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Immune responses to Tissue Transglutaminase in Coeliac Disease

A thesis submitted for the degree Doctor of Philosophy

by

Ross Comerford, BSc. (Hons)

Trinity College

University of Dublin

2013
Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

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Ross Comerford
Summary

Coeliac disease is a common inflammatory disease of the small intestine caused by an inappropriate immune response to wheat gluten and gliadin in genetically predisposed individuals. Investigation into the pathogenesis of coeliac disease reveals a complex interplay between environmental factors, genetics, the adaptive and innate immune systems, and the presence of autoantigens, in a process which has still not been fully elucidated.

The principal autoantigen of coeliac disease is the ubiquitously expressed, multifunctional enzyme tissue transglutaminase; the demonstration of IgA autoantibodies directed to this self-protein is an integral component in the diagnosis of coeliac disease. Tissue transglutaminase is also intimately involved in coeliac disease pathogenesis by modifying gluten and gliadin peptides by deamidation, enhancing their recognition by the immune system. Autoantibodies of the IgG class directed against tissue transglutaminase may also be present in coeliac disease, and in multiple autoimmune diseases, including type 1 diabetes. The underlying mechanism resulting in the production of anti-tTG autoantibodies has not been defined; it has been speculated that in coeliac disease this may be a result of an immune response to covalent complexes of gliadin and tTG, with an epitope spread from gliadin to tTG.

The aim of this study was to investigate various aspects of anti-tTG immunity, including an analysis of coeliac disease IgA and IgG anti-tTG epitopes, an investigation of differences in IgG anti-tTG epitope specificity and subclass usage between individuals with CD and those with other autoimmune diseases, and the development of tTG antigen-specific T cell lines.

In the first part of the study, five full-length recombinant human tissue transglutaminase proteins were generated; the wild-type protein, and four active-site mutants. These proteins were then applied as antigen in an ELISA system, where it was found that
coeliac disease serum IgA and IgG anti-tTG antibodies displayed a reduction in binding to all mutant proteins when compared to the wild-type protein. From this section it was concluded that an intact tTG active-site was required for coeliac disease autoantibody binding, an observation made in both adults and children with coeliac disease.

The second part of the study further dissected the IgG anti-tTG response, with a comparison of the epitope specificity and IgG anti-tTG subclass usage being made between individuals with coeliac disease, and individuals with autoimmune disease who were positive for IgG anti-tTG. A modified ‘epitope-masking’ ELISA was developed, in which potential differences in the epitope specificity of IgG anti-tTG in non-coeliac individuals were identified, an observation strengthened by the equal binding to wild-type and mutant tissue transglutaminases in these individuals. A profile of the IgG anti-tTG subclass usage was generated for all disease groups; that of coeliac disease and type 1 diabetes was dominated by IgG1, whereas this IgG subclass was infrequently a component of the IgG anti-tTG response in diseases such as Wegener’s granulomatosis and Crohn’s disease.

In the third part of the study, antigen-specific T cell responses to tissue transglutaminase were investigated, via the measurement of proliferation, and the generation of T-cell lines in response to tTG stimulation in coeliac disease patients and controls. Tissue transglutaminase-specific T cells were found to produce large amounts of IFN-γ, as well as IL-10, IL-17A, and IL-21. The production of IFN-γ, and the up-regulation of the T cell activation markers CD25 and HLA-DR by these cells were determined to be effective markers of response to stimulation with tissue transglutaminase. The implication of the findings of this study, and their relevance to coeliac disease pathogenesis are discussed in the final section.
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This thesis is dedicated to my parents and grandparents
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Publications


- Comerford, R., Byrne, G., Feighery, C., Kelly, J. "The epitope specificity and subclass usage of IgG antibodies to tissue transglutaminase differs between coeliac disease and other autoimmune diseases". (Manuscript in preparation).


Oral presentations

- Comerford, R., Byrne, G., Feighery, C., Kelly, J. "Molecular analysis of epitope specificity of anti-tTG antibodies in coeliac disease"
  - Irish Society for Immunology annual conference 2009, Institute of Molecular Medicine, St James’ Hospital, Dublin.

Poster presentations

- Comerford, R., Byrne, G., Feighery, C., Kelly, J. "Molecular analysis of epitope specificity of anti-tTG antibodies in coeliac disease"
  - Irish Society for Immunology annual conference 2009, Institute of Molecular Medicine, St James’ Hospital, Dublin.
  - 7th Mucosal Immunology Group meeting, Amsterdam, The Netherlands, 2010.

- Comerford, R., Byrne, G., Feighery, C., Kelly, J. "Analysis of IgG autoantibody responses to tissue transglutaminase"
  - Irish Society for Immunology annual conference 2010, Queen’s University, Belfast.
- 7th Mucosal Immunology Group meeting, Amsterdam, The Netherlands, 2010.

- Comerford, R., Dunne, M., Coates, C., Kelly, J., Feighery, C. "A study of tissue transglutaminase-sensitised T cells in coeliac disease"
  - Irish Society for Immunology annual conference 2011, University College Galway.
  - 14th International Coeliac Disease Symposium, Oslo, Norway, 2011.
<table>
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<tr>
<td>%CV</td>
<td>Percentage coefficient of variation</td>
</tr>
<tr>
<td>Aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AD</td>
<td>Anno domini</td>
</tr>
<tr>
<td>AID</td>
<td>Autoimmune disease</td>
</tr>
<tr>
<td>ANCA</td>
<td>Anti-neutrophil cytoplasmic antibody</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>BC</td>
<td>Before Christ</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCL3</td>
<td>Chemokine (C-C motif) ligand 3</td>
</tr>
<tr>
<td>CD</td>
<td>Coeliac disease</td>
</tr>
<tr>
<td>CFSE</td>
<td>5,6-carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T-lymphocyte-associated protein 4</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-Diaminobezidine</td>
</tr>
<tr>
<td>DH</td>
<td>Dermatitis herpetiformis</td>
</tr>
<tr>
<td>DGP</td>
<td>Deamidated gliadin peptides</td>
</tr>
<tr>
<td>dh20</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>1,4-Dithiothreitol</td>
</tr>
<tr>
<td>EATL</td>
<td>Enteropathy-associated T cell lymphoma</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent assay</td>
</tr>
<tr>
<td>EMA</td>
<td>Endomysial antibody</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-associated cell sorter</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
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<td>Glutathione-S-transferase</td>
</tr>
<tr>
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<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IA-2</td>
<td>Islet cell autoantigen 2</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible co-stimulator</td>
</tr>
<tr>
<td>IEL</td>
<td>Intraepithelial lymphocyte</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>INR</td>
<td>International normalised ratio</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D thiogalactoside</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LMW</td>
<td>Low molecular weight</td>
</tr>
<tr>
<td>mA</td>
<td>Milliamp</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosal-associated lymphoid tissue</td>
</tr>
<tr>
<td>MB</td>
<td>Million bases</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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<td>Millilitre</td>
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</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomole</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBA</td>
<td>Phosphate buffered saline with albumin</td>
</tr>
<tr>
<td>PBC</td>
<td>Primary biliary cirrhosis</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
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<td>Polymerase chain reaction</td>
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<td>Phytohaemaglutinin</td>
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<tr>
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<td>Isoelectric point</td>
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<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified protein derivative</td>
</tr>
<tr>
<td>PT</td>
<td>Partially treated</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>rh</td>
<td>Recombinant human</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Surface epitope masking</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel</td>
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<tr>
<td></td>
<td>electrophoresis</td>
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<tr>
<td>SI</td>
<td>Stimulation Index</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosis</td>
</tr>
<tr>
<td>T</td>
<td>Treated</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate EDTA</td>
</tr>
<tr>
<td>TCM</td>
<td>T cell medium</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylethylenediamine</td>
</tr>
<tr>
<td>Tfh</td>
<td>Follicular T helper cell</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TG</td>
<td>Transglutaminase</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethyl-benzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Tr1</td>
<td>Type 1 regulatory T cells</td>
</tr>
<tr>
<td>tTG</td>
<td>Tissue transglutaminase</td>
</tr>
<tr>
<td>UT</td>
<td>Untreated</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VDJ</td>
<td>Variable diversity joining</td>
</tr>
<tr>
<td>wt</td>
<td>Wild-type</td>
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</tbody>
</table>
Chapter 1
General Introduction
1.1 Coeliac disease

1.1.1 Introduction

Coeliac disease (CD) is a chronic inflammatory disorder of the small intestine characterised by an immune response to ingested gliadin and related cereal proteins (Wieser 1995), affecting approximately 1 in 100 Caucasian individuals (Dube, Rostom et al. 2005). Traditionally considered a disease of childhood, the classical presenting symptom in individuals suffering from CD is malabsorption, due to intestinal villous atrophy and loss of absorptive surface, with associated chronic diarrhoea, fatigue and a failure to thrive (Marine, Farre et al. 2011). Increasingly, patients are being diagnosed in the absence of these ‘classical’ CD symptoms, often presenting with more subtle and variable clinical symptoms (Ravikumara, Tuthill et al. 2006). The mean age at diagnosis has also risen, with screening methods often detecting asymptomatic individuals (McGough and Cummings 2005). The diverse clinical heterogeneity in coeliac disease patients, therefore, ranges from those who are asymptomatic, to extremely symptomatic (Scherer 2008), including extraintestinal symptoms such as osteoporosis (Cashman 2008), and neurologic disorders (Leggio, Abenavoli et al. 2004). An investigation into the pathogenesis of coeliac disease reveals a complex interplay between environmental factors, genetics, the adaptive and innate immune systems, and the presence of autoantigens, in a process which still has not been fully elucidated.

1.1.2 The history of coeliac disease

It has been suggested that coeliac disease developed after the last ice age, in the fertile crescent of the Middle East (Losowsky 2008). The agricultural revolution of this period (circa 8000-5000 BC) involved the domestication of animals and cultivation of grains,
resulting in the generation of dietary antigens previously unseen to the human mucosal immune system. The first record of identification, and naming of, coeliac disease was made at the start of the first millennia AD by a Greek physician, Aretaeus of Cappadocia. In an 1856 translation of Aretaeus' work at the Sydenham Society by Francis Adams, the term 'coeliac' was first coined, based on Aretaeus' description of the disorder as 'koiliakos' - originating from the Greek word for abdomen, 'koelia' (Adams 1856).

In 1888, a prominent English paediatrician, Dr. Samuel Gee published what has become the milestone modern description of coeliac disease, documenting cases in children and adults (Gee 1888). Gee noted at the time that '...if the patient can be cured at all, it must be by means of diet'. With the causative agent remaining unknown, it was noted that coeliac patients could tolerate fats better than carbohydrate (Herter 1908), and that a diet consisting of bananas was beneficial (Haas 1924). The identification of wheat as the dietary agent responsible for CD was made by Dicke, a Dutch paediatrician who noted remission of the disease in coeliac children during the bread shortages of World War II, and the rapid return of symptoms upon re-introduction of bread into the diet (Dicke 1941). A series of key publications followed, detailing the beneficial effects of removal of cereals from the diet of coeliac patients, the identification of wheat flour, but not starch, as toxic, the detrimental effects of the inclusion of rye, flour and oats in the diet of patients, and the identification of wheat gluten as the causative agent in coeliac disease (van Berge-Henegouwen and Mulder 1993). The 'gluten-challenge', described by Anderson et al. (Anderson, French et al. 1952) to provide further evidence of the aetiological agent in CD, details the re-occurrence of symptoms following reintroduction of gluten into the diet of a coeliac patient in remission, and became part of the
diagnostic criteria for coeliac disease in 1969, as the 'Interlaken criteria' (McNeish, Harms et al. 1979).

### 1.1.3 Epidemiology

Coeliac disease was once thought of as a rare disease, with incidences such as 1/8000 being reported in England and Wales (Davidson and Fountain 1950). The development of biopsy apparatus capable of sampling the distal duodenum by Shiner in the mid-fifties (Shiner 1956) provided a valuable tool for coeliac disease diagnosis (Girdwood, Delamore et al. 1961), reflected in the subsequent rises of incidence. Incidences of 1/1000 were reported in Sweden from 1966-75 (Berg and Lindberg 1979), and of 1/597 in children, and 1/303 in adults, in the west of Ireland (Mylotte, Egan-Mitchell et al. 1973).

With the advent of serological screening and recognition of atypical forms of the disease, the incidence of coeliac disease has risen dramatically, with an incidence of 1/100 being generally reported in large population-based studies (Tommasini 2004; Rewers 2005; Mustalahti, Catassi et al. 2010). Until recently, coeliac disease was thought to exclusively affect Europeans, or people of European origin, however high incidences of 1/355 in Tunisian healthy blood donors (Mankai, Landolsi et al. 2006), 1/167 in Argentinean adults (Gomez, Selvaggio et al. 2001), and 5/100 in Saharawi children (Catassi, Ratsch et al. 1999) have been described. Cases have also been reported in China (Wu, Xia et al. 2010), India (Yadav, Das et al. 2011), and Japan (Cummins and Roberts-Thomson 2009).

Whilst traditionally considered a paediatric disorder, it has become evident in the last few decades that CD can occur in individuals of any age. The majority of new cases are being diagnosed in adults (Hawkes, Swift et al. 2000), with a peak of incidence in the fifth decade of life (Feighery 1999). As is the case for most autoimmune diseases there is a
Due to the often subtle, and varying, clinical symptoms shown by coeliac individuals, CD is thought of as remaining largely under-diagnosed (Hin, Bird et al. 1999; Lebwohl, Kapel et al. 2011). The varying degrees of disease severity are recognised by the use of the ‘coeliac iceberg’ (Ferguson, Arranz et al. 1993) as an epidemiological model, where patients with overt symptoms form the tip of the iceberg visible above the waterline, i.e. the smallest proportion (Fig 1.1).
Individuals with characteristic intestinal changes of coeliac disease and positive serology, which both return to normal upon commencement of a gluten-free diet, and who are asymptomatic, are deemed to have ‘silent’ coeliac disease (Goddard and Gillett 2006). The presence of serological markers for coeliac disease, but normal intestinal mucosa is termed ‘latent’ coeliac disease (Ludvigsson, Montgomery et al. 2009). Those with latent coeliac disease may later develop symptoms and/or intestinal lesions (Gasbarrini, Malandrino et al. 2008).

CD is linked with other autoimmune conditions, such as type 1 diabetes mellitus (Bao, Yu et al. 1999), autoimmune thyroid disease (Sategna-Guidetti, Bruno et al. 1998), Addison’s disease (Biagi, Campanella et al. 2006), systemic lupus erythematosis (Komatireddy, Marshall et al. 1995), and autoimmune hepatitis (Caprai, Vajro et al. 2008).

1.1.4 Clinical symptoms

Classically, CD presents in the first years of life with symptoms of diarrhoea, failure to thrive, malnutrition, abdominal pain and distention (Barker and Liu 2008). Currently, there is a trend towards a delayed onset of symptomatic CD in older children (5-7 years old) (Fasano 2005). In contrast to paediatric patients, CD in adults often presents with more variable and subtle symptoms (Westerberg, Gill et al. 2006), with diarrhoea present in roughly 50% of cases (Green and Cellier 2007). Typically, symptoms improve or resolve upon commencement of a gluten-free-diet (Armstrong, Hegade et al. 2012). Many of the extra-intestinal or atypical symptoms of CD may be attributed to malnutrition due to loss of absorptive surface in the small intestine. Anaemia, caused by deficiencies in iron, folate, or vitamin B12 is a frequent manifestation of CD (Nelsen 2002). A recent study in Beaumont hospital, Dublin, found that 21% of newly-diagnosed
adult CD patients presented with anaemia (Tajuddin, Razif et al. 2011). Deficiencies of vitamins D and K may result in rickets/hypocalcaemia (Nield, Mahajan et al. 2006), and coagulopathy such as prolonged prothrombin time/abnormal INR (Djuric, Zivic et al. 2007), respectively. Perturbations of calcium homeostasis secondary to vitamin D deficiency is likely responsible for the low bone mineral density found in some patients (Mager, Qiao et al. 2012). Reproductive disorders such as infertility in both men and women, miscarriages, and amenorrhea have been reported (Collin, Vilska et al. 1996; Rostami, Steegers et al. 2001). Table 1.1 lists presenting symptoms of CD. Arthritis (Lubrano, Ciacci et al. 1996), hypertransaminasaemia (Rubio-Tapia and Murray 2007), and neurological disorders such as ataxia, peripheral neuropathy and epilepsy, related to gluten sensitivity have also been described (Hadjivassiliou, Sanders et al. 2010). The related gluten sensitive skin disorder, dermatitis herpetiformis may also be present, although this is infrequently found in paediatric CD (Fasano 2005).

Table 1.1: Presenting symptoms of coeliac disease

<table>
<thead>
<tr>
<th>Gastrointestinal symptoms</th>
<th>Non-gastrointestinal symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diarrhoea</td>
<td>Anaemia</td>
</tr>
<tr>
<td>Steatorrhoea</td>
<td>Infertility</td>
</tr>
<tr>
<td>Occult blood</td>
<td>Dental enamel defects</td>
</tr>
<tr>
<td>Constipation</td>
<td>Osteoporosis</td>
</tr>
<tr>
<td>Vomiting</td>
<td>Neuropathy</td>
</tr>
<tr>
<td>Dyspepsia</td>
<td>Depression</td>
</tr>
<tr>
<td>Abdominal distension</td>
<td>Seizures</td>
</tr>
<tr>
<td>Hypertransaminasaemia</td>
<td>Fatigue</td>
</tr>
<tr>
<td>Recurrent pancreatitis</td>
<td>Folate/B12/Iron deficiency</td>
</tr>
<tr>
<td>T cell lymphoma (EATL)</td>
<td>Dermatitis herpetiformis</td>
</tr>
</tbody>
</table>
1.1.5 Diagnosis

Due to the classical presenting symptoms of CD such as diarrhoea, constipation, abdominal cramping and distension being frequently absent (Lo, Sano et al. 2003), CD may only be suspected due to non-classical gastrointestinal symptoms such as anaemia, osteoporosis, mouth ulcers, or vitamin and mineral deficiencies, suggesting that there is a high prevalence of undiagnosed CD. Whilst the mean age at diagnosis has risen, there is great variation, the disease may present at any stage in life (Armstrong, Robins et al. 2009). It has been proposed by Silano (2007) that diagnosis later in life confers a greater risk of malignancy associated with CD (Silano, Volta et al. 2007).

Duodenal biopsy

The 'gold standard' for the diagnosis of CD is a representative distal duodenum biopsy revealing significant villous atrophy and mucosal crypt hyperplasia (Walker and Talley 2011) (Fig. 1.2). Increased numbers of intraepithelial lymphocytes (IEL) are usually present in the coeliac lesion (Collin, Wahab et al. 2005), however this is not CD-specific and occurs in giardiasis, tropical sprue, and various autoimmune diseases (Kakar, Nehra et al. 2003). Recent reports have also recommended additional sampling of the duodenal bulb, which may frequently reveal villous atrophy (Leeds, Hopper et al. 2008; Nenna, Pontone et al. 2012).
Figure 1.2: Small intestinal mucosal biopsy viewed through a dissecting microscope. a) is from normal small intestinal mucosa with numerous surface villi visible, whilst b) is taken from an untreated CD patient and displays total villous atrophy, with surface openings to the underlying crypts visible. Image Adapted from Kagnoff (2005).

Lesions are graded using the staging system devised by Marsh in 1992 (Marsh 1992), and modified by Oberhuber in 1999 (Oberhuber, Granditsch et al. 1999) (Fig. 1.3). Normal intestinal histology is graded as Marsh 0, whilst the presence of increased (>30 lymphocytes per 100 enterocytes) IELs alone defines marsh I. Marsh II describes a lesion with intraepithelial lymphocytosis accompanied by crypt hyperplasia. Lesions with any degree of villous atrophy, which may vary from blunting to total loss of villous architecture, are classified as Marsh III. Marsh stages II and III are present in the majority of patients with untreated coeliac disease (Hopper, Cross et al. 2007), although in keeping with the heterogeneous nature of clinical presentation, this may vary.
Figure 1.3: The Marsh staging system of intestinal damage in CD. H&E staining of CD small intestinal mucosa. a) Marsh I: increased intraepithelial lymphocytes. b) Marsh II: intraepithelial lymphocytosis and crypt hyperplasia. c) Marsh III villous atrophy and lymphocytosis. Image adapted from Volta, 2011.
From the 1970s onwards, the European Society of Paediatric Gastroenterology and Nutrition criteria were applied in the diagnosis of CD. This involved the taking of three biopsies: the first demonstrating a loss of mucosal architecture, the second displaying restoration or improvement of the intestinal lesion following commencement of a gluten-free diet, and a third showing a return of the intestinal lesion upon gluten-challenge (Meeuwisse 1970). With the dawn of more sensitive and specific serological testing for CD, these guidelines were subsequently revised, the presence of CD-related symptoms and autoantibodies accompanied by a single positive biopsy result is now deemed sufficient for diagnosis (Fasano and Catassi 2001). A strategy of serial biopsy may still be useful in the diagnosis of CD in asymptomatic or seronegative symptomatic patients (Walker and Murray 2011). Currently there is an increasing debate on the requirement for duodenal biopsy in the presence of typical symptoms and high levels of anti-tissue transglutaminase antibodies (Hill and Holmes 2008; Mubarak, Wolters et al. 2011).

**Serology**

Serological tests to recognise CD are usually in the first line of investigations performed on symptomatic individuals, at-risk individuals such as first-degree relatives of CD patients (Biagi, Campanella et al. 2008), or those with autoimmune disease such as T1DM (Chand and Mihas 2006). Positive CD-related serology is frequently an indicator of the need to perform a duodenal biopsy. The automation of anti-tTG measurement has resulted in the application of this test for mass-screening purposes (Ress, Harro et al. 2011).
Endomysial antibodies

The most specific serological tool to aid in the diagnosis of coeliac disease is the detection of IgA antibodies directed against the endomysium, a connective tissue layer in the muscularis mucosa, which have a sensitivity of 93%, and a specificity of 99% (Wong, Steele et al. 2003). Initially, monkey oesophagus was used as a tissue substrate; however this has widely been replaced with human umbilical cord tissue (Volta, Molinaro et al. 1995). Endomysial antibodies (EMAs) may be visualised by indirect immunofluorescence, with positive sera displaying a typical green fishnet pattern (Tumer, Hasanoglu et al. 2001). EMAs may be titrated, in order to gauge the level of the autoantibody response. Due to its labour intensity requirement and operator subjectiveness, the measurement of EMAs is not used as a mass-screening tool, but serves as an important confirmatory test in CD diagnosis (Sinclair, Pearce et al. 2003).

Anti-tissue transglutaminase antibodies

The autoantigen contained in the endomysium was identified as the enzyme tissue transglutaminase, (termed tTG or TG2), by Dieterich et al. in a landmark paper published in 1997 (Dieterich, Ehnis et al. 1997). This allowed the design of diagnostic ELISA tests for screening for IgA autoantibodies to tTG, initially based on tTG extracted from guinea pig liver, which shares 80% homology with human tTG (Dieterich, Laag et al. 1998). Anti-tTG measurement using guinea pig tTG had high rates of false positivity, possibly due to contaminating non-tTG liver proteins (Fabiani, Peruzzi et al. 2004). The use of recombinant human tTG based ELISA testing now serves as an excellent population screening test for detection of IgA autoantibodies to tTG, and hence predict the presence of CD, with optimized assays giving a sensitivity and specificity of 94 and 97% respectively (Rostom, Dube et al. 2005).
The measurement of IgG anti-tTG traditionally suffers from poor sensitivity (Ankelo, Kleimola et al. 2007), but is of use in the detection of CD in children <2 years of age, who are frequently IgA anti-tTG/EMA negative (Burgin-Wolff, Gaze et al. 1991; Ghedira, Sghiri et al. 2001), and in patients with selective IgA deficiency (Korponay-Szabo 2003).

**Anti-gliadin antibodies**

Identified by Berger in 1964, ELISA assays measuring IgA or IgG anti-gliadin antibodies were one of the first serological tests developed for CD diagnosis (Berger, Buergin-Wolff et al. 1964). Whilst IgG anti-gliadin assays are useful for detecting CD in IgA-deficient patients, and young children, in general anti-gliadin measurement displays unacceptable sensitivity and specificity and is prone to generating false positive results, with one study estimating ten times as many positive results as there are biopsy-proven CD positives (McMillan, Watson et al. 1996).

Recently, new ELISAs measuring IgA and IgG anti-deamidated gliadin peptides (DGP) have been described, revealing high sensitivity and specificity for CD (Basso, Guariso et al. 2008; Mubarak, Gmelig-Meyling et al. 2011). The appearance of anti-DGP antibodies in CD parallel that of anti-tTG, however anti-DGP resolve more quickly upon commencement of a gluten-free diet, indicating a possible use as a more sensitive monitor of treatment (Liu, Li et al. 2007).

**1.1.6 Genetics**

There is evidence of a strong genetic influence which will predispose an individual to the development of CD. The prevalence amongst first-degree relatives of an affected individual is 10% (Koning, Schuppan et al. 2005), whilst the concordance rate between monozygotic twins is high at 75% (Bevan, Popat et al. 1999). CD primarily involves an
inappropriate immune response to gluten, and accordingly many of the genetic linkages identified relate to gene products that influence the immune system.

**The Human Leukocyte Antigen (HLA) complex**

The HLA complex is encoded by a 4MB region on the short arm of chromosome 6 (6p21), with the majority of the 200 genes contained therein associated with immune function (McCluskey, Kanaan et al. 2003). The first reporting of an association between CD and the HLA complex was made in the early 1970s, initially with the MHC class I molecule B8 (Falchuk, Rogentine et al. 1972). Stronger associations with the MHC class II molecules DR3 and DQ2 were later observed, both contained within the B8-DR3-DQ2 and B18-DR3-DQ2 extended haplotypes associated with CD (Sollid 2000). This haplotype is also associated with autoimmune diseases such as T1DM and autoimmune thyroid disease, which likely accounts for the increased risk of these disorders in patients with CD (Volta, De Franceschi et al. 1997). Approximately 90% of CD patients express the HLA DQ2 variant DQ2.5, with the remaining 10% expressing HLA-DQ8 (Fallang, Bergseng et al. 2009).

