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Characterizing TG2 autoantibody response in Celiac Disease

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Cycle XXVIII

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Characterizing TG2 autoantibody response in Celiac Disease

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By Szilvia Bakó

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ABSTRACT

Characterizing TG2 autoantibody response in Celiac Disease

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Background: Celiac Disease (CD) is a gluten sensitive enteropathy with autoimmune features, characterized by the destruction of intestinal epithelium and the production of IgA and IgG antibodies against gluten and self antigen tissue transglutaminase (TG2). Because of the fact that TG2 acts not only as key player in the disease but also as a self antigen studying the autoimmune response against this enzyme is crucial for understanding CD. Numerous studies aimed to investigate the antibody response due to the possible role anti TG2 antibodies play in the onset of the disease, however there are still many questions to be answered. Phage display antibody technology have been proved to be an efficient tool in the study of autoimmune diseases, and because of this we selected this method for dissecting the autoimmune response in CD.

Results: We were able to generate and classify antibodies in phage expressed scFv format first due to their specificity and second by competing them with reference antibodies. Antibodies were clustered into four Epitope groups, with great majority belonging to Epitope 1., the VH5 cluster. Antibodies representing each epitope cluster were analyzed in scFv-Fc format. It was shown that Epitope 1. antibodies recognize a region in TG2 molecule overlapping with fibronectin binding site while other epitope groups recognized a different region. Three out of four epitope clusters were able to stain cell surface TG2 (csTG2). Of the four epitope groups only Epitope 2. had inhibitory effect on TG2 enzymatic activity *in vitro*.

By reconstructing a VH5 antibody from IgG1 format into scFv format we generated two constructs with different VH/VL orders that retained specificity. The VH chain was substituted with Vk chains originating from three cDNA libraries generated from CD patients. The Vk usage was restricted IGKV1-5*03 F, IGKV1-39*01 F, IGKV1-12*01 F chains, IGKV1-39*01 being most abundant chain. Two construct with original VH/VL and four selected clones with Vk chains coming from cDNA libraries were characterized in scFv-Fc format. All clones were shown to recognize the region on TG2 overlapping with fibronectin binding site. Most but not all of scFv-Fcs recognized csTG2. None of the antibodies showed inhibitory effect on TG2 enzymatic activity corresponding to what we saw in the case of VH5 antibodies .

Conclusion: In this work we demonstrated that phage antibody display is an extraordinary tool to study the autoantibody response in CD. The generated antibodies in phage expressed scFv format were able to mimic the natural antibody response, and a straightforward reconstruction in scFv-Fc format allowed the precise analysis of the disease. The study not only provided an insight into the pathology of CD but also revealed the possibilities of a novel technology to analyze autoimmune diseases.

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INTRODUCTION

Chapter 1. Tissue transglutaminase

1. 1. Transglutaminases

Transglutaminases (TGases) are widely distributed group of enzymes catalyzing the post-translational modification of proteins through acyl transfer reactions involving γ-carboxamide group of peptide-bound glutamine and the ε-amino group of peptide-bound lysine, resulting in a ε-(γ-glutamyl) lysine isopeptide bond [1]. The cross linked products often of high molecular mass are highly resistant to proteolysis and their accumulation can be found in various tissues and processes where they play an important role. The term transglutaminase was first introduced by Clarke et al in 1957 [2] where they were describing the transamidating activity observed in guinea pig liver. Since these findings enzymes having TGase activity have been identified in micro organisms [3], plants [4], amphibians [5], fish [6], and birds [7]. In mammals nine TGase isoenzyme have been identified at the genomic level, however only eight have been characterized at protein level. All mammalian forms of TGases having structural homology are members of the papain -like superfamily of cysteine proteases [8], and the tissue content of them is tightly regulated at transcriptional level (Table1.1). Transglutaminase 2 (TG2) is the most abundant and studied member of the TGase enzyme family including TG1, TG3 and TG5 isoforms expressed in epithelial tissue, TG4 expressed in prostate gland, factor XIII expressed in blood, TG6 and TG7 most prominently distributed in the testis and lungs [9]. A further member of TGase family is band 4.2 an enzymatically inactive protein component of erythrocyte membrane [10]. The characteristics of each member of the family include:

- **Keratinocyte transglutaminase (TG1):** localized in squamous epithelia, keratinocytes and cytosolic membrane, activated by proteolysis, has a barrier function in stratified squamous epithelia and plays a central role in keratinocyte differentiation.

- **Tissue transglutaminase (TG2): e**xists in extracellular and intracellular form, ubiquitously distributed in various tissue types. Plays role in many biological processes including apoptosis, cell survival, signaling, cell differentiation, matrix stabilization, endocytosis and so forth.

- **Epidermal transglutaminase (TG3):** cytosolic protein, **r**equires proteolysis to become active and get involved in the terminal differentiation of keratinocytes, hair follicles.

- **Prostate transglutaminase (TG4): e**xpressed in prostate gland, prostatic fluids, extracellular. Plays key role in the fertility of rodents where it is involved in semen coagulation.

- **TG5:** Ubiquitously expressed cytosolic protein, predominant in female reproductive tissues and skeletal muscle, playing role in epidermal differentiation.

- **TG6:** Showing high homology with TG2, distributed in testis, lungs, brain involved in the development of central nervous system and late stage cell envelope formation in the epidermis and hair follicle.

- **TG7:** Ubiquitously expressed , prominent in testis and lungs.

- **Factor XIII:** Converted into the active TGase Factor XIIIa (plasma TGase) by a thrombin-dependent proteolysis, distributed in chondrocytes, platelets, astrocytes, macrophages and dendritic cells in the dermis. Involved in wound healing, blood clotting and bone growth.

- **erythrocyte protein band 4.2 (Band 4.2):** distributed in the surface of erythrocyte membranes, bone marrow, fetal liver and spleen. Key component of erythrocyte skeletal network, maintaining erythrocyte shape and mechanical properties.

Identified forms of Tgase	Synonyms	Residues (molecular mass in kDa)	Gene	Gene map locus	Prevalent function
Factor XIII A	Catalytic A subunit of Factor XIII found associated with B subunit in plasma as A2B2 heterotetramer. Fibrin stabilizing factor	732 (83)	F13A1	6p24-25	Blood clotting and wound healing
Type 1 Tgase	Keratinocyte Tgase	814 (90)	TGM1	14q11.2	Cell envelope formation in the differentiation of keratinocytes
Type 2 Tgase	Tissue Tgase	686 (80)	TGM2	20q11-12	Cell death and cell differentiation, matrix stabilization, adhesion protein
Type 3 Tgase	Epidermal Tgase	692 (77)	TGM3	20q11-12	Cell envelope formation during terminal differentiation of keratinocytes
Type 4 Tgase	Prostate Tgase	683 (77)	TGM4	3q21-22	Reproductive function involving semen coagulation particularly in rodents
Type 5 Tgase	Tgase X	719 (81)	TGM5	15q15.2	Epidermal differentiation
Type 6 Tgase	Tgase Y		TGM6	20q11 15	Not characterized
Type 7 Tgase	Tgase Z	710 (80)	TGM7	15q15.2	Not characterized

Table 1.1. Transglutaminases characterized at protein level [11]

1. 2. Tissue transglutaminase a multifunctional enzyme

Tissue transglutaminase (TG2) is the most ubiquitous and diverse member of the transglutaminase family with a great variety of biological functions. The human transglutaminase gene localized to the chromosome 20q11-12 is composed of 13 exons and spams 37kb.[12] The monomeric TG2 protein is composed of 687 amino acids, (MW ~ 78kDa) including four distinct domains. N terminal β-sandwich with the fibronectin and integral binding site, catalytic core containing the catalytic tridad Cys277-His335-Asp358 for the acyl- transfer reaction, a conserved Trp essential for catalytic activity [13] a Ca^{2+} binding region and two C terminal β-barrels, with β-barrel 1 containing the GTP binding site, while β-barrel 2 containing a phospholipase C binding sequence [14] (Figure 1.1.)

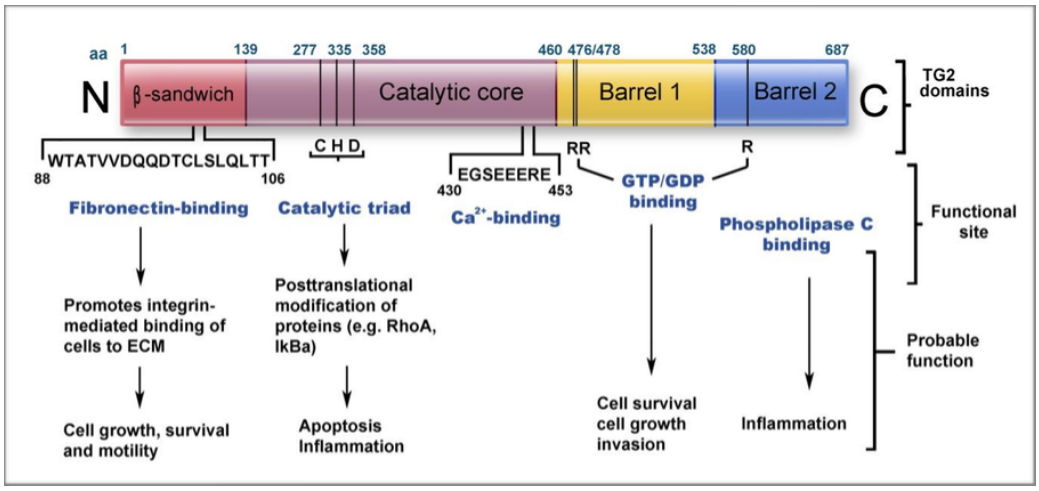

Figure 1.1. Structural and functional domains of TG2 [15]

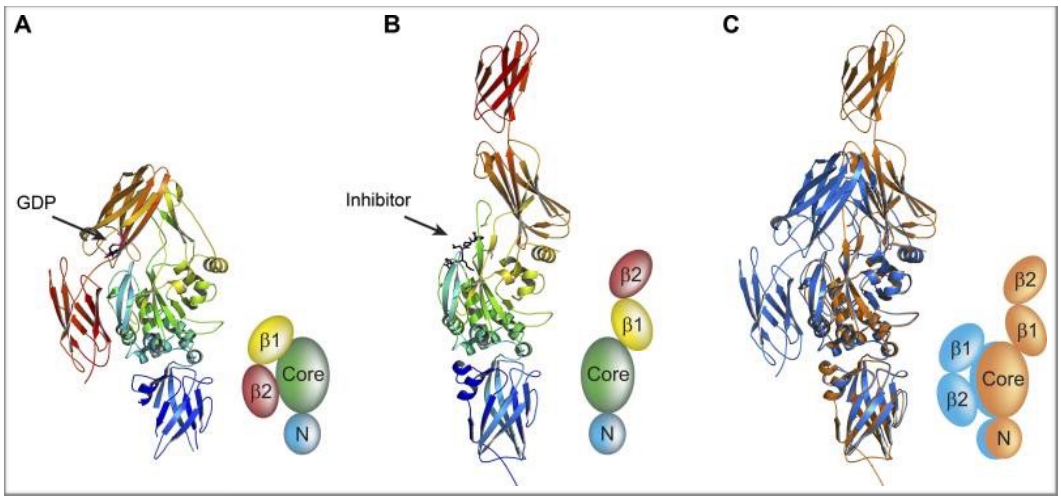

Figure 1.2. TG2 conformations. The N-terminal β-sandwich is shown in blue (N), the catalytic domain (Core) in green, and the C-terminal β-barrels (β1 and β2) in yellow and red, respectively. (A) GDP-bound TG2 [16]. (B) TG2 inhibited with the active-site inhibitor [17]. (C) The N-terminal β-sandwich and catalytic domains of the two structures are superimposed, highlighting the conformational change. The GDP-bound structure is shown in blue and the inhibitor-bound structure in gold.

In spite of the fact that TG2 was found with high expression in various tissues such as in endothelial cells, red blood cells and smooth muscle cells, it is still considered as a stress related protein, with its expression up regulated by physiological and pathological stimuli. Retionic acid (RA) is a well known inducer of TG2 expression both at mRNA and protein level [18]. A number of inflammatory cytokines and growth factors can induce TG2 expression such as transforming growth factor β1 (TGFβ1) [19], interleukins [20] and tumor necrosis factor α (TNFα) [21], thought to up regulate TG2 by activating nuclear factor κB (NF-κB). The activity of TG2 is tightly controlled within the intracellular environment, due to the presence of low Ca^{2+} and the inhibitory effects of GTP and GDP on its cross-linking ability [22]. In normal physiological conditions TG2 exists in a closed conformation where the active site of TG2 is hidden in a cleft preventing substrate binding and the C-terminal β-barrel domains tightly fold back for the catalytic core domain [23] (Figure 1.2.). Under stress, disturbance of the intracellular Ca^{2+} balance may cause transient loss of Ca^{2+} homeostasis activate TG2 into its catalytically active, open conformation. Ca^{2+} binding activates TG2 by inducing a conformational change increasing the inter domain distance between the catalytic core and the two C-terminal β-barrel domains up to 15nm. This results in the relaxation, widening of the whole molecule making the active site accessible for TG2 substrates [16, 17, 24]. In contrast, GTP binding likely stabilizes the closed conformation. Although GTP is considered to be a negative regulator of TGase activity, it has been indicated that the GTP binding is required to display transamidation activity of TG2 [25].

1. 3. Enzymatic functions of TG2

1. 3. 1. Transamidating activity

Two main biological functions of TG2 are transamidation and GTP-binding, with Ca^{2+} levels acting as a switch between them. When the transamidating activity is turned on the catalysis of post-translational modifications of proteins is hallmark of TG2 activity [26]. The two step process involves as a first step the formation of a thioester bond with the enzyme's active cysteine site via the transamination of γ-carboxamide group of a specific protein-bound glutamine substrate, accompanied by the release of ammonia. The second step includes the transfer of acyl intermediate to a nucleophilic substrate either the ε-amino group of a distinct protein-bound lysine residue [27] or primary amines such as polyamines and histamine. These bonds are resistant to chemical and physical degradation and are essential for stabilizing the ECM [28, 29]. Isopeptide bonds formed by TG2 also play a key role in apoptosis where they prevent inflammation by ensuring that intracellular contents of dying cells are not released to the extracellular environment [30]. Water can also act as a nucleophile causing deamidation where the acyl-donor glutamine residue is converted to a glutamate residue [31] (Figure 1.3.).

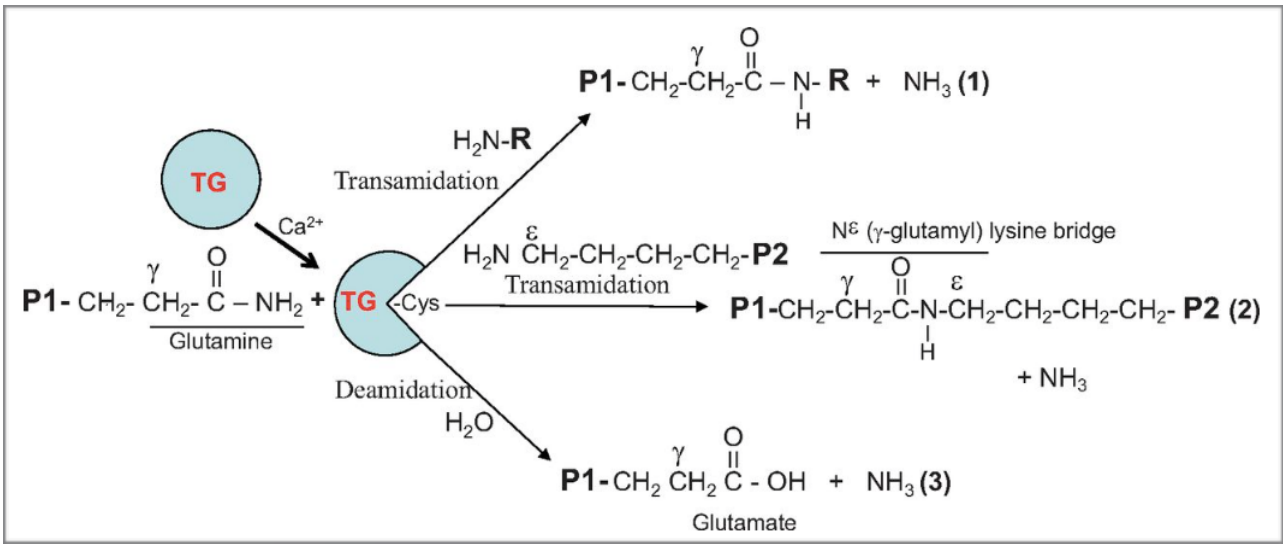

Figure 1.3. Enzymatically active TG2 catalyzes Ca^{2+}-dependent acyl-transfer reaction between γ-carboxamide group of a protein-bound glutamine and either the ε-amino group of a distinct protein-bound lysine residue (covalent protein cross-linking) or primary amines such as polyamines and histamine. Water can replace amine donor substrates, leading to deamidation of the recognized glutamines. [32].

1. 3. 2. GTPase activity of TG2

Under normal conditions in the intracellular environment the transamidating activity of TG2 is turned off due to the low level of calcium and the high level of GTP, inhibiting this function of the enzyme. TG2 is involved in transmembrane signaling by its GTPase activity combined with its ability to interact and signal through receptors [33-35]. TG2 was shown to interact with several receptors, facilitating hormone-receptor mediated transmembrane signaling pathways, involving the classical G-protein-coupled receptors.TG2 has been shown to directly interact with PLC δ1, a key player in the signal transduction process for many receptors [36]. A region of 12 amino acids between Leu^{661} and Lys^{672} in TG2 was identified as the PLC interaction site while the TG2 interaction site in PLC δ1 was shown to be located within the C2 domain of the enzyme [14, 37]. PLC δ1 not only serves as a guanine nucleotide exchange factor (GEF) but also as GTP hydrolysis inhibiting factor (GHIF) for TG2. These functions of PLC δ1 for TG2 facilitate TG2-mediated signaling. Other receptors, which interact with TG2 and mediate transmembrane signaling, are the thromboxane receptor [38] and the oxytocin receptor [39]. Both of the interactions are regulated by activation of PLC.

1. 3. 3. Pro and Anti-apoptotic effect of TG2

TG2 was reported to be involved in apoptosis [40] and it has been proposed that the cell type, the kind of stressor, intracellular localization, and transamidation activity of TG2 determine whether it promotes pro-apoptotic or anti- apoptotic responses [41]. The pro-apoptotic effect of TG2 is due to its cross linking activity, which requires millimolar concentration of calcium. Stressful conditions can generate reactive oxygen species (ROS) and also trigger the release of Ca^{2+} from the endoplasmic reticulum (ER), resulting in the activation of TG2 and cross linking of intracellular proteins initiating the apoptotic process [42, 43]. TG2 initiates apoptosis by mediating the crosstalk between dying and phagocytic cells and also ensures that once apoptosis initiated it is completed without inflammation or tissue injury [44]. TG2 can indirectly promote apoptosis by prompting the activation of TGF -ß release by macrophages, which can lead to the death of various cells [45, 46] . Additionally, TG2 can promote chemoattractant formation and the release of phosphatidylserin, to aid macrophage migration to the site of apoptosis and eventually the recognition of apoptotic cell [47].

TG2 may also have anti-apoptotic effect, which is independent from its transamidation and cross-linking activities. Nuclear TG2 protects cells by interacting with retinoblastoma protein pRb, polymerizing the alpha-inhibitory subunit of the transcription factor NF-kappaß, regulating the transcription of several anti-apoptotic genes [48]. Similarly TG2 can translocate to the plasma membrane where it serves as a co-receptor for integrin, promoting its interaction with fibronectin. A plausible scenario is that pro-apoptotic and anti-apoptotic effects of TG2 are dependent on the activation pathways and localization of the protein, with nuclear and extracellular TG2 as anti-apoptotic and cytosolic TG2 is pro-apoptotic [49].

1. 3. 4. PDI activity of TG2

TG2 was proposed to have protein disulphide isomerase (PDI) activity. PDI is a typical resident protein of the lumen of the endoplasmic reticulum (ER) on the surface of eukaryotic cells, catalyzing the formation, breakup, and exchange of disulphide bonds via cysteine residues in various proteins [50-52]. The PDI activity of TG2 was found to be independent from Ca^{2+} and GTP [53] but required free sulfhydryl groups of the protein for catalysis. Oxidants/antioxidants influence PDI activity, which is strongly amplified by oxidized glutathione but inhibited by its reduced form. These indicate that TG2 might be able to function as PDI in cytosol, where the majority of the enzyme is found in cells and where the concentrations of Ca^{2+} are very low and of nucleotides fairly high. The distribution of PDI is generally believed to be specific in the lumen of the ER, but there have been recent reports of its distribution in non-ER fractions including cytosol, nucleus and cell-surface fractions [54]. PDI function of TG2 was also supported by analysis of *TG2-/-* mice which had abnormalities in the mitochondrial respiratory chain and ATP production [55]. The underlying molecular mechanism may depend on defective disulphide bond formation in the ATP synthase complex and other components of the respiratory chain [56], including ADP/ATP transporter adenine nucleotide translocator (ANTI) which was incorrectly assembled and dysfunctional in the absence of PDI activity of mitochondrial TG2 [57].

1. 3. 5. Protein kinase activity of TG2

Evidence shows that cytosolic TG2 under specific physiological conditions can be translocated into the nucleus where it either crosslinks proteins or interact with them non-covalently [58]. The increase in Ca^{2+} concentration can result in the translocation of TG2 into the nucleus and subsequent increase in its transamidation activity [59]. Sequence analysis of TG2 showed the presence of a putative bipartite nuclear localization sequence (NLS) which indicates the active transport of TG2 to the nucleus [60]. The p53 oncoprotein was implied to be a substrate for the protein kinase activity of TG2 in the nucleus. TG2 induced phosphorylation was shown to interfere with Mdm2 binding suggesting that this mechanism can facilitate apoptosis [61]. Additional substrates of nuclear TG2 protein kinase activity include histones H1 and H3, indicating that TG2 night be involved in modulating the conformation of nucleosomes [62]. It is proposed that the interaction is involved in the condensation of chromatin in apoptotic nuclei [63]. TG2 was also reported to catalyze the cross-linking of polyamine binding proteins [64] and polyamines, involved in the modulation of chromatin structure and function [65]. Because putrescine, a polyamine, is incorporated by histones via a transglutaminase reaction, TG2 could contribute to polyamine-mediated chromatin condensation by direct interaction and covalent modification of histones.

1. 4. Functions of extracellular TG2

TG2 is predominantly localized within cells in the cytosol but can also be found in other cell compartments such as plasma membrane and the nucleus. TG2 is externalized from various cell types including fibroblasts, osteoblasts, endothelial cells, smooth muscle cells and monocytes/ macrophages [66, 67]. However it is not fully understood how TG2 is secreted as there are no secretory signal sequences and hydrophobic or transmembrane domains in the protein. Available data suggests that TG2 is externalized by an unconventional mechanism. A plausible scenario is that the unconventional secretion pathway of TG2 involves phospholipid dependent delivery into recycling endosomes [68] (Figure 1.4).

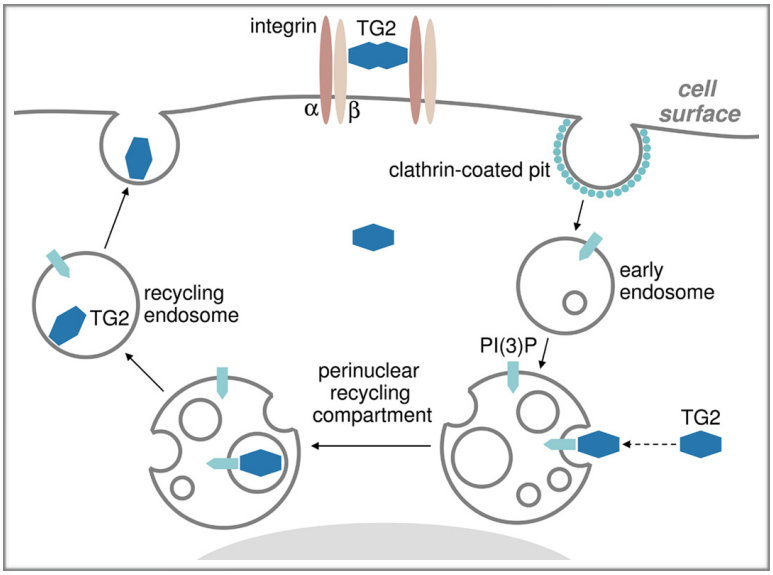

Figure 1.4 Unconventional secretion pathway of TG2 involving phospholipid-dependent delivery into recycling endosomes. [29]

The process occurs most likely in two steps where TG2 initially tethers to the endosomal phosphoinositides followed by binding the endosomal membrane through unidentified membrane proteins. Once secreted outside the cell TG2 promotes cell adhesion, migration and stabilization of the ECM by cross-linking several ECM proteins. A number of proteins are potential substates of TG2 cross-linking including fibronectin (FN), vitronectin, osteonectin, osteopontin, laminin, fibrillin, collagen I, collagen II, collagen V, collagen VI and XI. [69-71]. Collagen I can also be cross-linked by TG2 leading to high resistance to protease degradation and matrix turnover. TG2 cross-liked collagen shows low toxicity to cells providing an ideal biomaterial in wound healing, enhancing cell adhesion, proliferation and differentiation compared to native collagen [72] ECM and cell–matrix interactions are regulated by cross-linking of ECM proteins and also by proteolysis of them, where matrix metalloproteinases (MMPs) are playing a key role [73]. These proteinases, such as MT1-MMP, participate not only in ECM degradation, but also degradation of TG2 on tumor cell surfaces [74]. MT1-MMP is also activator of MMP2 [75, 76] which interact with the core domain of TG2 and direct its cleavage leading to the elimination of the catalytic and adhesion activity [77]. The degradation of TG2 by MMP2 plays a significant role in adhesion/migration related physiopathological conditions.

1. 5. Interacting partners of TG2

TG2 is a multifunctional enzyme with several substrates and interacting partners.Until now, 159 TG2 substrates, and 46 interaction partners, have been identified, according to TRANSDAB online database (http://genomics.dote.hu/wiki/). The two-third of the intracellular TG2 substrates in TRANSDAB are reported to be primarily in the cytoplasm, supporting the fact that TG2 is predominantly a cytoplasmic protein. STRING, the database of known and predicted protein interactions (http://string-db.org/), predicts over 100 possible protein interaction for TG2 with 50% confidence. Identified interacting partners of TG2 include proteins involved in interaction with ECM such as fibronectin and cell signaling as integrin α subunit and PLC-δ1 (Figure 1.5)

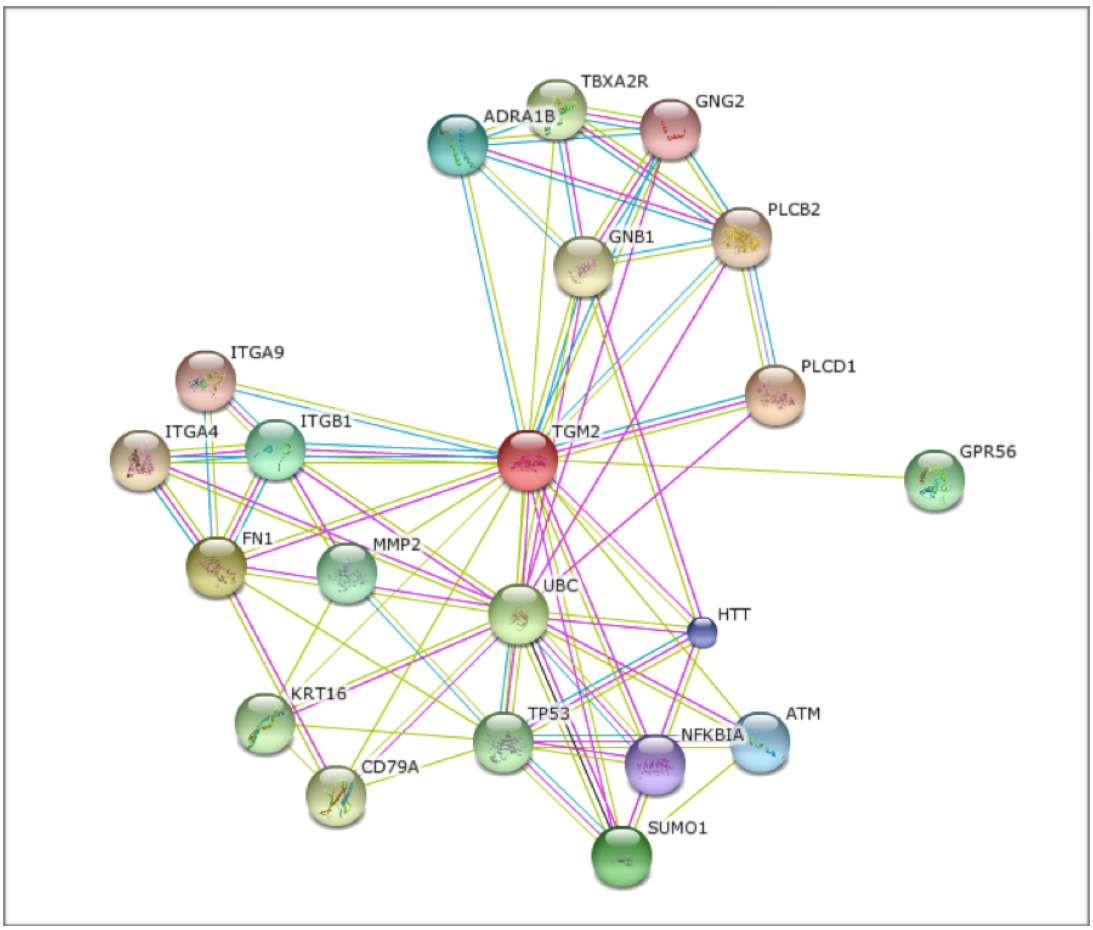

Figure 1.5. TG2 interacting protein network generated by STRING.

1. 5. 1. Fibronectin and TG2 interaction in the ECM

Fibronectin (FN) is a high molecular weight glycoprotein present in plasma and extracellular matrix (ECM) produced by hepatocytes and many other cell types [78, 79]. FN is a dimer of 230-250 kDa subunits joined by disulfide bonds in physiological conditions, consists of highly structural domains separated by flexible polypeptide segments. Each subunit is composed of three type of modules of which there are 12 type 1 (F1), two type 2 (F2) and 15-17 type 3 (F3) modules per subunits depending on splice variation (Figure 1.6) [78, 80]. These modules compose various functional domains, from the N-terminal there are heparin and fibrin-binding domain (30 kDa), collagen-binding domain (40 kDa), fibrin-binding domain (20 kDa), cell-binding domain (57 kDa), heparin-binding site (35 kDa) and fibrin-binding site (30 kDa). The domains are relatively resistant to proteases and contain the binding sites for macromolecules such as collagen, fibrinogen, fibrin, and proteoglycans, as well as cells [81].

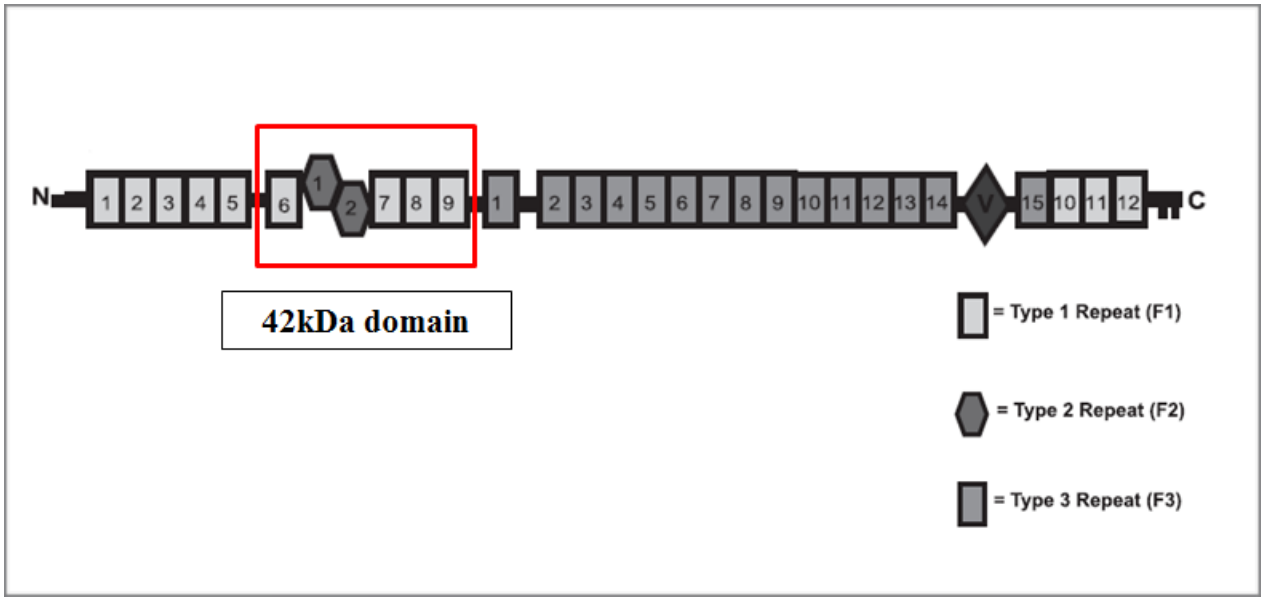

Figure 1.6. Structural domains of fibronectin molecule. The 42kDA domain responsible for TG2 binding is indicated in red. (modified from [82] **)**

FN has been reported to be a substrate for TG2 enzymatic activity and high molecular weight covalently cross-linked FN complexes may be formed by TG2 [83, 84]. However the formation of matrix fibrils does not require the enzyme's cross-linking function but depends on the binding to FN. TG2 binds FN with high affinity (K_d ~8nM) and 2:1 stoichiometry [85]. The binding involves the 42 kDa gelatin binding domain of FN consisting of modules $I_6II_{1,2}I_{7-9}$ [84, 86].

The N-terminal of TG2 is involved in the interaction with FN and the functional sequences required for the binding are located within the amino acids 89-140 of the protein [87]. The major functional sequence has been demonstrated that the β5/β6 hairpin of the first domain represents the major recognition site on TG2 molecule for the interaction with FN. This hairpin forms a prominent "finger" which is extended well beyond the globular first domain with its tip located aside from any other parts of TG2 molecule [16] making the β5/β6 hairpin well positioned for interaction with the large FN molecule. Surface TG2 on isolated hepatocytes and endothelial cells was shown to bind FN and mediate it's cross-linking into high molecular weight complexes [88]. Reduced expression of TG2 in endothelial cells led to an inhibition of the cross-linking of Fn [89] and, conversely, up-regulation of TG2 in Swiss 3T3 fibroblasts increased the amount of the cross-linked FN.

1. 5. 2. Other extracellular interacting partners of TG2

TG2 is involved in cell adhesion and migration by interacting with integrins mediating their association with FN which potentiates signaling. TG2 interacts with integrins through non covalent interaction involving the β1, β3 and β5 intern subunits of integrins [90, 91]. The exact mapping of integrin binding site on TG2 molecule has not been reported yet, however its most likely involves the first and fourth domains of TG2 whereas the TG2 binding site in integrins includes several membrane-proximal epidermal growth factor (EGF) like repeats of β subunit away from the FN binding site. Integrins are relatively low affinity receptors for ECM proteins including FN. In contrast TG2 binds high with affinity the 42 kDa fragment of FN, lacking the integrin binding site and this way creates additional binding sites between the two molecules.[90]. This potentially doubles the number of sites in the FN matrix that cells can access in adhesion and spreading. There are different models proposing the role of TG2 in cell adhesion (Figure 1.7). In one scenario TG2 serves merely as a bridge between integrin and FN. This strengthen adhesion because of the higher affinity and by allowing a second integrin molecule to access the FN chain. However there is also the possibility for even more stable ternary complexes where each protein interact with two other. Either scenario provides an explanation for increased cell adhesion and spreading on FN.

