin, the protein-preparations were purified as described previously (11) and cell cultures were incubated in very low endotoxin media. $CD4^+$ T cells from a healthy and preimmunised HLA-DR1*11 donor were stimulated with $TT_{947-966}$ or Hsp70 molecules alone or Hsp70:$TT_{947-966}$ complexes, respectively. As shown in figure 6, stimulation with peptide or Hsp70 molecule alone resulted in low T cell proliferation. Similar proliferation could be obtained with mutant Hsp70:$TT_{947-966}$ complexes, comprising A405G, V437G and AS fragment. However, proliferation of T cells could be significantly enhanced by respective stimulation with complexed wild type Hsp70:$TT_{947-966}$ as expected.

**Discussion**

In this study HSP70 interactions were investigated by point mutations in the substrate binding site allowing insights into the HSP70:peptide and the HSP70:HLA-DR interplay. In earlier studies an interaction of Hsp70 and HLA-DR independent of a peptide binding could be demonstrated (19).

Members of the HSP70 family consist of an N-terminal ATPase unit of 45 kDa and a C-terminal substrate binding domain of 25 kDa which is further subdivided into a β-sandwich subunit of 15 kDa and a C-terminal alpha-helical subdomain. The ATPase subunit and the substrate binding domain are linked by a short α-helical linker sequence (hinge) (23). HSP70 molecules undergo conformational changes during the binding and release of substrate (3, 7, 8, 20, 21, 23-26). Substrate exchange at the substrate binding cleft on the β-sandwich subunit is thereby facilitated or handicapped by the α-helical subdomain of the substrate binding domain. This subdomain forms a lid that covers the peptide binding site and opens and closes in an ATP-dependant manner.

As an interference of HLA-DR binding with the Hsp70:peptide binding could be excluded, we postulate an interaction of HLA-DR and Hsp70 independent of the Hsp70 substrate binding domain. This hypothesis could be affirmed by the findings that the point mutations alanine 405 to glycine and valine 437 to glycine resulted in a significant reduction of the substrate binding affinity whereas HLA-DR binding remained unaffected. In contrast to the results of Mayer et al. (20) with altered bacterial Hsp70 homologue DnaK, a complete lack of peptide binding could not be observed for mutant V437G which might be due to the fact that a steric effect cannot be achieved by replacement of valine to the much smaller glycine. For the C-terminal shortened AS-fragment, the substrate binding domain remains unaffected but

due to its lacking covering lid presents itself in an all open conformation in contrast to wild type Hsp70 with a randomly distributed (1:1) open or closed covering lid. A similar or even slightly higher substrate binding affinity could be observed for AS compared to wild type Hsp70. This might be in consequence of the all open conformation allowing a statistically facilitated enzyme-substrate interaction. Substrate affinity of human Hsp70 to peptide fragments both $TT_{947-966}$ and $HA_{307-319}$ has already been approved in our previously performed assays (11). The ATPase subunit of HSP70 proteins is involved in the binding and release of substrates. Addition of ATP not only leads to an all open conformation of the covering lid of the substrate binding domain but also increases substrate binding and release rates due to diverse conformational changes (7). It could be demonstrated that ATP leads to a distinct reduction of peptide binding. Interestingly, the C-terminal shortened AS-fragment, which lacks the covering lid, also revealed a reduced binding of both $HA_{307-319}$ and $TT_{947-966}$ in contrast to incubation without ATP. Therefore we conclude that the C-terminal lid does not primarily regulate the substrate access to the substrate binding cavity but probably exerts influence on substrate release.

Substrate binding of Hsp70 does not influence the binding of HLA-DR (19). Hence we hypothesize that the interaction of HLA-DR and Hsp70 takes place outside the peptide binding domain. Our findings demonstrated in this study affirm this hypothesis. Point mutations of the substrate binding cavity A405G and V437G did not interfere with HLA-DR binding giving evidence that amino acids 405 and 437 are not involved in HLA-DR binding. Furthermore, it could be demonstrated that the removal of the C-terminal lid did not influence the HLA-DR interaction either, indicating that HLA-DR binding is neither located in the substrate binding cavity nor the C-terminal lid. Low binding activity of tetanus toxin $TT_{947-966}$ to plate bound HLA-DR*04:02 is considered as unspecific binding to the microtiter plate.

