Doctoral Thesis

Engineering TCR-like Antibodies

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Oslo, March 2019

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Abbreviations

ADCC	Antibody dependent cell-mediated cytotoxicity
AMA-II	Second Antibody Modeling Assessment
APC	Professional antigen presenting cell
BCR	B-cell receptor
CAR	Chimeric antigen receptor
CDC	Complement dependent cytotoxicity
CDR	Complementarity determining region
CLIP	Class II-associated invariant chain peptide
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
Fv	Fragment variable
HLA	Human leukocyte antigen
HV	High valence
IFN-γ	Interferon γ
Ig	Immunoglobulin
Ii	Invariant chain
LV	Low valence
MHC	Major histocompatibility complex
NK	Natural killer
NMR	Nuclear magnetic resonance
PDB	Protein database
pMHC	Peptide:MHC complex
RMSD	Root mean square deviation
scFv	Single-chain fragment variable
SPR	Surface plasmon resonance
TCR	T-cell receptor
TG2	Transglutaminase 2
VH	Variable heavy

Vα	Variable α
Vβ	Variable β
VL	Variable light
WT	Wild type

List of publications

Manuscripts included in the thesis

Plasma cells are the most abundant gluten peptide MHC-expressing cells in inflamed intestinal tissues from patients with celiac disease
Lene Støkken Høydahl, Lisa Richter, Rahel Frick, Omri Snir, Kristin Støen Gunnarsen, Ole JB. Landsverk, Rasmus Iversen, Jeliazko R. Jeliazkov, Jeffrey J. Gray, Elin Bergseng, Stian Foss, Shuo-Wang Qiao, Knut EA. Lundin, Jørgen Jahnsen, Frode L. Jahnsen, Inger Sandlie, Ludvig M. Sollid, Geir Åge Løset.
Gastroenterology 2019 Apr;156(5):1428-1439.e10

Engineering gliadin-specific TCR-like antibodies with picomolar affinities using docking models and phage display
Rahel Frick, Lene Støkken Høydahl, Ina Hodnebrug, Jeliazko R. Jeliazkov, Kristin Støen Gunnarsen, Shraddha Kumari, Grete Berntsen, Terje Frigstad, Erik S. Vik, Knut E. A. Lundin, Jørgen Jahnsen, Jeffrey J. Gray, Ludvig M. Sollid, Inger Sandlie, Geir Åge Løset.
Manuscript

A confined *TRAV26-1* encoded recognition motif focuses the biased T-cell response in celiac disease
Rahel Frick, Kristin Støen Gunnarsen, Shiva Dahal-Koirala, Louise Fremgaard Risnes, Ludvig M. Sollid, Inger Sandlie, Lene Støkken Høydahl, Geir Åge Løset.
Manuscript

Review article

Part of the introduction is based on the following review article:

Targeting the pMHC ligandome by use of engineered antibodies
Lene Støkken Høydahl, Rahel Frick, Inger Sandlie, Geir Åge Løset.
in preparation

Other publications

A TCRα framework-centered codon shapes a biased T cell repertoire through direct MHC and CDR3β interactions
Kristin Støen Gunnarsen*, Lene Støkken Høydahl*, Louise Fremgaard Risnes, Shiva Dahal-Koirala, Ralf Stefan Neumann, Elin Bergseng, Terje Frigstad, Rahel Frick, M. Fleur du Pré, Bjørn Dalhus, Knut E.A. Lundin, Shuo-Wang Qiao, Ludvig M. Sollid, Inger Sandlie, Geir Åge Løset.
* contributed equally
JCI Insight, 2(17), 2017

Modeling and docking antibody structures with Rosetta
Brian D. Weitzner, Jeliazko R. Jeliazkov, Sergey Lyskov, Nicholas Marze, Daisuke Kuroda, Rahel Frick, Jared Adolf-Bryfogle, Naireeta Biswas, Roland L. Dunbrack Jr., Jeffrey J. Gray.
Nat. Protocols, 12(2):401-416, feb 2017

Consolidation of proteomics data in the Cancer proteomics database
Magnus Ø. Arntzen, Paul Boddie, Rahel Frick, Christian J. Koehler, and Bernd Thiede.
PROTEOMICS, 15(22):3765-3771, nov 2015

1 Introduction

The immune system serves to protect us from potentially harmful pathogens, including parasites, bacteria, and viruses, as well as dangerously altered self as in cancer. It is finely balanced to ensure efficient removal of threats but at the same time maintain self-tolerance.

The immune system of vertebrates consists of innate and adaptive immunity. The innate immune system comprises a series of physical barriers, such as skin and mucus layers, but also immune cells and molecules. They serve as a first line of defense but lack antigen specificity and memory. These tasks are fulfilled by the adaptive immune system, which provides the framework for this thesis and will therefore be reviewed in greater detail.

1.1 Adaptive immunity

The hallmarks of the adaptive immune system are antigen specificity and long lasting memory. The cells that confer both properties are B and T cells. Both cell types originate from a common lymphoid progenitor cell in the bone marrow. They use unique antigen specific receptors on their surfaces called B-cell and T-cell receptors that can be specific to virtually any pathogen. Their development is highly regulated to ensure exclusive reactivity to foreign antigens.

1.1.1 Immunoglobulins

Each B cell expresses a unique antigen specific immunoglobulin (Ig) called B-cell receptor (BCR) on its surface. Antibodies are a soluble version of these BCRs and are important molecules of the adaptive immune system. Mammalian antibodies are classified into 5 isotypes: IgA, IgD, IgE, IgG, and IgM. IgG is the most abundant isotype in human blood and also most commonly used for therapeutic applications [1, 2].

IgGs are symmetric Y-shaped glycoproteins consisting of two identical heavy chains and two identical light chains (Fig. 1.1). The constant region of an IgG consists of domains CH1, CH2, CH3, and CL. It is independent of antigen specificity but determines the effector functions of the antibody and is constant for antibodies of the same isotype. The variable region is unique for each B-cell clone and consists of a variable heavy (VH) and a variable light (VL) domain. They come together to form the paratope, the region where antigen is bound. The six complementarity determining region (CDR) loops typically make up most of the paratope [3].

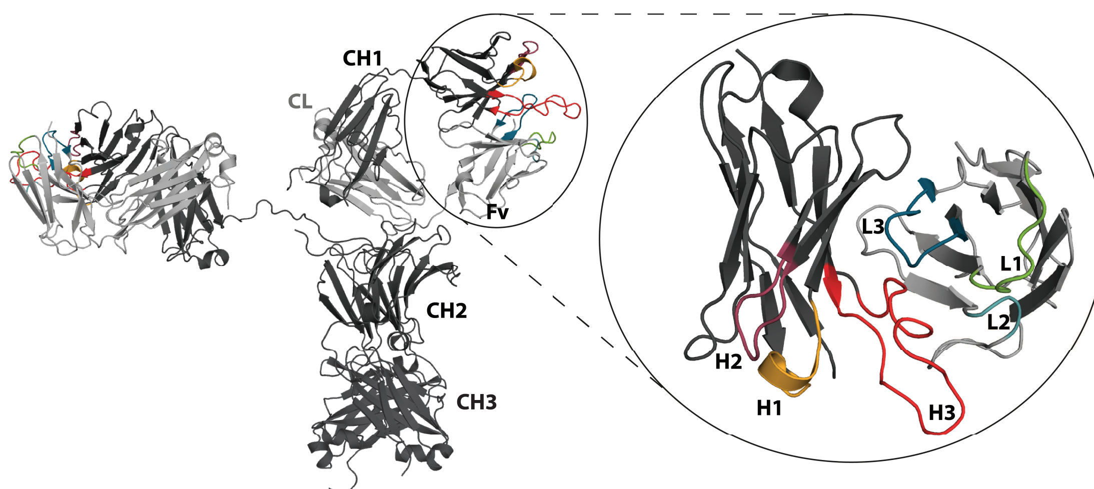

Figure 1.1: **Crystal structure of antibody:** The crystal structure of a full length IgG is depicted as a cartoon. The light chain is shown in light gray, the heavy chain in black. The six CDR loops are colored and annotated (PDB ID: 1HZH [4]).

Having specificity for any pathogen requires an extremely diverse set of antibodies. This is achieved through processes called V(D)J recombination [5] and somatic hypermutation [6, 7] during B-cell development. The gene segments encoding different parts of an antibody variable domain are organized in an unusual way. They are not functional in their germline conformation, but need to be rearranged to encode a functional antibody variable region. The VH domain segments are located on the Ig heavy locus and the light chain can be rearranged using segments from either the Ig κ or the Ig λ locus. A VL domain is formed by joining V (*IGKV* or *IGLV*) and J (*IGKJ* or *IGLJ*) gene segments, while the VH domain consists of V, D, and J segments (Fig. 1.2). In order to rearrange the genes, highly controlled double strand DNA breaks occur. At the junctions between the segments, palindromic P nucleotides and random N nucleotides are inserted, further increasing the diversity generated by the recombination events. All junctions are located in the CDR3 loops, making them the most diverse.

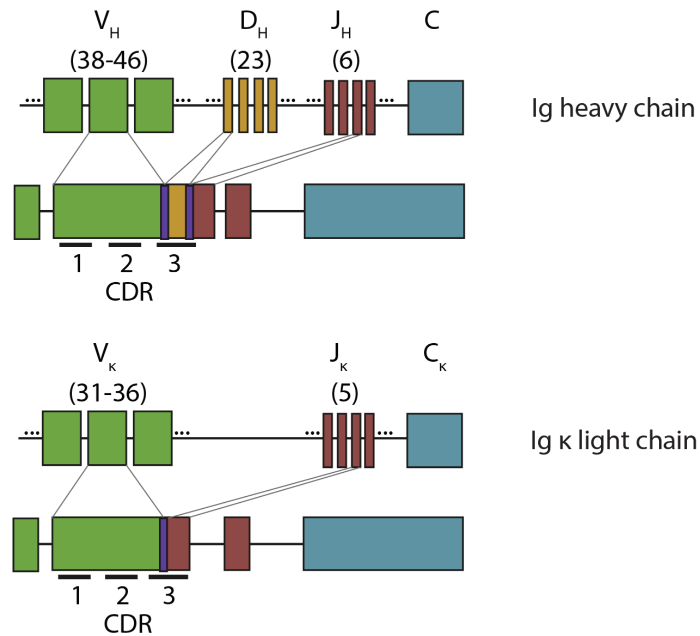

Figure 1.2: **V(D)J recombination of antibodies:** Gene segments for a VH domain are chosen from 38–46 *IGHV*, approximately 23 *IGHD* and 6 *IGHJ*, whereas gene segments for a VL domain come from 31–36 *IGKV* and 5 *IGKJ* for a κ light chain or 29–33 *IGLV* and 4–5 *IGLJ* for a λ light chain (not shown), giving rise to massive combinatorial diversity. Random nucleotide insertions at the junctions increase the diversity further (purple).

When B cells are activated, they go through somatic hypermutation and class switch recombination [8] in organized lymphoid tissues. The enzyme activation-induced cytidine deaminase initiates a process that ultimately results in the introduction of point mutations primarily in the CDR loops at a rate approximately 10^4–10^6 times higher than the basal mutation rate in the human genome [9, 10]. This generates additional diversity in the antibody repertoire and allows for modulating the specificity [11] and increasing the affinity to the antigen, and is therefore referred to as affinity maturation.

IgG effector functions

Affinity matured antibodies are typically highly specific for their antigens and bind with at least nanomolar affinities. When bound to their targets, they have different functional activities in an immune response depending on the isotype [12]. Human IgG can be further divided into the four subclasses IgG1, IgG2, IgG3, and IgG4 [13]. They have intracellular and extracellular effector functions [14, 15], part of which are exploited in therapeutic antibodies.

Antibody dependent cellular cytotoxicity (ADCC) depends on natural killer (NK) cells. NK cells are innate immune cells that do not possess antigen specific receptors, but express FcγRIIIA (CD16), which binds to the Fc region of IgG. Binding activates the NK cell and cell lysis is induced by exocytosis of cytotoxic granules [16–18].