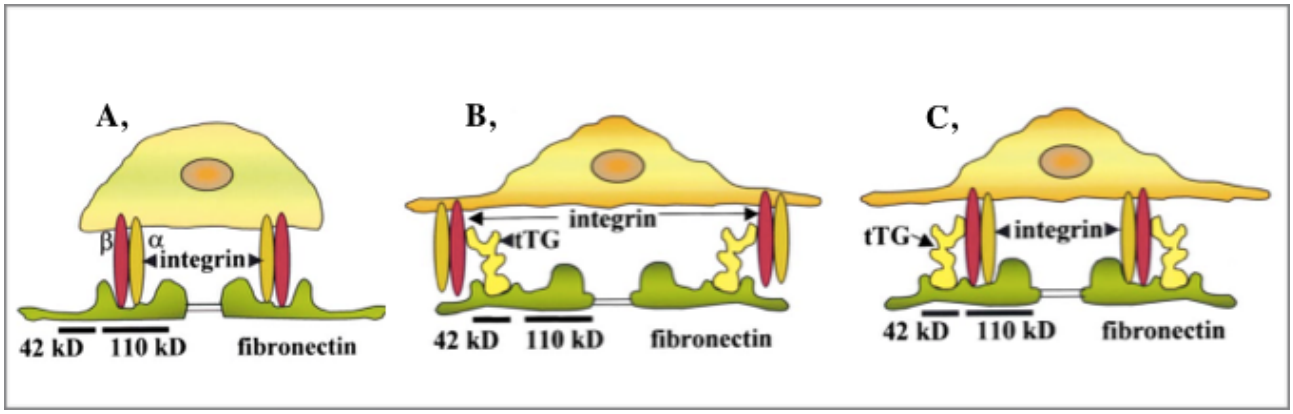

Figure 1.7. **Model proposing the role of TG2 in cell adhesion and spreading by forming ternary complexes with integrin and FN. (A)** Integrin-mediated adhesion to FN in the absence of TG2. **(B)** TG2 enhancing adhesion acting as a bridge between integrins and FN. **(C)** TG2 enhancing adhesion by mediating the formation of ternary complexes where all three proteins interact with each other. [90]

Increased expression of TG2 has been described in epithelial malignancies, specifically in ovarian, breast and pancreatic cancers [92-94]. TG2 has been linked to various functions in cancer but most importantly it acts as a promoter of chemotherapy resistance [95, 96] and a facilitator of metastasis [94, 97, 98]. It has been demonstrated that TG2 increases peritoneal metastasis [94, 98] and linked this process to β integrin mediated ovarian cancer cell adhesion to the peritoneal matrix. TG2 induces epithelial-to-mesenchymal transition (EMT) [98] which is a critical step in the initiation of metastasis and that the FN-binding domain of TG2 is sufficient to initiate this process [99, 100]. In addition, the TG2-mediated interaction between β integrin and FN activates cell survival pathways [90] and contributes to doxorubicin resistance in breast cancer cells [101], as well as cisplatin and dacarbazine resistance in melanoma cells [102]. Because of these findings TG2-FN interaction has become a potential target for cancer therapies. TG2 and FN interaction has been analyzed by AlphaLISA assay [103] and the application was used to study a ChemDiv library of chemical compounds leading to the discovery of potent TG2 inhibitors [104]. TG2-FN interactions characterized by a TG2 hairpin inserting a deep pocket of FN generating an attracting target for small molecular inhibitors, the most potent a diamino-pyrimidine derivate TG53 [104].

Another important binding partner of TG2 is the heparin sulphate proteoglycan, syndecan-4 (Figure 1.8). Syndecan-4 transmembrane component present together with integrins in focal adhesions where it interacts with the Hep-2 region of fibronectin. [105]. The high affinity interaction of TG2 and syndecan-4 is thought to maintain the activation of protein kinase Cα, important for controlling integrin levels and clustering throughout cell surface [106, 107]. Furthermore the fibronectin-TG2 heterocomplexes interact with syndecan-4 what may serve as a parallel adhesive/signaling platform cells may utilize in the case of integrin-deficiency [108, 109]. TG2 also reported to act as a scaffolding protein between platelet-derived growth factor receptor (PDGFR) and integrin, amplifying signaling from the membrane to the cell's interior this way stimulating cell adhesion and migration [110] (Figure 1.8).

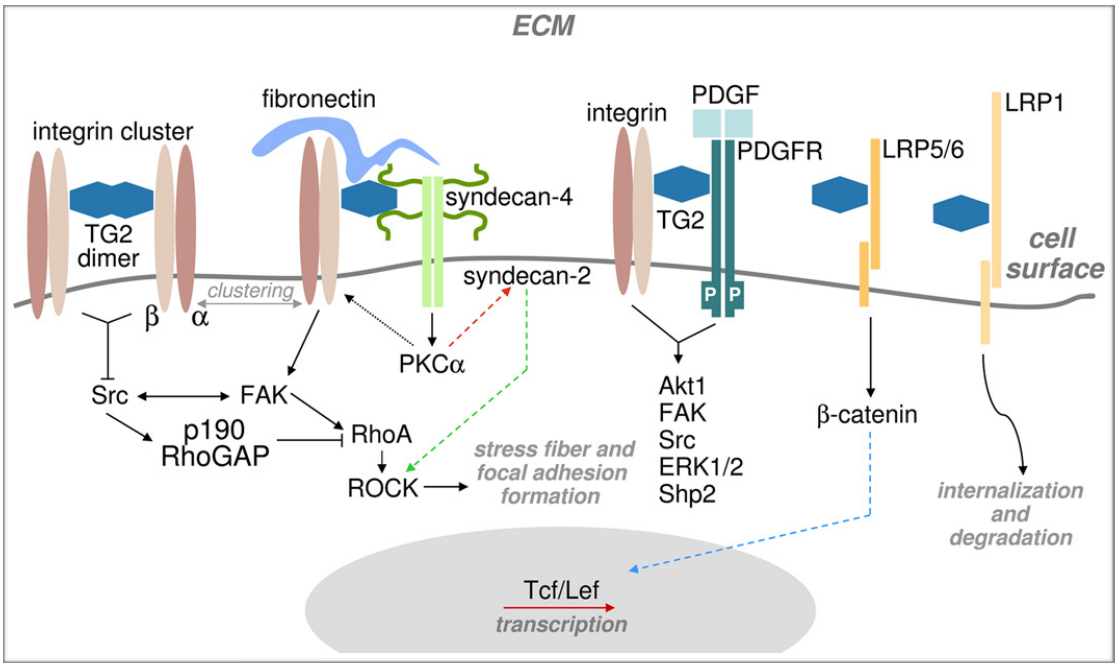

Figure 1.8 Types of TG2-containing adhesive/signaling complexes present on the cell surface involving main interactors of extracellular TG2. [29]

1. 6. Pathological implications of TG2

TG2 has been implicated in a wide variety of pathological states:neurodegenerative disorders (Huntington‟s, Alzheimer‟s), fibrosis and scarring, cancer and autoimmune disease such as Celiac disease. One of the most well known and studied pathological role of the enzyme is involved in Celiac disease, which will be discussed more detailed in the next chapter.

1. 6. 1. TG2 in neurodegenerative diseases

TG2 has been implied to be involved in several neurological diseases such as Huntington's disease [111, 112], Alzheimer's disease [113, 114] and Parkinson's disease [115].

Huntington's disease

Huntington's disease (HD) is a dominant, monogenic, neurodegenerative disorder affecting 1 in 10,000 individuals. The disease is characterized by progressive loss of gross motor skills, development of chorea (involuntary movements), subcortical dementia and emotional disturbance. The disease is caused by a mutation in the huntingtin gene, an expanded CAG repeat, which encodes an abnormally long polyglutamine (polyQ) repeat in the N-terminus of huntingtin protein. When the length of the polyQ domain exceeds 35-40 glutamines, the disease occurs. Despite the widespread expression of huntingtin, the brains of HD patients show selective neuronal loss in the striatum and the deep layers of the cerebral cortex. Aggregation of mutated huntingtin, transcriptional dysregulation, altered energy metabolism, impaired axonal transport and altered synaptic transmission culminate in neuronal dysfunction and cause death. Huntingtin was found to be primarily associated with microtubules as well as other huntingtin-associated proteins [116] [117]. TG2 was shown to co-localized with huntingtin and β-tubulin and it was proposed that microtubules could serve as binding site, bringing together TG2, huntingtin, and other TG2 substrates for TG2 dependent cross-linking [118]. Another protein reported to interact with TG2 and contribute to HD pathology was a calcium binding protein, calmodulin. Calmodulin was found to co-localize with TG2 and mutant huntingtin in the intranuclear inclusions in the HD brain. It has been shown that calmodulin is might increase TG2 activity by regulating calcium concentration [119].

Because mutant huntingtin interacts with both TG2 and calmodulin in HD brain, it was suggested that huntingtin might be also involved in increasing TG2 activity and subsequently its own cross-linking by bringing calmodulin and TG2 in close proximity. Thus, calmodulin indirectly regulates TG2-mediated cross-linking of huntingtin and formation of stable aggregates and inclusions in HD [120].

Alzheimer's disease

Alzheimer‟s disease (AD) is the most common age-related neurodegenerative disorder, associated with the selective damage of brain regions and neural circuits, including neurons in the neocortex, hippocampus, and amygdala. Dysfunction and loss of neurons in these neural circuits results in impaired memory, thinking and behavior. Many factors likely involved in the pathogenesis of AD, like traumatic brain injury [121], aging [122], inflammation [123], ischemic damage [124] and brain stress [125] overly induce TG2 expression and/or activity. AD is characterized by pathological lesions such as intraneuronal neurofibrillary tangles (NFTs), extracellular senile plaques and cerebral amyloid angiopathy. Major component of neurofibrillary tangles is aggregated hyperphosphorylated tau protein [126], whereas senile plaques and cerebral amyloid angiopathy largely consist of aggregated amyloid beta (Aβ) peptide [127]. Conformational changes of both Aβ [128] and tau [129] may lead to their aggregation. In addition, both of these proteins are particularly neurotoxic when in such an aggregated state [130]. It has been hypothesized that TG2 may be involved in the pathogenesis of AD by facilitating the formation of one or both of these insoluble lesions. Senile plaques contain amyloid fibrils composed of the Aβ, and it has been shown that TG2 can cross-link Aβ1-28 [131], Aβ1-42 [132] and APP [133]. It has been demonstrated that TG2 induces monomeric Aβ to rapidly form protease-resistant oligomers and aggregates in a time- and concentration-dependent manner similar to self-assembly, and lowers the concentration for Aβ oligomerization, so it can occur at physiological Aβ levels [134]. Tau protein is an excellent TG2 substrate both *in vitro* and *in vivo* [135] and Tau protein cross-linking catalyzed by TG2 has been confirmed in tau transgenic mice that develop neurofibrillary tangles and have cross-linked tau protein [136].

1. 6. 2. TG2 in fibrosis and scarring

TG2 is involved in many pathological conditions where it is thought that the wound-healing response does not process normally. Chronic stress resulting in fibrotic disease is caused by an excess of ECM, cross-linked by TG2 [137-139]. In addition to causing increased matrix deposition by activating TGFβ1, TG2 cross-linking in the ECM has been shown to result in a reduction of matrix turnover leading to net deposition and accumulation [140]. TG2 has also been reported to be responsible for the progression and stabilization of atherosclerotic plaques, where it prevents plaque rupture with it's cross-linking activity [141]. Hypertropic scarring, characterized by excessive collagen deposition by prolonged activation of myofibroblasts, also involves TG2 [142], in a process that can be prevented by applying TG2 inhibitors [143]. The importance of TG2 in renal fibrosis has been highlighted by the beneficial effects of membrane soluble irreversible TG2 inhibitor R283 or membrane-impermeable irreversible TG2 inhibitor R281 [144] caused in this condition. Targeting extracellular TG2 with membrane-impermeable inhibitors did not affect the transcription of major ECM proteins or MMP-1 nor the activity of TGFβ1 suggesting that the cross linking activity of the enzyme on the ECM was responsible for the effect [145].

1. 6. 3. TG2 in cancer

The ability of malignant cells to proliferate and invade surrounding tissues is characterized by insensitivity to growth signals and resistance to apoptotic cell death, in which the GTP binding and transamidating activity of TG2 have potential role [146]. Over expression of TG2 in malignant hamster fibrosarcoma cells led to delayed progression from S-phase to G_2/M [34] supporting that reduced TG2 activity is associated with tumor growth and metastasis [147]. In spite of the fact that intracellular TG2 has suggested to have pro- and anti-apoptotic effect, the increased TG2 expression has been correlated with increased apoptotic index in human breast carcinomas [148]. Cytosolic TG2 can be anti-apoptotic by activating NF kappa B pathway [92]. In the extracellular environment TG2/fibronectin is also associated with cell survival via interaction with integrins [149] and syndecan 4 [110]. It has been reported that MT-MMPs regulate cancer cell attachment and motion by digesting cell surface TG2. While the digestion of cell surface TG2 suppressed cell adhesion and locomotion on fibronectin, the interaction of fibronectin and TG2 protected cell surface TG2 from digestion, this way prompting cell adhesion and locomotion [74].

The effect of exogenously added TG2 to angiogenesis models demonstrated that there was an accumulation of ECM proteins without causing an increase in cell death [150]. It has also been implied that increase in matrix rigidity caused by TG2 cross-linking is responsible for alteration in cell behavior in mouse model with CT26 colon carcinoma. In a study involving 200 cases of human breast cancer, an increased TG2 level in the stroma surrounding the tumor was associated reduction in lymph node metastasis [151]. Increase TG2 expression has also been associated with the development of drug resistance and metastatic phenotype [152, 153]. Aberrant expression of TG2 in breast cancer has been linked to the fact that *TGM2* gene is among the selected genes whose expression is altered by hypo- and hypermethylation in drug resistant breast cancer cells [154]. TG2 has been implied to induced epithelial-to-mesenchymal transition which associated with metastatic spread and poor disease outcome in patients with breast cancer [155] and malignant pleural mesothelioma.

1. 7. Inhibition of TG2

TG2 is implicated in a range of pathologies, demonstrating a need for potent TG2-specific inhibitors. The pathogenic role of TG2 is primarily linked to its cross-linking and deamidation activity, while the use of inhibitory molecules in biological systems gave promising results in a number of disease models, proposing a potential use for therapeutic treatment of human diseases.
Upon their mechanism of inhibition, TG2 inhibitors can be divided into two classes: reversible inhibitors and irreversible inhibitors.

1. 7. 1. Reversible TG2 inhibitors

Reversible TG2 inhibitors prevent TG2 activity by blocking substrate access to the active site without covalently modifying the enzyme. In the early studies aiming to inhibit TG2, primary amines were used [156, 157], which inhibit the native function of the enzyme by competing substrates in the cross-inking reaction. Zn^{2+} as a bivalent cation was also used as an early reversible inhibitor for competing Ca^{2+} for the binding sites [158, 159]. Cystamines are inhibitors, capable of competitive amine inhibition [160].

It was also shown that cystamines able to inactivate TG2 in time-dependent manner by forming mixed disulphide bonds between TG2 active site cysteine and cystamine [161-163]. Despite their lack of selectivity, particularly when applied in biological settings [164], cystamines are still widely used inhibitors. Recently used reversible inhibitors may also be competitive to the acyl-donor substrate. Cinnamoyl inhibitors such as trans-cinnamoyl derivates [165], cinnamoyl benzotriazolyl amides [166], and azachaltones were shown to be potent inhibitors (Figure 1.9). These inhibitors were shown to be competitive in respect to the donor substrate, supporting that inhibitors bind to the hydrophobic groove of the acyl-donor binding site, this way blocking the substrate access to the active site. Acylideneoxoindoles [167] are another scaffold of reversible TG2 inhibitors based on the structure of isatin, whose analogs are widely used as reversible inhibitors of Cys-dependent proteases [168]. Another examples for reversible inhibitors are TG2 cofactors, such as GTP and GDP; GTP analogues, such as GTPγS and GMP-PCP [169].

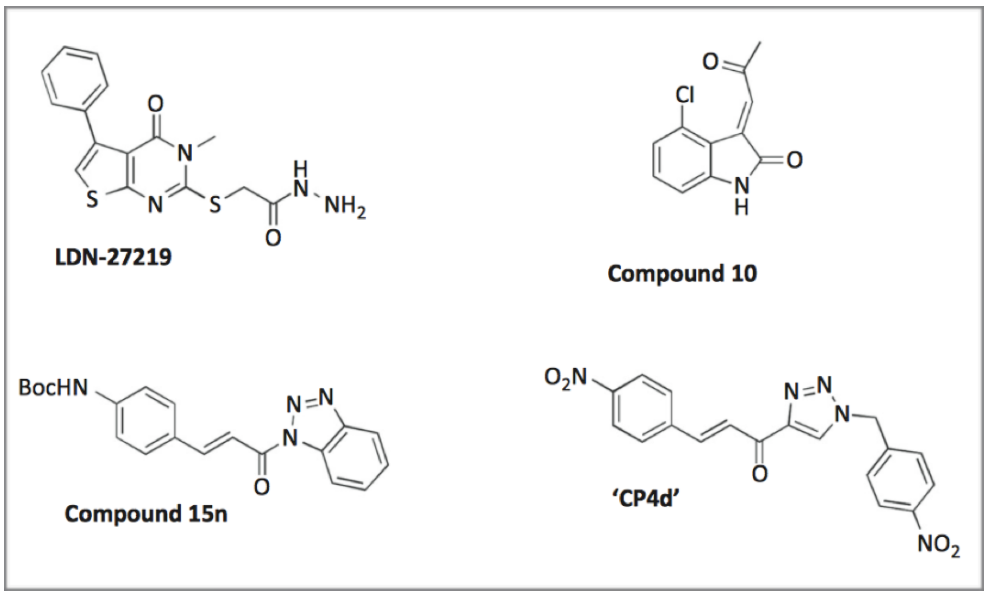

Figure 1.9. Earliest reversible (allosteric) inhibitor **LDN-27219**, cynnamoyl based reversible inhibitor **Cp4d, Compound 15n,** acylideneoxoindole based reversible inhibitor **Compound 10. modified from [170]**

1. 7. 2. Irreversible TG2 inhibitors

Over the course of research many irreversible inhibitors have been developed against TG2. Certain electrophilic functional groups that react with the active site nucleophile of the enzyme have emerged as privileged warheads. The most portent irreversible inhibitors can be sorted by the class of warhead. Halomethyl carbonyl inhibitors, such as iodoacetamide, were one of the first irreversible inhibitors tested on TG2, when it was shown that was able to inactivate guinea pig liver TG2 [159]. More recently chloromethyl ketones were generated [171], based on a Cbz-Phe scaffold [172] (Figure 1.10). 3-Halo-4,5-dihydroisoxazole inhibitors are another group of irreversible inhibitors. Acivicin, a natural product of Gln isostere is known to inhibit several cysteine-dependent enzymes [173]. By incorporating its 3-halo-4,5-dihydroisoxazole warhead into a Cbz-Phe dipeptide scaffold, irreversible inhibitors against TG2 were made [174]. More recently a large series of 3-bromo analogs were synthesized [175], with he most potent ones optimized with respect to stereochemical configuration, aromatic side chain and *N*-terminal carbamoyl group [176]. These compounds were evaluated *in vivo* and showed good oral bioavailability and efficient extracellular TG2 inhibition in small intestinal tissue with toxicity [175].

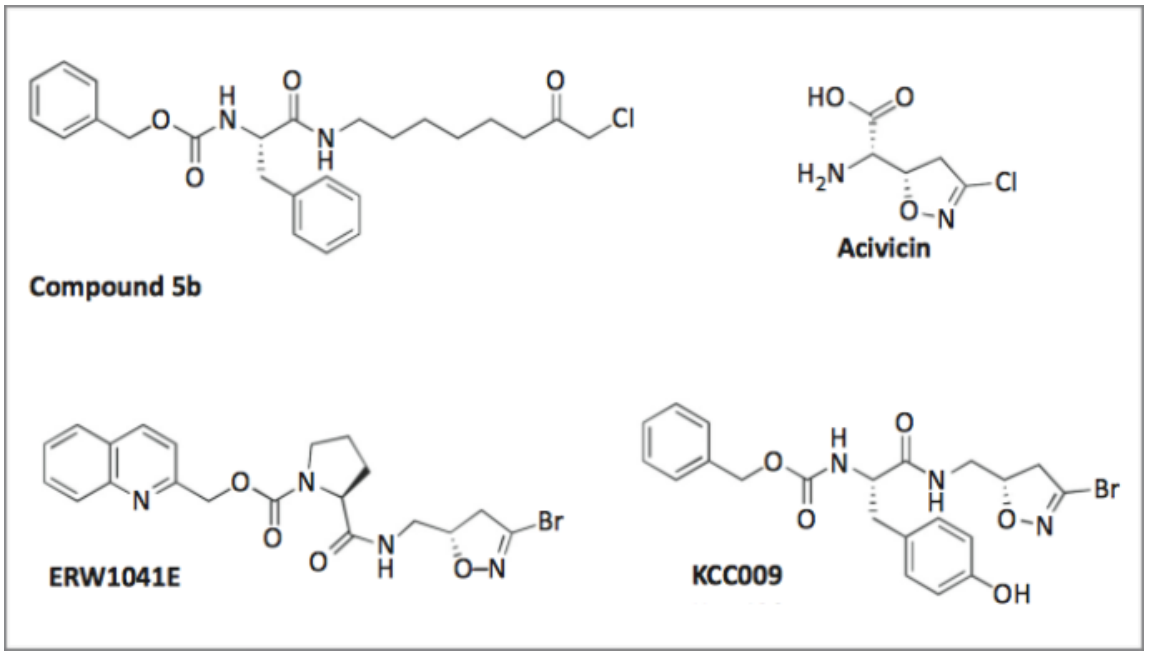

Figure 1.10. Chloromethyl ketone based inhibitor **Compound 5b**, dhydroisooxazole-based inhibitor **KCC009, ERW1041E. Avicidin modified from [170].**

A quinolyl carbamate functionalized (S)-proline compound was also developed, based on a TG2 specific gluten derived peptide sequence (PQPQPLY) [176] and found to inhibit small intestine TG2 activity when tested in a mouse model [177]. Michael acceptors are α,β-unsaturated carbonyl derivates that undergo 1,4,-addition reactions. Michael acceptors have been used to inhibit cysteine protease [178], some going for human clinical trials for cancer [179]. Irreversible inhibitors with acrylamide warhead [171] or acrylamide group on the side chain [180] of the dipeptide scaffold were generated and proved to be excellent inhibitors of TG2. Sulfonium inhibitors are bearing a dimethyl sulfonium warhead and reported to inactivate epidermal transglutaminase and TG2 [181]. The design of these inhibitors was based on the familiar Cbz-Phe scaffold, to which electrophilic warhead was attached via a spacer of varied length. A derivate was prepared, bearing an extra carboxyl group for enhanced aqueous solubility [144]. These inhibitors which are predominantly negatively charged at physiological pH (due to the added carboxylate group), are less favored to enter the cell membrane and, therefore rather target extracellular TG2.

Chapter 2. Celiac disease

Celiac disease (CD) is a gluten-sensitive enteropathy that develops in genetically susceptible individuals by exposure to cereal gluten proteins. The first description of CD was found in the first and second centuries where CD was described as an intestinal disorder associated with diarrhea and malabsorption occurring in children and adults. The idea that the disease was linked to food ingestion was brought forward in 1888, confirmed in 1950s when Dicke and colleagues established that consumption of wheat and rye brought CD and removing these grains from the diet resulted in the improvement of the patients condition. In 1954 Paulley [182] reported that the clinical manifestation of CD is linked to the destruction of the lining of small intestine.

It has been revealed that the expression of CD is strictly dependent on dietary exposure to gluten and similar cereal proteins [183]. Gluten is the name of wheat proteins only, but it is increasingly used to describe proteins of wheat, rye and barley that are rich in proline (Pro) and glutamine (Gln) residues. Of gluten proteins, both gliadins (alcohol soluble) and glutenins (alcohol insoluble) are harmful [184]. CD can occur at all ages following the introduction of gluten to the diet. Similarly to most autoimmune disorders, CD is more frequently (twice as often) found in women than in men [185]. CD is primarily the disease of caucasians, affecting 1% of the population [186], most frequently recognized among Europeans. The disease exhibits a very strong HLA association, in which the relative risk to the disease development for carriers of certain alleles is increased 30 fold [187]. Susceptibility is strongly associated with MHC class II molecules HLA-DQ2 and HLA-DQ8 [188, 189] and the immune response directed against specific gluten antigens leads to the destruction of intestinal epithelial cells. CD commonly present in early childhood with classic symptoms including chronic diarrhea, abdominal distension and failure to thrive [190]. The disease may also present later in life with symptoms that tend to be more vague, including anemia, fatigue, weight loss, diarrhea, constipation and neurological symptoms [191]. The celiac lesion is localized in the proximal part of the small intestine. The alterations such as villous atrophy, crypt cell hyperplasia, lymphocytic infiltration of the epithelium and increased density of various leukocytes in the lamina propria characterize one end of a spectrum that has classified into three stages: the infiltrative, hyperplastic and the destructive lesions [170]. The infiltrative lesion is characterized by the infiltration of small non mitotic lymphocytes in the villous epithelium without any signs of mucosal pathology.

The hyperplastic lesion is similar to the infiltrate lesion but in addition has hypertrophic crypts whose epithelium may be infiltrated with lymphocytes. The destructive lesion is synonymous to the classic lesion of CD. Oral challenge experiments with gluten revealed that these stages are dynamically related [192]. Patients with CD typically also develop antibodies against gluten and for the endogenous enzyme tissue transglutaminase. Despite the fact that gluten is the single causative agent, CD can be viewed as an organ-specific autoimmune disease.

2. 1. Antigen presentation in Celiac disease

Both genetic and environmental factors contribute to the development of the disease. CD is a polygenic disorder with involvement of many genes but the HLA locus encoding human major histocompatibility complex (MHC) molecules is far the most important genetic factor contributing about 40% of the genetic variance of the disease [193-196]. The primary HLA association for CD is conferred by class II HLA-DQ genes. Approximately 90% of CD patients express HLA-DQ2.5 molecule encoded by DQA1*05/DQB1*02 genes, and the majority of the remaining patients express HLA-DQ8, encoded by DQA1*03/DQB1*03:02 genes [188, 197]. If genes encoding the α- and the β-chains of the HLA-DQ2.5 heterodimer are carried on the same chromosome (in cis position), they are most often found as part of the highly conserved A1-B8-DR3-DQ2 haplotype. This particular haplotype is associated with several autoimmune diseases such as type 1 diabetes and myasthenia gravis and because of this, sometimes referred to as the "autoimmune haplotype" [187]. Patients that are DR5-DQ7/DR7-DQ2 heterozygous also express the HLA-DQ2.5 molecules, but then the two encoding genes are carried on different chromosomes (in trans position). The relevant DQA1 allele (DQA1*05) is encoded by the DR5- DQ7 haplotype and the relevant DQB1 allele (DQB1*02) is encoded by the DR7-DQ2 haplotype. There have been 39 non-HLA loci associated with CD so far, estimated to contribute about 14% of the genetic variance in the disease [194, 195]. The relative contribution of each of these genes is minor compared to the HLA genes. Many of the non-HLA polymorphisms seem to act by influencing gene expression [195]. Furthermore, many non-HLA CD risk loci are shared with other immune-related diseases, such as type 1 diabetes and rheumatoid arthritis [198-201]. The shared genetic background among these diseases points to common pathogenic pathways in them [202]. In CD different HLA types associated with the disease have been shown to bind proline rich gluten peptides that harbor glutamic acid, however all HLA types have distinct peptide binding preferences (Figure 1.11.).

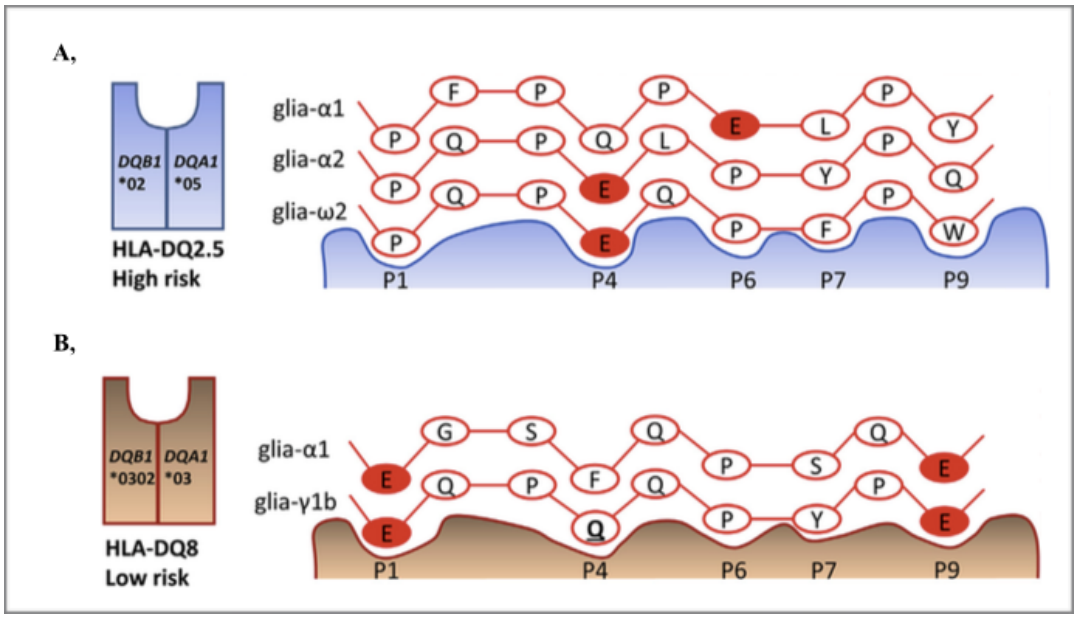

Figure 1.11. HLA-DQ2.5 and HLA-DQ2.8 present different type of gluten peptides. HLA molecules bind proline-rich gluten peptides that harbor glutamic acid residues **(E)**. **A.** HLA-DQ2.5 presents a high risk for CD and bind peptides deamidated at positions P4 and P6. **B.** HLA-DQ8 low risk variant prefers binding gluten peptides with deamidation at position P1 or P9. [203]

Gluten peptides have preferred binding to DQ2.5 molecules compared to other HLA class II molecules [204]. Peptide binding studies have shown that contact between DQ2.5/DQ8 and the side chains of the peptides, so-called anchor residue and pocket interactions, takes place at the P1, P4, P6, P7, and P9 position [205-208]. Peptides that bind HLA-DQ2.5 preferentially have deamidation at positions P4, P6, and occasionally P7, whereas peptides binding HLA-DQ8 have negatively charged residues at P1and/or P9 [209]. To date, more than 15 different gluten-derived T-cell epitopes have been reported in CD [210-212] and all contain a deamidated residue in at least one of these pocket positions. The great majority of these T-cell epitopes contain glutamine residues that are targeted by TG2 and converted into glutamate residues by a deamidation reaction. In general T cells of celiac disease patients recognize deamidated epitopes with greater efficiency than native ones. This effect of PTM appears to be stronger for HLA-DQ2.5 restricted epitopes than for HLA-DQ8 restricted epitopes, as many of the HLA-DQ2.5 restricted epitopes are not recognized in their native form whereas some HLA-DQ8 restricted epitopes are equally well recognized as native peptides [213].

There is a correlation between how frequently T-cell epitopes are recognized by CD patients and their propensity to be targeted as substrates for TG2 [214] (Figure 1.12.). The idea that TG2 is one of the most important factor selecting gluten T-cell epitopes is supported by an experiment where TG2 was used to select peptides from a proteolytic digest of gluten containing several thousand different peptides [215]. Peptides, which were targeted by TG2, were purified and sequenced. Strikingly, of 31 selected peptides, more than 75% contained celiac disease related T-cell epitopes. The negative charges introduced by the TG2-mediated deamidation increase the binding affinity of gluten peptides to HLA-DQ2.5 and HLA-DQ8. Majority of extracellular TG2 is inactive, however it can be transiently activated by certain types of inflammation or injury signals [216].

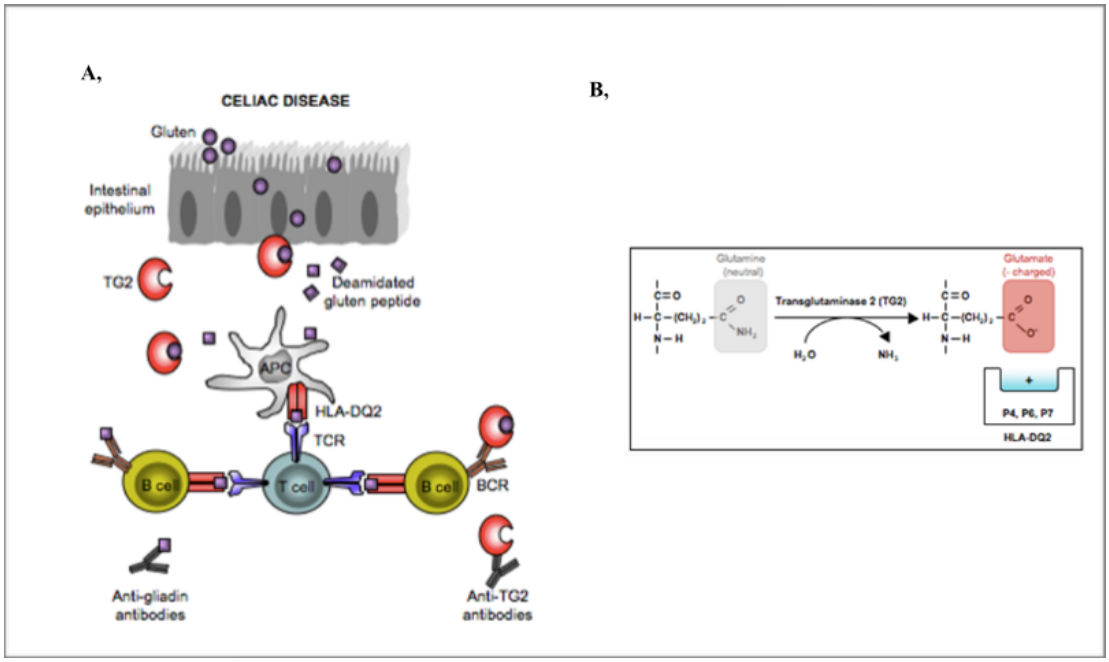

Figure 1.12. Pathogenesis in celiac disease. **A,** TG2 mediates the post-translational modifications of gluten peptides which in turn are presented on the surface of HLA molecules to T cells. In turn, the generation of antibodies start against gluten and TG2. **B,** TG2 mediated deamidation turns the protein bound, neutral glutamine into negatively charged glutamate which can be presented on the surface of HLA molecules. modified from [217]

The enzymatic activity of TG2 is found to be tightly regulated by the redox potential of the environment, mediated by a redox-sensitive cysteine triad consisting of Cys^{230}, Cys^{370}, and Cys^{371} [218]. TG2 has been shown to reversibly inactivated by oxidation, however Ca^{2+} can protect the enzyme from oxidation and inactivation.