Normally, HLA-DQ facilitates presentation of exogenous peptide antigens to helper T cells, and is therefore expressed on the surface of antigen-presenting cells such as dendritic cells, macrophages, and monocytes (Ichikawa, Baba et al. 1994). In CD, gliadin peptides are presented to the immune system via HLA-DQ; the discovery that gliadin-specific T cells isolated from the CD intestinal lesion are HLA-DQ-restricted confirmed a functional mechanism for the association of DQ genes with CD (Lundin, Scott et al. 1993). Most patients with CD express the HLA-DQ2.5 heterodimer, which may be encoded in two ways. Individuals with the DR3-DQ2 haplotype express the HLA-DQ2.5 heterodimer
encoded in cis by two adjacent gene alleles HLA-DQA1*0501 and HLA-DQB1*0201 which encode the two subunits DQ-α⁵ and DQ-β², respectively (Sollid 2000) (Fig 1.1.X). The HLA-DQ2.5 heterodimer may also be formed by those with the combined DR5-DQ7/DR7-DQ2 haplotype. The DR5-DQ7 haplotype carries the DQA1*0501 and DQB1*0301 alleles, and the DR7-DQ2 haplotype carries the DQA1*0201 and DQB1*0202 alleles, which are encoded in trans (Sollid 2000) (Fig 1.4). The DQB1*0201 and DQB1*0202 alleles vary at a residue in the membrane proximal domain of the DQ β chain; however this is not predicted to affect antigen presentation capability (Hall, Lanchbury et al. 1993).

Of the CD patients who do not express the HLA-DQ2.5 heterodimer, the majority express the HLA-DQ8 heterodimer associated with the HLA-DR4 haplotype, encoded by HLA-DQB1*0302 and HLA-DQA1*03 (Karell, Louka et al. 2003).

**Figure 1.4: The HLA-DQ2 molecule may be encoded in cis or in trans.** In DR3 individuals, the HLA-DQA1*0501 and HLA-DQB1*0201 genes are located on the same chromosome (cis), whereas in DR5/DR7 heterozygotes they are located on opposite chromosomes (trans). Image adapted from Sollid, 2000)

15
The relative level of HLA-DQ expression may also influence CD development. CD is more prevalent in those whom 100%, or 50% of the HLA-DQ heterodimers are DQ2 than in those whom only 25% of the HLA-DQ heterodimers are DQ2 (Kagnoff 2007). Polymorphisms of other HLA complex genes, such as those coding for C₂, C₄, TNF-α and TNF-β have also been associated with CD (Woolley, Mustalahti et al. 2005). A variant of TNF-α, with a polymorphism in the promoter region resulting in increased TNF-α production occurs at an increased frequency in patients with CD (McManus, Moloney et al. 1996; Garrote, Arranz et al. 2002), which may have a functional relevance given the role of TNF-α in inflammation and activation of tissue mettalloproteinases (Nagase and Woessner 1999).

Non-HLA complex genes

Although the combined distribution of HLA-DQ2 or DQ8 in the general population is approximately 40% (Sollied and Thorsby 1993) the prevalence of CD is only 1% (Freeman, Chopra et al. 2011), suggesting the contribution of additional genetic or environmental factors contribute to disease development. The advent of genome-wide association studies has allowed the search for, and identification of linkage regions associated with CD. To date, candidate regions identified contain genes largely related to immune function; this has led to the description of four loci of interest, termed COELIAC1-4. COELIAC1 refers to the HLA complex on chromosome 6p21, with an attributable genetic effect of 53% (Sollied and Lie 2005). COELIAC2 (5q31-33) contains the gene clusters for Th2 cytokines, and has also been associated with T1DM, Crohn’s disease, asthma, psoriasis, and rheumatoid arthritis (Koskinen, Einarsdottir et al. 2009). Molecules involved in T-cell activation and regulation of function such as the negative co-stimulatory molecule CTLA4, and the stimulatory CD28 and ICOS are encoded for in
COELIAC3 (2q33) (Holopainen, Arvas et al. 1999). COELIAC4 (19p13.1) codes for an unconventional myosin IXB variant that alters epithelial actin remodelling (Monsuur, de Bakker et al. 2005; Koskinen, Korponay-Szabo et al. 2008).

Recently, the results of genome-wide studies have identified risk variants in the region of chromosome 4q27 harbouring the genes for IL-2 and IL-21, two cytokines implicated in intestinal inflammation and CD pathogenesis (Hunt, Zhernakova et al. 2008). Especially pertinent to CD-associated disease mechanisms may be the discovery of genes of the IL-18 pathway, which may participate in regulation of IFN-γ levels (Leon, Garrote et al. 2006), as a genetic risk variant (Koskinen, Einarsdottir et al. 2009; Brophy, Ryan et al. 2010).

1.1.7 Environmental factors

Unlike most autoimmune diseases, the principal environmental antigen in CD, wheat gluten has been identified. As wheat is contained in many food products, exposure to dietary gluten starts at a relatively young age (~6 months) (Koning, Schuppan et al. 2005); however, the fact that almost the entire population will ingest wheat regularly but will not develop CD means that there must be interplay between other environmental or genetic factors. The quality and quantity of cereal foods in the diet, whether an individual has been breast-fed or not, and the age at introduction to solid foods are all thought to be contributory (Hernell, Ivarsson et al. 2001).

Cereals

Cereals belong to the grass family Graminaceae, which is further subdivided into the subfamilies Triticeae, containing wheat, barley and rye, and Pooidea, containing oats (Kagnoff 2007). The safety of the inclusion of oats in the CD diet has generated much
debate (Lundin, Nilsen et al. 2003; Holm, Maki et al. 2006; Srinivasan, Jones et al. 2006), but their relative non-toxicity may reflect evolutionary distance from the more harmful Triticeae. Cereal proteins may be separated into four groups (Osborne fractions) based on their solubility. The albumins are water soluble, the globulins are soluble in salt solutions of weak ionic strength, the prolamins are alcohol soluble, and the glutelins are insoluble in solvents (Bean, Bietz et al. 1998). Prolamins function as cereal storage proteins, constituting 50% of the total grain protein (Shewry and Halford 2002).

The prolamin fractions of differing cereals have been named individually as gliadin in wheat, hordein in barley, secalin in rye, and avenin in oats (McGough and Cummings 2005). The glutelin fraction of wheat has been termed glutenin (Kawakatsu, Yamamoto et al. 2008). The term ‘prolamin’ originated from the words ‘proline’ and glutamine’, due to the relatively high proportions of these two amino acids found in wheat storage proteins. Triticeae prolamins contain approximately 19% proline and 35% glutamine, compared to 10% proline and 25% glutamine in oats (Arentz-Hansen, Fleckenstein et al. 2004).

Gluten

Gluten, formed by gliadin and glutenin, is the water-insoluble material from wheat flour that forms an elastic mass after dough has been washed out (Shewry, Halford et al. 2002). In baking, gluten is thought to be responsible for the elasticity and strength of bread dough. The gliadins are subdivided into α, γ, and ω-gliadins, while the glutenins consist of low molecular weight (LMW) and high molecular weight (HMW) glutenins (Camarca, Anderson et al. 2009). Although a number of toxic CD epitopes are derived from glutenins, the majority reside in the α and γ gliadin fractions (Koning, Gilissen et al. 2005). The unusually high proline content renders prolamin fragments resistant to
complete proteolytic digestion by gastric, pancreatic, and brush border proteases and peptidases as these enzymes are deficient in prolyl endopeptidase activity (Stenman, Venalainen et al. 2009). This results in fragments of proline and glutamine rich peptides up to 50 amino acids in length reaching the small intestine and being transported across the epithelium, by unknown mechanisms (Uibo, Tian et al. 2011). Infectious agents affecting the gastrointestinal tract may also play an important role in the passage of toxic gliadin fragments through the epithelium, as gastrointestinal infection can transiently affect mucosal permeability, and hence the intensity of exposure to dietary antigens (Schuppan 2000).

1.1.8 Immune responses in coeliac disease

The immunopathogenesis of CD reveals a complex interplay between genetics, environmental antigens, and the innate and adaptive immune systems, much of which remains unresolved. Working backwards, the end-point of this interaction is signified by the failure of oral tolerance towards dietary gluten. Kagnof, in 2007 proposed an attractive model for CD pathogenesis, describing three major series of events: luminal and early mucosal events, the activation of pathogenic CD4⁺ T cells, and events leading to tissue damage (Kagnoff 2007), summarised in Fig 1.5.

Luminal and early mucosal events

During the first phase of events an individual ingests gluten, which due to its high proline content is relatively resistant to normal digestive processes. Large glutamine and proline rich peptides arrive in the duodenum, where they cross the epithelial barrier to the lamina propria. How the partially digested gluten peptides gain access to the lamina propria is unknown, but a number of mechanistic scenarios have been proposed.
Paracellular transport through an already damaged epithelial cell layer may occur; over expression of zonulin, an epithelial tight junction protein that increases intestinal permeability has been noted in CD (Fasano, Not et al. 2000). Gluten peptides may be translocated across the epithelial layer, either by an IFN-γ dependant mechanism, or bound to secretory IgA which can be translocated via the transferrin receptor, CD71 (Matysiak-Budnik, Moura et al. 2008). Peptides may be transported across the epithelial layer by dendritic cells directly sampling luminal antigen (Niess, Brand et al. 2005). Given that only 1% of individuals who ingest gluten develop CD, the presence of pre-existing factors which lead to an increased number of gluten peptides reaching the lamina propria is plausible. The most likely factor in this scenario is infection in the small intestine, possibly of viral origin (Troncone and Auricchio 2007).

Figure 1.5 Proposed model for the pathogenesis of coeliac disease. CD pathogenesis may be divided into three major series of events: luminal and early mucosal events; the activation of pathogenic CD4+ T cells; and the subsequent events leading to tissue damage. Partially digested gluten peptides cross the intestinal barrier, where they are deamidated by tTG, and presented to the immune system. Gluten-specific T cells then orchestrate the subsequent IFN-γ driven immune response, ultimately resulting in tissue damage. Image adapted from Kagnoff, 2007.
Gluten may also directly activate the innate immune system through induction of IL-15 expression on epithelial cells and dendritic cells in the lamina propria (Barone, Zanzi et al. 2011). IL-15 has many pro-inflammatory effects, including increasing intestinal permeability by disruption of tight junctions (Heyman, Abed et al. 2011), the activation and survival of CD8\(^+\) T cells (Steel, Waldmann et al. 2012), upregulation of cell surface ligands on intestinal epithelial cells that render them as a target for cytotoxic NK-like cells (Garrote, Gomez-Gonzalez et al. 2008), and the indirect modulation of CD4\(^+\) T cell responses by influencing APC differentiation (Ohteki, Suzue et al. 2001). Increased IL-15 levels have been detected in the intestinal epithelium and lamina propria of untreated CD patients (Di Sabatino, Ciccocioppo et al. 2006), and p31–43 of \(\alpha\)-gliadin induced CD68\(^+\) APC from CD patients, but not controls, to produce IL-15 (Maiuri, Ciacci et al. 2003).

In the intestinal lamina propria, the gluten peptides encounter tTG, highly expressed in this region (Sakly, Sriha et al. 2005), and deamidation (removal of an amino group, see section 1.2.2) of specific sequences in the peptides may occur. tTG expression may be increased due to infectious stimuli, or indirectly by the gluten peptides which can induce nitric oxide production by enterocytes, which in turn up-regulates tTG expression (Beckett, Dell'Olio et al. 1999).

**Activation of mucosal APCs and CD4\(^+\) T cells**

Professional APCs, whilst essential in protective immune responses in the mucosa, are also central to the induction and maintenance of oral tolerance (Goubier, Dubois et al. 2008). Under normal conditions, sub-epithelial macrophages and dendritic cells are in a state of quiescence, expressing low levels of the co-stimulatory molecules B7 (CD80) and B7.2 (CD86) (Abbas, Lichtman et al. 2012), and displaying poor T cell stimulatory capacity
Due to an unknown trigger such as infection or innate activation signals, mucosal APCs trigger the immune response towards gliadin and related peptides through the induction of gliadin-specific Th1 cells (Koning 2003). CD11c⁺ dendritic cells are believed to be central to the activation of gliadin-specific T cells, being upregulated in the CD mucosa, and more efficient at activating gluten-reactive T cell clones than their macrophage counterparts (Raki, Tollefsen et al. 2006). The active CD lesion is characterised by an infiltration of IFN-γ producing CD4⁺ T cells to the lamina propria displaying markers of memory (CD45RO), and activation (CD25) (Sollid 2000). Intraepithelial lymphocytosis also occurs. Both the CD8⁺ αβ and γδ populations are expanded in CD, with the γδ population remaining expanded during a gluten-free diet (Borrelli, Maglio et al. 2010). Whilst regulatory T cell numbers are increased in the CD intestinal lesion (Tiittanen, Westerholm-Ormio et al. 2008), a recent report by Hmida (2012), has shown them to be functionally impaired (Hmida, Ahmed et al. 2012).

**Events leading to tissue damage**

The dominant cytokine in the coeliac lesion is IFN-γ, with mRNA increased 1000 fold in untreated disease (Nilsen, Jahnsen et al. 1998). IFN-γ is essential in the maintenance and development of mucosal damage by increasing intestinal permeability, inducing macrophages to produce TNF-α, activating matrix metalloproteinases, and contributing to the expansion of plasma cells. Gut-derived, gluten-specific CD4⁺ T cell clones secrete a wide variety of cytokines in addition to IFN-γ, such as IL-4, IL-5, IL-6, IL-10, TNF-α, and TGF-β (Sollid 2000). The involvement of two newly described T cell subsets Th17, and T follicular helper (Tfh) cells in CD was confirmed by the observation that IL-17A is produced in response to gliadin stimulation in both the peripheral and intestinal T cells (Harris, Fasano et al. 2010; Monteleone, Sarra et al. 2010), and that gluten-specific T cells
can produce IL-21 (Bodd, Raki et al. 2010). There is also a massive influx of plasma cells, secreting antibodies of the IgA, IgG, and IgM class to gliadin and tTG (Brandtzaeg 2006). Locally deposited anti-tTG may contribute to the pro-inflammatory environment, with the possibility of complement activation (Maglio, Tosco et al. 2011).

1.1.9 Related gluten-sensitive disorders

Gluten sensitivity

Gluten may induce pathological conditions other than CD, such as IgE-mediated wheat allergy (Komata, Soderstrom et al. 2009). Recently, there is an increasing recognition of gluten sensitivity as a condition distinct from CD. Gluten sensitivity describes disorders that miss one or more of the key CD diagnostic criteria (HLA haplotype, enteropathy, and anti-tTG positivity), but respond to a gluten-free diet (Troncone and Jabri 2011).

Dermatitis herpetiformis (DH)

The CD-related gluten sensitive enteropathy, DH is characterised by a blistering skin rash in response to ingested gluten, with less severe gut involvement (Karpati 2012). IgA deposits directed against epidermal transglutaminase are found at the dermo-epidermal junction (Karpati 2004), along with circulating autoantibodies to epidermal, and tissue transglutaminases (Rose, Armbruster et al. 2009).
1.2 Tissue transglutaminase

1.2.1 The transglutaminase family of enzymes

Transglutaminases are members of a family of enzymes related by structure and functionality which catalyse the post-translational modification of proteins via crosslinking, amine incorporation, and sequence-specific deamidation (Griffin, Casadio et al. 2002). Transglutaminases, universally dependant on Ca\(^{2+}\) for enzymatic activity, are found in diverse organisms such as eukaryotes, plants, and mammals (Lorand and Graham 2003). To date nine transglutaminases have been described in humans, sharing common structural features such as a lack of disulphide bonds and glycosylation, despite the presence of cysteine residues and sites with potential for \(N\)-linked glycosylation (Ikura, Nasu et al. 1988). Despite being secreted by cells, transglutaminases lack the hydrophobic leader sequence required for transport through the endoplasmic reticulum, and ultimate secretion from the cell (Ichinose, Bottenus et al. 1990). Enzymatic activity is mediated by an active-site cysteine residue, which forms a papain-like triad of amino acids with a histidine and an aspartic acid or asparagine residue (Griffin, Casadio et al. 2002).

The human transglutaminase family consists of transglutaminases 1-7, the coagulation factor XIII\(^a\), and the catalytically inactive erythrocyte membrane protein Band 4.2. Transglutaminase 1 (keratinocyte transglutaminase) is responsible for the crosslinking of epidermal proteins, and is involved in the terminal differentiation of keratinocytes (Egberts, Heinrich et al. 2004). Transglutaminase 2 (tissue transglutaminase), is ubiquitously expressed and has multiple functions supplemental to its protein crosslinking ability such as GTPase, ATPase, and protein kinase activities (Wang and
Griffin 2012). Transglutaminase 3 (epidermal transglutaminase), expressed in the epidermis and hair follicles is crucial for the generation of hair fibres, and epidermal cell differentiation (Hitomi, Presland et al. 2003). The function of transglutaminase 4 (prostate transglutaminase) in humans is unknown, but may play a similar role as to that in rodents, where the enzyme is involved in semen coagulation (Tseng, Tang et al. 2011). Transglutaminase 5, important for keratinocyte differentiation, can also trigger cell death if over-expressed intracellularly (Cadot, Rufini et al. 2004). Transglutaminase 6 (neuronal transglutaminase), is predominantly expressed by a subset of neurons in the central nervous system (CNS) (Hadjivassiliou, Aeschlimann et al. 2008). Transglutaminase 7, expressed in testis and lung, is of unknown function (Mehta and Eckert 2005).

Factor XIII®, or plasma transglutaminase, exists as an inactive zymogen whose catalytic activity is regulated by thrombin and Ca^{2+}. Factor XIII® functions in the stabilisation of the fibrin clot formed during coagulation via the crosslinking of fibrin molecules, as well as playing a role in inflammation and bone growth (Muszbek, Bereczky et al. 2011). Band 4.2, a major component in the erythrocyte skeletal network shares sequence homology with various transglutaminases, and is rendered catalytically inactive by an active-site amino acid substitution (Mehta and Eckert 2005).
1.2.2 Transglutaminase enzymology

The Enzyme Commission on Nomenclature describes transglutaminases as an R-glutaminyl-peptide: amine γ-glutaminyl transferase (EC 2.3.2.13) (Greenberg, Birckbichler et al. 1991). The transamidation reaction, involves an acyl-transfer between the γ carboxyamide group of peptide-bound glutamine (donor) and acyl acceptor residues of amines such as the ε amino group of peptide bound lysine residues, resulting in the formation an ε-(γ-glutamyl)lysine isopeptide bond, or covalent incorporation of an amine into a glutamine residue of some polyamines and histamines (Fig 1.6). The cross-linking reaction proceeds in two steps. In the first, the active-site cysteine reacts with the γ carboxyamide of the target glutamine residue, forming an acyl-enzyme intermediate with ammonia release. In the second, an isopeptide bond is formed from the acyl-enzyme intermediate and the target primary amine, with release of the enzyme.

![Figure 1.6: Biochemical reactions catalysed by transglutaminases.](image)

The transamidation reaction can result in (1) covalent incorporation of an amine (R-NH₂,such as polyamines, histamines) into the glutamine residue of the acceptor protein (P₁),(2) covalent cross-linking of proteins by introducing an Nε(γ-glutamyl)lysine isopeptide bond between the lysine donor residue of one protein (P₂) and the acceptor glutamine residue of another (P₁), and (3) in some instances water can replace amine donor substrates leading to deamidation of the protein (P₁)-bound glutamine residue. (Adapted from Mehta, 2005)
Transamidation will only occur at a pH above 7, and whilst there is a high specificity for certain protein-bound glutamine residues as glutamine donor substrates, lysine-containing glutamine acceptor substrates are numerous (Telci and Griffin 2006). When there are no lysine residues available as glutamine acceptors, or at low pH (below 7) in the presence of a water molecule, deamidation of the target glutamine in the substrate protein may occur, resulting in the neutral glutamine being changed to a negatively charged glutamic acid residue (Griffin, Casadio et al. 2002) (Fig1.6).

1.2.3 Tissue transglutaminase

Tissue transglutaminase (tTG) is a ubiquitously expressed, multi-functional protein that is the best-characterised member of the transglutaminase family due to its involvement in a number of human diseases. The ability of tTG to cross-link proteins by amine incorporation led to its discovery (Sarkar, Clarke et al. 1957), however tTG also functions in GTP hydrolysis, cell signalling, CD4+ T cell migration, apoptosis, and as a cell-surface adhesion mediator. The gene for tissue transglutaminase (tTG) is found on the long arm of chromosome 20, (locus 20q11-12) (Lorand and Graham 2003).

1.2.4 Structure of tissue transglutaminase

Human tTG is a 77 kDa protein, consisting of 687 amino acids. The enzyme has four domains - an N-terminal β-sandwich, spanning amino acids 1-139, the core domain, spanning amino acids 140-454, and two C-terminal β-barrels, which span amino acids 479-585 and 586-687, respectively (Fesus and Piacentini 2002). The first domain contains binding sites for fibronectin and integrin molecules, comprising of a 28 KDa docking site on the N-terminal (Gaudry, Verderio et al. 1999). The catalytic core domain is folded in a prevalently α-helical secondary structure, and contains the active-site triad of amino
acids, Cysteine^{277}, Histidine^{335} and Aspartic acid^{358}, which in the absence of bound calcium ion is buried within a narrow cleft with walls formed by this domain and domains 3 and 4 (Griffin, Casadio et al. 2002).

The main calcium-binding residues, Serine^{449}, Glutamic acid^{451} and Glutamic acid^{452} are also located in the core domain. Amino acids 454-478 act as a flexible hinge region between domains 2 and 3, allowing movement of these regions (Casadio, Polverini et al. 1999). Calcium binding to the protein unwinds this loop, and results in an alteration in the shape of the protein and the relative positions of domains 3 and 4 (Casadio, Polverini et al. 1999) (Fig. 1.7). GTP binding involves Lysine^{173}, Phenylanalanine^{174}, Arginine^{476}, Arginine^{478}, Valine^{479}, Methionine^{483}, Arginine^{580} and Tyrosine^{583} (Liu, Cerione et al. 2002). Serine^{171} and Lysine^{173} are essential for GTP binding, as mutated forms of tTG lacking these two residues retain transamidating activity, but are devoid of GTPase activity (Griffin, Casadio et al. 2002).

The first tTG protein domain contains binding sites for fibronectin and integrin molecules, comprising of a 28 KDa docking site on the N-terminal (Hoffmann, Annis et al. 2011). Phospholipase C interaction involves amino acids 655-672, located in β-barrel 2, close to the carboxy terminus of the protein (Hwang, Gray et al. 1995). tTG lacks the hydrophobic leader sequences required for secretion via the conventional endoplasmic reticulum/golgi pathway, a shared characteristic of all human transglutaminases. As tTG activity can be detected extracellularly, one may deduce that tTG externalisation occurs either by an as-yet unidentified alternative pathway, or by leakage of the enzyme from stressed or damaged cells. tTG conformation may be an important factor for secretion; mutations which hold tTG in its 'open' conformation prevent tTG translocation to the cell surface (Johnson and Terkeltaub 2005). A recent publication by Zemskov (2011) indicates
that tTG may be targeted to integrin β1-rich perinuclear recycling endosomes prior to externalisation (Zemskov, Mikhailenko et al. 2011).

Figure 1.7: Structure of tissue transglutaminase. a) shows the enzyme in its catalytically inactive conformation, whilst b) is the open, active conformation. Image adapted from Wang, 2012, and Mhaouty-Kodja, 2004.
1.2.5 Regulation of tissue transglutaminase activity

The transamidase and GTPase activity of tTG is reciprocally regulated by the combined action of two ligands, calcium and GTP. GTP binds to residues from the first and last strands of \( \beta \)-barrel 1, and to two core region residues that protrude on a loop into \( \beta \)-barrel 1; Lysine\(^{173} \) and Phenylalanine\(^{174} \) (Begg, Carrington et al. 2006). Binding of GTP stabilises the enzyme in its 'closed' conformation by stabilising \( \beta \)-barrel 1 loops that prevent substrate access to the active site, and permits the formation of a hydrogen bond between Tyrosine\(^{516} \) or Cysteine\(^{336} \) and the active-site Cysteine\(^{277} \), rendering it inactive (Begg, Carrington et al. 2006). GTP-bound tTG displays GTPase activity, but is incapable of transamidation or deamidation (Fesus and Piacentini 2002).

Calcium binding to residues in the core region \( \alpha \)-helix \( H_4 \) causes a conformational shift in the tTG molecule to the 'open' conformation which allows access of substrate to the active-site and procedure of the transamidation or deamidation reactions. Investigation of the activation of tTG by calcium carried out by Casadio et al. using small-angle neutron and X-ray scattering reveal that the interactions between domain 2, which contains the catalytic triad, and domains 3 and 4, break down upon the binding of calcium, opening access to the active site. In the same paper, they also describe an increase in gyration radius of the protein, from 3.0nm to 3.9nm upon activation, which is dependent on the hinge flexible hinge region connecting the N-terminal and C-terminal regions of the protein (Casadio, Polverini et al. 1999). Site-directed mutagenesis has identified Tryptophan\(^{241} \) as an essential residue in the stabilisation of transition states between the closed to open conformations (Murthy, lismaa et al. 2002).

A third mechanism of regulation of tTG activity has recently been described, relating to calcium-activated tTG in the extracellular environment. In an oxidative environment, tTG
becomes inactivated through the formation of a disulphide bond between Cysteine\textsuperscript{370} and Cysteine\textsuperscript{371} (Stamnaes, Pinkas et al. 2010) (Fig. 1.8), a process that can be reversed by thioredoxin, a potent activator of tTG (Jin, Stamnaes et al. 2011).

![Figure 1.8: Regulation of transglutaminase activity by redox status.](image)

**Figure 1.8: Regulation of transglutaminase activity by redox status.** In the open, inactive state the catalytic cysteine residue forms a disulphide bond with a neighbouring residue.

### 1.2.6 Regulation of tissue transglutaminase expression

tTG could be considered as a stress-related protein, due to up-regulation of its expression by numerous physiological and pathological stimuli (Fesus 1982). Cytokines secreted during the early phase of cell injury can induce expression of tTG. Transforming growth factor-\(\beta\) (TGF-\(\beta\)) induces tTG expression in keratinocytes and dermal fibroblasts, through binding to a TGF-\(\beta\) response element in the *TGM2* gene promoter region (Quan, Choi et al. 2005). The 5’ promoter region of the *TGM2* gene also contains response elements to IL-6, which upregulates tTG expression in hepatocytes (Suto, Ikura et al. 1993), and NF-\(\kappa\)B (Mehta, 2005), which is activated downstream to release of pro-inflammatory TNF-\(\alpha\). Kim and colleagues (2002) have demonstrated increased tTG mRNA expression in rat small intestinal cells following treatment with IFN-\(\gamma\) (Kim, Jeong et al. 2002), a relevant finding given that CD could be described immunologically as an ‘IFN-\(\gamma\)-driven’ condition.
Retinoic acid, which may also influence immunity to dietary antigens (DePaolo, Abadie et al. 2011), is a potent inducer of tTG expression at protein and mRNA levels in multiple cell types (Moore, Murtaugh et al. 1984; Rebe, Raveneau et al. 2009; Garabuzi, Kiss et al. 2011). Binding of trans-retinoic acid, or of cis-retinoic acid to their respective nuclear receptors RAR-β/RAR-γ and RXR triggers transcription of tTG (Kliewer, Umesono et al. 1992), due to activation of a retinoic acid response element located 1.7kb upstream from the initiation site of the TGM2 gene (Szegezdi, Szondy et al. 2000). Vitamin D, and steroid hormones can also induce expression of tTG (Szegezdi, Szondy et al. 2000).