Furthermore, antibodies bound to cell surface molecules can activate the classical pathway of the complement system leading to **complement dependent cytotoxicity (CDC)**. Starting with C1q deposition on cell surface bound antibodies, a cascade of complement proteins becomes activated. This leads to release of pro-inflammatory molecules, opsonization of the target, and to formation of a membrane-attack complex, resulting in target cell lysis [19]. IgM are the most potent subclass for induction of CDC. However, IgG1 and IgG3 are also able to induce CDC if C1q is bound to IgG multimers [20, 21]. Therefore, antigen density and distance are crucial.

1.1.2 Antigen presenting cells and MHC molecules

The MHC is a highly polymorphic genetic locus that encodes two classes of MHC molecules, also termed human leukocyte antigens (HLA) in humans. Both classes of MHC are transmembrane proteins and serve the purpose of presenting peptides. All nucleated cells of the body express MHC class I molecules to present intracellular self peptides for T-cell surveillance. This allows for detection and removal of virus infected cells or cancer cells by cytotoxic CD8+ T cells. MHC class II molecules, however, are only expressed by professional antigen presenting cells (APCs), including dendritic cells (DCs), macrophages, and cells of the B-cell lineage. They are expressed as heterodimers of an α-chain and a β-chain that are both encoded on the MHC locus. The peptide binding groove of MHC class II consists of a β-pleated sheet flanked by two α-helices. It is open on both ends and can accommodate peptides of different lengths (Fig. 1.3) [22]. The MHC chains are assembled in the endoplasmic reticulum (ER) together with a polypeptide termed invariant chain (Ii). The complexes enter late endosomal compartments where Ii is digested, leaving only a short class II-associated Ii peptide (CLIP), which serves as a place-holder in the peptide binding groove of MHC class II. CLIP is then exchanged for foreign peptides with higher affinity for the MHC, in a process catalyzed by the non-classical MHC molecule HLA-DM [23]. B cells express HLA-DO, a modulator of HLA-DM activity, which causes preferred loading of peptides

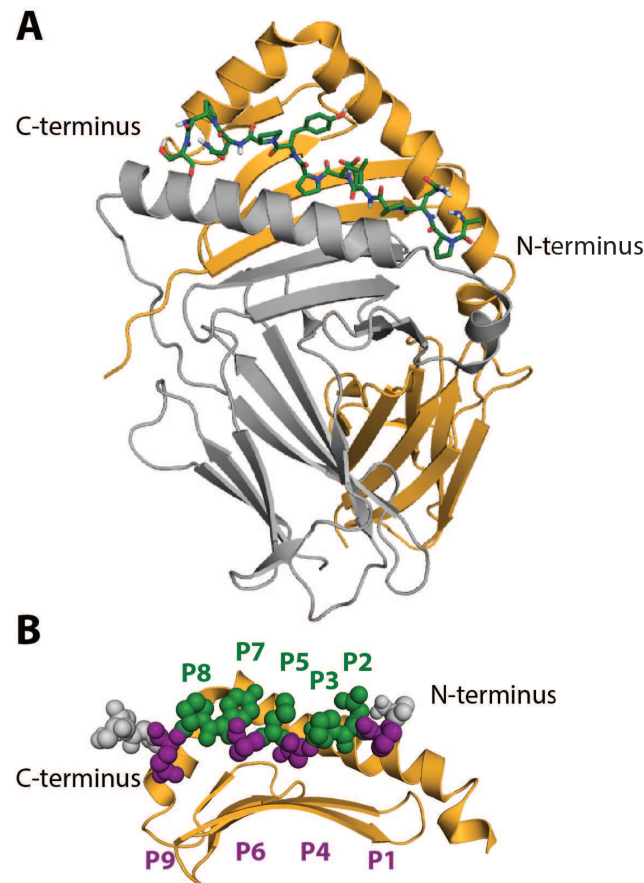

Figure 1.3: **Representative crystal structure of MHC class II** (PDB ID 4OZF, [26]). **A:** MHC class II molecules consist of an α-chain (gray) and a β-chain (orange) that both contribute to forming the peptide binding groove. The peptide is shown in stick representation (green). **B:** Peptide shown in sphere representation, along with the MHC β-chain (orange). Especially positions 1, 4, 6, and 9 (purple) form contacts with the MHC molecules. The remaining side chains of the 9-mer core region are exposed (green) and able to directly interact with the TCR. Flanking residues outside the 9-mer core region are shown in grey.

that have been internalized by the surface BCR in an antigen specific manner [24, 25].

MHC class II molecules are encoded by three sets of genes (HLA-DR, HLA-DQ, and HLA-DP) that are highly polymorphic. Every MHC molecule is able to bind and present many different peptides, but affinities of the interactions vary [27]. The polymorphisms are mainly located in the region contacting the peptide or the TCR [28, 29], thus shaping the peptide and T-cell repertoires and explaining the strong correlation between MHC haplotype and disease risk seen in many autoimmune diseases [30].

APCs display peptide:MHC (pMHC) complexes on their surface and prime naïve CD4+ helper T cells in organized lymphoid tissues. DCs express 10–100 times more MHC class II than other APCs [31] and are particularly efficient at activating naïve T cells [32–34]. Depending on the cytokine profile of the APC, activated T cells differentiate into regulatory T cells or effector T cells and home to the site of antigen exposure. Some of these T cells, and in particular follicular CD4+ T cells (T_{FH}), in turn give help to B cells which allows them to differentiate into memory B cells and antibody producing plasma cells. APCs also activate effector CD4+ T cells locally at the site of infection.

While DCs, macrophages and B cells are the classical APCs, other cell types may play a role in antigen presentation to CD4+ T cells. Activated T cells themselves were found to have MHC class II on their surface and efficiently present to other CD4+ T cells [35, 36]. Furthermore, it has been demonstrated that interferon γ (IFN-γ) can induce various cell types to express MHC class II [37–39].

A role of plasma cells as APCs has been under debate. Cells from the B lineage constitutively express MHC class II from an early stage but it has been assumed that expression is rapidly down-regulated upon plasma cell differentiation [40, 41]. The mechanism for this differential expression is dependent on B lymphocyte-induced maturation protein 1 (BLIMP-1). BLIMP-1 represses expression of class II transactivator (CIITA) [42]. Therefore, CIITA is expressed in B cells but down-regulated in plasma cells [43].

However, MHC class II expressing plasma cells have been detected in the bone marrow of multiple myeloma patients [44] and in blood of systemic lupus erythematosus (SLE) patients [45]. Pelletier et al. found high expression levels of MHC class II as well as costimulatory molecules CD80 and CD86 along with other components needed for antigen processing and presentation in terminally differentiated plasma cells in mice [46]. These cells successfully activated naïve antigen specific CD4+ T cells but had a negative effect on previously activated CD4+ effector T cells and T_{FH} cells. These findings suggest that plasma cells can play a role in antigen presentation and T-cell regulation.

1.1.3 T cells and $\alpha\beta$ T-cell receptors

T cells originate from stem cells in the bone marrow and mature in the thymus, where they are equipped with T-cell receptors (TCRs). $\alpha\beta$ TCRs are membrane-

bound heterodimers. Each chain consists of a membrane spanning α-helix and two extracellular domains that together form an antigen receptor that is structurally and functionally similar to Fab fragments. The membrane proximal domains are constant and the distal domains are variable. The variable domains of a TCR are composed of framework regions and six CDR loops - three in the α-chain and three in the β-chain.

The T-cell repertoire in an individual is extremely diverse with approximately 2×10^7 different TCRs expressed in 10^{12} T cells in the human body and a theoretically possible diversity of 10^{15} [47–49]. This is achieved through combinatorial diversity and junctional diversity introduced during V(D)J recombination, highly similar to the process described for antibody variable domains (1.1.1).

Priming of naïve CD4+ T cells by APCs in peripheral lymphoid tissues is probably the most central interaction in the adaptive immune system. Primed CD4+ T cells will expand and differentiate into different T cells subsets, that confer memory, release cytokines, or give help to B cells. Therefore, the generation of the naïve T-cell repertoire is strictly regulated in the thymus. During positive selection, only those thymocytes receive a survival signal, that weakly bind to self-MHC and are able to induce TCR signaling. During negative selection, TCRs binding with high affinity to self-MHC are removed from the repertoire. By the time naïve T cells leave the thymus, they are restricted to bind peptide presented by MHC. They express either a CD4 co-receptor if they are MHC class II restricted, or a CD8 co-receptor if they are MHC class I restricted [50].

1.2 Antibody engineering

Antibodies are highly specific, have a long serum half life, and naturally come with several effector functions (see 1.1.1). These properties are exploited for development and use of antibodies in treatment of cancers, autoimmune and infectious disease, and others [51].

Traditionally, therapeutic antibodies are generated by hybridoma technology [52]. Animals, usually mice, are immunized with a target antigen and launch an immune response against it. B cells from the spleens of the immunized animals are isolated and fused with myeloma cells, yielding immortal hybridoma cells that produce antibodies. During screening, clones with desired specificities can be identified.

Hybridoma technology has enabled generation of antibodies with desired specificities and is the basis of the first therapeutic antibody Muromonab-CD3 [53] and many others. However, fully murine antibodies were immunogenic when injected into human patients, leading to anti-drug antibodies with unwanted effects on safety and pharmacokinetics [54, 55]. Chimeric antibodies, where the rodent constant region is replaced with a human constant region, were generated in an effort to decrease immunogenicity [56], but it was found that a foreign Fv domain can be sufficient to induce an immune response [57]. In humanized antibodies the rodent CDR loops are usually grafted onto a human framework [58, 59]. Immunogenicity is reduced compared to fully murine formats, but the antibodies may require further engineering to maintain desired biophysical properties. These formats have been successful and have led to the development of several therapeutic antibodies, but fully human antibodies remain the gold standard. To this end, transgenic mice containing human V gene segments were generated starting in the 1990s [60–62], and allow for isolation of human variable regions after vaccination. *In vitro* display technologies, including phage [63], ribosome [64], and yeast display [65] are complementary platforms used for isolation of fully human antibodies. Both hybridoma technology and *in vitro* display systems have been used to generate antibodies currently used for therapy and enabled the extraordinary success of immunotherapy.

The majority of therapeutic antibodies are of the human IgG1 (hIgG1) subclass, as it combines excellent pharmacokinetics with potent effector functions. Other isotypes may have complementary advantages [66, 67]. Both IgG2 and IgG4 antibodies are on the market, but so far IgG3s are not [1, 2]. This is likely due to problems with stability and manufacturability as well as a short serum half-life. IgG1/IgG3 combination variants, however, have been suggested to have increased cytotoxic potential [68, 69].

Their natural effector functions make antibodies with desired specificities potent therapeutics for cancer and other indications. Fc engineering can further improve an antibody's pharmacokinetics and tailor its effector functions and biodistribution. IgG Fc mutations for increased half life [70, 71] and modified CDC [72, 73] or ADCC [74, 75] have been described. To achieve an effect beyond natural IgG effector functions, different antibody formats have been used. These included antibody-drug conjugates (ADC) [76–79], chimeric antigen receptor (CAR) T cells [80], and bispecific T-cell engagers (BiTE) [81] (Fig. 1.4).

