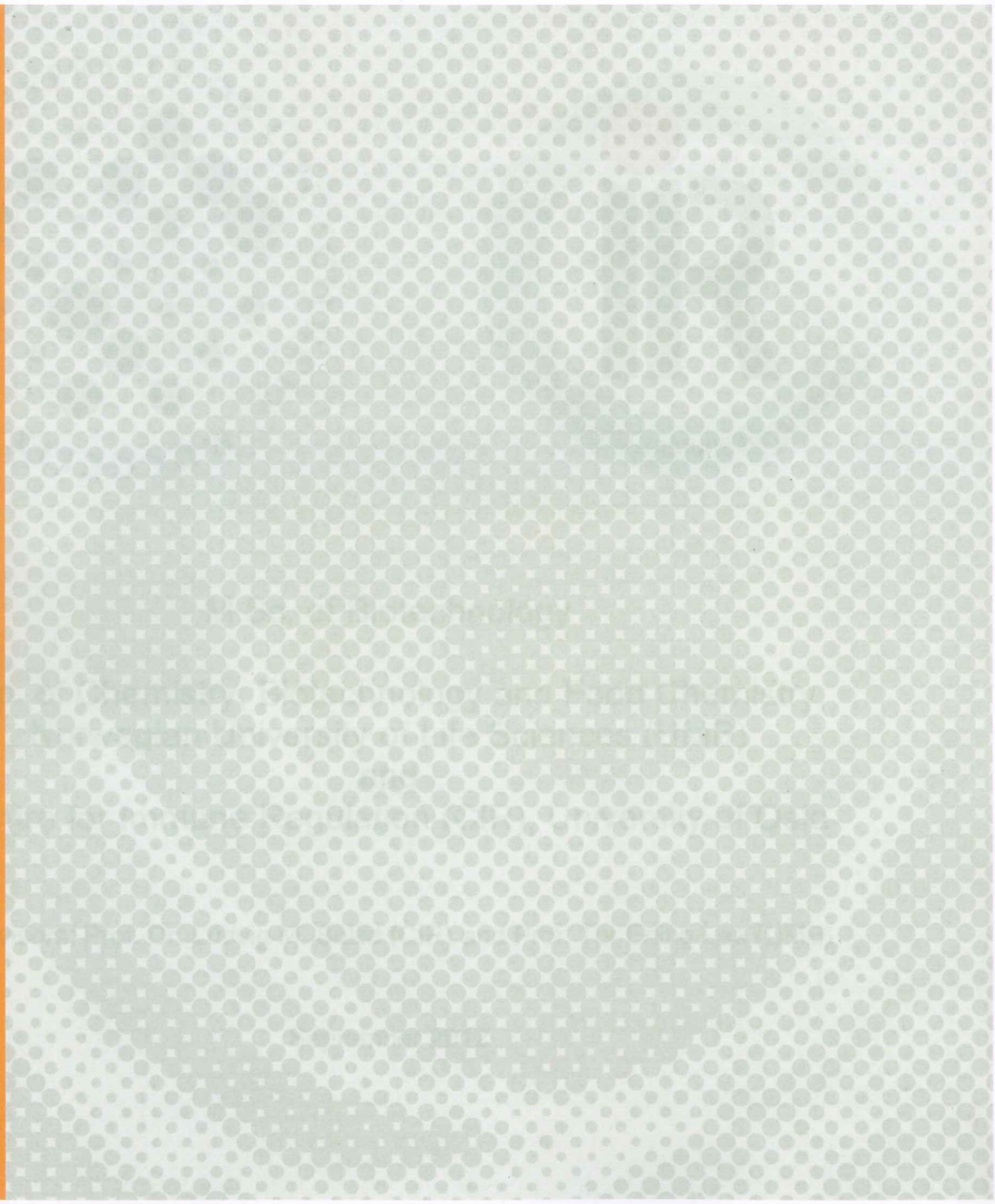
Generation of MHC II Streptamers for gluten-specific CD4+ T cells

Louise Fremgaard Risnes



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Master Thesis
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Abstract

Coeliac disease is a chronic inflammatory disorder of the small intestine due to inappropriate immune responses to gluten. Disease-associated HLA-DQ2.5 molecules are complexed with deamidated gluten epitopes displayed on antigen-presenting cells and presented to $CD4^+$ T cells. The investigation of T cells and their interaction with MHC molecules has revolutionised the research of cell-mediated immunology by the multimer technology. This technology was developed by Altman et al. in 1996. Gluten-specific $CD4^+$ T cells have been detected and identified by a similar MHC II tetramer technology. Soluble recombinant HLA-DQ2.5 molecules with covalently linked epitope peptides are expressed in the baculovirus expression vector system and purified in their folded state. Biotinylated MHC II reagents are multimerised on streptavidin prior to T-cell staining in flow cytometry.

A reversible multimer technology based on the tetramer technology and the Strep-tag:streptavidin interaction has been established for MHC class I molecules and $CD8^+$ T cells. Basic principle of the streptamer technology is that multimerised MHC molecules on streptavidin (Streptactin) are left on the T-cell surface as monomers in the presence of d-biotin. D-biotin binds to streptavidin and leads to dissocation. Hence, the interaction of the Streptag and Streptactin is reversible. This technology is also basis for a recently developed k_{off}-rate assay that measures the dissociation of monomeric MHC molecules from TCR. To date, there are no publications on the so-called Streptamer technology applied on MHC class II molecules and antigen-specific $CD4^+$ T cells.

Here, we have generated recombinant, soluble HLA-DQ2.5 molecules fused with the affinity peptide StreptagII. Based on the well-established recombinant DQA and DQB gene sequences in the Sollid lab, the BirA site was replaced with a tandem repeat of the StreptagII sequence (StreptagIII) and a dye conjugation site at the C-terminus of the β chain. Dye-conjugated MHC II can therefore be observed in the absence of the Streptactin backbone. The new MHC II multimer reagents (Streptamers) were quality checked prior to flow cytometry. They were tested in flow cytometry to verify specific staining to gluten-reactive $CD4^+$ T-cell clones (TCCs). Two specific HLA-DQ2.5-Streptag constructs were made. One contruct demonstrated positive staining of the majority of the cognate DQ2.5-reactive TCCs while the other construct showed variable staining signals. Possible interference with the dye

conjugation was considered. Subsequently, TCCs that showed high staining with the dye-conjugated Streptamers were tested in the human k_{off}-rate assay in Munich.

The performance of the k_{off}-rate assay did not give any useful data, but problems were addressed and investigated in order to improve the MHC II reagents. These variable findings remain to be elucidated and must be further investigated. The Streptamer-positive TCCs remain to be retested in the k_{off}-rate assay.

List of Abbreviations

AP	Alkaline phosphatase
APC	Allophycocyanin
APC	Antigen-presenting cell
APS	Ammoniumpersulfate
β_2m	Beta-2-microglobuline
BEVS	Baculovirus expression vector system
BSA	Bovine serum albumine
C-terminus	Carboxyl terminus
CD	Cluster of differentation
CD	Coeliac disease
CMV	Cytomelagovirus
CDR	Complementary determining region
CIP	Calf intestine phosphatase
CLIP	Class II-associated invariant chain peptide
CPM	Counts per minute
CTL	Cytotoxic T lymphocyte
DC	Dentritic cell
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOL	Degree of labelling
EBV	Epstein-Barr virus
EDTA	Ethylendiaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluoresceinisothiocyanate
FPLC	Fast protein liquid chromatography
GAM	Goat anti-mouse
GFD	Gluten-free diet
Glia	Gliadin
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
kDa	Kilodalton
LB	Luria Bertoni medium
M	Molar
MALDI	Matrix-assisted laser desorption ionisation
MHC	Major histocompatibility complex
MS	Mass spectrometry
MQ	Milli-Q
N-terminus	Amino terminus
OD	Optical density
P	Passage
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell

PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PFU	Plaque-forming units
pMHC	Peptide-loaded MHC
RBC	Red blood cell
RPMI	Roswell Park Memorial Institute
RPM	Revolutions per minute
RT	Room temperature
SDS	Sodiumdodecylsulfate
SD	Standard deviation
Sf	*Spodoptera frugiperda*
SPR	Surface plasmon resonance
TBE	Tris/Borate/EDTA buffer
TOF	Time-of-flight
TCR	T-cell receptor
TEMED	Tetramethylethylendiamine
tTG2	Tissue transglutaminase 2

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1 Introduction

1.1 The Immune System

We are continuously exposed to the microbial world of bacteria, virus, fungi and parasites of which many can cause disease due to their pathogenic features. There is an ongoing battle between the pathogenic microorganisms and the immune system. As they have encountered one another during evolution, their battle strategies have co-evolved. A well-functioning immune system has the ability to distinguish self from non-self and has to protect the body from malignant growth. Pathogenic microbes are considered as non-self, so are food-derived antigens. Thus, the immune system must also distinguish harmful microbial antigens from innocent food antigens. During evolution, there has also been developed non-harmful or beneficial relationships between human and bacteria. In fact, there are ten times more commensal bacteria than human cells in the gastrointestinal tract (1). The immune system has therefore an important regulatory task in order to maintain a normal gut flora.

The human immune system is highly sophisticated and tightly regulated network consisting of different units of lymphoid organs, tissues, cells and molecules throughout the body. In general, the immune system can be divided into the innate and adaptive immune systems. The innate system functions as second line of defense and comes into action when foreign invaders cross the physical epithelial barrier. This barricade serves as first line of defense. Cells of the immune system are known as white blood cells (leukocytes) and originate from hematopoietic stem cells (HSCs). HSCs differentiate into lymphoid and myeloid precursors. Myeloid precursor lineage differentiates into innate immune cells, e.g. macrophages, granulocytes, dendritic cells (DCs). Red blood cells (erythrocytes) and platelets also originate from this lineage. The lymphoid precursor lineage gives rise to lymphocytes and natural killer (NK) cells. NK cells are considered as innate immune cells. The innate immune system is able to combat common infections due to recognition of different conserved molecular patterns of pathogens by using a large set of germ-line encoded receptors. But due to lack of memory, the fighting strategy remains the same during successive immune responses.

The intricate communication between the different cells of the immune system is essential to coordinate immune responses or to provide tolerance. As it is important to induce an appropriate immune response, it is crucial to turn off the immune response and return to

homeostasis. Communication can be based on cell-to-cell contact or it can be provided by production of a variety of cytokines, such as interleukines (IL) and chemokines. These hormone-like signalling molecules can bind to cognate receptors on the surface of recipient cells triggering new cellular events. They affect the cell environment either in a pro-inflammatory or anti-inflammatory manner. Another important role of cytokines is to provide survival signals to maintain lymphocyte populations. The immune system is a network made up by a multitude of contributors and is regulated by at many levels. Consequently, the immune system is therefore susceptible to induce inappropriate and undesirable immune responses. The regulation must be balanced between immune response and immunological tolerance. A system out of balance can lead to allergies, autoimmunity and cancer.

1.2 The adaptive immune system

The adaptive immune system provides a more versatile and specific immune response and serves as special forces to defend against distinct invaders as well as infected and damaged cells. Key players in this system are B and T lymphocytes, which give rise to humoral and cell-mediated immunity, respectively. In contrast to antibody-secreting B cells responsible for eradication of extracellular pathogens, T cells are specialised in the eradication of pathogens existing intracellularly and infected or damaged cells. Immunological memory is a key feature of these lymphocytes. (2)

T cells develop in the thymus and mature into naïve T cells through negative and positive selection before circulating to peripheral lymphoid organs to complete maturation. There are three major subsets of T cells that can be distinguished by surface markers. Cytotoxic T cells (CTLs) express the CD8 co-receptor ($CD8^+$ T cells) while helper T cells (T_h-cells) express CD4 co-receptor ($CD4^+$ T cells). The former has the ability to kill infected or damaged cells directly while T_h-cells secrete cytokines and interact with effector cells such as CTLs, B cells and macrophages. The third subset of T cells is $CD4^+$ regulatory T cells. This subset expresses the CD25 marker and functions to suppress immune responses and to maintain tolerance. There are several subsets within all types of T cells giving rise to a variety of specific T-cell responses. When naïve T cells have encountered its cognate antigen in lymph nodes in the presence of co-activating signals, they proliferate and differentiate by clonal expansion into

effector T cells. A small population of antigen-specific T cells differentiates into resting memory T cells that can counteract a re-infection. In re-infection, memory cells will be activated and rapidly eliminate the invaders by antigen recognition. An adaptive immune response is shaped by information given from the innate system. Although unable to combat all invading pathogens, the innate system detects uncommon pathogens providing essential signals with regard to activate the most suited branch of the adaptive immune system (2).

1.3 Antigen-presenting cells

Antigen-presenting cells (APCs) play an important role in initiating an adaptive immune response due to the ability to capture, process and present antigens to T cells. APCs connect the innate and adaptive immune system. Macrophages, B cells and dendritic cells (DCs) express MHC class II and costimulatory molecules. Thus, these cells can display antigens to $CD4^+$ T cells which make them professional APCs. The most effective are DCs and these are the only cells that can prime and activate naïve T cells. DCs are present in lymphoid organs and throughout the tissue where they can encounter antigens, e.g. the gastrointestinal tract.

1.4 MHC molecules

Major histocompatibility complex (MHC) are clusters of gene loci that encode for the most polymorphic proteins in human (3). These membrane glycoproteins that spesialise in display peptides to T cells. There are two main types – MHC class I and MHC class II molecules. In humans, the MHC molecules are called human leukocyte antigen (HLA) molecules. They show a high degree of similarity in their three-dimensional structure but not in the sequence comparison. The heavy chain of human MHC class I molecules are encoded by the three genes HLA-A, HLA-B and HLA-C in addition to a separate gene encoding the β_2 microglobulin chain. There are seven genes encoding the human MHC class II molecules. Each of the gene loci HLA-DR, HLA-DQ and HLA-DP give rise to both the α chain and β chain, e.g. DPA1 and DPB1 genes, whereas the DR encodes for an additional β chain. All

together, individuals express six MHC I molecules and eight MHC II molecules due to codominant expression.

MHC class I molecules are expressed on any nucleated cells and display intracellular-derived antigens to cytotoxic $CD8^+$ T cells. MHC class II molecules are only expressed on the surface of professional antigen-presenting cells and some epithelial cells. Extracellular components are endocytosed in order to be processed and presented as peptides on the MHC II molecules to $CD4^+$ T helper cells. The main focus in this thesis is on MHC class II molecules, and will be described in more detail.

MHC II molecules consist of α chain and β chain where both having a transmembrane region and a C-terminal cytosolic tail. The α and β polypeptide chains have a molecular weight of approximately 35 kDa and 28 kDa, respectively. Each chain has two extracellular domains. The open N-terminal ends of the α1 and β1 domains interact non-covalently and make up the binding groove for the processed peptide. These domains are responsible for the polymorphic feature of the MHC molecules (4, 5). The walls of the peptide-binding groove are made up by α helices while β sheets form the floor. There are distinctive pockets in the floor and these pockets can interact with side chains of the peptide, often through hydrophobic interactions. These amino acid residues of the peptide serve as anchor residues. Other important interactions occur in the α-helical walls through hydrogen bonds and salt bridges (5). The residues interacting with the MHC molecules are not the same that are recognised by the T cell receptor, as shown in figure 1.1 below. In contrast, the two other extracellular domains of the MHC class II molecules, α2 and β2, are nonpolymorphic where the latter provides a binding site for the CD4 co-receptor expressed on T helper cells (6).

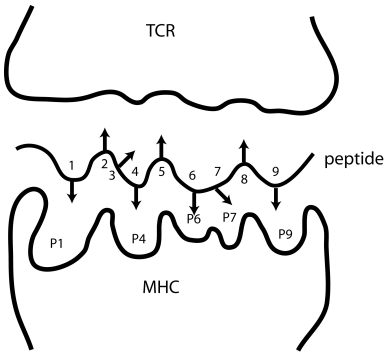

Figure 1.1: Schematic presentation of the TCR:MHC interface with the peptide.

Each individual has a limited number of MHC molecules but each MHC molecule can accommodate a multitude of different peptides. Due to the structure of the peptide-binding groove of MHC class II molecules, these can accommodate larger peptides than MHC class I which is limited to about 11 residues. Peptides binding to MHC II can be about 10-30 residues with optimal length of 12 to 16 residues. The peptide-dissociation rate is slow which provides stability to the peptide-MHC complexes. This is particularly important for APCs in order to provide sufficient amount of time to present the antigens to their respective T cells (7). For that reason, a functional MHC class II molecule is a heterotrimer consisting of α chain, β chain complexed with a stably bound peptide.

1.5 T cell receptor

Most of the time the MHC molecules display self-peptides. Hence, T cells have a crucial surveillance role to discriminate self-peptides from foreign peptides displayed on MHC molecules, which lack this ability. T cells through its antigen receptor also recognise conserved and polymorphic residues of the α helices of the MHC molecule itself leading to self-MHC restriction. This recognition system ensures that antigens are presented by self-MHC and has a central part in inducing graft rejection of transplanted organs.

The antigen recognition receptor of T cells, T cell receptor (TCR), is responsible for the recognition of the peptides displayed on MHC molecules. The receptor is made up by an α chain and β chain (αβTCR) where each consists of three variable loops. These loops are termed ’complementary determining regions’ (CDR) which determine the specificity of the TCR. The development of the antigen-specific T cells is very similar to development and maturation of antigen-specific B cells. The chain loci of the TCR genes consist of several gene segments – V (variable), J (joining) and C (constant) regions. The β chain has an additional gene segment that makes up D (diversity) region. There is a vast number of alleles within each region. Prior to clonal expansion, the TCR genes are subject to gene rearrangement and recombination giving rise to functional TCRs containing a unique combination of V(D)J gene segments. (2)

The interacting part of the antigen peptide binding to the TCR is termed the epitope while the complementary interacting residues of the TCR is called the paratope. To fully function as a receptor, TCR is non-covalently complexed with heterodimeric γε CD3 which serves as a surface marker of T cells and ζ proteins (homodimer) which have activating motifs (ITAMs) within the cytosolic domains. These motifs are important in transducing signals leading to activation and immune response (8). The coreceptor CD4 assists the TCR complex in the antigen recognition and enhances TCR signalling by binding to β_2 domain of MHC class II (9, 10).

1.6 Coeliac disease

Coeliac disease (CD) is a chronic inflammatory disease that affects the small intestine due to hypersensitivity to gluten protein found in wheat, rye and barley. The CD prevalence is estimated to about 1 % in the western population (11). Inappropriate immune response caused by food-derived gluten peptide antigen is a feature of allergy (food hypersensitivity) but the presence of autoantibodies in peripheral blood makes coeliac disease also an autoimmune disease (12). There is a strong genetic predisposition related to coeliac disease. More than 90 % of all affected individuals express the HLA-DQ2 (DQ2.5) or HLA-DQ8 (5 %) (13, 14). HLA-DQ2.5 molecules are encoded by the alleles HLA-DQA1*0501 and HLA-DQB1*0201. HLA-DQ8 molecules are encoded by HLA-DQA1*0301 and HLA-DQB1*0302. The prevalence of HLA-DQ2.5 in the Norwegian population is about 25 % (15). In addition, other non-HLA linked genes also contribute to disease development.

Gluten protein is relatively resistant to digestive enzymes in the gastrointestinal tract due to its high contents of proline. Gluten is a mixture of gliadin and glutenin polypeptides depending on their solubility in water and alcohol, respectively. The most immunodominant epitopes are within gliadin peptides. The gluten peptides are rich in glutamine, which make them a suitable target to the enzyme tissue transglutaminase 2 (tTG2). This enzyme was identified as the autoantigen in coeliac disease by Dietrich et al. (16). tTG2 converts glutamine to glutamate via a deamidation reaction. The acquired negative charges of the deamidated peptides are favourable when loaded onto MHC class II molecules. APCs expressing HLA-DQ2 molecules can display these modified gliadin peptides to naïve T cells leading to expansion

and activation of gluten-specific effector T helper cells (17). These $CD4^+$ T cells dominate in the lamina propria and their key feature is to secrete proinflammatory IFN-γ (18). Disease-associated B cells are primed to produce anti-gluten antibodies and anti-TG2 autoantibodies in the immune response to gluten (15).

The inflammatory environment of the intestinal mucosa leads to villous atrophy, crypt hyperplasia and intraepithelial lymphocyte (IEL) infiltration (19). Thus, classical symptoms are malnutrition, diarrhoea and anaemia. However, there are huge variations in affected individuals. The diagnostics of CD is based on serological tests for the presence of disease-associated antibodies, such as IgA autoantibodies for TG2 (20), and intestinal biopsy analysis. The only treatment to coeliac disease is a strict gluten-free diet (GFD). In the absence of gluten, the levels of TG2-autoantibodies decrease correlated to gluten intake. GFD leads to complete remission in most cases.

When investigating disease mechanisms that lead to chronic inflammation and autoimmunity, coeliac disease has shown to be an excellent disease model. In many diseases the key factors are unknown, but in CD the antigen gluten and the HLA class II genes are well-known factors. A main focus in the investigation of CD pathogenesis is the role of gluten-reactive $CD4^+$ T cells and their interaction with the disease-associated HLA-DQ molecules.

1.7 Production of recombinant soluble MHC class II molecules in BEVS

In the investigation of the TCR:pMHC interaction, recombinant MHCs are used as a reagent to study specificity and responses of intact TCRs on living T cells. In contrast to membrane-bound MHC class II, the recombinant version is soluble due to replacement of the transmembrane regions with leucine zippers. This modification facilitates the pairing of the chains increasing the stability of the dimer (21). Another feature of recombinant soluble MHCs is that the bound peptide in the groove is covalently linked at the N-terminus of the β chain. Due to the inherent low affinity of TCR to pMHC, recombinant pMHCs must be biotinylated and multimerised on streptavidin in order to function as a staining reagent in flow cytometry (in more details in next section). The molecules have been engineered with a BirA site at the C-terminus of the β chain. In this thesis, the BirA site has been replaced by Streptag

sequence as described in more detail below and in the Methods section. The production of recombinant soluble MHC class II molecules takes place in a eukaryotic expression system mediated by baculovirus.

Baculovirus expression vector system (BEVS)

In contrast to MHC class I, protein expression of MHC class II has been troublesome in prokaryotic *E. coli* expression system. For decades, the molecular biology of the baculovirus has been elucidated due to its insecticide properties. The baculovirus is an enveloped and double-stranded DNA virus belonging to the family *Baculoviridae.* The circular and supercoiled DNA (80-220 kb) is packed into nucleocapsids. The most studied baculovirus is of the genus nucleopolyhedrovirus. Insects are natural hosts for this virus and it was originally isolated from the nocturnal moth *Autographica californica* which gives rise to the short name of the baculovirus strain used in this expression system – AcNPV. The baculovirus can be used as a molecular tool to express recombinant proteins. Baculovirus genes such as polyhedrin and p10 genes encode for the most predominant proteins and can be replaced by heterologous genes using viral promoters. Occlusion bodies consisting of a polyhedrin-based matrix can be observed in wild-type virus but not in the recombinant virus. In the recombinant virus, the occluded virus (OV) production is affected due to the removal of the polyhedrin gene in which reduces the pathogenicity of the virus. This protein is not essential in *in vitro* cell culture, seeing that viral replication and embedded virus (EV) production are preserved. The viral infection leads to shut-off of host cell gene expression and newly synthesised virions are released from infected cells due to lytic capability. (22)

The BaculoGold DNA is a modified and linearised AcNPV Baculovirus DNA that contains a lethal deletion. This improved DNA does not code for viable virus but can be rescued by co-transfection with a transfer vector. There are transfer vectors, e.g. pAcAB3, that can carry multiple gene inserts simultaneously and cloning regions are flanked by essential viral sequences, which lead to *in vivo* homologous recombination. The result is rescue of virus and successful insertion of heterologous genes and the recombination efficiency is therefore close to 100 %. The polyhedrin gene in the linearised baculovirus DNA is replaced by the *lacZ* gene and plaques are therefore colourless. (23)

Sf9 cells are commonly used in the baculovirus expression system. These cells originate from Sf21 cell lines that were first established from ovarian tissues of the fall armyworm,

Spodoptera frugiperda (Sf), in its caterpillar life stage. They are highly susceptible to AcNPV virus infection and can be cultured either in monolayer or in cell suspension. Sf9 cells can also be adapted to grow in serum-free medium that is very convenient in the purification process and they go under the name of ExpresSf+ cells. (23)

There are several advantages using this expression system. Proper folding is essential in order to obtain functional proteins. A common issue using prokaryote systems is production of insoluble and aggregated proteins. This eukaryotic system resembles mammalian protein synthesis better than prokaryote systems increasing the chance of producing biologically active proteins. This is very important in particular for complex proteins that have post-translational modifications, e.g. glycosylations. It has been shown that insect cells and mammalian cells may have certain differences in the nature of glycosylation (24). However, biological activity is maintained.

1.8 Multimer technology

Previously, antigen-specific lymphocytes stained with their cognate antigen have only been demonstrated with B cells. The detection of T cells has been possible to a certain extent but staining with the TCR for the detection of antigen-specific T cells has been troublesome. In contrast to antibody:antigen interaction, the TCR:pMHC interaction is of relatively low affinity and single MHC complexes to TCR have a fast dissociation rate (25). To overcome this problem, MHC molecules can be multimerised on streptavidin in order to form stable MHC-peptide complexes. MHC multimerisation increases the relative binding avidity to TCR leading to slower dissociation rate. However, the binding affinity remains unchanged. The multimer technology for MHC class I molecules was developed by Altman et al. in 1996 (26). In flow cytometry, fluorochrome-conjugated MHC I multimers function as staining reagent allowing direct visualisation and identification of antigen-specific $CD8^+$ T cells. The multimer technology is based on the biotin:streptavidin interaction which is one of the strongest non-convalently interaction in nature and has a widespread use in biochemistry and biotechnology (27, 28). Streptavidin has a homotetrameric structure and can bind up to four biotin molecules giving rise to the term tetramer technology. In the generation of recombinant MHC molecules, a substrate peptide (GLNDIFEAOKIEWHN) for BirA-dependent biotinylation is

engineered at the C-terminal end of the heavy chain of MHC (29). These recombinant molecules can be biotinylated at the BirA site (BSP tag) in the presence of the enzyme BirA. Furthermore, biotinylated MHC molecules can bind to streptavidin labelled with a fluorochrome.