1.2.7 Physiological roles of tissue transglutaminase

80% of tissue transglutaminase is located in the cytosol of the cell, with 10-15% located in the plasma membrane, and 5% in the nuclear membrane (Lorand and Graham 2003). Secreted tTG localizes to the cell surface and the extracellular matrix, but can also be transported to the nucleus by interaction with nuclear transport proteins (Lesort, Attanavanich et al. 1998).

The ubiquitous expression of tTG, combined with its various intra and extracellular locations, and numerous substrates and interacting proteins mean that multiple physiological roles for the enzyme have been described. The fact that the amount of calcium required to activate the transamidating activity of tTG falls into the supraphysiological range and not the physiological range associated with most intracellular processes, would indicate that the enzyme is virtually inactive under normal cellular conditions, and are only activated following disruptions of physiological homeostatic mechanisms. This means that under normal conditions tTG exists in a latent GTP-bound form which is also important in protecting the enzyme from intracellular
proteases such as calpain, which cleaves calcium-bound, but not GTP-bound tTG (Zhang, Lesort et al. 1998). The fact that tTG can bind and hydrolyse GTP with an affinity and catalytic rate similar to the α subunits of large heterotrimeric G proteins (Fesus and Placentini 2002) would indicate its importance in the transmission of extracellular signals. In 1994, a novel G protein, Gha, observed in rat liver plasma membrane was identified as tTG (Nakaoka, Perez et al. 1994). In the GTP-bound state, the last C-terminal amino acids in domain 4 of tTG will interact with phospholipase C, participating in the transmission of extracellular α1-andrenergic signals via the seven-transmembrane helix receptor system (Baek, Kang et al. 2001). Gh complex interaction with α1-andrenergic receptors dissociates Gha from the Ghβ subunit, identified by Feng as calreticulin (Feng, Readon et al. 1999), resulting in activation of phospholipase C (Murthy, Lomasney et al. 1999).

Transport of tTG to the nucleus by the nuclear transport protein importin-α3 (Peng, Zhang et al. 1999) clearly illustrates its bifunctionality. Here it can act either as a G protein, or it can crosslink histone proteins and hence play a role in the regulation of gene expression via subsequent chromatin modification (Ballestar, Boix-Chornet et al. 2001).

During the process of apoptosis, where the cell becomes permeable to calcium and GTP generation is impaired, tTG crosslinking activity may be activated. This theory is confounded by the detection of ε-(γ-glutamyl) lysine crosslinks in apoptotic bodies (Fesus, Thomazy et al. 1987). In the late stage of apoptosis where there is a massive influx of calcium into the cell, tTG may crosslink cytoskeletal proteins such as actin, stabilizing the structure of the cell prior to phagocytosis, and hence preventing the release of intracellular components which could possibly cause inflammatory or
autoimmune responses. Studies using tTG knockout mice seem to contradict this, but this could be explained through redundancy between tTG and other transglutaminase isoforms. It has been suggested that tTG could ‘prime’ cells for apoptosis by interacting with mitochondria, altering their redox status (Piacentini, Farrace et al. 2002). Tissue transglutaminase also contributes significantly to the organization of the extracellular matrix (ECM) through its involvement in cell adhesion and spreading, wound healing, angiogenesis, tissue mineralisation, and activation of TGF-β (Kim 2006). Cell-surface tTG is present in a 1:1 complex with integrins (Akimov and Belkin 2001) and can bind to fibronectin, a molecule which will enhance cell adhesion and amplify adhesion-dependant phosphorylation of focal adhesion kinase (Verma and Mehta 2007). Interaction of tTG with integrins and fibronectin is facilitated via a 28 KDa docking site on the N-terminal of tTG (Jeong, Murthy et al. 1995).

The bone formation process requires the crosslinking of Ca^{2+}-binding matrix proteins by tTG; ε-(γ-glutamyl) lysine crosslinks are abundant in the bone matrix (Kaartinen, El-Maadawy et al. 2002). Chondrocytes also express and externalize tTG throughout various stages of their differentiation (Aeschlimann, Mosher et al. 1996). tTG is directly involved in the activation of TGF-β, a cytokine which promotes transcriptional regulation of ECM genes, and of tTG itself. tTG crosslinks latent TGF-β binding protein to the extracellular matrix, which is required for the release and activation of the mature cytokine (Nunes, Gleizes et al. 1997).

There are no known inherited deficiencies of tissue transglutaminase. Given its ubiquitous expression and multiple functions, one would suspect that its absence could have lethal consequences. Homozygous null mutants of tTG are normal at birth, but show defective wound healing, macrophage activity, and clearance of apoptotic cells.
(Nanda, Lismaa et al. 2001). tTG deficient mice develop autoimmune reactions, as well as a glucose intolerance and hyperglycaemia, due to reduced insulin secretion, which resembles maturity-onset diabetes of the young that occurs in humans (Bernassola, Federici et al. 2002). The explanation for these less radical than expected consequences of tTG deletion may be redundancy between the actions of tTG and other members of the transglutaminase family, e.g. TG5, TG6 and TG7, which have not been fully characterised.

1.2.8 Tissue transglutaminase in pathology

Inflammatory states

A consequence of the physiological role of tTG in tissue repair and wound healing is a contribution to fibrosis and scarring in chronic inflammatory states such as hepatic (Grenard, Bresson-Hadni et al. 2001), renal (Schelling 2009), and pulmonary (Olsen, Sapinoro et al. 2011) fibrosis. The involvement of tTG in rheumatoid arthritis and osteoarthritis may be linked to its activation of TGF-β, which can stimulate pyrophosphate release in diseased joints leading to mineralization and progression of the arthritis (Lotz, Rosen et al. 1995). Interestingly, IgG anti-tTG antibodies have been detected in the sera of patients with rheumatoid arthritis (Feighery, Collins et al. 2003). tTG may contribute to the formation of atherosclerosis, as it can crosslink lipoprotein-A into insoluble atherosclerotic plaques in vitro (Kim, Jeitner et al. 2002). Transglutaminase activity has been detected in aortas containing atherosclerotic lesions of cholesterol-fed rabbits (Wiebe, Tarr et al. 1991).

tTG may also promote acute inflammatory states such as septic shock. tTG-knockout mice display increased resistance to lipopolysaccharide (LPS)-induced septic shock.
compared to wild-type counterparts, an effect associated with decreased NF-κB activation and reduced neutrophil recruitment to the peritoneum and kidney (Falasca, Farrace et al. 2008).

*tTG and cancer*

Elevated tTG expression has been demonstrated in many differing types of cancer cells, including lung carcinoma (Kawai, Wada et al. 2008), pancreatic adenocarcinoma (Elsasser, MacDonald et al. 1993), ovarian carcinoma (Singer, Hudelist et al. 2006), and malignant melanoma (Fok, Ekmekcioglu et al. 2006). Increased expression of tTG in cancerous cells has been linked with metastasis and drug resistance, and poor patient survival (Budillon, Carbone et al. 2011). Gene expression analysis of tumour samples from patients with pancreatic adenocarcinoma revealed TGM2 as one of the most highly expressed genes (Mehta 2009). Downregulation of tTG expression, or inhibition of tTG function by small interfering RNA (siRNA), antisense RNA, ribozyme, or small molecule tTG inhibitors has been shown to increase sensitivity of tumour cells to chemotherapy and reduce metastasis in various *in vitro* and animal models (Verma and Mehta 2007).

*Neurodegenerative disease*

Huntington's disease is a progressive neurodegenerative disorder caused by pathological mutations of the *huntingtin* gene involving expansion of trinucleotide CAG repeats (Shoulson and Young 2011). This results in stretches of polyglutamine residues in the coded protein, huntingtin, polymers of which are found in the affected regions of Huntington's disease brains. Huntingtin is a tTG substrate, and it is hypothesised that the elongated polyglutamine domain favours polymerisation of huntingtin by tTG. Increased numbers of polyglutamine repeats are associated with improved ability to act as a tTG
substrate, which may explain the relationship between disease severity and the number of CAG repeats (Kim, Jeitner et al. 2002). Increased transglutaminase activity has also been detected in the striata of Huntington’s disease brains (Lesort, Chun et al. 1999).

Increased transglutaminase activity has also been described in the brains of patients with Alzheimer’s disease, a disease characterised by cognitive impairment due to loss of neurons and protein aggregates in the brain (Khairallah and Kassem 2011). In studies of insoluble proteins from Alzheimer’s disease brains, Kim et al. found that the number of ε-(γ-glutamyl)lysine linkages found were elevated up to 50-fold compared to normal controls, combined with increased tTG and TG1 mRNA and protein expression (Kim, Grant et al. 1999).

**Coeliac disease**

tTG is intimately involved in the pathogenesis of CD, modifying the immunostimulatory properties of the causative cereal agent, gliadin (Feighery 1999). In addition to this, tTG is the major autoantigen in CD; autoantibodies to tTG are an invaluable tool in CD diagnosis, and are increasingly emerging as having a pathogenic role in the disease (Caja, Maki et al. 2011).

**tTG and tTG substrates as autoantigens**

Autoantibody responses to tTG occur in a number of autoimmune conditions such as the CD-related gluten-sensitive skin disorder dermatitis herpetiformis (Byrne, Ryan et al. 2007), type 1 diabetes mellitus (T1DM) (Lampasona, Bonfanti et al. 1999), rheumatoid arthritis (Picarelli, Di Tola et al. 2003), primary biliary cirrhosis (PBC) (Bizzaro, Villalta et al. 2003), and Crohn’s disease (Sjoberg, Eriksson et al. 2002). Anti-tTG reactivity has also
been detected in patients with human immunodeficiency virus (HIV) (Pereda, Bartolome-Pacheco et al. 2001), and children suffering from infectious diseases (Ferrara, Quaglia et al. 2010). While the prototypical example of a combined tTG and its modified substrate eliciting an immune response is that of tTG and deamidated gliadin, an ever increasing list of autoantigens are being identified as tTG substrates, including the typical diabetic autoantigens insulin, glutamic acid decarboxylase, and islet-cell autoantigen 2 (IA-2) (Bach 1994). In autoimmune hepatitis, the keratin and actin autoantigens are tTG substrates, as are a number of SLE autoantigens such as histone H2B and tubulin (Kim, Jeitner et al. 2002). It has been hypothesised by Kim et al. (2001) that tTG may contribute to autoantibody generation by crosslinking potential autoantigens and acting as a hapten.

1.2.9 The role of tissue transglutaminase in coeliac disease

The isolation of HLA-DQ restricted, gliadin-specific T cells from intestinal biopsies of CD patients by Lundin and co-workers in 1993 identified the pathogenic role of the CD associated MHC class II molecule (Lundin, Scott et al. 1993). Analysis of the peptide-binding groove of HLA-DQ2 and HLA-DQ8 revealed a preference for negatively charged residues in key anchor positions, a finding which confounded researchers due to the lack of such residues in gliadin (Sollid 2000). The identification of the first gliadin peptides capable of being recognised by gliadin-specific T cells in 1998 solved this mystery by revealing the presence of target glutamine residues capable of being deamidated by tTG. The deamidation of glutamine to the negatively charged glutamic acid by tTG facilitates presentation of gliadin peptides to the immune system by the HLA-DQ molecule (Sjostrom, Lundin et al. 1998).
The specificity of gluten deamidation by tTG is a crucial factor in the generation of toxic peptides which will bind to the HLA-DQ peptide-binding groove. Vader (2002) determined that the ability of tTG to deamidate synthetic gluten peptides is influenced by the relative spacing of glutamine and proline residues contained therein (Vader, de Ru et al. 2002). For gluten peptides, in the sequences QP and QXXP the Q is not a target for deamidation by tTG, whereas in the sequences QXP, QXXF(Y,W,M,L,I, or V) and QXPF(Y,W,M,L,I, or V) the Q is a target for deamidation. Algorithms to predict the identity of stimulatory peptides were designed from this data and also identified sequences from rye and barley (Vader, de Ru et al. 2002), cereals toxic to CD patients.

T cell stimulatory peptides have been identified in the α and γ gliadins, and the low and high molecular weight glutenins, with most but not all requiring deamidation to induce a T cell response (Koning, Gilissen et al. 2005). The best-characterised of the CD-relevant peptides is the α2-gliadin-33mer fragment, which harbours six partly overlapping DQ2-restricted epitopes (Shan, Molberg et al. 2002).

The exact location where gliadin deamidation occurs has not been conclusively determined, but the small intestine with its pH <7 and strong tTG expression in the epithelial brush border and subepithelial regions seems a plausible candidate. There is also the possibility that membrane-bound tTG on the surface of antigen-presenting cells is endocytosed and that deamidation takes place internally in these cells, with the deamidated peptides binding to DQ molecules in the endosome before being externalized (Sollid 2002).
CD is characterised by an autoantibody response to tTG of the IgA and IgG class (Reddick, Crowell et al. 2006). Synthesis of these antibodies is induced at mucosal level (Valletta, Fornaro et al. 2011), with the coeliac intestinal lesion being characterised by an influx for anti-tTG secreting plasma cells (Brandtzæg 2006). Epitope mapping studies have indicated the core region of tTG as being recognised by CD anti-tTG. In 2007, Byrne and colleagues demonstrated that removal of the active-site catalytic triad of amino acids abrogated IgA anti-tTG binding in adults with CD (Byrne, Ryan et al. 2007).

The presence of highly-specific, class-switched autoantibodies to tTG in active CD is indicative of a humoral immune response that has received help from autoreactive T cells. A dogma of CD pathogenesis, however, has been that evidence for the existence of a tTG antigen-specific T cell has been lacking, leading to the proposition of the hapten-carrier theory for the formation of anti-tTG antibodies in CD (Sollid, Molberg et al. 1997). According to this theory, gliadin/tTG complexes can be processed by tTG-specific B cells, with presentation of gliadin peptides to gliadin-specific T cells, which in turn provide T cell help to the tTG-specific B cell for the production of anti-tTG antibodies. A recent publication by Ciccocioppo et al. (2010) has, for the first time, demonstrated the presence of tTG-specific T cells in the periphery of CD patients with active disease (Ciccocioppo, Finamore et al. 2010). By weekly stimulation of peripheral blood mononuclear cells (PBMCs) with tTG it was possible to establish T-cell lines and clones that were specific for, and proliferated in response to, tTG. These tTG-specific T cells were classified as being in the T-helper 1 type category, mainly due to their production of large amounts of IFN-γ and IL-6, which is in agreement with CD being traditionally classed as a Th1-driven disease.
1.2.10 Project aims

The overall aims of this project were:

- To further dissect the anti-tTG response in coeliac disease by the generation of novel recombinant tTG active-site mutant proteins, and using said proteins as antigens in ELISA with serum from paediatric and adult coeliac disease patients.

- To determine and compare the epitope specificity and subclass usage of IgG anti-tTG antibodies in patients with coeliac disease, and patients with autoimmune diseases such as type 1 diabetes and Crohn’s disease, who are positive for these antibodies.

- To investigate the presence of tTG antigen-specific T cells in patients with treated coeliac disease and normal control individuals, through the generation of tTG antigen-specific T cell lines.
Chapter 2

Mutagenesis and protein production of recombinant human tissue transglutaminases
2.2 Introduction

2.1.1 Polymerase chain reaction

The polymerase chain reaction (PCR) is a technique that is based on the ability of DNA polymerase to synthesise a new strand of DNA, complimentary to the template strand (Vosberg 1989). Using template strands of DNA, the sequence of interest may be amplified, generating millions of copies. Described by Mullis et al. in 1984, PCR has revolutionised molecular biology due its ability to rapidly generate amplified DNA sequences from minute sources of DNA. As DNA polymerase is only capable of adding nucleotides to DNA in a 5' to 3' direction, the specific sequence of interest can be selectively amplified by using a primer flanking an adjacent sequence to initialise DNA replication (Goodman, Creighton et al. 1993). PCR involves three cycles: in the first, the template DNA is denatured to single-strand DNA at a temperature of 94°C. In the second PCR step, the primers are annealed at ~55 °C, followed by the third step, DNA extension at 72 °C (Bej, Mahbubani et al. 1991). Crucial to the success of the PCR reaction is the ability of the DNA polymerase from Thermus aquaticus (taq) to remain stable throughout the various cycling temperatures (Saiki, Gelfand et al. 1988). In order to amplify gene transcripts, which are expressed as RNA, reverse transcription PCR must be applied (Stahlberg and Bengtsson 2010). The RNA sequence of interest is reverse transcribed into its complimentary DNA, which is then applied as template DNA in a standard PCR reaction (Nollau, Jung et al. 1995).

2.1.2 Site-directed mutagenesis

Site-directed mutagenesis is a PCR-based method which results in the production of an altered DNA sequence. DNA mutagenesis is a useful biological tool, allowing for
investigations at both a molecular and protein level (Ling and Robinson 1997). Its application in the study of transcriptional regulatory sequences has been well documented (Zaret, Liu et al. 1990; Piens, Muller et al. 2010). The importance of specific amino acids for protein functions such as enzymatic activity (Huang, Horiuchi et al. 1997; Chave, Galivan et al. 1999; Gu, Yang et al. 2011) and protein-protein interactions (Kube, Becker et al. 1992) may also be investigated. From an immunological viewpoint, DNA mutagenesis is of significant use in the field of epitope mapping of antigens in both infectious (Sun, Lu et al. 2009), and autoimmune disease (Lubin, Healey et al. 1997; Pedchenko, Bondar et al. 2010).

Oligonucleotide site-directed mutagenesis is based on the use of mismatched oligonucleotide primers to produce a mutated DNA sequence by PCR. Individual amino acids can be changed by mutating their coding codon to that of the desired replacement amino acid (Carter 1986). A common method is to replace the target amino acids with alanine, termed alanine screening (Lefevre, Remy et al. 1997). Due to its small size and simple structure, alanine substitution can potentially remove any steric or functional properties of the target amino acid.

Site-directed mutagenesis has been recently used by Byrne et al. to demonstrate the importance of the three catalytic triad amino acids of tTG for autoantibody binding in CD and the related gluten-sensitive disorder, dermatitis herpetiformis (Byrne, Ryan et al. 2007). The catalytic triad Cys^{277}, His^{335}, and Asp^{358} is contained in the core region of tTG (Griffin, Casadio et al. 2002), which had previously been implicated as a potential CD epitope in some studies (Sblattero, Florian et al. 2002; Tiberti, Bao et al. 2003), whilst not in others (Seissler, Wohlrab et al. 2001; Nakachi, Powell et al. 2004). The study by Byrne et al. had the advantage of using full-length tTG, whereas the aforementioned studies all
used truncated fragments of tTG which may not have allowed for correct protein conformation. Previous studies have also demonstrated that the presence of calcium ions in ELISA coating buffers results in increased CD autoantibody binding to tTG (Feighery, Collins et al. 2003; Roth, Sjoberg et al. 2003), presumably due to the conformational shift and subsequent exposure of the active-site, which occurs upon calcium binding. In 2011, Lindfors et al. demonstrated the increased sensitivity of an ELISA using 'open' conformation tTG as antigen (Lindfors, Koskinen et al. 2011).

In the study by Byrne et al. the entire catalytic triad of tTG was replaced with alanine residues, thus not allowing an investigation of the contribution of individual residues to CD autoantibody epitopes. An aim of this study was to further dissect the specificity of the anti-tTG response through the generation of further, novel, tTG active-site mutants. Using site-directed mutagenesis and various permutations of alanine screening, three further unique tTG mutants were generated (Table 2.2). As well as the wild-type (wt tTG), and triple mutant (CHΔ tTG), the proteins generated were; CΔ tTG, CHΔ tTG, and HΔΔ tTG. The CΔ tTG had a single amino acid substitution at Cys^{277}, the amino acid responsible for the catalytic activity of tTG via its reaction with the γ-carboxamide group of target glutamine residues during transamidation (Griffin, Casadio et al. 2002). Cys^{277} is also known to be structurally important, representing the beginning of a helical loop structure in the tTG molecule. In contrast to this, HΔΔ tTG had conservation of Cys^{277}, and replacement of His^{335}, and Asp^{358} with alanine residues.

The template for the mutagenesis PCR employed in this study was the wt tTG sequence contained in the pGEX-4T-1 vector, previously constructed in the study by Byrne et al. (Byrne, Ryan et al. 2007). Mismatched primers were designed to introduce the desired
mutations to the tTG catalytic triad, with the amplified PCR product then ready for transfection to a suitable host, and produced as a protein. The Quickchange® system (Stratagene) was chosen to perform the mutagenesis reaction. The kit employs a restriction enzyme, Dpnl, to digest any remaining bacterial DNA post mutagenesis. Dpnl is an endonuclease with a specificity for methylated or hemimethylated DNA, such as found in Dam⁺ strains of Escherichia Coli. (de la Campa, Springhorn et al. 1988).

2.1.3 Recombinant protein production

In order to produce the recombinant protein coded for in the PCR product, ligation to an appropriate vector is needed. There are a number of possible vectors available for expression of recombinant proteins, including bacteria, human cells, insect cells and transgenic plants (Kangas, Cooney et al. 1982; Wurm and Bernard 1999; Hellwig, Drossard et al. 2004; Drugmand, Schneider et al. 2011). In this study, the vector of choice was Escherichia Coli (E.coli) because of its ability to grow rapidly, and at high density on relatively inexpensive substrates (Tolia and Joshua-Tor 2006). As bacteria have no post-translational modification capabilities such as disulphide bond formation or glycosylation, one of the problems often associated with the production of mammalian proteins is that the product may be incorrectly folded. However, in the case of tTG, the protein does not contain disulphide bonds and is not glycosylated (Folk and Finlayson 1977), and therefore, these problems do not arise. The E. coli strain BL-21, commonly used for recombinant protein production was chosen for expression of the recombinant human tTG proteins.

Due to overproduction of the heterogeneous recombinant protein in bacteria, some of the protein can be misfolded and can aggregate in insoluble bodies, termed inclusion
bodies (Villaverde and Carrio 2003). The formation of inclusion bodies during tTG production has previously been reported by Shi et al. (Shi 2002). In order to minimise inclusion body formation, and hence maximise protein yields, the pGEX-4T-1 vector (Figure 2.1) was chosen to produce the recombinant tTG proteins. One advantage of using a pGEX vector is the production of recombinant proteins fused to Glutathione-S-transferase (GST), a system which has been shown to increase protein solubility (Geng and Carstens 2006). GST is a 26 kDa protein from the bacteria Shistosoma japonicum that is widely used in the manufacture of recombinant proteins (Smith, Davern et al. 1986). Upon lysis of the host bacterial cell, the recombinant protein can be purified due to the high affinity of GST for glutathione. For use in assays such as ELISA, the GST tag may be removed by thrombin cleavage (Jenny, Mann et al. 2003). Although antibody responses to GST have been detected in autoimmune diseases such as autoimmune hepatitis (Kato, Miyakawa et al. 2004), thrombin cleavage of the GST tag was not performed, as it may reduce protein yields.
For screening of successful transformants, the pGEX-4T-1 vector contains a *lacIq* gene, which is interrupted upon insertion of DNA into its cloning site. β-galactosidase, the enzyme coded for by the *lacIq* gene turns X-gal, its substrate, into an insoluble blue dye (Messing, Crea et al. 1981). Therefore, bacterial colonies can be screened based on colour - blue colonies possess β-galactosidase activity, and therefore do not have a DNA insert. To ensure selective growth of transformed bacteria during protein production, the pGEX-4T-1 vector also contains an ampicillin resistance gene.

Recombinant protein expression by the pGEX-4T-1 vector can be induced by isopropyl β-D thiogalactoside (IPTG). IPTG mimics the effects of allolactose, disrupting the binding of repressor proteins of the *tac* promoter and initiating transcription of the *lac* operon.
contained in the vector (van Hoek and Hogeweg 2007). In the study by Byrne et al. (Byrne, Ryan et al. 2007), it was observed that milder induction conditions resulted in increased recombinant protein yields by preventing the formation of inclusion bodies in *E. coli*.

### 2.1.4 SDS-PAGE and Western blot analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used throughout the project to visualize and assess recombinant tissue transglutaminase (tTG) expression and purification. SDS-PAGE is an electrophoretic method that separates proteins based on molecular weight only. This is achieved through the use of an anionic detergent, sodium dodecyl sulphate, which will denature secondary and non-disulphide linked tertiary structures in proteins, and confer a negative charge on the protein. The components of an SDS-polyacrylamide gel are acrylamide, ammonium persulphate, which acts as an initiator, TEMED; (N,N,N',N'-tetramethylethylethylenediamine), which catalyses the polymerization, and N,N'-methylene bis-acrylamide, which will join the linear polymers of acrylamide together, resulting in the formation of a three dimensional mesh.

Western blotting is a routinely used technique for the specific detection of proteins (Burnette 1981). Proteins are first separated electrophoretically by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane, and probed with antibody. Due to the highly specific nature of the antigen-antibody interaction, western blotting is an extremely useful tool for ensuring the correct identity of recombinantly produced proteins (Pogge von Strandmann, Zoidl et al. 1995).
Proteins are transferred from the SDS-PAGE gel to the nitrocellulose or PDVF membrane electrophoretically by sandwiching the gel and membrane between layers of soaked filter paper between two electrodes. The electrical field causes migration of the protein from the gel to the membrane, and allows subsequent exposure to the primary antibody. In order to visualise the blot, a secondary antibody coupled to a fluorescent, radioactive, or enzymatic label is added, with subsequent signal detection.

### 2.1.5 Chapter aims

The aim of this chapter was to produce wild-type recombinant human tTG, and four variant versions thereof. All of the tTG variants had selected amino acids of the catalytic triad Cys$_{277}$, His$_{335}$, and Asp$_{358}$ replaced with alanine residues.
2.2 Materials and Methods

2.2.1 Restriction digest of plasmid DNA

A restriction digest was performed prior to protein expression, in order to confirm the presence of the wild-type tTG sequence in the pGEX-4T-1 vector. Plasmid DNA was isolated using the Wizard miniprep kit (Promega). The digest contained 1µl EcoR1, 1µl Xho1, 10µl plasmid DNA, 6µl nuclease free H2O and 2µl of 10x reaction buffer. Samples were incubated at 37°C for one hour, and subsequently run on a 0.8% agarose gel at 100V for one hour.