A truncated recombinant ATPase-subunit of bovine constitutively expressed Hsc70, which is 99% homologue to the human Hsp70 ATPase fragment, was used to further confine the binding area of HLA-DR. The ATP-fragment is 541 amino acids long. Only 6 amino acids are changed within human and bovine Hsp70 ATPase fragment. According to the manufacturer, this fragment maintains its ATPase activity, whereas the C-terminus and the entire peptide-binding domain is lacking. In binding assays comparable binding affinities of HLA-DR molecules to this ATPase-fragment and the complete Hsc70 molecule could be detected. Unfortunately, isolated human Hsp70 ATPase-fragment is neither commercially available nor able to be expressed in a correct folding manner. The results of bovine ATPase-fragment further endorse that the HLA-DR binding site can be ascribed to structures on the N-terminal ATPase domain of the HSP70 molecules.

In T cell proliferation assays Hsp70 enhanced stimulation of $CD4^+$ T cells with tetanus toxin fragment $TT_{947\text{-}966}$ could be confirmed using wild type Hsp70:$TT_{947\text{-}966}$ complexes. Stimulation with peptide alone, Hsp70 molecules alone or preincubations of $TT_{947\text{-}966}$ with mutated Hsp70 molecules induced similarly low proliferation of $CD4^+$ T cells. Considering the amount of bound peptide as a pivotal factor we would have expected that the stimulation with AS:$TT_{947\text{-}966}$ would result in a similar proliferation effect as complexed wild type Hsp70 A1A:$TT_{947\text{-}966}$. Unexpectedly, the impact of AS:$TT_{947\text{-}966}$ on T cell proliferation was not significantly different from the control groups or the incubations with peptide complexed to A405G and V437G. This effect might be due to the physiological dependencies on Hsp70-uptake into $CD4^+$ T cells, implying that C-fragment shortened AS cannot be bound by Hsp70 specific receptors such as CD91 and diverse scavenger receptors (10).

Taken together, the results of this study affirm HLA-DR binding to Hsp70 outside the substrate binding groove. The mechanism proposed from our data for the Hsp70:HLA-DR interaction might allow that Hsp70-chaperoned peptides are transferred in a ternary complex directly into the binding groove of HLA-DR resulting in enhanced HLA-DR presentation of the peptide.

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**Footnotes**

[a] This work was supported by the Deutsche Forschungsgemeinschaft (DFG HO 2340/3-1) and SFB 685.

[b] HSP: Heat shock proteins

[c] HSP70: Heat shock proteins of the 70 kDa family

[d] HSP90: Heat shock proteins of the 90 kDa family

[e] ATPase: adenosine triphosphatase

[f] CD: cluster of differentiation

[g] $TT_{947-966}$: Tetanus toxin fragment FNNFTVSFWLRVPKVSASHL

[h] $HA_{307–319}$: Hemagglutinin fragment PKYVKQNTLKLAT

[i] DELFIA: dissociation-enhanced lanthanide fluorescent immunoassay

## Figure legends

### Figure 1. Coomassie Staining of Hsp70 purification.

SDS-PAGE analysis of .purificated recombinantly expressed Hsp70 molecules wild type Hsp70 A1A, A405G and V437G with mutated substrate binding site and C-fragment shortened AS. Coomassie Staining was performed after gel electrophoresis. A1A, A405G and V437G show protein bands at approx. 70-72 kDa, whereas AS is detectable at approx. 60 kDa. One of three independent experiments with similar outcome is represented.

### Figure 2. Binding affinity anti-Hsp70 mAb to Hsp70 molecules

Binding affinity of Hsp70-molecules to anti-Hsp70 monoclonal antibody was detected in an indirect ELISA. Microtiter plates were precoated with Hsp70 molecules A1A, A405G, V437G and AS (20 μg/mL) and subsequently incubated with anti-Hsp70 mAb (2 μg/mL). Bound anti-Hsp70 mAb was detected with HRP-conjugated secondary antibody. Binding affinity of the mutated Hsp70 molecules A405G, V437G and AS to anti-Hsp70-antibody was not significantly different ($p>0.05$) from wild type Hsp70 A1A. Sample size n=5. Data show mean + standard deviation (SD). a: significantly different to blank; $p<0.001$; ANOVA analysis of variance.