In a model of TG2 activation, TG2 is released extracellular matrix upon cell wounding and remains catalytically active for a short period of time but then becomes silenced through oxidation. A change in the reductive environment may rescue TG2 from inactive state as it happens when ongoing immune reactions alter the redox state of lymphoid tissue. The thiol content is increased after antigen stimulation, particularly in mesenteric lymph nodes after intraperitoneal immunization [219]. An ongoing immune response, including those directed against infectious agents, may thus facilitate TG2 activity and an immune response to deamidated gluten. Because of its high Pro content gliadin is remarkably resistant to luminal and brush border proteolysis and large fragments remain intact after digestion. The most illustrative fragment is the 33-mer produced by the digestions of certain α-gliadin proteins. The 33mer fragment remains intact even after extended incubation with gastric, pancreatic, and intestinal brush-border membrane enzymes [220]. It contains six overlapping copies of two different DQ2.5-restricted T-cell epitopes and is recognized by T-cell lines from nearly all adult CD patients. The two epitopes contained within this 33mer peptide, the DQ2. 5-glia-α1 and DQ2.5-glia-α2 epitopes, are often referred to as the dominant T-cell epitopes in CD. Intriguingly, the 33mer peptide can bind DQ2.5 molecules directly on the surface of APCs and can thus be presented to T cells without further intracellular processing [221]. The gliadin-derived epitopes cluster in the Pro-rich regions of gliadin, a property that both confers relative proteolytic resistance and favors TG2-mediated deamidation [210].

The adaptive immune response is initiated by APCs, primarily dendritic cells (DCs) but also macrophages and B cell subsets, which present to T-cell antigenic fragments in complex with cell surface MHC class II molecules. This antigen priming usually takes place in organized lymphoid tissues. APCs travel from peripheral tissues to lymph nodes where they generate effector or tolerogenic T cells that operate in the peripheral tissues. The re-activation of effector and tolerogenic T cells in peripheral tissues will again require interaction with APCs. Antigen presentation is a key initial step during the pathogenesis of CD both in mesenteric lymph nodes and the intestinal lamina propria.

Macrophages are tissue-resident cells that differentiate from circulating monocytes [222, 223]. These cells are long-lived with low turnover in the tissue. They occupy the cellular zone beneath the enterocytes and also can be found scattered in the lamina propria. Human jejunal macrophages have been reported to have a decreased capacity to produce cytokines and do not express receptors typical for innate immune responses.

The role of activated macrophages in CD is elusive, since they were not found to be effective presenters of gluten peptides to gluten reactive T-cell clones [224]. However activated macrophages may play role in the pathogenesis by producing cytokines. In contrast to macrophages DCs are messenger cells with a rapid turnover. Precursors of DCs have not yet been described in the human blood, but three distinct cell subsets may generate tissue DCs: monocytes, classical myeloid DCs, and plasmacytoid DCs.

The two main subgroups of DCs, myeloid and plasmacytoid DCs, are characterized respectively by their abilities to prime naïve T cells and to secrete large amounts of IFN-α in response to viral infections. In the lamina propria of patients with CD, the density of classical myeloid DCs is reduced [225]. Lower tissue density may be due to inflammation-induced expansion of the tissue volume. Another plausible scenario is that genuine cell depletion is due to migration to lymph nodes, diminished recruitment, cell death, or a combination of these events. Human plasmacytoid DCs (PDCs) typically reside in lymphoid tissues and rarely infiltrate inflamed tissues. Plasmacytoid DCs residing in the mesenteric lymph shown to induce mucosal T-cell-independent IgA synthesis through the cytokines APRIL and BAFF [226]. Plasmacytoid DCs probably do not migrate in intestinal or hepatic lymph, distinguishing these cells from myeloid DCs that carry intestinal antigens to mesenteric lymph nodes [227]. Plasmacytoid DCs are producers of IFN-α which has been reported to induce development of CD. This finding supports their role in the pathogenesis of the disease. Intermediate dendritic cells are a subset of antigen presenting cells in the normal duodenum [225]. These cells have an intermediate phenotype and express markers typical for both DCs and macrophages. In mice intermediate DCs appear to originate from monocytes rather than DC precursors and believed to be tissue resident cells playing an important role in modifying local immune responses. Several lines of evidence suggest that intermediate DCs play a direct role in the pathology of CD. Unlike classical DCs and macrophages that show a decrease in the tissue concentration, the density of these cells increases after 3-day gluten challenge of CD patients. Beyond this, the increased density of intermediate DCs proceeds the typical inflammation related changes of the intestine and the surge of intraepithelial lymphocytes and eosinophils, suggesting that they have a role in the initiation of CD [228]. These findings indicate a plausible scenario where myeloid DCs traveling from the tissue to the mesenteric lymph nodes, carry gluten antigens and inflammatory signals for priming the naive T-cells into effector T-cells.

This causes a depletion of myeloid DCs in the lamina propria. In the other hand an influx of blood monocytes that differentiate into intermediate DCs can be observed in the. These cells mediate the reactivation of memory T cells in the intestinal mucosa. B cells likely play a role as antigen presenting cells in the mesenteric lymph nodes for the amplification of gluten T cell response.

2. 2. T cell response in Celiac Disease

$CD4^+$ T cells that recognize gluten peptides can readily be isolated from biopsies of CD patients, but not from individuals without the disease [229, 230]. These T cells recognize epitopes of gluten presented by disease associated HLA-DQ molecules [231, 232]. The MHC association in CD is linked to the preferential binding by HLA-DQ2 and HLA-DQ8 molecules to the proteolysis resistant gluten peptides that have negatively charged glutamate residues introduced by TG2 [220, 233-235]. Moreover, it has been proposed that gluten peptide-MHC complexes, expressed on the surface of antigen presenting cells are defining the magnitude of the gluten-specific $CD4^+$ T cell response and consequent induction of intestinal tissue damage [236]. This was based on a finding that susceptibility to CD is higher for those individuals who are homozygous for HLA-DQ2 or HLA-DQ8 alleles [237]. Unlike in healthy individuals, $CD4^+$ T cell responses to dietary gluten in the small intestinal mucosa can be observed for CD patients [230] (Figure 1.13). In CD an alteration in the intestinal environment can be observed which affects the differentiation/function of forkhead box P3 $FOXP3^+$ T_{Reg} cells. FOXP3 cells are responsible for oral tolerance [238] by secretion of anti-inflammatory cytokines such as transforming growth factor-β (TGFβ), IL-10 and IL-4 and promote the production of IgA antibodies [239].

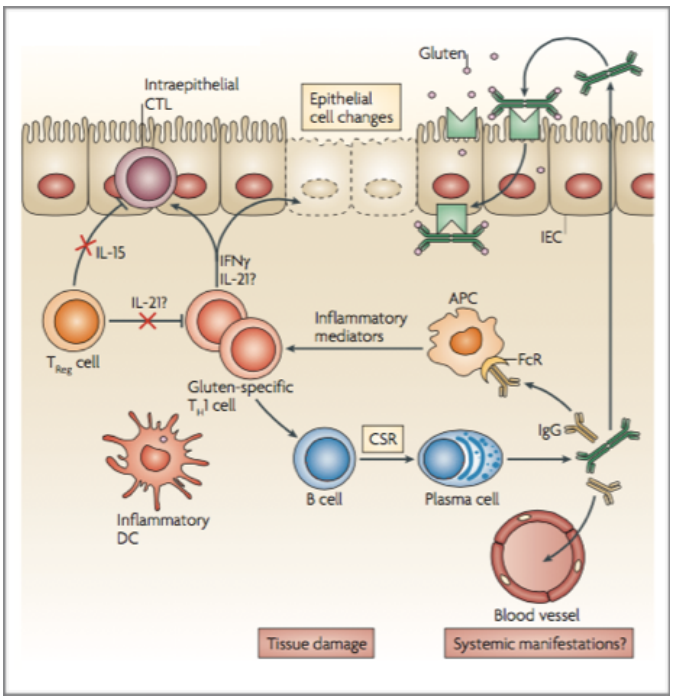

Figure 1.13. In the pathogenesis of CD gluten-specific $CD4^+$ T helper 1 (T_H1) cells secrete pro-inflammatory mediators such as interferon-γ (IFNγ) or interleukin-21 (IL-21), which promote activation of intraepithelial cytotoxic T lymphocytes (CTLs) and block the inhibitory effects of forkhead box P3 $(FOXP3)^+$ T_{Reg} cells. In addition, gluten-specific T_H1 cells help B cells to produce gluten- and TG2-specific IgG and IgA antibodies [240].

The intestinal mucosa in CD is characterized by presence of high levels of pro-inflammatory cytokines such as IL-15 [241-243] and IFNα [244-246]. It has been observed that in HLA-DQ8-transgenic mice IL-15 is able to alter the phenotype of intestinal DCs and prevent the activation of $FOXP3^+T_{reg}$ cells after oral challenge with gluten. Based on these observations it has been proposed that intestinal DCs when stimulated by pro-inflammatory factors as IL-15 or IFNα might loose their tolerogenic phenotype, promoting the differentiation of pro-inflammatory T cells. Beyond this effect IL-15 was shown to prevent the inhibitory effect of TGFβ [247] supporting that effector T cells in the gut mucosa of CD patients might be insensitive to the regulatory effect of TGFβ and $FOXP3^+$ T_{Reg} cells. $CD4^+$ T cells have been implicated to help to set up the inflammatory environment which allows the intraepithelial cytotoxic T lymphocytes (CTL) to induce tissue damage. By the secretion of pro-inflammatory cytokines such as IFNγ and IL-21, which may promote epithelial cell destruction by intraepithelial CTL activation. After activation by IL-15, intraepithelial CTLs were reported to expand early in the disease process [248], leading to the destruction of intestinal epithelial cells and villous atrophy [249, 250]. In humans CTLs has been shown to express the activating receptor NKG2D [251].

Expression of this receptor conferred potent co-stimulatory and direct cytotoxic functions on the CTLs, in particular following stimulation with IL-15 [252]. In healthy individuals, intraepithelial CTLs express the inhibitory receptor CD94–NKG2A, the C-type lectin CD161 and low levels of the activating receptors NKG2D and CD94–NKG2C [253] by contrast, intraepithelial CTLs from patients with celiac disease lose their expression of the inhibitory receptor CD94–NKG2A and acquire high levels of expression of the activating receptors NKG2D and CD94–NKG2C. In addition, IL-15, produced by intestinal epithelial cells in patients with celiac disease, has recently been shown to induce signaling in CTLs and can alter their function, in particular by up regulating the expression of NKG2D and co-stimulating the NKG2D cytotoxic signaling pathway [254].

Activation of intraepithelial CTLs by NKG2D and IL-15 could therefore not only contribute to the destruction of intestinal epithelial cells but also promote nonspecific inflammation in the intestinal mucosa of patients with celiac disease. Intraepithelial CTLs in patients with celiac disease were also proved to undergo a profound genetic reprogramming of NK cell functions, becoming NK cell-like cells. This NK cell-like transformation of intraepithelial CTLs may be a crucial step that precedes refractory sprue and enteropathy-associated T cell lymphoma (EATL), which are rare but major complications of celiac disease that are characterized by the presence of high numbers of intraepithelial CTLs with a NK cell-like phenotype and persistent villous atrophy despite a gluten-free diet [255, 256]. It is a plausible scenario that chronic NKG2D activation participates in NK cell reprogramming and malignant transformation of CTLs in CD. Although intraepithelial CTLs in celiac disease seem not to be specific for gluten, they respond to the indirect effects of gluten that cause up regulation of IL-15 and non-classical MHC class I molecules on intestinal epithelial cells.

2. 3. Antibody response in Celiac disease

In addition to T cells reactive to gluten, CD patients produce antibodies specific for gluten and TG2 [257]. These antibodies are excellent markers for celiac disease, as both anti-gluten and anti-TG2 antibodies disappear from the circulation within months after the introduction of gluten-free diet [258, 259]. Serologic testing for CD specific antibodies has become increasingly important tool in the diagnostic workup of patients. The first serologic tests were developed 50 years ago based on anti-gliadin antibodies binding to native gliadin antigen [260, 261], but these tests showed relatively poor sensitivity and specificity. In 1997, TG2 was identified as the antigen recognized by anti-reticulin and anti-endomysium antibodies, leading to the development of diagnostics test measuring serum anti-TG2 antibodies by ELISA [262]. At the moment, anti-TG2 antibodies serve as the most specific and sensitive marker for active CD and their detection is the preferred diagnostic tool [263, 264]. New tests to detect IgG anti-gluten antibodies using synthetic deamidated gliadin peptides as substrates perform almost as well as the IgA anti-TG2 test [259] and proved to be especially useful in the diagnoses of infants and IgA-deficient patients [265]. This paragraph will dissect the anti-gulten B-cell response while the autoantibody response against TG2 will be discussed in the next chapter. The epitopes recognized by gluten-reactive CD4+ T cells of CD patients include glutamate residues, which have been introduced by TG2-mediated deamidation of certain glutamine residues [215]. Gluten B-cell epitopes have been characterized by studying polyclonal serum antibody reactivity to synthetic peptides of gliadin proteins [266] and by bacterial cell-displayed peptide libraries [267]. Deamidation is also relevant for the B-cell epitopes, and serum antibody reactivity is higher to deamidated than to native (non-deamidated) peptides [266, 268]. Gliadin B-cell epitopes appear to be located in proximity and/or to overlap with gliadin T-cell epitopes IgA antibodies to gliadin only occur in subjects with HLA-DQ2 or HLA-DQ8 [269]. The HLA dependence of the antibody production and the colocalization of T-cell epitopes and B-cell epitopes suggest that the antibody response to gluten in CD is T cell dependent. In a recent study, gluten (gliadin)-reactive antibodies expression was characterized by cloning IgA+ PCs isolated from small intestinal biopsies [270]. Antibodies had a limited number of somatic hyper mutation (SHM) which is common feature with IgA antibody responses to gluten and TG2 in CD. This suggests that the factor(s) causing the limited SHM in IgA of gliadin specific PCs is likely involved in both antibody responses. Anti TG2 and anti-gliadin antibodies have a parallel fluctuation [258] suggesting that the production of these antibodies is regulated in a coordinated way.

It has been suggested that gluten-specific T cells could provide help to TG2-specific B cells. Gluten-specific T-cells may also provide help to gluten-specific B cells. As gluten-specific T cells preferentially recognize deamidated gluten peptides, B cells with surface immunoglobulin that bind and internalize deamidated gluten peptides would be better situated to receive T-cell help. Some anti-gliadin antibodies are specific to deamidated gliadin peptides and do not recognize non-deamidated counterparts, indicating that gliadin-specific B cells must have encountered deamidated gliadin [270]. Gliadin specific antibodies have also been reported to cross-react with different gliadin peptides [270] and display several different T-cell epitopes. This could lead to B cells receiving help from several different T-cell clones, as recently described [271]. In addition to the limited SHM, a restricted VH/VL usage was observed in the panel of gluten-specific antibodies with he same VH/VL pairings appearing in different subjects. Limited VH usage is a sign of T-cell-dependent responses [272]. The restricted VH/VL usage also raises the possibility that might be genetic effects at the immunoglobulin loci for the development of this disease.

2. 4. TG2 specific autoantibody response

2. 4. 1. TG2 specific antibody production and response to gluten

In addition to gluten reactive T cells, CD patients produce antibodies specific for gluten and self antigen TG2 [257]. In 1997 TG2 was identified as the antigen recognized by anti-reticulin and anti-endomysium antibodies [262], however antibodies specific for other autoantigens as actin, collagen have also been described [273]. In addition of the massive CD4+ T-cell response toward deamidated gluten, the celiac lesion is characterized by the expansion of plasma cell (PC) population [274, 275] and enhanced local immunoglobulin secretion [276, 277]. The specific antibody response against TG2 in the serum has become the most sensitive and specific serological assay used fro the diagnosis of CD [263]. Upon commencement of gluten free diet anti-TG2 antibody titer drops below the cut off value within six month [278]. Following a short term gluten challenge they reappear in the in the blood within 2-4 weeks [279]. Serum titers of anti-TG2 antibodies seem to correlate with intestinal damage [280], however antibodies can be detected in patients with no histological findings [281-283].

It was possible to visualize TG2-specific antibodies in the duodenal mucosa of CD patients [284]. TG2 specific antibodies can be found as IgA deposits in the basement membrane of small intestine [285] and these deposits are present even in the absence of detectable anti-TG2 serum level serving a predictive value for the disease [281]. This indicates that anti-TG2 antibodies appear at an early stage of CD and TG2 specific B-cells are activated in response to dietary gluten prior to any clinical symptom. Phage display libraries of single chain antibody variable regions obtained from the IgA producing intestinal biopsy lymphocytes and peripheral blood lymphocytes of CD patients were used to study the antibody response in CD [286]. It was possible to isolate anti-TG2 antibodies from intestinal lymphocytes libraries but not from those generated from peripheral lymphocytes. This indicates that the response against TG2 occurs at a local level in the small intestine and their presence in the serum is attributable to spill over into the blood compartment. These antibodies target the same antigen in the extracellular matrix and endothelium of small blood vessels [285], supporting the local antibody production.

2. 4. 2. Biased V gene usage and limited somatic hypermutations of Abs

Analysis of the TG2 specific antibody repertoire from single chain immunoglobulin phage display libraries from intestinal biopsies [286] revealed that VH gene usage was restricted to three (VH 5, VH 3, VH1) of the seven human Ab VH families with a biased usage VH5 family gene segment among IgA producing cells. The preference was not found in naive B-cell library indicating that VH5 family selection is not due to the intrinsic TG2 affinity associated with the heavy chain variable region. Recombinant antibodies has been generated from the single plasma cells of celiac patients. It was found that TG2 specific PCs represent 5-25% of total PCs in the celiac lesion [284, 287]. The PCs had strong bias in their VH and VL usage with a preference of *IGHV5-51, IGHV3-48, IGHV4-59, IGHV1-69, IGKV1-39, IGKV1-5* with a dominance of *IGVH5-51*. It was found that 44% of TG2 specific antibodies were encoded by *IGHV5-51* family. It is an important feature that a number of *IGHV5-51* antibodies with different specificity did not bind TG2, thus showing that the anti-TG2 reactivity is encoded by CDR and not depends on the unspecific binding of the VH5 framework region [284]. The biased usage of certain genes in antibody response has been observed in other autoimmune diseases such as rheumatoid arthritis [288] and also in responses towards foreign antigens such as HIV [289] and influenza [290]. In spite the extensive switch to IgA anti-TG2 antibodies also have few somatic hypermutations [284], less than half amount of mutations were observed compared to the rest of the intestinal PC compartment.

This is in contrast to what is observed in IgA responses toward pathogens such as influenza and rotavirus [284, 291] and also in autoimmune diseases such as rheumatoid arthritis [288]. Reversion of mutated anti-TG2 antibody sequences into presumed germline configuration leads to reduced affinity of antibodies, indicating that the affinity maturation happens during the generation of these antibodies [284]. The fact that none of the anti-TG2 antibodies have polyreactivity indicate that TG2 reactive B-cells recognizing the antigen with high specificity and reasonable affinity must exist in the naive B-cell repertoire of CD patients. Gut derived TG2 specific PCs have a small but detectable memory compartment [284] which is supported by the fact that TG2 reactive PCs in the small intestine do not disappear completely in gluten free diet indicating that al least a number of the PCs are long lived as also observed in *in vitro* culture [284, 292]

2. 4. 3. Targeted epitopes of anti TG2 antibodies

Epitopes recognized by TG2 specific antibodies are known to be conformational, and a study with polyclonal sera of CD patients indicated that there is an important conformational epitope which was targeted by both serum antibodies and tissue-derived monoclonal antibodies [293]. In contrast, TG2 antibodies from subjects with other autoimmune diseases preferred other binding sites. The main anchors points of this celiac epitope reported to be Glu153 and Glu154 on the edge of the first alpha helix of the core domain of TG2, but they also need one more anchor point either on the N-terminal or C-terminal domains (Figure 1.14.). The N-terminal anchor point is formed by the first helix containing Arg19 while the anchor point Met659 can be found on the C-terminal domain.

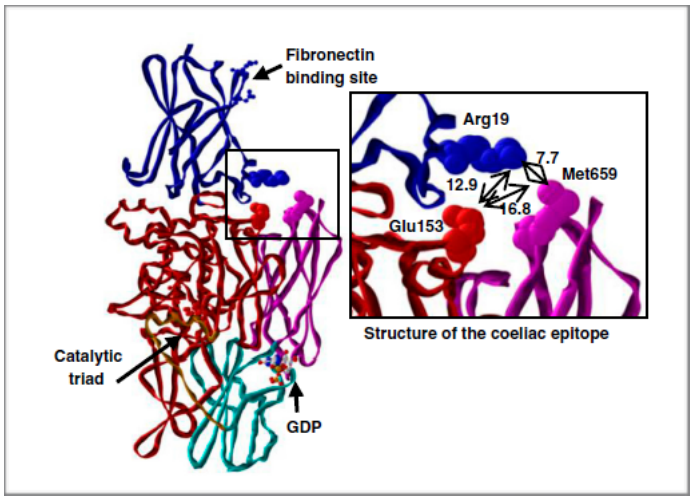

Figure 1.14. Three-dimensional view of TG2 in the closed conformation with the N-terminal β-sandwich shown in blue, catalytic (core) domain in red, β-barrel 1 in cyan and β-barrel 2 in pink. The amino acids of the putative celiac epitope with their lowest distances in angstroms (Å) are illustrated as represented on surface. [293]

Epitope mapping studies revealed that TG2 specific antibodies target at least four different epitopes clustered in the N-terminal of TG2 [294] (Figure1.15). These epitopes were also represented among the polyclonal anti-TG2 IgA antibodies present in the serum. Epitope recognition correlated with the VH usage, epitope 1. antibodies mainly used VH5 gene segment, epitope 2 antibodies used VH3 segments and epitope 3 antibodies used mainly VH4 gene segments [294]. Antibodies representing each epitope cluster were tested for their ability to stain cell surface TG2 (csTG2) expressed on the surface of immature dendritic cells (iDCs). None of the antibodies were able to stain suggesting that the epitopes are hidden when TG2 is expressed on cell surface. It was also revealed that the region recognized by epitope 1. antibodies is overlapping with the fibronectin binding site of TG2 [71, 294]. A more recent study by hydrogen/deuterium exchange and subsequent mutational analysis of TG2 demonstrated that residues Lys-30 and Glu-8 are part of epitope 1., while Arg-19 is part of epitope 2 [295]. The binding interface of antibodies and TG2 was further analyzed by small angle x-ray scattering, *ab initio* and rigid body modeling using the known crystal structure of TG2 and an epitope 1. antibody Fab fragment. The results, which were supported by ELISA made on TG2 point mutants, implicated that Arg-116 and His-134 are critical for binding epitope 1. antibodies, but not for epitope 2 and epitope 3. antibodies [296].

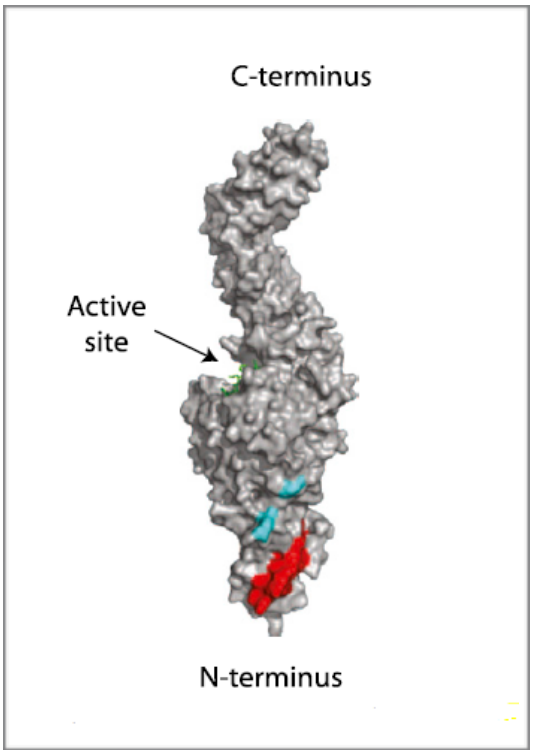

Figure 1.15. Regions targeted by TG2-specific autoantibodies. Surface representation of the open TG2 structure with a bound active site inhibitor shown in green. The region in the N-terminal domain implicated in binding FN representing epitope 1/4 is shown in red. The region representing epitope 2/3 is shown in cyan. [294]

2. 4. 4. Collaboration of TG2 specific B-cells and gluten specific T-cells in CD

TG2 specific autoreactive B-cells may receive help from gluten specific T-cells as stated in the most widely accepted, 'hapten carrier model' [297] (Figure 1.16)**.** The model relies on the fact that TG2 specific B-cells are able to internalize gluten T-cell epitopes by taking up TG2-gluten complexes through B cell receptor (BCR) mediated endocytosis. Two types of covalently linked complexes of gluten peptides and TG2 can be formed: either by coupling to the active site cysteine *via* thioester-bond or by coupling to surface exposed lysine residues *via* isopeptide-bonds [298]. The lysine residues of TG2 which are involved in forming isopeptide-bonds are predominantly situated on the C-terminal part of the molecule, leaving the N-terminal part recognized by TG2 specific B-cells untouched [299]. Internalization of gluten-TG2 complexes is followed by endosomal processing and presentation of gluten peptides on MHC class II molecules on the cell surface to gliadin specific T-cells. This would result in TG2 specific B-cells receiving activation signals from $CD4^+$ T-cells. The uptake of TG2 cross-linked to a variety of different T-cell epitopes could provide TG2 specific B-cells help from T-cells with different specificities. The model is supported by that TG2 specific antibodies rapidly disappear when gluten is removed from the diet [258, 300]. In addition the strict HLA dependence of the antibody response strongly implies the involvement of T-cells. A population based study on Swedish children compared the presence of TG2 specific antibodies in individuals with or without DQ2 or DQ8 HLA-risk alleles [269], and found that TG2 specific antibodies are only detectable in those who are having specific HLA types associated with CD.

Another possible scenario for activation of TG2 specific B-cells relies on TG2 utilizing the BCR as a substrate for covalent cross-linking of gluten peptides [301] (Figure 1.16). TG2 cross-links peptides to TG2 specific BCR of the IgD but not to the IgA isotype, with a preference to *IGHV5-51*. It is know that cross-linking of BCRs or covalent linkage of antigen stimulate B cell activation [302], indicating a plausible scenario for activation of naive TG2-specific B-cells compared to isotype-switched IgA- or IgG-expressing memory B cells. The model could explain the preferential usage *IGHV5-51* and the limited number of somatic hypermutations.

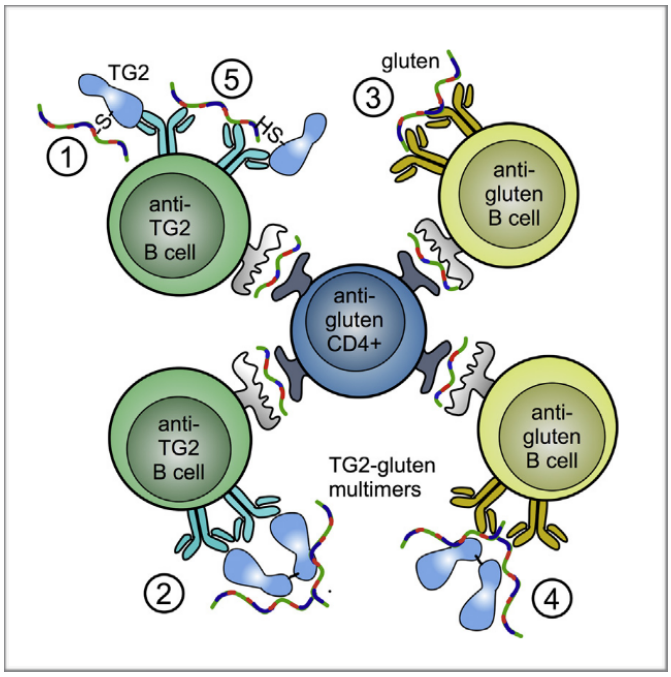

Figure 1.16. Models for activation of disease specific B cells by gluten specific CD4+ T cells in CD. Gluten T-cell epitopes (shown in green) are deamidated (shown in red) by TG2, and overlap with or are in close proximity to gluten B-cell epitopes (blue). TG2-specific B cells may internalize gluten T-cell epitopes through BCR mediated uptake of TG2, with gluten transiently bound to the active site **(1)** or through uptake of isopeptide linked TG2-gluten complexes **(2).** Gluten-specific B cells preferentially bind long, multivalent peptides **(3).** Multivalent TG2-gluten complexes may cross-link the BCRs of both TG2-specific B cells (2) and gluten-specific B cells **(4)**. In addition, gluten T-cell epitopes may be cross-linked directly to the TG2 specific BCR **(5)**.

The model requires that TG2 remains active when bound to the BCR. From 47 antibodies generated from single intestinal PCs of CD patients none had inhibitory effect on TG2 enzymatic activity [284]. A contradictory data was shown with IgA and IgG antibodies purified from CD patients, the IgG antibodies showing inhibition between 8.3-24% and IGA antibodies having 11.1-39% inhibition over TG2 enzymatic activity [303]. The experiment repeated with monoclonal antibodies derived from the intestinal lymphocytes of CD patients had the same result with an inhibitory effect ranging from 20% to 40%. Apart from the providing a possible explanation for the gluten-dependent production of TG2 specific B-cells, the hapten carrier model propose a plausible scenario where TG2 specific B-cells act as antigen presenting cells for gliadin specific T-cells and this way contribute to the pathogenesis of CD. There has been speculations whether the antibodies themselves are pathogenic.

2. 4. 5. Pathogenic roles of anti TG2 antibodies

Celiac disease is hallmarked by increased small-intestine epithelial cell proliferation and decreased differentiation. It has been shown that anti-TG2 antibodies inhibit intestinal epithelial cell differentiation and also increase the permeability of the intestinal epithelial cell [304-306] The conception of anti-TG2 antibodies being pathogenic is further supported by studies performed on CD patient small-bowel biopsy organ culture suggesting that patient IgA enhances passage of gliadin peptides into the lamina propria [307]. Patient IgA induced gliadin translocation into the tissue could promote the development of small-intestinal damage by enabling and amplifying the T-cell response. Anti-TG2 IgA antibodies also may detach TG2 from fibronectin and the complexes could be transported across the epithelium where TG2 or the antibodies themselves contribute to the sampling of gliadin peptides. This model is supported by the fact that epitope 1. anti-TG2 antibodies recognize the fibronectin binding site on TG2 [294]. Anti-TG2 autoantibody deposits are located around the mucosal blood vessels in the small-bowel in patients with CD [285] and considering the fact that they have been show to inhibit angiogenesis *in vitro* [308, 309] they might contribute to the aberrant organization of the mucosal vascular network. Anti-TG2 antibodies have been reported to increase blood vessel permeability to both macromolecules and lymphocytes *in vitro* and this effect could potentiate the small-bowel mucosal inflammatory response by enhancing the invasion of lymphocytes and macromolecules into the small intestine.

Celiac disease is occasionally reported to manifest in the nervous system including peripheral neuropathy, epilepsy and cerebral ataxia [310]. Gluten ataxia is a sporadic cerebellar ataxia with majority of the patients with enteropathy having circulating anti-TG2 antibodies [311]. It has been reported that all gluten ataxia patients, even seronegative had IgA-class anti-TG2 antibodies deposited in the small intestinal mucosa and the antibodies were also present in brain vasculature of a gluten ataxia patient [312]. The passive transfer of anti-TG2 antibodies derived from CD patient to the mouse brain has been reported to cause ataxia like symptoms in the recipient animals [313]. These data suggest that anti-TG2 antibodies might lead to the neurologic pathologies occurring in association with CD. Celiac disease has been associated with a wide range of reproductive disorders in women. A shorter duration of fertile life spam in women with untreated CD because of an older age of menarche and younger age of menopause and an increased prevalence of secondary amenorrhea have been shown in several studies [314, 315].

The length of the reproductive period seems to correlate with the state of CD since studies have shown that celiac women of long term GFD show a duration of fertility life spam analogue to healthy women [314, 316]. The mean number of children born from celiac patients is significantly less compared to healthy controls and the overall difference in fertility is due to an infertility period in celiac women [315]. Anti-TG2 antibodies have been implied in placental related pregnancy complications. It has been demonstrated that TG2 is expressed in endometrial cells as well as in stromal and trophoblast cells with higher level in late pregnancy[317]. Since extracellular TG2 is involved in cell adhesion and migration [66, 318] it is likely to play a critical role in the implantation process. TG2 on syncytiotrophoblasts may be the target of maternal anti-TG2 antibodies in CD. Anti-TG2 antibodies of IgA class have been reported to bind to the syncytial surface of placenta and inhibit TG2 activity [319], explaining the functional impairment of placental development. It has been demonstrated that polyclonal fractions of anti-TG2 antibodies as well as the monoclonal anti-TG2 antibody were able to directly bind to trophoblast cells and significantly reduce trophoblast invasiveness through apoptotic damage [320]. Beyond these findings a significant decrease in MMPs activity was observed in the study which could be an indirect effect of the increase in trophoblast apoptosis. After the stimulation of angiogenic factors the basement membrane is degraded by MMPs and proteolytic enzymes secreted by endothelial cells allowing cells to invade, migrate and proliferate into the underlying interstitial matrix and form new capillary structures [321, 322]. The angiogenesis induces crucial changes in the endometrium, enabling it to accept blastocysts and initiate implantation. Anti-TG2 antibodies have been implicated to interfere in the endometrial angiogenesis. IgA and IgG polyclonal immunoglobulins isolated from the sera of celiac patients as well monoclonal anti-TG2 antibody have been reported to bind the membrane of human endometrial endothelial cells (HEECs) isolated from placental explants [323]. The binding was followed by a significant decrease of *in vitro* angiogenesis. The mechanism causing this decrease was investigated and a significant level of reduction of both pro- and active MMP-2 level was observed. These data suggest a plausible scenario of anti-TG2 antibodies inhibiting angiogenesis by reducing MMP secretion and this way extracellular matrix degradation. A dramatic disarrangement in the F-actin cytoskeleton in the anti-TG2 antibody treated cells have been reported [324, 325]. FAK and ERK are key kinases of the intracellular pathway regulating cytoskeleton rearrangement and the transcription of pro-angiogenic factors [326, 327]. Anti-TG2 antibodies have been demonstrated to inhibit FAK and ERK providing this way another mechanism how they inhibit angiogenesis in endometrial cells [328].

Chapter 3. Expression libraries

Gene expression technologies are important tools for the study of expression profiles of a great variety of biological systems. These technologies provide a direct physical association between phenotype (protein under analysis) and genotype (gene encoding the protein). Expression libraries are used for two main goals in research, to screen at the same time thousand of proteins expressed by the library and to detect such characteristics as interaction, sensitivity even when the interactors in the biological sample or the proteins of the library are present in low level. cDNA libraries are the most common source of DNA for screening approaches. These libraries are prepared from the total single-stranded mRNA extracted from human tissue as well as other organisms such as plants or yeast [329-333]. The single-stranded mRNA is converted into double-stranded DNA by the enzyme reverse transcriptase. After this step the cDNA fragments can be cloned to an appropriate vector, where the final population of vectors will represent the entire set of genes expressed in the source of mRNA. A number of formats can be used to study the complex mixture of proteins. Bacterial systems, such as E. coli are preferred because of their ability to grow rapidly and high density on inexpensive substrate, well-characterized genetics and availability of cloning vectors [334]. In spite of these advantages bacterial systems might have problems in expressing eukaryotic proteins due to aggregation, formation of inclusion bodies and the lack of post-translational modifications [335]. Eukaryotic systems, such as baculovirus system [336] overcome these drawbacks, however have issues such as lower yield, high demands of sterility and time consuming cloning procedures. cDNA libraries can be tested by PCR, DNA hybridization [337], two-hybrid systems [338], enzymatic activity [339], high-throughput structure determination [340], and by recognition by antibodies [341].

3. 1. Display systems

Display systems allow us to create libraries of biomolecules and to screen them for certain characteristics in a high throughput format.This approach not only makes possible to analyze protein-protein, protein-substrate interactions, but also to isolate antibodies for the desired antigen. Complex libraries can be enriched by selection strategies for clones with the desired specificity. The advantages of the method is that selected proteins can be rapidly identified by DNA sequencing and manipulated by molecular biology techniques.