2.2.2 Site-directed mutagenesis primer design

The primers used in the site-directed mutagenesis PCR were designed using the Stratagene online labtools website (Table 2.1). The codon GCT was used to code for alanine in the C277A and H335A substitutions, whilst GCC was used in the D358A substitution.

Table 2.1: Primers used for site-directed mutagenesis. (Sigma-Genosys)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C277A-F</td>
<td>5' - GTCAAGTATGGCCAGGCTTGTTGCTTTCCGGG- 3’</td>
</tr>
<tr>
<td>C277A-R</td>
<td>5' - CGGCGAAGACCCAAGGCTTGCCCATACCTGAC- 3’</td>
</tr>
<tr>
<td>H335A-F</td>
<td>5' - CGAGATGATCTGGAACTTCGCTTGCTGGGTGGAGTCGTG- 3’</td>
</tr>
<tr>
<td>H335A-R</td>
<td>5' - CACGACTCCACCCAGCAAGGGAAGTTCCAGATCATCTCG- 3’</td>
</tr>
<tr>
<td>D358A-F</td>
<td>5' - GCAGGCCCTGGCCCAAGGAGGGGCGTTGGGGCCAGGG- 3’</td>
</tr>
<tr>
<td>D358A-R</td>
<td>5' - GGCCCAGGGCTGCAGGGCGCC- 3’</td>
</tr>
</tbody>
</table>

The underlined sequences in bold indicate the mutated codons. Primers were ordered from Sigma Genosys.
2.2.3 Site-directed mutagenesis polymerase chain reaction

Site-directed mutagenesis was performed using the Quickchange® system (Stratagene). The only modification of the manufacturer’s recommendations was the use of *Escherichia Coli* (E. Coli) BL21 as competent bacterial cells. The mutagenesis reaction mixture contained 5µl of 10x reaction buffer (Stratagene), 1µl DNTP mix (Stratagene), 50ng DNA template, 125ng of forward and reverse primers, 1µl *PfuUltra* DNA polymerase, and 31.5µl MilliQ dH₂O. The thermal cycling conditions were 16 cycles of denaturing at 95°C for 30 seconds, annealing at 55°C for 1 minute, and elongation at 68°C for 7 minutes. After the mutagenesis PCR was complete, the reaction mixture was placed on ice for 2 minutes. Bacterial chromosomal DNA was then digested by the addition of 1µl (10U) of DpnI, and incubation at 37°C for 1 hour. As a positive control, a control plasmid containing a stop codon in the β-galactosidase gene was included in each mutagenesis PCR. A successful mutation cycle repairs the β-galactosidase gene, and allows for downstream blue/white screening.

2.2.4 Agarose gel electrophoresis of mutagenesis PCR products

The products of the mutagenesis PCR were visualised by Agarose gel electrophoresis. A 1% gel was prepared by adding 1g of agarose to 100mls tris-borate-EDTA (TBE) buffer and heating in a microwave until the agarose was dissolved. The solution was allowed to cool to <60°C before the addition of 5µl of 10mg/ml ethidium bromide, in a fume hood. The gel was then poured into the template, and the comb inserted. Once the gel had set, the comb was removed. 10µl of the mutagenesis PCR product was mixed with 2µl of 6xloading buffer (Appendix i) and added to the well. A 100 base-pair marker (Invitrogen) was run with each gel in order to estimate the size of the mutagenesis PCR products. The
gel was then electrophoresed at 100V for ~45 minutes, and visualised using a ultra-violet (UV) box.

2.2.5 Preparation of chemically competent *Escherichia Coli BL 21*

50mls of LB broth was incubated with *Escherichia Coli* BL21 in a sterile flask. The culture was then incubated at 37°C for 3 hours, until an optical density at 600nm (OD_{600}) of ~0.5 was reached. The flask was placed on ice for 15 minutes, and then pelleted at 3000rpm for 10 minutes. The supernatant was discarded; the pellet was gently resuspended in 10mls of ice-cold 100mM MgCl\(_2\), and incubated on ice for 15 minutes. The bacteria were pelleted as before and resuspended in 10mls of cold 100mM CaCl\(_2\). The mixture was then incubated on ice for at least an hour, and was subsequently ready for use.

2.2.6 Transfection of *E. coli BL 21* with mutagenesis PCR products

1μl of the PCR product from Section 2.2.3 was added to 100μl chemically competent *E.coli* in a sterile, pre-chilled tube. The mixture was gently swirled, to mix, and incubated on ice for 30 minutes. The mixture was then heat-shocked at 42°C for 2 minutes, and placed on ice for a further 2 minutes. 1ml of SOC medium (Sigma) was then added, with subsequent incubation for 1 hour at 37 °C. Plates of LB agar, containing 100μg/ml ampicillin, were prepared by lawning with a mixture of 100μl of X-gal (2%) and 100μl of IPTG (10mM) for blue/white screening purposes. This was allowed to dry on the plate before plating of 250μl of the *E.coli/PCR* product mixture, and incubation overnight at 37 °C. Colonies were selected based on a blue appearance, and subjected to subsequent plasmid isolation and restriction digest. Selected transformants were lifted from colonies on the plate using an autoclaved toothpick, and were cultured overnight in LB broth.
containing 100μg/ml ampicillin. Bacterial cryobeads (Key Scientific) were inoculated with a sample of each of these cultures, and stored at -80°C.

2.2.7 Plasmid isolation

Plasmid isolation was performed using the Wizard Miniprep® (Promega), as per the manufacturer’s instructions. Briefly, 5ml of each tTG-plasmid containing *E.coli*, which had been cultured overnight at 37 °C in LB broth containing 100μg/ml ampicillin, was pelleted at 10,000xg. The supernatant was poured off, the pellet resuspended in 300μl of cell resuspension solution (50 mM Tris-HCl, 10 mM EDTA, 100 μg/mL RNase A, pH 7.5), and transferred to a sterile 1.5ml centrifuge tube. 300μl of cell lysis solution (1% SDS, 0.2 M NaOH) was added, and the tube was mixed by gentle inversion. The mixture was incubated on ice for 5 minutes, before the addition of 300μl of neutralisation buffer (1.32 M Potassium Acetate, pH 4.8) with subsequent mixing by inversion and incubation on ice for 5 minutes. The mixture was then centrifuged at 3500 rpm for 5 minutes, with the plasmid-containing supernatant being transferred to a new 1.5ml centrifuge tube.

2.2.8 DNA sequencing

In order to confirm the sequence identity of, and to ensure the successful introduction of desired mutations to each recombinant tTG, DNA sequencing of the isolated plasmid DNA was performed commercially by MWG Eurofins Operon Ltd. Sequencing results were then analysed using the Geneious® program.
2.2.9 Recombinant protein expression and purification

One litre cultures were grown in nutrient broth to OD_{600} ~0.5, and protein expression was induced by addition of 1μM IPTG, followed by incubation overnight at room temperature with 150 rpm agitation. The bacteria were pelleted and resuspended in lysis buffer (20mM Tris, 150mM NaCl, 1mM EDTA, 1mM DTT, 15% glycerol, pH 8.0), and pelleted at 3,500xg for 15 minutes. Pellets were stored at -80°C prior to purification. For purification of the recombinant tTGs, pellets were resuspended in 50mls CelLytic reagent (Sigma-Aldrich) containing 1ml protease inhibitor cocktail (Sigma-Aldrich), 10mg lysozyme (Sigma-Aldrich), and 10μl Benzonase endonuclease (Sigma-Aldrich). Lysate was incubated at room temperature for 20 minutes with mixing and subsequently pelleted at 15,000 xg for 15 minutes at 4°C. Two mls of Glutathione Sepharose (GE Healthcare) was added to the resulting supernatant, and incubated for one hour at 4°C, with mixing. The sepharose was washed 4 times with PBS, and the GST-fusion protein eluted from the beads with 1ml elution solution (100mM glutathione, 20mM Tris, pH 8.0). Recombinant tTG was mixed with 50% glycerol to prevent freezing, and stored at -20°C. Some of each produced protein batch was not mixed with glycerol, for use in Bradford assays, SDS-PAGE analysis, and Western blotting.

2.2.10 Recombinant protein quantification

A Bradford assay was used for the quantification of the recombinant tTG proteins. Described in 1976 by Bradford, the method is based on the shift in the absorbance maximum of Coomassie Brilliant Blue from 465nm to 595 nm that occurs upon binding to protein (Bradford 1976). The assay was performed using Bradford dye reagent (Sigma) pre-warmed to room temperature. A seven point standard curve was generated using BSA concentrations of 4 to 0.0625 mg/ml. Each standard, unknown sample, and a blank
of dH₂O was then diluted 200-fold in the Bradford reagent to a final volume of 750μl, and incubated in the dark at room temperature for at least five minutes. 200μl of each sample was then added in triplicate to wells of a 96 well plate (Nunc), and the absorbance at 595nm measured using a Biotek ELx800 spectrophotometer. The standard curve was generated, and used to estimate the protein concentration of the unknown samples.

2.2.11 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

A 12% SDS-PAGE gel was prepared according to the following steps: firstly the ATTO mini gel system was assembled and clamped into place. 12% resolving gel was then prepared, (Appendix i), poured into the assembled gel system, overlaid with distilled H₂O, and allowed to set for 20-30 minutes. When the resolving gel had set, the overlay was poured off. The 5% stacking gel was then prepared (Appendix i) and poured into the system, overlaying the resolving gel. A comb was inserted, and removed after 15 minutes, to form the sample wells. The gel rig was assembled, filled with tris-glycine buffer (Appendix i), and the wells of the gel washed with buffer to remove any unpolymerised acrylamide. Samples were prepared by adding 2x loading buffer, and boiled in a water bath for 5 minutes to further reduce disulphide bonds and disrupt the proteins folding pattern. Samples were loaded into the wells of the gel, with a broad range (6-175 kDa) molecular size marker (New England Biolabs), being loaded into lane 1 of every gel performed. The gel was run at 30mA for 1 hour, until the dye front had reached the bottom of the gel. Upon completion, the protein in the gel was visualized by overnight staining with Coomassie Blue; with excess Coomassie Blue being removed by destain solution. (Appendix i)
2.2.12 Western blotting

To characterise each recombinant tTG protein, recognition by the monoclonal anti-tTG antibody CUB 7402 (Abcam) was assessed by Western blotting. SDS-PAGE was carried out as per Section 2.2.11, applying 0.3μg of each tTG to the gel. The protein was blotted onto a PDVF membrane by semi-dry transfer at a current of 0.8 mA/sq cm for 2 hours. The membrane was then blocked with 0.1 ml/sq cm of 5% non-fat dried milk in 0.5% PBS-Tween for 2 hours at room temperature with gentle agitation. The primary antibody (CUB 7402) was diluted 1:1000 in 5% non-fat dried milk in 0.5% PBS-Tween, and incubated overnight at 4°C with gentle agitation. After overnight incubation, rabbit anti-mouse conjugated to horseradish peroxidase (HRP) (Sigma) was added, and the blot was visualised using 3,3’-Diaminobenzidine (DAB) (Sigma), and hydrogen peroxide. A thorough washing with PBS plus 0.1% Tween was performed between each step, in order to remove any unbound antibody.
2.3 Results

2.3.1 Restriction digest of plasmid DNA

Upon digestion of the pGEX-4T-1 vector with the restriction enzymes EcoRI and XhoI, the tTG PCR product was clearly visible (Figure 2.2). The tTG insert is 2067bp whereas the pGEX-4T-1 genome is 4.9kb in size.

Figure 2.2: Restriction digest analysis of the wild-type tTG-containing plasmid. 1: 100bp ladder, 2: Uncut plasmid, 3: Restriction digest of pGEX-4T-1-tTG with EcoRI and XhoI
2.3.2 Site-directed mutagenesis PCR

Five differing p-GEX-4T plasmids, containing the sequence for wild-type and four mutagenic variants of tTG were generated (Table 2.2). The mutant tTG proteins had single (C277A), double (C277A, H335A and H335A, D358A), or triple (C277A, H335A, D358A) alanine substitutions of their catalytic triad amino acids. Each PCR product was successfully transfected into chemically competent *E. coli* BL-21.

Table 2.2: Recombinant human tTG proteins generated.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mutations Performed</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt tTG</td>
<td>None</td>
</tr>
<tr>
<td>CA tTG</td>
<td>C277A</td>
</tr>
<tr>
<td>CHA tTG</td>
<td>C277A, H335A</td>
</tr>
<tr>
<td>CHDA tTG</td>
<td>C277A, H335A, D358A</td>
</tr>
<tr>
<td>HDA tTG</td>
<td>H335A, D358A</td>
</tr>
</tbody>
</table>

Desired mutations were introduced using the Quickchange™ system. CA indicates the cysteine residue of the catalytic triad has been replaced with alanine, CHA indicates the cysteine and histidine residues have been replaced with alanine, CHDA indicates the cysteine and histidine and aspartic acid residues have been replaced with alanine, whilst HDA indicates the histidine and aspartic acid residues have been replaced with alanine.
2.3.3 DNA sequencing

DNA sequencing confirmed the identity of each tTG coding sequence, contained in the pGEX-4T-1 plasmids. The successful introduction of alanine substitutions into the catalytic triad of each mutant tTG was also observed. No PCR errors were detected by DNA sequencing. A screenshot from the Geneious® program is shown in Figure 2.3, detailing the C277A mutation introduced into the CA tTG protein.

![Geneious® screenshot showing C277A mutation](image)

**Fig 2.3:** Example of the introduction of the C277A mutation into the catalytic triad of tTG. The red square indicates the codon shift. The top three sequences represent three different stocks of transfected *E.coli* BL-21 containing the CA tTG plasmid, whilst the bottom sequence is of the wild-type protein (the black rectangle representing the codon for the C277 residue). Screenshot taken from the Geneious® program.

2.3.4 Recombinant protein expression and purification

All five recombinant human tTG proteins were successfully purified from *E. coli* BL-21. Due to the tendency for tTG to form inclusion bodies in *E. coli*, mild induction conditions of 1µM IPTG per litre of culture were applied, as per the method of Byrne et al. Initial protein yields were poor (see section 2.3.5), prompting the inclusion of a bacterial protease inhibitor cocktail, which was successful in increasing the amount of protein purified. In order to estimate both the molecular weight, and purity, of each batch of recombinant tTG purified, a sample of each was run on a 12% SDS-PAGE gel. The 12% SDS-PAGE gel of all five recombinant human tTGs in Figure 2.4 shows all proteins to be of
a similar molecular size, indicating that the introduced mutations have not affected translation of the full length tTG molecule. All protein batches were routinely >95% pure, as visually estimated by SDS-PAGE analysis, reflecting the efficiency of the purification process, and success of the numerous washing steps to remove any contaminating \textit{E. coli} proteins.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure24.png}
\caption{12\% SDS-PAGE of wild-type and mutant tTGs. Two micrograms of each protein was visualised on a 12\% SDS-PAGE gel. The molecular weight ladder can be seen in the first lane. The tTG band at 103 kDa consists of both tTG (77 kDa) and the GST tag (26 kDa). As expected, the wild-type and mutant tTGs migrate to the same area of the gel.}
\end{figure}
2.3.5 Recombinant protein quantification

A standard Bradford assay (Bradford 1976) was performed to quantify yields of the recombinant wild-type and mutant tTG proteins. A BSA standard curve was generated with a range of 4 to 0.0625 mg/ml (Figure 2.5), and used to calculate the protein concentration of the unknown samples.

Fig 2.5: Example of Bradford assay standard curve. BSA was used to make the standard curve, generated using GraphPad Prism software.

Initial tTG yields were low, as can be seen in Table 2.3, with an average yield of 0.25mg of wild-type tTG per litre of *E. coli* cultured. Yields of the mutant tTG proteins were similar. The subsequent inclusion of the protease inhibitor cocktail during the purification process dramatically improved the amount of recombinant protein purified (Tables 2.4, 2.5).
Table 2.3: Yields of wild-type and mutant tTGs pre inclusion of a bacterial protease inhibitor during purification.

<table>
<thead>
<tr>
<th>Date</th>
<th>wt tTG yield</th>
<th>CA tTG yield</th>
<th>CHΔ tTG yield</th>
<th>CHΔΔ tTG yield</th>
<th>HΔΔ tTG yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>14/02/2008</td>
<td>0.26 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21/04/2008</td>
<td>0.23 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10/10/2008</td>
<td>0.27 mg</td>
<td>0.10 mg</td>
<td>0.33 mg</td>
<td>0.31 mg</td>
<td>-</td>
</tr>
<tr>
<td>Mean</td>
<td>0.25 mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Yields are expressed as mg per litre of E. coli. The dashed line indicates that the respective tTG was not purified.

The average yield of the wild-type tTG protein when the protease inhibitor cocktail was used was 1.57mg per litre of E. coli. From Table 2.4 it can be seen that there was a large degree of variation in yields.

Table 2.4: Yields of wild-type tTG post inclusion of a bacterial protease inhibitor during purification.

<table>
<thead>
<tr>
<th>Date</th>
<th>wt tTG yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>21/01/2009</td>
<td>0.84 mg</td>
</tr>
<tr>
<td>01/07/2009</td>
<td>0.49 mg</td>
</tr>
<tr>
<td>20/04/2010</td>
<td>2.80 mg</td>
</tr>
<tr>
<td>20/04/2010</td>
<td>1.87 mg</td>
</tr>
<tr>
<td>09/08/2010</td>
<td>0.99 mg</td>
</tr>
<tr>
<td>10/11/2010</td>
<td>1.02 mg</td>
</tr>
<tr>
<td>20/10/2010</td>
<td>0.96 mg</td>
</tr>
<tr>
<td>20/10/2010</td>
<td>0.79 mg</td>
</tr>
<tr>
<td>28/07/2011</td>
<td>4.35 mg</td>
</tr>
<tr>
<td>Mean</td>
<td>1.57 mg</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.26 mg</td>
</tr>
<tr>
<td>Median</td>
<td>0.99 mg</td>
</tr>
</tbody>
</table>

Yields are expressed as mg per litre of E. coli.

In general, the yields of the mutant tTG proteins was lower than that of the wild-type protein, however the inclusion of the protease inhibitor cocktail during purification increased yields (Table 2.5). The average yield of mutant tTG proteins was 0.87mg per litre of E. coli, again with some fluctuation in yields.
Table 2.5: Yields of mutant tTGs post inclusion of a bacterial protease inhibitor during purification.

<table>
<thead>
<tr>
<th>Date</th>
<th>CA tTG yield</th>
<th>CHA tTG yield</th>
<th>CHDA tTG yield</th>
<th>HDA tTG yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>21/01/2009</td>
<td>0.43 mg</td>
<td>0.56 mg</td>
<td>0.43 mg</td>
<td>-</td>
</tr>
<tr>
<td>01/07/2009</td>
<td>-</td>
<td>-</td>
<td>0.87 mg</td>
<td>-</td>
</tr>
<tr>
<td>06/08/2009</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.94 mg</td>
</tr>
<tr>
<td>12/11/2009</td>
<td>-</td>
<td>-</td>
<td>1.61 mg</td>
<td>-</td>
</tr>
<tr>
<td>10/11/2009</td>
<td>0.11 mg</td>
<td>0.78 mg</td>
<td>0.99 mg</td>
<td>0.83 mg</td>
</tr>
<tr>
<td>10/02/2011</td>
<td>-</td>
<td>0.50 mg</td>
<td>0.56 mg</td>
<td>0.53 mg</td>
</tr>
<tr>
<td>28/07/2011</td>
<td>1.37 mg</td>
<td>1.36 mg</td>
<td>1.45 mg</td>
<td>1.45 mg</td>
</tr>
<tr>
<td>Mean</td>
<td>0.64 mg</td>
<td>0.80 mg</td>
<td>0.98 mg</td>
<td>0.93 mg</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.66 mg</td>
<td>0.39 mg</td>
<td>0.47 mg</td>
<td>0.38 mg</td>
</tr>
<tr>
<td>Median</td>
<td>0.43 mg</td>
<td>0.67 mg</td>
<td>0.93 mg</td>
<td>0.88 mg</td>
</tr>
</tbody>
</table>

Yields are expressed as mg per litre of *E. coli*. The dashed line indicates that the respective tTG was not purified.

2.3.6 Western blotting

In a western blot, the monoclonal anti-tTG antibody CUB 7402 bound to wild type and mutant tTGs (Figure 2.6). This further confirmed the identity of each recombinant human tTG protein produced in section 2.2.9. The murine antibody CUB 7402 targets a region on the tTG molecule at amino acids 447 to 478. Its successful recognition of the mutant tTG proteins indicates the conservation of this epitope despite the active-site mutations.

![Western blot of the recombinant tTG proteins](image)

Fig 2.6: Western blot of the recombinant tTG proteins. 0.3µg of each recombinant tTG protein was probed with the murine monoclonal anti-tTG antibody, CUB 7402.
2.4 Discussion

2.4.1 Site-directed mutagenesis

Controversy exists as to the target of anti-tTG autoantibodies in CD. Previous work has indicated that the removal of the tTG active-site abrogated CD IgA anti-tTG binding. In order to further explore this observation, we have generated further novel tTG mutants, with varying levels of alanine substitution of the tTG active-site residues. Selected mutations were introduced into the tTG catalytic triad, using the wild-type sequence inserted into the pGEX-4T-1 vector as template DNA. Three novel tTG mutants (CΔ tTG, CHΔ tTG, and HDΔ tTG), and the previously described CHDΔ tTG mutant (Byrne, Ryan et al. 2007), were generated. All of the selected active-site codons were mutated to code for alanine residues.

Applying site-directed mutagenesis, the introduction of new atoms may have a structural effect on the epitope of interest (Cunningham and Wells 1989). Due to its simple chemical structure, using alanine as a replacement amino acid allows for the removal of most atoms from a given position without the subsequent introduction of new atoms or charges. For site-directed mutagenesis, typically a template of single-stranded DNA is required (Kunkel 1985) - an advantage of using the Quickchange® kit was that it allowed for mutation of double stranded plasmid DNA, such as the wild-type tTG-containing plasmid used in this chapter.

2.4.2 DNA sequencing

The identity of each recombinant tTG was confirmed by DNA sequencing of the tTG sequence-containing plasmids generated in the site-directed mutagenesis PCR. Analysis
of the DNA sequencing results was carried out using the Geneious® program, which allowed for alignment of the recombinant tTG sequences with the tTG sequence published by Gentile (Gentile, Saydak et al. 1991). The correct catalytic triad codon shifts were observed for each mutant tTG protein. No PCR errors were detected by the DNA sequencing.

2.4.3 Recombinant tTG expression and purification

The method for the expression and purification of recombinant wild-type and mutant tTGs used in this study was based on that developed by Byrne et al. (Byrne, Ryan et al. 2007). Production of recombinant tTG has been shown to be problematic, due to its tendency to form insoluble inclusion bodies and need for gentle induction conditions (Shi 2002). The expression of recombinant tTG as a GST-fusion protein represents a superior alternative to the use of a His-tag, as the solubility-enhancing properties of GST-fusion have been reported (Esposito and Chatterjee 2006). Cleavage of the GST-tag by thrombin post-purification was avoided in an attempt to avoid further manipulation, and potentially lower yields of the recombinant tTGs.

Several studies have reported the necessity for mild induction conditions in order to generate adequate amounts of soluble tTG (Cunningham and Wells 1989; Murthy, Iismaa et al. 2002; Shi 2002). In this study, 1μM of IPTG per litre of culture was used to induce protein production, it is possible that even smaller, or staggered, gradually increasing amounts of IPTG could have resulted in further increased yields of recombinant tTG. Although early attempts at tTG purification resulted in low yields - averaging 0.25mg per litre of E. coli (n=6), the inclusion of a bacterial protease inhibitor cocktail caused a dramatic increase in the amount of tTG purified. The protease inhibitor works by
inhibiting the activity of protease enzymes that are present in cell extracts, and are capable of degrading the proteins present in the extract (Murby, Uhlen et al. 1996). The increased yields of both recombinant wild-type and mutant tTGs post inclusion of the protease inhibitor cocktail can be seen in Tables 2.4 and 2.5. Some fluctuation in levels of protein yields were observed, a possible explanation for this may be the fluctuation in room temperature during induction. In keeping with the observation by Byrne et al. (Byrne, Ryan et al. 2007), yields of the mutant tTG proteins were roughly half that of the wild-type protein. The mean yield of all mutant proteins was however increased, when compared with the yield of CHΔ tTG in the study by Byrne et al. (Byrne, Ryan et al. 2007). The difference in yield between wild-type and mutant tTGs may represent problematic folding efficiency, and insolubility of mutant tTGs. SDS-PAGE analysis showed that all tTG preparations were highly pure (Figure 2.4), with little contaminating material, whilst in a Western blot; the monoclonal anti-tTG antibody CUB 7402 bound each recombinant tTG, and variant thereof, in a similar pattern (Figure 2.6).

2.4.4 Conclusions

Five recombinant human tTG proteins were generated – the wild type protein, and four mutants with differing alanine substitutions of their Cys^277, His^335, and Asp^358 active-site residues, three of which were novel. Site-directed mutagenesis was used to introduce the desired mutations into the tTG active-site, with sequence-containing plasmids being transfected into E. coli BL-21 for protein expression. In order to maximise the solubility, and hence, yields of the recombinant tTGs, GST-tagging was chosen as the purification method.

SDS-PAGE and western blotting analysis demonstrated that highly pure, and antigenic tTG had been produced. Yields fluctuated, but were satisfactory, with an average yield of 67
1.57 mg per litre of *E. coli* for wild-type tTG, compared to 0.87 mg per litre of *E. coli* for mutant tTGs. Despite the difficulties in solubility, sufficient amounts of each recombinant protein were generated for use in a range of experiments to dissect the interaction between CD autoantibodies and tTG.
Chapter 3

Analysis of coeliac disease autoantibody responses to wild-type and mutant tissue transglutaminases
3.1 Introduction

3.1.1 Epitope mapping

The study and characterisation of binding sites on protein antigens for products of cellular and humoral immunity is termed epitope mapping. Antigens are highly diverse, with differences in primary sequence, size, and post-translational modifications such as glycosylation contributing to this diversity (Abell and Denney 1985; Trujillo, Kumpula-McWhirter et al. 2004). Epitopes may be composed of either a continuous linear amino acid sequence, or when amino acids that are distant in the linear protein sequence are situated in close proximity upon protein folding. T lymphocytes recognise linear epitopes (Cyster, Shotton et al. 1991; Deraos, Chatzantoni et al. 2008), whilst epitopes recognised by antibodies produced by B lymphocytes are structurally dependant on correct protein folding (Enshell-Seijffers, Denisov et al. 2003; Rubinstein, Mayrose et al. 2008).