### Figure 3. Peptide binding is reduced for A405G and V437G.

Overnight precoating with 5 μg/mL anti-Hsp70 antibody was followed by either incubation with Hsp70 molecules (10 μg/mL) alone or preincubated Hsp70-peptide (10 μg/mL:20 μg/mL) complexes. Biotinylated peptides $TT_{947-966}$ (**A**) or $HA_{307-319}$ (**B**) were detected via DELFIA fluorescence assay. Additionally, Hsp70-peptide binding was measured in the presence or absence of 2 mM ATP as indicated. Experiments were carried out in triplicates

and represent one of four independent experiments with similar outcome. Data show mean + standard error of the mean (SEM) of triplicates. *** $p<0.001$, a: $p<0.001$ compared to respective incubations without ATP; ANOVA analysis of variance.

**Figure 4. Titration curves for Hsp70:$TT_{947-966}$.**

Overnight precoating with 5 µg/mL anti-Hsp70 antibody was followed by incubation with preincubated Hsp70-$TT_{947-966}$ complexes. A1A, A405G, V437G and AS (10 µg/mL) were incubated with increasing amounts of biotinylated $TT_{947-966}$ (0.01 µg/mL to 40 µg/mL). Bound $TT_{947-966}$ was detected via DELFIA fluorescence assay. Sample size n=3. Experiments were carried out in triplicates. Data show nonlinear regression. ***: $p<0.001$; n.s.: not significant.

**Figure 5. Binding of Hsp70 and A405G to HLA-DR*0402 is not reduced.**

Microtiter plates were precoated with Hsp70 molecules (20 µg/mL) or $TT_{947-966}$ (40 µg/mL). Biotinylated HLA-DRB*0402 (6 µg/mL) was added and incubated for one hour. Bound HLA-DRB*0402 was detected in a DELFIA assay. Binding affinity of the mutated Hsp70 molecules A405G, V437G and AS to HLA-DR*0402 was not significantly different ($p>0.05$) from wild type Hsp70 A1A. Sample size n=7. Data show mean + SD. *:$p<0.1$, a: significantly different ($p<0.001$) compared to blank and $TT_{947-966}$-control; ANOVA analysis of variance.

**Figure 6: T-cell proliferation with Hsp70 molecules and $TT_{947-966}$.**

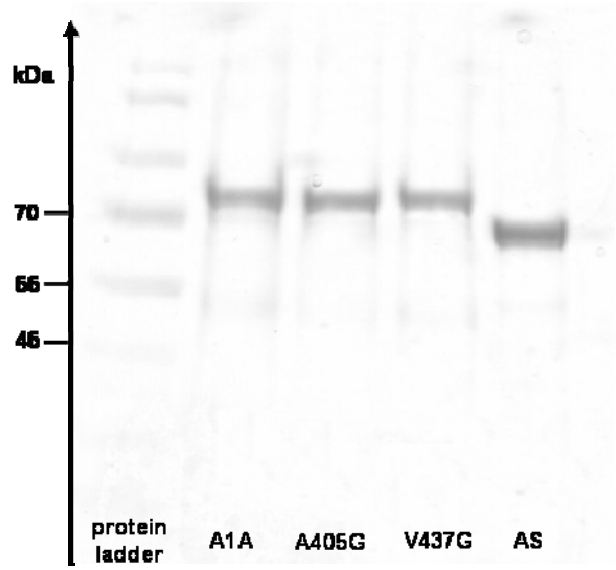
Purified $CD4^+$ T cells were incubated for 7 days with irradiated APC. Prior to co-incubation APC were incubated with (**A**) Hsp70:$TT_{947-966}$ complexes (0.1 µg/mL:0.01 µg/mL), (**B**) Hsp70 molecules (0.1 µg/mL) or (**C**) $TT_{947-966}$ (0.01 µg/mL). $CD4^+$ T cells were stained with