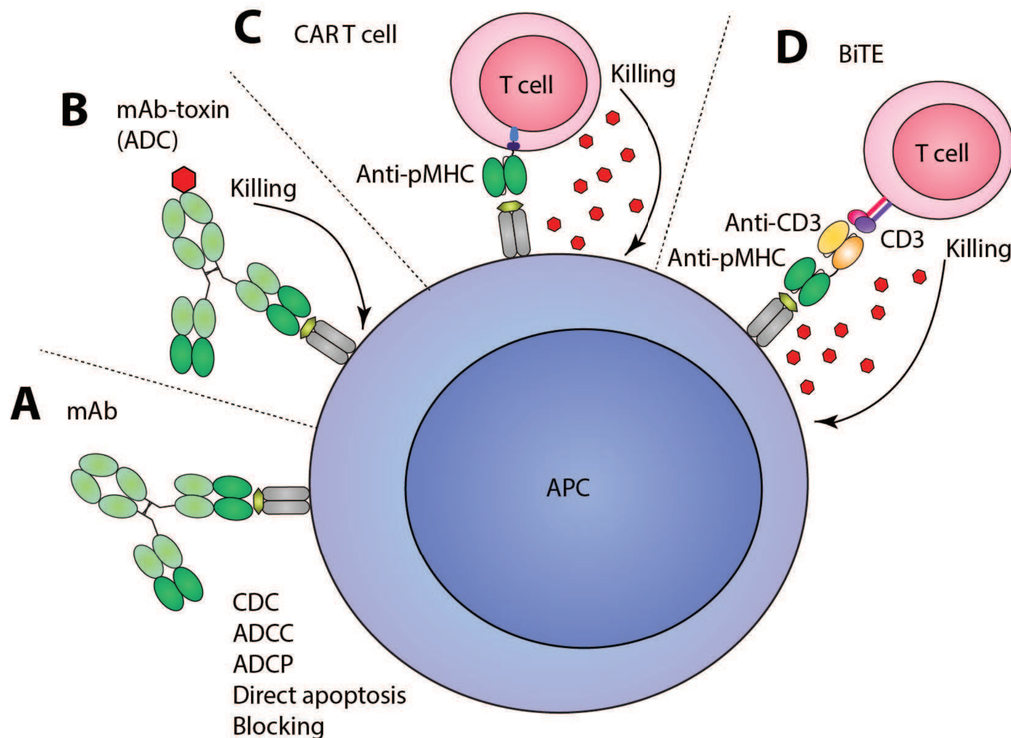

Figure 1.4: **Effector functions of therapeutic antibodies exemplified by pMHC specific antibodies:** **A:** Monoclonal antibodies (mAbs) come with isotype dependent effector functions that can directly have a therapeutic effect (CDC, ADCC, antibody dependent cellular phagocytosis (ADCP), direct apoptosis or blocking). **B:** ADCs deliver a toxic cargo to the target cell. **C:** CAR T cells are re-engineered cytotoxic T cells where target specificity is conferred through an antibody fragment targeting a surface antigen. **D:** BiTEs are bispecific antibody fragments that bind to a cell surface antigen on the target cell, and CD3 of T cells to recruit cytotoxic T cells which induce target cell killing. Figure modified from unpublished work by L. S. Høydahl.

1.2.1 Phage display

Phage display is the most widely used *in vitro* display technology for isolation of proteins and peptides with desired specificities. It was first described in the 1980s [63] and has since been applied to a wide range of purposes including protein interface engineering and analysis [82], epitope mapping [83], directed evolution of enzymes [84], and antibody engineering [85]. The first approved fully human antibody generated using phage display is adalimumab (Humira by Abbvie) [86], which is currently the best-selling drug on the market, generating $18 billion in sales globally in 2017 [87].

Phage display technology is based on the genetic fusion of an antibody library to

a coat protein on bacteriophage. Filamentous Ff phages used for this purpose are rod-shaped particles consisting of a single-stranded DNA molecule surrounded by a coat of proteins - approximately 2,700 copies of the major coat protein pVIII, and 3-5 copies of the minor coat proteins pIII, pVI, pVII, and pIX. They infect *E. coli* via an interaction between pIII and the bacterial F-pili [88] and replicate as episomes [89]. New virions are assembled and leave the host cell without killing it [90].

If an antibody fragment is fused in frame to one of the coat proteins, the phage particle will display the fusion protein on its surface and carry the genetic information on the inside, thus providing a phenotype-genotype link. For antibody selections, pIII fusions are most widely used, however, fusions on all coat proteins have been made successfully [91]. Our group has reported improved antibody selection outcomes using pIX display with strong enrichment of functional full-length clones and retrieval of higher affinity antibodies compared to display on pIII [92, 93]. Display of a fusion protein on phage is most commonly achieved by use of a phagemid in combination with a helper phage. The phagemid encodes the selected coat protein fused to the protein of interest (e.g. a scFv) and the helper phage encodes all other components needed to assemble a phage. To achieve low valence (LV) display, the coat protein fusion is complemented with a wild type (WT) version on the helper phage. This means that there are two sources of this coat protein and only a fraction of them will display the protein of interest. Deletion of the WT sequence from the helper phage results in high valence (HV) display, because the only available source of the coat protein contains the fusion.

Antibody libraries displayed on phage can then be selected for specific binding in a process called biopanning (Fig. 1.5), where the phage library is selected on the target of choice, and only selected phage are propagated in *E. coli* and packaged. Selection over multiple rounds enriches binders and reduces background.

Antibody libraries for phage display selection can be generated by different approaches:

1. **Naïve libraries** allow for the selection of fully human antibodies. Naïve B cells from healthy donors are isolated and the transcribed V gene repertoires are amplified and cloned into vectors for phage display. This results in large libraries, and binders against a wide range of targets can be isolated from these libraries. Retrieved binders typically have low to medium affinity for the target.

2. **Immunized libraries** provide an alternative strategy. These libraries are constructed from the antibody repertoire of immunized humans or animals

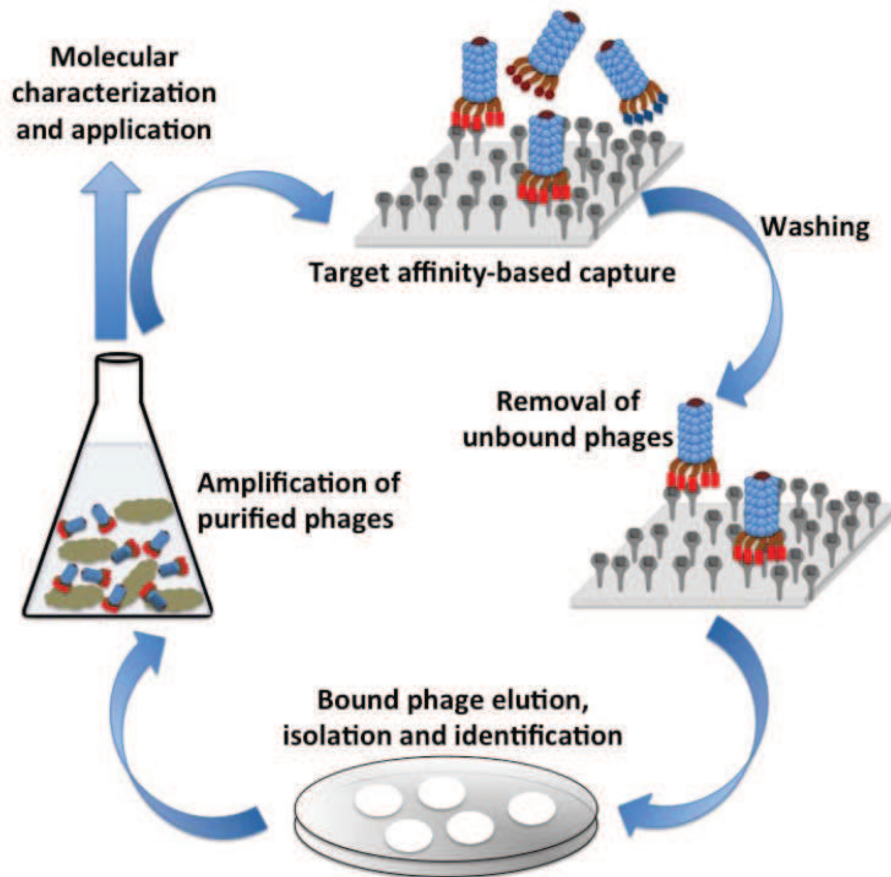

Figure 1.5: **Biopanning of a phage library:** A phage library is incubated with immobilized target molecules. Weak binders or non-binders are washed away. Binders are eluted and allowed to infect *E. coli*. Phage are amplified by the host and enter the next round of panning or are used for analysis and screening. Figure reprinted from [94].

that have launched an immune response, including somatic hypermutation and affinity maturation. This enables direct selection of high affinity binders.

3. **Synthetic libraries** typically rely on one template sequence. The sequence is then randomized in a fully random or targeted manner, to generate diversity for the selection.

The most common antibody formats displayed on phage are single-chain Fv (scFv) fragments or Fab fragments. Fab fragments have the advantage of containing the CH1 and CL domains and therefore, reformatting to full length antibodies is more reliable [95]. However, expression of large Fab fragments negatively affects the host cell and can be technically challenging. Other formats, including nanobodies [96], have been used successfully in phage display.

1.2.2 *In vitro* affinity maturation of antibodies

An increased affinity of a protein-protein interaction correlates with a reduction of the difference in Gibb's free energy upon binding.

$$\Delta G \propto T \ln K_{\mathrm{d}}$$

where ΔG is the difference in Gibb's free binding energy, T is the temperature, and K_{d} is the dissociation constant. The change in Gibb's free energy upon binding is the sum of an enthalpic and an entropic term:

$$\Delta G = \Delta H - T\Delta S$$

where ΔH is the change in free enthalpy and ΔS is the change in entropy. A decrease in Gibb's free energy at fixed temperature can therefore be achieved by reducing the free enthalpy or by reducing the loss of entropy upon binding, often referred to as the entropic penalty. The enthalpic term reflects the change in polar or hydrophobic intermolecular interactions such as hydrogen bonds, van-der-Waals interactions, or salt bridges [97]. Improving these interactions across the protein-protein interface, will therefore improve the affinity. Increasing the buried surface area will also positively affect affinity because it leads to a larger gain in entropy of the solvent upon binding. This is known as the hydrophobic effect. The conformational entropy of proteins on the other hand decreases upon binding, as they can populate a smaller number of conformational states if they are part of the complex [97]. This entropic penalty can theoretically be reduced by rigidifying the monomeric proteins leading to enhanced affinity. In practice, rigidification during *in vivo* affinity maturation may be restricted to certain parts of the Fv and not applicable to all antibodies [98–100].

The goal of *in vitro* affinity maturation is typically the isolation of antibodies with increased target affinity but without compromising specificity. Since protein-protein interactions are complex, the most widely used approach is the generation of mutant libraries based on a lead antibody in combination with *in vitro* display technologies and selection for high affinity variants. The mutations can be introduced randomly, e.g. by error-prone PCR, or in a targeted approach, e.g focused on the CDR3 loops. Another strategy is chain-shuffling, where the light and heavy chains or parts of them are sequentially replaced with libraries and then selected for improved binders [101, 102]. Reduction of protein stability during the process of affinity maturation is typically unwanted, as it would cause problems with manufacturability

and handling. Therefore, stability and affinity engineering are often done in parallel [103].

1.2.3 Computational methods in antibody engineering

Computational antibody Fv structure prediction tools are being actively developed, and their performance is constantly improving [104, 105]. These tools provide a fast and cheap alternative to experimental methods for obtaining atomic-scale structural information, albeit at the cost of accuracy. They are particularly useful, when experimental methods for structure determination like x-ray crystallography or nuclear magnetic resonance (NMR) are not feasible. Structure prediction of Fv regions is very successful due to the high level of sequence and structure conservation found across antibodies. Framework regions are the most homologous and can be reliably predicted to backbone root mean square deviations (RMSD) below 1 Å [104]. Even five out of the six CDR loops, which are less homologous across antibodies, can be predicted with high accuracy because they adopt canonical conformations [106, 107]. But prediction of the heavy chain CDR3 loop [104] as well as the relative orientation of light and heavy chains is more challenging and the focus of ongoing development [108–110].

Structural models of antibodies bound to their antigen can be particularly useful for antibody engineering purposes. Software packages to generate docking models are available and have been used successfully to guide antibody engineering [111, 112]. In addition to structure prediction, computational methods are used to design variants with novel specificity or improved affinity or stability [113–116]. *In silico* prediction of mutations that affect protein stability or affinity, has lead to the design of refined antibody libraries [117], or directly to the design of enhancing mutations [118, 119]. However, for the latter, construction of combination mutants and laborious screening is typically necessary due to inaccuracies in the predictions.

1.3 pMHC specific reagents

Reagents with specificity for defined complexes of peptides and MHC molecules are valuable tools for studies of peptide presentation and for precise targeting in therapeutic strategies. They can be used to distinguish transformed cells from healthy cells

in cancer and infectious disease, or detect presentation of self-peptides in autoimmunity.