Site-directed mutagenesis is a powerful epitope mapping tool, allowing for the investigation of the contribution of individual amino acids to both linear and structural epitopes. Site-directed mutagenesis has been used to map evolutionary variants of hen egg lysozyme (Smith-Gill, Wilson et al. 1982), natural escape mutants of the influenza virus (Varghese, Webster et al. 1988), and a multitude of studies mapping epitopes recognised by monoclonal antibodies (Notley, Hillier et al. 1994; Bodker, Wind et al. 2003; Hong, Li et al. 2009). The use of site-directed mutagenesis to map epitopes recognised by autoantibodies in human autoimmune disease has particular relevance to this study (Sekiguchi, Futei et al. 2001; Wolin and Reinisch 2006; Kim, Jeong et al. 2007).

As epitopes are highly dependent on charge, and correct protein folding, alanine screening, in which the target residue is replaced with alanine, is commonly applied (Lefevre, Remy et al. 1997). The side chain of alanine consists of a single methyl group,
corresponding to the carbon in all amino acids except glycine and proline. Alanine substitution will therefore remove any steric or electrostatic contributions from side chain bonds and salt bridges to the epitope of interest by the target amino acid.

3.1.2 Tissue transglutaminase epitope mapping in coeliac disease

Prior to the study by Byrne et al. in which mutagenesis of the tTG catalytic triad abrogated CD IgA autoantibody binding (Byrne, Ryan et al. 2007), site-directed mutagenesis had not been applied as a tTG epitope mapping technique. tTG epitope mapping studies have largely been based upon using truncated fragments of tTG containing specific domains or sequences of tTG. In the study performed by Sblattero et al., using cloned fragments of tTG as antigen, it was found that CD serum anti-tTG, and antibodies cloned from phage antibody libraries created from intestinal lymphocytes of CD patients, bound exclusively to a conformational-dependant antigenic region of tTG which contained the active-site catalytic triad of amino acids, Cysteine\(^{277}\), Histidine\(^{355}\) and Aspartic acid\(^{358}\) (Sblattero, Florian et al. 2002). Byrne et al. expanded this observation by demonstrating the requirement of the three active-site residues for CD and dermatitis herpetiformis (DH) IgA anti-tTG binding, using full length tTG constructs (Byrne, Ryan et al. 2007). The complete replacement of the tTG catalytic triad by Byrne et al. did not allow an assessment of the contribution of individual residue to autoantibody binding in CD and DH. By applying various permutations of active-site residue mutation, a further dissection of this highly specific autoantibody response is possible.

Previous studies have indicated longitudinal changes in the epitope specificity of autoantibodies to self-proteins, such as glutamic acid decarboxylase 65 (GAD65) in type 1 diabetes mellitus (Hampe, Hall et al. 2007). In order to examine the dynamics of the
evolution of the anti-tTG response in CD, binding to wild-type and mutant tTG proteins was measured in both paediatric and adult CD patients. The recombinant proteins produced in chapter 3 were applied as antigen in an ELISA system, with CD IgA and IgG autoantibody binding to mutant tTGs being compared to that of the wild-type protein. In order to ensure that the introduction of mutations to the catalytic triad did not adversely affect protein folding and structure, the ability of both monoclonal and polyclonal antibodies directed against tTG to bind to each recombinant protein purified was also assessed by ELISA.

3.1.3 Enzyme-linked Immunosorbent Assay (ELISA)

The ELISA assay is a biochemical assay capable of quantifying the amount of antigen or antibody in a given sample. One of either the antigen or antibody is labelled with an enzyme, and addition of substrate leads to a measurable colourmetric change. The intensity of this colour change is proportional to the amount of antigen or antibody in the sample, as determined by simultaneously measuring standards of known concentration (Engvall, Jonsson et al. 1971). Due to the high specificity of the antigen-antibody interaction, and signal amplification capabilities of the enzymatic label, the ELISA assay is capable of detecting analytes present in minute quantities (Carlsson, Lindberg et al. 1972). There are three major types of ELISA: indirect ELISA, in which the antigen is immobilised on a solid support followed by addition of the primary antibody; sandwich ELISA, in which the antigen of interest is 'captured' between two detection antibodies; and, competitive ELISA which can be used to quantify antigen (Ma, Zhang et al. 2011). A non-competitive indirect solid-phase ELISA was developed in this study in order to compare autoantibody binding to the recombinant wild-type and mutant tTG proteins generated in Section 2. This involved coating the relevant tTG antigen to an
ELISA well, followed by addition of the anti-tTG-containing patient sera. Antibodies bound to their antigen could be detected by further probing with a labelled isotype-specific secondary antibody (Dieterich, Laag et al. 1998).

In order to develop an in-house anti-tTG ELISA assay, optimisation of a number of variables including primary and secondary antibody concentrations was required. Antigen coating is affected by factors such as pH (Kim, Park et al. 1998), which for tTG should generally be slightly alkaline (Sblattero, Berti et al. 2000), concentration used for coating (Giardina, Evans et al. 2003), and the physical properties of the antigen (Bantroch, Buhler et al. 1994). Optimal ELISA conditions can be devised by employing a series of checkerboard experiments representing different combinations of each variable (Lee, Ahn et al. 2001). Horseradish peroxidase (HRP) is commonly used to enzymatically label secondary antibodies (Shigematsu, Suda et al. 2007), and was chosen for use in this study. Addition of a substrate such as 3,3',5,5'-tetramethyl-benzidine (TMB), o-phenylenediamine or 5-aminosalicylic acid results in a coloured compound (Rye, Saper et al. 1984) that is detectable spectrophotometrically.

The results of ELISA assays can be reported in either absolute amounts of analyte present e.g. microgram per microlitre, or arbitrary ELISA units. For reporting in arbitrary units (AU), a standard curve is generated from a strongly positive, or pooled positive samples (Yoon, Angov et al. 2005). As creating a quantified anti-tTG standard is unfeasible, the ELISA assays developed in this chapter are reported in AU. The standard curve may be generated by plotting absorbance versus the log of concentration, however in ELISA assays it is more appropriate to use a spline curve constructed from polynomials, that runs through each point (Karpinski 1990).

In order to establish the threshold of positivity for an ELISA assay, sera from groups of control individuals must be tested for reactivity to the antigen. A common strategy for
determination of the cut-off points for positivity is to set the value at the mean + 2 standard deviations of the control response (Aggarwal, Dabadghao et al. 1994). Ideally the control and disease populations should be age-matched, with age-specific cut-off points calculated (Baldas, Not et al. 2004).

### 3.1.4 Chapter aims

The aims of this chapter were to characterise the antigenicity of the four mutant tTG proteins generated in the previous chapter, in a comparison with the non-mutated wild-type protein. In order to investigate the correct folding of, and conservation of epitopes on the mutant tTG proteins, their reactivity to a range of polyclonal and monoclonal control antibodies was also examined. The use of various tTG active-site mutants was to investigate the contribution of each active-site residue to CD autoantibody epitopes. IgA and IgG reactivity to each recombinant protein was assessed in order to dissect the anti-tTG response of these autoantibody isotypes, and determine potential isotype differences in anti-tTG specificity. Anti-tTG epitope mapping results were compared for both adults and children with CD, in order to inform as to the nature of the anti-tTG response early in disease course, and map the evolution of the autoantibody response to tTG.
3.2 Materials and Methods

3.2.1 Serum samples

Serum samples from 93 patients with coeliac disease were used to assess tTG autoantibody binding. The adult group consisted of 30 patients (M:F 1:2.8; age 22-85 years, median = 59), whilst the paediatric group consisted of 63 patients (M:F 1:1.7; age 2-15 years, median=8) (Table 3.1). The diagnosis of coeliac disease was based upon positive IgA EMA and anti-tTG serology, reduction in levels of these autoantibodies with gluten withdrawal, and duodenal histology which was available for 41 of the paediatric CD patients, and for all of the adult CD patients. Both CD study populations were further subdivided by disease activity status, based on serial IgA anti-tTG levels measured by the Celikey™ assay, serial IgA EMA levels, and duodenal histology. In the paediatric CD group, 23 patients had untreated CD, 15 patients had partially treated CD, and 25 patients had treated CD (Table 3.2). In the adult CD group, 12 were untreated, 13 were partially treated, and 5 were treated (Table 3.2). Individuals with partially treated CD were defined as patients with low to medium levels of IgA anti-tTG (20-50 AU, Celikey™ ELISA system), in which serial tTG measurement (Celikey™) has demonstrated falling titres of IgA anti-tTG levels post-commencement of a gluten-free diet.
Table 3.1: Study groups used to investigate anti-tTG binding.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>n=</th>
<th>Sex</th>
<th>Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
<td>Mean</td>
</tr>
<tr>
<td>Paediatric CD</td>
<td>63</td>
<td>23</td>
<td>40</td>
</tr>
<tr>
<td>Paediatric controls</td>
<td>57</td>
<td>29</td>
<td>28</td>
</tr>
<tr>
<td>Adult CD</td>
<td>30</td>
<td>9</td>
<td>21</td>
</tr>
<tr>
<td>Adult controls</td>
<td>30</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

Cut-off points for ELISA positivity (mean+2SD) were established by assaying serum from groups of normal adult (n=30, M:F 1:2, age 24-82 years, median = 52) and paediatric (n=57, M:F 1:1, age 1-15 years, median =8) individuals with negative IgA anti-tTG serology (Table 3.1). All adult non-coeliacs had normal intestinal histology. For ELISA standard curve generation, 4 strongly positive (tTG >100 AU by Celikey™ assay) adult CD samples were pooled, aliquoted, and stored at -70°C until needed. All serum samples were obtained from the Immunology laboratory, St. James Hospital. Ethical approval for this study was granted from St. James’ Hospital and Our Lady’s Children’s Hospital, Dublin.

Table 3.2: Treatment status of both CD study populations.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Treatment Status (n=)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Partially Treated</td>
</tr>
<tr>
<td>Paediatric CD</td>
<td>23</td>
<td>15</td>
</tr>
<tr>
<td>Adult CD</td>
<td>12</td>
<td>13</td>
</tr>
</tbody>
</table>

Treatment status was determined based upon serial IgA anti-tTG/IgA EMA levels, and contemporaneous duodenal biopsy specimens, where available.
3.2.2 Characterisation of wild-type and mutant tissue transglutaminases

In order to assess any potential structural differences between the wild-type and mutant tTGs, each recombinant protein was used as antigen in an ELISA, with binding of various monoclonal and polyclonal anti-tTG antibodies assessed. Certified 96-well Maxisorb plates (Nunc) were coated with 0.3μg of each recombinant protein in coating buffer (50mM Tris, 150mM NaCl, 5mM CaCl₂, pH 7.5) overnight at 4°C. Wells were blocked with 5% casein (Sigma) in PBS for 1 hour, and washed four times with PBS containing 0.01% Tween between each step. Control antibodies CUB 7402 (Abcam), polyclonal anti-tTG (Abcam), 5G7G6 (mouse monoclonal anti-tTG, a gift from Fernando Chirdo), and anti-GST (Abcam) were used as primary antibodies to characterise each recombinant tTG protein. CUB 7402 was diluted 1:5,000 in PBS plus 0.01% Tween, followed by HRP-conjugated rabbit anti-mouse (Dako) diluted 1:2,500 in PBS plus 0.01% Tween. Polyclonal anti-tTG was diluted 1:1,000 in PBS plus 0.01% Tween, followed by HRP-conjugated swine anti-rabbit (Dako) diluted 1:2,500 in PBS plus 0.01% Tween. 5G6 was diluted 1:5,000 in PBS plus 0.01% Tween, followed by HRP-conjugated rabbit anti-mouse (Dako) diluted 1:2,500 in PBS plus 0.01% Tween. Rabbit anti-GST was diluted 1:5,000, followed by HRP-conjugated swine anti-rabbit (Dako), diluted 1:2,500. All antibody incubations were performed for one hour at room temperature. ELISAs were developed using the 3,3',5,5'-tetramethylbenzidine liquid substrate system (Sigma-Aldrich), and OD₄₅₀ measured with a Biotek ELx800 spectrophotometer.
3.2.3 IgA anti-tissue transglutaminase ELISA optimisation

In order to establish a reliable ELISA assay, it was necessary to optimise certain parameters. An antigen coating concentration of 0.3μg per well, as described by Byrne et al. was chosen for all ELISA assays (Byrne, Ryan et al. 2007). In order to determine the optimal concentration of primary and secondary antibodies to be applied, checkerboard experiments were carried out using wild-type tTG as antigen. Differing combinations of primary and secondary antibody (HRP-conjugated rabbit anti-human IgA, Dako) concentration were measured in a single plate, using negative and positive IgA anti-tTG serum (as measured by the Celikey™ assay) as test samples. A sample layout of such a plate can be seen in Fig 3.1.

Figure 3.1: IgA ELISA checkerboard experiment. In order to optimise primary and secondary dilution, checkerboard experiments were performed. (+ve1/2=known anti-tTG positive CD sera, -ve=known anti-tTG negative serum, B=blank)
3.2.4 IgG anti-tissue transglutaminase ELISA optimisation

Checkerboard experiments were also carried out to determine the optimal concentrations of primary and secondary antibody (HRP-conjugated rabbit anti-human IgG, Dako) for use in the IgG anti-tTG ELISA. As IgG is found in higher concentrations than IgA in human serum (Gonzalez-Quintela, Alende et al. 2008), larger dilutions of primary antibody were investigated. Again, a coating concentration of 0.3μg per well of wild-type tTG was used. Fig 3.2 gives an example of such a plate.

![Figure 3.2: IgG ELISA checkerboard experiment. In order to optimise primary and secondary dilution, checkerboard experiments were performed. (+ve1/2=known anti-tTG positive CD sera, -ve=known anti-tTG negative serum, B=blank)](image-url)
3.2.5 Standard curve generation

Four strongly positive CD IgA anti-tTG samples (>100AU, Celikey™ assay) were pooled for the generation of standard curves for both the IgA and IgG anti-tTG assays. Once secondary antibody concentration had been established, a range of doubling dilutions of the pooled positive sera was applied to plates coated with wild-type tTG. The seven dilutions that gave an approximate range of OD<sub>450</sub> values between 0.2 and 2.0 were then chosen for use as the standard curve.

3.2.6 Final IgA anti-tissue transglutaminase ELISA protocol

Certified 96-well Maxisorp plates (Nunc) were coated with 0.3μg recombinant protein in coating buffer (50mM Tris, 150mM NaCl, 5mM CaCl<sub>2</sub>, pH 7.5) overnight at 4°C. Wells were blocked with 5% casein (Sigma) in PBS for 1 hour, and washed four times with PBS containing 0.01% Tween between each step. Human serum was diluted 1:100 in PBS plus 0.01% Tween, followed by HRP-conjugated polyclonal rabbit anti-human IgA (Dako) diluted 1:2,500 in PBS plus 0.01% Tween. The ELISA was then developed by the addition of TMB liquid substrate (Sigma-Aldrich), the reaction stopped by the addition of 2M H<sub>2</sub>SO<sub>4</sub>, and read at OD<sub>450</sub> in a Biotek ELx800 spectrophotometer. All antibody incubations were performed for one hour at room temperature.

3.2.7 Final IgG anti-tissue transglutaminase ELISA protocol

Certified 96-well Maxisorp plates (Nunc) were coated with 0.3μg recombinant protein in coating buffer (50mM Tris, 150mM NaCl, 5mM CaCl<sub>2</sub>, pH 7.5) overnight at 4°C. Wells were blocked with 5% casein (Sigma) in PBS for 1 hour, and washed four times with PBS containing 0.01% Tween between each step. Human serum was diluted 1:1000 in PBS plus 0.01% Tween, followed by HRP-conjugated polyclonal rabbit anti-human IgG (Dako)
diluted 1:2,000 in PBS plus 0.01% Tween. The ELISA was then developed by the addition of TMB liquid substrate (Sigma-Aldrich), the reaction stopped by the addition of 2M H$_2$SO$_4$, and read at OD$_{450}$ in a Biotek ELx800 spectrophotometer. All antibody incubations were performed for one hour at room temperature.

3.2.8 Inter and intra assay variability

Inter-assay variability was measured by the inclusion of control sera in each assay. The percentage co-efficient of variation (%CV) was then calculated from the formula:

$$\%CV = \left( \frac{\text{Standard deviation}}{\text{Mean}} \right) \times 100$$

Intra-assay variability, or variations within a single plate, was established by randomly distributing the same sample at ten locations in a plate coated with wild-type tTG, followed with detection by the appropriate isotype-specific secondary antibody. The %CV was calculated, with levels <15% desired (Kerrigan and Brooks 1998).
3.3 Results

3.3.1 Characterisation of wild-type and mutant tissue transglutaminases

The ability of control antibodies to tTG to bind to each recombinant protein was assessed by ELISA. As a control for conformational integrity and coating efficiency of the wild-type and mutant proteins, both monoclonal murine (CUB 7402, 5G7G6) anti-tTG and polyclonal anti-tTG were assayed with each protein. Similar binding patterns were observed for each recombinant protein (Fig 3.3). To ensure equal amounts of the GST-fusion proteins were being coated to each well, reactivity of all recombinant tTG proteins to a mouse monoclonal anti-GST was measured, with similar OD values obtained for all samples (Fig 3.3d).

![Graphs showing binding patterns of control antibodies to wild-type and mutant tTGs](image)

**Figure 3.3: Characterisation of wild-type and mutant tTGs.** The control antibodies used are: (a) CUB 7402, (b) 5G7G6, (c) Polyclonal anti-tTG, and (d) Anti-GST. Similar binding patterns were observed for each recombinant tTG.

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3.3.2 Optimisation of the IgA anti-tissue transglutaminase ELISA

An optimal ELISA system is based on a configuration of coating concentration and a primary and secondary antibody dilution that yields optical densities (OD$_{450}$) of $>1.0$ for the positive control, $<0.2$ for the negative control, and $<0.1$ for the blank. As an antigen coating concentration of 0.3µg tTG per well, as per Byrne et al. was chosen, it was necessary to perform checkerboard experiments in order to determine the optimum dilution of patient sera, and secondary antibody.

Dilutions of patient sera of 1:50, 1:100, and 1:200 were investigated, in combination with secondary antibody (HRP-conjugated polyclonal rabbit anti-human IgA, Dako) dilutions of 1:1000, 1:2500, and 1:5000. Samples used as positive controls were from EMA-positive adult CD patients, one with an IgA anti-tTG level of 83 AU, and one of 11.2 AU, as measured by the Celikey™ assay. Serum from an EMA-negative control individual (IgA anti-tTG 1.3 AU, Celikey™ assay, (cut-off=1.8 AU)) was used as a negative control, with 5% casein used as a blank. The OD$_{450}$ values generated as a result of this experiment are shown in table 3.3.
## Table 3.3: IgA anti-tTG ELISA primary and secondary antibody dilution optimisation

<table>
<thead>
<tr>
<th>Sample Dilution</th>
<th>Secondary Antibody Dilution</th>
<th>OD450</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:1000</td>
<td>1:2500</td>
</tr>
<tr>
<td>+ve Ctrl 1 (AU:83)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:50</td>
<td>2.945</td>
<td>2.244</td>
</tr>
<tr>
<td>1:100</td>
<td>2.714</td>
<td><strong>1.513</strong></td>
</tr>
<tr>
<td>1:200</td>
<td>2.029</td>
<td>1.223</td>
</tr>
<tr>
<td>+ve Ctrl 2 (AU:11.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:50</td>
<td>0.916</td>
<td>0.718</td>
</tr>
<tr>
<td>1:100</td>
<td>0.787</td>
<td><strong>0.507</strong></td>
</tr>
<tr>
<td>1:200</td>
<td>0.636</td>
<td>0.345</td>
</tr>
<tr>
<td>-ve Ctrl (AU:1.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:50</td>
<td>0.324</td>
<td>0.228</td>
</tr>
<tr>
<td>1:100</td>
<td>0.298</td>
<td><strong>0.182</strong></td>
</tr>
<tr>
<td>1:200</td>
<td>0.224</td>
<td>0.130</td>
</tr>
<tr>
<td>Blank</td>
<td>0.089</td>
<td><strong>0.032</strong></td>
</tr>
</tbody>
</table>

IgA anti-tTG AU values were generated using the Celikey™ assay. HRP-conjugated polyclonal rabbit anti-human IgA (Dako) was used as the secondary antibody. The red values indicate the combination of dilutions chosen as optimal.

From this checkerboard experiment, it was concluded that a serum dilution of 1:100, combined with a secondary antibody dilution of 1:2500 resulted in discriminatory \( \text{OD}_{450} \) values of negativity, and low and high positivity. The use of secondary antibody at a 1:2500 dilution also yielded satisfactory \( \text{OD}_{450} \) values for the blank wells.

### 3.3.3 Optimisation of the IgG anti-tissue transglutaminase ELISA

Checkerboard experiments for the IgG anti-tTG assay investigated higher dilutions of patient sera. EMA-positive, IgA anti-tTG (Celikey™ assay) adult CD samples were again used as a positive control (2.7 and 46.3 AU, respectively), with serum from an EMA / IgA anti-tTG control individual as a negative control. Casein at 5% was also used as a blank in this experiment, the results of which are shown in table 3.4.
Table 3.4: IgG anti-tTG ELISA primary and secondary antibody dilution optimisation

<table>
<thead>
<tr>
<th>Sample Dilution</th>
<th>Secondary Antibody Dilution</th>
<th>+ve Ctrl 1</th>
<th>-ve Ctrl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:1000</td>
<td>1:2000</td>
<td>1:5000</td>
</tr>
<tr>
<td>1:500</td>
<td>1.322</td>
<td>0.801</td>
<td>0.712</td>
</tr>
<tr>
<td>1:1000</td>
<td>0.884</td>
<td><strong>0.590</strong></td>
<td>0.385</td>
</tr>
<tr>
<td>1:2000</td>
<td>0.769</td>
<td>0.507</td>
<td>0.154</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>1:2000</td>
<td>1:5000</td>
</tr>
<tr>
<td>1:500</td>
<td>2.101</td>
<td>1.580</td>
<td>1.104</td>
</tr>
<tr>
<td>1:1000</td>
<td>1.477</td>
<td><strong>1.069</strong></td>
<td>0.755</td>
</tr>
<tr>
<td>1:2000</td>
<td>1.291</td>
<td>0.882</td>
<td>0.448</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>1:2000</td>
<td>1:5000</td>
</tr>
<tr>
<td>1:500</td>
<td>0.894</td>
<td>0.251</td>
<td>0.228</td>
</tr>
<tr>
<td>1:1000</td>
<td>0.727</td>
<td><strong>0.160</strong></td>
<td>0.113</td>
</tr>
<tr>
<td>1:2000</td>
<td>0.531</td>
<td>0.144</td>
<td>0.087</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>1:2000</td>
<td>1:5000</td>
</tr>
<tr>
<td></td>
<td>0.077</td>
<td><strong>0.033</strong></td>
<td>0.015</td>
</tr>
</tbody>
</table>

IgA anti-tTG AU values were generated using the Celikey™ assay. HRP-conjugated polyclonal rabbit anti-human IgG (Dako) was used as the secondary antibody. The red values indicate the combination of dilutions chosen as optimal.

From this experiment, a serum dilution of 1:1000 and secondary antibody (HRP-conjugated polyclonal rabbit anti-human IgG, Dako) dilution of 1:2000 were chosen for future IgG anti-tTG measurement. As expected, a larger serum dilution than the IgA anti-tTG assay was required, presumably the higher levels of the IgG antibody subclass found in human serum.
3.3.4 Standard curve generation

In order to generate a standard curve for both anti-tTG assays, 12 doubling dilutions of the pooled positive sample was applied to an ELISA plate, and probed with secondary antibody. Table 3.5 shows the results of these experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>OD$_{450}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.037</td>
</tr>
<tr>
<td>1:163,840</td>
<td>0.091</td>
</tr>
<tr>
<td>1:81,920</td>
<td>0.107</td>
</tr>
<tr>
<td>1:40,960</td>
<td>0.162</td>
</tr>
<tr>
<td>1:20,480</td>
<td>0.248</td>
</tr>
<tr>
<td>1:10,240</td>
<td>0.491</td>
</tr>
<tr>
<td>1:5,120</td>
<td>0.849</td>
</tr>
<tr>
<td>1:2,560</td>
<td>1.370</td>
</tr>
<tr>
<td>1:1,280</td>
<td>1.928</td>
</tr>
<tr>
<td>1:640</td>
<td>2.422</td>
</tr>
<tr>
<td>1:320</td>
<td>2.725</td>
</tr>
<tr>
<td>1:160</td>
<td>2.913</td>
</tr>
<tr>
<td>Blank</td>
<td>0.023</td>
</tr>
<tr>
<td>1:40,960</td>
<td>0.038</td>
</tr>
<tr>
<td>1:20,480</td>
<td>0.058</td>
</tr>
<tr>
<td>1:10,240</td>
<td>0.084</td>
</tr>
<tr>
<td>1:5,120</td>
<td>0.132</td>
</tr>
<tr>
<td>1:2,560</td>
<td>0.206</td>
</tr>
<tr>
<td>1:1,280</td>
<td>0.348</td>
</tr>
<tr>
<td>1:640</td>
<td>0.575</td>
</tr>
<tr>
<td>1:320</td>
<td>0.923</td>
</tr>
<tr>
<td>1:160</td>
<td>1.329</td>
</tr>
<tr>
<td>1:80</td>
<td>2.101</td>
</tr>
<tr>
<td>1:40</td>
<td>2.443</td>
</tr>
</tbody>
</table>

Table 3.5: Optimisation of the ELISA standard curves.

a) shows the dilutions chosen for the IgA anti-tTG standard curve, whilst b) shows those chosen for the IgG anti-tTG standard curve (both highlighted in bold).

In order to provide discriminatory values for test samples assayed, both the IgA and IgG anti-tTG assays used a six-point curve, with OD$_{450}$ values of roughly 0.2 to 2.0 for the lowest and highest points of the curve, respectively, being desired. For the IgA anti-tTG assay the optimum dilution range of the pooled positive sample was from 1:640 to 1:20,480, whilst for the IgG anti-tTG assay it was 1:80 to 1:2,560. Standard curves were generated using the Multicalc software (Wallac), an example of which can be seen in Fig 3.4.
Figure 3.4: Six point ELISA curves. Standard curves for the IgA (a), and IgG (b) anti-tTG ELISAs were generated using doubling dilutions of pooled, strongly tTG-positive CD sera incubated with wild-type tTG. These curves, generated using Multicale software (Wallac), were used to assign AU values to test sera.
3.3.5 Intra-assay variability

Both the IgA and IgG anti-tTG ELISAs displayed excellent intra-assay variability (Table 3.6), and were well within the recommended range of <15%. Using the CD sample 54, the intra-assay %CV for the IgA anti-tTG assay was calculated as 6.35%; the intra-assay variability for the IgG anti-tTG assay, calculated using CD sample 13, was smaller at 3.98%.