3. 1. 1. In vitro display systems

Ribosome and RNA display are *in vitro* display systems, where genes are coupled to the proteins they encode after a translation step in an in vitro translation mix. These display systems rely on PCR for amplification and *in vitro* translation of RNA to produce the binding ligand, which is attached to the encoding RNA. In RNA display the RNA is covalently linked to the encoded protein by a puromycin adaptor, while in ribosome display the gene and the encoded protein are non-covalently linked by the ribosome itself [342, 343]. These methods allow size as large as 10^{15} different members increasing the probability to select rare sequences and to improve the diversity of the selected ones. In vitro selection methods overcome such problems as poor protein expression and protein degradation which might reduce the diversity of selected sequences.

3. 1. 2. Cell based display systems

In cell based display systems such as phage and yeast display the amplification, display and coupling of genotype and phenotype is carried out by living organisms. Phage display in the oldest and the most widely used cell based display system. In this system a large number of genes are cloned upstream the coat protein gene and displayed on the surface fused to the coat protein of the filamentous phage. This system will be described in detail in the next paragraph. The yeast cell provides the linkage as the protein of interest is expressed as a fusion to a cell wall protein. Different yeast strains and cell surface receptors have been used for cell surface display, however *S. cerevisiae* Aga2p system remains the most commonly used. In this system, proteins are expressed as cell surface fusions to theAga2p subunit of the mating protein a-agglutinin in *S. cerevisiae.*

3. 2. Selection methods

3. 2. 1. Physical selection methods.

Physical selection involves the sequential enrichment of specific binding clones from a large excess of non-binding clones. In order to select the binding ligands against a specific protein using physical selection methods, the selector has to be used in a purified form and a significant quantity 200-1000 ug is usually required.

There are two main types of these selection methods. In one, the selector is fixed to a solid support, such as polystyrene tube or pin and incubated with the library of polypeptides. After several rounds of wash steps those that bind to the selector can be eluted, while the unbound ones are washed away. In the second method the selector is labeled with biotin or fluorescein and selection is carried out using streptavidin coated magnetic beads (MACS) [344] in case of biotin and fluoresence-activated cell sorting (FACS) [345] in case of fluorescein labelled selectors. Physical selection methods are used in *in vitro* and also in cell based display systems, where usually more than one round of selection is required.

3. 2. 2. Genetic selection methods

Physical selection methods are ideal for selecting binding ligands when a selector is available in a purified format in large quantity. Genetic selection methods allow to avoid the use of physical selection by using DNA encoding the gene of interest. One of the most widely used genetic selection method is the **yeast two-hybrid (YTH) system** [346], where each of the two interacting polypeptides is fused with one of the two functional subunits of a transcription factor. Interaction between the two polypeptides reconstitutes the transcription factor, enabling the transcription of a reporter gene, normally represented by the antibiotic resistance or a chromogenic enzyme. The problem with the system is that the polypeptide libraries may contain more than five billion clones which makes a primary selection round necessary before cloning to the yeast two-hybrid vector [347, 348]. **Protein complementation assay (PCA)** might serve as an alternative for the physical selection [349, 350]. In this method the enzyme required for cell survival, such as dihydrofolate reductase and β-lactamase, is divided into two fragments [350, 351]. The refolding of the reporter protein from its fragments is catalyzed by the binding of the supposed interacting proteins, and is detected as reconstitution of the enzymatic activity.

3. 3. Phage Display

Phage display has proven to be a powerful tool to display libraries containing millions of different peptides and proteins. In this method the coupling of phenotype and genotype is achieved through the specific properties of bacteriophages. The concept of displaying polypeptides on the surface of the filamentous M13 bacteriophage (phage) was introduced by Smith and colleagues in 1985 [352]. Smith demonstrated that phage genome could be manipulated by the insertion of a foreign DNA into a gene encoding phage coat protein without interfering with their infectivity.

This way phages displaying proteins fused to their surface could be affinity purified against an immobilized specific immunoglobulin, thereby allowing over 1000 fold enrichment of correct phages from a background of phage particles not displaying the antigen. Depending on the application different types of phages can be used for phage display such as Lambda [353], T7 [354] and filamentous phage (M13, f1, fd). By far the most popular phage that has been used for display is the non-lytic filamentous phage M13 [355, 356] (Figure 1.17). This type of phage infect only gram-negative bacteria and unlike lytic species such as Lambda and T7 replicate and assemble without killing the *E. coli* host [357]. The genome of M13 phage consists of a single stranded DNA molecule (6407 base pair long) encapsulated approximately 2700 copies of the major coat protein pVIII and with 5 copies of the four minor coat proteins (pIX, pVII, pVI, pIII) at distal end.

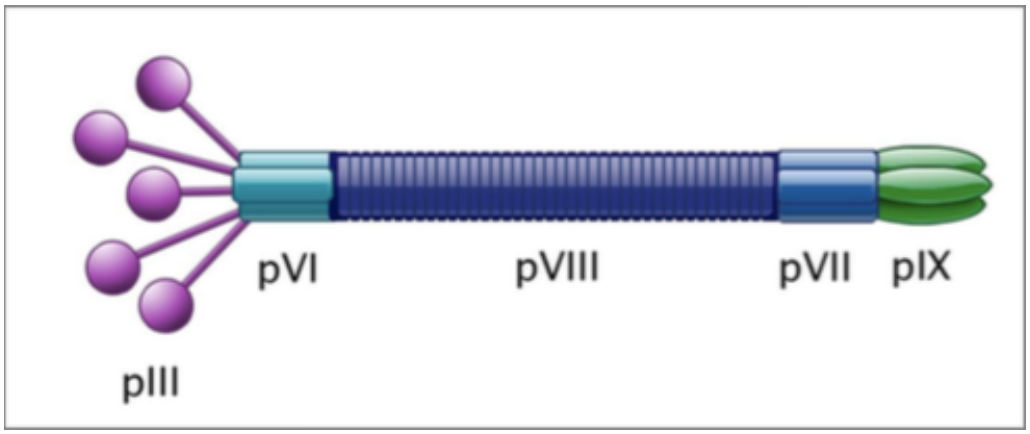

Figure 1.17. M13 bacteriophage structure. Coat proteins are indicated. (Sulic. A. 2010. PhD thesis)

During infection the minor coat protein pIII binds to the F-pilus of *E. coli* [358]. This protein consists of two N-termial domains (N1, N2) and one C-terminal domain (CT). The infection is mediated through the N2 domain and the F-pilus, which brings the TolA membrane protein of *E. coli* close proximity to N1 resulting in the interaction of the two. This process leads to the deposition of phage single stranded DNA into the cytoplasm and later converted into double stranded DNA by bacterial enzymes. The C-terminal domain is responsible for terminating phage assembly in the periplasm and the subsequent release from the cell membrane.

In the phage display system pIII, pVIII and rarely pVI proteins are used. The DNA fragments of interest are cloned upstream of gene encoding pIII or pVIII, resulting in 3-5 recombinant proteins at one end of the phage int the case of pIII and 2700 copies of the original coat and recombinant proteins in the case of pVIII. The type of coat protein used as fusion partner should be considered, since pVIII is beneficial for the display of large number of smaller proteins, while pIII is more efficiently displays a fewer number of large proteins [356]. Excluding the display of short peptides pIII based systems are more commonly used.

Phagemid vectors have been developed to overcome the difficulties working with phage genomes. The two basic type of polypeptide display is the polyvalent and the monovalent display. In polyvalent phage display the libraries are based on vectors derived from phage genome encoding all necessary proteins fro replication and assembly. Each copy of the coat protein displays the polypeptide, however the system is limited to smaller polypeptides since larger ones would interfere with the function of the coat proteins and eventually lead to the decrease in the infection property.

Monovalent phage display the inserted DNA are cloned upstream the gene encoding coat protein pIII or pVIII and are presented in a single copy. This method has been used for cDNA libraries where the encoded protein domains are too large to be expressed on the coat protein without interfering with its function. In monovalent display the virion contains the phagemid carrying the antibiotic resistance marker, bacterial and phage origin of replication, the inserted DNA containing phage promoter upstream the gene encoding the coat protein and a packaging signal. The excess of wild type coat protein, necessary for phage replication, ssDNA production and assembly is provided by the helper phage, such as M13KO7 or VCS-M13, which has disabled packaging signal [359, 360]. The infection with the helper phage initiates the assembly using phagemid DNA carrying the packaging signal and thus makes the phages functional.

Since the helper phage genome encodes wild-type coat proteins, typically 90% of phages display no recombinant protein at all and the vast majority that do display the fusion product will only contain a single copy. This way the recombinant proteins represent only a small portion of the total coat protein, allowing the phage overcoming the issues of polyvalent phage display.

3. 3. 1. Phage antibody libraries

Antibodies were the first proteins to be successfully displayed on the surface of the phage (d28), which was achieved by fusing the coding sequence of the antibody variable (V) regions encoding a single-chain fragment variable (scFv) to the amino terminal of pIII minor coat protein of the phage. Since then, phage display has been efficiently used to generate antibody libraries and eventually isolate monoclonal antibodies from them. Phage antibody libraries can be divided into three type, namely: immune, naive and synthetic.

Immune phage antibody libraries can be generated from the spleen B-cells of mice immunized with antigen [361] or from immune donors. The phage antibody repertoire will have antibodies enriched for a specific antigen and some of them will have been affinity maturated by the immune system [362]. This system provides antibodies with higher affinity and also higher quantity of antibodies might be obtained from the material originating form a single donor, which can be easily produced and manipulated. The construction of immune libraries have been reported from various species, including mouse [363, 364], human [365], chicken [366], rabbit [367], and camel [368]. The bottleneck of the system is that active immunization is not always possible due to ethical concerns, tolerance mechanisms and if toxic antigen is involved. Providing suitable sources of antibody producing B-cells or plasma cells, immune phage antibody libraries are ideal for analyzing autoimmune diseases [286, 369] or viral infections [370].

Naive phage antibody libraries can be generated by harvesting the V-genes from the IgM mRNA of B-cells from unimmunized donors, isolated from peripheral blood lymphocytes [286], bone marrow or from animal sources [371].

V-genes are amplified from B-cells by family based oligonucleotides [372] where heavy and light chains are randomly combined and cloned to encode a combinatorial library of scFv or Fab antibody fragments. The affinity of the selected antibodies is proportional to the size of the library. This method gives access to antibodies which have not encountered the antigen yet. If sufficiently large and diverse naive libraries may be used to generate a large panel of antibodies against self, non-immunogenic or toxic antigens [373, 374].

Synthetic phage antibody libraries are built artificially, by *in vitro* assembly of V-gene segments and D/J segments. V-genes might be assembled by introducing a level of randomization to the CDR regions of germline V-gene segments [375] or rearranged V-genes [376]. The regions and the degree of diversity might be chosen in a way to correspond to t areas with the highest natural diversity of the antibody repertoire. Most of the natural structural diversity is found in the loop, most central to the CDR3 of the heavy chain, while the other five CDRs have less variation [377], therefore this has been the target for introducing diversity in synthetic libraries.

Phage antibody selection involves the sequential enrichment of specific binding phage from a large excess of non-binding clones (Figure 1.18.). This is achieved by multiple rounds of binding to the target, washing to remove unbound clones and elution to retrieve specific binders for the target. Several different selections methods have been developed including panning on immobilized antigen coated onto solid support, selection using biotinilated antigen, affinity purification on columns and direct panning on cells. Phage antibodies bound to antigen can be separated from non specific clones by wash steps and positive clones can be subsequently eluted using several methods. These elution ways involve: acidic solutions such as HCl or glycine buffers, with chaotropic agents, with DTT by enzymatic cleavage or by competition with antibodies specific to the antigen. In a similar fashion what happens in vivo in the course of B-cell selection, phage antibodies with higher affinity might be enriched in successive rounds of selection by decreasing the quantity of antigen. This selection might be chosen to favor affinity or kinetic parameters such as off-rate, based on the use of limited and decreasing amounts of antigen and on performing the selection in solution rather than by using panning on coated antigen.

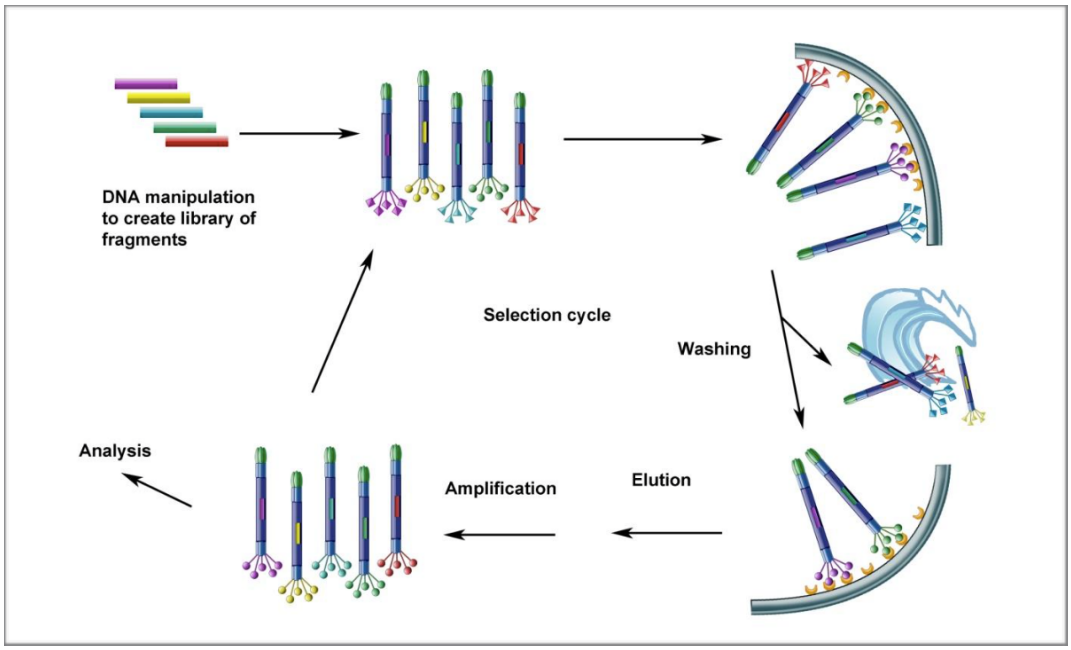

Figure 1.18. Phage display selection cycle. The isolation of a specific phage for it's binding to a ligand leads to the isolation of the corresponding gene, while the unreactive clones are eliminated from the selection. Up to five rounds of selection can be performed, resulting in enrichment for phages that are represented in very low numbers in the original library. (Sulic. A. 2010. PhD thesis)

Antibodies selected from phage antibody libraries may be useful for analytic applications, however often need to be improved in terms of affinity to be used for therapeutic purposes or sensitive diagnosis. A possible solution for this problem is the *in vitro* affinity maturation of antibodies involving three steps: introducing diversity int he V-genes, selecting higher affinity variants and screening to discriminate between antibody variants regarding affinity or kinetics of binding. If the concern is not the affinity of individual antibodies but rather to generically increase effective affinities downstream methods can be applied. One system is to fuse genetically multimerization domains (e.g., jun/fos dimerization) [378] to the end of selected scFvs, which significantly improves sensitivity in ELISA and Western blot. Antibodies originating from phage display can be used for all application that use monoclonal antibodies, however they require an extra step to reveal them. This is usually achieved by the binding of a monoclonal antibody to the antibody-binding tag (e.g., myc) [379] or SV5 [286]. Some scFvs selected from phage antibody libraries have low yields and stability which can be improved when scFv genes are re-cloned into downstream vectors. These vectors might be for enhanced expression [380], mini antibodies, dimerization domains and Fabs [381], targeting intracellular immunization and enabling the transfer of V regions from scFvs to full-length immunoglobulins for mammalian expression [382].

3. 3. 2. Phage display for the study of celiac disease

Phage display proved to be an effective tool to study the autoimmune response in Celiac disease [383-385]. This technique provides a tool to dissect the autoantibody response, select interacting proteins and also to identify specific antigens of the disease. In phage display of human Ab segments the patients antibody repertoire is fused to the coat protein of the phage vector carrying the encoded antibody gene [386], with each phage having a single Ab specificity.

A system was developed in 2000 [387] for making large libraries exceeding the limits of traditional phage display technology. It is known that the affinity of the antibodies isolated from a library is proportional to the initial size of library used for selection [388, 389] and due to this large libraries have become essential sources of high-affinity antibodies [390, 391]. In the study first a relatively small primary library was created in a phagemid vector pDAN5 where VH and VL genes were separated by two non homologous lox sites. VH and VL genes of the primary library were recombined by infecting the phagemid to Cre expressing bacteria at high multiplicity of infection. The library created with this method was validated by selecting high affinity antibodies against various antigens. The same construct was used for production and analysis of six phage Ab libraries from the peripheral and intestinal lymphocytes of three CD patients [286]. VL chains were amplified using a set of oligonucleotides recognizing all human V genes, while oligonucleotides amplifying VH genes were specific to IgA [392]. VH and VL chains were cloned to the phagemid vector pDAN5 [387] to generate libraries to be studied.

It was possible to isolate Abs to gliadin from all libraries while Abs to TG2 were only isolated intestinal lymphocytes libraries but not from those obtained from peripheral lymphocytes, revealing that humoral response against TG2 occurs at local level while against gliadin occurs both peripherally and centrally. Antibodies generated from the IBL of all three patients had a biased usage of the VH5 variable region family, which was supported in later studies [284, 294].

Selected ones of the isolated antibodies were cloned and expressed in scFv-Fc format [393] where the anti TG2 scFvs were genetically fused to human, mouse and rat Fc domains, allowing in vivo expression. The scFv-Fc molecules were efficiently secreted as homodimeric, mimicking the original structure of the antibody. The reactivity towards the target antigen TG2 was retained for all different constructs indicating that epitope recognition did not change due to expression as scFv-Fc. The *in vivo* expression in mice led to detectable serum levels even 40 days after the injection, with no immune response by the host.

Phage display is effective tool to identify interacting proteins to a specific target, however the presence of frame-shifted DNA sequences has been an issue of the system. To overcome this problem a method for the selection of DNA encoding open reading frames (ORFs) from non-coding DNA was developed [394] within the context of pPAO2 phagemid vector. In this system DNA fragments were cloned in a fusion gene upstream of β-lactamase gene flanked by two homologous *lox* sites in frame with gene 3. Only phages that contained ORFs in frame with β-lactamase gene conferred ampicillin resistance and survived. After the selection of ORFs the lactamatase gene could be removed by Cre recombinase-induced recombination, allowing full display of in-frame ORF-g3p fusion products on phage surface. The pPAO2 vector based system was used to study the monoclonal antibody epitopes by filtered TG2 fragments [395]. Three monoclonal antibodies derived from the spleen cells of immunized mice were used for the selection of random fragments of TG2 gene expressed in pPAO2 phagemid vector. The epitopes recognized by the antibodies were determined by DNA sequencing, revealing that the epitopes overlap with each other and localized on the surface of TG2 protein. The phage display library of ORF fragments created from the mRNA of various tissues was used to study the interaction network of TG2 [396]. After two rounds of selection on TG2 the frequency of different ORFs in the page population was assayed by 454 sequencing allowing the identification and ranking of several potential interactors. Several new interacting proteins were identified and it was also possible to determine the specific domains involved in the interaction for example for fibronectin. In a study made in 2013 sera from celiac patients were used to select immunoreactive antigens from cDNA phage-display library constructed from human tissues [397]. Clones encoding ORFs were selected using sere from celiac patients. Using multiple analysis provided by protein microarray 42 putative antigens were identified, 13 of which were confirmed to be specific for celiac sera.The majority of the identified clones encoded nuclear antigens and mostly involved in interaction networks with one another as well as with nucleic acids and carbohydrates. Several antigens identified have been implied to be involved in various diseases, however none have been described previously to be targets of autoimmune responses. The antibody response against these antigens was not abolished on a gluten free diet and was not shared with other autoimmune diseases, indicating that the response is CD specific and independent from gluten. The results of the study provided not only a deeper understanding of the autoantibody response in CD but also showed the potential of phage display technology for identifying new targets in autoimmunity research.

Chapter 4. Recombinant antibody technology

4. 1. Early systems for therapeutic antibody generation

Nowadays antibodies are used for several applications in research, diagnostics, and therapy. They are widely used in many standard assays such as immunoblot, flow cytometry, or immunohistochemistry. In addition this, the emerging field of proteome research has a huge need for binders against different protein antigens[398, 399]. Beyond this, recombinant antibodies are used for the diagnosis of different pathogens [400-402] or toxins [403, 404]. In the past decade, several antibodies have been developed for therapeutic applications [405], primarily targeting inflammatory or tumor diseases [406]. Polyclonal antibodies have been used for research and diagnostics, however non-human polyclonal antibodies might exhibit immune response in humans interfering with therapeutic use for example after snake bites [407]. The first developed and most widely used technology for generation of monoclonal antibodies (mAbs) for therapeutic use was the hybridoma technology [408]. This technology was based on the fusion of antibody producing spleen cell from immunized mice or rats with immortal myeloma cell lines. The bottleneck of the system is that mice immunization cannot be used for highly toxic or conserved antigens. Another issue is the high immunogenicity of these foreign proteins in humans as well as the weak interaction that mouse antibodies typically have with human complement and FcγRs, resulting in inefficient effector functions. In addition, mouse antibodies do not bind the human salvage receptor FcRn [409] resulting in a terminal half-life that is typically less than 20 hours [410, 411]. These limitations of mouse antibodies have largely been overcome by their chimerization or humanization. A great success in antibody engineering was the possibility to generate chimeric antibodies, where the binding activity of IgG molecules is generated by the variable domains of the heavy and light chains. As antibodies are well conserved through evolution, it was possible to create chimeras by fusing murine variable domains, responsible for the binding activity, with human constant domains [412] leading to the development of a new generation of therapeutic candidates [413]. These chimeric antibodies were 70% human and possessed a fully human Fc portion, which made them considerably less immunogenic in humans allowing them to interact with human effector cells and the complement cascade.

The development of antibody engineering techniques it has made it feasible to decrease further the murine part of the generated mAbs by replacing the hypervariable loops of a fully human antibody with the hypervariable loops of the murine antibody of interest, using an approach called complementarity-determining region grafting [414]. This resulted in the generation of the first humanized antibodies which were 85–90% human and are even less immunogenic than chimeric antibodies. Most of the approved mAbs in current use are either chimeric or humanized. Another major improvement came with the development of *in vitro* selection methods, the most successful one being phage display which was discussed in the previous chapter. With the ever increasing power of antibody engineering, it became possible to clone entire repertoires of antibody fragment genes, from immunized or non-immunized animals or humans.

4. 2. Recombinant antibody formats

New antibody generation technologies have increased the amount of antibodies for different applications and, therefore, also the need of efficient production systems. IgG is a heterotetrameric molecule consisting of two heavy and two light chains connected and stabilized by disulfide bonds [415]. Because of this, IgG molecules require correct folding apparatus and an oxidizing environment for the generation of disulfide bonds. Since many traditional expression hosts do not provide these mechanisms, for efficient production, smaller antibody fragments have been developed which combine easier production with full antigen binding capacity of an IgG (Figure 1.19). In addition, the development of smaller fragments was the basis for most of the *in vitro* antibody generation systems [416-419]. These antibody fragments can be used for applications, where epitope binding is sufficient for the desired effect including therapeutic applications such as virus neutralization or receptor blocking.

4. 2. 1. Single domain antibodies

The smallest antigen binding fragment of immunoglobulins maintaining its complete antigen binding site is the Fv fragment, which consists only of variable (V) regions (Figure 1.19). In 1989, it was demonstrated that mouse variable domains could be used as single binding units [420], however the vast majority of these domains aggregate spontaneously.

Later it was found that camelids and sharks express a type of antibodies devoid of light chains [421] and new antigen receptor antibodies (IgNAR) [422]. These antibodies have a single variable domain (called VHH for camelids and V-NAR for sharks), which generates high affinities towards a large spectrum of antigens. These small domains (13 kDa) can be easily produced in bacteria or yeast and called domain antibodies (dAbs), single-domain antibodies (sdAbs) or nanobodies.

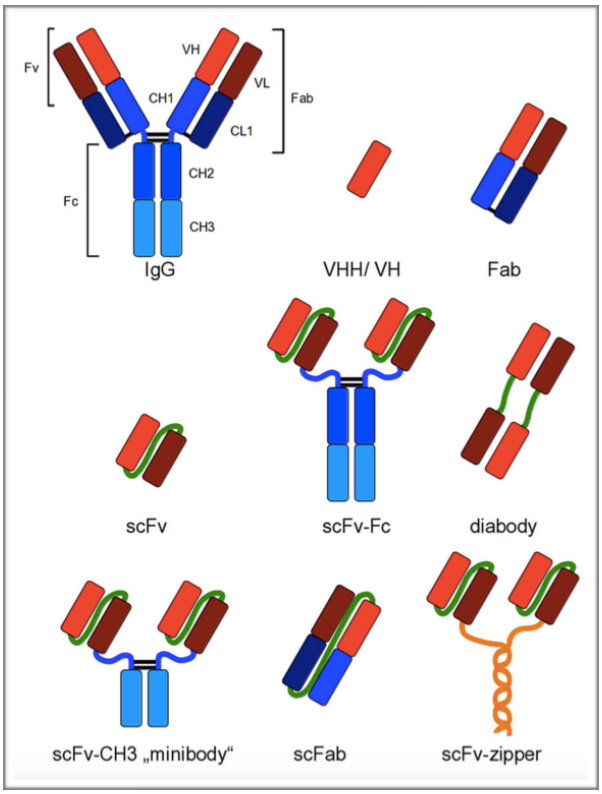

Figure 1.19. Recombinant antibody formats for different applications compared to IgG. Red and dark red: variable regions; blue: constant regions; green: artificial peptide linkers.yellow: dHLX represents amphiphatic helices used for dimerization of scFv fragments. [423]

4. 2. 2. Multi domain antibodies

The scFv fragments are composed of the variable domains of heavy and light chain connected by a flexible linker peptide (Figure1.19). These molecules were described as small fragments capable of retaining the binding activity of the full IgG molecule in a monovalent fashion. Fab fragments are composed of one constant and one variable domain of each of the heavy and the light chain, connected also by a flexible linker peptide (Figure 1.19.).

Nowadays scFvs and Fabs are the most widely used antibody fragments produced in prokaryotes. The issue of scFvs and Fabs is the very short half-life in serum (~2 h). By decreasing the length of the linker between the two domains, a dimer format was introduced called diabody [424] (Figure 1.19). Diabodies are compact, medium-size (60 kDa) molecules and can be a good choice for imaging purposes or radioimmunotherapy. Besides increasing the molecular weight, the dimerization provides bivalency, which leads to a higher avidity and higher tumor retention. Because of these features diabodies provide rapid tissue penetration, high target retention and rapid blood clearance. Because of the fact that they are rapidly eliminated through the kidneys, they limit the exposure to the bone marrow, which is most often the dose-limiting organ with intact radio-labelled mAbs. Other antibody formats have been produced in prokaryotic and eukaryotic cells, for example, disulfide-bond stabilized scFv (ds-scFv) [425], single chain Fab fragments (scFab) combining scFv and Fab properties [426] (Figure 1.19.).

For most therapeutic applications, the Fc moiety of an immunoglobulin is essential for the method of action as it mediates the effector functions such as cellular dependent cytotoxicity or the activation of the complement system. Therefore, antibody fragments have been fused to the Fc domain to regain effector functions and avidity [427, 428]. These constructs include scFv-Fc where the scFv fragment is substituted of Fc domain composed of CH2 and CH3 domain (Figure 1.19.) and minibody where scFv fragments are connected with CH3 domain (Figure 1.19.). The main importance of the Fc portion is that hey can recruit the complement cascade through interaction with C1q, ultimately leading to the formation of pores in the targeted cell membrane, or they can recruit effector cells through interaction with the C4b/C2b/C3b complex bound to the target cell surface and the receptor CR1 (complement receptor). Another important mechanisms by which IgG antibodies activate the cellular immune system is via interaction of the Fc domain with Fcg receptors (FcgRs). The human FcgR family contains six known members in three subgroups, including FcgRI (CD64), FcgRIIa,b,c (CD32a,b,c) and FcgRIIIa,b (CD16a,b), expressed by various effector cells of the immune system, including macrophages, neutrophils, dendritic cells and natural killer (NK) cells. NK cells are the main agent of antibody-dependent, cell-mediated cytotoxicity (ADCC). These cells can be recruited and activated through the interaction between FcgRIIIa and the Fc region, leading to the formation of an immunological synapse, the release of perforin/ granzyme and the establishment of the Fas/FasL interaction, both leading to apoptosis of the target cells.

4. 3. Recombinant antibody expression

Recombinant antibodies can be produced in large quantity and good quality in several different hosts. Prokaryotic hosts include Gram-negative bacteria such as *Escherichia coli* and also Gram-positive bacteria as *Bacillus brevis* [429] *Bacillus subtilis* [430] and *Bacillus megaterium* [431]. The most important eukaryotic hosts are mammalian cells, however antibodies can be efficiently produced in yeast such as *Pichia pastoris* [432], filamentus fungi as *Trichoderma* and *Aspergillus* species [433] or insect cells like *Spodoptera frugiperda*, or *Drosophila melanogaster* [434]. In this chapter prokaryotic production of recombinant antibodies will be described through *Escherichia coli* host. For eukaryotic expression mammalian hosts will be studied in more detail.

4. 3. 1. Prokaryotic expression system

Escherichia coli is the most important prokaryotic production system for recombinant proteins [435, 436]. For production of functional antibody fragments, the key feature is the secretion of both V chains into the periplasmic space of *E. coli* where the oxidizing environment allows the correct formation of disulfide bonds and the assembly to a functional Fv fragment [437]. This strategy was the first one allowing the expression of functional Fab fragments in *E. coli* described in 1988 [438]. The production of recombinant antibodies in the reducing cytoplasmic compartment results mostly in non-functional aggregates [439] and recovery of functional antibody fragments from cytoplasmic inclusion bodies by complete denaturation and refolding [440] is often not efficient. Nowadays most antibody fragments are produced in the periplasm of *E. coli* using N-terminal leader sequences targeting the periplasmic *Sec* pathway [441], for example signal peptides derived from outer membrane protein A (*OmpA*), alkaline phosphatase A (*PhoA*), or pectate lyase B (*PelB*) [442, 443]. After expression, recombinant antibodies are usually isolated from the periplasmic fraction [444, 445] but also from the culture supernatant [446]. Expression of recombinant antibodies can also be increased by optimization of cultivation parameters, such as temperature, media, or additives which depend on the individual antibody fragment [447, 448]. The production system itself influences the production rate. Very high yields of antibody fragments produced in *E. coli* are mainly provided by high-cell density fermentation in bioreactors [449] or optimized shake flasks [450].

5. 3. 2. Eukaryotic expression in mammalian cells

Today, 95% of the currently approved therapeutic antibodies are still produced in mammalian cell lines despite relatively high production costs and difficult in handling. This is because of the advanced mammalian folding, secretion and post-translational apparatus is capable of producing antibodies indistinguishable from those in the human body with least concerns for immunogenic modifications. The system is also capable of highly efficient secretion of large and complex IgGs and in combination with the folding and post-translational control it results in high product quality. Chinese hamster ovary (CHO) cells are the most common cells applied in the commercial production of recombinant antibodies. This cell line isolated in the 1950s gave rise to a range of genetically different progeny, such as K1-, DukX B11-, DG44- cell lines and others which differ in protein product quality and achievable yield. In addition, Per.C6 cells, mouse myeloma NS0 cells, baby hamster kidney (BHK) cells and the human embryonic kidney cell line HEK293 has proved to be efficient production hosts. In order to have an effective production the antibody gene expression cassettes have to be stably integrated into the host cell genome. Strong promoters like the immediate early cytomegalovirus (CMV) or the cellular elongation factor (EF) 1-alpha promoter and polyadenylation sites from the simian virus (SV) 40 or the bovine growth hormone (BGH) for improved mRNA stability and translation efficiency are usually implemented into the expression vector. The generation of high producer cell lines has been dramatically improved and accelerated [451], however it is still too expensive, time-consuming and laborious for research applications, or if large numbers of individual antibodies have to be produced. Transient and semi-stable mammalian antibody expression is much more suitable in many cases since it allows fast and parallelized production without any need to generate producer cell lines [452]. Transient antibody production is suitable for small-scale production in antibody screening [453], but also capable to generate grams of antibodies [454, 455].

RESULTS

Chapter 1. Developing TG2 inhibitor antibody

Recombinant antibody technology provides an effective tool for generating therapeutic antibodies for a great variety of diseases including cancer and autoimmune diseases. Celiac disease is among the most widespread of the autoimmune diseases affecting the 1% of western population. Despite the efforts no effective therapeutic has been generated for the disease, therefore the only known treatment is lifelong gluten free diet. Based on this there is an increasing need to generate recombinant antibodies with the ability to inhibit the enzymatic function of TG2 and this way contribute to the treatment of the disease.

The main objective of our project is to dissect the autoantibody response in CD. One feature of the antibodies that we would like to determine is the inhibitory effect on TG2 enzymatic activity. To achieve this goal we had to develop a system to efficiently test TG2 in vitro transamidating activity and also a reliable inhibitor antibody that we could use as control in the experiments. In 2013 a patent has described the generation of a series of TG2 inhibitory antibodies (ref:WO2013/175229 A1). Antibodies were created by immunizing mice with recombinant human TG2 encompassing amino acids 143-473 of the TG2 core. The sequence of the best TG2 inhibitor was retrieved and used to generate the antibody in scFv-Fc format, widely used in our systems. The purified antibody was tested for its specificity and used to develop the inhibition assay on TG2.

Workflow:

- Construction of antibody in scFv-Fc format in pMA-T vector
- Cloning and expression in pMB-SV5 vector.
- Developing TG2 inhibition assay on solid surface and in solution

1. 1. Construction of antibody in scFv-Fc format in pMA-T vector

In order to generate a control inhibitory antibody we used the sequence of the best inhibitor described in the patent (ref:WO2013/175229 A1) (Table 2.1). The antibody with code hAb004 comprised of variable heavy chain IGHV3-23*01 F and variable light chain IGKV1-16*01 F. The antibody was designed by GENEART synthesis in scFv format into pMA-T conventional plasmid. In the construct the variable chains were cloned in an order VL followed by VH and the two chains were separated by a flexible loxP511 protein linker to form the functional scFv. At the N-terminal of the VL a BssHII restriction site, while at the C-terminal of the VH an NheI site was introduced for the efficient subcloning into pMB-SV5 vector.

Clone ID	VH	VL
hAb004	IGHV3-23*01 F	IGKV1-16*01 F

Table 2.1. The sequence and VH/VL usage of hAb004 anti TG2 antibody was determined.

1. 2. Cloning and expression in pMB-SV5 vector.

In order to express the antibody in the scFv-Fc format widely used in our systems hAb004 was subcloned into pMB-SV5 vector (Figure 2.1). The sub-cloning was characterized by the use of BssHII-NheI cassette. For the mammalian expression a CMV promoter and a leader sequence was introduced at the N-terminus. At the C-terminus the CH2 and CH3 domains of Fc from Human IgG1 were connected to the scFv fragment by a Hinge region. An SV5 tag at the 3' of the construct was present for uniform detection.