With the recently developed tetramer technology in mind, Crawford et al. (1998) developed a similar system for MHC class II molecules (30). The production of the soluble MHC class I and MHC class II molecules differs in the choice of expression system. Another main difference is that the peptide of MHC class II is covalently linked to the β chain (31). T cell research has been greatly improved with the use of multimer technologies. But as research has progressed and increased the basic structural and functional knowledge of the TCR:pMHC interaction, the underlying technologies can be developed to a greater extent. In 2002, Knabel et al. published a paper in Nature Medicine where they established a reversible multimer technology (32). This novel technology, also termed Streptamer technology, combines the tetramer technology developed by Altman et al. and the novel Streptag:streptavidin system described below (33).

Originally, Streptag was developed as an affinity tag to streptavidin due to the widespread use of biotin:streptavidin interaction. In the early days of Streptag, Streptag was used as a molecular tool in purification of recombinant proteins. In contrast, affinity tags such as myc tag and Flag peptide are derived from epitope peptides for monoclonal antibodies. Recombinant fusion proteins can be purified due to specific interaction of the antibody and the integrated affinity tag. However, the binding dissociation often involves harsh conditions for the purified protein. Purification and separation under physiological conditions are prerequisites for many recombinant proteins to function. The search for a new peptide tag based on affinity to streptavidin started. The short peptide nine-amino acid peptide (Ala-Trp-Arg-His-Pro-Gln-Phe-Gly-Gly) making up the Streptag was discovered in a random peptide library (34). Peptides were expressed at C-terminus of variable heavy chain of a F_V fragment and screened with streptavidin-alkaline phosphatase. The Streptag peptide and streptavidin turned out to form a stable complex. However, the Streptag showed to dissociate rapidly in the presence of biotin. This feature allows the dissociation of recombinant fusion proteins with Streptag under physiological conditions. In order to improve the Streptag:streptavidin interaction even further, the Streptag was optimised after new insights when analysing the co-crystal structure. The new Streptag was termed StreptagII with the sequence Trp-Ser-His-Pro-

Gln-Phe-Glu-Lys (WSHPQFEK). The affinity peptide was found to bind close to the biotin binding site of streptavidin but not as deeply as biotin does. An important feature of StreptagII was that the peptide could be expressed anywhere in the protein. Streptavidin was also engineered to improve the binding strength. Streptactin is a modified streptavidin with three mutations in the flexible loop close to the biotin site. The binding affinity to biotin is not altered ($K_d = 10^{-13}$ M) but StreptagII binds to Streptactin with a higher affinity compared ($K_d = 10^{-6}$ M) to streptavidin (35).

Conventional MHC multimer technology has provided a way to identify and detect antigen-specific T cells. But further investigation of T cells, i.e. functional T cell analysis, is interfered due to the irreversible staining conditions. Identification and purification of antigen-specific T cells without altering their functional status has been of great scientific and clinical interest. Knabel et al. used a murine model system of *Listeria monocytogenes* infection to study the *in vivo* T-cell (CTLs) responses and protective immunity against the pathogen. The immunodominant epitope used in this system originates from the virulence factor listeriolysin-O (LLO). Both conventional tetramers and the novel reversible Streptamers (see figure 1.2) were used as staining reagents for LLO-specific CTLs. This group postulated that T cells might maintain their functional status and phenotype if procedures are performed at low temperatures. It had been previously reported that higher temperatures might improve the staining intensity in flow cytometry. On the other hand, the cellular events that can take place at physiological temperatures include T cell receptor internalisation, activation, overstimulation and apoptosis (36). TCR internalisation was also demonstrated with bound MHC II oligomers (37). They wanted to elucidate the temperature effects of these biological events. Data showing reduction in maximum lysis and decreased peptide sensitivity was possibly due to TCR internalisation and early apoptotic events. Knabel et al. concluded that it is crucial to perform the staining at 4°C in order to preserve the functional status of the T cells analysed. They also showed that labeling CTLs with conventional MHC tetramers affect their cytotoxic profile *in vivo* independent of temperature.

The novel Streptamer technology is based on the Streptag:streptavidin system as explained earlier. The Streptag peptide sequence is fused to C-terminal end of chain of MHC class I molecule replacing the BirA site. Using two Streptag sequences (termed StreptagIII) separated by a GS-flexible linker were found to provide stabilisation to the streptamer complex. In the absence of biotin, streptagged MHC I can be loaded on to the Streptactin

backbone. Addition of biotin leads to targeted disruption of Streptactin and the MHC multimers turn into MHC monomers. Due to relatively low affinity to TCR ligand, the MHC monomers will rapidly dissosiate from the surface-bound TCRs.

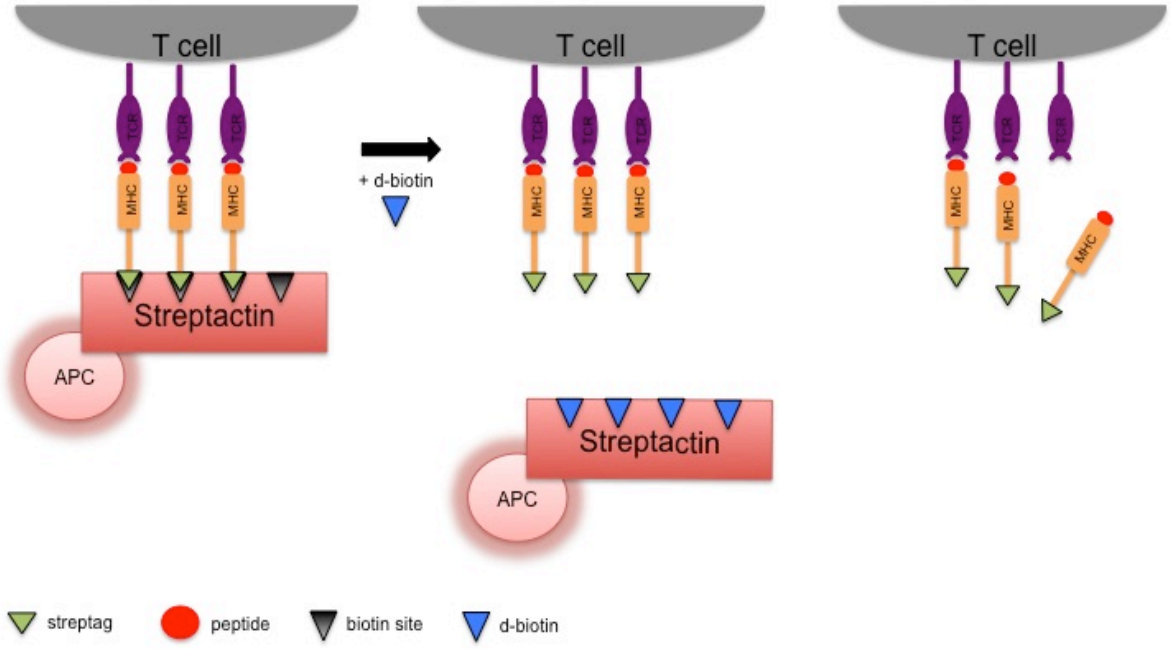

Figure 1.2: Principle of Streptamer technology. Several pMHC-Streptag can multimerise on Streptactin-APC which make the Streptamer complex. These Streptamers can be used to stain T cells specific for the pMHC molecules. In the presence of d-biotin, the Streptactin backbone will dissociate due to higher affinity for d-biotin. The pMHC molecules are left as monomers on the T-cell surface bound to cognate TCRs. Subsequently, the pMHCs dissociate due to inherent low TCR affinity.

With this in mind, recombinant H2-StreptagII fusion proteins (murine MHCs) were generated and incubated with the Streptactin backbone. CTLs were stained with Streptamers followed by addition of biotin leading to dissociation of the backbone and decrease of monomeric surface-bound MHC molecules. The reversible multimerisation of LLO-specific T cells (biotin treated) were compared with Streptamer-stained T cells without multimer dissociation. As for the MHC multimers, these CTLs were also transferred into naïve recipient mice to test the *in vivo* function. The reversibly stained CTLs proved to show protection, which was comparable to positive controls.

Neudorfer et al. first demonstrated the isolation of human antigen-specific $CD8^+$ T cells by reversible multimers in 2007 (38). They showed that the use of Streptamer technology has a

great advantage in adoptive cell transfer due to preservation of T cell function. The use of different multimer technologies for detection and adoptive transfer of antigen-specific T cells was reviewed by Casalegno-Garduno et al. (2010) (39). In this review, they favour Streptamers for T cell isolation for clinical application.

1.9 Kinetics of the TCR:pMHC interaction

The cell-mediated immune response provided by T cells is believed to be correlated with the strength of the TCR interaction with its pMHC ligand. Therefore, investigating the binding kinetics of this interaction can lead to greater insights about the importance of T cell antigen recognition and T cell responsiveness. How efficiently T cells respond to an encountered antigen can be defined as T cell avidity. The quality of a T cell can be described by the antigen dose required for T cell activation. High avidity T cells respond to lower antigen doses than low avidity T cells (40).

1.9.1 Kinetics assays

The rate constant (k) of a reaction directly measure how fast the reaction is. It has been of interest to investigate receptor:ligand (1:1) interactions such as the TCR:pMHC interaction of both association (k_a or k_{on}) and dissociation (k_d or k_{off}) rates. There have been different approaches to study the kinetics of TCR:pMHC interaction. Two-dimensional (2D) kinetics is based on cell to cell contact while in three-dimensional (3D) kinetics one of the binding partners can move in three dimensions (in solution).

2D kinetics assay of the TCR:pMHC interaction is a mechanical assay where TCR and MHC are anchored on 2D membranes of apposing cells. These cells are aspirated by micropipettes and brought in close proximity. TCRs are surface exposed on intact T cells while streptavidin-coupled MHC molecules are bound to biotinylated red blood cells (RBCs). These RBCs serve as surrogate APCs and a force sensor. TCR:pMHC binding is observed by elongation of the RBC. The adhesion probability per contact is measured and the adhesion tests of the same cell pair are multiple repeated giving rise to an adhesion frequency (41). Adhesion frequency assay provides a monomeric pMHC presentation by using divalent streptavidin. Analysis of

obtained data points are fitted into a mathematical model of the interaction, measuring 2D binding affinity, on-rate and off-rate.

The BIAcore system is a well-known kinetics assay and is based on the principle of surface plasmon resonance (SPR). SPR is an optical phenomenon that occurs when incident light reflects off thin metal films. At a defined angle (SPR angle) at the interface, electrons in the metal layer can be excited in resulting in electron charge density waves called surface plasmons. BIAcore is an optical and sensitive technique that can be used to measure binding specificity and kinetics of protein:protein interactions (42), including the TCR:pMHC interaction (43, 44). One partner in the binding pair is immobilised in a matrix on the surface of a chip while the other interacting partner is in solution. Binding causes changes in the SPR angle of the reflected light. Changes in the intensity of the reflected light are proportional to the total protein concentration and are measured over time. Both association and dissociation rates can be measured.

Kinetics assays based on MHC multimer technology have tried to correlate tetramer staining intensity with T cell avidity. In 1999, Yee et al. demonstrated isolation of high-avidity tumor-reactive CTL clones based on their tetramer staining intensity in flow cytometry (45). MHC multimer technology has also led to tetramer dissociation assays. In this case, a blocking reagent is used to prevent rebinding of MHC on tetramer-stained T cells. Competitive binding with the blocking reagent leads to decay in fluorescence over time. The selection of different blocking reagents was investigated by Wang and Altman (46). The use of anti-MHC Fab fragments showed to be the most suitable blocking reagent compared to intact anti-MHC antibodies.

1.9.2 A novel Streptamer-based k_{off} rate assay

A novel assay measuring the dissociation rate (k_{off}) of the TCR:pMHC interaction has been developed, but yet not published, based on the Streptamer technology published in 2002 (47). This German research group hypothesised that observation of dissociation of fluorescently labelled monomeric MHCs to surface expressed TCRs, after addition of d-biotin, could provide an accurate and reliable method to measure and determine binding kinetics on living T cells. By realtime confocal microscopy, the dissociation can be measured as decay in fluorescence intensity over time. The disruption of the Streptag:Streptactin interaction by d-biotin leads to removal of the Streptactin backbone and consequently the fluorescent signal.

The unlabelled pMHC complexes are bound to their cognate TCRs as monomers. To be able to detect the monomeric pMHC complex, a fluorescent dye is directly conjugated to the molecule. An engineered cystein residue after the StreptagIII region at C-terminus of the heavy chain can be coupled to the fluorescent dye in a maleimide reaction. T cells stained with these dichromatic Streptamers will therefore be double stained due to the dye on the Streptactin backbone and the dye on the MHC molecules and sorted by FACS.

In the microscope setup, a customised metal insert is sealed with a cover slip placed on a cooling device to provide a buffer reservoir. The cooling device ensures that the subsequent events occur at 4°C. A drop of Streptamer-stained T cells in FACS buffer is pipetted on the cover slip. To preserve the densed cell distribution in the drop, the cell suspension is captured in a thin layer between the cover slip and a pore membrane before cold buffer is added. This membrane is semi-permeable allowing free diffusion of d-biotin (when added). The observation of the stained cells takes place in real time by confocal microscopy connected to a computer. In a given time series, pictures are taken every 10 seconds. When d-biotin is added and diffuse into the captured cell suspension, the dissocation of the Streptactin backbone and the subsequent MHC dissociation can be observed visually. The obtained fluorescence data is analysed by a customised software and the final data is fitted into a mathematical model showing decay in the fluorescence over time.

The $t_{1/2}$ (half-life time) value obtained provides a means to determine T cell avidity to a given peptide epitope displayed on the MHC molecule. They demonstrate a correlation of slow dissociation rate with high avidity. This research group focuses on cytotoxic T cells (CMV^+) and their potential in adoptive cell transfer. During the PhD work of Magdalena Nauerth, they have demonstrated that high-avidity CMV^+ CTLs T cells determined by their Streptamer-based k_{off}-rate assay confer protective immunity and erradication of CMV-infected cells (47).

2 Aims of the Thesis

The main focus of this thesis is the interaction between disease-associated HLA-DQ2.5 molecules complexed with gliadin epitope peptides and gliadin-specific $CD4^+$ T helper cells. The Streptamer technology is well established for MHC class I molecules and $CD8^+$ T cells. Streptamer technology with multimerised MHC class II molecules to detect antigen-specific $CD4^+$ T cells has so far not yet been successful. There are no publications of this technology applied on MHC class II molecules and $CD4^+$ T cells.

There are three main aims of this project. Firstly, we generated new reversible MHC II reagents (HLA-DQ2.5-Streptag molecules) that can be multimerised on Streptactin (HLA-DQ2.5-Streptamers). Secondly, we wanted to test if these HLA-DQ2.5-Streptamers would stain gluten-reactive $CD4^+$ T cell clones in flow cytometry. Thirdly, we aimed to test the Streptamer-positive T cells in a novel Streptamer-based k_{off}-rate assay in Munich.

3 Materials

3.1 Reagents and Chemicals

Reagent	Manufacturer
Ampicillin	Sigma-Aldrich, St. Louis, MO, USA
Ammonium persulfate (APS)	Sigma-Aldrich
ATTO565-maleimide	ATTO-TEC GmbH, Siegen, Germany
β-mercaptoethanol	Sigma-Aldrich
Bromophenol blue	Sigma-Aldrich
BioSafe Coomassie	BioRad, Hercules, CA, USA
Crystal Violet C-3886	Sigma-Aldrich
d-biotin	Sigma-Aldrich
Diethanolamine	Sigma-Aldrich
Diethylamine	MERCK KGaA, Darmstadt, Germany
Dimethylsulfoxid (DMSO)	Sigma-Aldrich
Dipotassium phosphate (K_2HPO_4)	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Ethanol, 70 %	Sigma-Aldrich
Glucose	Sigma-Aldrich
Glycerol ≥ 86 %	Sigma-Aldrich
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich
Monopotassium phosphate (KH_2PO_4)	Sigma-Aldrich
Phosphatase substrate (5mg tablet)	Sigma-Aldrich
Potassium chloride (KCl)	Sigma-Aldrich
Rinderalbumin 30 % (BSA)	Biotest, Dreieich, Germany
Sodium acetate ($NaCHCOO^-$)	Sigma-Aldrich
Sodiumdodecylsulphate (SDS)	BioRad
Sodium chloride (NaCl)	Sigma-Aldrich
Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich
Streptavidin-AP	Southern Biotech, Birmingham, Alabama, USA
Streptactin-APC	IBA, Göttingen, Germany
Tetramethylethylenediamine (TEMED)	AMRESCO LLC, Solon, OH, USA
Thrombin Restriction Grade	Novagen (Merck)
Trichloroacetic acid (TCA)	MERCK KGaA
Trypan blue solution (0.4%)	Sigma-Aldrich
Tween-20	Sigma-Aldrich

3.2 Plasmid construction

Plasmid construction	Manufacturer
Plasmid	
pUC18	Stratagene, La Jolla, CA, USA
pUC57_DQB1*0201_α1_streptag	GenScript, Piscataway, NJ, USA
pGEM-11Z(+)	Promega
pAcAB3	Becton, Dickinson and Company (BD Biosciences), Franklin Lakes, NJ, USA

Plasmid construction	Manufacturer
DNA modifying enzyme/buffer	
BamHI	New England Biolabs, Ipswich, MA, USA
BglII	New England Biolabs
Nar1	Promega, Fitchburg, WI, USA
Sac1	New England Biolabs
Turbo Nar1 buffer	Promega
NE Buffer 3	New England Biolabs
BSA	New England Biolabs
T4 DNA ligase	Promega
Ligase buffer	Promega
Calf Intestine Phosphatase (CIP)	New England Biolabs
Oligomer hybridisation	
CLIP2.1 Forward oligomer (p5' CC GGA ATG GCC ACC CCC CTC CTT ATG CAG GCA CTG CCT ATG GGC GCT CTG GG 3')	MWG Biotech AG, Eberberg, Germany
CLIP2.2 Reverse oligomer (p5' C GCC CAG AGC GCC CAT AGG CAG TGC CTG CAT AAG GAG GGG GGT GGC CAT T 3')	MWG Biotech AG
PCR	
Forward primer (CLIP2.1)	MWG Biotech AG
Reverse primer (DQB-ampB) (CTG GTA GTT GTG TCT GCA CAC)	Eurogentec S.A., Seraing, Belgium
dNTP mix	Thermo Fisher Scientific Inc, Waltman, MA, USA
DyNAzyme II DNA polymerase	Finnzymes (Thermo Fischer Scientific Inc)
10X DyNAzyme II Buffer	Finnzymes (Thermo Fischer Scientific Inc)

3.3 Cells and cell clones

Cells	Manufacturer
XL10-GOLD Ultracompetent cells	Stratagene , La Jolla, CA, USA
Sf9 cells	Novagen (EMD Millipore)
ExpresSf+ cells	Protein Sciences Coop., Meriden, CT, USA
EBV transformed B cells	In house; CD patient (CD114)

T cell clones (TCC)

Origin	DQ2.5-glia-α1-specific TCC	DQ2.5-glia-α2-specific TCC
Gut	TCC535.3.23	TCC678.3.22
Peripheral blood (EM)	TCC CD1030.64 TCC CD1030.65 TCC CD1030.66 TCC CD1030.63	TCC CD1030.38 TCC CD1030.41 TCC CD1030.43 TCC CD1030.44
Peripheral blood (Naïve)	TCC BC02.16 TCC BC02.19	TCC BC02.29 TCC BC02.30 TCC CD1030.53 TCC CD1030.60

3.4 Equipment

Chemical/compound/equipment	Manufacturer
Plasmid construction	
Agarose powder	Sigma-Aldrich
6X Loading buffer	TaKaRa Bio Inc., Shiga, Japan
1 Kb DNA Ladder	Fermentas, Vilnius, Lithuania
50 bp DNA Ladder	Fermentas
30 % Bis-Acrylamide (Duracryl)	NextgenSciences, Ann Arbor, MI, USA
Ethidium Bromide	Invitrogen (LifeTechnologies), Carlsbad, CA, USA
SYBR Safe DNA gel stain	Invitrogen (LifeTechnologies)
Insect cell culture	
Spinner Flasks (CELLspin1000/250)	Integra Biosciences, Zizers, Switzerland
Grace's Insect Medium Supplemented	GIBCO (LifeTechnologies)
Grace's Insect Medium 2X Supplemented	GIBCO (LifeTechnologies)
Insect-Xpress Medium	Lonza, Basel, Switzerland
Fetal calf serum (FCS)	GIBCO (LifeTechnologies)
Gentamicin (Gensumycin 40 mg/mL)	Sanofi, Paris, France
Pluronic F68	Sigma-Aldrich
Baculovirus transfection buffer A/B	BD Biosciences
BaculoGold –Plaque Assay Agarose	BD Biosciences
Baculovirus DNA	BD Biosciences
Protein purification	
GF-D 2.7 μm filter	Whatman (GE Healthcare), Kent, UK
GF-F 0.7 μm filter	Whatman
0.45 μm filter	EMD Millipore, Billerica, MA, USA
Sepharose CL-4B	GE Healthcare
Protein A Sepharose CL-4B	GE Healthcare
Superdex 200 GL 10/30	Amersham Biosciences, Uppsala Sweden
Syringe 50 μL	Hamilton Bonaduz AG, Bonaduz, Switzerland
Syringe 250 μL	Hamilton Bonaduz AG
Nap-25 column Sephadex G-25 DNA Grade	GE Healthcare
T cell culture	
RPMI 1640	GIBCO (LifeTechnologies)
LymphoPrep	Axis-shield PoC AS, Oslo, Norway
Human serum (HS)	In house production
mCi ^{3}H-Thymidine	Hartmann Analytic GmbH, Glattbrugg, Switzerland

3.5 Buffers and Media

Buffer	Composition
Phosphate-buffered saline (PBS)	100 mM NaCl, 4.5 mM KCl, 7 mM Na_2HPO_4, 3 mM KH_2PO_4 (pH 7.2)
Laemmli's sample buffer (4X)	0.25M Tris-HCl pH 6.8, 8 % SDS, 40 % glycerol, 0.02 % bromophenol blue, 4 % β-mercaptoethanol
Plasmid construction	
Crush&Soak buffer	300 mM sodium acetate, 1mM EDTA, 0.1 %SDS,H_20
SOC medium (Super Optimal Broth (SOB) with Catabolite repression)	20 g Bacto-tryptone, 5 g Bacto-yeast extract, 0.5 g NaCl, 10 mL of 250 mM KCl , 5 mL of 2M $MgCl_2$ + 20 mM glucose
LB (Luria-Bertani) medium	10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl, deionized H_2O to 1L
LB agar	15 g agar to 1L LB Medium
TBE (Tris/Borate/EDTA) Buffer	108 g Tris base, 55g boric acid, 40 mL 0.5 M EDTA (pH 8.0) to 1L
TE8 buffer	10 mM Tris-HCl, 1 mM EDTA
Insect cell culture	
Complete Grace's medium	Grace's Insect Medium, 10 % FCS, 24 μg/mL gentamicin, 0.1 % Pluronic
2X Grace's medium	Grace's 2X Insect medium, 20 % FCS, 24 μg/mL gentamicin
Plaque assay staining solution	1% crystal violet in 20 % EtOH, 20 % TCA
Protein purification and ELISA	
Elution buffer (2.12.E11 column)	0.05M diethylamine, 0.15M NaCl pH 11.5
Elution buffer (Protein A column)	0.1M citrate buffer pH 3
Neutralising buffer	2M Tris-HCl pH 6.3
Blocking buffer	3 % BSA in PBS
Dilution buffer (samples)	0.5 % Tween-20 in PBS (PBST)
Dilution buffer (substrate)	1M diethanolamine buffer pH 9.8, 0.5 mM $MgCl_2$
T-cell culture	
Culture medium	10 % human serum in MEPS (HS/MEPS)
Freezing medium	50 % FCS/RPMI1640 20 % FCS, 20 % DMSO, 60 % RPMI1640
MEPS medium	3.5 % 2-Mercaptoethanol, 120μg/mL Penicillin, 100μg/mLStreptomycin in RPMI 1640 + (10 U/mL IL-2, 1 ng/mL IL-15,1 μg/mL PHA)
Thawing medium	20 % FCS/RPMI1640
Streptamer staining	
d-biotin buffer	5 mM d-biotin in dH_2O pH7.0
FACS buffer	3 % FCS/PBS

3.6 Gels

Gel	Composition
20 % PAGE	8.0 mL 30 % Bis/Acrylamide, 1.2 mL10X TBE, 2.8 mL MQ water, SDS, 60 µL APS, 6 µL Temed
1 % Agarose gel	Agarose powder, dH_2O
2 % Baculovirus agarose gel	BaculoGold Agarose powder, dH_2O
Separating gel (12 % SDS PAGE)	4.8 mL Bis/Acrylamide, 3.0 mL 1.5M Tris pH 8.8, 4.06 mL dH_2O, 60 µL 20 % SDS, 40 µL10 % APS, 6.0 µL TEMED (3 gels)
Stacking gel (6 % SDS PAGE)	1.2 mL Bis/Acrylamide, 1.5 mL 0.5M Tris pH 6.8, 3.3 mL dH_2O, 30 µL 20 % SDS, 40 µL 10% APS, 40 µL TEMED (3 gels)

3.7 Antibodies

Antibody	Manufacturer
2.12.E11 antibody	Diatec Monoclonals AS, Oslo, Norway
SPV-L3 antibody	Diatec Monoclonals AS
Goat anti-mouse (GAM)-IgG2a-BIOT	Southern Biotech
Anti-CD4-PE	eBioscience, Inc., San Diego, CA, USA
Anti-CD3-APC	eBioscience, Inc.