anti-CD4 antibody and CFSE dilution in $CD4^+$ T cells was measured in a flow cytometric analysis. (**D**) Proliferation of $CFSE^{low}CD4^{+high}$ cells after treatment with Hsp70:peptide-complexes was compared to control groups $TT_{947-966}$ and Hsp70 molecules alone. Sample size n=4. Data show mean + SD. ***: $p<0.001$; **: $p<0.01$; a. significantly different compared to peptide alone, $p<0.001$; b: significantly different compared to respective Hsp70-molecule without peptide, $p<0.001$; ANOVA analysis of variance.

**Figure 7. ATPase domain of HSP70 takes part in the HLA-DR:HSP70 interaction.**

Microtiter plates were precoated with 5 µg/mL Hsp70, Hsc70, ATPase fragment of Hsc70 (Hsc70-ATPf) or human serum albumin (HSA). Binding of 10 nM biotin-labelled purified HLA-DRB1*0401 was analysed in 150 mM phosphate buffer pH 5 (0.05 % Tween-20) as described in the materials and methods section. Non-specific binding represents binding of biotinylated HLA-DR to the blocking agent bovine serum albumin. Experiments were carried out in triplicates and represent one of at least three independent experiments with similar outcome. Data show mean + SEM of triplicates. n.s.: not significant; a: significantly different from both HSA and non-specific binding, $p<0.01$; ANOVA analysis of variance.

**Figure 1.**



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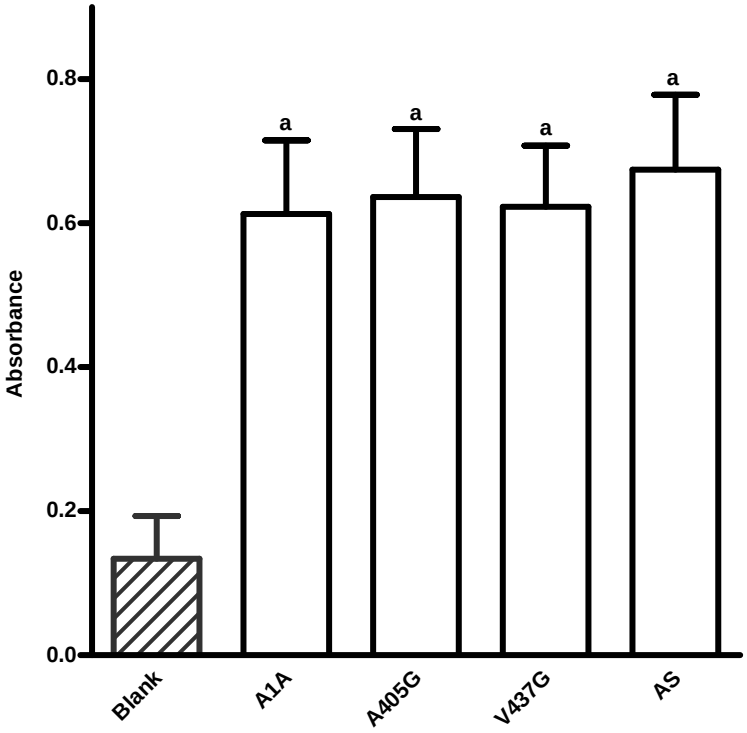