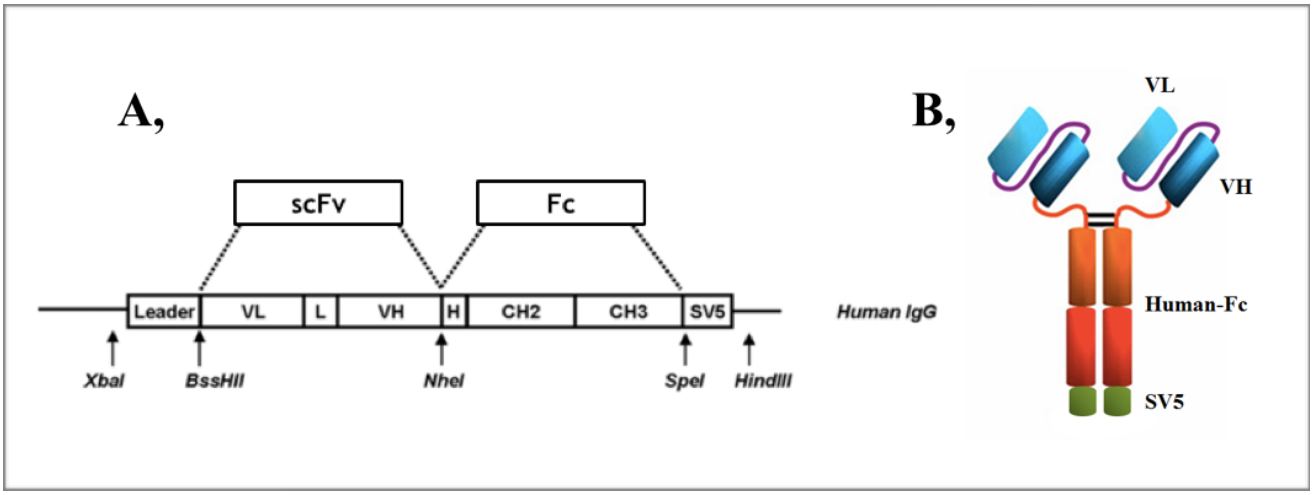

Figure 2.1. Schematic representation of the cloning vector (A) modified from[393] and expressed scFv-Fc (B).

After cloning vector was checked by DNA sequencing and the purified plasmidic DNA was transfected into CHO-S cells. The secretion of the scFv-Fc in the culture medium was analyzed after 72 h by ELISA on plates coated with hTG2, mTG2 and unrelated control protein (BSA). The results were determined by recognition of the SV5 tag at the C-terminus of the construct. hAb004 had reactivity on hTG2 with no cross-reactivity with the murine protein, as it was reported in the patent (ref:WO2013/175229 A1) (Figure 2.2).

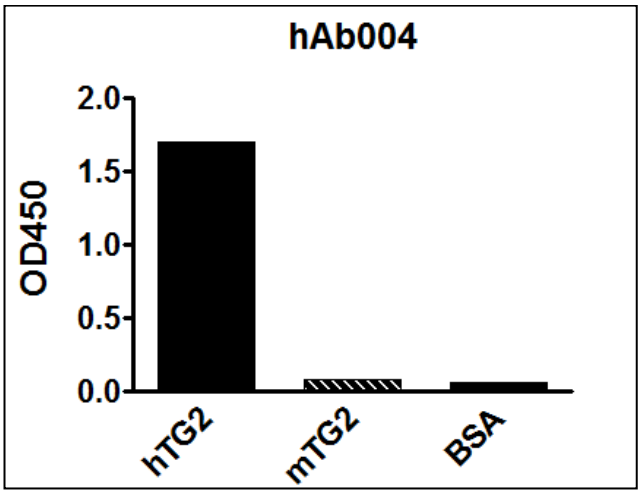

Figure 2. 2. TG2 inhibitory antibody hAb004 expressed in CHO cells was tested as primary antibody in ELISA on hTG2/mTG2/BSA. As C(+) an scFv-Fc with murine cross-reactivity was used. As C(-) a non TG2 specific antibody was used.

Stable cell line was made by growing cells in the selective agent for the Hygromycin resistance. Purification of the scFv-Fc hAb004 was done using Protein A agarose with a production yield ranging 3 mg/l of culture. Purified hAb004 analyzed by Comassie and Western blot under reducing conditions (Figure 2.3.). The antibody was revealed by a mouse antibody recognizing the SV5 tag, which was followed by anti Mouse-AP secondary antibody. Purified hAb004 appeared at predicted molecular weight which was around 55 kDa due to the fusion of the scFv with constant domains and SV5 tag and additionally the loss of interchain disulfide bond in the Hinge region as result of the reducing condition.

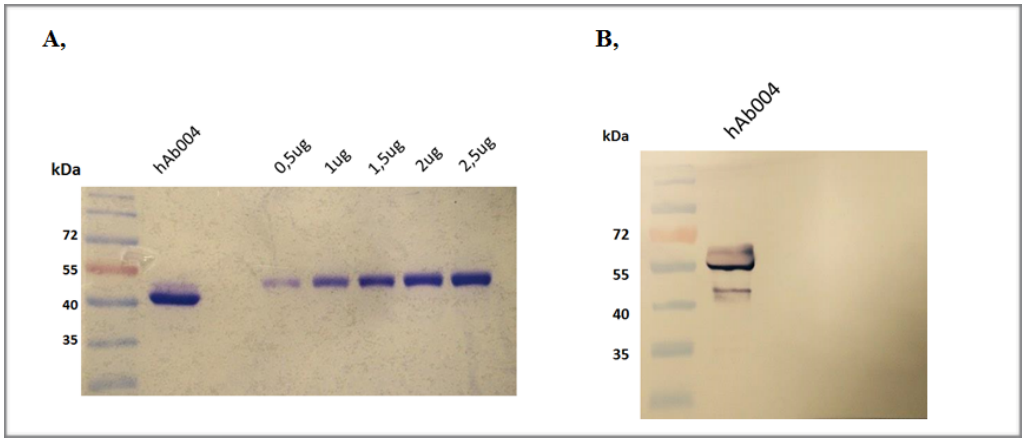

Figure 2.3. Comassie staining **(A)**, and Western blot **(B)** image of purified hAb004 antibody in scFv-Fc format.

The specificity of purified hAb004 was dissected by ELISA made on hTG2 and mTG2 **(**Figure 2.4.). The antibody was used in different concentrations (0,1-0,25-0,5 ug/ml) to further investigated the reactivity. As is has been seen before hAb004 had specific reactivity towards hTG2.

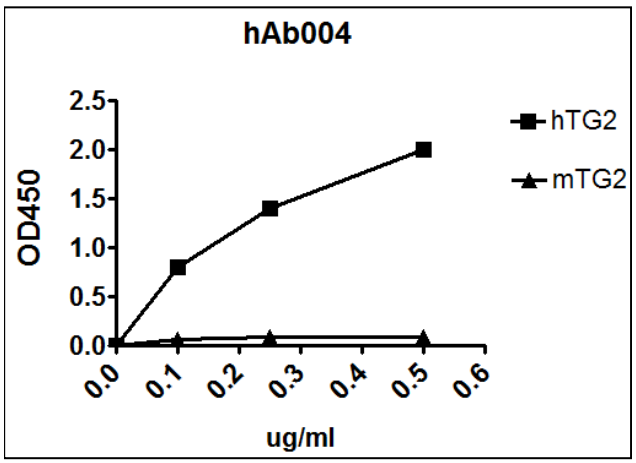

Figure 2.4. ELISA on hTG2 and mTG2 using three dilutions of hAb004: 0.1-0.25-0.5ug/ml.

1. 3. Developing TG2 inhibition assay on solid surface and in solution

In order to develop an inhibition assay first an ELISA test measuring TG2 *in vitro* transamidating activity was done. The test was based on the coupling of 5 - (biotinamido)-pentyalamine to NN-dimethylcasein which was catalyzed by recombinant hTG2. The biotinilated primary amine and DMC were incubated for 1 hour at 37^0C in the presence of TG2, DTT and Ca^{2+}. The incorporation of biotin into NN-dimethylcasein was revealed by Streptavidin conjugated with peroxidase. The inhibitory effect of hAb004 was investigated by introducing a preliminary incubation step of TG2 and the antibody. This step allows the antibody to bind TG2 molecule and inhibit it's enzymatic activity before that could meet its substrates. In the case of inhibition TG2 transamidating activity is blocked causing a significant decrease in the signal (Figure 2.5).

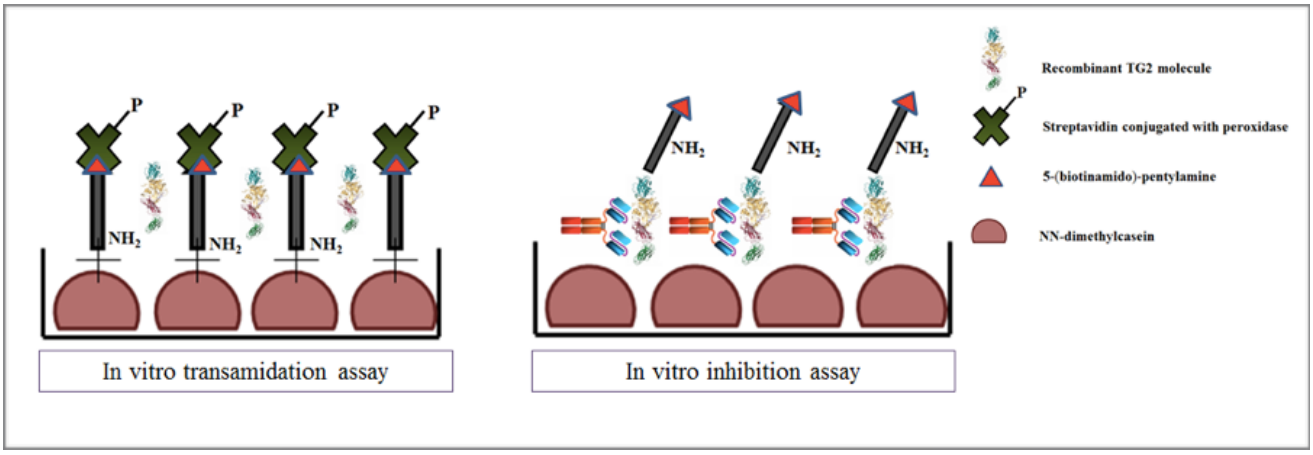

Figure 2.5 Schematic representation of in vitro assays. DMC indicated in brown, 5'-Streptavidin(biotinamido)-pentyalamine in dark green stick while Streptavidin conjugated peroxidase with an X. **A.** *In vitro* transamidation assay where the coupling of biotinilated primary amine and DMC is catalyzed by recombinant hTG2. **B.** *In vitro* inhibition assay where the scFv-Fc hAb004 is blocking the enzymatic effect of hTG2 molecule, interfering with the coupling of the two substrates.

In the inhibition assay hAb004 was used in different concentration ranging from 24nM-0,188nM (Figure 2.6). As negative control for inhibition a non TG2 specific antibody was used in the same scFv-Fc format. In the experiment hAb004 proved to have a significant inhibitory effect on TG2 enzymatic activity in a dose dependent manner. The inhibitory effect was similar what was seen in the patent (ref:WO2013/175229 A1) meaning that hAb004 retained its inhibitory potency in scFv-Fc format.

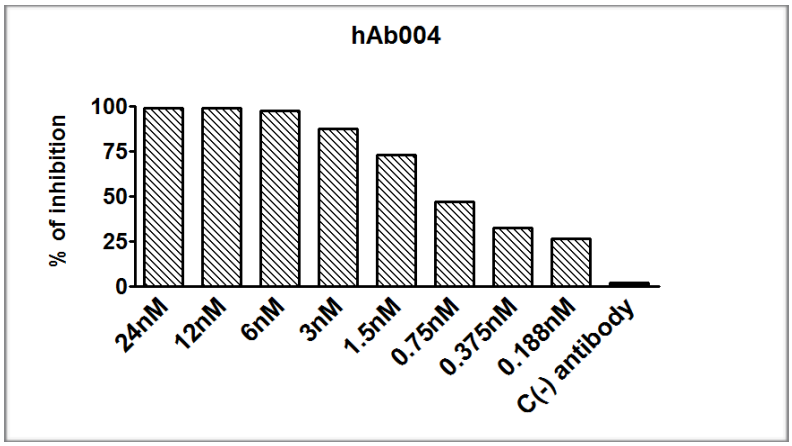

Figure 2.6 *In vitro* transamidation assay performed on solid surface using hAb004 in concentrations ranging from 24-0,188nM.

Antibody hAb004 was tested for the inhibitory effect in solution. In the assay TG2 and hAb004 were pre-incubated to allow the antibody to bind and inhibit TG2. After incubation the mix was substituted with 5'- (biotinamido)-pentyalamine and N N-dimethylcasein. A volume of the mix which corresponded to 0,5ug of DMC was separated by SDS/PAGE and transferred onto nitrocellulose membrane. Incubation was done with Streptavidin-Alkaline Phosphatase antibody and revealed by NBT/BCIP. As control positive for transamidation TG2 used in the presence of Ca^{2+} without a pre-incubation step with the antibody, while negative control was TG2 with no Ca^{2+} added. Control negative antibody was a non TG2 specific antibody in same scFv-Fc format. The reduction in the signal closely mirrors the ratio of antibody added to the reaction. The inhibitory effect is corresponding what we saw in ELISA experiments (Figure 2.7.).

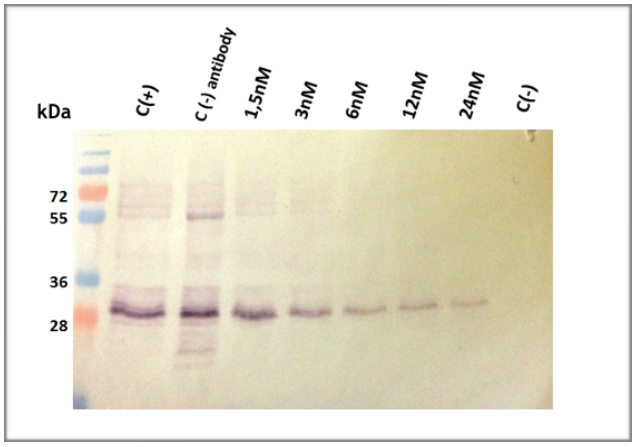

Figure 2.7. *In vitro* transamidation assay in solution. C(+) is hTG2 used in the presence of Ca^{2+} while C(-) is hTG2 with no activating Ca^{2+} added. C(-) antibody was a non TG2 specific antibody in scFv-Fc format. hAb004 was used in different concentrations ranging from 1.5-24nM.

Chapter 2. Dissecting anti TG2 antibody response in CD by phage display technology

Aim of the research

Celiac disease (CD) is characterized by destruction of intestinal epithelium and the secretion of gluten-and TG2-specific IgA and IgG antibodies. TG2 acts not only as the main trigger of the disease but also as a self antigen and therefore the study of autoimmune response against this enzyme is crucial for understanding the disease. The aim of the research is to dissect the molecular basis underlying the biological properties of TG2 via the characterization of autoantibody specificities to TG2 epitopes in celiac disease. Antibodies in scFv format were derived from phage antibody libraries originating from the intestinal biopsy lymphocytes of CD patients. These antibodies were clustered into epitope groups according to their cross-reactivity with murine TG2 and by competing them with reference antibodies representing different VH families. All together four epitope clusters were determined, and one antibody representing each epitope cluster was further characterized in scFv-Fc format expressed in eukaryotic cells. The interaction between antibody and TG2 in the extracellular environment was analyzed by staining TG2 expressed on cell surface (csTG2) and TG2 bound to fibronectin (FN). All but one epitope groups were able to stain csTG2 indicating that most epitopes are displayed on the surface of cells. VH5 antibodies representing the most dominant antibody gene family in CD were not able to stain FN-bound TG2 suggesting that this epitope is overlapping with FN binding site on TG2 molecule. The inhibitory effect of antibodies on TG2 enzymatic activity was tested by in vitro transamidating assay, where only one epitope group had inhibitory effect on the activity of the enzyme.

Workflow:

- Construction of phage antibody libraries
- Characterizing antigen specificity in scFv format
- Clustering antibodies into epitope groups in scFv format
- Cloning and expression in scFv-Fc format
- Dissecting TG2-autoantibody interaction in the extracellular environment
- Investigating inhibitory effect of antibodies in scFv-Fc format

2. 1. Introduction to the results

The aim of the project was to dissect the autoantibody response against the self antigen TG2 in the onset of Celiac disease. In the course of the project we used a combination of molecular biological tools that enabled a deeper understanding of the response **(**Figure 2.8).

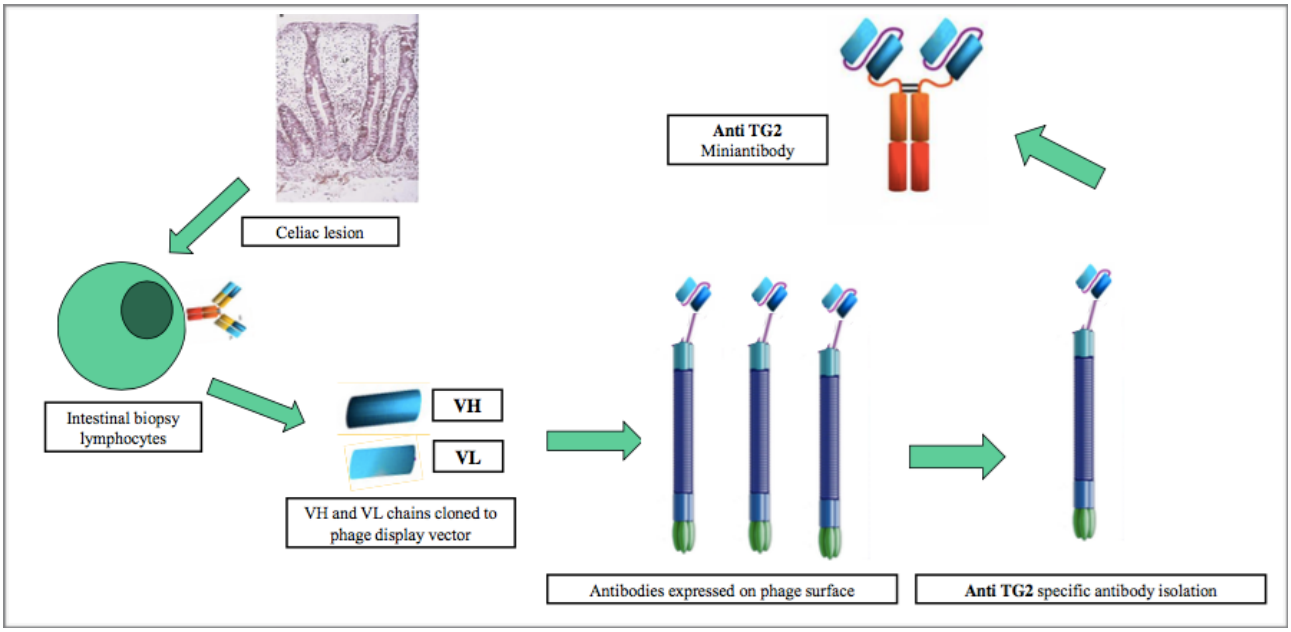

Figure 2.8. Schematic representation of workflow used to dissect autoantibody response against TG2.

A. Construction of phage antibody libraries: Phage antibody library generated from the intestinal biopsy lymphocytes of 7 CD patients, where the antibody repertoire in scFv format was displayed on filamentous phage.

B. Characterizing antigen specificity in scFv format: Antibodies coming from phage antibody libraries were tested for their reactivity and specificity. The selection output of the six libraries were first screened on hTG2 by ELISA. The clones found to be positive were tested for their specificity using mTG2 in ELISA experiments.

C. Clustering antibodies into epitope groups in scFv format: Antibodies in scFv format were clustered into epitope groups by competing them with reference antibodies. The experiment was performed as competition phage ELISA where reference antibodies in scFv-Fc format, representing different VH families were used to compete the positive clones of phage antibody libraries expressed in scFv format.

D. Cloning and expression in scFv-Fc format: Reference clones representing each epitope cluster were cloned into mammalian expression pMB-SV5 vector and expressed in scFv-Fc format in Chinese hamster ovary (CHO) cells. Antibodies were tested for retaining reactivity in scFv-Fc format by performing ELISA on different TG2 proteins.

E. Dissecting TG2-autoantibody interaction in the extracellular environment: The effect of TG2-FN interaction on antibody recognition was investigated. Competition ELISA was performed using FN 45kDa domain to compete antibodies for binding TG2.
Antibodies were tested to recognize cell surface TG2 by using them for staining various TG2 expressing cell lines.

F. Investigating inhibitory effect of antibodies in scFv-Fc format: The inhibitory properties of antibodies was determined by using them *in vitro* transamidation assays performed in solution and in solid surface.

2. 2. Construction of phage antibody libraries

Phage display of human antibody fragments proved to be an extraordinary tool to study the autoantibody response in celiac disease. In this method the patients antibody repertoire is expressed fused to the coat protein of a filamentous phage carrying the encoded antibody gene. In the system each phage carries a single antibody specificity allowing to dissect the antibody response against self antigen TG2.

Phage antibody libraries used in this study were constructed as described in Marzari et al. [286] where libraries were generated from the IBL of three untreated adult CD patients. The biopsy materials were obtained from patients undergoing intestinal biopsy in order to confirm the diagnoses. Total mRNA preparation of IBL was performed and followed by cDNA synthesis using random primer reverse transcription and normalization. The VH and VL chains were amplified from cDNA by PCR using a set of oligonucleotides that recognize human V genes [392]. The 3' primer used for the VH chain was specific for IgA antibodies, the most abundant ones in the anti TG2 response. Amplified VH and VL chains were assembled by PCR [380] and cloned in scFv format into pDAN5 phagemid vector [387] (Figure 2.2). In the vector variable chains are cloned in an order VL followed by VH and the two chains are separated by loxP511 protein linker to form the functional scFv (Figure 2.9). The scFv genes are cloned upstream the gene encoding pIII minor coat protein allowing the antibody expression fused to the phage surface. Antibodies were affinity selected on hTG2 and after every cycle of selection the eluted phages were re-amplified for the next cycle and tested by ELISA against hTG2.

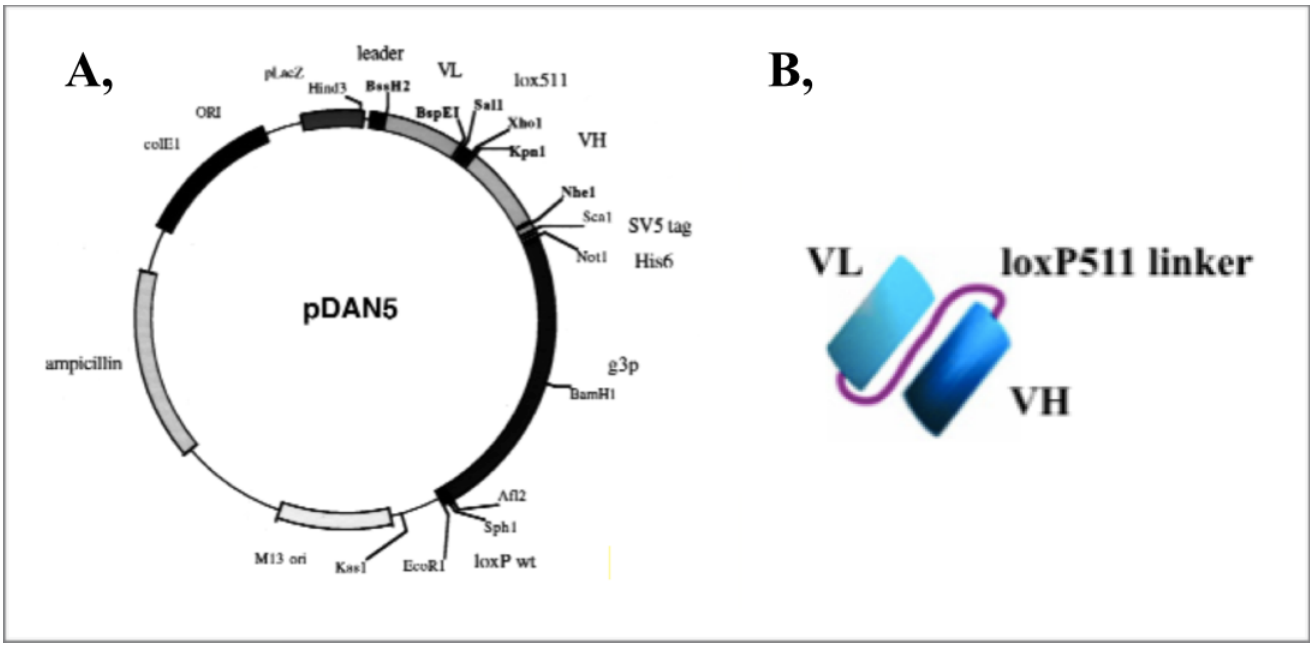

Figure 2.9. (A,) Map of phagemid vector pDAN5 with an scFv cloned [387]. **(B,)** Structural image of scFv. The VL and VH chains are separated by the loxP511 flexible linker peptide.

2. 3. Characterizing antigen specificity in scFv format

The selection output of phage antibody libraries generated from the IBL of seven patients was analyzed for the positivity on the selecting antigen by screening in phage ELISA on hTG2. A number of 28 clones proved to be hTG2 positive. The positive clones were tested for their specificity first, by testing reactivity on recombinant TG2 with human and mouse origin (Figure 2.10.).

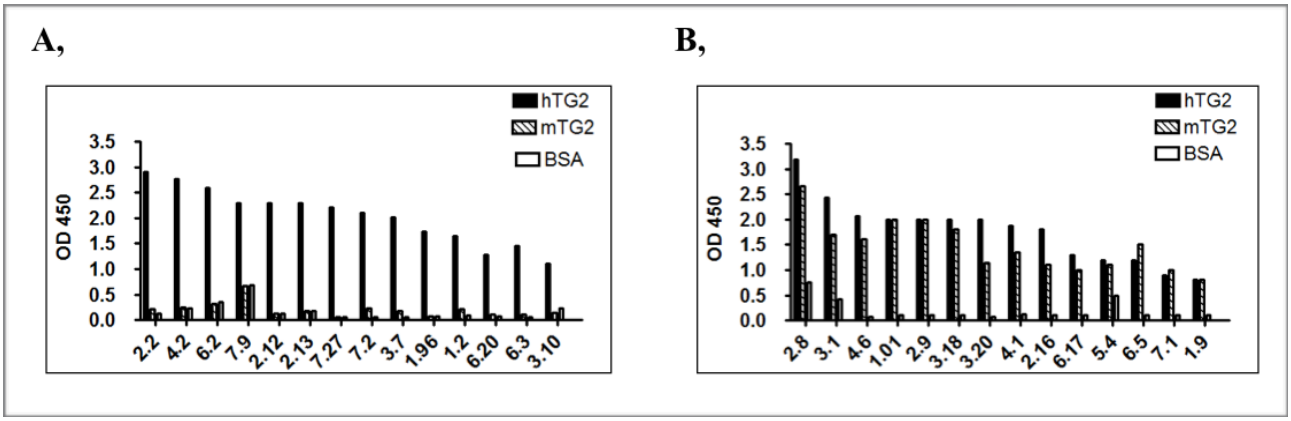

Figure 2.10. Reactivity of the 28 positive clones of the selection output examined by phage ELISA. **(A,)** 14 clones proved to be hTG2 specific. **B.** Total number of 14 clones showed cross-reactivity with mTG2.

It is known that there is a significant homology between the human and mouse TG2 and due to this phage ELISA experiments were performed using these proteins and an unrelated control (BSA). As it was expected a significant portion of the clones had cross-reactivity. Out of the 28 hTG2 positive clones 14 showed cross-reactivity with mTG2 while 14 clones proved to hTG2 specific. This ratio of the clones correlates what has been shown in other studies [286, 294].

2. 4. Clustering antibodies into epitope groups in scFv format

Anti TG2 antibodies have been reported to recognize distinct epitopes located on TG2 molecule [286, 294]. In order to investigate the epitope usage of the 28 selected clones competition phage ELISA experiments were carried out using reference antibodies. The reference antibodies used for the test were coming from the phage antibody library described in a previous paper by our group [286] were expressed in scFv-Fc format [393], by the genetic fusion of anti TG2 scFv to human IgG1 Fc domain. Three reference antibodies represented two epitope groups [286]. Two VH5 antibodies in scFv-Fc format (2.8 and 4.1) [393] used as reference antibodies for competing clones with mTG2 cross-reactivity belonged to Epitope 1 [286] (Figure 2.11).

All VH5 antibodies from CD patients were shown to recognize this epitope which was shared by murine TG2. From the 14 tested mTG2 reactive clones 11 had competition with reference antibody 2.8 and 9 with reference antibody 4.1. As most of the tested clones had high competition with both VH5 antibodies we can consider Epitope 1. as the reference epitope for clones having cross-reactivity with the murine protein.

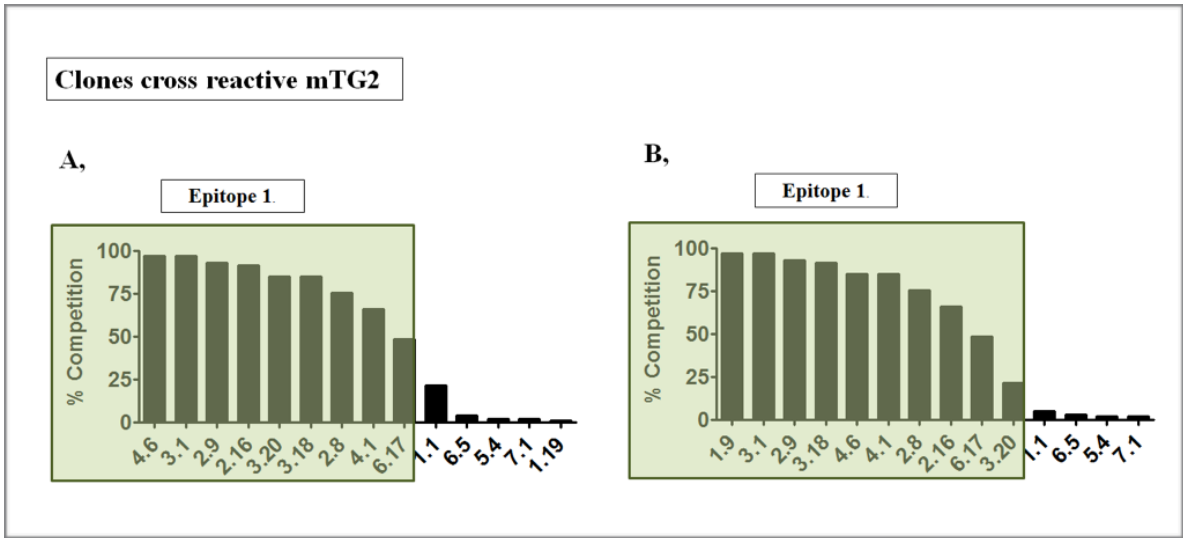

Figure 2.11. Competition pattern of mTG2 cross-reactive clones on VH5 reference antibodies. **(A)** Competition result using reference antibody 2.8 **(B)** Competition result using reference antibody 4.1 .Antibodies considered to represent Epitope 1. indicated in green.

Antibodies specific for hTG2 were tested with a VH3, Epitope 2. antibody (4.2) [456] in scFv-Fc format (Figure 2.12.). Antibodies belonging to this cluster were directed to a second, less well-defined epitope, characterized by an exclusive presence on hTG2, in spite of the high homology between hTG2 and murine TG2. From the 11 tested clones 4 had significant competition with the reference antibody while 7 had no competition indicating that there are at least two epitope clusters among the TG2 specific clones. The antibodies having competition with the reference antibody represented Epitope 2. while those which were not competing represented Epitope 3. cluster. The last competition study was carried out using reference antibody 4 (7.12), a VH3 antibody which has been shown to have different epitope recognition than the ones used previously (Figure 2.12.). Competition experiment was carried out using two Epitope 1. antibody (2.8, 6.17) one Epitope 2. (4.2) and two Epitope 3. antibody (7.27 and 3.7). Only one antibody, 7.27 had competition with reference antibody 4. and became representative of Epitope 4.

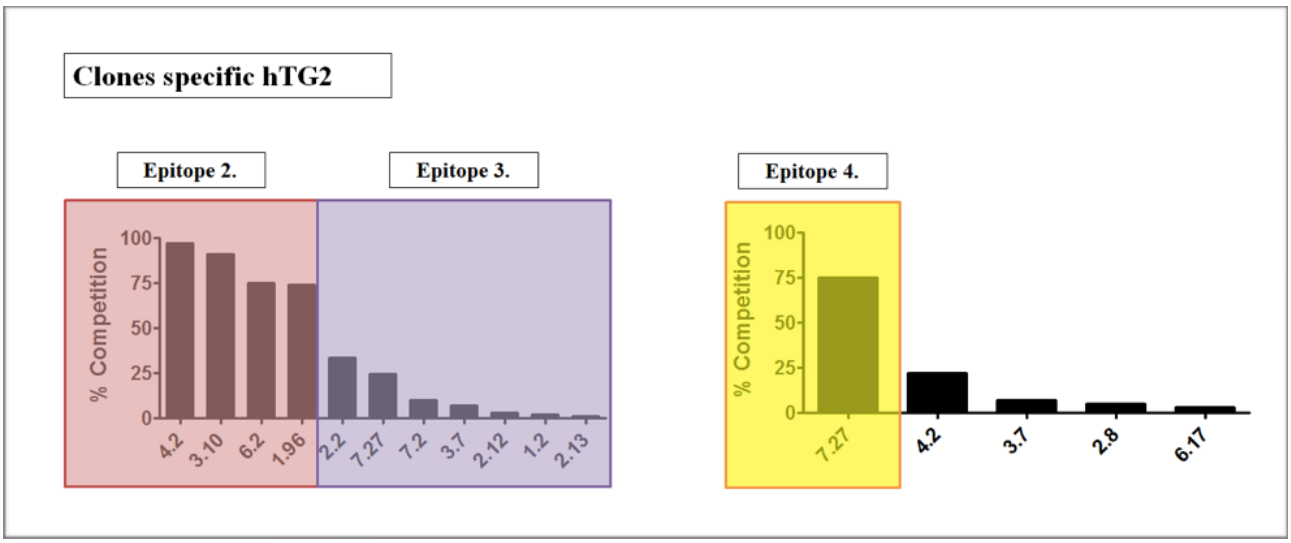

Figure 2.12. Competition pattern of TG2 specific clones on hTG2 specific clones. Clones competing with Reference antibody 3. clustered into Epitope 2. (indicated in red) while clones not competing clustered to Epitope 3 (indicated in violet). Reference antibody 4. competed only with one antibody which represented Epitope 4. (indicated in yellow).

The results of the competition experiments indicate that there are at least four epitope groups among the TG2 positive clones (Figure 2.13). Epitope 1., found to be the largest, contained all the clones showing mouse cross-reactivity and competition with two VH5 antibodies. The clones specific to hTG2 were tested with two VH3 antibodies and divided into three clusters. The findings are supported by data from the literature [294] where similar conclusions were made on mAbs generated from single plasma cells of CD patients.

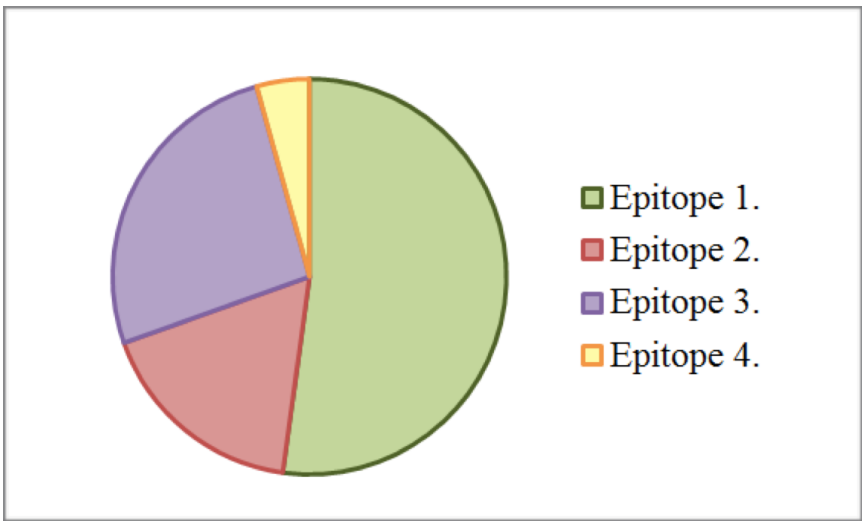

Figure 2.13. Grouping of TG2-reactive scFvs according to epitope binding.