3.8 Kits

Kit	Manufacturer
Wizard *Plus* SV MiniPreps DNA Purification Systems	Promega
PureYield Plasmid MidiPrep	Promega
QIAquick Gel Extraction Kit	QIAGEN, Hilden, Germany
QIAquick PCR Purification Kit	QIAGEN

3.9 Instruments

Instrument	Manufacturer
FACSCalibur	BD Biosciences
LSR II Yellow Laser *	BD Biosciences
SmartSpec 3000 spectrophotometer	BioRad
FPLC (Äkta)	Amersham Biosciences
Scintillation and Luminescence 1450 Microbeta TriLux	Perkin Elmer Life Sciences, Waltham, MA, USA
Ultraflex II (MALDI-TOF-MS)	Bruker Daltonics, Bremen, Germany
Multiskan ascent	Thermo Scientific
Gammacell 3000 Elan	MDS Nordion, Ottawa, ON, Canada
HydroFlex ELISA washer	Tecan, Group Ltd., Männedorf, Switzerland
NanoDrop 1000 Spectrophotometer v3.7	Thermo Scientific
Megafuge 1.0 Heraeus centrifuge	Thermo Scientific
Varifuge 3.0R Heraeus sepatech	Thermo Scientific
Sorvall RC5C PLUS Superspeed centrifuge	Thermo Scientific
Sorvall SLA-3000 Super-Lite (rotor)	Thermo Scientific
Microcentrifuge 5415R	Eppendorf, Hamburg, Germany

**Flow Cytometry Core Facility, OUS Montebello, Oslo, Norway*

3.10 Software

Software	Manufacturer
EndNote X5	Thomson Reuters Cooperation, New York, NY, USA
FlowJo	Tree Star, Ashland, OR, USA
GraphPad Prism5	GraphPad Software, La Jolla, CA, USA
Microsoft Office Mac 2011	Microsoft, Redmond, WA, USA
PyMOL Molecular Graphics System v1.5.0.4	Schrödinger LLC, New York, NY, USA
UNICORN v5.01	GE Healthcare (Life Sciences)

4 Methods

4.1 Plasmid Construction

Recombinant HLA-DQA1*0501 (DQA) and HLA-DQB1*0201 (DQB) gene sequences encoding α and β chains that make up the dimeric MHC class II molecules (HLA-DQ2.5) are well established in the Sollid group, originally created by cDNA synthesis from a B-cell line (48). Gluten-derived peptide sequences are integreated within the DQB sequence and are therefore covalently linked to the expressed HLA molecule. In this project these sequences were used as basis to generate a new construct. The BirA site positioned within the DQB sequence was replaced by two Streptag sequences (One STrEP-tag, StreptagIII) and a GGSC-sequence for dye conjugation. The DQA1*0501 sequence remained unmodified. A comparison of the modified and the conventional HLA-DQ2.5 molecule is illustrated in figure 4.1.

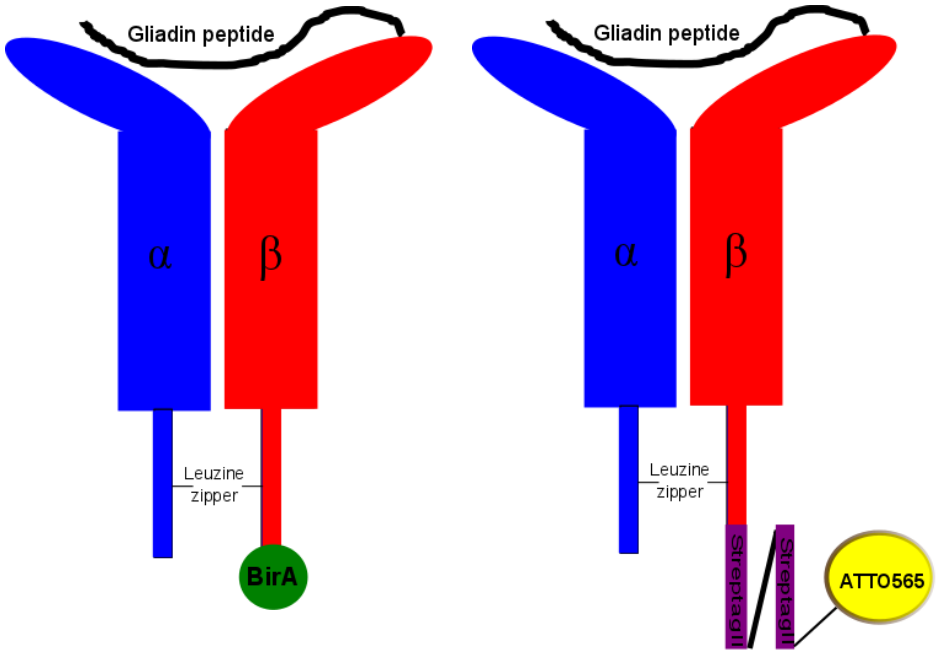

Figure 4.1: Modification of the recombinant HLA-DQ2.5 molecule. The BirA site is replaced with a StreptagIII region and dye conjugation site for ATTO565-maleimide. The BirA site of conventional DQ2.5 is marked in green (*left*) and StreptagIII region and dye conjugation site of DQ2.5-Streptag are marked in purple and yellow, respectively (*right*).

The new nucleotide sequence, DQB1*0201_α1_streptag, was synthetically generated and purchased from GenScript. The sequence was placed in pUC57 vector (1068 bp), flanked by

BamHI restriction sites. From this product, we created three constructs with different peptides covalently linked to the β chain as listed in **table 4.1**. Two constructs have gluten-derived peptides while the third has endogenous peptide derived from invariant chain. The latter was made to function as a control reagent in future experiments.

Table 4.1: List of plasmid constructs and the corresponding peptide sequences.

Construct	Peptide sequence
DQ2.5-glia-α1a (α1)*	Q L Q P F P Q P E L P Y
DQ2.5-glia-α2 (α2)*	P Q P E L P Y P Q P E
DQ2.5-CLIP2	M A T P L L M Q A L P M G A L

* Nomenclature of the T-cell epitopes (underlined) (49).

4.1.1 Gene cloning – restriction enzyme digestion and ligation

The modified DQB1*0201 sequence containing the DQ2.5-glia-α1a (α1) peptide-encoding sequence was directly cloned into the cloning vector, pGEM, after BamHI restriction digestion. The pUC57 vector was digested for 3 hours in 37 °C water bath with BamHI mixed with buffer 3 and BSA in a reaction volume of 40 μL. 6X loading buffer was added to the sample prior to loading onto a 1 % agarose gel containing ethidium bromide (EtBr). 6 μL 1kb DNA ladder was also included to determine fragment size. The 1kb DQB fragment was separated from rest of the vector in 1X TBE buffer, excised and isolated from the gel according to the microcentrifuge protocol of the QIAquick Gel Extraction Kit. The DNA concentration was measured by Nanodrop and stored at -20°C.

The purified fragment was then ligated into already BamHI –and phosphatase treated pGEM vector (see figure 4.2). 1 μL T4 ligase and ligase buffer were mixed with pGEM and DQB-α1-streptag in a insert:vector ratio of 3:1. In a reaction volume of 20 μL, the mixture incubated over the weekend at 4°C, alternatively at 16°C over night. A religation reaction with vector only was set up in parallel as a control.

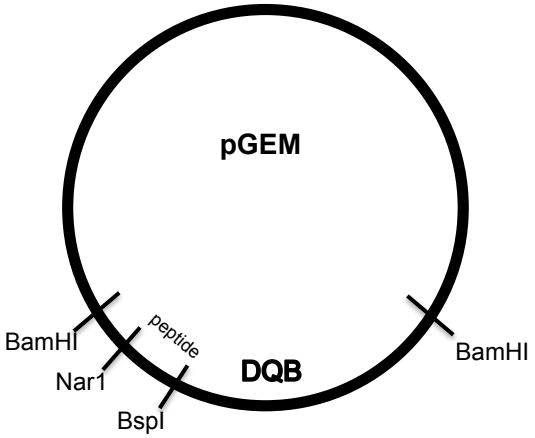

Figure 4.2: Schematic pGEM vector with key restriction enzymes. The DQB gene sequence is flanked by BamHI restriction sites. The peptide sequence within the DQB sequence is flanked by Nar1 and BspE1 restrisction sites.

4.1.2 Transformation of pGEM vector

In order to amplify the newly created pGEM plasmid, the plasmid was transformed into XL10 competent cells. These cells are calcium chloride ($CaCl_2$)-treated *E. coli* that make them prone to DNA uptake due to reduction of membrane potential. Three samples were set up which include the ligation plasmid product, the plasmid with no insert as a control (religation), and a control tube containing pUC18 plasmid. The pUC18 plasmid was used to determine the transformation efficiency of the XL10 cells. 95 μL of thawed XL10 cells was added to prechilled BD Falcon round-bottom tubes. While kept on ice, 4 μL of experimental DNA and 1 μL of pUC18 plasmid were added to the respective tubes. After 30 minutes incubation on ice, the tubes were heated in a 42°C water bath for 45 seconds. 500 μL of SOC medium was added after another 2 minutes on ice followed by 1 hour shaking (225 rpm) at 37°C.

150 μL of the experimental transformation mixtures and 50 μL of the pUC18 control were plated on ampicillin-containing (50 μg/μL) LB agar plates (LB Amp). The plates were kept at 37°C over night. Only cells that have taken up the plasmid will grow in the LB Amp plates due to Amp-resistant marker within the plasmid. Single colonies on ligation plates were picked to obtain pure cultures after comparing the ligation plates with the religation plates. A high ligation:religation ratio reduces the probability of picking colonies containing empty vectors. 3 mL LB medium supplemented with 50 μg/μL ampicillin for each selected colony was added to 50 mL tubes and shaked (225 rpm) over night at 37°C. A tube with medium only was included as a negative control. 500 μL 20 % glycerol stocks were made as back up and stored at -70°C. The amplified plasmids were then purified by a Miniprep procedure,

performed according to the centrifugation protocol of Wizard *Plus* SV Miniprep DNA Purification System. Only exception to the protocol was the elution volume of the purified DNA – 60 μL nuclease-free water was used instead of 100 μL. DNA concentration was measured by Nanodrop (approximately 200 ng/μL).

In a reaction volume of 20 μL, 500 ng DNA was used in a control restriction enzyme digestion with 0.2 μL BamHI (37°C, 3 hours) followed by gel electrophoresis to verify that the ligation and transformation had taken place.

4.1.3 Exchange of peptide-encoding fragment in pGEM vector

Two restriction enzymes were used to excise the fragment encoding the peptide – BspE1 and Nar1. As a standard, this restriction enzyme digestion is performed in a two-step procedure in a 40 μL reaction volume with 3 hours incubation at 37°C. Specific enzyme buffers are included for the BspE1 and Nar1 enzymes (TurboNar1). The latter also requires BSA. QIAquick PCR Purification Kit is used to remove the enzyme and buffer before the second enzyme is added. After 2 hours, 0.5 μL calf intestine phosphatase (CIP) is added to prevent religation. The linearised plasmid is eluted in H_2O instead of ethanol prior to step 2.

In order to obtain constructs that contain modified DQB sequences with glia-α2 and CLIP2 peptide-coding fragments, the glia-α1 fragment could have been excised from the pUC57-DQB1*0201_α1_streptag vector and inserted the CLIP2 and glia-α2 fragments in its place. But this could not take place directly due to an internal Nar1 restriction site in the pUC57 vector. Instead, the newly made pGEM-DQB1*0201-α1-streptag was used since this vector only contains Nar1 site within the DQB insert.

4.1.4 Hybridisation of oligomers

In the generation of the two other constructs, DQ2.5-glia-α2-Streptag and DQ2.5-CLIP2-Streptag, two different approaches were used. The first approach was used to generate the CLIP2-peptide-encoding fragment by hybridisation of oligomers. 10 μL of 100 μM 5'-phosphorylated forward and reverse CLIP2 oligomers were mixed in TE8 buffer in a final volume of 100 μL. The reaction mixture was heated at 85°C for 30 seconds and slowly cooled down to 28°C before put on ice. A 5 μL sample mixed with 6X loading buffer was run on a

20 % polyacrylamide gel to detect if the hybridisation had occured. 50 bp DNA ladder and unhybridised samples (forward and reverse) were included.

4.1.5 DNA Extraction from Gel

The approach for generating the glia-α2 fragment was to excise the fragment from an excisting construct in the laboratory, pGEM-DQB1*0201-α2. This was performed by a BspE1/Nar1 digestion as described in section 4.1.3. The fragments were detected and separated in a 20 % polyacrylamide gel with a 50 bp DNA ladder and purified from the gel with a crush and soak procedure. The excised gel fragment was crushed and mixed with the Crush&Soak buffer (elution buffer) and gently shaked over night at room temperature. After 1 minute centrifugation at full speed (eppendorf), 0.5 volume of elution buffer was added to the supernatant before a 10 minutes centrifugation at 4°C. After adding 2 volumes of cold ethanol and 1 μL of pink pellet paint, the supernatant was left on ice for 30 minutes. The ethanol-precipitated DNA pellet was rinsed once with 70 % ethanol. DNA concentration was measured and the DNA was stored at -20°C.

The pGEM-DQB-α1-streptag vector was BspE1/Nar1 digested and the linearised pGEM-DQB-streptag without the α1 fragment could be extracted from gel using the QIAquick Gel Extraction Kit. DNA was eluted in 40 μL H_2O and concentration was measured by Nanodrop. The hybridised CLIP2 sample was 100-fold diluted prior to ligation. With an insert:vector ratio of 3:1, α2 and CLIP2 fragment were ligated with 50 ng purified 3.2 kb pGEM vector using T4 ligase in a 20 μL volume. The plasmid amplification in XL10 competent cells and a control gel electrophoresis were performed as previously described.

4.1.6 PCR Amplification

Amplification of the CLIP2 fragment of the pGEM-DQB-CLIP2-Streptag was performed by PCR to verify that the plasmid contained the proper fragment. As DNA template, single cells colonies were picked and added to PCR strips with 15 μL DNase/RNase free water. 1 μL DNA template was mixed with 20 μM of forward primer, CLIP2.1 and reverse primer, DQB-ampβ, 2 mM dNTPs, 0.3U Dynazyme (DNA polymerase) and $MgCl_2$-containing dynazyme buffer in a final volume of 10 μL. Negative control without DNA was included. Stage 1 of the PCR reaction was set to 1 cycle of 95°C for 1 minute, stage 2 had 27 cycles of 95°C, 55°C,

72°C with 30 seconds at each temperature. Stage 3 had 1 cycle of 72°C for 7 minutes ending with 4°C. Afterwards, the samples were run in a 1 % agarose gel.

4.1.7 Transformation of pAcAB3 vector

The recombinant HLA genes are expressed in the baculovirus expression vector system with the use of the transfer vector, pAcAB3. This vector already contains the corresponding DQA1*0501 sequence of the HLA-DQ2.5 molecule. The three modified DQB sequences with fragments encoding three different peptides were placed in pGEM cloning vector and was subsequently cloned into the appropriate vector.

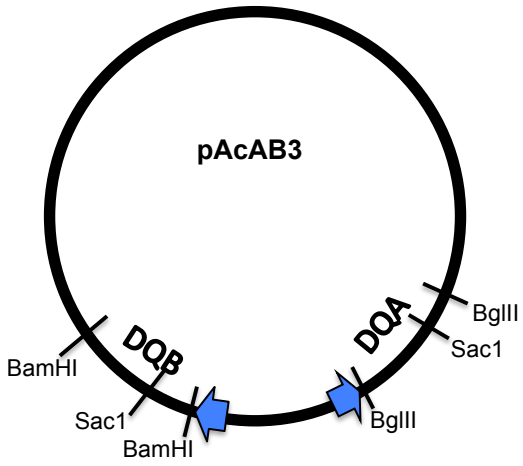

Figure 4.3: Schematic pAcAB3 vector with key restriction enzymes. DQA and DQB sequences are flanked by single BglII and BamHI restriction sites, respectively. There is a Sac1 restriction site within each gene sequence. p10 gene promoters are indicated with blue arrows.

The pGEM vector was digested with BamHI in order to excise the 1kB DQB sequence. The isolated fragments were inserted into the pACAB3 vector (see figure 4.3) as described in section 4.1.1. The expected transformation efficiency of this vector is much lower than pGEM vector due to its size. The restriction digestion was performed with only one restriction enzyme, BamHI, so there was a 50:50 probability achieving a plasmid with correct orientation of insert. Therefore, a multiple of single colonies had to be picked for obtaining pure colonies due to the chance of having plasmids with insert with incorrect orientation. Miniprep was performed and the samples were digested with Sac1 enzyme and buffer 1. After 2-3 hours incubation at 37°C, the samples were monitored on gel eletrophoresis. Sac1 restriction digestion gives slightly different fragment sizes of plasmids with correct and incorrect

orientation of insert. A control plasmid with correct orientation was included to be able to select the appriopriate clones.

Retransformation of selected clones was performed as described in 4.1.2. For the addition of experimental DNA to the XL10 competent cells, 1 μL of 25 to 50-fold diluted DNA miniprep samples were added. The selected single colonies incubated at 37°C for 5-6 hours serving as primary cultures. The pure cultures were transferred to 1L Erlenmeyer flasks containing 100 mL LB medium supplemented with 200 μL ampicillin and shaked over night at 37°C. The purification of pAcAB3 plasmids was performed according to the Plasmid Midiprep System Protocol. DNA concentration was measured by Nanodrop (appr. 300-400 ng/μL). The purified plasmids were digested with BamHI and BglII and tested on gel electrophoresis. 30 μL (60 ng/μL) of the DNA plasmids were sent for sequencing.

4.2 Protein Expression in Insect Cells

The expression of the DQA and DQB gene sequences into functional HLA-DQ2.5 molecules takes place in baculovirus-infected insect cells. Baculovirus-infected Sf9 cells produce both the virus and the recombinant DQ proteins. Prior to large-scale protein production, three recombinant baculovirus stocks were prepared – P0, P1 and P2 virus stocks. The initial, passage-zero (P0) stocks consist of the transfection supernatants while the P1 virus stocks are positive plaques selected from plaque assay. P2 virus stocks are amplified from P1 and are used in the large-scale protein production.

4.2.1 Sf9 insect cells

Sf9 insect cells are semi-adherent and will attach to surfaces, e.g. plastic. These insect cells can either be cultured as monolayer in culture flasks or in cell suspension in spinner flasks. In the latter case, Pluronic is added as a protective surfactant to the cell suspension to protect against cellular damage by shear forces and to reduce cell attachment (50). We used Sf9 cells in suspension and they are placed in a humid 27 °C incubator on a rotator for optimal growth conditions. The optimal cell density is between 1.0 to 4.0 x 10^6 /mL. A suspension stock of Sf9 cells was placed in a small spinner flask while large spinner flasks were used in expansion of the cells. Maximum volume used in the small and large spinner flasks was 100 mL and 350

mL, respectively. The cells were typically split every third or firth day with complete Grace´s medium and 0.1 % Pluronic. The health of the cells and the cell density were examined under the microscope and dead cells were stained with Trypan Blue. ExpresSf^{+} cells are Sf9 cells adapted to grow in serum-free medium – Xpress insect medium. In cell maintenance they are treated as the Sf9 cells except for the medium.

4.2.2 Baculovirus transfection (P0)

Sf9 cells were transfected with linearised baculovirus DNA along with the newly generated pAcAB3 vectors. Linearised baculovirus DNA and transfection buffers A and B of the BD BaculoGold Transfection kit were used. 600 µL à 1x10^{6} Sf9 cells/mL in complete Grace's medium (no Pluoronic) was added to four wells in a 12-well plate – one for each construct and one for a transfection without plasmid. After the Sf9 cells had attached to the wells, the medium was extracted from the wells and 200 µL of transfection buffer A was added.

1 µL (0.1µg) linearised baculovirus DNA was mixed with 4 µL (0.1 µg/µL) plasmid. 200 µL transfection buffer B was added after 5 minutes. The DNA mix in buffer B was dropwise added to the wells containing Sf9 cells in transfection buffer A. To allow transfection of baculovirus DNA and plasmid into the Sf9 cells, the plate incubated for four hours in a humid container at 27°C. The medium was extracted from all wells and washed with 1 mL complete Grace's medium. 600 µL of fresh complete Grace's medium was added before placed in the 27°C incubator for 4 to 6 days. The P0 transfection supernatants were collected and stored at 4°C.

4.2.3 Plaque assay (P1)

2 mL of 1 x 10^{6} Sf9 cells in 10 % FCS/Grace's medium was added to 15 60 mm Falcon petridishes and incubated for 30 minutes at room temperature to allow cells to attach. Dilution series of the P0 samples from 10^{-2} to 10^{-6} were performed. Medium was aspirated with Vacusafe aspiration unit and 1 mL diluted P0 virus supernatants were carefully added to the dishes and incubated for an hour at room temperature. A 2 % agarose gel of BD BaculoGold Plaque Assay Agarose and milli-Q water was prepared by boiling the mix in microwave oven followed by cooling in a 44°C waterbath. 20% FCS in 2x Grace's medium supplemented with gentamicin was also incubated at the same temperature for 15 minutes. The medium and

agarose were mixed and placed in a 42°C waterbath. The virus supernatants were extracted before 4 mL 1% agarose mix was carefully added to the plates to form an overlay. The plates were left for 30 minutes to allow solidification of the gel and then placed in the moist chamber at 27°C for 5 to 10 days. After 10 days plaques in the agarose gel were observed formed due to cytolysis of virus-infected Sf9 cells. From plates with 20 to 100 plaques, 10 single plaques were marked and picked for each construct. By using sterile 1 mL pipette tips, one by one were transferred to small culture flasks containing 3 mL Sf9 cells à 1 mill/mL. First, the cells were allowed to attach to the flasks for 15 minutes. After 6 days incubation at 27°C, the cell suspensions were centrifuged at (1100 x g, Megafuge1.0) for 10 minutes and the virus supernatants were collected and stored at 4°C. The P1 baculovirus stocks (amplified from P0) were tested in ELISA with anti-DQ2 antibody to confirm positive plaques, i.e. the plaques containing HLA-DQ2.5-producing virus.

Plaque assay was performed in order to amplify baculovirus carrying our genes of interest. P1 virus stocks were produced from a clonally expanded single infected Sf9 cell These P1 supernatants were subsequently amplified into P2 baculovirus stocks. Another purpose of performing a plaque assay was to determine the virus titer. The virus titer was used to decide the amount of P2 virus stocks necessary for optimal infection of ExpresSf+ cells in the protein production step. Lower amount of the P2 virus supernatant is needed for optimal protein production with high-titer virus stocks. The multiplicity of infection (MOI) is the number of virions that are added per cell during infection and should be between 3 and 10. A plaque assay with a serial dilution from 10^{-2} to 10^{-9} was performed. A 10-fold increase in number of plaques should be observed starting with the plate harbouring one single plaque. This plate determines the virus titer. If one plaque is observed at the 10^{-8} plate, the virus titer is 10^{-8} meaning there are 10^{8} plaque forming units per mL (PFU/mL). For instance, adding 27 mL P2 virus stock to 7.0 x 10^{8} ExpresSf+ cells, gives a MOI (PFU/cell) of 3.86.

In the determination of virus titer, the number of plaques (PFU) was counted with the naked eye. An alternative was to stain the plaque assay plates with 1 % crystal violet to make it easier to visualise the plaques and thereby get a more reliable counting. 2 mL 20 % TCA was added to each plate for 5 minutes. The solution and the agar layer were removed before 2 mL 1% Crystal violet was added to each plate for 30 minutes. The plates was rinsed gently with H_2O and let to dry prior to counting the plaques.

4.2.4 Amplification from P1 to P2 virus stocks

In order to produce sufficient amounts of DQ proteins, the positive P1 baculovirus plaques are amplified into P2 virus stocks. A selected P1 supernatant was transferred to a spinner flask with 350 mL complete Grace's medium containing 2 x 10^6 Sf9 cells. After six days the cell suspension were spun down (1100 x g, Megafuge1.0) for 10 minutes and the amplified baculovirus supernatants were collected and stored at 4°C.

4.3 Protein Production and Purification

4.3.1 Protein expression and supernatant filtration

In the production stage of the HLA proteins ExpreSf+ cells replace Sf9 cells. Insect Xpress medium is ready to use when supplemented with gentamicin. One protein batch is approximately 1L in 3 spinner flasks á 350 mL with cell density of 2 x 10^6 cells/mL. P2 supernatant is added to the cell suspension calculated from the virus titer obtained by plaque assay.

After 4 to 6 days of incubation at 27°C, the virus infected cell suspension was transferred to 500 mL centrifuge bottles and centrifuged at 1800 rpm using a SLA-3000 rotor for 30 minutes at 4°C (Sorvall). The supernatants were filtered through three filters – GF-D 2.7 µm filter, GF-F 0.7 µm filter and 0.45 µm filter – and kept on ice.

4.3.2 Affinity chromatography

In affinity chromatography proteins are isolated from a cell free extract by the specific interaction with the binding partner of the protein, e.g. antibody and its antigen or enzyme and its substrate. A gel matrix consisting of cross-linked sepharose makes up the columns. This beaded gel filtration medium can be coupled to antibodies bridged by protein A and monoclonal antibodies specific for the protein of interest. Only proteins with the physical and chemical features of the columns will be trapped while the rest will flow through. The immobilised proteins can be released in elution buffer. This buffer alters the pH conditions of the column leading to interruption of the specific antibody-antigen interaction.

The filtered supernatants were run through three serial coupled columns. First column containing sepharose CL-4B adsorbs non-specific proteins present in the cell free extract. The second column containing protein A immobilised on sepharose CL-4B adsorbs IgG antibodies. The third column packed with protein A sepharose CL-4B cross linked to the monoclonal antibody 2.12.E11 traps the DQ2 proteins. 1X PBS pH7.2 was used as wash buffer. The columns were washed with approximately 200 mL 1X PBS before the supernatant was run through overnight with a velocity of 1 drop per 2 seconds.

After the supernatant had run through, the columns were washed with 150 mL PBS and then disassembled. The trapped DQ2.5 molecules in the 2.12.E11 column were eluted with 25 mL 0.05M diethylamine, 0.15M NaCl pH 11.5 directly into 3 mL neutralising buffer (2M Tris-HCl pH 6.3) in a 50 mL tube. The column was regenerated to neutral pH with PBS afterwards. The Protein A column was eluted with 50 mL 0.1M citrate buffer pH 3 before regenerated to neutral pH. 4-5 mL of PBS containing 0.05% azide was added to the columns for storage.