T-cell receptors (TCRs) are the natural binding partners for pMHC complexes. Their affinity is low (dissociation constant K_d 1-100 μM) [120], as high affinity TCRs are negatively selected *in vivo* [121] and tend to be cross-reactive [122]. Therefore, they typically require affinity or avidity engineering to allow for usage as research tools or therapeutics. Furthermore, TCRs are transmembrane proteins that do not naturally occur as soluble molecules. Nevertheless, they have been successfully engineered to be stable high affinity pMHC specific reagents using *in vitro* display technologies [123–127]. However, their higher affinities and favorable biophysical properties make antibodies an attractive alternative.

1.3.1 Structural aspects of pMHC specific reagents

The docking orientation of TCRs onto pMHC complexes has been found to be conserved (Fig. 1.6). They bind in a diagonal mode, positioning the variable region of the α-chain (Vα) over the N-terminal half of the peptide, and the variable region of the β-chain (Vβ) over the C-terminal half [128]. The germline encoded CDR1 and CDR2 loops are positioned over the α-helices that form the peptide binding groove of the MHC molecule. The CDR3 loops are focused on the peptide, typically centered on the p5 position, and thus provide antigen specificity. Even relatively small deviations from this canonical binding mode have been associated with non-canonical peptides [129], self-reactivity [130] and inability to induce TCR signaling [131].

For MHC class I, the only clear exception is a TCR contacting a bulged 13 residue long viral peptide (PDB: 4JRY) [133]. This TCR is shifted towards the peptide N-terminus compared to the canonical orientation. For MHC class II, again almost all TCRs dock in the conserved orientation across the peptide binding groove. Exceptions are two TCRs from human induced T regulatory cells in complex with HLA-DR4 presenting a self peptide (PDB: 4Y1A and 4Y19) [134]. These two TCRs adopt a conformation that is rotated by 180° and shifted towards the MHC α-chain. Another two structures are markedly tilted away from the canonical orientation towards the peptide N-terminus and the MHC β-chain (PDB: 1YMM and 2WBJ) [135, 136].

It is striking that almost all TCRs with solved co-crystal structures have highly similar binding modes and the reasons for this are still debated [137, 138]. One

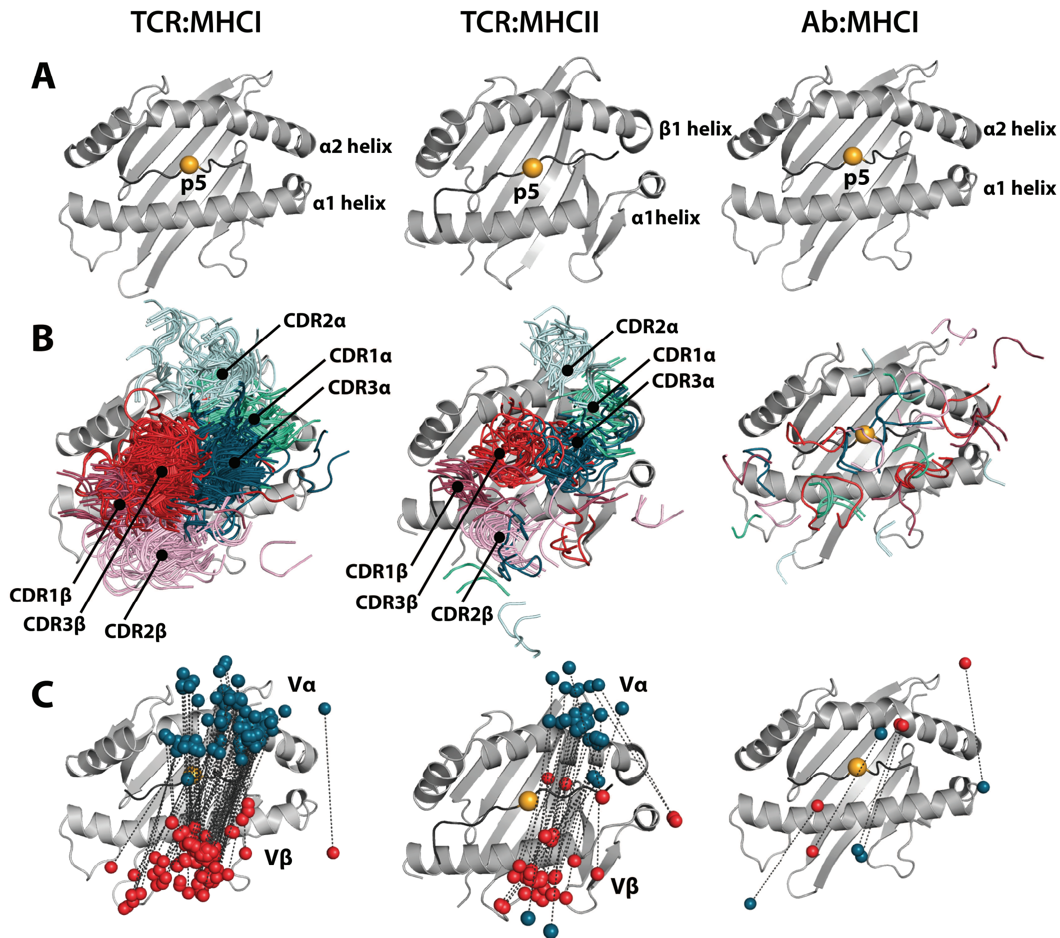

Figure 1.6: **MHC class I and class II complexes bound to TCRs or antibodies:** Overlay of publicly available co-crystal structures of human TCRs in complex with MHC class I (103 structures) or class II (33 structures) were retrieved from the STCRDab [132]. Structures of antibodies in complex with MHC class I were collected from the PDB (5 structures). **A:** Representative pMHC complexes (PDB IDs: 1AO7, 4OZF, 1W72) are illustrated and the central p5 positions of the peptides are highlighted. The MHC molecule is represented in light gray, the peptide in dark gray. **B:** CDR loops of TCRs or antibodies are represented as cartoons and annotated. Coloring of the antibody CDR loops is as annotated for the TCRs with the heavy chains in shades of red and the light chains in shades of blue. **C:** The centers of mass of the variable domains are represented as spheres (red: variable β/ variable heavy, teal: variable α/ variable light) and connected with dashed lines to illustrate orientations.

explanation is that the conserved orientation is the result of thymic selection where only those T cells are recruited to the mature repertoire that have a TCR:MHC interaction that allows for simultaneous engagement of CD4 or CD8 co-receptors necessary for signaling. Orientations deviating from the canonical binding mode may fail to induce TCR signaling due to a lack of MHC specificity or a lack of co-receptor engagement [139, 140]. Another explanation is that TCRs are predisposed to interact with certain MHC alleles via germline encoded features in their CDR1 and CDR2 loops. These residues then form interactions with the MHC helices, which have acquired complementary residues via co-evolution. These interactions bias the TCR repertoire towards a diagonal orientation even before thymic selection [141–143].

Crystallization and mutagenesis studies of TCR-like antibodies have suggested that they either recognize pMHC in a similar way to the corresponding TCRs or utilize non-canonical binding modes [144–149]. A comparison of the five available co-crystal structures (PDB: 1W72, 3CVH, 3GJF, 3HAE, 4WUU) of antibodies bound to pMHC, illustrates the more diverse binding modes (Fig. 1.6) [144, 145, 149, 150]. Two of the antibodies (PDB: 3GJF, 3HAE) show highly similar orientations, however they differ at only 3 and 6 amino acid positions in the light and heavy chain, respectively, and are specific for the same epitope. Their binding mode is similar to the canonical orientation of TCRs, where the antibody VL domain corresponds to the TCR Vβ domain and the antibody VH domain corresponds to the TCR Vα domain. The CDR3 loops form direct interactions with the peptide. All other structures differ significantly from each other and from the canonical binding mode of TCRs. However, all antibodies appear to rely on their CDR3 loops to form direct interactions with the peptide, albeit to different degrees.

Antibodies are naturally not restricted to MHC molecules and have not co-evolved with them. Therefore, the germline encoded recognition motifs found in the V gene segments of TCRs [143] do not exist in antibody genes. Unlike TCRs, antibodies are purely selected based on their ability to bind target specifically and are not restricted by interactions of co-receptors like CD4 and CD8. For these reasons, antibodies can take on diverse binding conformations to achieve specificity, whereas TCRs are largely limited to the diagonal canoncial conformation.

1.3.2 Applications of pMHC specific antibodies

TCR-like antibodies specific for MHC class I and II complexes have been engineered by use of immunization strategies and *in vitro* display [151–158]. The majority of these antibodies was raised against MHC class I complexes presenting viral peptides or tumor antigens.

In 2000, the first antibody specific for MHC class I with the tumor-derived antigen MAGE-A1 was isolated directly from a naïve human phage library [158]. Since then several similar antibodies have been reported. They have enabled peptide detection on tumor cells and have potential for targeted cancer therapy. One example is the generation of antibodies against mutation-associated neoantigens ("MANAbodies") [159]. Skora et al. selected these antibodies from a synthetic phage library based on the HER2/neu specific antibody trastuzumab. Antibodies specific for HLA-A2 in complex with a KRAS mutant peptide were selected and induced CDC in peptide pulsed cells.

There are also several examples of TCR-like antibodies against MHC class II complexes. Krogsgaard et al. engineered an antibody specific for a pMHC complex in multiple sclerosis (HLA-DR2 in complex with a myelin basic protein self-peptide (MBP85-99)) by combining immunization and phage display [154]. The MK16 antibody successfully stained peptide pulsed cells and APCs from multiple sclerosis lesions, and inhibited T-cell activation *in vitro*. Furthermore, microglia/macrophages were determined as the most abundant MBP presenting cells in tissue sections from multiple sclerosis patients. Another study describes the isolation of antibodies against a complex of HLA-DR4 with a type 1 diabetes autoantigen. These antibodies were selected from a naïve phage library and inhibit T-cell activation *in vitro* and in a mouse model [160]. A different TCR-like antibody against a T1D autoantigen was obtained using an immunization strategy and delayed diabetes onset and progression in an animal model, suggesting that TCR like antibodies may be applicable for treatment of autoimmune disorders [161]. Different formats for TCR-like antibodies, including CAR T cells and antibody drug conjugates are being explored [162–164].

1.4 Celiac disease

Celiac disease (CeD) is an inflammatory condition of the small intestine that affects approximately 1% of people in Europe and the USA [165–167]. Prevalence varies con-

siderably between countries and ethnicities and was found to be increasing over time [165, 168, 169]. CeD patients typically experience gastrointestinal symptoms, such as diarrhea, bloating, and abdominal pain, but also extraintestinal symptoms, including fatigue, failure to thrive, and anemia. These symptoms may be partly or completely absent in patients, and definite diagnosis usually requires a small intestinal biopsy. The celiac lesion is characterized by villous atrophy, crypt hyperplasia, scalloping and infiltration of lymphocytes [170].

Symptoms are triggered by dietary uptake of gluten from wheat, barley, or rye. But what looks like a food intolerance at first glance, has been found to be strikingly similar to autoimmune diseases. One of the autoimmune features is the presence of autoantibodies against the enzyme transglutaminase 2 (TG2) [171]. TG2 specific IgA titers in blood serve as a diagnostic marker [172, 173]. Furthermore, there is a genetic predisposition for CeD, that is linked to the MHC locus. More than 90% of patients express HLA-DQ2.5 (*DQA1*05*/ *DQB1*02*) and the majority of the remaining patients are positive for HLA-DQ8 (*DQA1*03*/ *DQB1*03:02*) or HLA-DQ2.2 (*DQA1*02:01*/ *DQB1*02*) [174]. This makes the HLA locus the single most important genetic factor accounting for 40% of the genetic variance [175]. At least 39 other disease associated genetic loci have been identified explaining another 13.7% of the genetic variance [175], some of which are shared with other autoimmune diseases [176, 177]. Environmental factors, including mode of delivery at birth and viral infections may influence disease risk [178–180]. Infant feeding practices and age at gluten introduction have been studied, but no correlations were found [181, 182].