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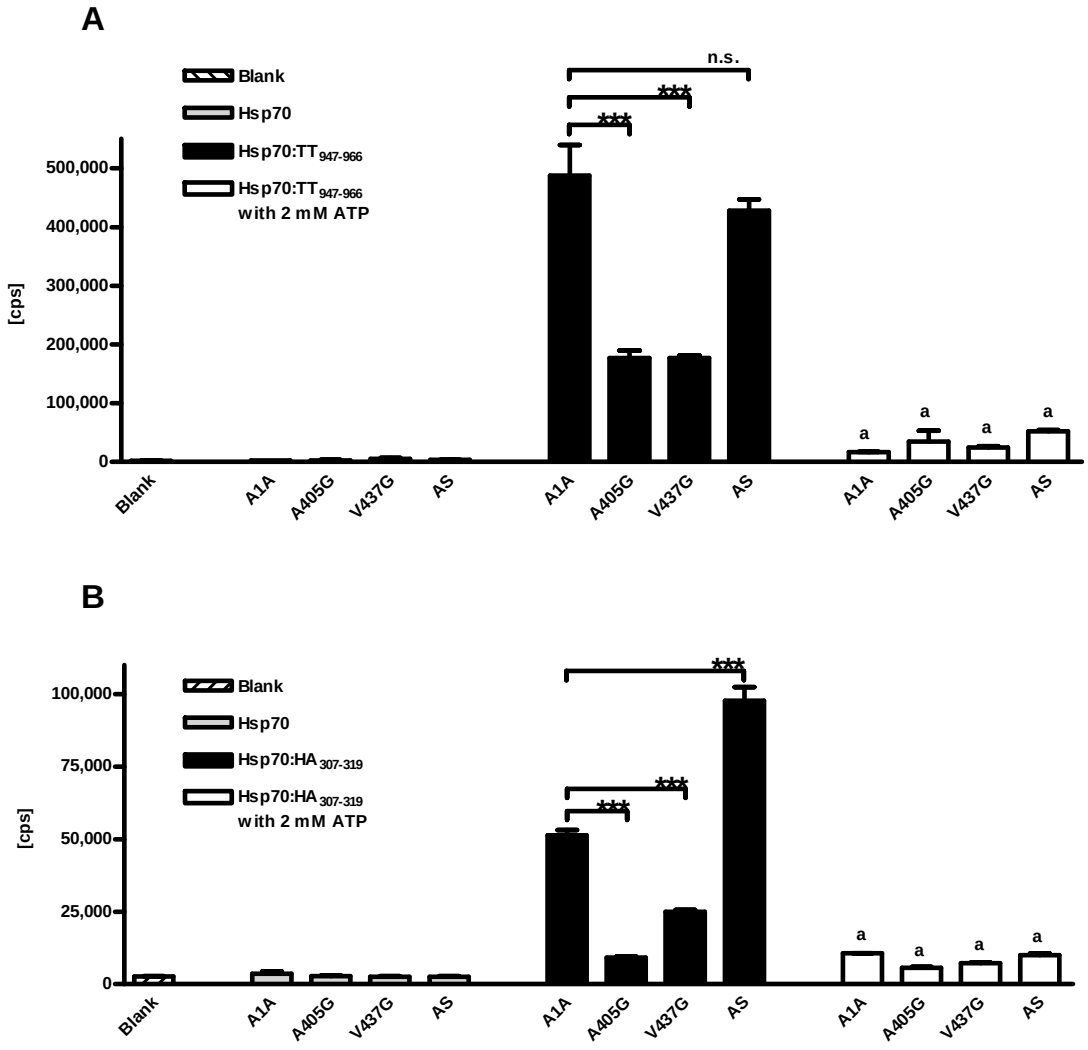
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**Figure 4.**

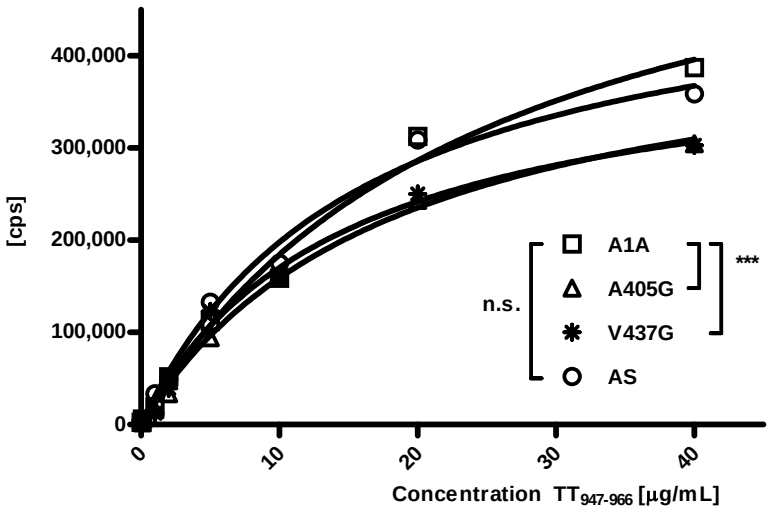
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**Figure 5.**