The eluted protein sample was concentrated to 500 μL in Vivaspin centrifugal concentratators (10-kDa cut off). The concentrated sample was then washed with 2x15 mL PBS by centrifugation at 4°C (2100 x g, Varifuge3.0R). The protein concentration was measured by Nanodrop with extinction factor of 75.7 M^{-1} cm^{-1} and molecular weight of 65 kDa. 0.09 % azide was added before the aliquots of the samples were freezed in liquid nitrogen and stored at -70 °C.

4.4 Quality Control of Recombinant DQ Molecules

4.4.1 ELISA

Enzyme-linked immunosorbent assay (ELISA) is a sensitive detection method based on the specific antibody-antigen interaction. This method was used for two purposes. The first purpose was to identify positive plaques in plaque assay while the other purpose was a part of the quality check of the purified proteins in order to confirm the specificity of DQ2.5 molecules produced.

A flat-bottomed 96-well plate was first coated with 10 µg/mL of unconjugated primary antibody, 2.12.E11, in a final volume of 50 µL in PBS. This monoclonal antibody of IgG1 isotype is an anti-HLA-DQ2 antibody (51). The coated plate incubated for two hours at 37°C. The plate was washed three times with PBST (0.05% Tween-20) with a HydroFlex ELISA washer. 100 µL blocking buffer (3% BSA in PBS) was added to each well and incubated overnight at 4°C. The washing procedure with PBST was performed prior to addition of a new reagent and this buffer was also used as dilution buffer in a final volume of 50 µL for samples and the other reagents. The plate incubated for an hour at 37°C for the samples, the enzyme and for each antibody step.

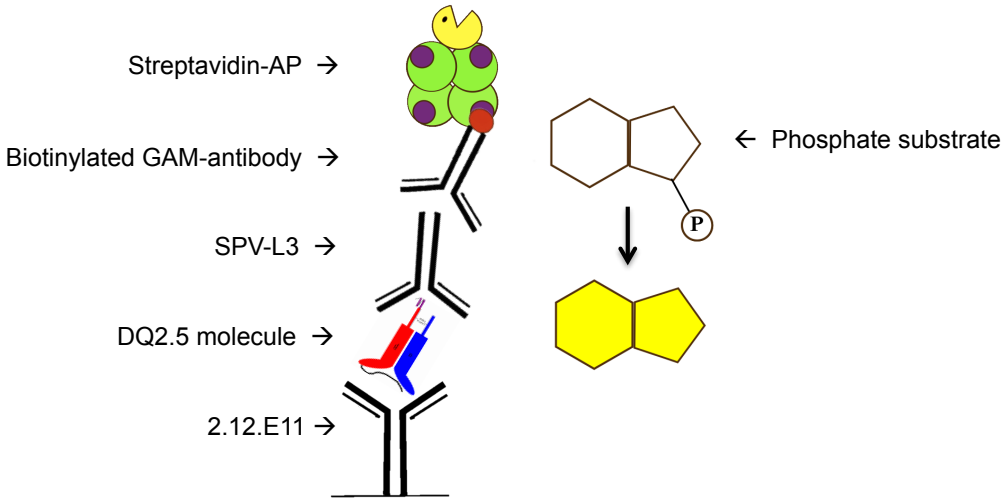

Figure 4.4: Schematic presentation of reagents in ELISA. The plate is coated with the human monoclonal anti-DQ2 antibody, 2.12.E11, which can capture HLA-DQ2.5 molecules (antigen) present. Another monoclonal anti-DQ antibody (murine), SPV-L3, functions as an unconjugated secondary antibody and binds to another epitope of the DQ2.5 molecule. The conjugated secondary polyclonal goat-anti mouse (GAM) antibody binds subsequently to SPV-L3. Enzyme-coupled streptavidin with biotin-binding sites will bind to the biotinylated GAM. In the presence of phosphate substrate, alkaline phospatase (AP) dephosphorylates the substrate leading to yellow colour development.

Dilution series of the samples were performed and added to the wells in duplicates. Conventional DQ2.5-α1 molecules were used as positive control while 10% FCS in Grace's medium and PBST were included as negative controls. After a new wash step, 1 µg/mL of the unconjugated secondary antibody, SPV-L3 was added to the wells. This monoclonal antibody is of murine origin and binds to HLA-DQ proteins present in the sample. Next, 0.25 µg/mL of biotinylated goat anti-mouse (GAM) antibody was added. This polyclonal antibody conjugate

of IgG2a isotype binds to the murine secondary antibody to several epitopes and is human adsorped to avoid cross reactivity.

Streptavidin-AP (alkaline phosphatase) was diluted a 1000-fold and added to the wells for 30 minutes before addition of 100 μL phosphatase substrate (2 mg/mL) in 1M diethanolamin buffer. In positive samples, colour development of the substrate caused by the enzyme-linked streptavidin was observed after five minutes and the intensity was measured at 405 nm using a Multiskan ascent analyser. The different steps are illustrated in figure 4.4.

4.4.2 SDS PAGE

Sodium dodecyl sulfat polyacrylamide gel electrophoresis (SDS PAGE) is a method to separate proteins and nucleotides primarily based on their molecular weight. SDS is a detergent added to the gel solutions and protein samples and will linearise the proteins due to denaturation of secondary and partly tertiary structures. This anionic detergent binds to the protein surface giving them a uniform negative electrical charge. The reducing agent β-mercaptoethanol is added to the sample buffer to reduce disulfid bonds of the proteins. This method is used to separate the α and β chain of the HLA dimer and to look at the purity after affinity purification.

The gel consists of bisacrylamide and the gel polymerisation is caused by free radicals formed by ammonium persulfat (APS). TEMED is added due to its catalytic ability in formation of these free radicals. The samples run through two gels of slightly different composition. The samples are applied onto the stacking gel and are allowed to form a linearised band. The separation of the different protein fragments does not take place until the samples enter the separating gel, which has higher polyacrylamide content. The narrower pore size of the gel makes it easier for the smallest fragments to travel and larger fragments are restricted to enter the pores due to size.

The 12 % separating gel solution and 6 % stacking gel solution were prepared according to the recipe described in section 3.6. When the gel apparatus was set up the 12 % solution was transferred to the glass plates. After polymerisation of the gel, 6 % solution was applied on top of the separating gel and allowed to polymerise.

To improve the separation of the chains making up the dimeric proteins, the covalently linked peptides were cleaved with thrombin. 5 to 8 μg protein in 14 μL volume was incubated with 1 μL thrombin for two hours at room temperature. 5μL 4X Laemmli's sample buffer was added to the samples before 5 minutes boiling. The gel tray was filled with 1X running buffer prior to loading to ensure a sealed system. The boiled 20 μL samples were loaded onto the gel and a protein ladder was included. A conventional DQ2.5 molecule was used as comparison and as a positive control. The gel was run at 150V and 20 mA for approxiamately 70 minutes. Biosafe CooMassie was used to stain the gel. After one hour of staining, the gel was destained with dH_2O and left overnight.

4.4.3 Size-exclusion Chromotography

Chromatography is a well-known laboratory technique to separate molecules in a mixture either planar or on a column. In liquid chromotography the mobile phase is liquid. In contrast to high-performance liquid chromatography (HPLC), which requires high pressure to drive the mobile phase through the column, separation with fast protein liquid chromatography (FPLC) can be carried out with low pressure conditions. For proteins and other biomolecules, both HPLC and FPLC can be used as analytical tool. The latter was developed to function as a preparative tool, e.g. purification of biomolecules. There is of great importance to handle proteins under physical conditions in order to retain their bioactivity. (52)

Size exclusion chromatography or gel filtration can be performed with a FPLC instrument (Äkta). The prepacked gel filtration column, Superdex 200 GL consists of beads of crosslinked dextran (sephadex) and agarose (sepharose) and make up the stationary phase. The mobile phase consists of the protein sample in PBS buffer. The Superdex200 column separates the molecules according to their size and has a separation range from 10 to 600 kDa. Larger proteins (or aggregates) will be excluded from entering the beads due to size and will be eluted first. The instrument and the connected computer with the software Unicorn 5.01 monitor the parameters UV (215 nm and 280 nm), conductivity, pH, pressure and flow rate. The settings for each sample run can be executed manually or be pre-programmed.

The purpose for using this method was first to analyse the affinity purified recombinant proteins to examine the stability of the protein by comparing monomeric peak with protein

aggregations. But the FPLC instrument was also used to fractionate the monomeric peak of the complete affinity purified protein sample.

Prior to loading the sample, the system including a system pump must be equilibrated with the same buffer as the sample buffer. Both the system and the column are stored in ethanol (70 % EtOH). The latter is also kept cold at 4°C when not used. EtOH must be exchanged with sonicated MQ water before changing to PBS. The volume needed for exchange corresponds to the column volume of 24 mL. In terms of keeping a stable pressure, it is critical to avoid any bubbles in the system especially when the column is assembled.

Loops and syringes used were also equilibrated in PBS. For analytical samples, 10 μg protein in 50 μL PBS was injected into a 100 μL loop using a 50 μL syringe. For fractionation of monomers, 700 to 1400 μg protein in 250 μL PBS was injected into 1 mL loop using 250 μL syringe. In the latter case, eluate was collected in a fraction collector with fraction size of 0.5 mL and tubes containing the peak of interest were selected. The column was regenerated and stored in EtOH after use.

4.4.4 MALDI-TOF mass spectrometry

Matrix-assisted laser desorption/ionisation (MALDI) is a technique used with time-of-flight mass spectrometry (MS-TOF) and can be used to identify peptides. MALDI is used as an ion source for the ionisation of the molecules. With MS-TOF, the single-charged molecules are separated as they accelerate through a magnetic field according to their mass-to-charge ratio. A detector measures the time of flight and the analysed data generates a mass spectrum.

The recombinant proteins are covalently linked to the epitope peptide at the N-terminus of the β chain. The peptide can be cleaved with thrombin due to a thrombin cleavage site adjacent to the peptide sequence. The purpose for using this method was to identify the epitope peptide sequences of each construct and to confirm the correct mass of the peptides. The spectra of the three peptides were compared with the theoretical mass of the peptide sequences (see **table 4.2**).

1-2 μL of the DQ molecules was treated with 0.2 μL thrombin for one hour at room temperature. The samples were mixed with a matrix dissolved in acetonitril and placed on a stainless steel plate. Prior to MALDI-TOF analysis, the mixture was allowed to crystallise.

Table 4.2: Peptide fragments after thrombin cleavage and their theoritical mass.

Peptide	Sequence	M+H
DQ2.5-glia-α1a	RDSGQLQPFPQPELPYGAGSLVPR	2609.35
DQ2.5-glia-α2	RDSGPQPELPYPQPEGAGSLVPR	2447.70
CLIP2	RDSGMATPLLMQALPMGALGAGSLVPR	2710.42

4.5 Dye conjugation

The generated HLA-DQ2.5 molecules are engineered with a GGSC sequence for dye conjugation. The selected dye is called ATTO-565 maleimide and can be coupled to free cystein residues of proteins at the pH range of 6.5 to 7.5 where the sulfhydryl group is kept at reduced state. The sulfhydryl group is added across the double bond of the maleimide group forming a stable thioether bond (see figure 4.5).

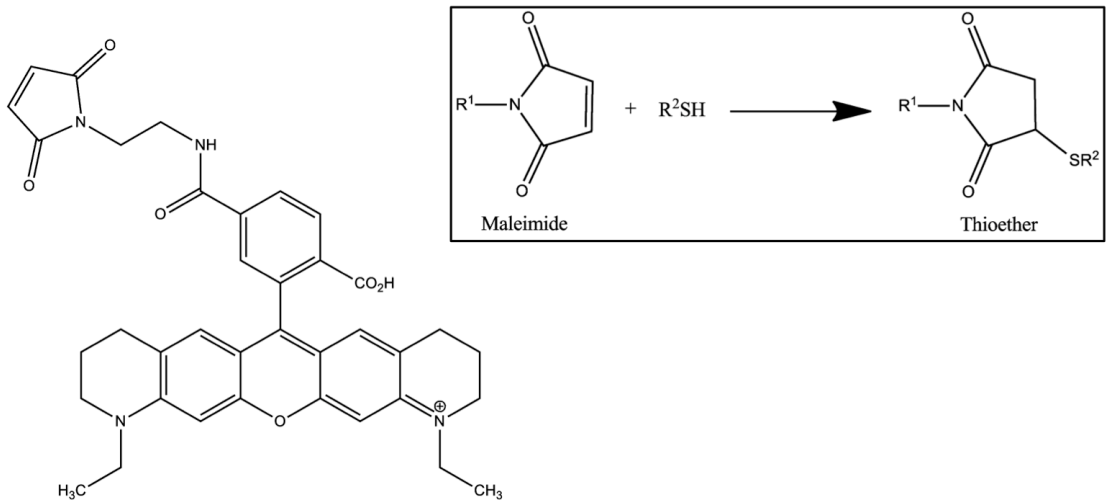

Figure 4.5: ATTO565-maleimide and the maleimide reaction. The aromatic ATTO565 with the functional group maleimide can react with sulfur groups (-SH) of cysteine residues and form stable thioether bonds in reducing conditions (Illustration created with ChemDraw).

1 mg ATTO565 was dissolved in 100 µL anhydrous, aminefree DMSO and stored at -20 °C. According to the protocol provided with reagent, the 1.3x molar excess of the reactive dye should be sufficient amount in the conjugation reaction. The amount of ATTO565 was calculated and 0.15 µL ATTO565 was mixed with 100 µg protein in 100 µL PBS pH7.2. The reaction incubated protected from light for two hours at room temperature or at 4°C overnight

before free dye (either hydrolysed or unbound) was removed. Two approaches for purification of the conjugate were made, either by gel filtration or centrifugation with 20 mL Vivaspin centrifugal concentrators (10-kDa cut off).

In gel filtration, 3.5 g of Sephadex G-25 was added to a 50 mL tube and let to swell in PBS. 1 mL of the Sephadex slurr was added to the small column and preequilibrated with PBS. The 100 μL sample was applied on the column and eluted in PBS. The first coloured and fluorescent zone to elute is the desired dye-protein conjugate. The eluate was upconcentrated using 500 μL Vivaspin centrifugal concentrators (10-kDa cut off) for 5 minutes (15 000 x g, Eppendorf). Protein concentration was measured and stored at -70°C. Purification of conjugate was also performed with Superdex 200 GL on FPLC as described in 4.4.3. The other approach to remove free dye was dilution of the sample with PBS (3x 15 mL) by centrifugation at 4°C (2100 x g, Megafuge 1.0).

It is of interest to estimate the number of ATTO565 molecules conjugated per DQ2 protein. In order to determine the degree of labelling (DOL), i.e. dye-to-protein ratio, the absorption at 280 nm and 565 nm was measured in spectrophotometry (SmartSpec3000) and then calculated according to the formula below. A titration of molar excess of ATTO565 to DQ2.5 molecules up to 100x molar excess was performed.

Determing the Degree of Labelling (DOL): dye-to-protein ratio

$$\frac{c_{dye}}{c_{prot}} = \frac{A_{max}/\varepsilon_{max}}{A_{prot}/\varepsilon_{max}} = \frac{A_{max} \times \varepsilon_{max}}{A_{280} - (A_{max} \times CF_{280}) \times \varepsilon_{max}}$$

c(dye) = concentration of bound dye		c(prot) = concentration of protein	
$A_{max} = \lambda_{abs}$ 565 nm		$A_{280} = \lambda_{abs}$ 280 nm	
ε_{max} (extinction coefficient)	1.2×10^5 M^{-1} cm^{-1}	ε_{prot} (extinction coefficient)	75700 M^{-1} cm^{-1}
CF_{280} (correction factor)	0.16		

4.6 T cell expansion

In order to maintain the *in vitro* reservoir of T cell clones, they must be expanded. For clonal expansion T cells are stimulated with IL-2, IL-15 and PHA to grow in the presence of feeder cells. Irradiated mononuclear cells from peripheral blood (PBMC) from 2 or 3 donors are used as feeder cells.

20 mL blood samples from each donor were transferred to 50 mL tubes and mixed with room-temperated RPMI1640 up to 40 mL before the density gradient lymfoprep was carefully added to the bottom of the tube. The tubes were centrifuged for 20 minutes with the brakes off (800 x g, Megafuge1.0). PBMCs forming a distinct band at the interface between medium and lymphoprep were transferred to new 50 mL tubes for each donor and washed twice with RPMI1640 and centrifuged at 250 x g for 10 minutes and a second round at 500 x g for 7 minutes. The cell pellets were resuspended in 10 % human serum in preheated RPMI1640 medium supplemented with 2-mercaptoetanol, penicillin, streptomycin (MEPS) before radiated at 50 Gray (Gammacell).

T cell clones are stored in 1 mL vials in liquid nitrogen and must be carefully thawed for successful expansion. 8 mL thawing medium per vial (20 % FCS in RPMI1640) was preheated to 37 °C. The vials were thawed in 37°C waterbath and transferred to 50 mL tubes. The thawing medium was dropwise added to cell suspension while the mix was gently swirled before centrifuged for 7 minutes (500 x g, Megafuge1.0). The pellets were resuspended in preheated 10% human serum (HS).

The irradiated PBMCs were combined and resuspended in 10 % HS in MEPS supplemented with 10 U/ml IL-2, 1 ng/ml IL-15 and 1 μg/ml PHA. TCCs were resuspended in this feeder mix in a final volume of 0.2 – 0.5 mill/mL. 1 mL was seeded for every flat-bottomed 24-well plate. The cells were split and fed with 10% HS / MEPS/IL-2/IL-15 (no PHA) during day 3 to 7 after restimulation when required. The cells were tested and frozen on day 7 to 10 depending on their growth. The cryovials were frozen in icecold 0.5 mL 50% FCS in RPMI1640 and 0.5 mL 20% FCS/20% DMSO in RPMI1640. Each cryovial typically contain 1-4 x 10^6 expanded T cells. After 24 hours at -70°C, the vials were transferred to the liquid nitrogen tank.

A T cell assay was performed to test if the TCCs were specific for their antigens, i.e. deamidated gluten peptides. Antigen dilutions from 10 μM to 0.1 μM were prepared in PBS and 25 μL of the antigen dilutions was co-incubated with 75 μL irradiated (75 Gray) $DQ2.5^{+}$, EBV-transformed B cells overnight in triplicates. A negative control with no antigen was included. 20-40 000 T cells were added the following day and 2 days later 20 μL (1μCi) ^{3}H-thymidine was added to the wells. 16 to 20 hours after added thymidine, the plates were harvested and CPM counted using a β-plate counter.

4.7 Flow Cytometry and Streptamer Staining

4.7.1 Flow Cytometry - background

Flow cytometry is a powerful technique for the analysis of multiple parameters of individual cells. A flow cytometer consists of several primary systems. The cell samples are conducted through channels in a fluidic system. At the interrogation point cells can pass through the laser beams one at a time. The lasers making up the light source for scatter light and fluorescense are controlled by an optic system. Light is detected by detectors while the connected computer system converts data from the electronics and analyses the data statistically.

Different cell types and phenotypes give rise to a certain cell profile, such as cell size and granularity, which is determined by forward and side scattered light, respectively. Fluorescent light can also be detected in flow cytometry giving us the opportunity to achieve greater insights of cells analysed. Fluorescence is the process of absorbing and re-emitting of light occuring with fluorescent substances called fluorophores. Due to internal conversion of the energy the absorbed energy is re-emitted as photons (light particles) at a longer wavelength than the absorbed photon. The fluorescent characteristics depend on the excitation state of the fluorophore and its wavelength.

A fluorophore can be coupled to monoclonal antibodies specific for cell surface molecules or molecules inside the cell. A laser beam consists of monochromatic light of a certain wavelength and the emitted light from a given fluorophore, the fluoroscent signal, can be

detected by the flow cytometer. Several signature proteins (surface/intracellular markers) coupled to a variety of fluorophores can be used as multiple parameters in order to characterise cells of interest. The selection of fluorophores is dependent on the compatibility of excitation/emission spectra by examining the emission peaks and spectral overlap. Staining cells with two fluorescent parameters gives rise to four distinct populations. Cells expressing two surface receptors will be double positive while expressing only one of the surface receptors cells will be positive for one marker and negative for the other and visa versa. On the other hand, cells not expressing the receptors will be double negative. The latter accounts for unstained cells as well.

4.7.2 Streptamer Staining

Cells can also be stained with fluorophore-conjugated MHC multimers prior to flow cytometry. In this thesis, gliadin-specific T cell clones were stained with Streptamers consisting of the newly generated HLA-DQ2.5 molecules and Streptactin-APC. The cryo-preserved clones were thawed as described in T cell expansion except for that cell pellets were resuspended in FACS buffer.

Two approaches for Streptamer staining were used. All steps were performed at 4 °C and incubations were carried out in the absence of light. The first approach was using the conventional tetramer staining procedure where molar streptavidin:protein ratio is calculated to find the tetramer concentration in a final volume of 50 μL. Titration of molar ratio and concentration was performed in addition to different T cell staining incubations from 15 to 180 minutes. 5 μg of DQ2.5 molecules was preconjugated for at least 45 minutes with Streptactin-APC prior to T cell staining. The amount of Streptactin-APC was stepwise added to optimise the saturation of the HLA2.5 molecules to the binding sites of the Streptactin backbone.

The other approach was using the protocol provided with the Streptactin-APC reagent. 5 μL Streptactin-APC was conjugated with 1-3 μg DQ2.5 molecules in a final volume of 50 μL in FACS buffer for at least 45 minutes and stained with T cells for another 45 minutes. The T cell staining was performed either in 96-well plate or directly in flow tubes. A titration of amount of DQ2.5 molecules required for optimal staining was performed. Cells were washed with 200 μL FACS buffer and centrifuged for 4 minutes (500 x g, Varifuge3.0R) and

resuspended in 200 μL buffer. T cells stained with Streptamers also incubated with FACS buffer containing 1 mM d-biotin twice for 20 minutes with a wash step in between, followed by 4 rounds of washing before resuspended in 200 μL FACS buffer.

As a positive control, T cells were stained with 2.5 μL the direct-conjugated antibodies, anti-CD4-PE and anti-CD3-APC, to verify that the T cell clones stain positively for T cell marker CD3 and T-helper cell marker $CD4^+$. Conventional tetramers were also used as positive controls. Staining T cells with a non-specific DQ2.5-gliadin peptide-epitope or DQ2.5-CLIP2 was included as negative controls. A fraction of the cells were left unstained. Finally, the samples were transferred to labelled flow tubes and run on BD Flow cytometer (FACScalibur). Parts of the flow cytometry experiments (LSR II Yellow Laser) were also performed at the Flow Cytometry Core Facility, Montebello, OUS. The data were analysed on FlowJo software.

5 Results

5.1 Generation of Recombinant DQ2.5-molecules

Plasmid construction

The dimeric recombinant HLA-DQ2.5 molecule consists of an α chain and a β chain encoded by the DQA and DQB genes, respectively. The gluten epitope peptide encoding sequence is integrated at the 5'end of the DQB gene sequence while a BirA site constitutes the 3'end of the gene segment. In this project, the BirA site was replaced by a One STrEP-tag sequence (StreptagIII), which encodes a tandem repeat of the eight-amino acid Streptag peptide separated by a flexible linker. In addition, a cysteine-encoded sequence for a dye conjugation site was also incorporated. Three HLA-DQ2.5 constructs with different peptides linked to the β chain were generated. The modified DQB1*0201-α1 gene sequence, synthesised by GenScript, was cloned and ligated into the pGEM cloning vector (see **figure 4.2**) in order to generate all three constructs. Amplified and purified pGEM plasmids carrying the DQB-streptag sequences were digested with BamHI restriction enzyme and subsequently cloned into the pAcAB3 expression vector. The insertion of the DQB sequence can have two orientations due to single restriction enzyme digestion. Both the DQA and DQB sequences harbour a Sac1 restriction site. The correct orientation of the DQB insert was determined with a Sac1 restriction digestion as shown in **figure 5.1**. A construct with confirmed correct orientation of DQA and DQB was included as a positive control in lane 12 and lane 24. pAcAB3 vectors with ligated DQB-streptag sequences were amplified in XL10 cells and several single colonies were selected to increase the probability to obtain constructs with correct orientations. Only 1 (lane 2) out of 6 pAcAB3-DQB1*0201-α1 constructs (lanes 2 to 7) had the correct orientation compared to the positive construct in lane 12. From the pAcAB3-DQB1*0201-α2 constructs (lanes 8-11, 14-17), 4 out of 8 constructs were positive (lanes 8, 11, 15 and 16). For the pAcAB3-DQB1*0201-CLIP2 constructs (lanes 18-23), 2 constructs were found to be positive (lanes 19 and 22). Finally, miniprep DNA of the positive constructs with highest DNA concentrations (40-130 ng/μL) were selected, retransformed, amplified and purified by Midiprep (330-400 ng/μL).