<table>
<thead>
<tr>
<th>Replicate No.</th>
<th>Sample #54 AU</th>
<th>Replicate No.</th>
<th>Sample #33 AU</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.9</td>
<td>1</td>
<td>20.2</td>
</tr>
<tr>
<td>2</td>
<td>13.6</td>
<td>2</td>
<td>21.7</td>
</tr>
<tr>
<td>3</td>
<td>12.6</td>
<td>3</td>
<td>21.9</td>
</tr>
<tr>
<td>4</td>
<td>11.9</td>
<td>4</td>
<td>21.5</td>
</tr>
<tr>
<td>5</td>
<td>11.7</td>
<td>5</td>
<td>22.3</td>
</tr>
<tr>
<td>6</td>
<td>12.1</td>
<td>6</td>
<td>20.5</td>
</tr>
<tr>
<td>7</td>
<td>13.2</td>
<td>7</td>
<td>21.4</td>
</tr>
<tr>
<td>8</td>
<td>12.1</td>
<td>8</td>
<td>22.7</td>
</tr>
<tr>
<td>9</td>
<td>11.2</td>
<td>9</td>
<td>20.1</td>
</tr>
<tr>
<td>10</td>
<td>11.4</td>
<td>10</td>
<td>21.6</td>
</tr>
<tr>
<td>Mean</td>
<td>12.277</td>
<td>Mean</td>
<td>21.391</td>
</tr>
<tr>
<td>SD</td>
<td>0.780</td>
<td>SD</td>
<td>0.852</td>
</tr>
<tr>
<td>%CV</td>
<td>6.359</td>
<td>%CV</td>
<td>3.981</td>
</tr>
</tbody>
</table>
3.3.6 Inter-assay variability

Inter-assay variability was calculated by including a positive control sample with each ELISA plate. Assays were performed over an extended period of time, and showed a high degree of reproducibility. For both IgA and IgG anti-tTG assays the positive control sample chosen was from an EMA positive, biopsy-proven CD patient, of medium anti-tTG positivity (70AU for IgA and 22AU for IgG). The results of the %CV calculations for both anti-tTG assays are shown in Tables 3.7 and 3.8. The %CV was 12.8% for the IgA anti-tTG ELISA, and 12.9% for the IgG anti-tTG ELISA – both within the acceptable level of <15%.

Table 3.7: Inter-assay variability, IgA anti-tTG ELISA

<table>
<thead>
<tr>
<th>Run #</th>
<th>Sample #18 AU</th>
<th>Run #</th>
<th>Sample #18 AU</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67.8</td>
<td>15</td>
<td>76.7</td>
</tr>
<tr>
<td>2</td>
<td>56.8</td>
<td>16</td>
<td>86.6</td>
</tr>
<tr>
<td>3</td>
<td>63.6</td>
<td>17</td>
<td>62.5</td>
</tr>
<tr>
<td>4</td>
<td>52.9</td>
<td>18</td>
<td>61.4</td>
</tr>
<tr>
<td>5</td>
<td>60.0</td>
<td>19</td>
<td>81.3</td>
</tr>
<tr>
<td>6</td>
<td>55.0</td>
<td>20</td>
<td>67.4</td>
</tr>
<tr>
<td>7</td>
<td>71.3</td>
<td>21</td>
<td>64.5</td>
</tr>
<tr>
<td>8</td>
<td>76.4</td>
<td>22</td>
<td>72.1</td>
</tr>
<tr>
<td>9</td>
<td>63.4</td>
<td>23</td>
<td>67.4</td>
</tr>
<tr>
<td>10</td>
<td>64.6</td>
<td>24</td>
<td>81.3</td>
</tr>
<tr>
<td>11</td>
<td>72.2</td>
<td>25</td>
<td>82.4</td>
</tr>
<tr>
<td>12</td>
<td>76.1</td>
<td>26</td>
<td>69.8</td>
</tr>
<tr>
<td>13</td>
<td>69.2</td>
<td>27</td>
<td>58.0</td>
</tr>
<tr>
<td>14</td>
<td>71.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (AU)</td>
<td>68.594</td>
<td></td>
<td>SD (AU)</td>
</tr>
<tr>
<td>% CV</td>
<td>12.770</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.8: Inter-assay variability, IgG anti-tTG EISA

<table>
<thead>
<tr>
<th>Run #</th>
<th>Sample #33 AU</th>
<th>Run #</th>
<th>Sample #33 AU</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.1</td>
<td>10</td>
<td>26.1</td>
</tr>
<tr>
<td>2</td>
<td>22.8</td>
<td>11</td>
<td>26.8</td>
</tr>
<tr>
<td>3</td>
<td>17.5</td>
<td>12</td>
<td>24.4</td>
</tr>
<tr>
<td>4</td>
<td>19.4</td>
<td>13</td>
<td>20.5</td>
</tr>
<tr>
<td>5</td>
<td>18.4</td>
<td>14</td>
<td>17.4</td>
</tr>
<tr>
<td>6</td>
<td>22.0</td>
<td>15</td>
<td>22.4</td>
</tr>
<tr>
<td>7</td>
<td>18.4</td>
<td>16</td>
<td>22.6</td>
</tr>
<tr>
<td>8</td>
<td>22.0</td>
<td>17</td>
<td>22.7</td>
</tr>
<tr>
<td>9</td>
<td>23.5</td>
<td>18</td>
<td>25.2</td>
</tr>
</tbody>
</table>

Mean (AU): 21.896
SD (AU): 2.830
% CV: 12.930

3.3.7 Cut-off point determination

Sera from 87 age-matched individuals, negative for IgA anti-tTG by the Celikey™ assay were used to establish cut-off points for positivity (Mean +2SD) for the wild-type and mutant transglutaminases for both IgA and IgG ELISAs. Table 3.9 lists the cut-off points for the IgA anti-tTG ELISA, which were similar for both adults and children.

Table 3.9: IgA anti-tTG ELISA cut-off determination

<table>
<thead>
<tr>
<th>Paediatric Controls</th>
<th>wt tTG (AU)</th>
<th>ΔtTG (AU)</th>
<th>CHΔ tTG (AU)</th>
<th>CHΔΔ tTG (AU)</th>
<th>HΔΔ tTG (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.6</td>
<td>1.3</td>
<td>1.5</td>
<td>2.0</td>
<td>1.4</td>
</tr>
<tr>
<td>SD</td>
<td>1.1</td>
<td>0.9</td>
<td>1.0</td>
<td>2.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Mean +2SD</td>
<td>3.9</td>
<td>3.0</td>
<td>3.4</td>
<td>5.9</td>
<td>3.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Adult Controls</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>2.3</td>
<td>2.3</td>
<td>2.0</td>
<td>1.8</td>
<td>1.9</td>
</tr>
<tr>
<td>SD</td>
<td>1.3</td>
<td>1.8</td>
<td>1.2</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Mean +2SD</td>
<td>4.8</td>
<td>5.8</td>
<td>4.5</td>
<td>3.7</td>
<td>4.2</td>
</tr>
</tbody>
</table>
Cut-offs for the IgG anti-\(t\)TG assay are shown in Table 3.10. The cut-off point for the paediatric IgG anti-\(t\)TG assay was higher than that for adults, due to increased variance in control sera binding to the recombinant \(t\)TG proteins.

<table>
<thead>
<tr>
<th></th>
<th>wt (t)TG (AU)</th>
<th>(\Delta t)TG (AU)</th>
<th>CH(\Delta t)TG (AU)</th>
<th>CHDA (t)TG (AU)</th>
<th>HDA (t)TG (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Paediatric</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>Mean</td>
<td>4.6</td>
<td>5.0</td>
<td>4.0</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>5.3</td>
<td>7.6</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>Mean +2SD</td>
<td>15.3</td>
<td>20.2</td>
<td>13.0</td>
<td>12.9</td>
</tr>
<tr>
<td><strong>Adult</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>Mean</td>
<td>2.2</td>
<td>1.1</td>
<td>1.7</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>1.6</td>
<td>0.8</td>
<td>1.4</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Mean +2SD</td>
<td>5.3</td>
<td>2.8</td>
<td>4.5</td>
<td>3.9</td>
</tr>
</tbody>
</table>

### 3.3.8 Recognition of wild-type and mutant \(t\)TGs by coeliac disease serum IgA anti-\(t\)TG

The wild-type \(t\)TG protein was antigenic to IgA anti-\(t\)TG antibodies from CD patients, being recognised at significantly higher levels than controls (\(p<0.001\), Mann-Whitney test). The combined sensitivity of the assay, based on binding to the wild-type protein, for both the adult and paediatric CD groups was 97\% (Table 3.11), whilst the combined specificity was 96\% (paediatric 95\%, adult 97\%). In untreated CD, the assay had a specificity of 100\% (Table 3.12).
Table 3.11: Sensitivity of the IgA anti-tTG ELISA

<table>
<thead>
<tr>
<th>Paediatric CD (n=23)</th>
<th>Sensitivity (%)</th>
<th>Adult CD (n=12)</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>100</td>
<td>Untreated</td>
<td>100</td>
</tr>
<tr>
<td>Partially treated</td>
<td>100</td>
<td>Partially treated</td>
<td>92</td>
</tr>
<tr>
<td>Treated</td>
<td>96</td>
<td>Treated</td>
<td>100</td>
</tr>
</tbody>
</table>

CD serum IgA reactivity to wild-type tTG correlated strongly with IgA anti-tTG results from the Celikey™ tTG ELISA system for all samples tested ($r = 0.785$). The range of IgA anti-tTG values to wild-type tTG was reflective of treatment status, with untreated patients showing the highest levels in both children and adults (Table 3.12).

Table 3.12: Mean IgA anti-tTG levels and disease status

<table>
<thead>
<tr>
<th>Paediatric CD (n=67)</th>
<th>Untreated</th>
<th>Partially treated</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>96.3 AU</td>
<td>47.5 AU</td>
<td>13.3 AU</td>
</tr>
<tr>
<td>Adult CD (n=30)</td>
<td>135.0 AU</td>
<td>19.1 AU</td>
<td>12.5 AU</td>
</tr>
</tbody>
</table>

All mutant tTG proteins displayed diminished reactivity, when compared to the wild-type protein (Fig 3.5). In paediatric CD, mean reductions in autoantibody binding compared to the wild-type protein were 89% for CΔtTG ($p<0.001$), 90% for CHΔtTG ($p<0.001$), 87% for CHDΔtTG ($p<0.001$), and 91% for HDΔtTG ($p<0.001$) (Wilcoxon signed ranks test) (Table 3.13, Fig 3.5a).
Fig 3.5: (a) Recognition of wt and mutant tTGs by paediatric CD IgA anti-tTG, (b) Recognition of wt and mutant tTGs by adult CD IgA anti-tTG. Replacement of even one amino acid of the catalytic triad of tTG resulted in a loss of autoantibody binding. p values were calculated using the Wilcoxon signed ranks test.

In adult CD the pattern of reduced IgA binding to mutant tTG proteins was replicated. The mean reduction in binding to mutant tTG proteins was 76% in the adult CD study group (p<0.001 for each mutant protein, Wilcoxon signed ranks test) (Table 3.14, Fig 3.5b).
Table 3.13: Percentage reductions in paediatric CD IgA anti-tTG binding to mutant tTG proteins, compared to the wild-type protein.

<table>
<thead>
<tr>
<th></th>
<th>CA tTG</th>
<th>CHA tTG</th>
<th>CHDA tTG</th>
<th>HDA tTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>-99</td>
<td>-99.1</td>
<td>-99.5</td>
<td>-99.1</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>-96.9</td>
<td>-97.8</td>
<td>-97.2</td>
<td>-97.5</td>
</tr>
<tr>
<td>Median</td>
<td>-94.6</td>
<td>-94.9</td>
<td>-94.2</td>
<td>-96.3</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>-87.1</td>
<td>-89.9</td>
<td>-85.2</td>
<td>-90.5</td>
</tr>
<tr>
<td>Maximum</td>
<td>32.4</td>
<td>-9.6</td>
<td>0</td>
<td>31.3</td>
</tr>
<tr>
<td>Mean</td>
<td>-88.69</td>
<td>-90.05</td>
<td>-86.51</td>
<td>-91.03</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>18.16</td>
<td>16.09</td>
<td>19.55</td>
<td>14</td>
</tr>
<tr>
<td>Std. Error</td>
<td>2.288</td>
<td>2.027</td>
<td>2.464</td>
<td>1.764</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>-93.27</td>
<td>-94.11</td>
<td>-91.44</td>
<td>-94.56</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>-84.12</td>
<td>-86</td>
<td>-81.59</td>
<td>-87.51</td>
</tr>
</tbody>
</table>

Table 3.14: Percentage reductions in adult CD IgA anti-tTG binding to mutant tTG proteins, compared to the wild-type protein.

<table>
<thead>
<tr>
<th></th>
<th>CA tTG</th>
<th>CHA tTG</th>
<th>CHDA tTG</th>
<th>HDA tTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>-99.3</td>
<td>-99.2</td>
<td>-99.2</td>
<td>-99</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>-95.55</td>
<td>-95.1</td>
<td>-95.08</td>
<td>-95.95</td>
</tr>
<tr>
<td>Median</td>
<td>-84.75</td>
<td>-85.75</td>
<td>-84.9</td>
<td>-86.4</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>-61.75</td>
<td>-54.53</td>
<td>-59.38</td>
<td>-74.7</td>
</tr>
<tr>
<td>Maximum</td>
<td>-27.8</td>
<td>7.5</td>
<td>-8.5</td>
<td>47.1</td>
</tr>
<tr>
<td>Mean</td>
<td>-79.51</td>
<td>-73.62</td>
<td>-76.14</td>
<td>-73.25</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>18.88</td>
<td>28.97</td>
<td>24.79</td>
<td>37.61</td>
</tr>
<tr>
<td>Std. Error</td>
<td>3.446</td>
<td>5.289</td>
<td>4.526</td>
<td>6.866</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>-86.56</td>
<td>-84.44</td>
<td>-85.4</td>
<td>-87.3</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>-72.46</td>
<td>-62.81</td>
<td>-66.89</td>
<td>-59.21</td>
</tr>
</tbody>
</table>
3.3.9 Recognition of wild-type and mutant tTGs by coeliac disease serum IgG anti-tTG

Unlike the IgA anti-tTG assay, the sensitivity of the IgG anti-tTG assay was poor, being 17% for paediatric CD patients, and 47% for adults. The combined specificity was 95% (paediatric 96%, adult 93%). In paediatric CD, sensitivity was improved for patients with untreated disease, however this was still poor at 30% (Table 3.15). Sensitivity of the IgG anti-tTG ELISA in adult CD was not affected by disease activity status (Table 3.15).

Table 3.15: Sensitivity of the IgG anti-tTG ELISA

<table>
<thead>
<tr>
<th>Paediatric CD</th>
<th>Sensitivity (%)</th>
<th>Adult CD</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (n=23)</td>
<td>30</td>
<td>Untreated (n=12)</td>
<td>50</td>
</tr>
<tr>
<td>Partially treated (n=15)</td>
<td>0</td>
<td>Partially treated (n=13)</td>
<td>46</td>
</tr>
<tr>
<td>Treated (n=25)</td>
<td>12</td>
<td>Treated (n=5)</td>
<td>40</td>
</tr>
</tbody>
</table>

Mean IgG anti-tTG levels to the wild-type tTG in paediatric CD were 61.2 AU for untreated patients, 4.4 AU for partially treated patients, and 7.6 AU for treated patients (cut-off 15.3 AU)(Table 3.16). There was significantly increased binding to wild-type tTG in untreated paediatric CD patients, compared to those who were partially treated (p=0.0063), treated (p=0.0042), or controls (p=0.0003) (Fig 3.6).
Figure 3.6: The relationship of IgG anti-tTG levels with CD treatment status. The highest levels of IgG anti-tTG in paediatric CD (a) were seen in untreated patients, whilst in adult CD (b), the highest levels were seen in untreated and partially treated patients. UT = untreated, PT = partially treated, T = treated, Ctrl = Control population. The black line represents the mean IgG anti-tTG value for each study group.

In adult CD, mean IgG anti-tTG levels were similar regardless of treatment status, with mean levels of 7.7 AU for untreated patients, 8.0 AU for partially treated patients, and 4.9 AU for treated patients observed (cut-off 5.3 AU) (table 3.16). There was significantly increased IgG anti-tTG binding to the wild-type protein in adult CD patients with untreated or partially treated disease, when compared to controls (p = 0.0006, 0.0002 respectively) (Fig 3.6).

<table>
<thead>
<tr>
<th>Table 3.16: Mean IgG anti-tTG levels and disease status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paediatric CD (n=67)</td>
</tr>
<tr>
<td>Untreated</td>
</tr>
<tr>
<td>61.2 AU</td>
</tr>
<tr>
<td>Adult CD (n=30)</td>
</tr>
<tr>
<td>7.7 AU</td>
</tr>
</tbody>
</table>

The highest levels of IgG anti-tTG were detected in untreated CD patients <4 years of age (Figure 3.7, Table 3.17), concurring with a previous report by Agardh et al. describing the high prevalence of this anti-tTG isotype in young children (Agardh, Borulf et al. 2003).
Indeed, for CD patients grouped by age, the sensitivity of the IgG anti-tTG ELISA was an improved 50% for patients <4 years of age (Table 3.17).

![IgG anti-tTG levels and age in paediatric CD. The black line represents the mean IgG anti-tTG value for each age group.](image)

Table 3.17: The effect of age on IgG anti-tTG levels

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Mean IgG anti-tTG (AU)</th>
<th>IgG anti-tTG ELISA sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4 (n=11)</td>
<td>97</td>
<td>50</td>
</tr>
<tr>
<td>4-8 (n=17)</td>
<td>6.5</td>
<td>5</td>
</tr>
<tr>
<td>8-12 (n=26)</td>
<td>7.6</td>
<td>14</td>
</tr>
<tr>
<td>12-16 (n=9)</td>
<td>2.1</td>
<td>0</td>
</tr>
<tr>
<td>Adults (n=30)</td>
<td>7.4</td>
<td>47</td>
</tr>
</tbody>
</table>

When cut-off points for positivity in the paediatric population were determined using age-matched controls, grouped in four increments of four years, the improved specificity of the IgG anti-tTG ELISA was maintained, with the cut-off level being similar to that generated using the entire paediatric control population (14.5 AU using controls <4 years of age vs. 15.3 AU using the entire paediatric control population) (Table 3.18).
Table 3.18: IgG anti-tTG sensitivity in paediatric CD using individual cut-offs for each age group

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>IgG anti-tTG cut-off (AU)</th>
<th>IgG anti-tTG ELISA sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>14.5</td>
<td>50</td>
</tr>
<tr>
<td>4-8</td>
<td>27.6</td>
<td>0</td>
</tr>
<tr>
<td>8-12</td>
<td>7.6</td>
<td>24</td>
</tr>
<tr>
<td>12-16</td>
<td>7.8</td>
<td>0</td>
</tr>
</tbody>
</table>

There was a mean reduction in binding to all mutant tTG proteins, compared to wild-type tTG, of 33% in paediatric CD (range -100% to +180%) (Table 3.19, Figure 3.8a). The mean reduction in binding was 47% for CA<sub>tTG</sub> (p<0.001), 43% for CH<sub>A</sub>tTG (p<0.001), 32% for CHD<sub>A</sub>tTG (p<0.001), and 32% for HD<sub>A</sub>tTG (p<0.001) (Wilcoxon signed ranks test).

Table 3.19: Percentage reductions in paediatric CD IgG anti-tTG binding to mutant tTG proteins, compared to the wild-type protein.

<table>
<thead>
<tr>
<th></th>
<th>CA&lt;sub&gt;tTG&lt;/sub&gt;</th>
<th>CH&lt;sub&gt;A&lt;/sub&gt;tTG</th>
<th>CHD&lt;sub&gt;A&lt;/sub&gt;tTG</th>
<th>HD&lt;sub&gt;A&lt;/sub&gt;tTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>-100</td>
<td>-100</td>
<td>-100</td>
<td>-99.6</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>-85.9</td>
<td>-78.6</td>
<td>-80.5</td>
<td>-73.1</td>
</tr>
<tr>
<td>Median</td>
<td>-60</td>
<td>-55.3</td>
<td>-54.3</td>
<td>-35.5</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>-21</td>
<td>-8.3</td>
<td>13.5</td>
<td>-0.8</td>
</tr>
<tr>
<td>Maximum</td>
<td>119.4</td>
<td>70.4</td>
<td>180.2</td>
<td>114.5</td>
</tr>
<tr>
<td>Mean</td>
<td>-47.16</td>
<td>-42.67</td>
<td>-31.97</td>
<td>-32.32</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>47.51</td>
<td>43.6</td>
<td>61.54</td>
<td>47.12</td>
</tr>
<tr>
<td>Std. Error</td>
<td>5.986</td>
<td>5.493</td>
<td>7.754</td>
<td>5.936</td>
</tr>
<tr>
<td>Lower 95% Cl of mean</td>
<td>-59.12</td>
<td>-53.65</td>
<td>-47.46</td>
<td>-44.19</td>
</tr>
<tr>
<td>Upper 95% Cl of mean</td>
<td>-35.19</td>
<td>-31.69</td>
<td>-16.47</td>
<td>-20.46</td>
</tr>
</tbody>
</table>
When the paediatric CD group was further sub-divided by treatment status, the mean reduction in binding to all mutant proteins was highest in patients with untreated disease (Figure 3.9 a/b/c). The mean reduction in binding to all four mutants, compared to the wild-type protein was 67% in untreated CD, 47% in partially treated CD, and 8% in treated CD.

Reduction of IgG anti-tTG binding to the mutant tTG proteins was also observed in adult CD, with a mean reduction of 22% for all mutated tTGs (Table 3.20). The highest level of reduction in IgG anti-tTG binding to the mutant tTG proteins was seen in partially treated disease (Figure 3.9 c/d/e). The mean reduction in binding to all four mutants, compared to the wild-type protein was 20% in untreated adult CD, 29% in partially treated adult CD, and 11% in treated adult CD.

Fig 3.8: (a) Recognition of wt and mutant tTGs by paediatric CD IgG anti-tTG, (b) Recognition of wt and mutant tTGs by adult CD IgG anti-tTG. All mutant tTGs displayed diminished antigenicity, when compared to the wild-type tTG protein. p values were calculated using the Wilcoxon signed ranks test.
Figure 3.9: Reduction of IgG anti-tTG binding to the mutant tTG proteins in the differing CD treatment groups. A, b, and c represent untreated, partially treated, and treated paediatric CD respectively, whilst d, e, and f represent untreated, partially treated, and treated adult CD.
Table 3.20: Percentage reductions in adult CD IgG anti-tTG binding to mutant tTG proteins, compared to the wild-type protein.

<table>
<thead>
<tr>
<th></th>
<th>CA tTG</th>
<th>CHA tTG</th>
<th>CHDA tTG</th>
<th>HDA tTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>-88.8</td>
<td>-87.6</td>
<td>-87.2</td>
<td>-89.4</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>-58.1</td>
<td>-41.58</td>
<td>-33.7</td>
<td>-50.85</td>
</tr>
<tr>
<td>Median</td>
<td>-41.65</td>
<td>-20.1</td>
<td>-9.4</td>
<td>-19.4</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>-17.93</td>
<td>-1.8</td>
<td>6</td>
<td>3.4</td>
</tr>
<tr>
<td>Maximum</td>
<td>10.1</td>
<td>59.6</td>
<td>68.8</td>
<td>85.4</td>
</tr>
<tr>
<td>Mean</td>
<td>-37.8</td>
<td>-22.11</td>
<td>-12.58</td>
<td>-17.38</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>26.52</td>
<td>33.57</td>
<td>38.2</td>
<td>40.29</td>
</tr>
<tr>
<td>Std. Error</td>
<td>4.842</td>
<td>6.13</td>
<td>6.975</td>
<td>7.356</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>-47.7</td>
<td>-34.64</td>
<td>-26.84</td>
<td>-32.43</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>-27.89</td>
<td>-9.57</td>
<td>1.685</td>
<td>-2.339</td>
</tr>
</tbody>
</table>

The reduction in IgG anti-tTG binding to the mutant tTG proteins for both CD populations is much less than that observed for IgA anti-tTG, however this is probably reflective of lower levels of IgG anti-tTG directed against the wild-type protein. In CD patients who are considered positive in the IgG anti-tTG assay, the average reduction in IgG anti-tTG binding to the mutant tTG proteins is 85.9% in paediatric CD (Table 3.21), and 47.5% in adult CD (Table 3.22), the difference possibly reflecting the magnitude of IgG anti-tTG responsiveness to the wild-type protein in the two groups.
Table 3.21: Reduction of IgG anti-tTG binding to mutant tTG proteins in IgG anti-tTG ELISA positive paediatric CD patients.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>wt tTG (AU)</th>
<th>CA tTG (AU)</th>
<th>CHA tTG (AU)</th>
<th>CHDAtTG (AU)</th>
<th>HDAtTG (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>45.5</td>
<td>0.6</td>
<td>0.5</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>8</td>
<td>27.2</td>
<td>1.1</td>
<td>5.0</td>
<td>6.2</td>
<td>6.0</td>
</tr>
<tr>
<td>14</td>
<td>70.8</td>
<td>1.6</td>
<td>1.5</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>19</td>
<td>390.5</td>
<td>1.4</td>
<td>0.9</td>
<td>1.1</td>
<td>1.4</td>
</tr>
<tr>
<td>41</td>
<td>341.5</td>
<td>2.8</td>
<td>3.7</td>
<td>4.5</td>
<td>5.9</td>
</tr>
<tr>
<td>47</td>
<td>42.5</td>
<td>1.0</td>
<td>1.5</td>
<td>1.6</td>
<td>2.6</td>
</tr>
<tr>
<td>57</td>
<td>422.5</td>
<td>4.3</td>
<td>6.0</td>
<td>6.0</td>
<td>6.8</td>
</tr>
<tr>
<td>63</td>
<td>24.0</td>
<td>10.5</td>
<td>27.9</td>
<td>24.5</td>
<td>24.8</td>
</tr>
<tr>
<td>75</td>
<td>29.1</td>
<td>2.1</td>
<td>2.9</td>
<td>1.8</td>
<td>3.1</td>
</tr>
<tr>
<td>80</td>
<td>21.1</td>
<td>1.5</td>
<td>2.1</td>
<td>1.4</td>
<td>3.1</td>
</tr>
<tr>
<td>81</td>
<td>19.1</td>
<td>2.8</td>
<td>4.1</td>
<td>1.8</td>
<td>5.1</td>
</tr>
<tr>
<td>Mean % Reduction</td>
<td>N/A</td>
<td>-92.3</td>
<td>-83.2</td>
<td>-85.6</td>
<td>-82.5</td>
</tr>
</tbody>
</table>

Table 3.22: Reduction of IgG anti-tTG binding to mutant tTG proteins in IgG anti-tTG ELISA positive adult CD patients.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>wt tTG (AU)</th>
<th>CA tTG (AU)</th>
<th>CHA tTG (AU)</th>
<th>CHDAtTG (AU)</th>
<th>HDAtTG (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>301</td>
<td>7.8</td>
<td>3.4</td>
<td>4.8</td>
<td>7.4</td>
<td>4.2</td>
</tr>
<tr>
<td>302</td>
<td>25.4</td>
<td>2.8</td>
<td>3.1</td>
<td>3.3</td>
<td>2.7</td>
</tr>
<tr>
<td>304</td>
<td>10.0</td>
<td>5.4</td>
<td>7.8</td>
<td>8.9</td>
<td>10.1</td>
</tr>
<tr>
<td>306</td>
<td>10.8</td>
<td>3.7</td>
<td>4.3</td>
<td>4.6</td>
<td>3.6</td>
</tr>
<tr>
<td>307</td>
<td>18.1</td>
<td>8.9</td>
<td>12.0</td>
<td>12.5</td>
<td>10.9</td>
</tr>
<tr>
<td>310</td>
<td>6.4</td>
<td>2.0</td>
<td>2.8</td>
<td>3.0</td>
<td>2.8</td>
</tr>
<tr>
<td>313</td>
<td>12.0</td>
<td>3.4</td>
<td>3.4</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>315</td>
<td>13.6</td>
<td>7.5</td>
<td>8.1</td>
<td>8.3</td>
<td>6.7</td>
</tr>
<tr>
<td>317</td>
<td>10.4</td>
<td>4.0</td>
<td>5.7</td>
<td>7.1</td>
<td>8.8</td>
</tr>
<tr>
<td>318</td>
<td>18.7</td>
<td>6.0</td>
<td>5.4</td>
<td>6.6</td>
<td>6.9</td>
</tr>
<tr>
<td>322</td>
<td>7.8</td>
<td>4.8</td>
<td>7.9</td>
<td>8.5</td>
<td>9.6</td>
</tr>
<tr>
<td>323</td>
<td>8.9</td>
<td>5.8</td>
<td>6.1</td>
<td>6.5</td>
<td>8.9</td>
</tr>
<tr>
<td>325</td>
<td>20.5</td>
<td>6.2</td>
<td>5.1</td>
<td>6.3</td>
<td>7.1</td>
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<tr>
<td>Mean % Reduction</td>
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<td>-58.9</td>
<td>-48.7</td>
<td>-41.1</td>
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3.3 Discussion

3.4.1 Tissue transglutaminase epitope mapping

Since the identification of tTG as the autoantigen recognised by endomysial antibodies by Dieterich et al. (Dieterich, Ehnis et al. 1997), multiple studies have been undertaken in order to identify the epitopes recognised by anti-tTG autoantibodies in CD. Using radiolabelled tTG fragments, Seissler concluded that amino acids 1-281 and 473-687 contained the major epitopes recognised by IgA anti-tTG in CD patient sera, with the catalytic region being antigenic for only 22.5% of CD patients (Seissler, Wohlrab et al. 2001).