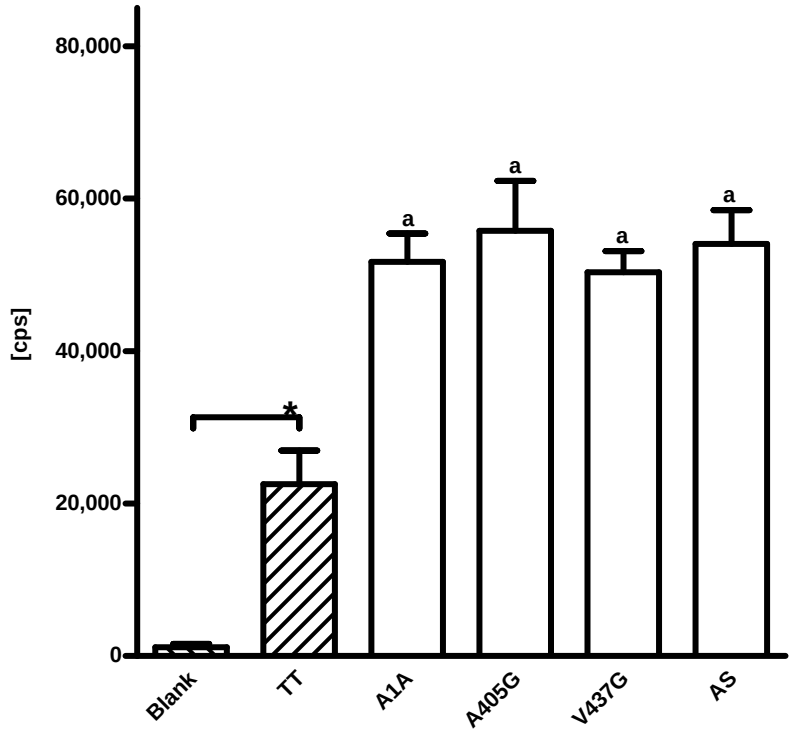
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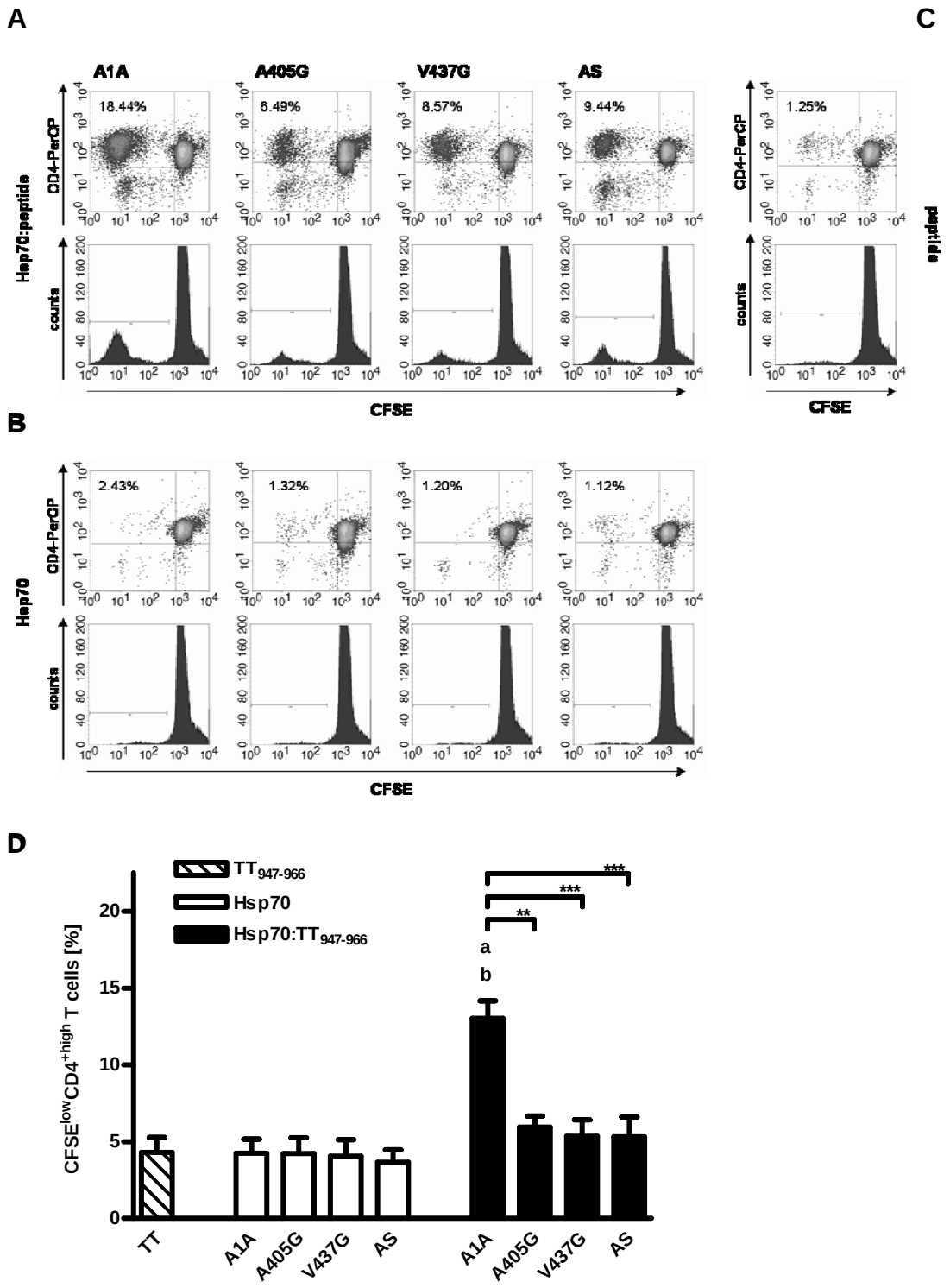
**Figure 6.**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D
TT947-966