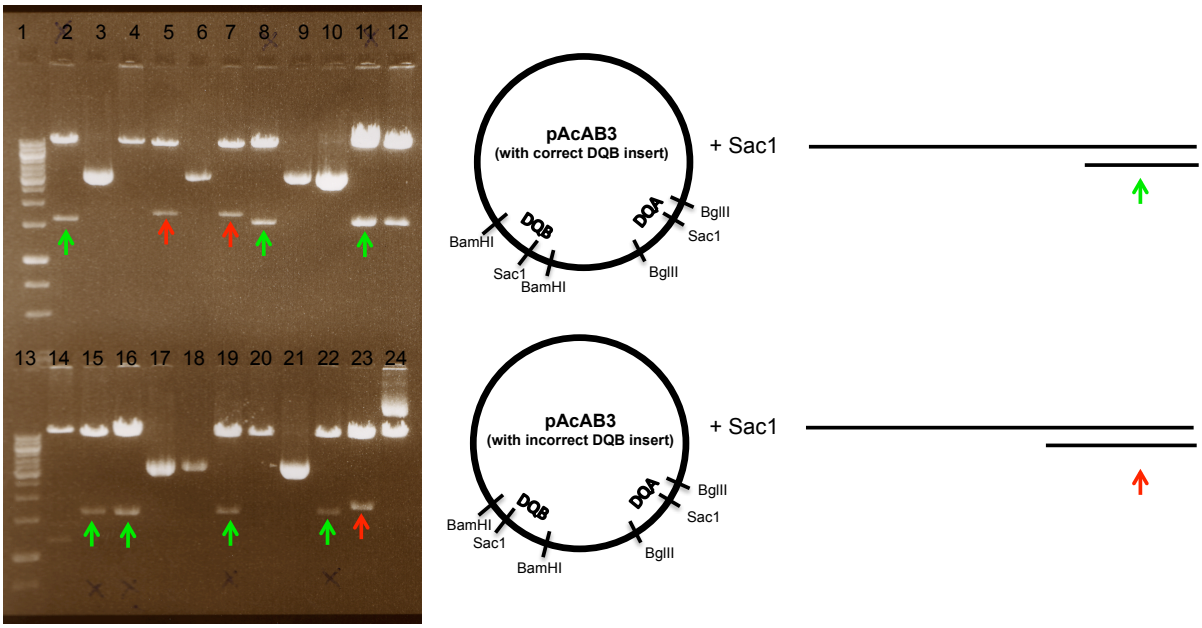

Figure 5.1: Gel electrophoresis – Sac1 digestion of pAcAB3 vectors. Lane 2-7; DQ2.5-glia-α1 plasmid constructs. Lane 8-11,14-17; DQ2.5-glia-α2 plasmids constructs. Lane 18-23; DQ2.5-CLIP2 plasmid constructs. 1kB DNA ladder in lane 1 and 13 and a positive control with correct orientation in lane 12 and 24. Constructs with correct orientation (compared with positive control) in lane 2, 8, 11, 15, 16, 19 and 22 (green arrows). Plasmids with incorrect DQB inserts are indicated with red arrows. The correct DQB insert is smaller in size compared to incorrect DQB insert (*right*) and migrates slightly faster in the agarose gel.

As a final control restriction enzyme digestion, the Midiprep-purified pAcAB3 vectors containing the correct inserts of DQA and DQB were digested with BamHI and BglII restriction enzymes. Four distinct fragment bands were expected from this restriction digestion – the DQB-streptag sequence (1070 bp), the unmodified DQA sequence (800 bp), the intermediate DQA/DQB region (500 bp) and the rest of the pAcAB3 vector (appr. 9400 bp). Lane 2, 3 and 4 in **figure 5.2** contain DQ2.5-α1, DQ2.5-α2 and DQ2.5-CLIP2 constructs, respectively. The positive control (lane 5) was only partially digested. However, the selected plasmid constructs were sent for DNA sequencing (GATC Biotech) in order to proofread the basepair sequences. The forward and reverse sequences were correlated by ClustalW multiple sequence alignment with the expected sequences (data not shown). Despite the partially digested positive control, the gel electrophoresis of the BamHI/BglII control digestion of the pAcAB3 constructs confirms the expected band pattern.

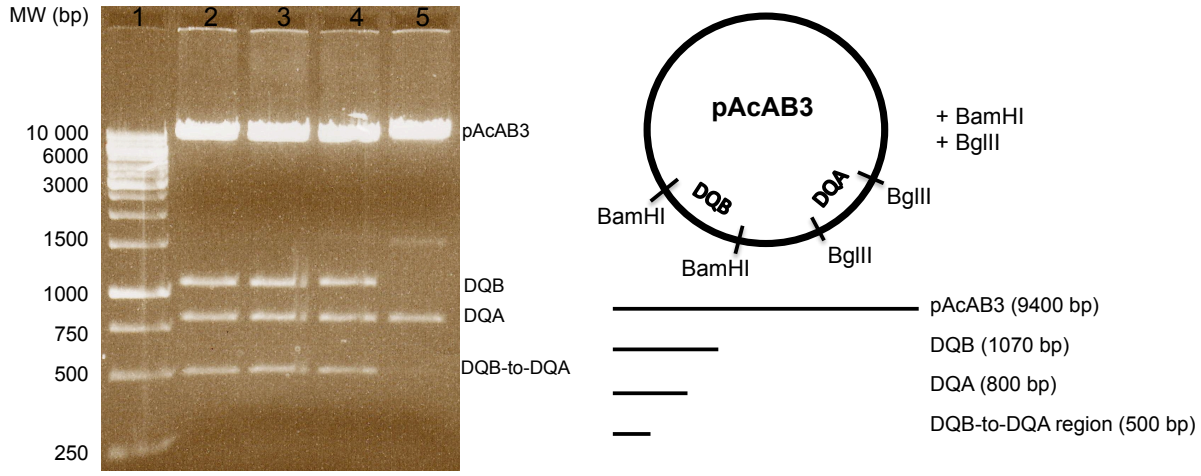

Figure 5.2: Gel electrophoresis – BamHI/BglII digestion of pAcAB3 vectors. Gel electrophoresis (*left*): Lane 1; 1kB DNA ladder. Lane 2; DQ2.5-α1-streptag construct, lane 3; DQ2.5-α2-streptag construct. Lane 4; DQ2.5-CLIP2-streptag construct, Lane 5; positive control. The expected Sac1-digestion fragments are illustrated to the right.

5.2 T cell Assay

T cell clones (TCCs) obtained from celiac lesions in the gut from CD patients had been expanded in vitro, and were then tested for their antigen-specificity in a so-called T cell assay. We used TCC535.3.23 and TCC678.3.22 and specific for the deamidated gluten-peptides HLA-DQ2.5-glia-α1a (P1269) and HLA-DQ2.5-glia-α2 (P1274). Diluted antigen peptides (10 μM to 0.1 μM) were co-incubated with DQ2.5+, EBV-transformed B cells (APCs) in triplicates. As a negative control we used APCs without adding the antigen. The TCCs were added to the APC:peptide mix on the following day. Using a β-plate counter, cell proliferaton was measured by the means of radioactivity as counts per minute (CPM) after ^{3}H-thymidine incorporation (which was added on day 4). **Figure 5.3** shows that P1269 is responsible for the cell proliferation of the DQ2.5-α1-restricted TCC. The same accounts for P1274 and the DQ2.5-α2-restricted TCC. Thus, TCC 535.3.23 was specific for the gliadin-α1 (P1269) peptide and TCC678.3.22 was specific for the gliadin-α2 (P1274) peptide. Thus, the T cell assay demonstrates the antigen-specificity of the TCCs used as test clones in flow cytometry.

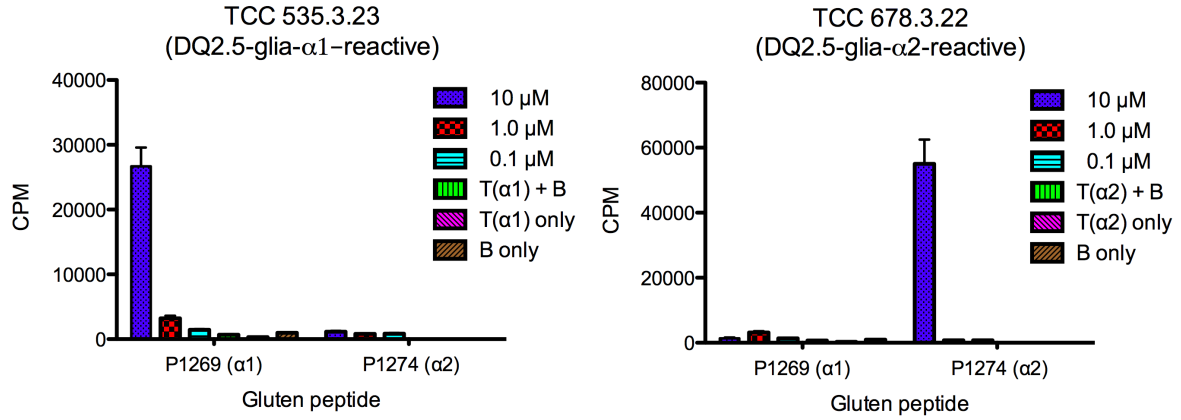

Figure 5.3: Testing the antigen specificity of TCCs. After expansion T cell clones were tested in a ^{3}H T proliferation assay. Triplicates were tested at 3 peptide concentrations, 0.1, 1.0 and 10 µM. The deamidated peptides, P1269 and P1274, are gliadin-α1 and gliadin-α2 peptides, respectively. DQ2.5^{+}, EBV B cells were co-incubated with the antigens and cells were measured for ^{3}H-thymidine incorporation. Negative controls with T and B cells were included. The error bars show mean ±SD.

5.3 Evaluation of Recombinant DQ2.5 molecules

The production of the recombinant HLA-DQ2.5 molecules took place in baculovirus-infected insect cells in serum-free medium. 7.0 x 10^{8} ExpresSf+ cells in 350 mL Insect Express medium were infected by 27 mL P2 virus supernatants for four to six days at 27°C. The cell suspensions were centrifuged and the supernatants were filtered prior to affinity purification. The affinity purified protein samples were evaluated to confirm specificity, purity, stability and functionality.

5.3.1 ELISA

ELISA was used to test the specificity of the affinity purified HLA-DQ2.5 molecules. The DQ2.5 molecules present in the samples were detected with the monoclonal capture antibody, anti-HLA-DQ2 antibody, 2.12.E11. Subsequently, the murine unconjugated secondary antibody SPV-L3 could bind to another epitope of the HLA-DQ molecules followed by addition of a biotinylated goat anti-mouse antibody (IgG2a) and streptavidin-AP. The affinity-purified samples were tested and compared with the filtered virus supernatants (before affinity purification) and the flow-through virus supernatants (after affinity purification) as shown in **figure 5.4**. Duplicates of the samples were 5-fold serial diluted. The affinity-purified samples had a start concentration of 10 µg/mL while the two virus supernatants were undiluted. The

colour development due to dephosphorylation of the phosphate substrate by the alkaline phosphatase-coupled streptavidin was measured as absorbance at 405 nm (Multiskan ascent). These experiments show the specific presence of HLA-DQ2.5 molecules in the filtered virus supernatants and in the affinity-purified samples. The low absorbance signals in the flow-through supernatants demonstrate the efficiency of the affinity purification with the anti-DQ2 antibody.

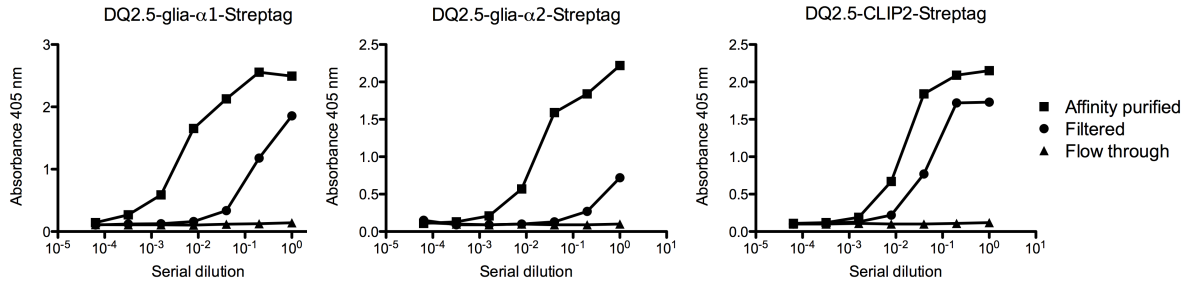

Figure 5.4: Detection of DQ2.5 molecules. The presence of DQ2.5 molecules were detected with an anti-DQ2 antibody. The start concentration of the affinity purified samples was 10 µg/mL followed by a 5-fold serial dilution. The highest concentrations of the filtered and flow-through supernatants were undiluted. The absorbance was measured at 405 nm.

5.3.2 SDS PAGE

The purity and size of the generated DQ2.5-Streptag molecules were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The covalently linked peptides of the DQ2.5-Streptag molecules were cleaved by thrombin. Uncleaved samples were run in parallel. The conventional DQ2.5-glia-α1 molecule harbouring a BirA-site was used as a positive control and used to compare size of the newly generated streptagged constructs as shown in **figure 5.5**. Thrombin cleavage of the covalently bound peptide in DQ2.5-Streptag molecules is necessary for chain separation in SDS PAGE.

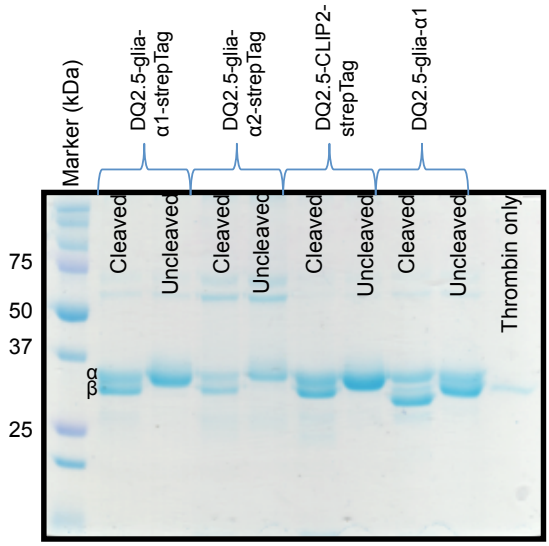

Figure 5.5: Comparison of size and purity of the recombinant DQ2.5 molecules. Thrombin-cleaved DQ2.5 constructs were run on an SDS PAGE in parallel with uncleaved constructs and compared with conventional DQ2.5 construct as a positive control. The DQ2.5 chains are in the 25-37 kDa range. A sample with thrombin only is included.

5.3.3 Size-exclusion Chromatography

Another parameter in the evaluation of the recombinant DQ2.5 molecules was to look at their stability. Size exclusion chromatography, or gel filtration, separates the proteins according to size. For analytical purposes, 10 µg of the affinity-purified proteins in 50 µL PBS were loaded on the Superdex200 column, protein peaks were monitored as illustrated in **figure 5.6** (left). The highest peaks represent unaggregated proteins (monomeric peak of dimeric proteins). Protein aggregations were eluted before the monomeric peaks due to larger size. The right diagram in **figure 5.6** compares the ATTO-conjugated DQ2.5-α1-Streptag with the corresponding unconjugated DQ2.5 molecule. The protein peaks in the analytical gel filtration is illustrated by UV absorbance at 215 nm, which monitors the signal from the peptide backbone.

Size exclusion chromatography was also used to fractionate the monomeric peaks of the affinity purified protein samples. The protein yields of the DQ2.5-glia-α1-Streptag and DQ2.5-glia-α2-Streptag were 1300 µg and 800 µg, respectively. ATTO conjugation (with 10x molar excess) took place prior to gel filtration. Thus, the protein fractionation also served to remove unbound ATTO565. The fractionations (0.5 mL volume) corresponding to the monomeric peaks, indicated by the black arrow in **figure 5.7**, were pooled and concentrated. Gel filtration was in this case used for preparative purposes. The protein peaks in figure 5.6

are illustrated by UV absorbance at 280 nm. The gel filtration experiments show protein aggregations that are consistent with the conventional DQ2.5 molecules (data not compared).

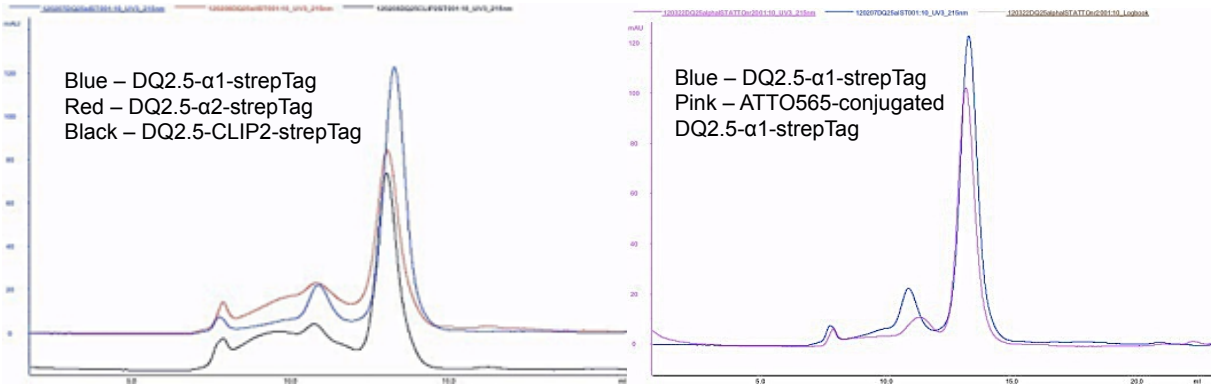

Figure 5.6: Stability of the DQ2.5 molecules. 10 µg of the DQ2.5 molecules were gel filtrated on a SuperDex200 column (FPLC) to check protein stability. Comparison of ATTO-conjugated (pink) and unconjugated DQ2.5-α1-Streptag (blue) is shown in right diagram.

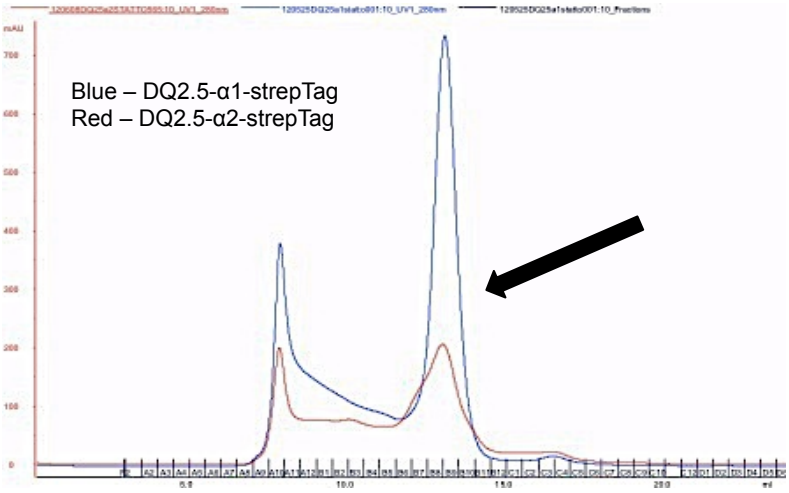

Figure 5.7: Monomeric fractionation and dye purification of ATTO-conjugated DQ2.5 molecules. Affinity purified and ATTO conjugated DQ2.5-α1-Streptag (blue) and DQ2.5-α2-Streptag (red) were gel filtrated on a SuperDex200 column (FPLC) and fractionated in 0.5 mL volumes. The samples of the monomeric protein peaks (black arrow) were pooled and concentrated.

5.3.4 MALDI-TOF MS

MALDI-TOF-MS (UltraflexII) was used to identify the epitope peptides linked to the β chain of the HLA-DQ2.5 molecules. As shown in **figure 5.8**, the generated mass spectrum provides observation of the thrombin-cleaved peptides (circled in green).

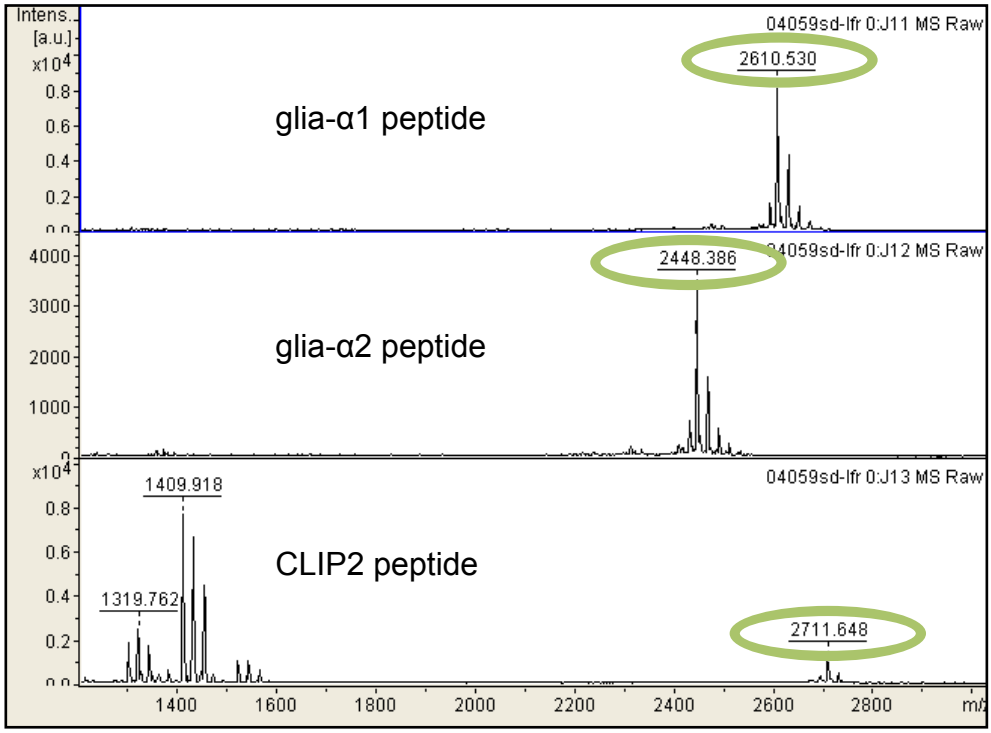

Figure 5.8: Identification of covalently linked epitope peptides. MALDI-TOF-MS was used to identify the covalently linked epitope peptides (green circle) of the three generated constructs. The observed mass corresponds to the theoretical mass of the peptides (table 4.2).

5.3.5 Flow Cytometry

Flow cytometry was used to evaluate the functional properties of the new HLA-DQ2.5 molecules harbouring a StreptagIII region at the C-terminus of the β chain. The DQ2.5 molecules were multimerised on APC-conjugated Streptactin and the Streptactin-DQ2.5-Streptag complexes (Streptamer) were used to stain the $CD4^+$ T cells of interest.

The cell distribution of the TCCs based on side-scattered light (SSC) and forward-scattered light (FSC) plot is illustrated in **figure 5.9a** with a typical gating strategy. To verify that the TCCs are CD4+ T cells, the cells were stained with anti-CD3 and anti-CD4 antibodies, as shown in **figure 5.9b**.

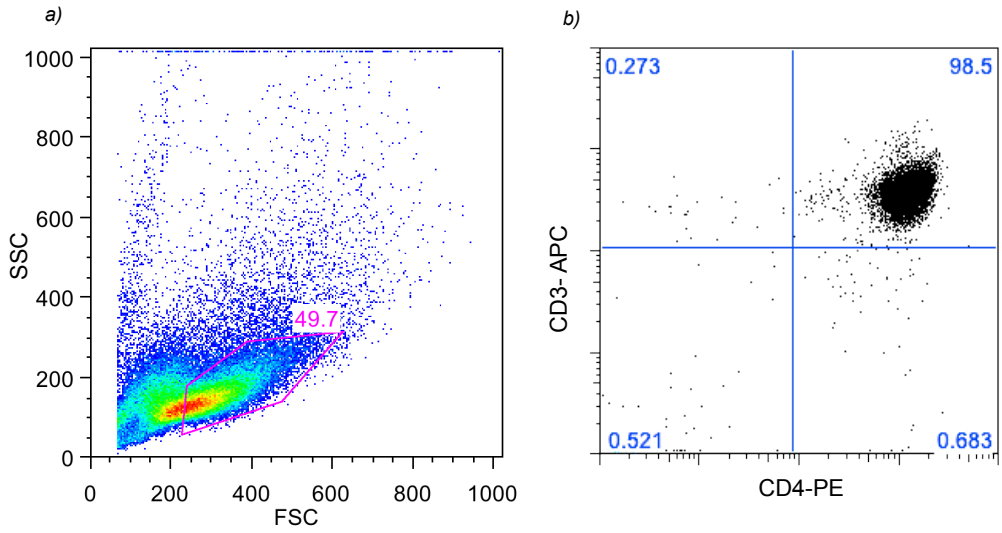

Figure 5.9: a) SSC/FSC plot. The distribution of the T-cell clone cells is represented in a SSC/FCS plot and was gated as indicated in pink. **b) $CD3^+$ $CD4^+$ TCCs.** The T cell clones (TCCs) were stained with anti-CD3 and anti-CD4 markers.

Titrations of the Streptamer reagents

Titrations of different conditions were tested in order to determine the optimal conditions for Streptamer staining of the TCCs. Increasing concentrations of Streptamers from 10 up to 40 µg/mL were tested (**figure 5.10**). 30 µg/mL were used in the subsequent stainings. The same TCCs were stained with Streptamers (30 µg/mL) with different incubation times. In **figure 5.11a**, comparison of Streptamer-stained TCC535.3.23 after 15, 45, 90 and 180 minutes are viewed as histograms. The right plot in figure 5.11a compares the Streptamer-stained TCC at time point 45 minutes with DQ2.5-α1 tetramer (black) and a Streptamer with the irrelevant peptide, CLIP2 (dark grey). **Figure 5.11b** shows the sufficient molar ratio of Streptactin-APC and DQ2.5-α1-Streptag for conjugation. A molar ratio of 1:4 means that four DQ2.5 molecules are co-incubated with each Streptactin-APC.

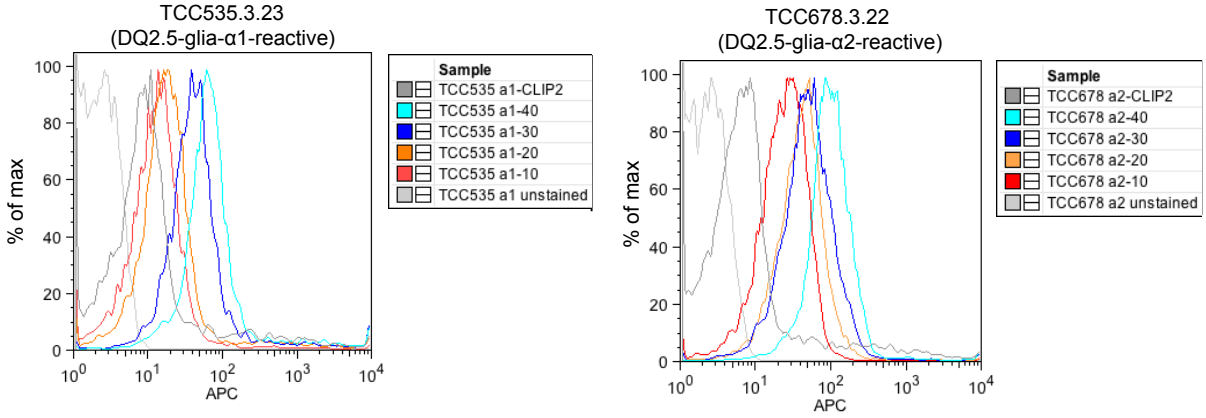

Figure 5.10: Titration of Streptamer concentration. Four different concentrations from 10 to 40 µg/mL streptamers were tested. 30 µg/mL Streptamer is marked in blue. Negative controls were unstained cells (grey) and DQ2.5-CLIP2 Streptamer (dark grey).