Comparing the antigenicity of $^{32}$S-radiolabelled full-length, and two truncated (aa 227-687, 473-687) tTG fragments, Tiberti demonstrated a bias in IgA anti-tTG binding to fragment 227-687 in female CD patients <4 years of age, and to fragment 473-687 in adult female CD patients (Tiberti, Bao et al. 2003). In 2004, Nakachi and colleagues produced nine $^{32}$S-radiolabelled tTG constructs, spanning the entire tTG sequence (Nakachi, Powell et al. 2004). Of the 15 CD sera tested, all required the N-terminal region of tTG (aa 1-89) for IgA anti-tTG binding, while all but one required the central region (aa 401-491).

In a study performed by Sblattero et al., twelve cloned deletion mutant fragments of tTG were generated, encompassing the entire length of the tTG sequence (Sblattero, Florian et al. 2002). These tTG constructs were then applied as antigen in an ELISA system, and probed with CD patient sera and monoclonal antibodies, cloned from phage antibody libraries created from intestinal lymphocytes of coeliac patients. It was demonstrated that the cloned antibody single-chain fragments, and IgA and IgG antibodies from coeliac patients bound exclusively to a conformational-dependant antigenic region of tTG which
contained the active-site catalytic triad of amino acids, Cysteine\textsuperscript{277}, Histidine\textsuperscript{335} and Aspartic acid\textsuperscript{358}.

All of the above tTG epitope mapping studies were carried out using truncated fragments of the protein, which may adversely affect correct assembly and conformational integrity of the produced recombinant protein. In order to avoid the potential structural issues of using incomplete protein fragments, site-directed mutagenesis is a superior tool for the investigation of immunoglobulin epitopes, which often require residues distant in the protein sequence to be brought together upon protein folding, as it results in the generation of the full-length protein. Site directed mutagenesis has been extensively used for the investigation of antigen-antibody interaction, identifying epitopes recognised during humoral immune responses to bacteria (Rementeria, Vivanco et al. 2009), allergens (Ferreira, Ebner et al. 1998), and self-components (Wen and Yaneva 1992). Of particular relevance to this study is the use of site-directed mutagenesis to map autoreactive B-cell epitopes on enzymes (Hampe, Hammerle et al. 2001; Lampasona, Belloni et al. 2008; Sutti, Vidali et al. 2010), which are frequently targeted by autoimmune responses.

Applying site-directed mutagenesis to the core region of tTG, previously implicated as a CD autoantibody epitope, Byrne et al. showed that the complete removal of the three active-site residues almost completely abolished adult CD and DH IgA anti-tTG binding (Byrne, Ryan et al. 2007). The results in this chapter expand on this observation through the investigation of CD IgA and IgG autoantibody responses to various active-site mutants, in both children and adults with CD. CD autoantibody recognition of the mutated tTG proteins produced in chapter 2 demonstrates that even a single amino acid
substitution (CA tTG) in the catalytic triad has the same effect as substitution of all three residues.

For antigen-antibody interaction, it has been shown that a typical structural epitope is formed from 15-22 amino acids (Dubska, Banga et al. 2006), with one to five residues contributing significantly to the binding energy of the complex (Chakrabarti and Janin 2002). Removal of any of these critical residues results in diminished antibody binding, likely due to the resultant disturbances in charge. Given the results in this chapter, it may be speculated that the amino acid substitutions introduced into the active-site of tTG caused a disruption of charge that either prevented correct assembly of the major CD autoantibody epitope, prevented normal antigen-antibody interaction, or both - confirming all three active-site residues as critical for CD autoantibody binding. Any electrostatic properties of the target amino acids will have been removed by their replacement with alanine residues, due to its neutral charge and simple methyl side chain. A replacement of the active-site residues with those from similar amino acid groups e.g. Cys\textsuperscript{277} with a nucleophilic amino acid or His\textsuperscript{335} with a basic amino acid, and the resultant effect on CD autoantibody binding, could prove informative in the future.

Production of anti-tTG in CD occurs at local level in the mucosal associated lymphoid tissue (MALT), a system that is under constant antigenic challenge and stimulation, and has evolved to preferentially produce IgA in order to neutralize pathogens, and build a tolerance to dietary antigens. Multiple rounds of somatic hypermutation and VDJ recombination occur at mucosal level, the result being affinity matured, and highly specific IgA antibodies (Cerutti 2008). It is possible that the generation of highly specific IgA anti-tTG antibodies is a result of affinity maturation and epitope focusing over a prolonged disease duration (Cucnik, Kveder et al. 2004). In order to examine this
hypothesis, we have compared the anti-tTG specificity of both adult and paediatric CD patients, with the paediatric population representing an earlier stage of disease evolution, and duration.

Many subtle immunological differences, which may have relevance in the aetiology and pathogenesis of coeliac disease, have been noted between adults and children. In studies investigating the T-cell responses to gliadin peptides, Vader et al demonstrated that T-cells from children with coeliac disease target a more diverse set of epitopes when compared to adult patients. Furthermore, paediatric T-cells reacted to non-deamidated gliadin peptides more frequently than adult T-cells, suggesting that the childhood T-cell response changes over time, focusing on immunodominant epitopes (Vader, Kooy et al. 2002). A more rapid response to gluten withdrawal has also been noted in children, with the possibility of regaining tolerance to gluten. (Matysiak-Budnik, Malamut et al. 2007).

In this study, we have demonstrated a highly focused and specific anti-tTG response in paediatric CD, indicating that the core region of tTG is targeted from early on in disease progression.

3.4.2 Characterisation of wild-type and mutant tissue transglutaminases

Given that here is a possibility that the introduction of mutations in the core region of tTG could have affected protein folding, and hence conformation-dependant autoantibody binding, the similar reactivity of various control antibodies to tTG to the wild-type and mutant tTGs in ELISA assays is reassuring. A polyclonal rabbit antibody, raised against recombinant human tTG bound wild-type and mutant tTG proteins at equal levels (Figure 3.3c). Mouse monoclonal anti-tTG antibodies CUB 7402 (epitope:aa 447-478) and 5G7G6 (epitope:aa548-558) also displayed similar recognition of all proteins (Figure 3.3a/b). The equal recognition by CUB 7402 may be especially pertinent,
as the epitope recognized by this antibody is in the core region of tTG. The inclusion of an anti-GST antibody ensured that equal amounts of each recombinant tTG were being coated to each ELISA well, providing evidence for correct protein quantification.

3.4.3 The IgA anti-tissue transglutaminase response in coeliac disease

The IgA anti-tTG ELISA developed in this chapter displayed the excellent sensitivity (97%) and specificity (96%) for CD expected from such an assay (Feighery, Conlon et al. 2006). Mean IgA anti-tTG levels in both paediatric and adult CD groups reflected disease status, with the highest levels being found in untreated disease (Table 3.12). Binding of CD IgA anti-tTG to the mutant tTG proteins was almost completely abrogated, confirming the importance of the three active-site residues in a highly specific autoantibody response (Figure 3.5). Whilst there are several IgA autoantigens in CD, such as calreticulin (Sánchez, Tučková et al. 2000), collagen (Dieterich, Esslinger et al. 2006), and actin (Clemente 2000), IgA autoantibody responses are generally rare—such as that directed against Ro/SSA and La/SSB in SLE (Pourmand, Wahren-Herlenius et al. 1999), or topoisomerase I in systemic scleroderma (Hildebrandt, Weiner et al. 1990).

The limited epitope recognition by IgA anti-tTG may be due to the mucosal origin (and high-class switching and affinity maturation) ability of IgA antibodies, or may reflect a specific break in immunological tolerance to the core region of tTG. There is a possibility that some enzymes, by their very nature are predisposed to becoming targets of autoimmunity. Their active-site may normally be sequestered from the immune system during inactivity, or new intermediates formed during catalysis can induce autoimmunity via neo-epitope formation. Certainly, a strong argument can be made for both of these processes occurring with tTG. It is known that under inactive conditions, the active site of
tTG is buried deep within a narrow cleft between domains 3 and 4 of the protein (Griffin, Casadio et al. 2002) and that calcium activation of tTG causes a conformational change which exposes the active-site (Pinkas, Strop et al. 2007). tTG/gliadin complexes occur, with a thioester bond formed between gliadin peptides and Cys$^{277}$, and/or cross-linking of gliadin peptides to lysine residues on the tTG molecule by isopeptide bonds occurring (Fleckenstein, Qiao et al. 2004). This could potentially freeze the enzyme in its ‘open’ configuration, with exposure of the active-site, and possible neo-epitope formation. The gliadin-dependant nature of anti-tTG antibodies (Setty, Hormaza et al. 2008), and the recently described increased sensitivity of CD IgA anti-tTG detection when tTG is in its ‘open’ conformation supports this notion (Lindfors, Koskinen et al. 2011).

3.4.4 The IgG anti-tissue transglutaminase response in coeliac disease

Whilst the measurement of IgG anti-tTG antibodies is of use in the detection of CD in individuals with selective IgA deficiency (Korponay-Szabo 2003), their utilisation for mass CD screening is of little value due to the poor sensitivity of IgG anti-tTG ELISAs (Rostom, Dube et al. 2005). The sensitivities of IgG anti-tTG ELISAs in various publications show a great degree of variation. In 2003, Feighery et al. reported a sensitivity of 13% (Feighery, Collins et al. 2003), with a similar result of 16% being observed by Ankelo and colleagues in 2007 (Ankelo, Kleimola et al. 2007). Improved sensitivities of 47% (Baldas 2000), and 67.6% (Sblattero, Berti et al. 2000) have also been described. The inherent variability of IgG anti-tTG sensitivity in CD was shown in a single paper by Villalta et al. in 2005, where using multiple IgG anti-tTG ELISA kits, a range of sensitivity of between 17-93% was found (Villalta, Crovatto et al. 2005). In IgA-deficient patients, sensitivities of 91% (Lenhardt, Plebani et al. 2004), 99% (Korponay-Szabo 2003), and 100% (Cataldo, Lio et al. 20108).
2000) have been noted. Interestingly in the study using multiple ELISA kits by Villalta et al., the degree of variation of the sensitivity of IgG anti-tTG for CD was less pronounced in patients with selective IgA deficiency, at 75-95% (Villalta, Alessio et al. 2007). It is likely that the increased magnitude of the IgG anti-tTG response (Dahlbom, Olsson et al. 2005) in IgA deficient CD patients is responsible for the increased sensitivity of the ELISA in this CD sub-group. It may be that the method of measuring IgG anti-tTG could affect the autoantibody binding, as a radiobinding IgG anti-tTG assay has shown an excellent sensitivity of 99% (Bazzigaluppi, Roggero et al. 2005). The specificity of IgG anti-tTG antibodies, whilst high at levels such as 86% (Ankelo, Kleimola et al. 2007), or 96% (Sblattero, Berti et al. 2000), does not mirror the almost absolute specificity of IgA anti-tTG (Rostom, Dube et al. 2005) due to the occurrence of IgG anti-tTG in various autoimmune conditions (Bizzaro, Villalta et al. 2003).

The IgG anti-tTG ELISA developed in this chapter showed the characteristic poor sensitivity of this method, being 17% and 47% for paediatric and adult CD, respectively. In the paediatric CD study group, sensitivity in untreated CD was improved marginally to 30%, whilst in adults with CD, the sensitivity of the assay remained relatively constant between the spectra of differing treatment statuses (Table 3.15). The poor sensitivity of the IgG anti-tTG assay for paediatric CD in this study may partly be explained by the high deviation of binding between normal control subjects, which resulted in a high cut-off level for the assay (15.3 AU, compared to 5.3 AU in adult CD) (Table 3.10). The combined specificity of the IgG anti-tTG assay developed in this chapter for CD was 95%, being almost equal regardless of age.

There was a clear relationship of mean IgG anti-tTG levels with treatment status in paediatric CD, which was not seen in adult CD. Indeed the effect of age on IgG anti-tTG
levels in paediatric CD was striking, with the highest levels measured in untreated CD patients <4 years of age (Figure 3.7). In this age group, the sensitivity of the IgG anti-tTG ELISA was 50% (Table 3.17), representing a marked improvement of the 17% noted for the entire paediatric CD group. Increased IgG1 anti-tTG levels in younger children with CD have been previously described (Agardh, Borulf et al. 2003), and the presence of higher numbers of IgG-producing plasma cells in the intestinal mucosa may be associated with an immature barrier function (Brandtzaeg 2006). The increased levels of IgG anti-tTG antibodies in young children may reflect an evolving immune system that is capable of (by virtue of IgA anti-tTG positivity), but not yet completely proficient, at class-switching to IgA. The local cytokine milieu in paediatric patients, which may be driving autoantibody class-switching or the levels of differing autoantibody isotypes, may differ between that of the mature immune system, which has fully developed and long-established tolerogenic mechanisms (Brandtzaeg 2002).

IgG anti-tTG binding to the mutant tTG proteins was diminished in both adult and paediatric CD (Figure 3.8), indicating that the IgG anti-tTG response is specifically dependant on an intact tTG active site, similar to the IgA anti-tTG response. This would concur with reports by Sblattero et al, who have demonstrated a similar binding pattern for both IgA and IgG anti-tTG to truncated fragments of recombinant human tTG (Sblattero, Florian et al. 2002). Generally speaking, autoimmune responses directed against self-proteins, particularly enzymes, are of the IgG class and involve the recognition of multiple antigenic sites. For example, multiple IgG autoantibody epitopes have been described for proteinase 3 in Wegener’s granulomatosis (Williams, Staud et al. 1994) and for GAD65 in type 1 diabetes (Fenalti and Rowley 2008). The dominance of one particular antigenic region on the tTG molecule for IgG autoantibodies in CD
represents an unusual specificity, however this must be viewed in the context of the potential mechanisms in which tTG becomes antigenic in CD, which taken together represent a unique phenomenon. The first important factor in this process is the location of events at an area of the body evolved to produce highly specific, neutralising antibodies to constant stimuli (Suzuki, Ha et al. 2007). The interaction of gliadin with tTG is also crucial; the deamidation process, which in turn stimulates an immune response contributing to the general local pro-inflammatory environment, occurs at the tTG active-site- allowing for possible antibody epitope-spreading. The possible effects of gliadin-tTG complexes on neo-epitope formation have been discussed earlier in this chapter.

The use of the tTG open/closed conformation ELISAs, as described by Lindfors et al. (Lindfors, Koskinen et al. 2011) to measure IgG anti-tTG antibodies in CD would prove useful as to a further dissection of this autoantibody response.

3.4.5 Conclusions

To conclude, the work described in this chapter provides a detailed dissection of the epitope specificity of CD anti-tTG responses, through the measurement of CD sera reactivity to novel tTG mutant proteins. We have demonstrated the presence of a highly conformational epitope in the core region of tTG that is specifically targeted by coeliac disease autoantibodies from early in disease course. Even disruption of a single amino acid of the tTG active-site abolished recognition by CD autoantibodies. The results of this chapter also identify the IgG anti-tTG response in CD as sharing this highly limited epitope specificity.
It is hypothesised that the anti-tTG response in CD occurs as a result of intramolecular epitope spreading from gliadin to tTG, possibly as a result of the formation of gliadin/tTG complexes at the active-site of tTG. In this scenario, the resulting immune response directed to a tTG epitope in the core region, involving the active-site seems logical, due to the potential of new epitope formation, and the fact that the active-site is normally hidden from the immune system.

The pathological role of anti-tTG antibodies in CD remains controversial. Conflicting reports exist as to the degree, if any, of inhibition of the enzymatic function of tTG by autoantibodies, and of the biological relevance of demonstrable inhibition (Dieterich, Trapp et al. 2003) (Kiraly, Vecsei et al. 2006) (Esposito, Paparo et al. 2002). In this study, we have confirmed the targeting of the tTG core region by CD autoantibodies, and one would expect some interference with enzymatic function, if only prevention of substrate access to the active site.

Apart from enzymatic inhibition, anti-tTG antibodies can potentially contribute to the general pro-inflammatory environment in the small intestine, either via circulating antibody/antigen complexes, or through autoantibody deposits in the small intestinal mucosa. CD autoantibodies have also been shown to activate monocytes via TLR4, providing a link between the innate and adaptive immune systems (Zanoni, Navone et al. 2006). The finding of IgA anti-tTG deposits in the liver, kidney, lymph node and muscle of CD patients has also been described (Caputo, Barone et al. 2009), and may be responsible for secondary organ-specific immunity in CD. These anti-tTG antibodies, directed against local tTG (ironically in a phenomenon mimicking the principle of the EMA test) represent a potential therapeutic target; an understanding of the tTG/anti-tTG interaction is key to any such development.
Chapter 4

Analysis of IgG autoantibody responses to tissue transglutaminase
4.1 Introduction

4.1.1 Autoantibody responses to tissue transglutaminase in non-coeliac disorders

A humoral immune response to tTG may also occur in non-coeliac individuals, with anti-tTG positivity being described in numerous autoimmune disorders (Kim, Jeitner et al. 2002), during viral infection such as HIV (Pereda, Bartolome-Pacheco et al. 2001), and in end-stage heart failure (Peracchi, Trovato et al. 2002). Autoantibodies directed against tTG in non-CD individuals are generally of the IgG class; however individuals positive for IgA anti-tTG but negative for IgA EMA have been described (Sárđy, Csikós et al. 2007). The source of the tTG antigen used is of importance- guinea-pig tTG is prone to giving false-positive results due to contaminating non-tTG proteins (Clemente, Musu et al. 2002).

Type 1 diabetes mellitus

The increased prevalence of CD in individuals with T1DM has been well established and is likely attributable to shared risk factors in the HLA DR3-DQ2 and DR4-DQ8 phenotypes (Barker and Liu 2008). IgA EMA positivity, and hence the likely presence of CD, has been estimated at 5% in patients with T1DM (Cronin and Shanahan 1997). In 1999, using a radiobinding anti-tTG assay, Lampasona and co-workers identified a sub-set of T1DM patients in which IgG, but not IgA, anti-tTG to a recombinant human tTG antigen could be demonstrated (Lampasona, Bonfanti et al. 1999). Anti-gliadin antibodies were rarely found in this sub-group of individuals, which strikingly, represented 34% (97/287) of the T1DM study population (Lampasona, Belloni et al. 2008).
**Crohn's disease**

There are conflicting reports with regard to the occurrence of anti-tTG in Crohn's disease. Using a guinea-pig tTG ELISA, Sjoberg observed increased levels of IgG (but not IgA) anti-tTG in sufferers of Crohn's disease, compared to healthy plasma donors and EMA-negative CD patients (Sjoberg, Eriksson et al. 2002). Using a recombinant human tTG based ELISA, two studies have identified increased levels of IgA anti-tTG in Crohn's disease; however, in both, IgG anti-tTG was not measured (Farrace, Picarelli et al. 2001; Sárdy, Csikós et al. 2007).

Screening multiple autoimmune disorders for tTG antibodies, Bizzaro and colleagues could not demonstrate IgA or IgG anti-tTG positivity in Crohn's disease patients (Bizzaro, Villalta et al. 2003), while IgA but not IgG anti-tTG positivity was observed by Ribeiro-Cabral (Ribeiro-Cabral, da-Silva-Patricio et al. 2011), both studies using recombinant human antigen.

**Rheumatoid arthritis**

While CD and Rheumatoid arthritis may share a pattern of genetic susceptibilities in common (Eyre, Hinks et al. 2010; Zhernakova, Stahl et al. 2011), a number of studies have demonstrated anti-tTG positivity in RA, independent of CD. Using recombinant human antigen in an ELISA system, Picarelli et al. detected IgA anti-tTG positivity in 42% of RA patients tested, all of whom were IgA EMA negative (Picarelli, Di Tola et al. 2003). In 2006, IgA and IgG anti-rh tTG positivity was found in 20% and 19%, respectively of an RA cohort by Roth (Roth, Stenberg et al. 2006). IgA, IgG, and IgM anti-tTG positivity to guinea-pig tTG has also been noted (Feighery, Collins et al. 2003), with all but one RA patient being IgA EMA negative. Interestingly, IgG anti-tTG levels in RA have been shown
to increase upon treatment with TNF-α blocking agents, the significance of which is unclear (Atzeni, Doria et al. 2008).

Autoimmune liver disease

False-positive IgA anti-tTG results in patients with liver disease were common in the guinea-pig tTG ELISA system, likely due to cross-reactivity with contaminating guinea-pig liver proteins in the antigen preparation (Clemente, Musu et al. 2002). Assays using recombinant human tTG antigen have detected IgA and IgG anti-tTG positivity in 8% of a group of 48 patients with primary biliary cirrhosis (PBC) (Bizzaro, Villalta et al. 2003). It may be that anti-tTG positivity is a reflection of non-specific hepatic damage, as levels of IgA anti-tTG have been shown to correlate with that of pro-inflammatory cytokines and markers of fibrogenesis in those with alcoholic liver disease (Koivisto, Hietala et al. 2007).

Other autoimmune conditions

Additionally, anti-tTG positivity to the recombinant human preparation has been described in systemic lupus erythematosus (both IgA and IgG isotypes) (Villalta, Bizzaro et al. 2002; Bizzaro, Villalta et al. 2003; Marai, Shoenfeld et al. 2004), Wegener’s granulomatosis (IgA) (Sárday, Csikós et al. 2007), ankylosing spondylitis (IgA and IgG) and psoriatic arthritis (IgA and IgG) (Riente, Chimenti et al. 2004).

Infection

There are also some reports of anti-tTG positivity during viral infection in non-CD individuals. Ferrara (2011) has described anti-tTG positivity in 8/222 non-coeliac children suffering from acute infectious diseases- one patient for IgA and IgG, one patient for IgA only, and six patients for IgG only; all of whom were negative for IgA EMA (Ferrara,
Quaglia et al. 2010). IgG anti-tTG positivity in a quarter of seropositive HIV-infected
individuals, which resolved upon commencement of highly active anti-retroviral therapy
was reported in 2001 (Pereda, Bartolome-Pacheco et al. 2001). HIV patients with villous
atrophy and positive IgA anti-tTG, but lacking IgA EMA and CD-associated HLA molecules
have also been identified (Kurien, Chalkiadakis et al. 2012).

Characterisation of the IgG anti-tTG response

Although IgG anti-tTG antibodies have been extensively demonstrated in differing
disease states, little is known about their epitope specificity and IgG subclass usage, in
comparison with CD, and in individual diseases. Given the importance of the tTG catalytic
triad in CD IgG anti-tTG binding observed in Chapter 3, which may be a possible
consequence of tTG/gliadin interaction in the development of CD, an investigation into
the epitope specificity of non-CD may inform as to the underlying mechanisms involved
in the generation of anti-tTG antibodies. Whilst the origin of the anti-tTG response in CD
may be intimately linked with gliadin, through the formation of neo-epitopes or epitope
spreading, it has been proposed that non-CD tTG autoimmunity may arise due to an
apoptotic defect resulting in inappropriate release of tTG from dying cells (van der Sluijs
Veer and Vermes 2001).