2. 5. Cloning and expression in scFv-Fc format

In order to further investigate the autoantibody response against TG2 five clones representing all the four epitope clusters were selected to be cloned and expressed in scFv-Fc format (Table 2.2.). Two antibodies (2.8, 6.17) representing Epitope 1. belonged to VH5, the most abundant antibody gene family of the epitope cluster. The antibody representing Epitope 2. (4.2) belonged to VH3, the antibody representing Epitope 3. (3.7) belonged to VH1 and the Epitope 4. antibody (7.27) belonged to VH3 antibody gene family.

Clone ID	VH	VL	Epitope cluster
2.8	IGHV5-51*01 F	IGKV1-5*03 F	Epitope 1
6.17	IGHV5-51*01 F	IGKV1-5*03 F	Epitope 1
4.2	IGHV3-30*03 F	IGKV3-7*02 ORF	Epitope 2
3.7	IGHV1-2*02 F	IGKV1-39*01 F	Epitope 3
7.27	IGHV3-30*02 F	IGLV1-44*01 F	Epitope 4

Table 2.2. The VH and VL genes usage of the reference antibodies representing all the four epitope cluster.

For the expression in scFv-Fc format reference scFvs from pDAN5 phagemid vector were cloned to modified pMB-SV5 mammalian expression vector [393]. As described before, the subcloning was characterized by the use of BssHII-NheI cassette. After cloning all vectors were analyzed by DNA sequencing and transfected into CHO cells. The secretion of the scFv-Fcs in the culture medium was analyzed after 72 h by ELISA on plates coated with hTG2, mTG2 and unrelated control protein (BSA). The results were determined by recognition of the SV5 tag at the C-terminus of the construct. All antibodies were expressed and had the same reactivity as in scFv format (Figure 2.14).

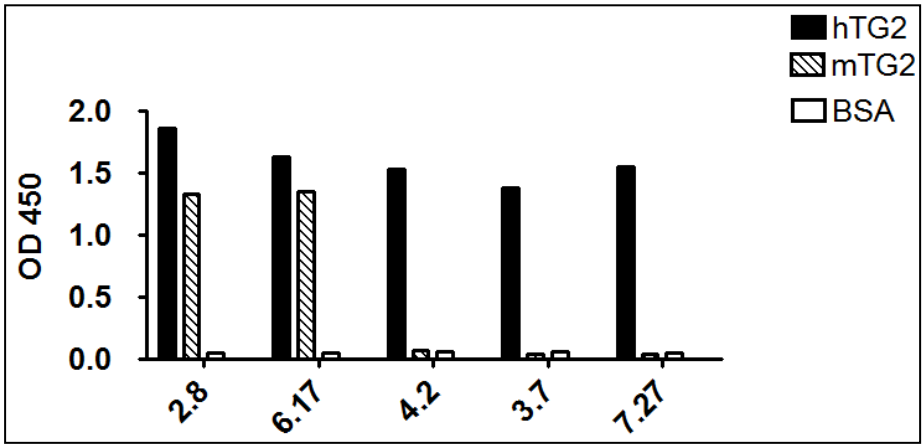

Figure 2.14. ELISA of supernatants of cultured CHO-s cells transfected with plasmid pCDNA3.1/Hygro(+) carrying scFvs fused to Fc domain of human IgG1.

Stable cell lines for each clone were made by growing cells in the selective agent for the Hygromycin resistance. Purification of the scFv-Fcs was done using Protein A agarose with a production yield ranging 4-7 mg/l of culture. Purified scFv-Fcs were analyzed by Comassie and Western blot under reducing conditions (Figure 2.15.). Antibodies were revealed by a mouse antibody recognizing the SV5 tag, which was followed by anti Mouse-AP secondary antibody. Antibodies appeared at predicted molecular weight which was around 55 kDa.

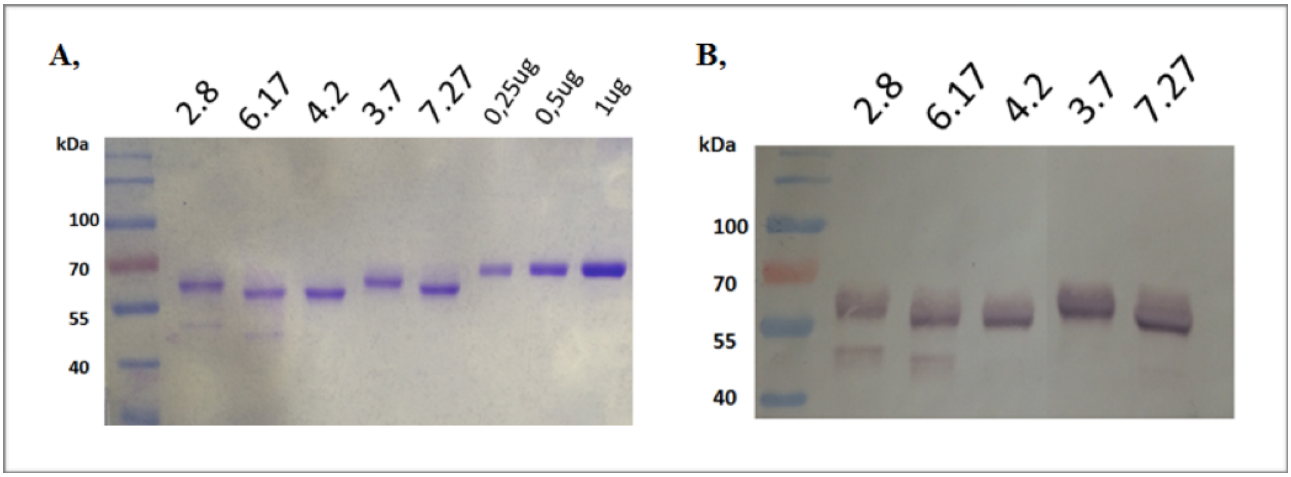

Figure 2.15. Comassie staining **(A)** and Western blot **(B)** of purified scFv-Fcs under reducing condition.

The specificity of purified scFv-Fcs was dissected by ELISA made on hTG2, mTG2 and an unrelated control protein (BSA) **(**Figure 2.16). Antibodies were used in different concentrations (0,1-0,25-0,5 ug/ml) to further investigated the reactivity. Specificity was retained for all antibodies in scFv-Fc format giving us two distinct groups. The Epitope 1. antibodies recognized both hTG2 and mTG2 in a concentration dependent manner. This correlates to the reactivity what they had as phage expressed scFvs and also what is typical for anti TG2 antibodies belonging toVH5 antibody gene family. The other three epitope groups also kept their specificity towards hTG2.

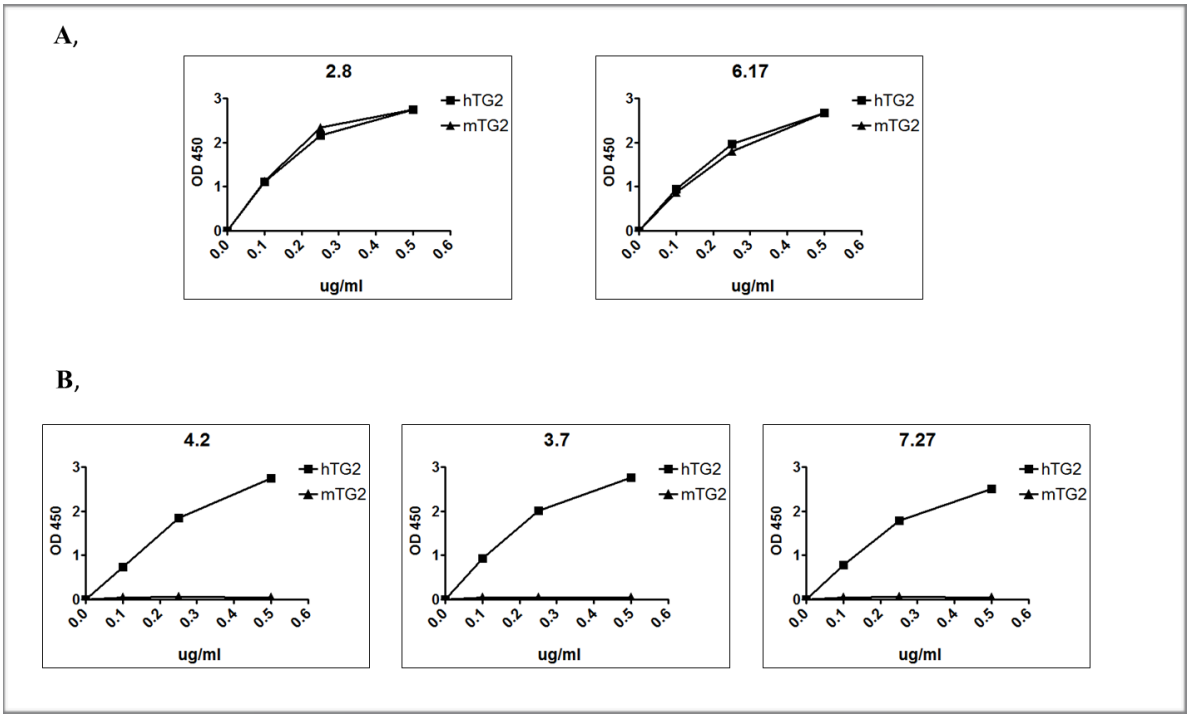

Figure 2.16. Saturation binding curves for selected scFv-Fcs representing two distinct groups. Antibodies used in increasing concentrations from 0,1-0,5 ug/ml. **A.** Epitope 1. antibodies having cross-reactivity with mTG2. **B.** Three antibodies representing Epitope 2, 3, 4 being specific to hTG2.

2. 6. Dissecting TG2-autoantibody interaction in ECM

2. 6. 1. Analyzing the effect of TG2-FN interaction on antibody binding.

In the extracellular environment TG2 interacts with the ubiquitous and abundant ECM protein fibronectin (FN) [85]. The specific binding between TG2 and fibronectin in the ECM involves the gelatin-binding domain of fibronectin [86] and the N-terminal β-sandwich domain of TG2. The formation of TG2-fibronectin complex has a significant impact on cell adhesion and migration signaling events [457]. The effect of TG2-fibronectin association on antibody binding has been reported by a study using monoclonal antibodies isolated from single plasma cells of CD patient [294]. To investigate if the association with fibronectin influences the binding between TG2 and Ab, we tested the ability of scFv-Fcs from each epitope group to react with fibronectin-bound TG2 (Figure 2.17). We found that scFv-Fcs representing Epitope 1. lost their reactivity when TG2 was associated with fibronectin. This indicates that this epitope overlaps with the fibronectin binding site in TG2. The other scFv-Fcs representing the other three epitope groups did not loose their reactivity suggesting that their epitopes are located in a different position in TG2 molecule.

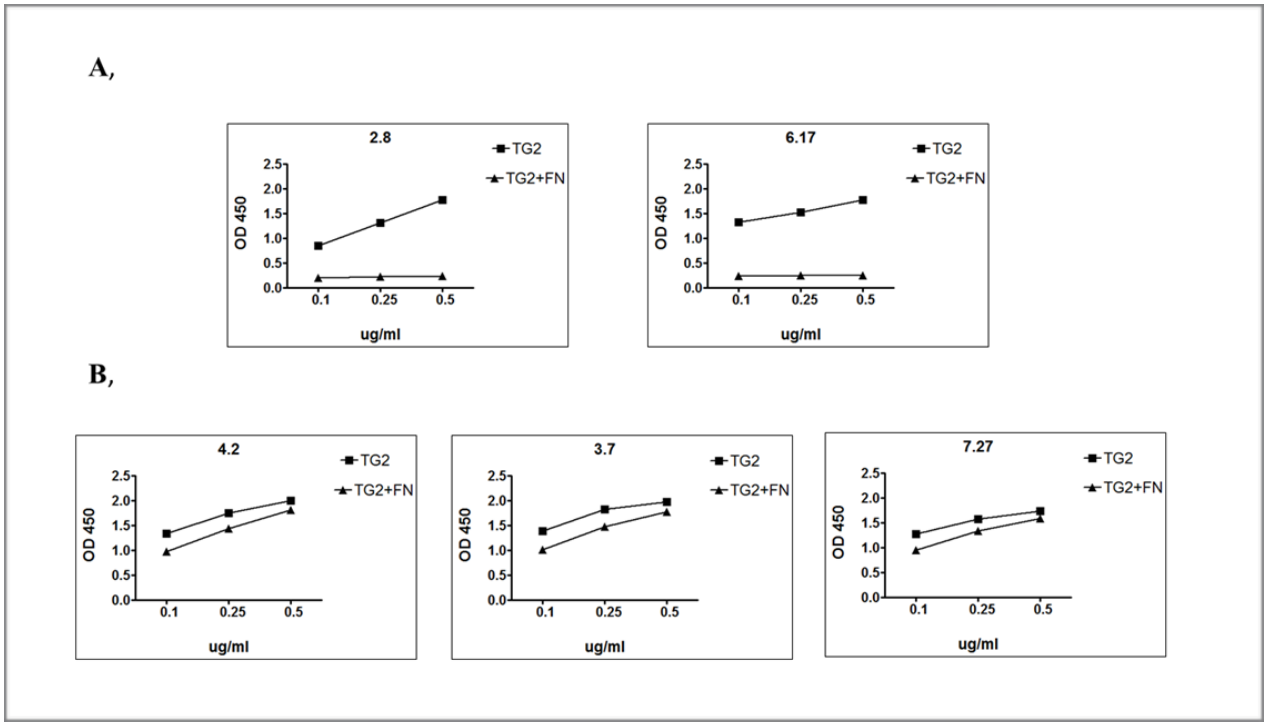

Figure 2.17. Comparison of scFv reactivity to TG2 alone and TG2 captured on human fibronectin. Antibodies in scFv-Fc format used in increasing concentrations ranging from 0,1-0,5ug/ml. **A.** Epitope 1. antibodies loosing their reactivity when TG2 is associated with fibronectin. **B.** Epitope 2., 3., and 4. antibodies have no difference in signal intensity when TG2 was bound to fibronectin.

2. 6. 2. Investigate the staining properties of antibodies on csTG2

TG2 has been found to be expressed on the surface of different cell types, where it interacts with fibronectin, integrins of the β1 and β3 subunit and heparan sulfate proteoglycans [85, 90, 105]. Anti TG2 antibodies isolated from single plasma cells of CD patients expressed in IgG1 format were tested for staining csTG2 on the surface of dendritic cells [294]. None of the antibodies showed staining on csTG2. We wanted to investigate if different origin and expression can have an effect on staining properties, since our antibodies are originating from phage antibody libraries and expressed in scFv-Fc format. It is possible to detect TG2 on the surface of various cancerous cell lines with mAb TG100. We used this antibody as a control to test the staining properties of our antibodies in flow cytometry using high TG2 expressing ovarian cancer cell lines such as SKOV3 and MCF7 (Figure 2.18). All except one epitope group (Epitope 4.) representatives were able to stain csTG2. This indicates that most of the epitopes are accessible when TG2 is expressed on cell surface. Both VH5 antibodies belonging to Epitope 1. were able to stain csTG2. Considering the biased usage of this VH family in CD we may conclude that the great majority of anti TG2 antibodies can bind to TG2 expressed on cell surface in the course of the disease.

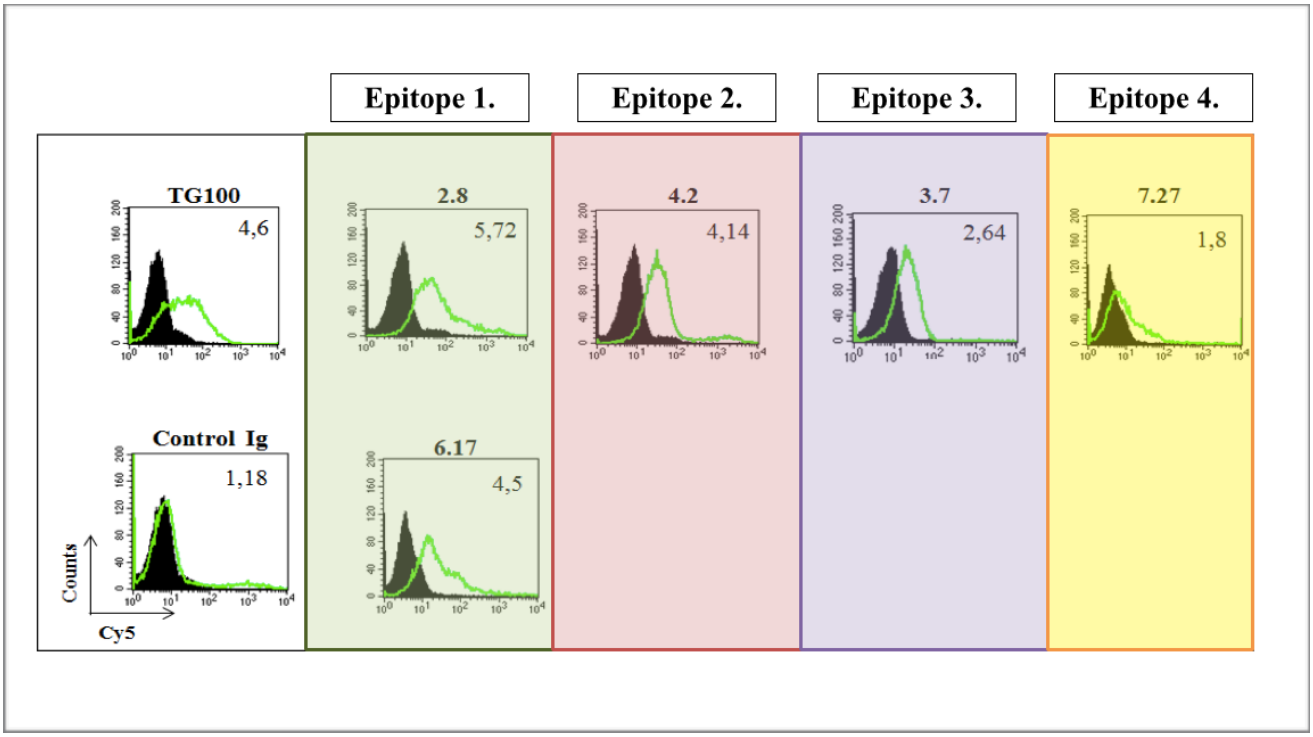

Figure 2.18. Reactivity of Abs with csTG2. Representative flow cytometry histograms showing staining of MCF7 cells with scFv-Fcs followed by Cy5-conjugated secondary antibody. Isotype control was an anti HPAG antibody expressed in scFv-Fc format.

2. 7. Investigating inhibitory effect of antibodies in scFv-Fc format

We wanted to investigate if the antibodies expressed in scFv-Fc format have an inhibitory effect on TG2 enzymatic activity. The inhibitory effect of the antibodies was tested by in vitro transamidation assay in solid surface and in solution. In the assay made on solid surface antibodies were used in increasing concentrations ranging from 12-48nM. As negative control a non TG2 specific antibody anti-HPAG, while as positive control an anti TG2 antibody (hAb004) with high inhibitory effect was used. Inhibition was considered significant over 60%. Epitope 1. antibodies representing the major epitope group of anti-TG2 antibodies had no inhibitory effect on the enzyme's activity. As, well as Epitope 1., Epitope group 3. and Epitope group 4. were also shown non inhibitory. Of the tested antibodies only Epitope 2. antibody (4.2) had considerable inhibitory effect (Figure 2.19.).

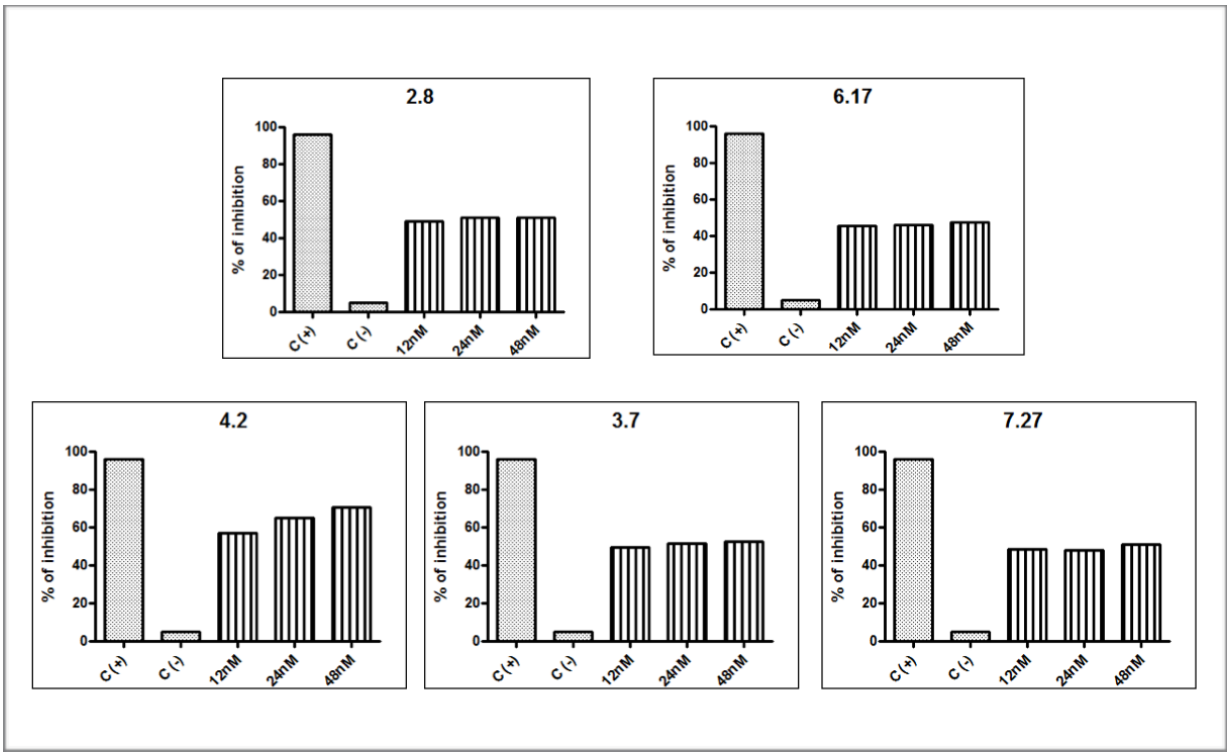

Figure 2.19. Inhibitory effect of antibodies in scFv-Fc format. ELISA plates coated with hTG2 and antibodies in scFv-Fc format. TG2 was incubated with increasing antibody concentration (12-48nM) .

Antibodies in scFv-Fc format were tested for their inhibitory effect also in solution. Also in the test made in solution Epitope 1. antibodies had no inhibitory effect on TG2 (data not shown) indicating that anti-TG2 antibodies belonging to the major epitope cluster do not inhibit the enzymes activity in the course of CD. The other two epitope clusters ,Epitope 3 and Epitope 4 had also no inhibitory effect just as in the previous experiment. The Epitope 2. antibody proved to be inhibitory also in solution supporting the results of the previous experiment. In the figure we can see the experiment done on Epitope 2. (4.2) and Epitope 4. (7.27) antibody using a TG2 inhibitory antibody (hAb004) as positive control (Figure 2.20).The antibodies just as the control were used at 48nM concentration.

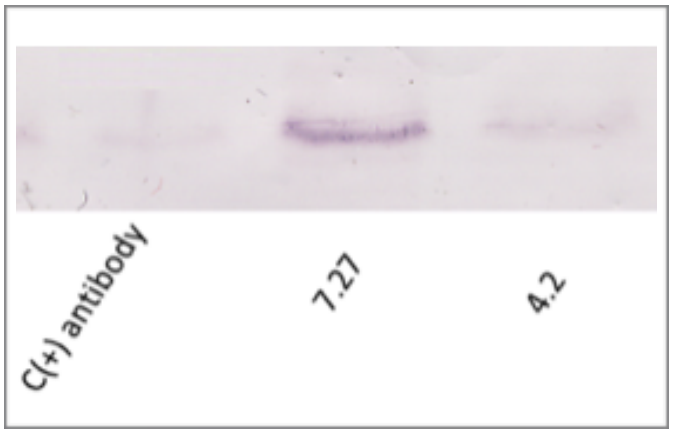

Figure 2.20. Antibodies were tested for their inhibitory effect on TG2 enzymatic activity in solution. **C(+)** positive control (hAb004).

Chapter 3. Defining the role of VH/VL pairing in antigen recognition

Aim of the project

Analyzing the autoantibody response against TG2 in CD has been in the center of attention in autoimmune research for many years. In the previous chapter we described the use of antibodies originating from phage antibody libraries of CD patients to dissect the response. In a different study a panel of mAbs have been generated from single plasma cells of CD patient small intestinal biopsies to characterize the targeting of TG2 specific antibodies [284]. Antibodies were generated in IgG1 format and represented the natural VH-VL coupling due to the single plasma cell origin. These antibodies undergone similar characterization regarding epitope clustering, fibronectin interaction, inhibitory effect and staining csTG2 [294]. The results of the two groups are consistent expect one striking difference, that none of the IgG1 antibodies were able to stain csTG2. We wanted to investigate the reason behind this difference. One explanation is that these antibodies are originating from single plasma cells representing the natural VH-VL coupling, while in phage display the coupling is random. The binding specificity of the antibodies might also be modified in scFv-Fc format compared to IgG1. In order to study the possible differences arising from expression in different format we decided to regenerate and characterize an Epitope 1. IgG1 antibody 693-10-B06 [294] in our scFv and scFv-Fc formats.

Celiac antibodies against TG2 have been reported to use a specific repertoire of VH and VL chains having similarities and using key residues which cannot be found in healthy individuals [286]. To further characterize this phenomena we kept the fix VH chain of Epitope 1. antibody 693-10-B06 [294] and paired it with Vk chains from libraries generated from the IBL of three celiac patients.The libraries were selected on hTG2 and clones proved to be positive on hTG2 by ELISA were identified by DNA sequencing.

3. 1. Introduction to the results

A. Reformat Epitope 1. antibody 693-10-B06 [294] from IgG1 into scFv format. The original antibody 693-10-B06 was rescued with specific oligos and cloned into phagemid vector pDAN5 in standard VL-VH and also in inverted VH-VL order.

B. Constructing clones in scFV format from three minilibraries of CD patients. The VH chain of the constructed Epitope 1. scFv in inverted format was substituted with Vk chains originating from three celiac libraries in phagemid vector pDAN5. The positive output generated from the Vk libraries were analyzed by DNA sequencing ad by phage ELISA on hTG2 and mTG2.

C. Cloning and expression in scFv-Fc format: Reference clones were cloned into mammalian expression vector pMB-SV5 and expressed in scFv-Fc format in Chinese hamster ovary (CHO) cells. Antibodies were tested for retaining reactivity in scFv-Fc format by performing ELISA on different TG2 proteins.

D. Dissecting TG2-autoantibody interaction in the extracellular environment: The effect of TG2-FN interaction on antibody recognition was investigated. Competition ELISA was performed using FN 45kDa domain to compete antibodies for binding TG2.
Antibodies were tested to recognize cell surface TG2 by using them for staining various TG2 expressing cell lines.

E. Investigating inhibitory effect of antibodies in scFv-Fc format: The inhibitory properties of antibodies was determined by using them *in vitro* transamidation assays performed in solution and in solid surface.

3. 2. Reformatting Epitope 1. IgG1 into scFv format

The IgG1 selected for the project was 693-10-B06 belonging to Epitope 1. cluster [294] and consisted of VH5 chain which is the most preferentially used in CD. The VL and VH chains of the antibody were rescued by PCR using a set of oligonucleotides that introduced the BssHII and NheI sites necessary for the cloning. VH and VL amplificates were assembled with loxP511 linker by PCR and cloned into the phagemid vector pDAN5 [387] in two different VH-VL orders (Figure 2.21). One of the scFvs, called scFv 1 was constructed VL-loxP511-VH order which has been widely used in our system. To analyze if a different order of V chains causes a difference in antibodies properties another construct scFv INV-1 was generated in VH-loxP511-VL order.

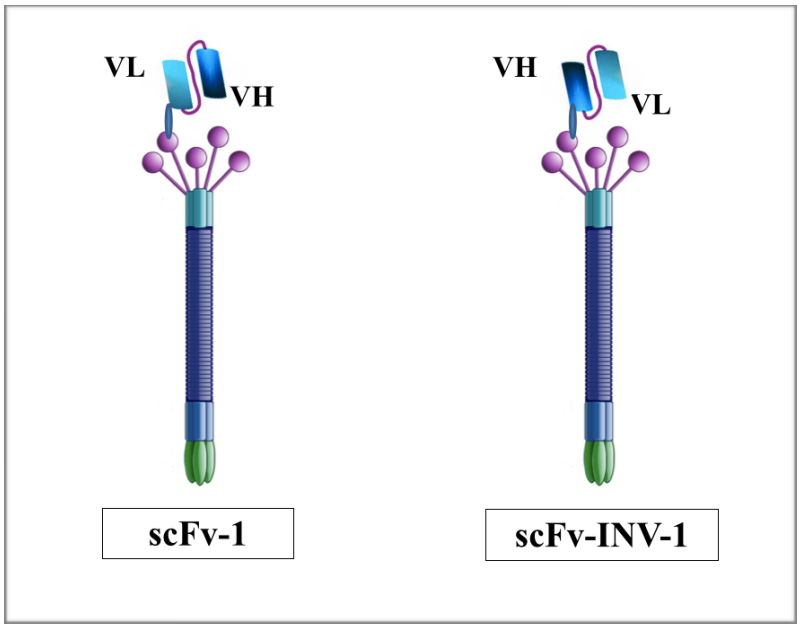

Figure 2.21. The two constructs scFv 1 with VL-LinkerVH order and scFv INV-1 with VH-LinkerVH order expressed by pDAN5 phagemid vector in M13 filamentous phage.

The two constructed antibodies were expressed fused to the coat protein P3 of the M13 filamentous phage and analyzed for retaining their specificity in scFv format by ELISA on hTG2, mTG2 and unrelated control protein (BSA) (Figure 2.22). Both antibodies had cross-reactivity with the murine protein which corresponds to the reactivity of the original Epitope 1. antibody in IgG1 format. This indicates that expression in the scFv format did not cause alterations in the reactivity to TG2 proteins with different mammalian origin. The experiment also revealed that the different VH-VL order in the scFv constructs did not affect the properties of the antibodies.

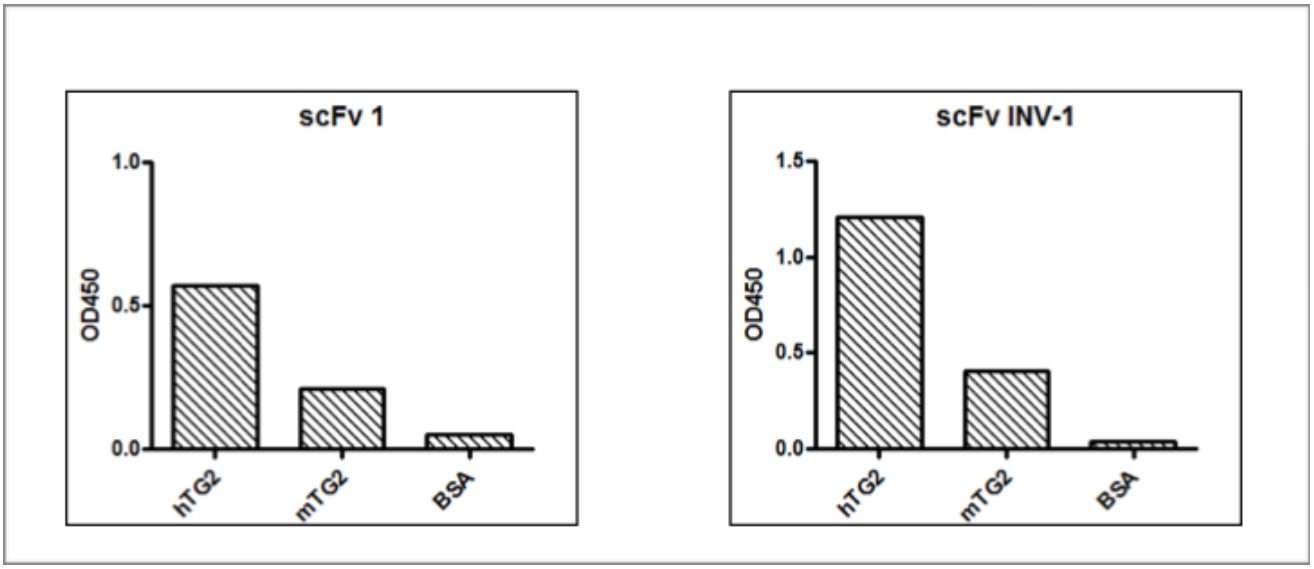

Figure 2.22. Phage ELISA made using scFv 1 and scFv INV-1 on hTG2, mTG2 and BSA.

3. 3. Constructing clones in scFv format from minilibraries of CD patients.

In order to study the VL usage of celiac antibodies the VH chain of scFv INV-1 was substituted with VL chains of three minilibraries generated from the IBL of Celiac patients (Figure 2.23). Total mRNA preparation of IBL was performed and followed by cDNA synthesis using random primer reverse transcription and normalization. The VL chains were amplified from cDNA by PCR using a set of oligonucleotides that recognize human Vk genes [392]. Amplified Vk chains were cloned into phagemid vector pDAN5 downstream loxP511 linker. Antibodies were affinity selected on hTG2 and after every cycle of selection the eluted phages were re-amplified for the next cycle and tested by ELISA against hTG2.

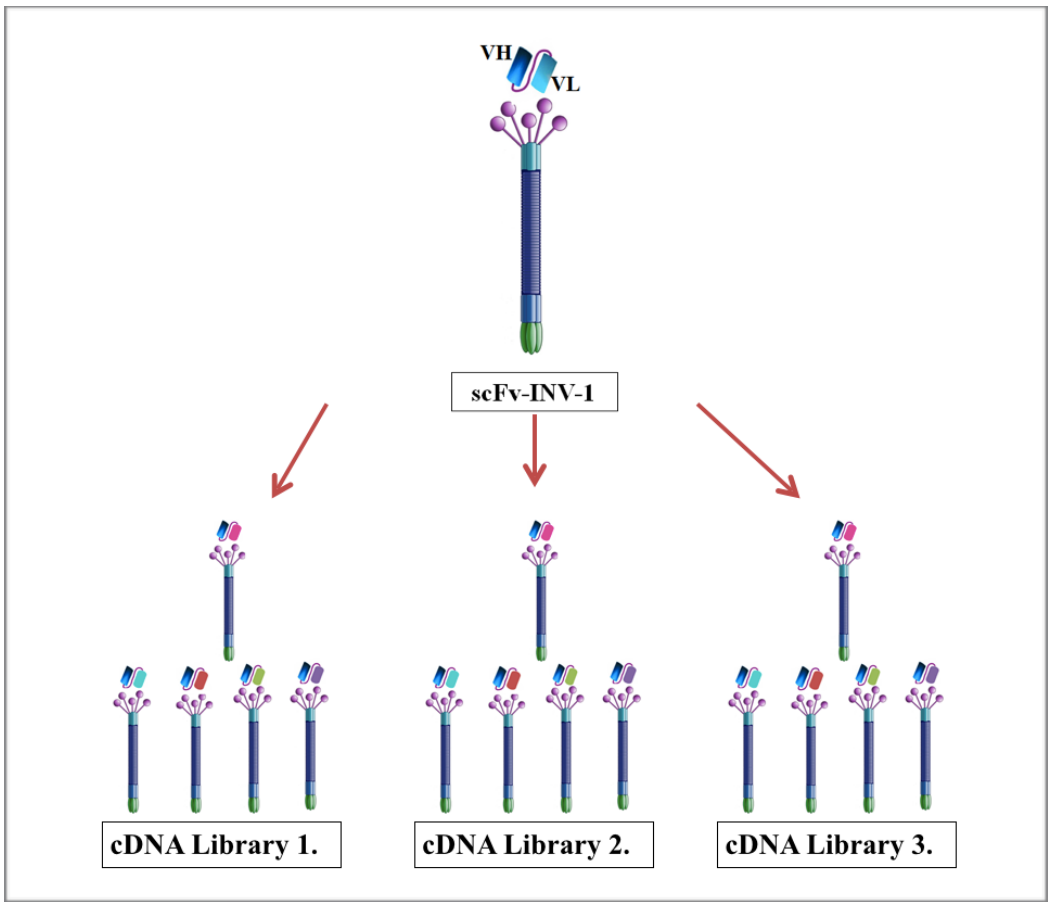

Figure 2.23. Generation of INV clones. The VH chain of scFv INV-1 was substituted with different Vk chains (indicated in different colours) coming from cDNA libraries of three celiac patients expressed in pDAN5 phagemid vector by M13 filamentous phage.