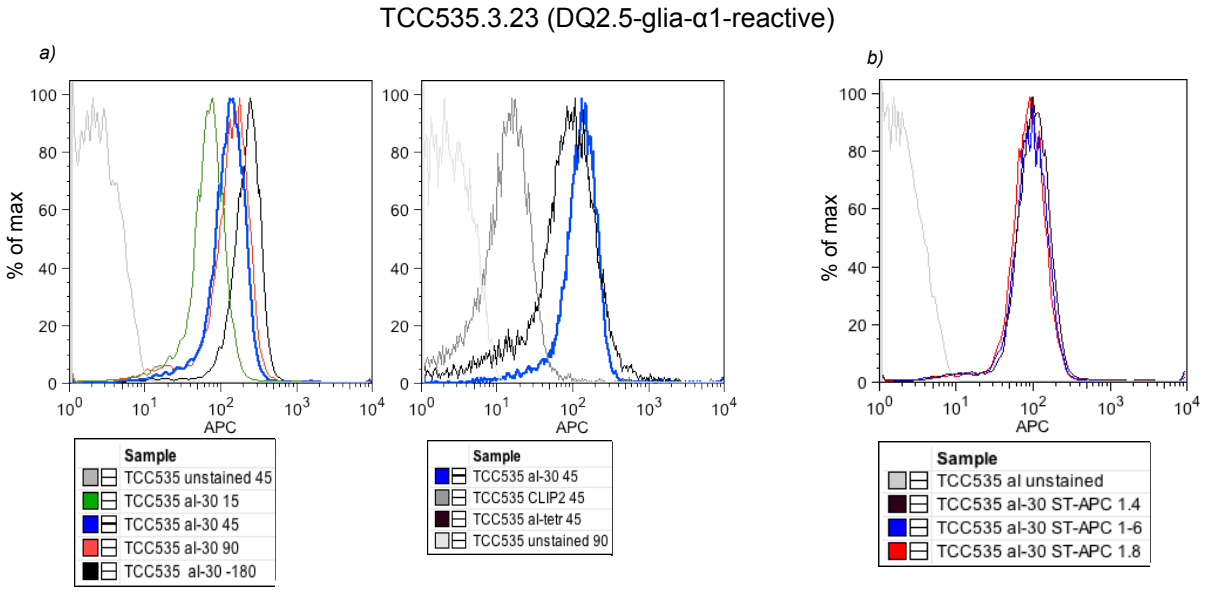

Figure 5.11: a) Incubation of T-cell staining. TCC535.3.23 was stained by Streptamers (30 µg/mL) at four time points, 15, 45, 90 and 180 min. Time point 45 min, shown in blue, was compared with specific tetramer (black), non-specific (α2) Streptamer (dark grey) and unstained cells (grey). **b) Streptactin-APC:DQ2.5 molar ratio.** DQ2.5 was titrated to determine sufficient amount to saturate the binding sites of Streptactin-APC. The tested molar ratios were 1:4, 1:6 and 1:8.

The titrations of Streptamer concentration, Streptamer molar ratio and staining incubation time were based on calculations of conventional tetramers. Another approach was the Streptamer staining protocol. 5 µL Streptactin-APC with 1 µg MHC protein can be used to stain up to 5.0 x 10^6 cells according to the protocol, but the Streptamer staining was typically of 1.0-4.0 x 10^5 cells. The amounts of the DQ2.5-Streptag molecules and Streptactin-APC

were therefore titrated. In **figure 5.12a**, 1 µg (blue) and 2.5 µg (black) of the DQ2.5 molecules were incubated with 5 µL Streptactin-APC for at least 45 minutes prior to 45 minutes T cell staining. The TCCs were also stained with Streptactin-APC only (no MHC) as shown as (light blue histograms, **figure 5.12a**) as a control. Notably, the staining intensity is slightly higher compared to non-specific Streptamer (red histogram). In **figure 5.12b**, 1-5 µL Streptactin-APC were incubated with 1 µg DQ2.5-α1-Streptag. For future experiments, 1 µg DQ2.5 molecule and 3 µL Streptactin-APC seem to be sufficient to stain TCCs without compromising the APC fluorescent signal.

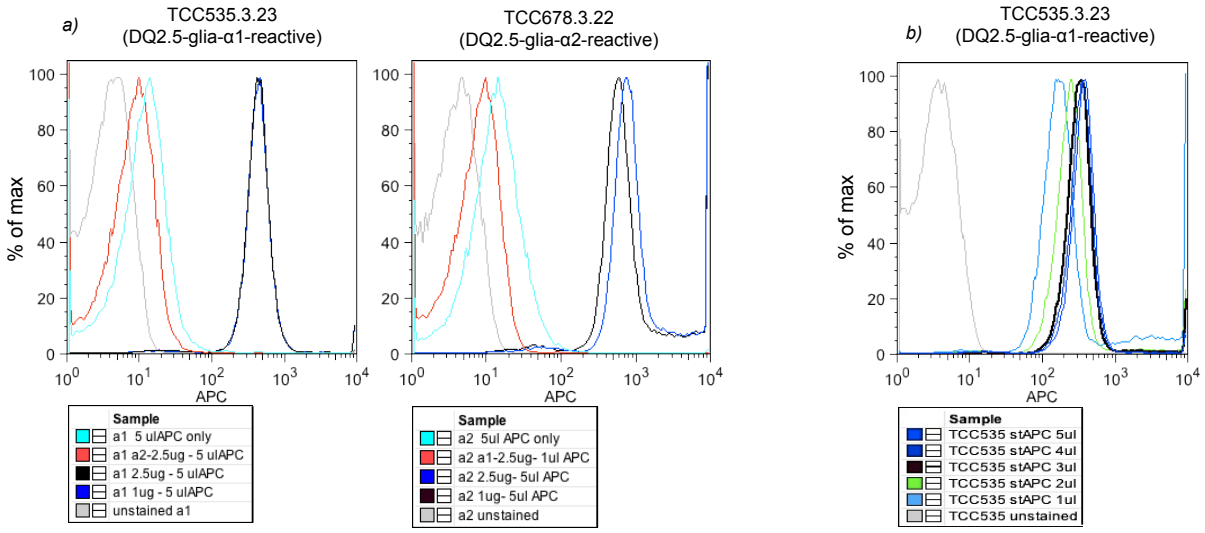

Figure 5.12: a) Titration of DQ2.5-Streptag. 1 and 2.5 µg (blue and black) of DQ2.5-glia-α1 and DQ2.5-glia-α2 incubated with 5 µL Streptactin-APC were used to stain TCC535.3.23 (DQ2.5-glia-α1-reactive) and TCC678.3.22 (DQ2.5-glia-α2-reactive). Non-specific Streptamers (α1/α2) are shown in red. TCCs stained with only 5 µL Streptactin-APC are shown in light blue. **b) Titration of Streptactin-APC.** 1-5 µL of Streptactin-APC was tested in flow cytometry to determine sufficient amount of the reagent for staining of TCC535.3.23. Unstained cells are shown in grey.

Initial flow cytometry experiments with ATTO565-conjugated Streptamers

The streptagged DQ2.5 molecules also harbour a GGSC sequence at the C-terminus for dye conjugation. The ATTO565 dye was conjugated to DQ2.5 molecules through a maleimide reaction with the C-terminal cystein residue. According to the ATTO565 protocol, 1.3x molar excess of ATTO565 should be sufficient to label the DQ2.5 molecules. **Figure 5.13** compares the APC signals given from Streptamers with ATTO-conjugated and unconjugated DQ2.5 molecules. Non-specific Streptamers (red) and irrelevant Streptamers (CLIP2, dark grey) are included as negative controls in addition to unstained cells.

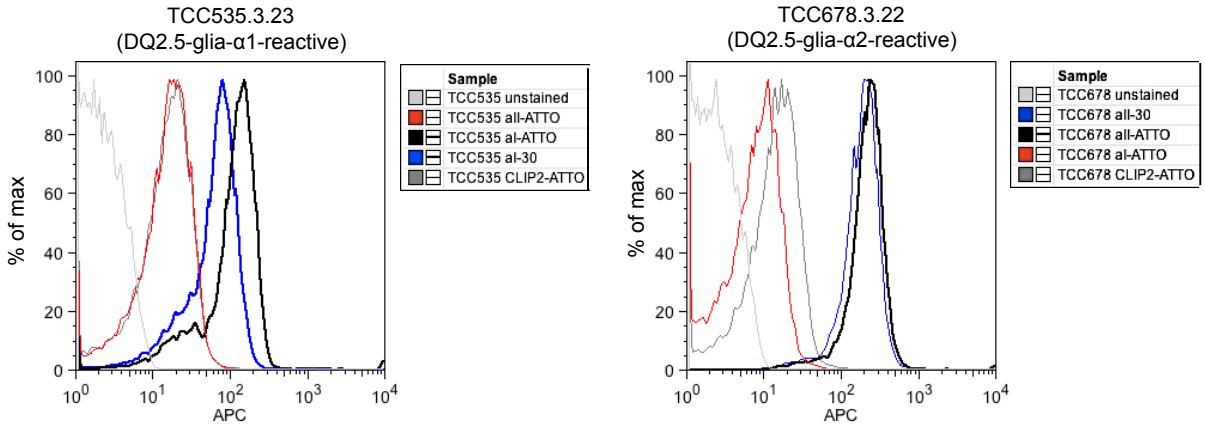

Figure 5.13: ATTO-conjugated Streptamers (1.3x molar excess). *Left:* TCC535.3.23 (DQ2.5-glia-α1-reactive). *Right:* TCC678.3.22 (DQ2.5-glia-α2-reactive clone). DQ2.5 molecules conjugated with 1.3x molar excess of ATTO565 are shown as black histograms. Blue histograms are streptamers with unconjugated DQ2.5 molecules. The negative controls , non-specific (α1/α2) Streptamer (red) and irrelevant Streptamer (dark grey) show low APC signal.

Comparison of ATTO565 signals with different molar excess of ATTO labelling

The ATTO-conjugated Streptamers (1.3x) were also tested in a flow cytometer with LSRII Yellow Laser (Core Facility, Montebello). This laser provides the detection of the fluorescent signal from ATTO565 at approximately 600 nm. The lower plots in **figure 5.14** show a small shift in the fluorescent signal in the ATTO565 channel for ATTO-conjugated Streptamers (black) compared with unconjugated Streptamers (blue). The APC signals in the upper plots show comparable intensity independent on ATTO565, especially for TCC678.3.22. Due to only minor shifts in ATTO565 intensity, higher molar excess of ATTO565 were tested. The titration of molar excess of ATTO565 plotted against degree of labelling (DOL) is shown in **figure 5.15**. The DOL estimates the number of ATTO565 molecules conjugated per DQ2.5 molecule and a DOL of 1 indicates that one ATTO565 molecule is conjugated to one DQ2.5 molecule. According to **figure 5.15**, a molar excess of 100 is necessary to obtain a DOL of 1. Streptamers with DQ2.5 molecules conjugated with 100x molar excess of ATTO565 were also tested as for the ATTO-conjugated Streptamers (1.3x). The comparison is shown in **figure 5.14**. These experiments show that increased molar excess of ATTO565 give higher ATTO signals in flow cytometry.

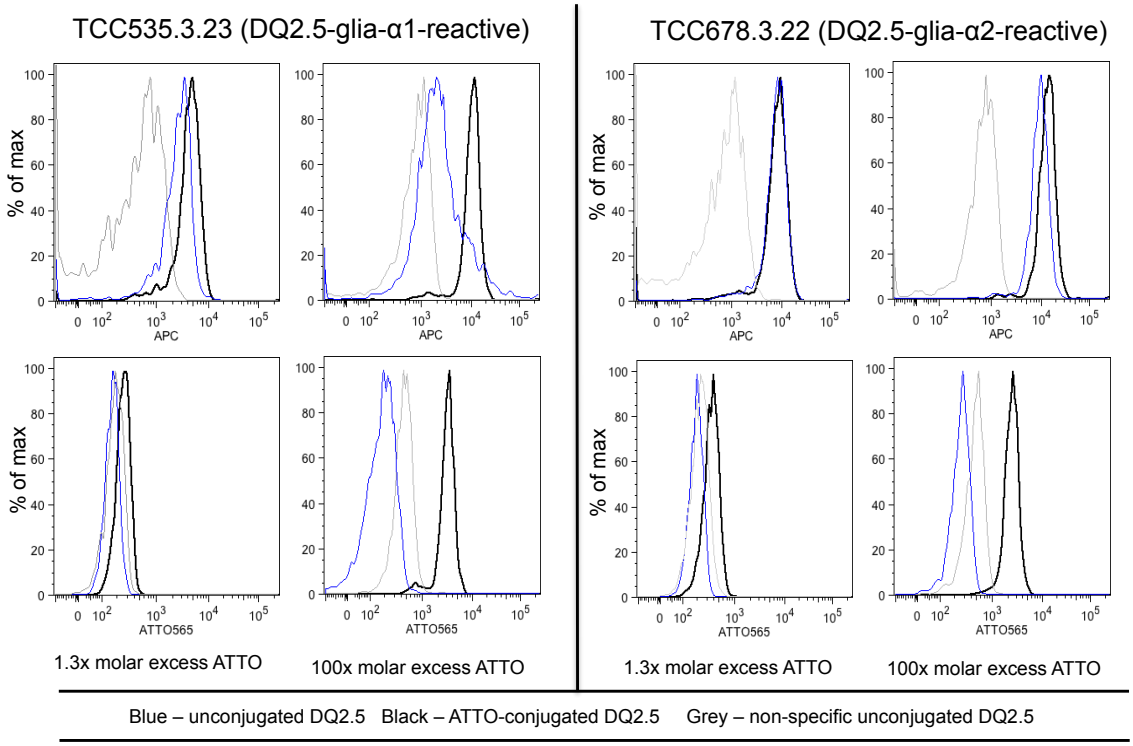

Figure 5.14: ATTO-conjugated Streptamers. Comparison of ATTO-conjugated Streptamers with different molar excess of ATTO565 in labelling of DQ2.5-Streptag molecules. APC and ATTO channels are shown in upper and lower histograms, respectively. Black histograms represent Streptamers with ATTO-conjugated DQ2.5 while blue histograms represent Streptamers with unconjugated DQ2.5.

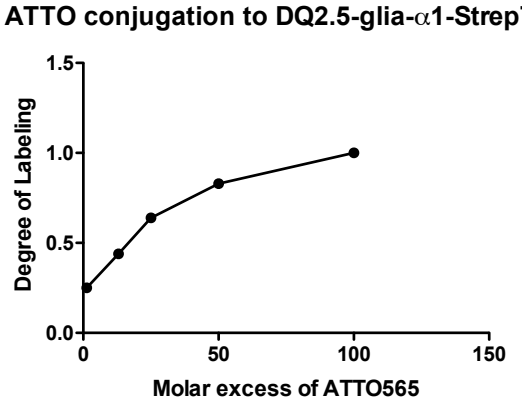

Figure 5.15: ATTO-dye titration. ATTO565 was conjugated to the DQ2.5-Streptag molecules with a molar excess ranging from 1.3 to 100-fold. The absorbance of the conjugated molecules was measured at two wavelenghts, 280 nm and 565 nm, on a spectrophotometer. The degree of labelling (DOL) was calculated using the formula shown in 3.2.6.

Testing panels of DQ2.5-glia-α1 and DQ2.5-glia-α2-reactive TCCs

TCC535.3.23 and TCC678.3.22 were used as test clones in order to establish procedures with the new multimer reagents (Streptamers). The use of Streptamers were expanded to two panels of eight TCCs reactive either to DQ2.5-glia-α1 and DQ2.5-glia-α2 epitopes as shown in **figure 5.16** and **figure 5.17**, respectively. These experiments were performed together with PhD student Asbjørn Christophersen. The upper row represents Streptamer staining while the mid row represents tetramer staining. In the lower row the cells were stained with anti-TCR antibody. Streptamer-stained TCCs were also washed with d-biotin (light blue, upper row) to visualise the reversible interaction between the DQ2.5-Streptag molecules and the Streptactin backbone in the presence of d-biotin. The Streptamer staining of DQ2.5-glia-α1-reactive TCCs (**figure 5.16**) was successful in 6 out of 8 TCCs (Streptamer positive is marked with red arrows). In the case of DQ2.5-glia-α2-reactive TCCs (**figure 5.17**) only 2 out of 8 TCCs were Streptamer positive. Streptamer staining of DQ2.5-glia-α2-reactive TCCs were repeated without any improvement (data not shown). These experiments demonstrate that most DQ2.5-glia-α1-reactive TCCs stain with the DQ2.5-glia-α1-Streptamers while DQ2.5-glia-α2-Streptamers have greater staining variations among the selected DQ2.5-glia-α2-reactive TCCs.

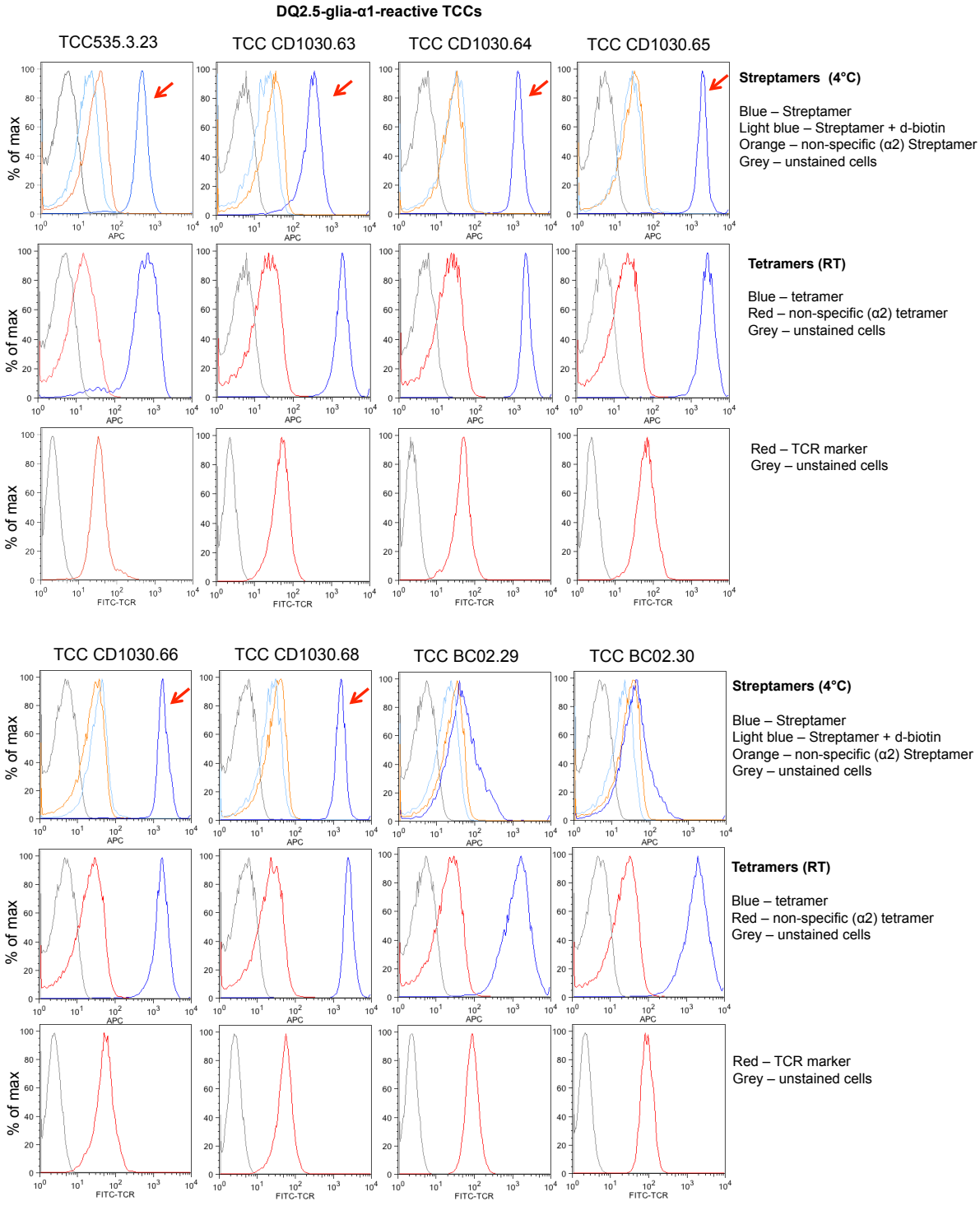

Figure 5.16: DQ2.5-glia-α1-reactive TCCs. 8 TCCs specific for glia-α1 peptide epitope stained with DQ2.5-α1 Streptamers and tetramers are shown in upper and mid row, respectively. D-biotin was added to Streptamer-stained TCCs shown as light blue histograms in upper row. All but TCC BC02.29 and TCC BC02.30 is Streptamer-positive (marked with red arrows). The TCCs were also stained with anti-TCR-FITC as shown in lower row. Non-specific (α1/α2) Streptamers (orange) and tetramers (red) were included and all unstained cells are shown as grey histograms. Streptamer and tetramer incubation were performed at 4°C and RT, respectively. The T-cell clones indicated with CD and BC (buffy coat) are obtained from peripheral blood.

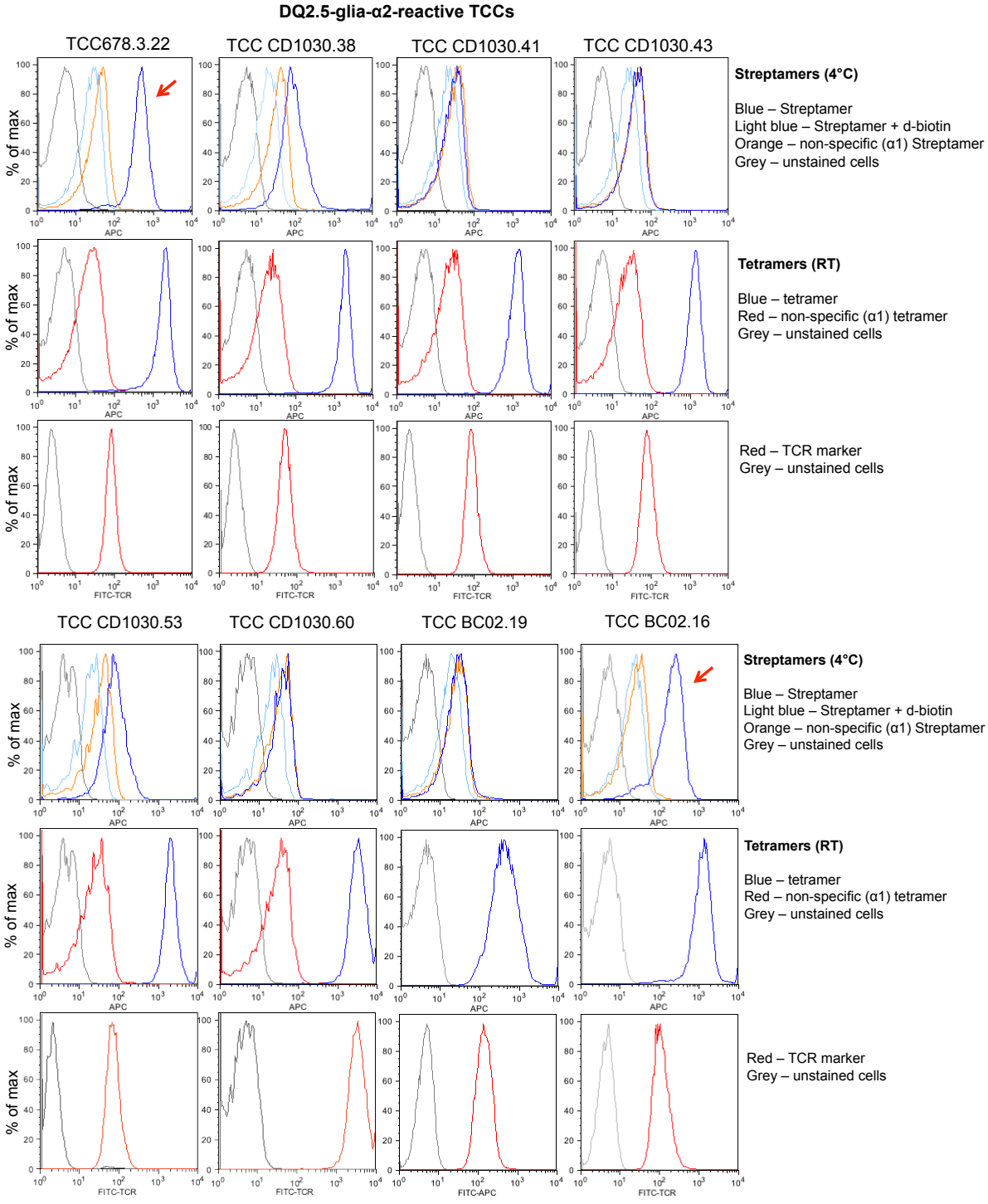

Figure 5.17: DQ2.5-glia-α2-reactive TCCs. 8 TCCs specific for glia-α2 peptide epitope stained with DQ2.5-α2-Streptamers and tetramers are shown in upper and mid row, respectively. All but TCC678.3.22 and TCC BC02.16 is Streptamer negative (Streptamer-positive TCCs are marked with red arrows). D-biotin was added to Streptamer-stained TCCs shown as light blue histograms in upper row. The TCCs were also stained with anti-TCR-FITC as shown in lower row. . Non-specific (α1/α2) Streptamers (orange) and tetramers (red) were included and all unstained cells are shown as grey histograms. Streptamer and tetramer incubation were performed at 4°C and RT, respectively. The T cell clones indicated with CD and BC (buffy coat) are obtained from peripheral blood. (Note: sample loss of the non-specific tetramers to TCC BC02.16 and TCC BC02.19)

Experiments done in Dr. Busch's research lab in Munich

In the following, Streptamer-positive $CD4^+$ T cell clones with ATTO565-conjugated DQ2.5 molecules were brought in person to Dr. Busch's research group in Munich and tested in their recently developed human k_{off}-rate assay (see 1.9.2). Interpretation of the data obtained from the fluorescence-based assay did not lead to any useful results but several suggestions to improve the ATTO-conjugated MHC II reagents were discussed (see 6.1.2, page 75).