The only available treatment is lifelong adherence to a gluten-free diet, which causes autoantibody titers and histological changes to revert to normal in most patients [183]. However, it is not effective in all cases [183, 184] and leads to poor patient compliance [185]. Therefore, new therapeutic options are needed.

1.4.1 Antigen presentation in celiac disease

Dietary proteins are broken down by proteases in the stomach and intestine. However, gluten proteins are rich in prolines and have been found to be exceptionally resistant to intestinal proteases [186, 187]. Gluten peptides cross the intestinal epithelium and enter the lamina propria, where they are selectively deamidated by TG2 (Fig. 1.7). The peptides are taken up by APCs and displayed on the disease associated MHC class II molecules. In the context of HLA-DQ2.5, HLA-DM mediated

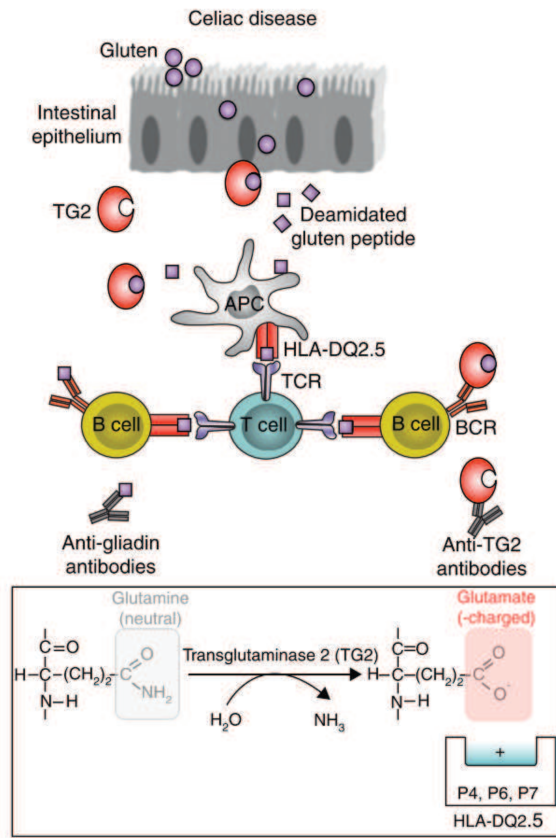

Figure 1.7: **Celiac disease mechanism:** After ingestion of gluten containing food, gluten peptides reach the small intestine and cross the epithelium. In the lamina propria they are specifically deamidated by transglutaminase 2 (TG2), taken up by APCs, and displayed on HLA-DQ2.5. Gluten specific T cells are activated and give help to B cells producing antibodies against gliadin or TG2. Figure modified from [198] with permission from Elsevier.

CLIP exchange is inefficient, making CLIP2 (core sequence: PLLMQALPM) the predominant peptide species presented on the cell surface [188, 189]. Negatively charged anchor residues in positions P4, P6, and P7 [190–192] are an advantage for binding to HLA-DQ2.5. These are introduced by TG2-mediated deamidation. The other disease associated DQ molecules HLA-DQ2.2 and HLA-DQ8 present peptides with different features: HLA-DQ2.2 favors a Ser in P3 of the peptide [193], and HLA-DQ8 preferentially binds peptides with glutamate residues in P1 and P9 [194]. The most important immunodominant gluten epitopes inducing T-cell responses across patients are HLA-DQ2.5 restricted and derived from α- and ω-gliadin (Table 1.1) [195–197].

APCs in the human small intestine carry out two distinct functions. Some APCs migrate to organized lymphoid tissues, such as the mesenteric lymph nodes, and activate

Table 1.1: **Immunodominant HLA-DQ2.5 restricted gliadin epitopes**

Epitope	9mer core sequence*
DQ2.5-glia-α1a	PFPQPELPY
DQ2.5-glia-α2	PQPELPYPQ
DQ2.5-glia-ω1	PFPQPEQPF
DQ2.5-glia-ω2	PQPEQPFPW

* Deamidated positions are underlined.

naïve CD4+ T cells. Other APCs stay at the site of antigen exposure and stimulate effector T cells locally. Several studies have compared frequencies and composition of HLA-DQ2+ APC populations in the CeD lesion with that in healthy duodenum.

In the healthy human duodenal mucosa, the majority of HLA-DQ+ APCs are CD68+ macrophages and most of the remaining are CD11c+ DCs [199]. B cells in the small intestine are typically located in secondary lymphoid tissues and there are only few B cells scattered in the lamina propria. The overall number of HLA-DQ2+ APCs in the active celiac lesion is increased by approximately 50% compared to healthy tissue, which is mainly due to an increase in CD11c+CD1c- monocyte derived DCs. An increased proportion of these cells display an activated CD86+ or matured DC-LAMP+ phenotype, suggesting that these cells may be functionally involved in CeD pathogenesis. After *in vitro* peptide pulsing, these cells efficiently activated gliadin specific T-cell clones [199]. A particular popluation of macrophages (CD163+CD11c+) is present at increased density in celiac lesions [200] and may be involved in activation of effector CD4+ T cells. CD163+CD11c- macrophages on the other hand, are significantly decreased but many of them are in an active state (CD86+) [199, 201]. They do not appear to be potent activators of gliadin specific T cells, but may contribute to inflammation by releasing cytokines [199, 202]. CD11c+ DCs co-expressing CD103 are also decreased in the celiac lesion [203]. This is detectable before onset of tissue alterations, and is therefore believed to be at least partly due to DC migration to lymph nodes [202, 203]. Therefore, this specific subset of DCs may be involved in disease initation.

B cells are scarce in the healthy small intestinal mucosa and have therefore not been considered to play an important role in local activation of T cells [199]. However, IgA secreting plasma cells specific for gliadin and TG2 are characteristically expanded in CeD [204, 205]. Interestingly, B cell depletion with rituximab has im-

proved CeD symptoms [206, 207], although no pathogenic role of antibodies has been established in CeD. This suggests, that B cells and plasma cells might contribute to CeD pathogenesis, apart from production of antibodies - possibly as APCs.

1.4.2 T-cell response in celiac disease

Gluten reactive CD4+ T cells are found in the small intestine [208] and in blood [209, 210] of both treated (i.e. on a gluten free diet) and untreated CeD patients, but they were not detected in healthy controls [209–211]. CeD patients have T-cell responses to different gluten epitopes. However, the majority has T cells specific for the α- and ω-gliadin derived immunodominant epitopes (Table 1.1).

Several gluten epitopes recruit public TCR responses with biased usage of V gene segments and in some of them preferential pairing of α and β-chains. The response against DQ2.5-glia-α2 is characterized by biased usage of *TRAV26-1* paired with *TRBV7-2* and the use of a canonical CDR3β loop [212–214]. A non-germline encoded central arginine residue in this loop [214] interacts directly with the peptide [26] and is central to this motif. The bias is only found in the effector memory compartment but not in the naïve T-cell population [215].

Activated gluten reactive CD4+ T cells in CeD patients give help to B cells specific for gliadin. They may further give help to B cells specific for TG2, leading to autoantibody production. This has been rationalized by a hapten-carrier model [216]. In this model TG2 crosslinks gluten to itself [217], mediating uptake by TG2 specific B cells via their BCR and presentation of gluten peptides to gluten specific CD4+ T cells. Furthermore, activated CD4+ T cells are thought to interact with intra-epithelial cytotoxic T lymphocytes, that mediate epithelial tissue destruction and inflammation [218]. CD4+ T cells and CD4+ T-cell activation are therefore central to CeD pathogenesis.

2 Aims of the thesis

The aim of this thesis was to combine computational and experimental methods to engineer high affinity gluten pMHC specific antibodies, validate them, and use them to study peptide presentation in CeD patients and explore their therapeutic potential. Furthermore, the aim was to dissect the interaction between gluten pMHC and a semi-public TCR to gain new insights into CeD and T-cell repertoire formation.

The specific goals were:

1. Selection and characterization of antibodies specific for HLA-DQ2.5 in complex with DQ2.5-glia-α1a and DQ2.5-glia-α2
2. Characterization of gluten peptide presentation in human biopsy material using the HLA-DQ2.5:DQ2.5-glia-α1a specific antibody
3. Engineering high affinity variants of the pMHC specific antibodies for increased sensitivity using computational models and phage display
4. *In vitro* characterization of the high affinity antibodies regarding their specificities and affinities
5. Exploring the therapeutic potential of the pMHC specific antibodies
6. Finemapping the interaction of HLA-DQ2.5:DQ2.5-glia-α2 with the prototypic antigen specific TCR to gain insights into CeD and the selection of biased antigen specific repetoires

3 Summary of individual papers

Paper 1

Plasma cells are the most abundant gluten peptide MHC-expressing cells in inflamed intestinal tissues from patients with celiac disease

In paper 1, we selected antibodies specific for deamidated DQ2.5-glia-α1a in complex with HLA-DQ2.5 from a human naïve scFv phage library, and confirmed their ability to discriminate between highly similar pMHC molecules. We then used these antibodies to study gluten peptide presentation in human small intestinal biopsies from untreated HLA-DQ2.5+ CeD patients and control subjects. Surprisingly, plasma cells and B cells were found to be the most abundant gluten peptide presenting cells in patients. No presentation of deamidated gluten peptides was detected in healthy or HLA-DQ2.5- controls. MHC class II expression, however, was found in plasma cells of both CeD patients and healthy individuals. A fraction of the gluten peptide presenting plasma cells expressed IgA specific for gliadin or TG2. Furthermore, these plasma cells expressed co-stimulatory molecules, suggesting that they may play a role in T-cell activation. The notion that plasma cells may act as APCs does not only provide a new perspective on their involvement in CeD pathogenesis but also on their general role in mucosal immunology.

Paper 2

Engineering gliadin-specific TCR-like antibodies with picomolar affinities using docking models and phage display

In paper 2, we aimed at improving and extending the repertoire of pMHC specific antibodies used in paper 1. An antibody specific for the second immunodominant epitope derived from α-gliadin, termed DQ2.5-glia-α2, was selected from a human naïve scFv phage library. This antibody bound target specifically but the interaction was characterized by a low affinity and a high off-rate. To enhance their sensitivity, we *in vitro* affinity matured the primary leads for both target epitopes using a semi-rational library design strategy complemented with a fully random strategy for one of the specificities. The targeted strategy was based on computational docking models and we chose poorly interacting CDR loops for randomization. We then selected antibodies from these second generation libraries using phage display on coat protein pIX. The matured variants had massively improved affinities over the mother clones and bound target specifically and with low off-rates. The highest affinity clone, which combines mutations from the random and the targeted strategy, had an affinity of 20 pM, which is the highest reported affinity for a human pMHC specific antibody. Staining of peptide loaded cells using these antibodies showed an increased sensitivity, and stainings of human biopsy material confirmed the findings from paper 1. Furthermore, the high affinity antibodies specifically inhibited T-cell activation *in vitro*, suggesting they may have therapeutic potential.

Paper 3

A confined *TRAV26-1* encoded recognition motif focuses the biased T-cell response in celiac disease

Paper 3 elucidates the molecular details underlying the recruitment of a biased antigen specific T-cell repertoire towards the DQ2.5-glia-α2 epitope in CeD. The prototypic TCR is characterized by usage of *TRAV26-1*, *TRBV7-2* and a canonical CDR3β loop. Building on a recent publication from our group [219], we studied the molecular details of this interaction and assessed whether it was conserved in other T cell clonotypes. We identified three *TRAV26-1* encoded framework residues, as well as a residue in the protoypic TCR β-chain and one in the MHC β-chain that are crucial for this interaction. We found that this recognition motif, centered on residue Y40$^{\mathrm{TCR}\alpha}$, is conserved in prototypic T-cell clones from two different patients, but differences in the CDR3α sequence can modulate the sensitivity of these TCRs. Furthermore, we studied a clonotype that occurs in the gluten specific repertoire of CeD patients at low frequency and pairs the prototypic TCR β-chain with a TCR α-chain encoded by *TRAV5* rather than *TRAV26-1*. This TCR α-chain shares two out of three residues involved in the recognition motif. The reconstructed *TRAV5* TCR specifically reacted to HLA-DQ2.5:DQ2.5-glia-α2. But notably, it appeared to use an altered recognition motif, which is largely independent of residue Y40$^{\mathrm{TCR}\alpha}$, suggesting that the proposed recognition motif is unique to *TRAV26-1*.