Selection output gave 28 positive clones on hTG2. Six clones belonged to Library 1., nine clones belonged to Library 2. and thirteen clones belonged to Library 3. The VL usage of the positive clones was analyzed (Table 4.1). The VL usage of clones coming from Library 1. consisted of IGKV1-5*03 F, IGKV1-39*01 F, IGKV1-12*01 F chains. Library 2. clones consisted of IGKV1-39*01 and IGKV1-12*01 chains. Library 3. was consisting of IGKV1-39*01 and IGKV1-5*03 F.

A,

cDNA library	Clone ID	VL	CDR
1	B1	IGKV1-5*03 F	CQQANSFPLTF
1	C1	IGKV1-5*03 F	CQQANSFPLTF
1	E9	IGKV1-12*01 F	CQQSYSTLWTF
1	D4	IGKV1-12*01 F	CQQSYSTLWTF
1	E1	IGKV1-39*01 F	CQQSYSSLWTF
1	E3	IGKV1-39*01 F	CQQSYSALWTF

B,

cDNA library	Clone ID	VL	CDR
2	C1	IGKV1-12*01 F	CQQAYSFPLTF
2	G1	IGKV1-39*01 F	CQQSYSTLVTF
2	G10	IGKV1-39*01 F	CQQSYSTLYTF
2	C12	IGKV1-39*01 F	CQQSYSTLYTF
2	D11	IGKV1-39*01 F	CQQTNSFPITF
2	F5	IGKV1-39*01 F	CQQSYSTPRTF
2	C3	IGKV1-39*01 F	CQQSYSTFYTF
2	D3	IGKV1-39*01 F	CQQSYSIPYTF
2	H3	IGKV1-39*01 F	CQQRNTLSLATF

C,

cDNA library	Clone ID	VL	CDR
3	D6	IGKV1-5*03 F	CQQSYSTPQWTF
3	F6	IGKV1-5*03 F	CQQSYSTPLTF
3	G8	IGKV1-5*03 F	CQHYNSYTLMYTF
3	D1	IGKV1-5*03 F	CQHYNSYTLMYTF
3	G1	IGKV1-5*03 F	CQHYNSYTLMYTF
3	C10	IGKV1-39*01 F	CQQSYSTPRTF
3	G4	IGKV1-39*01 F	CQQSYSTPRTF
3	E9	IGKV1-39*01 F	CQQSYSTPYSF
3	H6	IGKV1-39*01 F	CQQSYSSPVTF
3	B5	IGKV1-39*01 F	CQQSTTF
3	G7	IGKV1-39*01 F	CQQSTTF
3	A7	IGKV1-39*01 F	CQQSYSTLWTF
3	E7	IGKV1-39*01 F	CQQSYSTLWTF

Table 2.3. Vk family usage of anti-TG2 Abs from three libraries. Three different Vk chains: IGKV1-5*03 F (indicated in green), IGKV1-12*01 (indicated in violet) and IGKV1-39*01 F (indicated in orange).

The distribution of the three Vk chains points out some interesting facts (Figure 2.24). From all Vk chains present in the three libraries IGKV1-39*01 F was the far most used one. Not only being the most frequently used Vk chain, IGKV1-39*01was also the only chain which was present in all libraries. The results support the restricted VL usage reported in CD, with a biased usage of IGKV1-39*01.

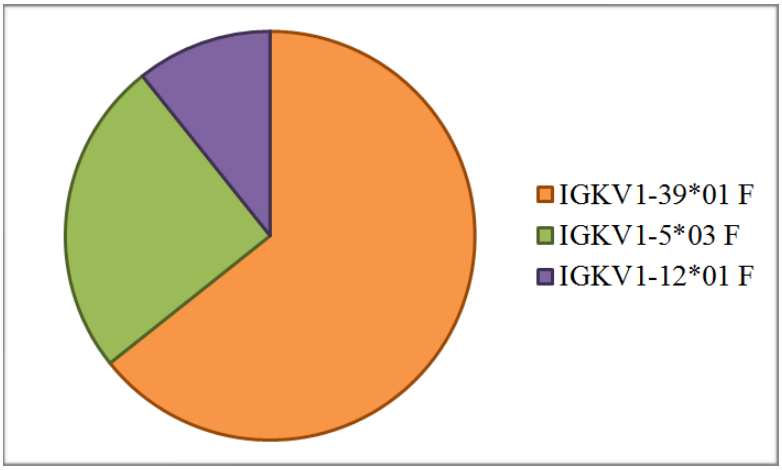

Figure 2.24. The distribution of three Vk chains in total selection output

A number of clones were tested for their reactivity on hTG2, mTG2 and unrelated control protein (BSA) by phage ELISA (Figure 2.25). It was observed that the great majority of clones has lost reactivity towards mTG2. This indicates that different VL chains impair different reactivity supporting the importance of VL chain in antigen recognition.

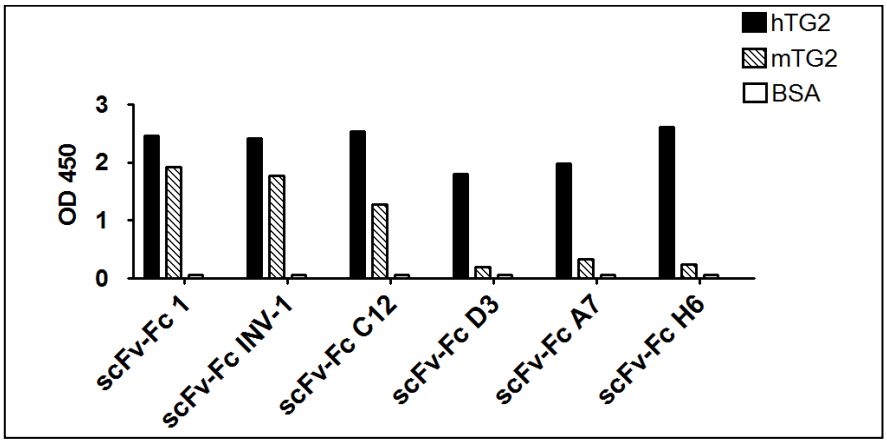

Figure 2.25. Phage ELISA results of four representative clones. **A.** Clone retaining specificity having cross-reactivity with mTG2. **B.** Clones loosing reactivity on mTG2 becoming specific on hTG2.

In order to study the response in deep we selected four representative clones (Table 2.4). The clones were coming from two cDNA libraries, from Library 2. clone C12 and D3 while from Library 3. clone A7 and H6. All four clones shared IGKV1-39*01 F which is the most used light chain in CD antibody response, and also was found to be the most abundant in the positive clones of the three cDNA library.

A,

Clone ID	VH	CDR3	VL	CDR3
1	IGHV5-51*03 F	CARLRMIGFDKNAPLDYW	IGKV1-5*03	CQQANSFPLTF
INV-1	IGHV5-51*03 F	CARLRMIGFDKNAPLDYW	IGKV1-5*03	CQQANSFPLTF

B,

Library	Clone ID	VH	CDR3	VL	CDR3
2	D3	IGHV5-51*03 F	CARLRMIGFDKNAPLDYW	IGKV1-39*01 F	CQQSYSIPYTF
2	C12	IGHV5-51*03 F	CARLRMIGFDKNAPLDYW	IGKV1-39*01 F	CQQSYSTLYTF
3	A7	IGHV5-51*03 F	CARLRMIGFDKNAPLDYW	IGKV1-39*01 F	CQQSYSTLWTF
3	H6	IGHV5-51*03 F	CARLRMIGFDKNAPLDYW	IGKV1-39*01 F	CQQSYSSPVTF

Table 2.4. Clones selected to be expressed as scFv-Fc. **A.** Clones harboring original VH and VL. **B.** Clones sharing original VH with Vks coming from two different libraries

However the CDR3 region was different for each clone allowing to more precisely dissect the effect of minor differences in CDR region cause in antibody properties. Beyond this selected clones had differences in specificity, C12 being the only one retaining mTG2 reactivity. The fact that clones with same type of Vk chain had altered reactivity on mTG2 indicates that different CDR3 regions support the recognition of different epitopes.

3. 4. Cloning and expression in scFv-Fc format

The two clones scFv 1 and scFv INV-1 composed of the VH and VL chains of the original IgG1 antibody [294] and the four selected clones (C12, D3, A7, H6) representing Library 2. and Library 3. were cloned into modified pMB-SV5 vector [393] as described in the previous chapter. After cloning all vectors were checked by DNA sequencing and the purified plasmidic DNAs were transfected into CHO cells. After 72 hours cultures supernatants were analyzed for antibody expression by performing ELISA on hTG2, mTG2 and unrelated control protein (BSA) (Figure 2.26.). All clones in scFv-Fc format retained the specificity experienced in the phage expressed scFv format.

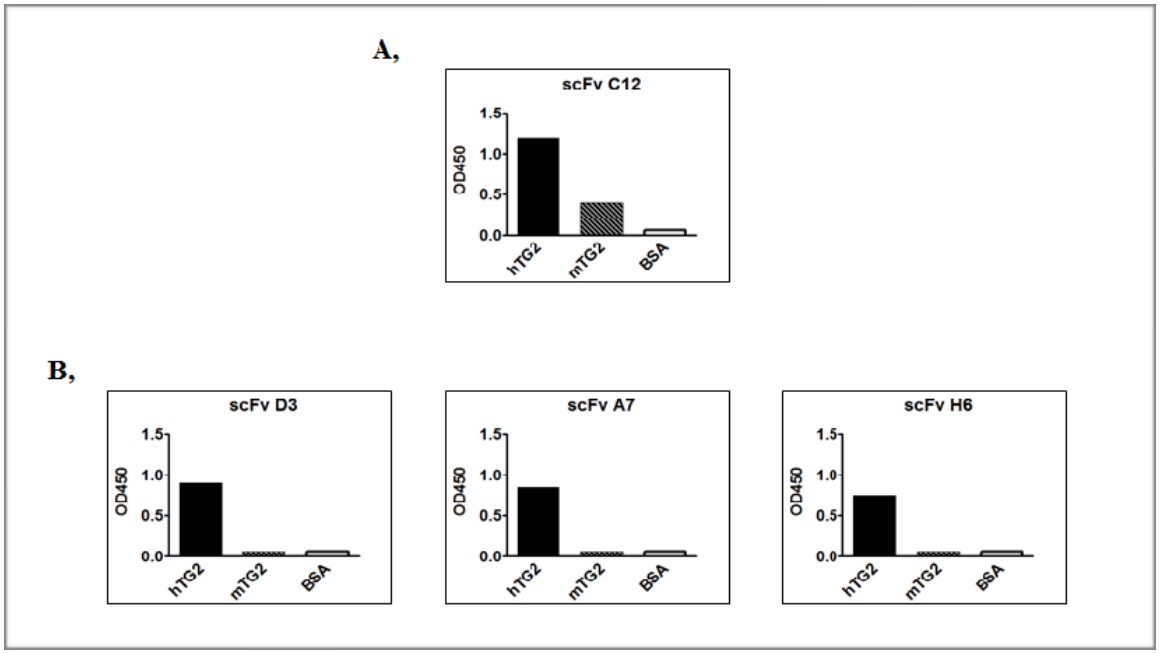

Figure 2.26. ELISA experiment performed on CHO supernatants 72 hours after transfection.

Purification was done using Protein-A agarose and production yield ranging 1-4 mg/l of culture. Purified scFv-Fcs were analyzed by Comassie and Western blot under reducing conditions (Figure 2.27). Antibodies appeared at predicted molecular weight which was around the expected 55 kDa.

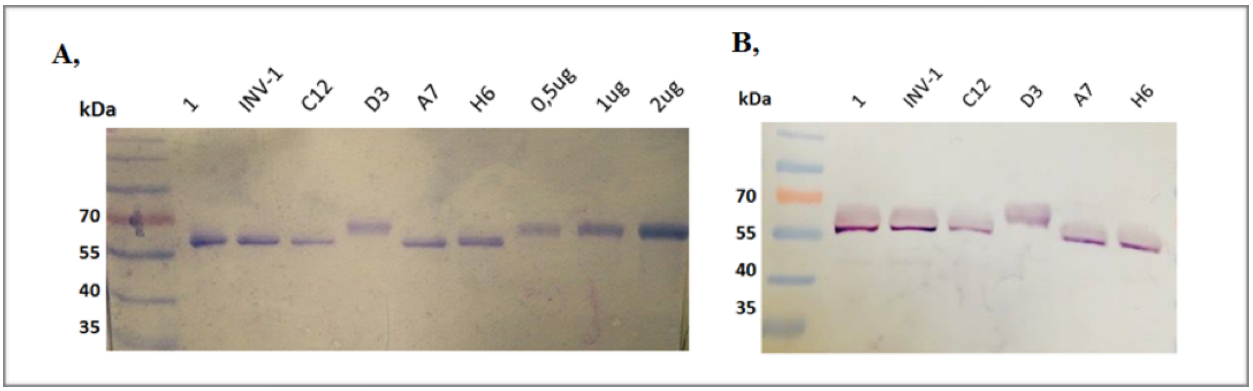

Figure 2.27. Comassie staining **(A)** and Western blot **(B)** of purified scFv-Fcs.

The specificity of purified scFv-Fcs was further dissected on hTG2 and mTG2 and by ELISA (Figure 2.28.) using antibody concentrations ranging from 0.1-0.5ug/ml. All of the antibodies were reactive on hTG2, but only scFv-Fc 1, INV-1 and C12 had cross reactivity on mTG2 as it was seen in the phage expressed scFv format. This indicates that the cloning and expression in scFv-Fc format did not alter antibody specificity.

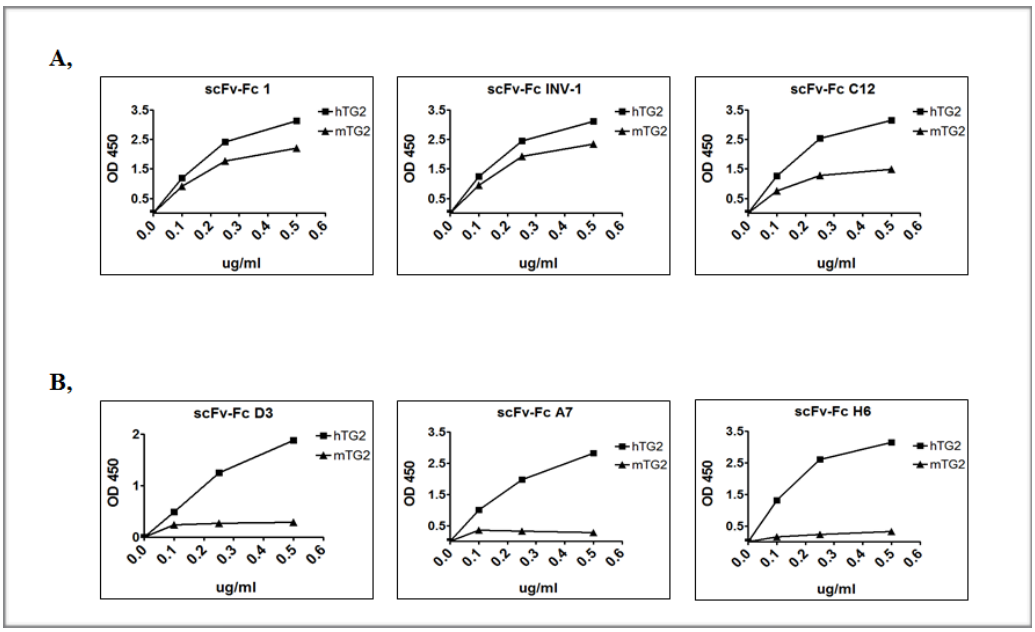

Figure 2.28. Saturation binding curves for selected scFv-Fcs representing two distinct group regarding specificity. **A.** Clones cross-reactive with mTG2. **B.** Clones specific to hTG2. Antibodies used in increasing concentrations from 0,1-0,5 ug/ml.

3. 5. Dissecting TG2-autoantibody interaction in the extracellular environment

3. 5. 1. Analyzing the effect of TG2-FN interaction on antibody binding.

Epitope 1. antibody 693-10-B06[294] has been reported to recognize the region on TG2 molecule which is overlapping with the FN-binding site. In order to study if the different expression format and different Vk chains caused differences in TG2-antibody interaction we tested the ability of scFv-Fcs to react with fibronectin bound TG2 (Figure 2.29.).

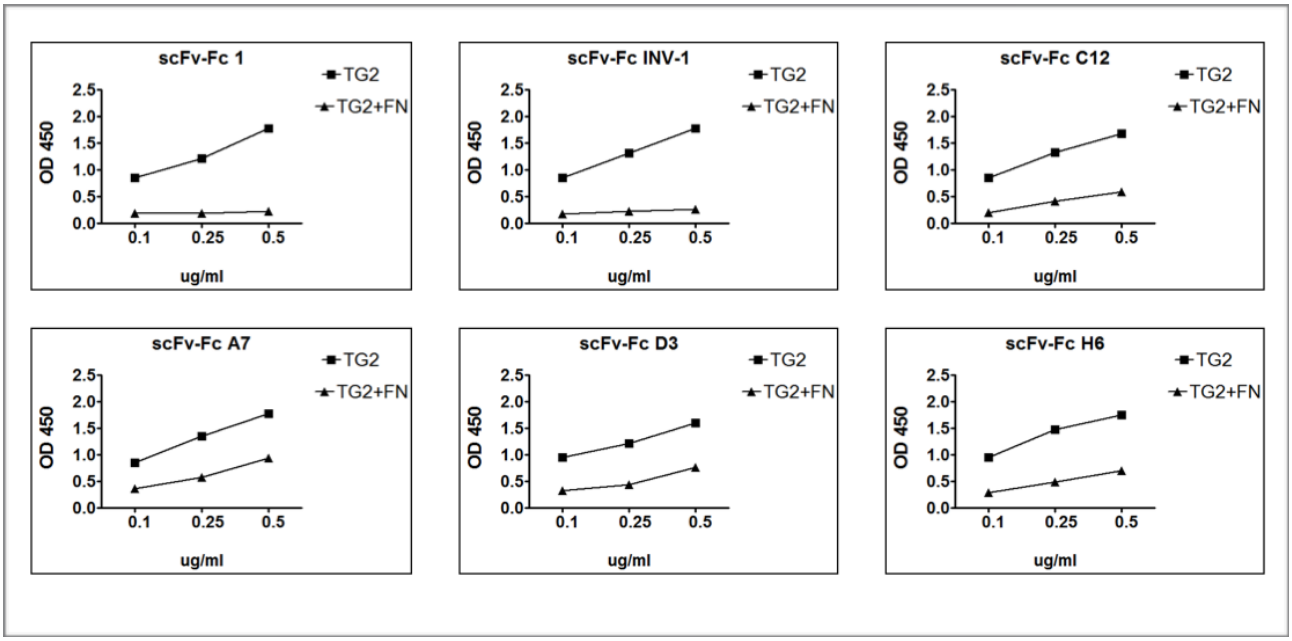

Figure 2.29. Competition ELISA between scFv-Fcs and 45kDa FN domain. TG2 was pre-incubated with antibodies in different concentrations from 0.05-5ug/ml. The graph indicates the decrease in signal intensity upon increasing antibody concentration.

All of the scFv-Fcs had decreased reactivity in the case of TG2-FN interaction. This indicates that the expression in different format did not interfere with recognizing the FN-binding site on TG2 molecule. However scFv-Fc1 and scFv-Fc INV-1 harboring the original VH and VL of Epitope 1. antibody [294] had a more significant decrease in reactivity for FN bound TG2 compared to the other clones. This support that the exchange in the VL did cause alterations in epitope specificity in some extent.

3. 5. 2. Investigate the staining properties of scFv-Fcs on csTG2

The antibodies in scFv-Fc format were tested for staining csTG2. This experiment had a particular interest since it has been reported that the original Epitope 1. antibody in IgG1 format hadn't recognized TG2 expressed on the surface of iDCs [294]. This was contradictory to our results showing that Epitope 1. antibodies (2.8, 6.17) in scFv-Fc format, originating from phage antibody libraries stained csTG2 on the surface of high TG2 expressing cancerous cell lines. We wanted to investigate if this difference is caused by the smaller size of scFv-Fc being able to access the binding site which is displayed on cell surfaceTG2 but is not possible for the bigger IgG1 molecule to reach. Another issue we came across is that the coupling of VH and VL chains in antibodies coming from phage antibody libraries is random while the antibodies generated from single plasma cells represent the natural coupling. All scFv-Fcs were tested for staining csTG2 on high TG2 expressing SKOV3 cell line (Figure 2.30). As a positive control scFv-Fc 2.8, described previously was used. As isotype control a nonTG2 specific antibody in the same scFv-Fc format was used.

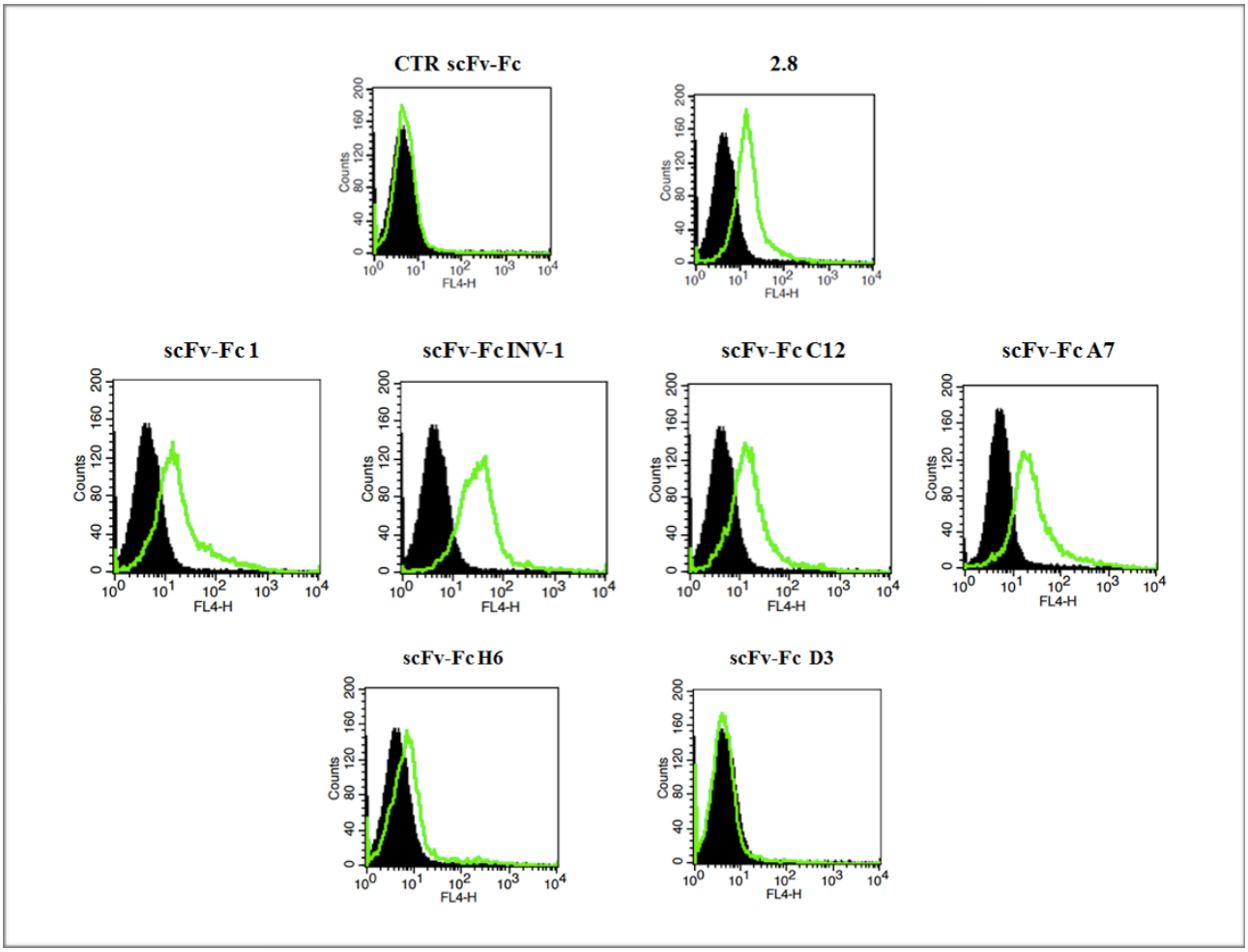

Figure 2.30. Reactivity of scFv-Fcs with csTG2. Representative flow cytometry histograms showing staining of MCF7 cells with scFv-Fcs.

Interestingly both scFv-Fc 1 and scFv-Fc INV-1 showed a strong staining on csTG2. This indicates that Epitope 1. antibodies can stains csTG2 and that the different expression format improved the staining property of the antibodies. Two clones with the same VH but different VL, scFv-Fc C12 and scFv-Fc A7 also had positive staining. However, the other two antibody scFv-Fc D3 and scFv-Fc H6 were negative for staining csTG2. This correlates to what we saw with antibodies coming from phage antibody libraries, that different, but not all epitope clusters can recognize csTG2.

3. 6. Investigating inhibitory effect of antibodies in scFv-Fc format

The inhibitory effect on TG2 *in vitro* transamidating activity was analyzed for all antibodies expressed in scFv-Fc format. The original Epitope 1. antibody 693-10-B06 was reported not to have inhibitory effect on TG2 enzymatic activity [284, 294]. These results correspond what we saw with our VH5 antibodies originating from phage antibody libraries. Experiments were performed as described before. The scFv-Fcs were used in three different concentrations of 6-12-24nM. As negative control a non TG2 specific antibody anti-HPAG, while as positive control an anti TG2 antibody (hAb004) with high inhibitory effect was used (Figure 2.31.).

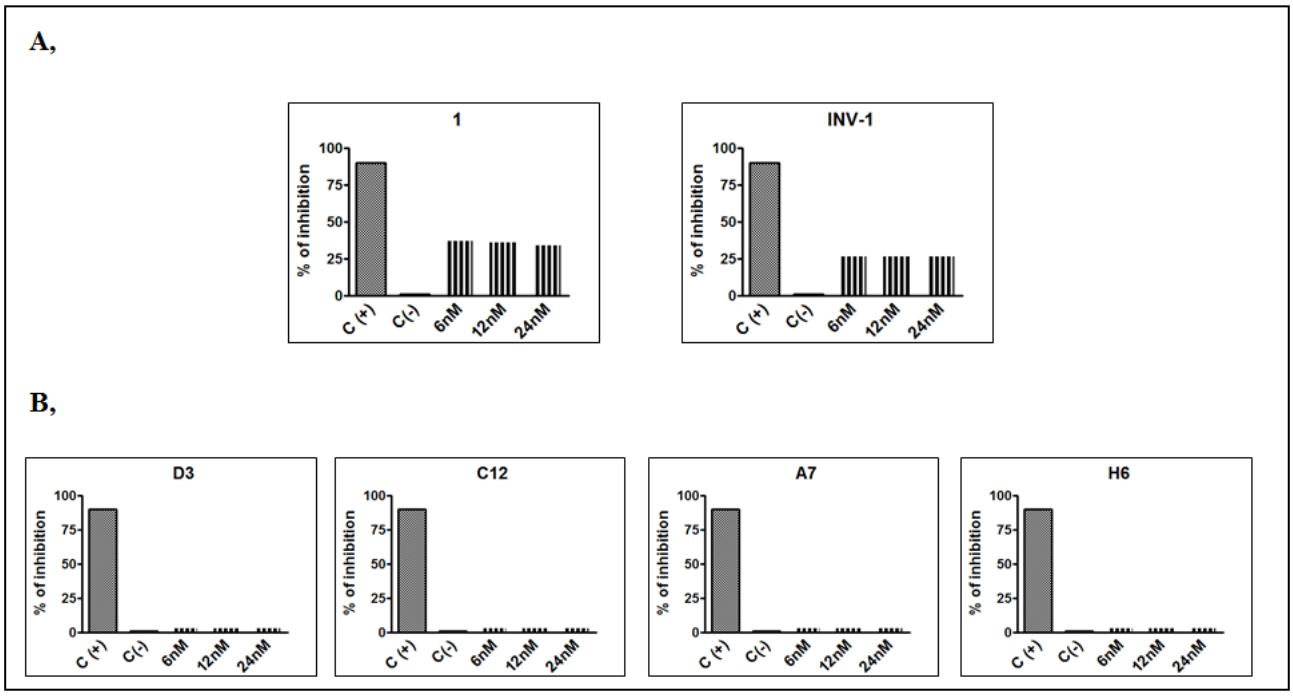

Figure 2.31. In vitro transamidation assay performed on solid surface. **A.** Clones harboring original VH and VL . **B.** Clones with different VL chains.

None of the antibodies had significant inhibitory effect on TG2 enzymatic activity. The very mild inhibition saw in scFv-Fc1 and scFv-Fc INV-1 is similar seen in other VH5 Epitope 1. antibodies that are not considered inhibitory. The remaining four antibodies harboring different VL chains had no inhibitory effect at all.

Antibodies in scFv-Fc format were tested for their inhibitory effect in solution. Antibodies were used in 24nM concentration for the experiment (Figure 2.32.). As positive control for inhibition a TG2 inhibitory antibody (hAb004) was used while ana negative control non TG2 specific antibody (anti-HPAG) was used in same concentration and scFv-Fc format.The results of the experiment supported what we saw in ELISA, confirming that none of the antibodies have a real inhibitory effect on TG2 enzymatic activity.

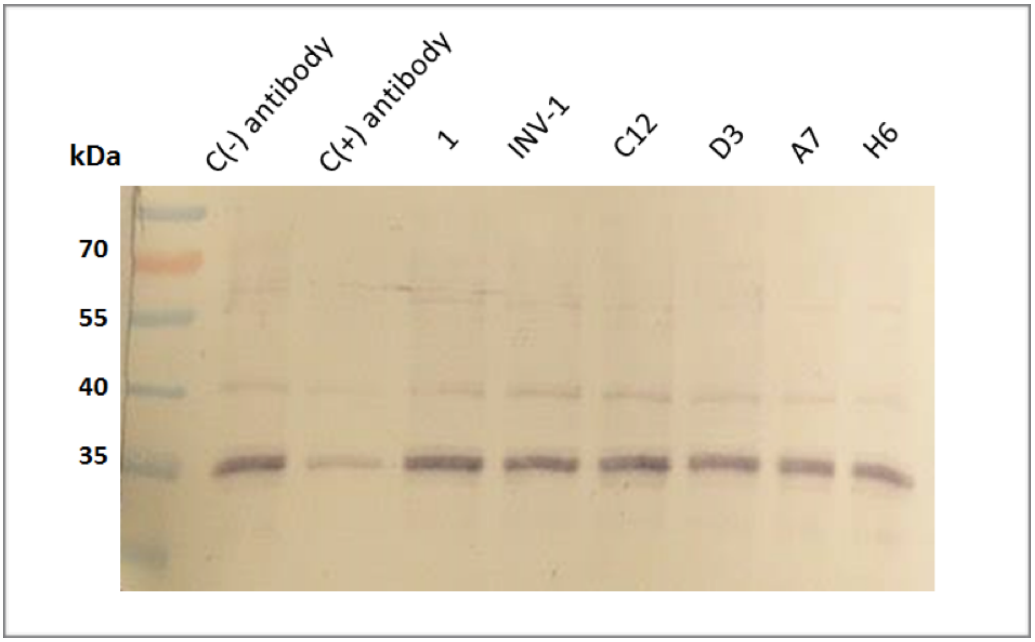

Figure 2.32. *In vitro* inhibition assay in solution. All scFv-fcs, including controls were used in 24nM concentration. **C(+)** positive control (hAb004). **C(-)**-negative control (anti-HPAG).

DISCUSSION

Chapter 1. Background

Celiac disease (CD) is a gluten-sensitive enteropathy that develops in genetically susceptible individuals by exposure to cereal gluten proteins. The disease characterized by symptoms like villous atrophy and crypt hyperplasia affects 1% of the western population [186]. It has been revealed that the occurrence of CD is strictly dependent on dietary exposure to gluten and similar cereal proteins [183]. Beyond this CD also exhibits genetic background with a very strong association with MHC class II molecules HLA-DQ2 and HLA-DQ8 [188, 189], with these alleles increasing the relative risk to the disease development 30 fold [187]. It has been revealed that CD patients produce antibodies specific for gluten and self antigen [257]. In 1997. TG2 was identified as the self antigen recognized by anti-reticulin and anti-endomysium antibodies [262].

TG2 is the most ubiquitous and diverse member of the transglutaminase family with a great variety of biological functions.TG2 has been shown to be an important player in many cellular functions as cell attachment and migration, wound healing, angiogenesis, apoptosis and cell and transmembrane signaling. As a result of diverse functions, TG2 has also been implicated in numerous pathologies including neurodegenerative disorders, cancer and autoimmune diseases. The main enzymatic function of TG2 is transamidation, where enzymatically active enzyme catalyzes Ca^{2+}-dependent acyl-transfer reaction between γ-carboxamide group of a protein-bound glutamine and either the ε-amino group of a distinct protein-bound lysine residue (covalent protein cross-linking) or primary amines such as polyamines and histamine. In certain conditions the amine donor substrates can be replaced by H_2O leading to deamidation of glutamine molecules by TG2, which makes the enzyme one of the key players in the onset of CD [32].

The specific antibody response against TG2 in the serum has become the most sensitive and specific serological assay used fro the diagnosis of CD [263]. TG2 specific antibodies can be found as IgA deposits in the small intestine of CD patients [285]. Anti-TG2 antibodies can be isolated from intestinal biopsy lymphocytes [286] even in the absence of detectable anti-TG2 serum level [281].

These findings pointed out that antibody response against TG2 occurs at a local level in the small intestine and Ab presence in the serum is attributable to spill over into the blood compartment. TG2 specific PCs have a strong bias in their VH and VL usage with a preference of *IGHV5-51, IGHV3-48, IGHV4-59, IGHV1-69, IGKV1-39, IGKV1-5* [284] with a dominance of *IGVH5-51* [286]. These antibodies have been shown to recognize a part of TG2 which overlaps with the FN binding site on the molecule [294]. TG2 specific antibodies target at least four different epitopes clustered in the N-terminal of TG2 [294] where epitope clusters correlate with the VH usage. So far none of the TG2 specific antibodies from human origin has been shown to be able to stain csTG2. [294].

Anti-TG2 antibodies have been implied to play a role in various pathologies. It has been shown that anti-TG2 antibodies inhibit intestinal epithelial cell differentiation, increase the intestinal epithelial cell [304-306] and blood vessel permeability. Anti-TG2 antibodies have been suggested to contribute to the onset of gluten ataxia [311-313] leading to the neurologic pathologies occurring in association with CD. Anti-TG2 antibodies also have been associated with a wide range of reproductive disorders in women.