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CFSElowCD4+high T cells [%]
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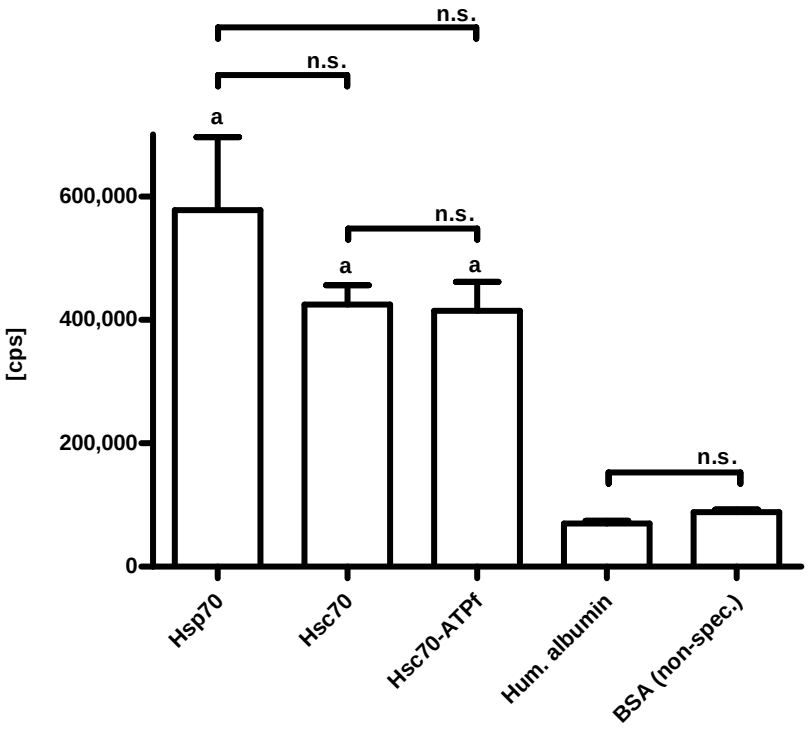
**Figure 7.**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