4 Methodological considerations

4.1 Computational modeling and docking of antibodies

We generated structural models of the TCR-like antibodies bound to their respective targets. The antigen-bound models of the mother clones served to rationalize the observed specificity of the DQ2.5-glia-α1a specific antibodies, and as a basis for the design of targeted scFv phage libraries. The models of the high affinity variants were analyzed to gain insight into the structural reasons for the improved affinities. We first generated structural models of the unbound Fv using RosettaAntibody [220–223] and then docked these models to crystal structures of the target pMHC using SnugDock [223–225].

For only antibody structure prediction, there are several other options for freely available software packages including Kotai Antibody Builder [226, 227], PIGS [228], and ABodyBuilder [229] as well as commercial options including Schrodinger and Accerlys Inc. These packages are relatively similar in their prediction performance and clearly out-compete general protein structure prediction software such as I-TASSER [104]. While models provide a fast and inexpensive method to obtain structural information, they come with limitations and cannot provide the same certainty as crystal structures. If template structures are available, RosettaAntibody typically predicts framework regions to below 1.0 Å backbone RMSD from the crystal structure, which is similar to other antibody prediction software. RosettaAntibody further predicted 42 out of 55 non-H3 CDRs within 1.0 Å RMSD during the Second Antibody Modeling Assessment (AMA-II) [104, 110]. Prediction of H3 loops is usually accurate for short loops ($<$ 12 residues) but less reliable for long loops [110]. The pMHC specific antibodies 107 and 206 (and their offspring) have CDR H3 loops with a length of 13 and 9 residues, respectively. Thus, prediction of the CDR H3 loop is expected to be more accurate for the DQ2.5-glia-α2 specific antibodies.

We opted to use RosettaAntibody over its competitors due to its integration into the Rosetta software suite for macromolecular modeling. This makes it possible to seamlessly proceed with docking using SnugDock, which we chose for its ability to model CDR loop motions upon binding more accurately than other antibody-antigen docking software [223, 224, 230]. The alternatives to SnugDock are PIPER's [231, 232] and ZDOCK's [233, 234] applications for antibody docking [230]. Unlike SnugDock, both employ a rigid-body docking algorithm, meaning they do not allow for CDR loop backbone flexibility or optimization of the VH/VL orientation. This reduces computational cost and allows these algorithms to search a larger space, which can be useful if the binding sites are unknown, but at the cost of modeling flexibility at the binding interface. For docking of a pMHC specific antibody however, the approximate binding site is known as it has to include both the peptide and the MHC molecule.

RosettaAntibody produces models that attempt to predict unbound structures of antibodies. Just like crystal structures, these models do not account for flexibility often seen in CDR H3 loops. During docking, we used an ensemble approach, where multiple antibody models are used as an input, to account for both flexibility and uncertainty in the antibody models [224]. In the most recent benchmark analysis of SnugDock, low-energy models accurately predicted at least 10% of interface contacts and had less than 4 Å interface RMSD for all 15 tested antibodies. In 11 out of 15, the models were significantly better, predicting more than 30% of contacts correctly and having less than 2 Å interface RMSD [223].

4.2 Phage display selection

TCRs are the natural binding partners for MHC molecules, but have low affinities [120] and are difficult to express as soluble molecules [235]. However, soluble high affinity TCRs have been engineered [236, 237] and used successfully for studies of peptide presentation [238, 239] and therapeutic approaches [240]. Attempts have been made to affinity engineer gluten pMHC specific TCRs in our group but with little success (L. S. Høydahl et al., unpublished). We therefore chose to select pMHC specific antibodies from a human naïve phage library.

4.2.1 Choice of antibody format and coat protein for display

The antibody formats most commonly used in phage display are scFv and Fab fragments. Fab fragments are generally more stable than scFv fragments and are less prone to dimerization. However, they are often displayed at lower efficiency on the phage surface and are toxic to the *E. coli* host, which can cause problems with growth bias. The scFv format has the advantage of allowing for fast and easy expression and display on phage resulting in more diverse libraries [241]. A potential problem with selection of scFv libraries is a loss or change in affinity or specificity after introduction of CH1 and CL domains. This was observed for the 206 antibody, isolated in the primary selection against HLA-DQ2.5:DQ2.5-glia-α2. While it bound its target strongly as a soluble or phage displayed scFv, it only exhibited weak binding as a full length antibody or Fab fragment. Furthermore, all HLA-DQ2.5:DQ2.5-glia-α2 binders, react to the related HLA-DQ2.5:DQ2.5-glia-ω2 complex as scFv but not as Fab or full-length IgG. This illustrates that conversion to Fab or full length IgG format may not only alter the affinity but also the fine specificity. It has been suggested that selection in Fab format may therefore be beneficial for *in vitro* affinity maturation attempts [95].

We chose to display scFv fragments on coat protein pIII for the primary selections. pIII fusions are well established and most widely used for phage display of antibodies. Our group has developed a novel helper phage for HV display on phage coat protein pIX called Deltaphage [92]. During the course of this project, a study from our group reported that display on coat protein pIX can have several advantages over display on pIII. It was found that pIX display leads to enrichment of full length clones, reduction of growth retardation, and more efficient selection for specific and stable binders compared to pIII display [93]. Therefore, we switched to pIX display for affinity maturation.

4.2.2 Design of phage libraries

The primary leads specific for HLA-DQ2.5:DQ2.5-glia-α1a were selected from the human naïve pL-NBL$_\kappa$ scFv phage library [242]. In the primary selection against the DQ2.5-glia-α2 complex we used a version of the library that was reformatted to the pFKPDN vector [93, 243]. The library was cloned from the IgM and IgD repertoire of healthy human volunteers. It was purified on protein L in order to enrich functional clones and therefore contains diverse pL binding V$_\kappa$ frameworks

but no V_λ frameworks. It has a high fraction of functional clones and a diversity of approximately $3{\times}10^8$ clones [242]. Both primary leads use highly similar light chain genes and were predicted to use the same residues for interaction with the MHC β-chain, suggesting that only a few germline sequences are suitable for interaction with the very confined epitope on HLA-DQ2.5:peptide complexes. Therefore, selecting the primary leads from a diverse naïve library containing many different V genes, rather than a synthetic library with variation limited to hot spots regions, may have been important for the success of the selections.

The targeted libraries were made using degenerate oligonucleotides with NNK codons (where N=A/C/G/T, and K=G/T). This gives rise to 32 codons, encoding all 20 natural amino acids. Use of NNK codons reduces the biased incorporation probabilities for the different amino acids, seen in NNN randomization, and reduces the frequency of stop codons. An alternative would have been NNB randomization (where B=C/G/T). This is expected to introduce fewer stop codons, but has a more biased distribution towards amino acids encoded by multiple codons [244]. Trinucleotide synthesis [245], MAX [246], or ProxiMAX [247] mutagenesis strategies aim at even distribution of the 20 amino acids for all positions and strict exclusion of stop codons. This allows for full coverage of the theoretically possible amino acid sequence space at a lower number of transformants, but these strategies become impossible, very laborious or very expensive when targeting longer sequence stretches.

4.2.3 Selection strategy

The target pMHC molecules were soluble, recombinant, biotinylated proteins, captured on streptavidin coated magnetic beads. Cell surface bound proteins have been used as selection targets before [248, 249], but introduce complexity and reduce control over the target. The soluble pMHC molecules have been used successfully to activate specific T cells, and are detected by anti HLA-DQ2 antibody 2.12.E11 and pan-DQ reactive antibody SPV-L3 when captured on Neutravidin coated ELISA plates. This suggests that they are of high quality and the T-cell epitope is accessible.

Both for the primary and the affinity maturation selections we aimed at isolating highly specific binders. Therefore, each round of selection was complemented with a

negative selection on HLA-DQ2.5:CLIP2. This is especially important, since HLA-DQ2.5:CLIP2 is likely to be prevalent on APCs *in vivo* (see 1.4.1) and cross-reactivity has to be excluded. This was apparently successful, as none of the antibodies tested, was cross-reactive to HLA-DQ2.5:CLIP2 after the selection.

The selection scheme for the affinity maturation was further aimed at isolating binders with high monomeric affinities and with retained or improved stability. The libraries were split into a thermal branch with heat challenges and a competition branch with an aggressive off-rate selection, loosely based on the hammer-hug selection protocol [250].

For off-rate selections, soluble antigen is typically added in excess after the library was incubated with the target. Once the binders dissociate, they are captured by a soluble antigen, and therefore removed from the selection output. Since soluble pMHC was limited, we used an excess of soluble mother clones for off-rate competition. In theory they should sequester unbound immobilized target and shield it from phage particles, after dissociation. However, this approach relies on the affinities and off-rates of the mother clones, which were especially unfavorable for 206. This may have affected the selection outcome. Most libraries except the 206 H1 library, were lost during the off-rate competition, with close to no selection output. The incubation time for off-rate selections and the concentration of competitor are expected to have a critical impact [251] and optimization of these parameters, might have improved the selection result even further.

4.2.4 Library screening

After the selections, single clones were screened in ELISA as described before [93]. Crude periplasmic fractions were analyzed for binding to target and CLIP background. The protein concentrations and functional fractions were not controlled for at this point, but affect the result. This was observed in the HLA-DQ2.5:DQ2.5-glia-α1a candidate clones after affinity maturation. They showed no binding to the target during screening but this could be attributed to poor expression levels of these scFvs when controlled for with an anti-myc antibody or protein L (data not shown). To identify favorable clones nevertheless, the selection outputs were screened in phage format, where scFv were displayed as fusions to coat protein pIX at HV [92]. HV display introduces avidity affects, that may mask

true monomeric affinity, but is sufficient to distinguish specific binders from non-binders.

4.3 Biophysical characterization of antibodies

In the primary selections we characterized the clones as scFv produced in *E. coli.* This allowed for the leads to be directly cloned from the phagemids into the vector for soluble expression as NcoI/NotI fragments and fast protein production.

After the affinity maturation selection, we also started out by characterizing the leads in scFv format as before, but most 107 offspring produced poorly in this system. Furthermore, we observed a tendency towards dimerization for some of the scFv clones in analytical size exclusion chromatography. Therefore, we decided to reformat to Fab fragments for careful characterization and identification of lead clones. This also allowed us to rank clones in a format that contains constant domains, which is important since we and others have described the possibility of drops in affinity or altered fine specificity after reformatting (see 4.2.1) [95]. To produce Fab fragments, we commercially obtained gene fragments of the light and heavy chain sequences flanked by BsmI and BsiWI restriction enzyme sites and cloned them into the pLNOH2 oriP and pLNOκ vectors containing constant human γ1 and C_κ domains, respectively. We expressed them by transient transfection of HEK293E cells [252]. They were produced with high yields and a predominant monomeric fraction, and thus this method proved ideal for assessing specificities in ELISA, estimating monomeric affinities in SPR experiments, and analyzing thermostability by nanoDSF.

4.4 Retroviral transduction of A20 B cells

We used murine A20 B cells retrovirally transduced to express pMHC constructs [219]. In these pMHC complexes, the peptide was covalently linked to the MHC β-chain. This ensures that all MHC molecules have a peptide of interest in their binding groove and makes it possible to compare expression levels with an HLA-DQ2 specific antibody. Effects of peptide affinity for the MHC molecule are excluded in this system. However, the linker introduces a non-natural component that might affect peptide orientation and the MHC expression level is artificially high compared to that found in natural APCs.