Improvements in the preparation of the staining reagents were performed by first producing new protein batches. The affinity purified protein samples were labelled with a 10-fold molar excess of ATTO dye. To minimise the protein loss due to removal of free dye and monomer fractionation, ATTO-conjugated DQ2.5 molecules were gel filtrated on Superdex200 column (Äkta FPLC) in order to remove unbound ATTO dye and fractionate the monomeric ATTO-conjugated HLA-DQ2.5-Streptag peak in one step (see **figure 5.7**). These gel-filtrated DQ2.5-Streptag molecules were used in the following experiments.

Exploring a native surface-exposed cysteine residue of the α chain

The crystal structure of the conventional HLA-DQ2.5-glia-α1 molecule (53) was examined to reveal the presence of a native and free cystein residue on the surface of the HLA-DQ2.5 molecule in addition to the engineered cysteine at the C-terminus. A model of the 3D-structure was created in PyMOL as illustrated in **figure 5.18a** (PDB-code: 1S9V). The protein surface is coloured blue while the bound peptide is coloured red. There are seven cystein residues (coloured yellow) in the entire molecule. Six of these are engaged in disulphide bridges within the structure while only cysteine 44 of the α chain (αCys44) is exposed to the surface. Experimentally, the conventional biotinylated DQ2.5-α1 molecule was labelled with ATTO565 (10-fold molar excess) and gel filtrated with prepacked Sephadex G-25 column to remove unbound dye prior to flow cytometry (LSRII Yellow Laser). ATTO-conjugated Streptamers (blue) and tetramers (black) are compared in an APC/ATTO565 dot plot in **figure 5.18b.** These experiments demonstrate that the ATTO-maleimide dye can be coupled to the surface-exposed cysteine residue of the α chain.

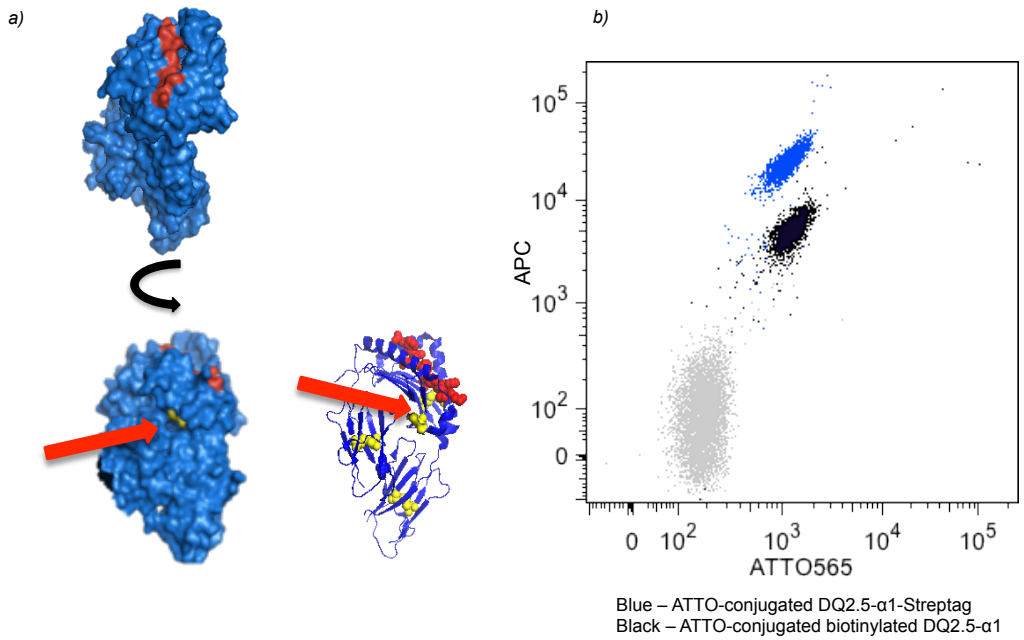

Figure 5.18. a) Free surface cystein. The crystal structure of DQ2.5-glia-α1 shows the presence of 7 Cys residues where one Cys residue is present on the surface, indicated by red arrows. This model was created in PyMOL (PDB code: 1SV9). **b) ATTO-conjugated biotinylated DQ2.5-α1.** Biotinylated DQ2.5-α1 was conjugated with ATTO565 and multimerised with streptavidin-APC. The ATTO565 signal in flow cytometry shown in black is comparable to ATTO-conjugated Streptamers shown as blue dots. Unstained cells are shown as grey dots.

Possible ATTO dye interference with TCR:MHC II binding

ATTO-conjugated (black histograms) and unconjugated Streptamers (blue histograms) were tested in parallel on a few TCCs as shown in **figure 5.19** and **figure 5.20**. TCC535.3.23 (α1-reactive) and TCC678.3.22 (α2-reactive) were included to verify positive staining. Streptamer-negative TCCs were selected due to negative/poor staining as shown in **figure 5.16 and 5.17**. The orange histograms in **figure 5.20** show the APC signal of d-biotin-washed Streptamer-stained TCCs, which are restained with Streptactin-APC only. These experiments demonstrate that the ATTO565 does not interfere with the TCR:MHC binding of the selected Streptamer-negative TCCs.

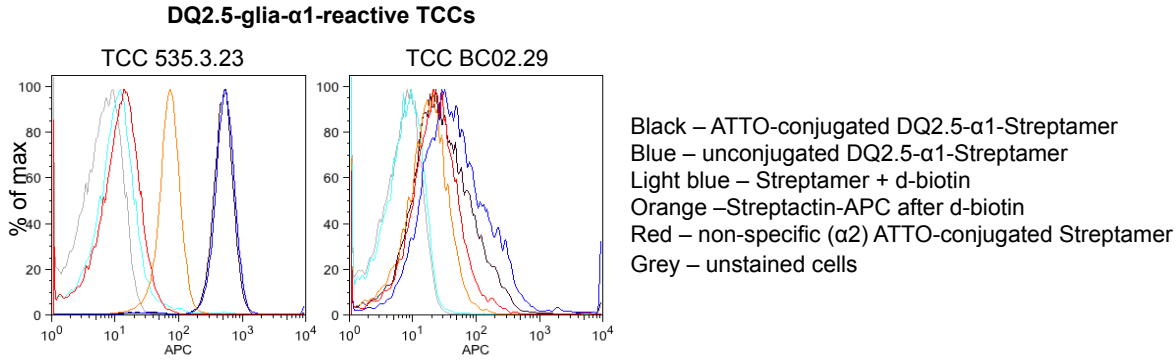

Figure 5.19: ATTO-conjugated vs unconjugated DQ2.5-glia-α1 molecules. DQ2.5-glia-α1-reactive TCCs stained with ATTO-conjugated Streptamers (black). TCCs stained with unconjugated Streptamers are shown as blue histograms. Stained TCCs treated with d-biotin are shown as light blue histograms. Streptactin-APC was added to biotin-washed cells and is shown as orange histograms.

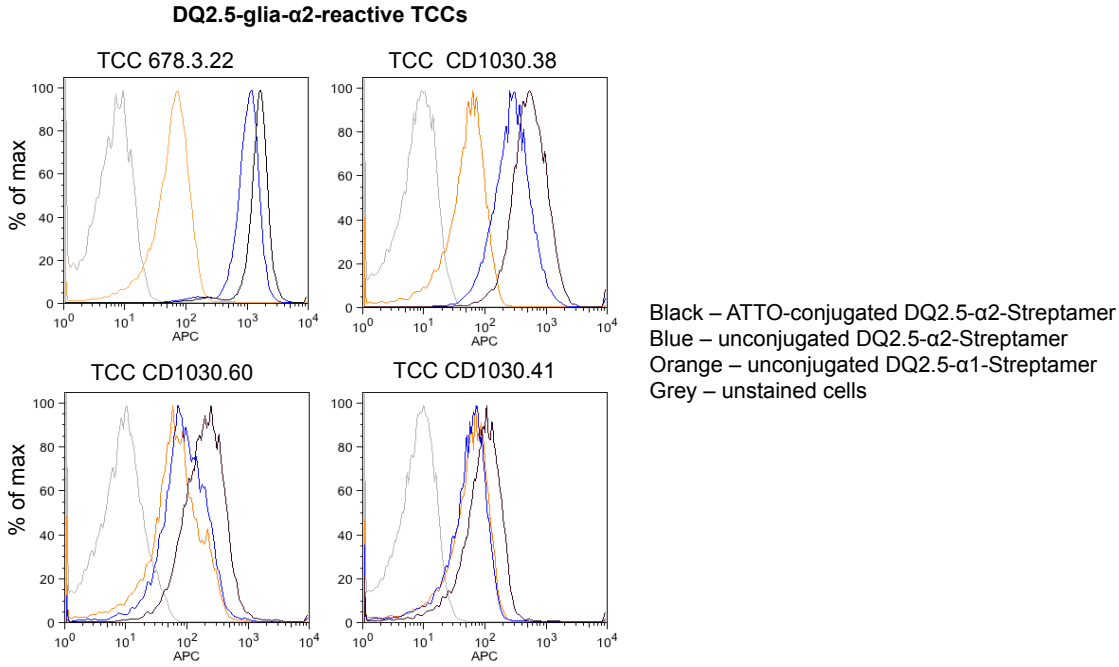

Figure 5.20: ATTO-conjugated vs. unconjugated DQ2.5-glia-α2 molecules. ATTO-conjugated DQ2.5-α2 Streptamers (black) were compared with unconjugated Streptamers (blue). Unconjugated DQ2.5-α1 Streptamers (orange) were included as negative control. Unstained TCCs are shown in grey.

6 Discussion

The use of Streptamer technology has so far only been successful for MHC class I molecules. In this thesis, we have generated new recombinant HLA-DQ2.5-Streptag molecules that can be used as staining reagents of gluten-reactive $CD4^+$ T cells in flow cytometry. The modified DQ2.5 molecules are mainly based on already existing recombinant DQ2.5 molecules (48). The quality of these Streptag fusion proteins was structurally and functionally evaluated in order to be tested in the recently developed Streptamer-based k_{off} rate assay. The variable stainings of the gluten-reactive TCCs with Streptamers in flow cytometry is discussed. In the last part of the Discussion, TCR:MHC kinetics assays are compared with the novel Streptamer k_{off}-rate assay presented in the Introduction.

6.1 Evaluation of Recombinant DQ2.5 molecules

6.1.1 Structural evaluation

Purification could alternatively be performed with Streptag affinity purification

Purification of the DQ2.5 proteins from the cell supernatant was performed by affinity purification. This is standard procedure in the Sollid lab. Captured HLA-DQ2.5 molecules to the monoclonal anti-DQ2 antibody, 2.12.E11, must be eluted at denaturing high pH. It would have been interesting to perform the purification of the streptagged HLA-DQ2.5 molecules by Streptag affinity chromatography. The original concept of the Streptag:Streptactin interaction was designed for protein purification. The elution procedure offers gentle conditions (physiological pH) due to d-biotin that replaces the bound Streptag peptide on Streptactin resin beads. Also, different HLA constructs vary in their stability. Perhaps, more gentle elution conditions might facilitate the stability. The biotin content in the serum-free expression medium must also be taken into account which could potentially disturb the Streptag:Streptactin interaction. Being aware of this issue, avidin can be added to the supernatant to capture free biotin prior to Streptag affinity purification. Streptag affinity chromatography provides a single-step purification method. The whole procedure has been reported to be completed within hours (54). In comparison, antibody affinity chromatography can take two days due to buffer exchange after elution and subsequent sample concentration.

Hence, this purification method can provide a faster procedure. Another aspect is the cost of antibody production compared to the cost of column beads (55). When considering the time consumption of the protein production of MHC class II molecules, an advantage of the Streptag fusion proteins is that the biotinylation step is no longer required for multimerisation.

By using the well-established antibody affinity chromatography in the Sollid lab, we could compare the protein yields from Streptag fusion and conventional HLA-DQ2.5 molecules. The protein yield of DQ2.5-α2 molecule varies from 0.3 to 0.7 mg per liter virus supernatant while DQ2.5-α1 and DQ2.5-CLIP2 molecules have a typical yield of 1 mg. These values are consistent with the production of conventional HLA-DQ2.5 molecules.

Specificity, purity, size and stability

Due to the available monoclonal anti-DQ2 antibody, 2.12.E11, ELISA provides a rapid method to screen for positive plaques, i.e. DQ2.5-producing viruses (data not shown). ELISA was also used to monitor the purification efficiency of the flow-through supernatant as shown in figure 4. In this indirect ELISA, minute amounts of the captured antigen (DQ2.5 molecule) can be detected due to increased sensitivity. Sensitivity is increased due to the polyclonal nature of the biotinylated secondary antibody, allowing for signal amplification. The signal of the flow-through supernatant was comparable to the signal of the negative controls (PBST and medium only). In addition to specific detection of the produced HLA-DQ2 molecules by ELISA, the peptide linked to the structure was investigated by MALDI-TOF MS to ensure that the new constructs carried the correct peptides (figure 5.8). This accurate method with short analysis time allowed for the identification of epitope peptide that is covalently linked to the β chain. These two methods allow for specific determination of the produced MHC II proteins.

Protein gel electrophoresis (SDS PAGE) was used to compare the molecular size of the generated HLA-DQ2.5 molecules with conventional HLA-DQ2.5 and to check purity of the affinity purified protein samples. The expected relative molecular masses were in the range of 25 to 37 kDa. According to the database IMGT-HLA, the amino-acid sequences of the membrane-bound HLA chains, α and β, (HLA-DQ2.5) are 254 and 261 amino acids long, respectively. The recombinant sequences are different due to exchange of the transmembrane regions with leuzine zipper sequences (encoded by fos and jun genes). The primary sequence

of the recombinant α chain is 241 amino acid long while the β chain consists of 323 amino acids. Notably, the β chain migrated faster than the α chain when examining the SDS-PAGE (figure 5). Glycosylation (post-translational modification) probably contributes to increase the molecular mass of the chains. Higher degree of glycosylation of the α chain might explain why the recombinant β chain is smaller in size.

The streptagged molecules showed poor separation of the α and β chains in SDS PAGE. This is mostly due to an increased molecular weight of the β chain fused with the StreptagIII sequence. The difference in molecular weight in the StreptagIII sequence and the BirA site is about 20 amino acid residues which gives an increased molecular weight of 2.2 kDa (average MW amino acid; 110 Da). The thrombin-cleaved DQ2.5 molecules reveal that the β chain migrates faster than the α chain when compared to uncleaved molecules. The separation of the chains is improved in the absence of the covalently linked peptide. We could also observe greater contamination for the sample containing DQ2.5-α2 compared to the other samples. However, the contamination bands were not observed to the same extent in later protein batches of streptagged DQ2.5-α2. Interestingly, the lowest protein yield of the DQ2.5-α2 batches gave the strongest contamination bands. Contaminating proteins in the range of 54-57 kDa and 65-68 kDa is a common problem and has previously been investigated (56).

The stability was examined by comparing the different gel filtrated peaks at 215 nm in FPLC in figure 5.6. It seems like the DQ2.5-glia-α2 sample has more aggregated proteins compared to DQ2.5-glia-α1. This is however consistent with the corresponding conventional HLA-DQ2.5 molecules. Backbone peptides can be observed with UV absorbance at 215 nm and aromatic amino acids, like tyrosine and tryptophan, can be observed at 280 nm. Due to small analytical samples the protein peaks were observed at UV absorbance at 215 nm. The preparative samples were observed at 280 nm. When comparing the peaks at 280 nm in figure 5.7, the protein peaks of DQ2.5-glia-α1-Streptag are much higher due to much higher protein amounts. The monomeric protein peak of DQ2.5-glia-α2-Streptag (red) seemed to have peak shoulders. Fewer peak fractions were therefore selected for this sample. However, a gel-filtrated protein batch of DQ2.5-glia-α2-Streptag must be performed to investigate this further. In all, the quality experiments for the structure of the DQ2.5-Streptag molecules show expected observations for specificity, stability and purity. Due to increased size of the β chain,

thrombin cleavage is necessary for HLA-DQ2.5-Streptag molecules is necessary for visualisation of the α and β chains in SDS-PAGE.

6.1.2 Functional evaluation

After having investigated the structural features of the soluble recombinant HLA-DQ2.5 molecules the remaining and most important parameter to investigate was the biological activity of the expressed protein. The human MHC class II molecules are expressed mainly on professional APCs and present peptide antigens to $CD4^+$ T cells through recognition by the TCR to provide cell-mediated immune responses. This *in vivo* biological setting was *in vitro* investigated by staining $CD4^+$ T cells with new MHC II multimers (Streptamers) in flow cytometry.

The experimental data generated by flow cytometry is mostly presented as overlaying histograms to easily visualise the staining results of a given experiment. Firstly, figure 5.9a represents the gating strategy based on an FCS/SSC plot of the T cells. Side scattered (SSC) and forward scattered (FCS) light provide information about the cell granularity and cell size, respectively. In a mixed cell sample consisting of monocytes, granulocytes and lymphocytes gives rise to a distinct cell distribution and distinct populations. However in this thesis, the samples consisted only of TCCs. These TCCs used as test clones were tested in a T cell proliferation assay to verify antigen-specificity (figure 5.3). In the distribution of the TCCs, cells were gated for viable cells to avoid contribitions from dead cells. Dead cells are typically small in size (low FCS intensity) and are therefore closer to the axis origin than the viable cells. Dead cells have a compromised membrane and tend to be more sticky and bind non-specifically to any reagent. Alternatively, dead cells can also be dicriminated and visualised by propium iodide (PI). PI is a staining agent used for staining the DNA of dead cells due to increased membrane permeability (57).

Optimalisation of the staining procedure – two approaches

It is important to investigate and determine optimal conditions for new MHC multimer reagents (58). The temperature conditions were not investigated due to the importance of keeping the Streptamer staining procedure at 4°C. In contrast, staining with conventional tetramers occurs at room temperature (RT). We tried two approaches to determine the optimal conditions due to the fact that Streptamer technology was developed with MHC I molecules

and the new streptagged MHC II reagents were similar in appearence to conventional MHC II tetramer reagents. In the case of conventional tetramers, a concentrated tetramer stock of biotinylated MHC II reagents multimerised on streptavidin can be prepared and kept at 4°C for weeks. In contrast, the preparation of streptamers should preferably be freshly made prior to T cell staining. The biotin:streptavidin interaction has a much higher affinity than the Streptag:Streptactin interaction. The reason for fresh Streptamer preparations might be that a concentrated Streptamer stock cannot provide the same stability as a tetramer stock with biotinylated MHC reagents.

In the MHC Streptamer protocol, the MHC reagent (protein) and the Streptactin backbone are refered as amounts (µg) or volume (µL). In contrast, common procedure for tetramers refer to concentration and molar ratio. It is interesting to compare streptavidin and Streptactin as reagent. The homotetrameric streptavidin can bind up to four biotinylated molecules. Streptactin is a modified version of streptavidin. Streptactin is however polymerised with dextran and is coupled via lysin residues or the free N-terminus to the carboxyl groups in the dextran backbone. The molar ratio was 60 moles Streptactin per mole of dextran in order to generate the Streptactin backbone (59). This implicates that Streptactin and streptavidin could behave differently as reagents. Multimerisation of conventional biotinylated DQ2.5 molecules on Streptactin-APC with subsequent TCC staining was not performed but could elucidate possible differences between Streptactin and streptavidin.

Different incubation times up to 180 minutes for the TCC staining were tested (figure 5.11a). Incubation of at least 45 minutes showed to provide acceptable staining intensity. Shortest possible time without comprimising the staining intensity too much is desirable. In practice, incubation of 3 hours (180 minutes) prior to flow analysis is not as desirable especially when considering subsequent events of T cell such as pMHC-induced cell activation and subsequent TCR internalisation. Streptamer incubation for at least 45 minutes was sufficient for positive staining with the test clones. In short, the MHC Streptamer protocol involves 45 minutes pre-conjugation of Streptamers in 50 µL FACS buffer and 45 minutes Streptamer staining of T cells prior to flow analysis. Due to the requirement of fresh Streptamer preparations, this procedure is simpler than taking molar calculations into account. Two amounts (µg) of DQ2.5-Streptag molecules for TCC staining was tested. To avoid excessive use of reagents, the amount (µL) of Streptactin-APC was also titrated. 1 µg HLA-DQ2.5 and 3 µL Streptactin

were found to be sufficient, which is consistent with the Streptamer protocol. In conclusion, staining procedure according to the Streptamer protocol is preferred.

ATTO565 conjugation

After labelling the HLA-DQ2.5-Streptag molecules with ATTO565, free dye was first removed by gel filtration (G-25 sephadex column) according to the protocol. Due to low protein yield after gel filtration, another method was tested for dye purification. The protein sample was diluted with PBS buffer using Vivaspin centrifugal concentrators (10-kDa cut off). With serial dilutions of the protein samples, the amount of free dye can be diluted down to an acceptable low level. This procedure was thought to be a suitable alternative to gel filtration to remove unbound ATTO dye. The estimated DOL of gel-filtrated or centrifuge-diluted samples were comparable indicating that free dye was also removed using the centrifugation procedure.

ATTO565 does not affect staining intensity of the test clones

From the initial flow cytometer experiments of the ATTO-conjugated HLA-DQ2.5 molecules, it did not seem like ATTO conjugation affected the MHC interaction to the TCR complex or to the Streptactin backbone. Figure 5.13 illustrates the APC fluorescence signal given by the ATTO-conjugated DQ2.5 molecules multimerised on Streptactin-APC. The same reagents were tested with LSRII Yellow Laser (Core Facility, Montebello) in order to detect the fluorescent signal from the ATTO dye (figure 5.14, left plots of each TCC). The small shift in ATTO fluorescence compared to unconjugated multimerised DQ2.5 was thought to be caused by the apparently low molar excess of the ATTO dye in the labelling procedure.

Increased protein:dye ratio correlates with increased ATTO565 signal in flow cytometry

The degree of labelling (DOL) was estimated to be approximately 0.25 which indicates that only every fourth DQ2.5 molecule is ATTO conjugated (with 1.3x molar excess). The molar excess of ATTO was titrated up to 100-fold as shown in figure 5.15 and retested in flow cytometry shown in figure 5.14. The ATTO signal was significantly improved with the extensively higher molar excess of ATTO in labelling the DQ2.5 molecules. In other words, the estimated DOL seemed to correlate well to the fluorescent intensity in flow cytometry.

Testing panels of DQ2.5-glia-α1 -and DQ2.5-glia-α2 reactive TCCs

The DQ2.5-glia-α1-reactive TCC535.3.23 and the DQ2.5-glia-α2-reactive TCC678.3.22, obtained from gut biopsies, were used as test clones to determine the optimal Streptamer conditions. Expanded panels of TCCs with origin from peripheral blood was selected to further verify the findings obtained from the gut-derived TCCs. Tetramer visualisation of gluten-reactive $CD4^+$ T cells in peripheral blood was demonstrated by Raki et al. in 2007 (60). The gluten-reactive $CD4^+$ TCCs (from peripheral blood) used in this project were originally cell sorted on flow cytometry (BD FACS Aria) with tetramers and specific surface markers such as CD45RA and CD62L in addition to CD3 and CD4 markers. Interestingly, a few $CD4^+$ T cells have been identified as naïve and showed to be reactive to DQ2.5-glia-α1 and DQ2.5-glia-α2 epitopes (unpublished data, A. Christophersen, 2012).

Positive Streptamer staining is reversed in the presence of d-biotin

The Streptamer-stained TCCs were also washed with d-biotin to verify reversibility of the Streptamer staining (light blue histograms, figures 5.16 and 5.17). Optimally should the biotin-washed TCCs have similar fluoroscence intensity as the unstained TCCs (grey histograms). The washing procedure was most likely incomplete. In comparison, TCC535.3.23 in figure 5.18 was sufficiently washed with d-biotin and shows comparable low signals with the unstained cells (light blue and grey histograms). The distruption of StreptagIII interaction with Streptactin-APC by d-biotin is the essence of the Streptamer technology. Streptamer-positive TCCs demonstrate reversibility of the high staining signals in the presence of d-biotin.

The majority of DQ2.5-glia-α1-reactive TCCs is Streptamer positive

As indicated in figure 5.16, the TCCs reactive to the DQ2.5-glia-α1-epitope show high APC signals for the DQ2.5-glia-α1-Streptamers (marked with red arrows). Notably, the two T-cell clones with naïve phenotype, TCC BC02.29 and TCC BC02.30, showed low staining signals. In contrast, they stain positively with conventional tetramers.

The majority of DQ2.5-glia-α2-reactive TCCs is Streptamer negative

Unfortunately, the TCCs reactive to DQ2.5-glia-α2-epitopes did not show the same trend as the DQ2.5-α1-reactive TCCs (figure 5.17). The two clones, TCC678.3.22 (test clone) and TCC BC02.16, show acceptable positive staining with Streptamers. In contrast to this naïve

TCCBC02.30, the naïve DQ2.5-glia-α1-reactive TCC BC02.29 and TCC BC02.30 was not Streptamer positive. The rest of the DQ2.5-glia-α1-reactive TCC panel gave high signals with specific tetramers but not to the specific Streptamers.

What could explain these variable findings? The conventional tetramers were used as positive controls to verify that the antigen-specificity of the selected TCC. The expanded panels of TCCs (all but the test clones) were cell-sorted by the tetramers used in these experiments. The temperature conditions for the tetramers and Streptamers are a main difference in the staining procedure of the TCCs. The staining of the TCCs with tetramers and Streptamers was performed at room temperature and at 4°C, respectively. Despite the importance of keeping Streptamer staining at cold temperatures, it would be interesting to investigate if elevated temperatures have a positive effect on the staining intensity of the Streptamer-negative DQ2.5-α2-reactive TCCs and naïve DQ2.5-α1-reactive TCCs. Previously, temperature effect was investigated and reviewed by Cameron et al. and different clones showed different temperature dependencies of staining with MHC II oligomers. Some clones showed no detectable or slight staining at 4°C but brighter staining at elevated temperatures (37, 61). Investigating the temperature effect would provide greater insights if conventional tetramers are more sensitive than Streptamers in order to detect gluten-reactive $CD4^+$ T cells.