The aim of this study was to dissect the autoantibody response against TG2 occurring in CD. This involved the generation and characterization of anti-TG2 antibodies in various format to obtain a more precise picture about the role they play and how they act in the disease.

Chapter 2. Developing TG2 inhibitor antibody

The main aim of the project is to dissect the autoantibody response in CD. One characteristic of anti-TG2 antibodies that need to be determined is the inhibitory effect on TG2 enzymatic activity.
In order to fulfill this we used the sequence of an Ab coming patent that has described the generation of a series of TG2 inhibitory Abs (ref:WO2013/175229 A1). The sequence of the best inhibitor, comprising of variable heavy chain IGHV3-23*01 F and variable light chain IGKV1-16*01 F with code hAb004, has been used to generate our antibody in scFv-Fc format [393] in the pMB-SV5 vector.

In the construct the anti-TG2 scFv is fused to the Fc domain of human IgG1, allowing to have activity similar to full length IgGs in most assays, and the advantages of dimerization and effector functions provided by the Fc domain along with the benefits of the smaller size of the molecule. The purified monoclonal hAb004 antibody in scFv-Fc format only found to be specific to hTG2 correlating to what has been reported in the patent.

In order to develop an inhibition assay first an ELISA measuring TG2 *in vitro* transamidating activity on solid surface was done, based on the coupling of 5'-(biotinamido)-pentyalamine to NN-dimethylcasein which was catalyzed by recombinant hTG2. The inhibitory effect of hAb004 was investigated in different concentration ranging from 24nM-0,188nM. In the experiment hAb004 proved to have a significant inhibitory effect on TG2 enzymatic activity in a dose dependent manner, reaching IC50 between 0,75-0,375nM when it was used to inhibit TG2 used in 1.5nM concentration. This inhibitory effect is comparable to the best TG2 inhibitors reported [170]. The same results were gained whet hAb004 was used to inhibit TG2 activity in solution, indicating that the Ab is an efficient inhibitor which can be used as a control in future experiments.

The significant inhibitory effect of the antibody also opens a wide range of applications in research and therapeutic purposes. TG2 is the key player in the onset of CD by deamidating glutamine molecules and this way generating gluten T-cell epitopes. Since the generation of the T-cell epitopes highly depend on the transamidation activity of TG2, targeting and inhibiting this activity of the enzyme provides a promising future perspective for the treatment of the disease.

Chapter 3. Dissecting anti TG2 antibody response

3. 1. Characterzing and clustering Abs in scFv format

Phage antibody libraries in this study were constructed as described in Marzari et al. [286], generating libraries from the IBL of seven untreated adult CD patients. After several rounds of selection individual colonies were analyzed on hTG2 from which 28 proved to be positive.To further characterize the clones positive on hTG2, the reactivity with mTG2 (sharing 84% sequence identity with hTG2) was assessed. From the 28 positive clones 14 showed cross-reactivity with mTG2 while 14 were specific to hTG2. This ratio can be explained with the great homology between the two proteins and correlates with former studies [286, 294] showing that the major portion of anti-TG2 antibodies cross-react with the murine protein.

In order to cluster the selected antibodies into epitope groups competition experiments were performed with reference antibodies belonging to different VH classes with different specificity. From the 14 clones with mTG2 cross-reactivity 11 showed competition with the two Epitope 1., VH5 antibodies indicating that these clones share one major epitope as it has been described before [294]. Epitope 1. antibodies added up to 44% of the clones and this way dominated the antibody response. TG2 specific clones were clustered into 3 different epitope groups by competing them with reference antibodies .The results indicate that while the antibodies with mTG2 cross-reactivity represent a homogenous Epitope 1. cluster, antibodies specific for hTG2 recognize at least three different epitopes on TG2 molecule. We can conclude that antibodies target at least four distinct regions, based on the competition pattern for TG2 binding. However, it is likely that each cluster is comprised of single overlapping epitopes, with competition results indicating that some of the epitope regions are partly overlapping.

3. 2. Cloning and expression in scFv-Fc format

Five antibodies representing the four epitope clusters were selected to be cloned and expressed in scFv-Fc format. Two VH5 antibodies represented the most abundant Epitope 1. cluster.
The other three less defined epitopes were represented by a single antibody. The antibody representing Epitope 2. belonged to VH3, the antibody representing Epitope 3. belonged to VH1 and the Epitope 4. antibody belonged to VH3 antibody gene family. The cloning into pMB-SV5 mammalian expression vector was characterized by BssHII/NheI cassette where selected scFvs were substituted with the Fc domain of human IgG1 [394]. After purification the reactivity on TG2 molecules with different origin was analyzed for each antibody in scFv-Fc format showing that all of them retained specificity. This indicates that the subcloning into scFv-Fc format did not alter the specificity of the antibodies, pointing out that this straightforward technology is appropriate for dissecting the antibody response in CD.

3. 3. Dissecting TG2-autoantibody interaction in ECM

3. 3. 1. Analyzing the effect of TG2-FN interaction on antibody binding.

In the extracellular environment one of TG2's major interacting partners is fibronectin (FN) [85]. FN has been reported to regulate cell adhesion, migration and tumorigenesis and contribute to multiple physiological processes including embryonic development, angiogenesis and wound healing. The specific binding between TG2 and FN in the ECM involves the gelatin-binding domain of FN and the N-terminal β-sandwich domain of TG2. The effect of TG2-FN association on antibody binding has been reported by a study using monoclonal antibodies isolated from single plasma cells of CD patients [294], showing that the mAbs assigned to epitope 1, the VH5 epitope, compete with FN for binding to TG2, while other epitopes were not. In order to investigate the effect of TG2-FN interaction on antibody binding, the selected representative antibodies were tested to react with FN bound TG2. The two VH5 scFv-Fcs, representing Epitope 1. lost their reactivity when TG2 was associated with FN, while the other three epitope clusters were not affected. Although we cannot rule out that the loss of reactivity in the case of Epitope 1. antibodies is due to conformational changes in TG2 induced by FN binding, it suggests that this epitope overlaps with the fibronectin binding site on TG2 molecule.

The results point out a possible pathogenic role of anti-TG2 Abs in CD. Anti-TG2 antibodies might act as competitors of FN which could lead to antibody induced displacement of TG2 from FN in the ECM. This may result in increased enzymatic activity due to the release of immobilized TG2. Increased activity of TG2 can lead to increased gluten deamidation and this way to the T-cell response against deamidated gluten.

Anti-TG2 antibodies have been implied in reproductive disorders in women with CD. Anti-TG2 antibodies have been reported to reduce trophoblast invasiveness through apoptotic damage [320] and implicated to interfere in the endometrial angiogenesis [323]. As TG2 mediated interaction between integrins and FN contributes to cell survival and angiogenesis the displacement of TG2 from FN meshwork by Epitope 1. anti-TG2 antibodies may lead to these pathological conditions.

TG2 has also been linked to various functions in cancer such as promoting β integrin mediated ovarian cancer cell adhesion to the peritoneal matrix. TG2 has been shown to induce epithelial-to-mesenchymal transition (EMT) [98] which is a critical step in the initiation of metastasis and that the FN-binding domain of TG2 is sufficient to initiate this process [99, 100]. Additionally, TG2-mediated interaction between β integrin and FN has been reported to activate cell survival pathways [90] and contribute to doxorubicin resistance in breast cancer cells [101], as well as cisplatin and dacarbazine resistance in melanoma cells [102]. Because of this the TG2-FN interaction might serve as a potential target for cancer therapies. Since the binding of Epitope 1. antibodies was inhibited when TG2 was in FN bound form this epitope group has been implied to interfere with TG2-FN interaction. Because this interaction plays an important role in adhesion and migration of cancer cells, Epitope 1. antibodies might be a potential tool to better understand the role of TG2 in cancer progression and also a potential therapeutic. The effect of an Epitope 1. antibody (2.8) has been studied in malignant pleural mesothelioma (data not shown) where matrigel adhesion was increased while invasion was inhibited by the antibody. This indicates that TG2-based interventions by Epitope 1. antibodies may be used to disrupt malignant invasion, growth and survival.

3. 3. 2. Investigate the staining properties of antibodies on csTG2

TG2 has been found to be expressed on the surface of different cell types, where it interacts with fibronectin, integrins of the β1 and β3 subunit [85, 90, 105]. There have been several studies aiming to generate and test mAbs with human origin to stain csTG2 until now with no success. Anti TG2 antibodies isolated from single plasma cells of CD patients expressed in IgG1 format were tested for staining csTG2 on the surface of dendritic cells [294] and none of them showed staining on csTG2. It has been concluded that N-terminal epitopes are not accessible when TG2 is found on cell surfaces, possibly due to the association of TG2 with integrins and fibronectin or other interaction partners. So far only two mouse monoclonal antibodies against TG2, CUB7402 and TG100, have been shown to be able to stain csTG2. Since these antibodies have been generated from mouse IgG1 by immunization with purified guinea pig liver TG2 (sharing 76% sequence identity with hTG2) both of these antibodies have limitations in certain research and therapeutic applications.

We wanted to investigate if the antibody expression in scFv-Fc format and the usage of higher TG2 expressing cell lines could improve the staining of mAbs with human origin. TG100 antibody was used as a control to test the staining properties of our scFv-Fc antibodies in flow cytometry using high TG2 expressing ovarian cancer cell lines such as SKOV3 and MCF7. All antibodies were able to recognize csTG2 except one representing Epitope 4. The results point several things. The fact that the Epitope 4. antibody showed no staining serves as a control for the scFv-Fc format, indicating that the staining is not due an unspecific binding of the Fc domain. The results also demonstrate that multiple but not all of the epitopes are accessible when TG2 is expressed on cell surface. The contradiction between our results and the previously published data [294] have raised questions about the cause of different staining properties. One reason might be the different size of antibodies since the ones described in the paper were expressed as IgG1 while ours were expressed in scFv-Fc format. The smaller size of the scFv-Fc molecule might allow to reach the binding site on TG2 not accessible for the IgG1 molecule. Another reason could be the different origin of antibodies since the ones described in the paper [294] were coming from single PCs representing a natural VL/VH coupling while our antibodies are originating from phage antibody libraries where the coupling of VH and VL chains is random. A number of experiments were carried out to investigate this phenomenon which will be described in chapter 4.

These antibodies also raise the possibility to have antibodies with human origin able to stain csTG2. This would open a whole new range of applications to understand the pathomechanism of CD and to find ways of therapeutic interventions.

3. 4. Investigating inhibitory effect of antibodies in scFv-Fc format

We wanted to investigate if the antibodies expressed in scFv-Fc format have an inhibitory effect on TG2 enzymatic activity. Of the tested antibodies only Epitope 2. antibody (4.2) had considerable inhibitory effect, which has been supported by the results of the experiment carried out in solution. This indicates that the great majority of anti-TG2 antibodies do not exert a pathogenic role by inhibiting TG2 enzymatic activity in the course of CD. The data correlates with findings of a previous paper [284] where a similar experiment was carried out using mAbs generated from single plasma cells positive on TG2.

Chapter 4. Defining the role of VH/VL pairing in antigen recognition

4. 1. Reformatting Epitope 1. [294] Ab 693-10-B06 from IgG1 into scFv format.

In the previous chapters we described the use of antibodies originating from phage antibody libraries of CD patients to dissect the antibody response against TG2. In a study a panel of mAbs have been generated from single plasma cells of CD patients to characterize the targeted epitopes of TG2 specific antibodies [284]. These Antibodies were generated in IgG1 format and represented the natural VH-VL coupling because of their single plasma cell origin. These antibodies undergone similar characterization regarding epitope clustering, fibronectin interaction, inhibitory effect and staining csTG2 [294]. These results were consistent with our findings except a striking difference, that none of the mAbs of different epitope groups was able to stain csTG2 expressed by iDCs. This was contradictory to our data showing that antibodies of three epitope clusters in scFv-Fc format were able to stain csTG2. Only the Epitope 4. antibody was not able to recognize the enzyme expressed on cell surface. This raised the question whether the different expression format or the difference in the VH/VL pairing is the reason behind the contradictory findings.

In order to investigate this phenomena,we reformatted and characterized an Epitope 1. VH5 antibody (693-10-B06) from IgG1 format [294] into scFv and scFv-Fc formats. The VH and VL chains were amplified and separately assembled with loxP511 linker before cloning into the phagemid vector pDAN5 [387] in two different VH-VL orders. scFv 1 was constructed in VL-loxP511-VH order which has been widely used in our system, while scFv INV-1 was generated in VH-loxP511-VL order to analyze the possible alterations caused by the different order of the V chains. The two constructs were tested for retaining specificity in scFv format by expressed fused to the coat protein P3 of the M13 filamentous phage. Both antibodies had cross-reactivity with the murine protein which corresponds to the reactivity of the original Epitope 1. antibody in IgG1 format. These results indicate that the expression in the scFv format did not cause alterations in the reactivity to the two TG2 proteins with different mammalian origin. The experiment also revealed that the different VH-VL order in the scFv constructs did not affect the specificity of the antibodies.

4. 2. Construction and analysis of clones in scFv format from Vk libraries of CD patients.

In order to dissect the VH/VL pairing of Celiac antibodies the pairing of Vk chains to a defined IGHV5-51*03 F chain was analyzed. In the experiment the VH chain of scFv INV-1 was substituted with Vk chains coming from three minilibraries generated from the IBL of Celiac patients. The VL chains were amplified from cDNA using a set of oligonucleotides that recognize human Vk genes [392]. Amplified Vk chains were cloned into phagemid vector pDAN5 downstream loxP511 linker.

After the selection on hTG2 from the three minilibraries all together 28 clones proved to be positive. It has been shown that the Vk usage was restricted to three family: IGKV1-5*03 F, IGKV1-39*01 F and IGKV1-12*01 F chains. IGKV1-39*01 was the most abundant chain which was present in all libraries. This correlates to previous findings showing that the VH/VL usage is restricted in CD and that the VL chains are dominated by the Vk1 [284].

The specificity of the positive clones was analyzed using hTG2 and mTG2. The results were different from what we expected, showing that the great majority of the clones lost their reactivity towards mTG2. Even the clones sharing the same IGKV1-39*01chain but had different specificity in case of differences in the CDR3 region. These findings indicate that different Vk chains impair different specificity. Considering that the VH domain has been reported to be the main source of binding energy and specificity in antigen recognition these results are particularly interesting and have to be considered in future studies. However, we have to note that this is not the natural VH/VL coupling and the effect might be considered when using phage display technology. We wanted to further analyze the clones in scFv-Fc format. Four representative clones were selected for investigation. Clones were coming from two cDNA libraries sharing IGKV1-39*01 F, the most used light chain in CD antibody response.The CDR3 region was different for each clone allowing to more precisely dissect the effect of the differences in CDR region have in antibody characteristics. Selected clones also had different specificity, only one out of four showing cross reactivity with mTG2.

4. 3. Cloning and expression in scFv-Fc format

The two clones scFv 1 and scFv INV-1 composed of the VH and VL chains of the original IgG1 antibody [294] and four selected clones harboring IGKV1-39*01 F chain were cloned into modified pMB-SV5 vector [393]. After purification the specificity of scFv-Fcs was dissected on hTG2 and mTG2. All of the antibodies were reactive on hTG2, only scFv-Fc 1, INV-1 and C12 had cross reactivity on mTG2 as it was seen in the scFv format.This demonstrates that the subcloning into the svFv-Fc format did not alter the specificity of the antibodies.

4. 4. Dissecting TG2-autoantibody interaction in the extracellular environment

4. 4. 1. Analyzing the effect of TG2-FN interaction on antibody binding.

Epitope 1. antibody 693-10-B06 [294] has been reported to recognize the region on TG2 molecule that is overlapping with the fibronectin binding site. We wanted to investigate if the expression in scFv-Fc format alters this property of the antibodies. Both scFv-Fc 1 and scFv-Fc INV-1 had the same amount of decrease in reactivity towards FN bound TG2 as reported for the original Epitope 1. antibody [294], indicating that neither the expression in scFv-Fc format nor the exchange of VH/ VL order caused alteration in this property.

The four reference clones with different Vk chains originating from the two cDNA libraries also had a decrease in reactivity, although less significant as scFv-Fc 1 and scFv-Fc INV-1 which had the VH and VL chains of the original Epitope 1. antibody. This indicates that the exchange in the Vk chain not only caused alterations in the specificity towards mouse TG2 but also in binding to FN binding site on TG2 molecule. These results provide further evidence for the change in epitope recognition caused by different Vk chains.

4. 4. 2. Investigate the staining properties of scFv-Fcs on csTG2

This experiment had a particular interest since in the previous chapter we showed data contradictory to the findings of a 2013 paper [294] where epitope 1. VH5 antibodies were not able to stain csTG2. It has been implicated that this contradictory data might be due to the different antibody origin, since the antibodies originating from single plasma cells represent the natural VH/VL coupling while in the case of antibodies from phage antibody libraries this coupling is random.

Both scFv-Fc 1 and scFv-Fc INV-1 harboring original VH/VL chains of epitope 1. antibody showed staining on csTG2. This result supports our findings that VH5 antibodies belonging to Epitope 1. are able to recognize TG2 when expressed on cell surface, if they are expressed in scFv-Fc format. Although we cannot completely rule out that the expression in scFv-Fc instead of IgG1 format causes alterations in epitope recognition, the cross-reactivity with mTG2 and the decreased reactivity towards FN associated TG2 indicates that it is not the case. It is more likely that the smaller size of the scFv-Fc format allows to reach TG2 even if it is not accessible to IgG1. With the use of scFv-Fc 1 and scFv-Fc INV 1 we have antibodies with human origin representing the natural VH/VL coupling that can be used for future research and therapeutic purposes.

The four reference clones harboring IGKV1-39*01 F chain with different CDR3 regions were also tested for staining csTG2. Two out of four had strong staining on csTG2 while the others were negative. Interestingly one of the antibodies able to stain csTG2 was specific to hTG2 while the other had cross reactivity to mTG2. This indicates that multiple, but not all epitopes are accessible when TG2 is expressed on cell surface, corresponding to what we have shown in the previous chapter.

4. 5. Investigating inhibitory effect of antibodies in scFv-Fc format

The inhibitory effect on TG2 *in vitro* transamidating activity on solid surface and in solution was analyzed for all antibodies expressed in scFv-Fc format. Original epitope 1. antibody (693-10-B06) has been reported not to have inhibitory effect on TG2 enzymatic activity [284, 294], corresponding to the results we had with our VH5 antibodies originating from phage antibody libraries.

Neither scFv-Fc 1 nor scFv-Fc INV-1 had significant inhibitory effect on TG2 enzymatic activity on solid surface. Both antibodies had a very mild inhibition, similar to other VH5 antibodies which are not considered inhibitory. The results were verified when the two antibodies were analyzed for the inhibitory effect in solution, where they showed no inhibitory effect at all.

The four antibodies harboring different Vk chains had no inhibitory effect at all on solid surface. The results were the same when the antibodies were analyzed in solution. These results show that even if the different Vk chains were able to cause alterations in epitope recognition, they were not improving the inhibitory property of the original antibody.

CONCLUSION

The autoantibody response in CD has been in the center of attention since TG2 has been identified as the antigen recognized by anti-reticulin and anti-endomysium antibodies in 1997 [262]. Great efforts have been made to investigate the antibody response due to the possible role anti TG2 antibodies play in the onset of the disease, however there are still many questions to be answered. One way to dissect the anti-TG2 antibody response is the use of "phage display antibody libraries" technology.

We were able to generate and separate antibodies in phage expressed scFv format first due to their specificity and second by competing them with reference antibodies. We were able cluster antibodies into four epitope groups with great majority of clones belonging to the VH5 cluster, Epitope 1. After cloning and expression in the scFv-Fc format antibodies were shown to retain their specificity, proving the effectiveness of the method. Epitope 1. antibodies were revealed to recognize a region in TG2 molecule overlapping with fibronectin binding site while other epitope groups recognized a different region. Three out of four epitope clusters were able to stain csTG2 indicating that multiple but not all the epitopes are accessible when TG2 is expressed on cell surface. Of all epitope groups only Epitope 2. showed inhibitory effect on TG2 transamidating activity, indicating that the majority of the antibodies do not inhibit the enzyme.

By reconstructing an Epitope 1. VH5 antibody (693-10-B06) from IgG1 format [294] into scFv format we generated two constructs with different VH/VL orders that retained specificity. The VH chain was substituted with Vk chains originating from three cDNA libraries originating from CD patients. The Vk usage was restricted IGKV1-5*03 F, IGKV1-39*01 F, IGKV1-12*01 F chains, IGKV1-39*01 being most abundant chain, present in all libraries and reported to be the most used Vk in CD. Interestingly clones with same Vk chain but altered CDR region had differences in reactivity towards murine TG2 indicating the role these small varieties play in antigen recognition. The two construct with original VH/VL and four selected clones with Vk chains coming from cDNA libraries were generated and characterized in scFv-Fc format. All clones were shown to recognize the region on TG2 which is overlapping with fibronectin binding site, although different Vk chains caused minor alterations in the location of the epitope. Most but not all of scFv-Fcs recognized csTG2, supporting the previous finding that multiple but not all epitopes are accessible on cell surface. Antibodies showed no inhibitory effect on TG2 enzymatic activity corresponding to the previous results with VH5 antibodies .

MATERIALS AND METHODS

Chapter 1. Abbreviations

Ab, antibody

AP, Alkaline Phosphatase

BCIP, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

BSA, Bovine Serum Albumine

DMC, N,N-Dimethylcasein

DMSO, Dimetilsulfoxide

DNase, deoxyribonuclease

dNTPs, deoxynucleotides

DTT, dithiothreitol

HRP, HorseRadish Peroxidase

IPTG, Isopropyl β-D-1-thiogalactopyranoside

MW, molecular weights

NBT, NitroBlue Tetrazolium

NC, nitrocellulose

O/N, over night

PBS, phosphate buffered saline

RT, Room Temperature

SDS-PAGE, sodium dodecyl sulphate - polyacrylamide gel electrophoresis

TG2, Tissue transglutaminase

TMB, Tetrametilbenzidine

Chapter 2. Materials

Solutions and Buffers

- **Phosphate buffered saline (PBS)**
  8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4 in 1000 ml H2O, final pH 7.4.

• **Phosphate buffered saline Tween 0.1% (PBST)**
PBS added with 0.1% Tween 20

• **Milk Phosphate buffered saline (MPBS)**
PBS added with 2% non-fat milk powder.

• **2xTY liquid broth for bacteria**
16 g Bacto-tryptone, 10 g Bacto-yeast, 5 g NaCl, final pH 7.0. If required, ampicillin 100 μg/mL, chloramphenicol 34 μg /mL, streptomycin 75 μg /mL

- **2xTY Agar plates**
  16 g Bacto-tryptone, 10 g Bacto-yeast, 5 g NaCl, 15 g Bacto-agar, final pH 7.0. If required, ampicillin 100 μg/mL, chloramphenicol 34 μg /mL, streptomycin 75 μg /mL.

- **CCMB80 for preparation of competent E.coli cells**
  11,8 g $CaCl_2$ (dihydrate), 4,0 g $MnCl_2$ (tetrahydrate), 2,0 g $MgCl_2$ (hexahydrate), 10mM K-acetate (pH7), 10% Glycerol, H_2O to 1L. Adjust pH to 6.4 . Filtration with 0.2 μm filter.

- **TAE buffer for DNA electrophoresis on agarose gels**
  0.04 M Tris-acetate, 0.001 M EDTA.

Bacterial strains

The bacterial strains used in this study were:

- *Escherichia coli DH5αF' (Gibco BRL), F'/endA1 hsd17 (rK− mK+) supE44 thi-1 recA1 gyrA (Nalr) relA1 _ (lacZYA-argF) U169 deoR (F80dlacD-(lacZ)M15)*

- *Escherichia coli BL21-CodonPlus(DE3)-RIPL strain B F– ompT hsdS(rB– mB–) dcm+ Tetr gal λ(DE3) endA Hte [argU proL/Camr] [argU ileY leuW Strep/Specr]*

Oligonucleotides

All primers were purchased from Biomers.

Oligo ID	Sequence
VLPT2	tac cta ttg cct acg gca gcc gct gga ttg tta tta ctc
VHPT2	tgg tga tgg tga gta cta tcc agg ccc agc agt ggg ttt g
PHYGRO-SEQ-SENSE	ctg ctt act ggc tta tcg
CH2 HUMAN ANTI	cgg tcc ccc cag gag ttc agg tgc

Table 3.1 Oligonucleotide sequences

Chapter 3. Standard protocols

PCR (Polymerase chain reaction)

Thermus aquaticus DNA polymerase (Thermo Fisher Scientific) was used.

Reaction mixture:

- Template DNA 0.01-1ng (plasmidic DNA)
- Sense primer 0.5 uM
- Antisense primer 0.5 uM
- Reaction buffer 10x 2.0 ul
- dNTPs (Promega) 0.25 mM
- Taq polymerase 0.025 unit/ul
- H_2O to 20 ul

The following cycles were performed:

- Denaturation step, 5’ at 94°C.
- 31 cycles of: denaturation, 45’’ at 94°C; annealing, 45’’ at 60°C; elongation, 1’’every 1000bp at 72°C.
- Final elongation step: 10’’ at 72°C.

DNA electrophoresis on agarose gels

Agarose (Sigma) gels with a concentration of 1.5 % in TAE buffer were used to separate PCR products; 0.8 % agarose gels were used to separate plasmidic DNA preparations, before and after digestions. 1 μL of ethidium bromide (2mg/mL, Sigma) was added to 50 ml of agarose gel.100 base-pairs plus and 1KB molecular weight markers were purchased from Fermentas.

DNA purification

The GeneJET PCR Purification Kit (Thermo Scientific) was used for purification of DNA from agarose gel and reaction mixtures, following the manufacturer instructions.

Plasmidic DNA extraction

The GeneJET Plasmid Miniprep Kit (Thermo Scientific) was used for plasmidic DNA mini-preparations (1 to 5 ml of O/N bacterial culture), following the manufacturer instructions.

DNA digestion with restriction endonucleases

All restriction endonucleases (BssHII, NheI) were purchased from New England Biolabs (NEB).

Reaction mixture:

- DNA
- NEB Buffer 10x
- BSA 100x
- Restriction endonuclease, 1 unit / μg of DNA
- H_2O to 50uL

Incubation for 1 hour at the temperature required by the specific endonuclease.

Ligation

Plasmidic vector DNA and insert DNA were mixed at a 1:3/1:5 ratio (number of molecules). T4 ligase was purchased from New England Biolabs (NEB).

Reaction mixture:

- DNA (about 50ng)
- T4 ligase Buffer (NEB) 10x
- T4 ligase (NEB), 1 Unit/reaction
- H_2O to 10ul

Incubation O/N at 16°C.

Preparation of competent E.coli

50 mL of E.coli culture, DH5α or BL21(DE3)RIPL strains, were grown at 37°C in 2xTY liquid broth to OD_{600} 0.5. Bacteria were chilled in ice for 10' to stop growth, centrifuged at 4°C for 10' at 3000 rpm and the supernatant was discarded. The bacterial pellet resuspended in 8 mL of CCMB80 solution and put in ice for 20'. After centrifuging for 10' at 4°C, supernatant was discarded, bacterial pellet resuspended in 2 mL of CCMB80 and dispensed in 80 µL aliquotes. Competent cells were immediately used or stocked at -80°C up to four weeks.

Bacterial transformation

5 µL of ligation reaction mixture or 10-50ng of plasmidic preparation were transferred into a tube containing 80 uL of competent cells. The mixture was incubated in ice for 20'. Heat shock was applied at 42°C for 1 minute and 30''; bacteria were then chilled in ice for 1 minute, resuspended in 1 ml of liquid broth and allowed to grow at 37°C in absence of selective antibiotic for 1 hour. Bacteria were then plated on antibiotic-containing agar plates and grown O/N at 30°C.

DNA Sequencing

PCR products were purified with GeneJET PCR Purification Kit (Thermo Scientific), following manufacturer instructions.

Reaction mixture for sequencing were composed of:

- 20-100ng of purified PCR product
- 1 µL primer (3,2µM)
- 1 µL Terminator Ready Reaction Mix (BigDye Terminator v 1.1 Cyle Sequencing Kit-Applied Biosystems)
- H_2O to 10µL

Sequencing program was performed as :

- 1'at 96°
- 25 cycles: 15'' at 96°; 5'' at 50°; 4' at 60°

Reactions mixes were purified with CENTRI SEP Spin Columns, following manufacturer instructions. 5 µL of purified sequences were loaded on sequencing plates with 10 µL of formamide, denaturated for 2' at 96° and analyzed with 3100 Genetic Analyzer sequencer (ABI PRISM-HITACHI).

Chapter 4. Methods

Intestinal Biopsy Lymphocyte RNA preparation and library construction

Total RNA was prepared from 10 ml of IBL from seven previously untreated CD adult patients. cDNA was synthesized by using random hexamers and SuperScript II reverse transcriptase (Life Technologies). Ig V regions were amplified by using specific V region primers and assembled into scFv as reported in [286] before cloning into pDAN5.

Recombinant TG2 production

Recombinant hTG2 was expressed in pET-28b vector by *E. coli* BL21-RIPL strain, harboring kanamycin resistance, while mTG2 was expressed pTrcHis-B (Invitrogen) vector by DH5αF' cells harboring ampicillin resistance. Bacteria were grown in corresponding antibiotics to 0.5 OD600, induced with 0.2 mM, isopropyl thio-b-D-galactoside (IPTG) and incubated at 20 °C O/N. The soluble cytoplasmic fraction was prepared by extraction of pelleted bacteria with lysozyme in lysis buffer followed by centrifugation. The supernatant was collected and purified fragments were obtained by affinity chromatography of the bacterial extract on HIS-Select® Nickel Affinity Gel (SIGMA).

Selection and testing of phage Abs

Phagemid particles were rescued as described in [286] and panning was performed by adding phages diluted in 2% nonfat milk in PBS (MPBS) to immunotubes (Nunc**,** Naperville, IL) coated with purified h TG2, washing 20 times with PBS 0.1% Tween 20, and 20 times with PBS, followed by elution with 1 ml of *E. coli* cells at 0.5 OD600 for 30 min at 37°C and overnight growth after addition of ampicillin, helper phage, and kanamycin. The panning procedure was repeated up to three times. After selection,individual clones were screened for reactivity to hTG2 by ELISA performed in 96 well plate.

Phage ELISA

Single clones were grown in 1 mL of 2xTY added with ampicillin 37°C to OD600 0.5 They were infected with a wild-type helper phage without agitation. Bacteria were then centrifuged, the supernatant discarded and the pellet resuspended in 1 ml of 2xTY added with ampicillin and kanamycin and grown O/N at 28°C. Costar ELISA strips were coated with hTG2, mTG2 or BSA as a control protein at 3 µg/ml. Wells were blocked with 2% MPBS at RT for 45'. Bacteria were centrifuged for 10' at 7000 rpm and supernatant with phages of individual clones were added to the wells, followed by extensive washes. Wells were added with horseradish conjugated anti-M13 monoclonal antibody (SIGMA) diluted 1:5000 and incubated which was followed by extensive washes. Immunocomplexes were revealed with the chromogenic substrate 3,3′,5,5′-Tetramethylbenzidine (Sigma), and the plate read at OD450.

Phage Competition ELISA

Preparation of phages as mentioned in „Phage ELISA". Costar ELISA strips were coated with hTG2, mTG2 or BSA as a control protein at 3 µg/ml. Wells were blocked with and then added with reference antibodies in scFv-Fc format, diluted in 1:1000 and incubated for 1h at RT. Bacteria were centrifuged 7000RPM and 15ul supernatant of individual clones was exchanged with 15ul of well solution and incubated 1.5 h at RT. After extensive wash steps wells were added with horseradish conjugated anti-M13 monoclonal antibody (SIGMA) diluted 1:5000 and incubated 1h at RT. Immunocomplexes were revealed after extensive wash steps with the chromogenic substrate 3,3′, 5,5′-Tetramethylbenzidine (SIGMA), and the plate read at OD450.

Cloning and Expression as Recombinant scFv-Fc in CHO cells

The cloning of scFv genes was performed by extraction from phagemid pDAN5 clones by cutting with BssHII and NheI (NEB) enzymes for 1h at 37 °C. Modified pMB-SV5 vector was cut with same enzymes. After cloning individual colonies were analyzed by fingerprinting with BstNI enzyme (NEB) and sequencing with 3130/3130xl Genetic Analyzer (Applied biosystems). Plasmid DNA has been extracted from positive clones (Thermo Scientific) and transfected into CHO cells with using Lipofectamine® 2000 Transfection Reagent (Invitrogen).

Western blotting

SDS/PAGE was performed according to standard techniques. Purified scFv-Fcs were separated by SDS/PAGE and transferred onto nitrocellulose (Whatman) by semi-dry blotting using the TRANS-BLOT SD SEMI-DRY TRANSFER CELL (BIORAD). The scFv-Fcs were revealed by anti-Human IgG conjugated with Alakaline Phosphatase (SIGMA).

TG2 Inhibition assay in solid surface

Costar ELISA strips were coated with Casein,N,N –dimethylated (SIGMA) in 15µg/ml concentration. A pre-incubation of the following mix(100mM Tris/HCl pH 8; 10mM DTT; 5mM $CaCl_2$; 150mM NaCl; XXµg of TG2 (0,1-0,01ug) and Xxug of scFv-Fc (0,7-0,1ug) was done at 30°C for 1h. After incubation the mix was substituted with 0,2 mM EZ-Link Pentylamine-Biotin, added to ELISA wells and incubated at 37°Cfor 1h. After extensive wash steps Streptavidin-HRP (R&D Systems) working solution was added to the wells. Incorporation was revealed with 3,3′,5,5′-Tetramethylbenzidine and H2SO4 as substrates and read at 450nm.

TG2 Inhibition assay in solution

A preliminary incubation step was done with the following mix: (100mM Tris/HCl pH 8; 10mM DTT; 5mM $CaCl_2$; 150mM NaCl; XXµg of TG2 (0,1-0,01ug) and Xxug of scFv-Fc (0,7-0,1ug)) at 30°C for 1h. After incubation the mix was substituted with 0, 2 mM EZ-Link Pentylamine-Biotin and DMC 30ug/ml. Corresponding amount of mix was separated by SDS/PAGE and transferred onto nitrocellulose (Whatman) by semi-dry blotting using the TRANS-BLOT SD SEMI-DRY TRANSFER CELL (BIORAD). Incubation was done with Streptavidin -Alkaline Phosphatase antibody and revealed by NBT/BCIP (Roche).

ELISA assay on Fn captured TG2

Plates were coated with 10ug/ml of full length Fn (SIGMA) or 3ug/ml TG2 protein. Incubation step was followed by addition of 3ug/ml TG2 for FN coated wells and PBS1x for wells coated with TG2. A 1h incubation step at 37°C was followed by extensive wash with PBST 0,1%. Primary antibodies (0,1/0,25/0,5ug/ml) were added and incubated 1h at 37°C. After wash step anti-Human-HRP was added as secondary antibody and incubated 1h at 37°C. Immunocomplexes were revealed after extensive wash steps with the chromogenic substrate 3,3′,5,5′-Tetramethylbenzidine (Sigma), and the plate read at OD450.

Surface staining and FACS analysis of TG2 expressing cells

MCF7 cells were washed with cold Staining Buffer and centrifuge 240 rcf for 5 min at 4°C. Cell pellet stained with saturating concentration of the antibody (1-2ug), diluted in 100ul of Staining Buffer. Cells were incubated with antibody for 20-30 min at 4°C. After wash and centrifuge step (240rcf for 5 min at 4°C) supernatant was carefully aspired. Cells were stained with the secondary antibody anti-Human Cy5 (Listarfish) diluted in 100ul of Staining Buffer, and incubate for 20-30 min at 4°C, washed and fixed.

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