4.5 B-cell lines and flow cytometry experiments

EBV transduced B-cell lines and Raji B lymphoma cells were used for peptide loading experiments to complement the experiments with engineered A20 cells. Here the MHC expression level is physiological and the peptides are soluble and non-linked. Using this system, we were able to assess target binding and specificity for the high affinity antibodies. However, the absence or reduction of binding to cells pulsed with different peptides, may or may not reflect antibody specificity and affinity. Different peptides have different affinities and half-lifes for binding to MHC molecules, which has been thoroughly described for CeD relevant gluten epitopes [192]. Thus, lack of detection may also reflect differential amounts of peptide presented on MHC. The ability of peptide pulsed B cells to activate T cells served as an indirect quality control, as it confirmed that the peptide is present in sufficient amounts to induce T-cell signaling. HLA-DQ2.5+ DC or macrophage cell lines may be an alternative for peptide loading experiments, as these are generally more efficient peptide presenters (see 1.1.2).

4.6 Staining of human biopsy material

Patients with suspected CeD underwent endoscopy to obtain duodenal biopsies for definite diagnosis based on intestinal histology. The diagnosis was based on the criteria by the American Gastroenterological Association [253] and scores were assigned using the modified Marsh classification. Furthermore, HLA status, anti-TG2 IgA serum titers, age and sex were known for all subjects. The patients were further grouped into treated (i.e. on a gluten-free diet) or untreated (i.e. on a gluten-containing diet). However, the amount of gluten consumption in untreated patients, or possible dietary transgressions in treated patients, are not controlled for. This likely contributed to the large variation seen across patients.

4.7 T-cell activation assays

Retrovirally transduced BW T cells were used for paper 3 and proved very useful to compare TCR reactivity. In a recent publication from our group, we reported that SPR experiments with soluble TCRs are not a suitable method to study TCR functionality [219]. This is in line with another recent publication that explains how T-cell

receptor signaling can be uncoupled from pMHC binding [254]. We therefore used engineered BW T cells for paper 3 and assessed them functionally in T-cell activation assays rather than relying on the use of recombinant soluble molecules in SPR or ELISA experiments.

In paper 2, we engineered human SKW3 T cells to express the desired TCR constructs. This has two advantages. First, it provides a human *in vitro* system for assessment of T-cell activation and inhibitory capacity of the pMHC specific antibodies. Second, SKW3 T cells can be assayed for CD69 expression in flow cytomtery, whereas BW cells cannot. CD69 is the earliest marker of T-cell activation [255] and this method is more sensitive than assaying IL-2, which is only secreted in a later stage of activation. In paper 3, we assayed IL-2 secretion of BW cells in ELISA to achieve a higher throughput than would be possible with a flow cytometry based method.

5 General discussion and future perspectives

The focus of this thesis is engineering TCR-like antibodies specific for the CeD associated MHC class II variant HLA-DQ2.5 in complex with two immunodominant α-gliadin epitopes. These antibodies have facilitated studies that provided new insights into gluten peptide presentation in CeD patients and have shown promise for therapeutic intervention. Furthermore, the molecular details of a semi-public TCR response towards one of these gliadin epitopes were investigated and provided new insights into the formation of biased antigen specific T-cell repertoires.

5.1 A novel method for *in vitro* affinity maturation

Many different strategies and selection protocols have been described for *in vitro* affinity maturation of antibodies [93, 149, 250, 256–263]. In paper 2, we engineered high affinity antibodies specific for gluten pMHC complexes based on molecular docking models. We targeted poorly interacting loops for sequence randomization and length variation, and used signal sequence independent phage display on coat protein pIX to select improved binders. Furthermore, we employed a selection protocol that was partly based on the hammer-hug selection strategy, which includes an aggressive off-rate selection and a heat challenge [250]. The affinity improvements for the lead clones derived from the targeted libraries, 4.7C and 3.C11, were 400-fold and 2,700-fold compared to the respective mother clones. The RF117 clone, which combines the most stable clone from the random mutagenesis library with the highest affinity CDR H3 loop sequence, has a final monomeric affinity of 20 pM, which is the highest reported affinity of a human TCR-like antibody. Due to the high affinity of the selected clones, and the large improvement compared to the mother clones, our affinity engineering strategy can be regarded as highly successful and could be applicable

to similar projects aiming at selecting antibodies specific for pMHC or other targets.

Typically, affinity maturation is achieved by introducing sequence diversity into a primary lead, followed by phenotypic selection using an *in vitro* display systems, such as phage, yeast, or ribosome display [63–65]. Targeting the CDRs for randomization mimics *in vivo* affinity maturation and promises to focus the mutations to the regions that are most likely to have a direct effect on antigen recognition. There are examples of successful *in vitro* affinity maturation achieved by diversification in the CDR loops, most commonly the H3, H2, or L3 loops [256, 260]. Other studies employed diversification strategies that introduce random mutations over the full Fv sequence, such as error-prone PCR or bacterial mutator strains [264–266].

Diversification strategies have been described that use structural information to design more focused libraries with the aim to increase the frequency of mutations that actually affect affinity. Stewart-Jones et al. engineered high affinity pMHC specific antibodies based on a Fab:pMHC co-crystal structure [149]. They randomized residues in close proximity to interacting "hot spot" residues, but kept the direct contacts invariant. Residues oriented away from the peptide were excluded to avoid increased affinity for MHC. Similarly, Barderas et al. and Chames et al. have describe affinity maturation of antibodies specific for protein hormones and steroids, where refined libraries were generated based on docking models [111, 112]. In paper 2, we also used a strategy based on docking models to design targeted libraries. However, unlike previous studies, we did not exclusively target suboptimal residues neighboring beneficial interactions, but also loops that were predicted to lack direct interactions with the target pMHC. Even though the light chain CDR loops were predicted to be responsible for a large part of the interaction, we kept them invariant. Since they were already contributing favorably in the mother clones, the possible gain in introducing mutations in these areas appeared lower than when targeting areas that did not contribute significantly. This strategy built on the observation that the size of the buried interface area is generally positively correlated with the affinity of an interaction [267, 268].

Therefore, we focused the mutagenesis on the CDR H1 and H3 loops of the heavy chains. The CDR H3 loop is a common choice for randomization in the absence of docking models, but the CDR H1 loop and the introduction of tailored length variations are more specific design strategies. However, random diversification of loop lengths has been successful for CDR H3 loops of Fvs and other binding domains and is known to affect affinity [261–263]. The 206 offspring retrieved after

the affinity maturation selection were all derived from the CDR H1 library, that included length variations of 2–3 additional amino acids compared to the mother clones. The selection output from both the competition and the thermal branch showed that clones with a loop length increased by 2 amino acids were favored. A docking model of the lead clone 3.C11 and analysis of the interface suggests that the gain in affinity is partly due to the increased interface area. Therefore, our strategy, including docking models and focusing on poorly interacting loops, was especially important for the success of this selection. The 107 offspring retrieved from the affinity maturation selection all stemmed from the CDR H3 targeted library. Based on the docking models, we had speculated that the central W100 is crucial for specific interaction with the peptide and had therefore kept it invariant in half of the library members. The fact that all selected clones with known sequence had retained this Trp residue, lends credibility to the docking models and confirms that there is value in including structural information, despite the uncertainty of docking models.

All affinity matured variants were retrieved from selections where scFv libraries were displayed as fusions to phage coat protein pIX. Importantly, the fusions were free of signal sequences. In a recent study [93] from our group it has been found that HV display on pIX has several advantages compared to display on pIII. HV display on pIX did not lead to enrichment of low affinity clones, as has been described for HV display on pIII. Furthermore, HV display on pIX was at least as efficient at retrieving high affinity binders as LV display on pIII in direct comparison. The absence of a signal sequence in the pIX display system was observed to cause enrichment of full-length functional clones, while display systems containing a pelB signal sequence cause growth retardation of functional clones, and thus enrichment of truncated non-functional clones. For these reasons, signal-sequence independent phage display on coat protein pIX appears particularly suitable for affinity maturation selections. Even though we have not included a direct comparison to pIII, it can be speculated that this choice contributed to the success of the selection.

5.2 Understanding antigen presentation in celiac disease

Antigen presentation and CD4+ T-cell activation are central to CeD pathogenesis. Our gluten pMHC specific antibodies offer a unique opportunity to study *in situ*

processed and presented gluten epitopes. Based on previous studies that have analyzed the composition of APCs in the small intestine of CeD patients and compared it to that of healthy control subjects, it was expected that DCs or macrophages would play a role, as their frequencies are characteristically altered in the CeD lesion [199, 200, 202]. In paper 1, we surprisingly detected B cells and plasma cells to be the most abundant peptide presenting cells, which was confirmed in paper 2.

Plasma cells are not typically regarded as APCs and have been described as MHC class II negative [269]. MHC class II expression is repressed by the transcription factor BLIMP-1 upon plasma cell differentiation [42]. The fact that we detect MHC class II expression along with co-stimulatory molecules CD80 and CD86 on plasma cells, suggests that they may activate gluten specific CD4+ T cells and therefore play an important role in CeD pathogenesis. However, expression of MHC class II and costimulatory molecules was also detected on plasma cells of healthy controls, suggesting a more general and previously unknown role for plasma cells in mucosal immunology.

In paper 1, we have used the 107 and 106 antibodies for staining of patient material. They have medium affinity and it is conceivable that they only detect cells with high levels of peptide presentation in flow cytometry experiments. Plasma cells expressing a surface BCR may specifically and efficiently take up gluten peptides via their antigen specific receptor, and therefore present high levels of pMHC despite the relatively low level of MHC expression. Gluten peptides may be taken up directly by cells expressing a gluten specific BCR or indirectly when gluten is crosslinked to TG2, a surface BCR or other proteins as suggested in the hapten carrier hypothesis [216]. And indeed, a fraction of pMHC+ plasma cells express BCRs specific for either gliadin or TG2. In the eight CeD patient samples included in paper 2, we directly compared staining with 107 and the high affinity variants 4.7C and 3.C11. While 4.7C stained a similar percentage of cells as its mother clone, 3.C11 appeared to stain slightly higher levels. The shift in histograms of individual patient samples was only slightly larger for the high affinity variants. However, it appears 4.7C and 3.C11 may have the capacity to detect cells with lower levels of peptide presentation, namely macrophages and dendritic cells. This is difficult to assess since these cells occur at low numbers in both healthy individuals and CeD patients, preventing efficient sampling. Furthermore, it has been suggested that subsets of these cells in CeD patients rapidly migrate to the lymph nodes after they have taken up antigen [202, 203]. This may hamper our attempts to sample them in the lamina propria.

5.3 Therapeutic potential of gluten pMHC specific antibodies

The first line of treatment of CeD is adherence to a strict gluten free diet. In most patients, symptoms will resolve and the intestinal mucosa will recover. However, approximately 4% of CeD patients do not improve on a gluten free diet [270] and will progress to refractory CeD, which puts them at high risk of developing lymphoma with poor 5-year survival rates [271, 272]. Treatment strategies of refractory CeD [273, 274] include corticosteroids (budesonide [275]) monoclonal antibodies (infliximab [276], alemtuzumab [277]), immunosuppressants (azathioprine [278, 279], prednisone[279], cyclosporine [280]), chemotherapeutics (cladribine [281], pentostatin [282]) and autologous stem cell transplant [283]. None of these therapeutic options is disease specific and severe side effects are expected for most of them. Therefore, the unmet need for targeted therapeutics to prevent progression to refractory CeD is high.

TCR-like antibodies have been suggested as therapeutics for other indications and the first therapeutic based on a TCR-like antibody is reported to enter clinical trials in the US in the first half of this year [284]. While there has been a lot of interest in targeting cancer associated and viral antigens, relatively few studies describe therapeutic potential for autoimmune disease [152, 160, 161, 259, 285, 286].

Immunotherapy using monoclonal antibodies is considered more specific than conventional treatment. However, treating pemphigus vulgaris with rituximab depletes all B cells, not only the autoreactive ones. Similarly, treating rheumatoid arthritis with adalimumab, targets all inflammatory immune responses, not only the pathogenic ones. TCR-like antibodies offer a new level of precision by specifically targeting cells that present a self-peptide [160, 161] or cells that express a protein with a tumor-associated mutation [159]. A drawback of pMHC specific therapy is that it is only effective in patients with a matching HLA-haplotype. In paper 1 and 2, we chose to generate antibodies specific for HLA-DQ2.5, which is the single most predisposing genetic risk factor for CeD and expressed in approximately 90% of patients [174, 175]. Furthermore, T-cell response against the two immunodominant α-gliadin derived epitopes are observed in most patients (see 1.4). Thus, a potential therapeutic could treat the majority of patients, rather than a small subgroup only.