The origin of the TCCs is also an interesting aspect. Most of the TCCs in the panels are from peripheral blood. Due to the fact that both of the test clones derive from gut biopsies, it would be interesting if the majority of gut-derived DQ2.5-glia-α1 and DQ2.5-glia-α2 reactive clones can demonstrate positive staining. Several clones of both origins should therfore be tested.

Despite the unexpected low staining of the majority of the DQ2.5-glia-α2-reactive TCCs, the DQ2.5-glia-α1-reactive TCCs and the cognate MHC II Streptamer reagents could be tested in the k_{off} rate assay in Dr. Busch's research group in Munich.

Performing human k_{off} rate assay in Munich: first attempt

DQ2.5-glia-α1-reactive TCCs were tested in the k_{off}-rate assay without applicable results

The ATTO-labelled and streptagged DQ2.5-glia-α1 molecules were tested in the k_{off} rate assay developed in Munich and the set-up was performed with the PhD student Bianca Weissbrich. The obtained data was interpreted without any applicable results. First

observation was that the fluorescence signal was surprisingly high for the ATTO dye. Contribution of free dye due to incomplete removal of unbound dye was thought to be the main reason for this observation. Since the HLA-DQ2.5 molecules have the potential to be labelled at two cystein residues (see below), this would also contribute to the high fluorescence intensity. Their standard procedure was to label their MHC I molecules with a 10-fold molar excess of ATTO dye followed by gel filtration without estimating the dye:protein ratio (DOL) (Bianca Weissbrich, personal communication, May 2012). Another observation was that the fluorescence signal by ATTO was sustained even after addition of d-biotin. In addition to the presence of free ATTO dye, a possible explanation for the high staining intensity after d-biotin could be caused by protein aggregations (dimers or trimers of the DQ2.5 molecules). It was later confirmed by the ATTO-TEC company that ATTO565 dye is very hydrophobic and can stick to the cell membrane. Hence, it is of great importance to remove the free dye completely. In order to perform a new attempt of the k_{off} rate assay, several discussed problems were addressed. In short, the improved ATTO-conjugated HLA-DQ2.5 molecules were gel filtrated (on Äkta FPLC) to allow separation of protein aggregates. Prior to gel filtration, the affinity-purified samples were labelled with a 10-fold molar excess of ATTO dye as it is the common procedure in Munich.

Improvements of the ATTO-conjugated MHC II reagents

ATTO565 can be coupled to a free surface-exposed cysteine residue of the α chain

The free surface accessible cystein residue in the α chain (αCys44) was identified early in the project (see figure 18a) (53). The issue concerning unstable TCR:MHC binding due to dye conjugation at αCys44 was previously considered. But this was not observed experimentally with the two test clones, TCC535.3.23 and TCC678.3.22. To address this potential problem experimentally, biotinylated DQ2.5-α1 molecules (conventional without the C-terminal cystein residue) were labelled with the ATTO dye. TCC535.3.23 (DQ2.5-glia-α1-reactive) was stained with the ATTO-conjugated biotinylated DQ2.5-glia-α1 tetramer. The ATTO fluorescence signal was comparable with ATTO-conjugated DQ2.5-glia-α1-Streptamers (figure 5.18b). This experiment supports the assumption that the ATTO-maleimide dye can be coupled to a surface-exposed cysteine residue in the original recombinant HLA-DQ2.5 molecule (conventional). However, there might be a possibility that cysteine residues that are supposed to be engaged in disulphid bridges are exposed at the surface. This possibility has

not been excluded. An HLA-DQ molecule without an expected surface-exposed cysteine residue could verify this issue even further.

Issues with estimation of dye:protein ratio by spectrophotometry

The observation that conventional DQ2.5-gliaα1 molecules can be conjugated with ATTO565 to the surface-exposed cystein led to the questioning about the reliability of estimating the dye:protein ratio (DOL) by spectrophotometry. Due to the fact that the ATTO-maleimide dye can potentially react with the sulfur group to more than one cystein residue, the maximum theoretical dye:protein ratio should be at least 2, but the highest experimentally obtained ratio has only been a ratio of 1. Even though the ATTO dye titration (figure 5.15) has not completely reached a plateau, the titration curve indicates that the dye:protein ratio has nearly reached a maximum. In practice, adding 100-fold molar excess of ATTO565 is not really feasible due to the extensive use of dye reagent. Thus, a 10-fold molar excess of ATTO was used without estimating DOL in the following labelling procedures.

Possible ATTO-dye interference with the TCR:MHC II interaction

The extended application of the Streptamer technology into a multimer-based kinetics assay was first developed for the murine MHC class I molecules (H2). However, the adaption of the murine k_{off} rate assay to a human setup was not straightforward. For the murine MHC class I molecules, the selected fluorescent dye for the k_{off}-rate assay was coupled to an inserted cysteine residue of the β_2 microglobulin. Even though the H2-1 and HLA class I molecules show a high degree of structural similarities, dye conjugated HLA-Streptamers proved not to stain the antigen-specific T cells. The human MHC I molecule was examined to find a suitable location for dye conjugation. The end result was an engineered site for dye conjugation at the C-terminus after the StreptagIII sequence. Although not demonstrated by the test clones, having this issue in mind, it might be a possibility that HLA-DQ2.5 molecules with a surface-exposed cystein residue close to the peptide-binding groove interferes with the TCR:MHC interaction of the Streptamer-negative TCCs.

ATTO is likely not responsible for low staining signals of DQ2.5-glia-α1/α2-reactive TCCs

The observed low staining of DQ2.5-glia-α1-reactive and DQ2.5-glia-α2-reactive TCCs were investigated to find out if the ATTO dye is responsible for this observation. As shown in figure 5.19, the gel-filtrated ATTO-conjugated and unconjugated Streptamers (no ATTO)

were tested in flow cytometry. TCC535.3.23 was the only clone that could demonstrate positive staining with both conjugated and unconjugated Streptamers, as previously demonstrated. Staining intensity of the naïve BC02.29 clone remained the same when compared to the APC signal in figure 5.16. As in figure 5.17, the same trend with great variation of the staining intensity was shown with the DQ2.5-glia-α2-reactive TCCs in figure 5.20. Only the test clone TCC678.3.22 showed positive staining independent of ATTO565 conjugation. Due to these observations, ATTO565 does probably not affect the Streptamer interaction with the selected TCCs. As previously discussed, a possible temperature dependency among the different $CD4^+$ T-cell clones with Streptamers remains to be investigated in order to clarify the variable stainings. Several TCCs should also be tested in order to obtain positive stainings, especially for the DQ2.5-glia-α2-Streptamer.

Streptamer-positive $CD4^+$ TCCs remain to be retested with the gel-filtrated DQ2.5-glia-α1 and DQ2.5-glia-α2 reagents in the k_{off} rate assay.

6.1.3 TCR:pMHC interaction and T cell responsiveness

As introduced in 1.9, the Streptamer-based k_{off} –rate assay has lead to promising findings for correlation of high-avidity antigen-specific $CD8^+$ T cells and protective immunity, a further comparison of this k_{off} -rate assay with other kinetics assays is discussed below. This recently developed Streptamer approach is an improved multimer-based kinetics assay without the limitations of conventional multimers. Conventional multimer-based k_{off} assays do not analyse the monomeric TCR:MHC interaction and is difficult to standardise due to the level of multimerisation. The Streptamer approach does not require a blocking reagent and the dissociation events occur in a monomeric fashion after addition of d-biotin. The monomeric TCR:pMHC interaction is also analysed with adhesion frequency assays (2D) and BIAcore assay (3D) and provide accurate calculation of both association and dissociation rates. In the BIAcore assay, both the TCR and pMHC must be expressed and purified as soluble molecules as opposed to the other assays. Important physiological conditions such as the cell membrane where the TCR is embedded and the contribution of the coreceptor are therefore not taken into consideration. In contrast, living T cells are used in 2D kinetics assay. Huang et al. (2010) compared the 2D kinetics data from the adhesion frequency assays with 3D kinetics data obtained based on surface plasmon resonance (62). They demonstrated that k_{off} rates of TCR:pMHC interaction with stronger ligands were progressively faster in 2D but slower in

3D. They could correlate the obtained 2D kinetics data with T cell proliferation. These opposite trends when comparing 2D and 3D kinetics data might be due to the cellular environment in which the TCR:pMHC interaction takes place. Determining the kinetics of TCR:pMHC interaction was favoured by 2D kinetics assays, as recently reviewed by Edwards et al. (63). However, 3D k_{off} rates demonstrated by the Streptamer-based k_{off}-rate assay show a correlation of slow dissocation rate with T cell responsiveness.

In the PhD work of Nauerth, they demonstrate that high-avidity T cells correlated with long half-life times of the TCR:pMHC interaction have a great potential in adoptive T cell transfer. Interestingly, the same research group has recently published an article on a novel enrichment and sorting strategy based on the Streptamer technology (64). Low-affinity streptagged Fab fragments have been engineered to serve as surface markers and can be used to enrich and sort the cells of interest. Due to the reversible nature of these reagents, the cells remain "untouched". This feature has shown to be very important in adoptive cell transfer.

The investigation of gluten-reactive $CD4^+$ T cells detected by DQ2 tetramers has shown to be a very important tool. For instance, detecting coeliac disease in HLA-$DQ2^+$ individuals on self-prescribed GFD is difficult. Recently, an HLA-DQ2 tetramer test was developed to assess possible coeliac disease in this group after a short oral gluten challenge (65). It would be interesting if a Streptamer-based k_{off} rate assay could provide useful insights with gluten-specific $CD4^+$ T cells and coeliac disease. Could there be a correlation with high-avidity gluten-specific $CD4^+$ T cells and development of coeliac disease? The gold standard for the diagnosis of coeliac disease is the Marsh score which is a categorisation of the pathological changes of coeliac disease in the small intestine (66). Could this Marsh score correlate to the avidity of the gluten-specific $CD4^+$ T cells? The research on regulatory $CD4^+$ T cells has been of great interest for their ability to suppress immune responses and provide peripheral tolerance. Their regulatory role has shown to be impaired in coeliac disease and other autoimmune disease (67). Perhaps functional $CD4^+$ regulatory T cells could serve as a suitable target in adoptive cell transfer in the future?

7 Conclusion

In this thesis, we have generated recombinant HLA-DQ2.5 molecules based on the Streptamer technology. These soluble class II HLA molecules were produced in insect cells and were affinity purified with the monoclonal anti-DQ2 antibody. Quality controls of the purified molecules demonstrated comparable structural features with the conventional DQ2.5 molecules. Panels of DQ2.5-glia-α1 and DQ2.5-glia-α2 reactive TCCs were stained with multimerised DQ2.5-Streptag molecules on Streptactin-APC in flow cytometry. It was demonstrated that DQ2.5-glia-α1-Streptamers stain the majority of DQ2.5-glia-α1-reactive TCCs. In contrast, DQ2.5-glia-α2-Streptamers showed highly variable staining signals of the DQ2.5-glia-α2 TCCs. A computer model of the DQ2.5-glia-α1 crystal structure revealed a native surface-exposed cysteine residue of the α chain. Dye conjugation with ATTO565-maleimide to the DQ2.5-Streptag molecules could potentially affect the staining. However, this was not demonstrated with the selected TCCs.

The DQ2.5-Streptamers were incubated with TCCs at 4°C as it is a requirement of the Streptamer technology and the k_{off}-rate assay. It may be that elevated temperatures have an effect on Streptamer-negative TCCs. In addition to test different temperatures, several T-cell clones (derived from the gut and peripheral blood) must be tested in order to further evaluate the quality of the new DQ2.5-Streptamers.

The Streptamer technology has provided great insights of the $CD8^+$ T-cells and MHC I molecules but it still remains to see if the Streptamer technology could be applied on HLA DQ2.5 and $CD4^+$ T cells. To further investigate the promising findings in this thesis, retesting the Streptamer-positive TCCs in the k_{off}-rate assay will therefore be the next step forward.

References

1. Berg RD. The indigenous gastrointestinal microflora. Trends in microbiology. 1996;4(11):430-5. Epub 1996/11/01.

2. Abbas AK. Cellular and Molecular Biology. 6th ed: Saunders Elsevier; 2010.

3. The MHCsc. Complete sequence and gene map of a human major histocompatibility complex. Nature. 1999;401(6756):921-3.

4. Stern LJ, Wiley DC. Antigenic peptide binding by class I and class II histocompatibility proteins. Structure (London, England : 1993). 1994;2(4):245-51. Epub 1994/04/15.

5. Brown JH, Jardetzky TS, Gorga JC, Stern LJ, Urban RG, Strominger JL, et al. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature. 1993;364(6432):33-9.

6. Cammarota G, Scheirle A, Takacs B, Doran DM, Knorr R, Bannwarth W, et al. Identification of a CD4 binding site on the [beta]2 domain of HLA-DR molecules. Nature. 1992;356(6372):799-801.

7. Lazarski CA, Chaves FA, Jenks SA, Wu S, Richards KA, Weaver JM, et al. The Kinetic Stability of MHC Class II:Peptide Complexes Is a Key Parameter that Dictates Immunodominance. Immunity. 2005;23(1):29-40.

8. Weiss A. T-cell antigen receptor signal transduction - A tale of tails and cytoplasmic protein-tyrosine kinases. Cell. 1993;73(2):209-12.

9. Doyle C, Strominger JL. Interaction between CD4 and class II MHC molecules mediates cell adhesion. Nature. 1987;330(6145):256-9.

10. Konig HR-Y, Germain RN. MHC class II interaction with CD4 mediated by a region analogous to the MHC class I binding site for CD8. Nature. 1992;356(6372):796-8.

11. van Heel DA, West J. Recent advances in coeliac disease. Gut. 2006;55(7):1037-46. Epub 2006/06/13.

12. Sollid LM, Jabri B. Is celiac disease an autoimmune disorder? Current opinion in immunology. 2005;17(6):595-600. Epub 2005/10/11.

13. Sollid LM, Markussen G, Ek J, Gjerde H, Vartdal F, Thorsby E. Evidence for a primary association of celiac disease to a particular HLA-DQ alpha/beta heterodimer. The Journal of experimental medicine. 1989;169(1):345-50. Epub 1989/01/01.

14. Sollid LM, Thorsby E. HLA susceptibility genes in celiac disease: genetic mapping and role in pathogenesis. Gastroenterology. 1993;105(3):910-22. Epub 1993/09/01.

15. Jabri B, Sollid LM. Mechanisms of disease: immunopathogenesis of celiac disease. Nature clinical practice Gastroenterology & hepatology. 2006;3(9):516-25. Epub 2006/09/05.

16. Dieterich W, Ehnis T, Bauer M, Donner P, Volta U, Riecken EO, et al. Identification of tissue transglutaminase as the autoantigen of celiac disease. Nature medicine. 1997;3(7):797-801. Epub 1997/07/01.

17. Molberg Ø, McAdam SN, Korner R, Quarsten H, Kristiansen C, Madsen L, et al. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. Nature medicine. 1998;4(6):713-7. Epub 1998/06/12.

18. Nilsen EM, Jahnsen FL, Lundin KE, Johansen FE, Fausa O, Sollid LM, et al. Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. Gastroenterology. 1998;115(3):551-63. Epub 1998/08/28.

19. Ferguson A, McClure JP, Townley RR. Intraepithelial lymphocyte counts in small intestinal biopsies from children with diarrhoea. Acta paediatrica Scandinavica. 1976;65(5):541-6. Epub 1976/09/01.

20. Sulkanen S, Halttunen T, Laurila K, Kolho KL, Korponay-Szabo IR, Sarnesto A, et al. Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. Gastroenterology. 1998;115(6):1322-8. Epub 1998/12/03.

21. Kalandadze A, Galleno M, Foncerrada L, Strominger JL, Wucherpfennig KW. Expression of recombinant HLA-DR2 molecules. Replacement of the hydrophobic transmembrane region by a leucine zipper dimerization motif allows the assembly and

secretion of soluble DR alpha beta heterodimers. The Journal of biological chemistry. 1996;271(33):20156-62. Epub 1996/08/16.

22. Miller LK. Baculoviruses as gene expression vectors. Annual review of microbiology. 1988;42:177-99. Epub 1988/01/01.

23. Instruction Manual: Baculovirus Expression Vector System. 6th ed1999.

24. Altmann F, Staudacher E, Wilson IB, Marz L. Insect cells as hosts for the expression of recombinant glycoproteins. Glycoconjugate journal. 1999;16(2):109-23. Epub 1999/12/28.

25. Matsui K, Boniface J, Reay P, Schild H, Fazekas de St Groth B, Davis M. Low affinity interaction of peptide-MHC complexes with T cell receptors. Science. 1991;254(5039):1788-91.

26. Altman JD, Moss PAH, Goulder PJR, Barouch DH, McHeyzer-Williams MG, Bell JI, et al. Phenotypic Analysis of Antigen-Specific T Lymphocytes. Science. 1996;274(5284):94-6.

27. Diamandis EP, Christopoulos TK. The biotin-(strept)avidin system: principles and applications in biotechnology. Clinical Chemistry. 1991;37(5):625-36.

28. Laitinen OH, Hytonen VP, Nordlund HR, Kulomaa MS. Genetically engineered avidins and streptavidins. Cellular and molecular life sciences : CMLS. 2006;63(24):2992-3017. Epub 2006/11/07.

29. Schatz PJ. Use of Peptide Libraries to Map the Substrate Specificity of a Peptide-Modifying Enzyme: A 13 Residue Consensus Peptide Specifies Biotinylation in Escherichia coli. Nat Biotech. 1993;11(10):1138-43.

30. Crawford F, Kozono H, White J, Marrack P, Kappler J. Detection of antigen-specific T cells with multivalent soluble class II MHC covalent peptide complexes. Immunity. 1998;8(6):675-82. Epub 1998/07/09.

31. Kozono H, White J, Clements J, Marrack P, Kappler J. Production of soluble MHC class II proteins with covalently bound single peptides. Nature. 1994;369(6476):151-4. Epub 1994/05/12.

32. Knabel M, Franz TJ, Schiemann M, Wulf A, Villmow B, Schmidt B, et al. Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. Nature medicine. 2002;8(6):631-7. Epub 2002/06/04.

33. Schmidt TG, Koepke J, Frank R, Skerra A. Molecular interaction between the Strep-tag affinity peptide and its cognate target, streptavidin. Journal of molecular biology. 1996;255(5):753-66. Epub 1996/02/09.

34. Schmidt TG, Skerra A. The random peptide library-assisted engineering of a C-terminal affinity peptide, useful for the detection and purification of a functional Ig Fv fragment. Protein engineering. 1993;6(1):109-22. Epub 1993/01/01.

35. Skerra A, Schmidt TG. Applications of a peptide ligand for streptavidin: the Strep-tag. Biomolecular engineering. 1999;16(1-4):79-86. Epub 2000/05/05.

36. Whelan JA, Dunbar PR, Price DA, Purbhoo MA, Lechner F, Ogg GS, et al. Specificity of CTL interactions with peptide-MHC class I tetrameric complexes is temperature dependent. J Immunol. 1999;163(8):4342-8. Epub 1999/10/08.

37. Cameron TO, Cochran JR, Yassine-Diab B, Sékaly R-P, Stern LJ. Cutting Edge: Detection of Antigen-Specific CD4+ T Cells by HLA-DR1 Oligomers Is Dependent on the T Cell Activation State. The Journal of Immunology. 2001;166(2):741-5.

38. Neudorfer J, Schmidt B, Huster KM, Anderl F, Schiemann M, Holzapfel G, et al. Reversible HLA multimers (Streptamers) for the isolation of human cytotoxic T lymphocytes functionally active against tumor- and virus-derived antigens. Journal of immunological methods. 2007;320(1-2):119-31. Epub 2007/02/20.

39. Casalegno-Garduno R, Schmitt A, Yao J, Wang X, Xu X, Freund M, et al. Multimer technologies for detection and adoptive transfer of antigen-specific T cells. Cancer immunology, immunotherapy : CII. 2010;59(2):195-202. Epub 2009/10/23.

40. Alexander-Miller MA. High-avidity CD8+ T cells: optimal soldiers in the war against viruses and tumors. Immunol Res. 2005;31(1):13-24. Epub 2004/12/14.

41. Chesla SE, Selvaraj P, Zhu C. Measuring two-dimensional receptor-ligand binding kinetics by micropipette. Biophysical journal. 1998;75(3):1553-72. Epub 1998/09/03.

42. Jason-Moller L, Murphy M, Bruno J. Overview of Biacore Systems and Their Applications. Current Protocols in Protein Science: John Wiley & Sons, Inc.; 2001.

43. Margulies DH, Plaksin D, Khilko SN, Jelonek MT. Studying interactions involving the T-cell antigen receptor by surface plasmon resonance. Current opinion in immunology. 1996;8(2):262-70. Epub 1996/04/01.

44. Khilko SN, Jelonek MT, Corr M, Boyd LF, Bothwell AL, Margulies DH. Measuring interactions of MHC class I molecules using surface plasmon resonance. Journal of immunological methods. 1995;183(1):77-94. Epub 1995/06/14.

45. Yee C, Savage PA, Lee PP, Davis MM, Greenberg PD. Isolation of High Avidity Melanoma-Reactive CTL from Heterogeneous Populations Using Peptide-MHC Tetramers. The Journal of Immunology. 1999;162(4):2227-34.

46. Wang XL, Altman JD. Caveats in the design of MHC class I tetramer/antigen-specific T lymphocytes dissociation assays. Journal of immunological methods. 2003;280(1-2):25-35. Epub 2003/09/16.

47. Nauerth M. Development of a novel TCR avidity assay for human CD8+ T cells. Germany: Technische Universitat München; 2012 (PDF: http://d-nb.info/1024567419/34)

48. Quarsten H, McAdam SN, Jensen T, Arentz-Hansen H, Molberg O, Lundin KE, et al. Staining of celiac disease-relevant T cells by peptide-DQ2 multimers. J Immunol. 2001;167(9):4861-8. Epub 2001/10/24.

49. Sollid LM, Qiao SW, Anderson RP, Gianfrani C, Koning F. Nomenclature and listing of celiac disease relevant gluten T-cell epitopes restricted by HLA-DQ molecules. Immunogenetics. 2012;64(6):455-60. Epub 2012/02/11.

50. Murhammer DW, Goochee CF. Scaleup of Insect Cell Cultures: Protective Effects of Pluronic F-68. Nat Biotech. 1988;6(12):1411-8.

51. Viken HD, Paulsen G, Sollid LM, Lundin KE, Tjonnfjord GE, Thorsby E, et al. Characterization of an HLA-DQ2-specific monoclonal antibody. Influence of amino acid substitutions in DQ beta 1*0202. Human immunology. 1995;42(4):319-27. Epub 1995/04/01.

52. Sheehan D, O'Sullivan S. Fast Protein Liquid Chromatography. Protein Purification Protocols2003. p. 253-8.

53. Kim CY, Quarsten H, Bergseng E, Khosla C, Sollid LM. Structural basis for HLA-DQ2-mediated presentation of gluten epitopes in celiac disease. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(12):4175-9. Epub 2004/03/17.

54. Junttila MR, Saarinen S, Schmidt T, Kast J, Westermarck J. Single-step Strep-tag purification for the isolation and identification of protein complexes from mammalian cells. Proteomics. 2005;5(5):1199-203. Epub 2005/03/12.

55. Lichty JJ, Malecki JL, Agnew HD, Michelson-Horowitz DJ, Tan S. Comparison of affinity tags for protein purification. Protein expression and purification. 2005;41(1):98-105. Epub 2005/04/02.

56. Ochs D. Protein contaminants of sodium dodecyl sulfate-polyacrylamide gels. Analytical biochemistry. 1983;135(2):470-4. Epub 1983/12/01.

57. Givan AL. Flow Cytometry: First Principles. 2nd ed. New York: Wiley 2001.

58. Altman JD. Flow Cytometry Applications of MHC Tetramers. Methods Cell Biol 2004;75:433-52. 2004.

59. Busch D, Wagner H, inventors; IBA GmbH (Göttingen, DE), assignee. Reversible MHC multimer staining for functional purification of antigen-specific T cells. United States2010.

60. Ráki M, Fallang L-E, Brottveit M, Bergseng E, Quarsten H, Lundin KEA, et al. Tetramer visualization of gut-homing gluten-specific T cells in the peripheral blood of celiac disease patients. Proceedings of the National Academy of Sciences. 2007;104(8):2831-6.

61. Cameron TO, Norris PJ, Patel A, Moulon C, Rosenberg ES, Mellins ED, et al. Labeling antigen-specific CD4(+) T cells with class II MHC oligomers. Journal of immunological methods. 2002;268(1):51-69. Epub 2002/09/06.

62. Huang J, Zarnitsyna VI, Liu B, Edwards LJ, Jiang N, Evavold BD, et al. The kinetics of two-dimensional TCR and pMHC interactions determine T-cell responsiveness. Nature. 2010;464(7290):932-6. Epub 2010/04/02.

63. Edwards LJ, Zarnitsyna VI, Hood JD, Evavold B, Zhu C. Insights into T cell recognition of antigen: Significance of two-dimensional kinetic parameters. Frontiers in Immunology. 2012;3.

64. Stemberger C, Dreher S, Tschulik C, Piossek C, Bet J, Yamamoto TN, et al. Novel Serial Positive Enrichment Technology Enables Clinical Multiparameter Cell Sorting. PLoS ONE. 2012;7(4):e35798.

65. Brottveit M, Raki M, Bergseng E, Fallang LE, Simonsen B, Lovik A, et al. Assessing possible celiac disease by an HLA-DQ2-gliadin Tetramer Test. The American journal of gastroenterology. 2011;106(7):1318-24. Epub 2011/03/03.

66. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). Gastroenterology. 1992;102(1):330-54. Epub 1992/01/01.

67. Granzotto M, dal Bo S, Quaglia S, Tommasini A, Piscianz E, Valencic E, et al. Regulatory T-cell function is impaired in celiac disease. Digestive diseases and sciences. 2009;54(7):1513-9. Epub 2008/11/01.