In paper 2, we demonstrated that the high affinity gluten pMHC specific antibodies

are able to specifically inhibit T-cell activation. A large number of gluten epitopes have been identified and CeD patients have CD4+ T-cell responses towards several of them [195, 197, 287]. Thus, it is unlikely that it is sufficient to block only T cells specific for the two α-gliadin derived epitopes targeted in this study. However, previous reports suggest that targeting one epitope with a TCR-like antibody may have an indirect effect on T-cell responses to other epitopes. Zhang et al. developed an antibody specific for an insulin derived autoantigen implicated in type 1 diabetes in complex with a murine MHC class II [161]. Their mAb287 antibody showed capacity to specifically inhibit T cells *in vitro* and delayed disease onset and progression when administered to a mouse model. Zhang and colleagues found a strong reduction of lymphocyte infiltration in the pancreatic islets of treated mice. Importantly, this was not limited to insulin specific CD4+ T cells, but also affected CD4+ and CD8+ T cells of different specificity as well as B cells. This suggests that potent inhibition of a single T-cell epitope may modulate the immune response to become more tolerogenic, which would be highly beneficial for the treatment of other autoimmune diseases, including CeD.

Different modes of action have been described for therapeutic antibodies that partly rely on their natural effector functions (e.g. CDC and ADCC) or other formats such as BiTEs, ADCs, or CAR T cells. All of these could be investigated for the pMHC specific antibodies. For most of them, the high affinity achieved in paper 2 will be beneficial. Only approaches involving CAR T cells may be hampered by the high affinities [163, 288, 289]. However, we have characterized several second generation clones as well as the mother clones, and therefore have a large panel of antibodies with high specificity and various affinities that could be employed.

5.4 The biased DQ2.5-glia-α2 specific T-cell response

The findings in paper 3, along with another recent study from our group [219], contribute to the understanding of how TCRs recognize pMHC, and how biased antigen specific T-cell repertoires are shaped. The CD4+ T-cell response to DQ2.5-glia-α2 has been analyzed carefully and is characterized by preferential usage and pairing of TCR chains encoded by *TRAV26-1* and *TRBV7-2* in combination with a canonical CDR3β loop (ASSx**R**xTDTQY). To understand the molecular details of this bias, we chose to dissect the interaction of the prototypic 364 TCR with

HLA-DQ2.5:DQ2.5-glia-α2. We identified a *TRAV26-1* encoded framework resident recognition motif centered on residue Y40$^{\mathrm{TCR}\alpha}$, that appears to anchor the 364 TCR onto the MHC β-chain and recruit the canonical CDR3β loop into an ideal position for peptide recognition [26, 219]. In paper 3, we fine-mapped this recognition motif and further demonstrated that it is conserved in a second prototypic TCR (S16) despite large sequence differences in the CDR3α loop. This suggests that this motif is a general characteristic of prototypic TCRs in the DQ2.5-glia-α2 response and may indeed be a driving factor for *TRAV26-1* overrepresentation in this context.

Interestingly, both in paper 2 and in paper 3, we observed a lower sensitivity of the S16 TCR compared to the 364 TCR. Both TCRs were reconstructed in two different cell lines and T-cell activation was assessed using different readouts, but the differences in sensitivity were consistently reproducible. This difference appears to be the result of the CDR3α loop contributions and suggests that this loop is able to modulate the interaction. This is further supported by co-crystal structures suggesting that, depending on their length, CDR3α loops in this context may be able to form direct interactions with the pMHC [26]. In previous reports, no recurrent motifs could be identified in the CDR3α loops of the prototypic DQ2.5-glia-α2 reactive TCRs and they varied in length and *TRAJ* gene usage [212]. However, analysis of a larger dataset may reveal patterns and possible restrictions in gene usage.

We then identified *TRAV* genes other than *TRAV26-1* that were found in combination with the prototypic TCR β-chain in the gluten specific CD4+ effector memory TCR repertoire of CeD patients. We chose *TRAV5* to assess if other germlines were able to use the same recognition motif for interaction with HLA-DQ2.5:DQ2.5-glia-α2. *TRAV5* has two out of the three residues, that are part of the proposed recognition motif, including the central Y40$^{\mathrm{TCR}\alpha}$. We reconstructed the *TRAV5* TCR based on a patient derived sequence and confirmed that it can be specifically activated by HLA-DQ2.5:DQ2.5-glia-α2. However, mutagenesis studies and structural modeling revealed that the *TRAV5* TCR uses a different binding mode which is largely independent of Y40$^{\mathrm{TCR}\alpha}$.

Even though the *TRAV5* TCR did not use the same recognition motif, it responded to stimulation with the cognate antigen with a sensitivity and specificity similar to the prototypic 364 TCR. Therefore, it is unclear why it is not more abundant in the antigen specific repertoire. Apart from antigen dependent selection, differences in precursor frequencies can contribute to the formation of a biased antigen specific repertoire. *TRAV5* gene segments were found at a similar frequency as

TRAV26-1 gene segments in the naïve repertoire [290, 291]. However, the prevalence of *TRAV5* in combination with the prototypic *TRBV7* gene segments is unclear. While there was no difference in pairing preference in a dataset reported by Howie et al. [291] (R. Neumann et al., unpublished), these combinations appeared to be rare in a dataset collected by Han et al. [292]. Thus, the frequency of such TCRs seems to vary across individuals and may be low in some. This is of importance, since pairing with the prototypic TCR β-chain appears crucial for recognition of the DQ2.5-glia-α2 epitope [212]. A low precursor frequency could be the result of negative selection in the thymus that may be affected by the HLA-haplotype of an individual [143]. Data about the naïve repertoire of HLA-DQ2.5+ individuals could illuminate to what extent precursor frequencies contribute to the observed bias. We observed epitope specific activation of the *TRAV5* BW T cells in response to plate bound recombinant pMHC and engineered A20 cells. However, in presence of HLA-DQ2.5+ Raji cells, *TRAV5* BW T cells secreted low levels of IL-2 independent of peptide or peptide concentration. Thus, this clonotype may be weakly self-reactive and a combination of central and peripheral tolerance mechanisms, may prevent higher abundance in the CD4+ effector memory T-cell repertoire [293–295].

The detailed characterization of the interaction between the prototypic TCRs and HLA-DQ2.5:DQ2.5-glia-α2 in paper 3, together with previous work [26, 219], appears to offer a molecular explanation for overrepresentation of *TRAV26-1* and the canonical CDR3β loop, specifically T115$^{\mathrm{TCR}\beta}$ encoded by *TRBJ2-3* (Fig. 5.1). However, it does not explain why *TRBV7-2* or *TRBV7-3* are required for recognition. *TRBV7-2* has been found to be strongly enriched and preferentially combined with the other prototypic features in the gliadin specific CD4+ T-cell response [212–214]. This was thought to be driven by the ability of *TRBV7-2* to harbor the canonical loop [219]. However, analysis of a large naïve paired TCR sequence dataset [291] revealed that the canonical loop is prevalent in combination with *TRBV* gene segments other than *TRBV7-2* or *TRBV7-3*. The canonical loop sequence (ASSx**R**xTDTQY) was found in 0.03% of all clonotypes in the naïve repertoire and only 20% of these canonical loops were part of a TCR β-chain that used *TRBV7-2* or *TRBV7-3* (R. Neumann et al., unpublished) [291]. This suggests that other interactions mediated by *TRBV7-2* germline residues, may contribute to drive their selection into the DQ2.5-glia-α2 reactive repertoire. The germline encoded CDR1 and CDR2 loops of *TRBV7-2* contribute little to binding in the S16 crystal structure [26] but these interactions may nevertheless favor them over other gene segments for binding the HLA-DQ2.5:DQ2.5-glia-α2 complex.

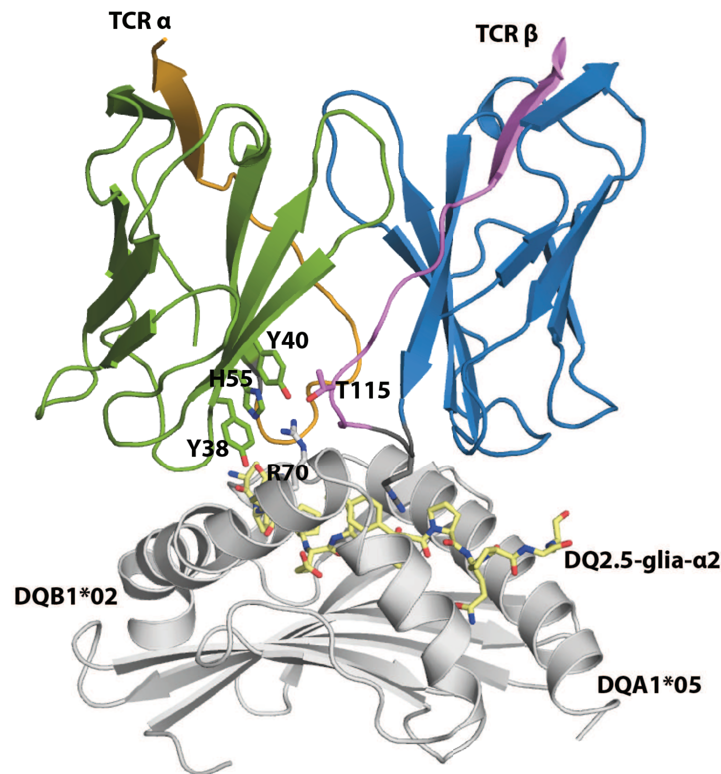

Figure 5.1: **Structural aspects of biased gene segment usage in S16 TCR [26]:** The co-crystal structure of the S16 TCR (*TRAV26-1* encoded: green, *TRAJ32* encoded: orange, *TRBV7-2* encoded: blue, *TRBJ2-3* encoded: purple) bound to HLA-DQ2.5:DQ2.5-glia-α2 (grey and yellow). The residues of the *TRAV26-1* encoded recognition motif and interacting residues of the TCR β-chain and the MHC β-chain are represented as sticks and annotated.

5.5 Future perspectives

The work presented in this thesis has contributed to an improved understanding of gluten antigen presentation and CD4+ T-cell activation in CeD. We have detected gluten pMHC complexes on small intestinal plasma cells in CeD patients and suggested a new possible role for plasma cells as APCs rather than antibody secreting cells. This has implications for our understanding of CeD pathogenesis and may point to plasma cells as a target for therapeutic intervention. We have demonstrated that plasma cells not only express gluten pMHC complexes, but also costimulatory molecules necessary for CD4+ T-cell activation. However, their ability to activate T cells remains to be confirmed. This is experimentally challenging as the number of pMHC+ plasma cells that can be sorted from human biopsy material for T-cell

activation experiments are low. Bead enrichment strategies or different model systems may offer solutions. A mouse model for CeD would be extremely beneficial to study antigen presentation *in vivo* and is currently being established (F. du Pré et al., submitted). The mice are transgenic for HLA-DQ2.5 and human TCRs specific for gliadin epitopes. Therefore, they would be ideal for studies of peptide presentation and T-cell activation.

We are also continuing studies to investigate the therapeutic potential of these antibodies. To this end, we will assess their capacity for ADCC or CDC to kill target cells. Since particularly the 3.C11 antibody has shown great promise for inhibition of T cells, it will also be interesting to assess its effect in the mouse model.

The method used for *in vitro* affinity maturation retrieved specific high affinity antibodies, which is the aim of many antibody engineering projects. The generation of docking models has supported the generation of targeted libraries and can be combined with different *in vitro* display technologies. This approach is expected to be applicable to any other affinity engineering project, with some knowledge regarding the binding site. Furthermore, the positive outcome of the affinity maturation selections supports previous findings from our group regarding the benefits of signal sequence free HV display on phage coat protein pIX and encourages its use for future selections.

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