Differing IgG subclass usage in the response to autoantigens is frequently observed in
autoimmune disease, and may be dominated by one subclass or be a mixture of two or
more (Xie, Gao et al. 2008). Differing rank orders of IgG subclass-specific responses to
autoantigens occur within disease groups, with subtype usage profiles being linked to
the mode of clinical presentation in SLE (Shoenfeld, Gershwin et al. 2007). IgG anti-tTG in
CD has been shown to be predominantly of the IgG1 subclass. In 2000, Cataldo and
colleagues detected IgG1 EMA in 20/20 CD patients with selective IgA deficiency
(Cataldo, Lio et al. 2000); however, IgG1 anti-tTG measurements in IgA-sufficient CD patients do not display this absolute sensitivity, with two different studies generating values of 51% (Agardh, Borulf et al. 2003) and 57% (Schilling, Sprekerkoetter et al. 2005), respectively. Apart from the study by Schilling et al., who measured IgG1 and IgG4 (detecting IgG4 anti-tTG in 1/20 patients with silent CD), an exploration of IgG anti-tTG subclasses other than IgG1 in CD has not been attempted, and nothing is known of the non-CD IgG anti-tTG subclass distribution. In CD, the IgG anti-gliadin response has also been shown to be dominated by IgG1, and to a lesser extent IgG3 (Hvatum, Scott et al. 1992), but positivity for all four subtypes has been noted (Engstrom, Sundin et al. 1992; Saalman, Dahlgren et al. 2001).

4.1.2 Immunoglobulin purification

The purification of immunoglobulins—historically a staple of immunological research, has gained increasing relevance due to the advent of hybridoma technology and the use of antibodies as biological therapeutic agents. Traditionally, antibodies are purified by methods of precipitation or affinity chromatography, with the latter frequently utilising immunoglobulin-binding proteins extracted from bacteria, such as protein A/G (Josic 2001).

Jacalin is a 40 KDa α-D-galactose binding lectin extracted from jack-fruit (Artocarpus integrifolia) seeds (Ske, Christopoulous et al. 1988), which binds to antibodies of the IgA1 subclass (Loomes, Stewart et al. 1991). Jacalin displays highly specific binding to the α-D-glycoside of the disaccharide Thomsen-Friedenreich antigen (Galβ1-3GalNAc) (Ma, Yoshida et al. 2009), making it an ideal ligand for the purification of O-linked glycoproteins such as human IgA1. Immobilised jacalin can be used to purify human monomeric and dimeric IgA1, and serum, colostrum, and secretory IgA by column-based
affinity chromatography (Kondoh, Kobayashi et al. 1987). In a competitive ELISA system, addition of GalNAc and Gal inhibit the jacalin-IgA1 interaction (Aucouturier, Mihaesco et al. 1987), the specificity of which is determined by carbohydrate moieties in the hinge region of the IgA1 molecule that are absent in the non-jacalin binding IgA2 subtype (Kabir 1998). Elution of jacalin-bound IgA1 may be achieved through the use of α-D-galactose or melibiose containing solutions (Aucouturier, Pineau et al. 1989). Once the total immunoglobulin isotype fraction has been purified, isotype-specific autoantibodies of interest may be isolated by further rounds of affinity chromatography by passing the total immunoglobulin fraction through a column coated with the antigen, and eluting the bound antibody under acidic conditions.

In order to affinity purify CD IgA anti-tTG for use as a further epitope mapping tool, column-based chromatography was used, using the recombinant wild-type tTG protein generated in Chapter 2 as a source of antigen. Affi-Gel® activated affinity media which couples to ligands containing primary amino groups by the formation of a stable amide bond, was used to immobilise the wild-type tTG.

4.1.3 Epitope masking ELISA as a tool for epitope mapping

Surface epitope masking (SEM) is an immunological technique extensively used in the generation of monoclonal antibodies. Briefly, the procedure involves silencing, or hiding, known epitopes from the immune system (either on a protein molecule, or cell surface) by pre-blocking them with specific antibodies, leaving the epitope of interest accessible (Su, Lin et al. 1996). The ‘blocked’ protein or cell may then be either used to immunize an animal and generate antibodies to the unblocked epitope only, or in an epitope mapping ELISA system to eliminate reactivity to the blocked epitopes.
In order to investigate differences in epitopes involved in IgG anti-tTG binding between CD and non-CD IgG positive individuals, a modified surface epitope mapping (SEM) ELISA technique was developed in this chapter. This system utilises the observation made in Chapter 3 that IgA and IgG anti-tTG antibodies in CD appear to target the same epitope in the core region of tTG. Given this observation, one could postulate that if the IgA anti-tTG epitope is masked, CD IgG anti-tTG antibodies will be prevented from binding due to the inaccessibility of this shared epitope (Fig 4.1). Using this system, differences in IgG anti-tTG epitope specificity between CD and non-CD individuals may be identified by their binding pattern to the masked tTG protein. Whilst this does not generate information as to the location of any additional non-CD IgG anti-tTG epitopes, it does permit the exclusion of the active site/core region.
Step 1: wttTG antigen coated to well

Step 2: Affinity-purified CD IgA anti-tTG is added to well, and will bind to, and occupy, the active-site of tTG. Well is incubated for one hour and washed.

Step 3: Patient sera containing IgG anti-tTG is added to well. If the antigenic targets of the IgG anti-tTG response are similar to that of IgA, binding will be reduced or abrogated due to occupation of these sites by pre-incubation with affinity-purified CD IgA. Well is incubated for one hour and washed.

Step 4: Addition of HRP-labelled anti-human IgG secondary antibody. Signal developed, and effect of pre-incubation with CD anti-tTG IgA on binding of IgG anti-tTG assessed.

Figure 4.1: The IgA anti-tTG epitope masking ELISA. A modified version of the IgG anti-tTG ELISA developed in Chapter 3 was used to further investigate IgG anti-tTG epitopes in CD, and in non-CD IgG anti-tTG positive individuals. The binding of IgG anti-tTG to epitopes recognised by IgA anti-tTG may be assessed, with shared epitopes potentially identified.
The aims of this chapter were to compare IgG anti-tTG antibodies from individuals with and without coeliac disease. Groups of patients with varying autoimmune diseases were screened for IgG anti-tTG in order to generate a cohort of non-CD individuals positive for these antibodies. Epitope analysis was performed by the establishment of an epitope masking ELISA utilising affinity-purified CD IgA anti-tTG, and by comparing binding of non-CD IgG anti-tTG to the wild-type and triple mutant tTGs synthesised in Chapter 2. In order to compare the IgG subclass profile of anti-tTG, ELISAs measuring all four IgG subclass antibodies to tTG were also established.
4.2 Materials and methods

4.2.1 Purification of IgA from human serum

Immobilised jacalin agarose (Pierce) was used to purify IgA from pooled CD (n=13), and control (n=5) serum, as per the manufacturer’s instructions. All CD patients were IgA EMA/tTG positive (levels >75, as per Celikey™ assay), with biopsy-proven disease, the controls used were all IgA anti-tTG negative healthy laboratory workers. Following equilibration to room temperature, 5mls of the 50% gel slurry was added to a 25ml chromatography column (Pierce) and washed with 5 column volumes of PBS. The pooled human serum was mixed 1:1 with PBS to a final volume of 10mls and added to the column, which was then washed with PBS to remove any unbound protein. Washing was deemed complete when monitoring of the flow-through at an absorbance of 280nm using a nanodrop™ spectrophotometer revealed a return to baseline levels. A solution of 0.1M melibiose was used to elute the bound IgA, with 8x 2ml fractions collected, which were tested for $A_{280}$ using the nanodrop spectrophotometer. Samples with the highest $A_{280}$ values were pooled, buffer-exchanged into PBS using Zeba™ desalt columns (Pierce) in order to remove any melibiose from the elution buffer, re-quantified, and visualised using SDS-PAGE. Different columns were used to purify CD and control IgA, in order to prevent the contamination of the control IgA with anti-tTG antibodies.

4.2.2 Affinity-purification of IgA anti-tTG from coeliac disease serum

CD IgA anti-tTG antibodies were affinity purified from the total CD IgA1 isolated in section 4.2.1 using tTG-bound Affi-Gel affinity medium (Bio-Rad). As the estimated pl of the recombinant GST-tagged tTG is 5.135 (EMBL WWW Gateway to Isoelectric Point Service, [http://www3.embl.de/cgi/pi-wraper.pl](http://www3.embl.de/cgi/pi-wraper.pl)) Affi-Gel 15 was used, as it binds
proteins with a pI <6.5. As tris-based buffers, such as those used to purify the recombinant wild-type tTG in Chapter 2 interfere with ligand coupling to Affi-gel, a protein desalting step was performed prior to coupling the wild-type tTG. Two milligrams of tTG was desalted and buffer-exchanged into 100mM hepes buffer (pH 7.2) using Zeba™ desalt columns (Pierce) to a final volume of 5mls. Two millilitres of the Affi-gel 15 was then washed five times with ice-cold dH₂O and incubated with the desalted tTG solution overnight at 4°C, with mixing. The post-coupling flow-through was retained in order to calculate coupling efficiency, and the column was washed with PBS until the baseline A_{280} value was reached. Total CD I(A1 was mixed 1:1 with PBS, added to the column, and incubated for 4 hours at 4°C, with mixing. The column was again washed with PBS until the baseline A_{280} value was reached, and the bound anti-tTG IgA was eluted using 200mM glycine-HCl at pH 2.5. The IgA anti-tTG was collected in 2ml fractions into a tube containing 50μl neutralisation buffer (1M Tris-HCl, pH 8) and buffer exchanged into PBS. Eluates were then quantified using the nanodrop, and stored at -70°C until required.

4.2.3 Western blot of coeliac disease IgA anti-tTG

In order to confirm the identity of the eluted protein, a western blot of the affinity-purified CD IgA anti-tTG was performed, in a modification of the procedure described in section 2.2.12. Non-denaturing SDS-PAGE was performed, with HRP-conjugated rabbit anti-human IgA (Dako) diluted 1:1000 used to directly visualise the blotted anti-tTG.
4.2.4 Screening of non-coeliac patients for IgG anti-tTG autoantibodies

Groups of patients with T1DM (n=25), Crohn's disease (n=53), and Wegener's granulomatosis (n=37) were screened for IgG anti-tTG positivity using the ELISA developed in Chapter 3. The T1DM group had a mean age of 40.6 years (range 3-66), and had a F:M ratio of 1.2:1. The WG patients had a mean age of 59.2 years (range 27-77), and had a F:M ratio of 1.25:1. No demographic information was available for the Crohn's disease sera, which had been collected for use in a previous study.

4.2.5 Optimisation of the IgA anti-tTG epitope masking ELISA

The effect of the addition of increasing amounts of affinity-purified CD IgA to the ELISA well prior to the addition of the IgG anti-tTG containing patient sample was investigated using 0.00025, 0.0005, 0.001, and 0.002 µg of affinity-purified autoantibody, which corresponded to dilutions of 1:100, 1:50, 1:20, and 1:10 of the stock IgA anti-tTG solution, respectively. The epitope-masking step was performed prior to the addition of patient serum by the addition of the required amount of CD IgA anti-tTG in 100µl PBS-Tween, incubation for an hour at room temperature, and subsequent washing. The IgG anti-tTG ELISA was then performed as per section 3.2.7, with AU results generated compared to that of the 'neat' values.

4.2.6 Final protocol for IgA anti-tTG epitope masking ELISA

The IgA anti-tTG epitope masking ELISA followed the same procedure as the IgG anti-tTG ELISA (Section 3.2.7), with the exception of an additional blocking step using affinity-purified CD IgA anti-tTG to silence epitopes on the tTG molecule. Briefly, 0.3µg wild-type tTG was coated to certified 96-well Maxisorp plates (Nunc) overnight at 4°C, followed by blocking with 5% casein in PBS. The CD IgA anti-tTG epitopes were then masked by
addition of 0.001 μg of affinity-purified CD IgA anti-tTG, and incubated for an hour at room temperature. Unbound IgA was then washed away, patient sera was added (containing IgG anti-tTG), and signal subsequently detected using HRP-conjugated rabbit anti-human IgG (Dako) as per section 3.2.7. Four washes with PBS containing 0.01% Tween were performed between each step. IgG anti-tTG values to the ‘epitope-masked’ wild-type tTG protein were compared to that directed against the native wild-type tTG, which were measured concurrently. Figure 4.1 gives an overview of the epitope masking ELISA system. As a control, experiments were performed in which the affinity-purified CD IgA anti-tTG was replaced with 1μg of control IgA, CUB 7402 (1:1000), or polyclonal anti-tTG (1:1000).

4.2.7 IgG anti-tTG binding to CHD ΔtTG

IgG autoantibodies to the triple active-site mutant CHDΔ tTG generated in Chapter 2 were measured in patients who were shown to be positive for IgG anti-tTG. The protocol followed that described in Section 3.2.7, with a comparison in binding to that of the wild-type tTG protein being made.

4.2.8 IgG anti-tTG subclass ELISA optimisation

In order to provide discriminatory values between positive and negative results, the dilution of patient serum and secondary antibody concentration was optimised for each IgG subclass in a series of checkerboard experiments. As the IgG anti-tTG ELISA developed in Chapter 3 employed a serum dilution of 1:1000, initial optimisation experiments measured the IgG anti-tTG subclasses in IgG anti-tTG positive patient sera at dilutions of 1:100, and 1:500. In these initial optimisation experiments, secondary antibody dilutions of 1:100, 1:500 and 1:1000 were investigated. Following these
experiments, which determined primary antibody dilution, groups of IgG positive CD patients, and normal control individuals were investigated as to the optimal secondary antibody concentration required to distinguish between both groups. Conditions such as incubation time, washing steps, and coating concentration used (0.3μg wild-type tTG per well) did not differ from the anti-tTG assays described in Chapter 3.

4.2.9 IgG anti-tTG subclass ELISA

The ELISA for each IgG anti-tTG subclass followed a similar procedure, with the exception of some differences in the secondary antibody dilution and development times. Certified 96-well Maxisorp plates (Nunc) were coated with 0.3μg recombinant protein in coating buffer (50mM Tris, 150mM NaCl, 5mM CaCl₂, pH 7.5) overnight at 4°C. Wells were blocked with 5% casein in PBS for 1 hour, and washed four times with PBS containing 0.01% Tween between each step. For each subclass specific assay, human serum was diluted 1:100 in PBS plus 0.01% Tween and incubated for one hour at room temperature. In the IgG1 anti-tTG assay, HRP-conjugated rabbit anti-human IgG1 (Cygnus Technologies) was diluted 1:250. HRP-conjugated rabbit anti-human IgG2, IgG3, and IgG4 (Cygnus Technologies) were diluted 1:100. All secondary antibody incubations were performed at room temperature for one hour. Assays were developed by the addition of TMB liquid substrate solution (Sigma-Genosys), stopped by addition of 2M H₂SO₄, and read at OD₄₅₀ in a Biotek ELx800 spectrophotometer. The development time for the IgG1 and IgG2 assays was 5 minutes, with that of the IgG3 and IgG4 assays being ten minutes.
4.3 Results

4.3.1 Purification of IgA from human serum

In separate experiments, total IgA1 was successfully purified from both pooled CD, and control sera using immobilised jacalin agarose (Pierce). Typically, the majority of the IgA was eluted in the second and third fractions recovered (Fig 4.2). A \textsubscript{280} analysis of the column washes indicated that the majority of serum protein had been washed away prior to elution, which was important to ensure a high purity of the IgA fractions.

![Figure 4.2: Purification of human IgA using jacalin agarose.](image)

After the pooling of the two most concentrated fractions and buffer exchange, the yield of CD IgA1 was 1.77mg/ml of serum applied to the column, and for controls was 1.04mg/ml of serum applied to the column. This is lower than the initial values generated for each fraction immediately post-elution, but may reflect contaminating melibiose in the initial reading, and the inevitable loss of some protein during desalting. Upon SDS-PAGE analysis, the heavy (55 KDa) and light (25 KDa) chains of immunoglobulin were clearly identifiable (Fig 4.3). The IgA anti-tTG levels in the total IgA fractions, using
the ELISA developed in Chapter 3 were 112.5 AU for the CD IgA, and 2.6 AU for the control IgA.

Figure 4.3: SDS-PAGE of CD and control total IgA. Two μg of jacalin-purified CD (left), or control (right) IgA were visualised on a 12% gel.

4.3.2 Affinity-purification of coeliac disease anti-tTG

The recombinant wild-type tTG synthesised in Chapter 2, attached to Affi-Gel 15 activated immunoaffinity media was used to affinity-purify IgA anti-tTG antibodies from the total IgA1 fraction of CD patient sera. The recombinant tTG displayed a coupling efficiency of 0.84mg tTG per ml of gel, which was approaching the maximum coupling efficiency of 1mg/ml of gel stated in the Affi-Gel product information sheet. CD IgA anti-tTG was collected into 2ml fractions, which were immediately neutralised and buffer-exchanged into PBS. The majority of bound antibody was eluted in the first two fractions, as measured by the nanodrop spectrophotometer (Table 4.1). Fractions 2.1 and 2.2 had the highest concentration of protein, and were chosen for anti-tTG analysis by ELISA. The yield of CD IgA anti-tTG was calculated as 45μg per mg of total IgA added to the column.
Table 4.1: Concentration of eluted CD IgA anti-tTG fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Concentration (µg/ml)</th>
<th>Fraction</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>0.02</td>
<td>2.1</td>
<td>0.10</td>
</tr>
<tr>
<td>1.2</td>
<td>0</td>
<td>2.2</td>
<td>0.05</td>
</tr>
<tr>
<td>1.3</td>
<td>0</td>
<td>2.3</td>
<td>0</td>
</tr>
<tr>
<td>1.4</td>
<td>0</td>
<td>2.4</td>
<td>0</td>
</tr>
</tbody>
</table>

Protein concentration was determined using the Nanodrop™ spectrophotometer using $A_{280}/\text{IgA}$ absorbance coefficient.

On analysis by ELISA, both CD IgA anti-tTG fractions displayed high levels of anti-tTG, which were proportional to the amount of protein added (Fig 4.4). The levels of anti-tTG at the dilution of 1:100 (which is used in the IgA anti-tTG assay developed in Chapter 2) are comparable with levels at or over the upper limit of the assay often seen in untreated CD patients. The IgA anti-tTG fractions were then pooled, and concentrated to 0.2µg/ml, as measured by the nanodrop, using a centrifugal concentrator column (Pierce).

Figure 4.4: IgA anti-tTG ELISA reactivity of affinity-purified IgA anti-tTG fractions. Both fractions display strong anti-tTG reactivity in a concentration-dependant manner. The amount of anti-tTG present shows strong correlation with the amount of protein added, as indicated by the correlation coefficient (r) values.
4.3.3 Western blot of coeliac disease IgA anti-tTG

Due to the low protein concentrations of the affinity-purified CD IgA anti-tTG, a modified Western blot was performed in order to visualise and confirm the identity of the affinity purification product. In order to preserve epitopes on the antibody molecule non-denaturing SDS-PAGE was used; probing with polyclonal rabbit anti-human IgA resulted in the detection of a band at ~160 KDa corresponding to that of human IgA (Fig 4.5).

![Western blot of affinity-purified CD IgA anti-tTG](image)

**Figure 4.5: Western blot of affinity-purified CD IgA anti-tTG.** 0.5µg of two differing aliquots of CD IgA anti-tTG (1, and 2) were visualised using non-denaturing SDS-PAGE and Western blot. HRP-labelled polyclonal rabbit anti-human IgA was used as used to detect the blotted antibody.

4.3.4 Screening of non-coeliac patients for IgG anti-tTG autoantibodies

The screening of non-CD patients for IgG anti-tTG antibodies detected positivity in 28% of patients with T1DM, 19% of patients with Crohn's disease, and 14% of patients with Wegener's granulomatosis (Table 4.2). The magnitude of IgG anti-tTG responsiveness in the autoimmune disease groups did not reach that seen in paediatric CD, but levels in positive individuals were comparable to that found in adult CD (Fig Fig 4.6).
Figure 4.6: Levels of IgG anti-tTG in autoimmune disease. IgG anti-tTG can be detected in patients with autoimmune disease. Levels in CD are included for comparison. The grey bar represents the mean IgG anti-tTG level in each disease group, with the stars representing the level of significance as calculated by the Mann-Whitney test. The miscellaneous autoimmune disease group consisted of 2 rheumatoid arthritis and 2 autoimmune liver disease patients. (AID=autoimmune disease).

Although positivity was generally in the lower range, levels of IgG anti-tTG were significantly increased when compared to control individuals (p=0.0004, 0.0079, and 0.0483 for T1DM, Crohn’s disease, and Wegener’s granulomatosis respectively, Mann-Whitney test) (Fig 4.6). Levels of IgG anti-tTG in the miscellaneous autoimmune group (two patients with rheumatoid arthritis, and two with autoimmune liver disease) were also increased compared to controls (p=0.0025, Mann-Whitney test) (Fig 4.6). Using both the ELISA established in Chapter 3, and the Celikey™ assay, IgA anti-tTG positivity was not observed in any of the IgG anti-tTG positive individuals with autoimmune disease.
with the exception of the Crohn’s disease patients, where low-level reactivity was detected in the ELISA established in Chapter 3.

**Table 4.2: IgG anti-tTG positivity in autoimmune disease.**

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>% IgG anti-tTG positive (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 Diabetes (n=25)</td>
<td>28 (9)</td>
</tr>
<tr>
<td>Crohn’s Disease (n=53)</td>
<td>19 (10)</td>
</tr>
<tr>
<td>Wegener’s Granulomatosis (n=37)</td>
<td>14 (5)</td>
</tr>
<tr>
<td>Adult controls (n=30)</td>
<td>7 (2)</td>
</tr>
</tbody>
</table>

4.3.5 Optimisation of the IgA anti-tTG epitope masking ELISA

The IgA anti-tTG epitope masking ELISA was optimised by investigating the effect of differing amounts of affinity-purified CD IgA anti-tTG on the binding of IgG anti-tTG in three IgG anti-tTG positive paediatric CD patients. From these experiments, an inverse relationship between the amount of IgA anti-tTG added and the amount of IgG anti-tTG detected was observed, with the addition of 0.002μg IgA anti-tTG per ELISA well resulting in the strongest inhibition of IgG anti-tTG binding (Fig 4.7). As a similar amount of inhibition was achieved with 0.001μg of the CD IgA anti-tTG, in order to preserve stock it was decided to pursue further experiments using this amount of autoantibody as a blocker.

![Figure 4.7: Optimisation of the IgA anti-tTG epitope masking ELISA.](image)

Figure 4.7: Optimisation of the IgA anti-tTG epitope masking ELISA. Serum from three IgG anti-tTG positive CD patients was added to ELISA wells pre-incubated with increasing amounts of affinity-purified CD IgA anti-tTG. The neat IgG anti-tTG value for each patient is indicated.
4.3.6 IgA anti-tTG epitope masking ELISA

In epitope masking experiments performed on IgG anti-tTG positive individuals, the addition of affinity-purified CD IgA caused a significant decrease in IgG anti-tTG binding in the paediatric and adult CD patient groups only (p<0.001 for both children and adults with CD, Wilcoxon signed ranks test) (Figure 4.8a/b). Masking the CD IgA anti-tTG epitope caused a mean reduction in IgG anti-tTG binding of 66% in paediatric CD, and 33% in adult CD.

![Figure 4.8: Epitope masking ELISA results in IgG anti-tTG positive CD patients.](image)

In contrast to this, the blocking of the CD IgA anti-tTG epitope did not dramatically reduce IgG anti-tTG binding in patients suffering from non-CD pathologies (Fig 4.9). In the T1DM patients, blocking caused an increase in IgG anti-tTG binding of 12%, compared to an increase of 17% in the WG/miscellaneous group (Fig 4.9b/c). There was a slight decrease in IgG anti-tTG binding post-blocking of 10% in the Crohn's disease patients, which did not reach statistical significance (Fig 4.9a).
Figure 4.9: Epitope masking ELISA results in IgG anti-tTG positive autoimmune disease patients. The pre-incubation with CD IgA anti-tTG did not affect IgG anti-tTG binding in individuals with Crohn’s disease (a), T1DM (b), and the miscellaneous group (c), indicating a difference in epitope specificity to that of CD. Significance was calculated using the Wilcoxon signed ranks test.

As a control for the epitope masking experiments, the affinity-purified CD IgA anti-tTG was replaced with control IgA, CUB 7402, or polyclonal anti-tTG. None of these antibodies replicated the reduction in CD IgG anti-tTG binding obtained by blocking with CD IgA anti-tTG in paediatric CD (4.10a/d/g), adult CD (Fig 4.10 b/e/h), or autoimmune disease (Fig 4.10c/f/i).
Figure 4.10: Epitope masking ELISA results using control antibodies. The pre-incubation of the tTG ELISA well with various control antibodies did not affect IgG anti-tTG binding in all of the patient groups studied. The results for control IgA (1 µg) in (a) paediatric CD, (b) adult CD, and (c) non-CD IgG anti-tTG positive individuals; CUB 7402 (1:1,000) (d) paediatric CD, (e) adult CD, and (f) non-CD IgG anti-tTG positive individuals; and polyclonal anti-tTG (1:1,000) (g) paediatric CD, (h) adult CD, and (g), non-CD IgG anti-tTG positive individuals are detailed. Statistics were calculated using the Wilcoxon signed ranks test.

4.3.7 IgG anti-tTG binding to CHD ΔtTG

The ability of IgG anti-tTG from non-coeliac individuals to bind to the mutant tTG generated in Chapter 2 which lacks the entire catalytic triad residues (CHD ΔtTG) was assessed by ELISA and compared with binding to the wild-type protein. In order to provide a comparison, 40 IgG anti-tTG positive CD patients (20 paediatric, 20 adult) were also tested against the wild-type and mutant tTG. The characteristic reduction in binding to CHD ΔtTG observed in the CD patients was not replicated in patients with Crohn’s disease, type 1 diabetes mellitus, Wegener’s granulomatosis, rheumatoid arthritis, and
autoimmune liver disease, with these individuals showing equal IgG anti-tTG responsiveness to the wild-type and mutant tTGs (Figure 4.11). A significant difference in IgG recognition of the wild-type and CHD ΔtTG was noted in CD (p<0.001 paediatric CD, p= 0.006 adult CD, Wilcoxon signed ranks test); no such difference was observed in the non-CD IgG anti-tTG positive disease groups.

Figure 4.11: Binding to wt and CHDΔ tTG in CD, and non-CD autoimmune disease. In CD, IgG anti-tTG binding to CHDΔ tTG was reduced in children (a) and adults (b), whilst for individuals with Crohn’s disease (a), T1DM (b), and WG/RA/PBC (c) removal of these amino acids did not affect IgG anti-tTG binding.

4.3.8 IgG anti-tTG subclass ELISA optimisation

As there was no standard available for the different IgG anti-tTG subclasses, the results of each assay were reported as raw OD values, and not in arbitrary units such as the assays developed in Chapter 3. ELISAs measuring IgG subclass-specific anti-tTG autoantibodies were established using a two-step optimisation procedure. In the first step, serum from three CD patients of known IgG anti-tTG CD positivity was used in
checkerboard experiments in order to gauge the range of dilutions needed for the generation of a positive signal (Table 4.3).

Table 4.3: IgG anti-tTG subclass ELISA optimisation 1.

<table>
<thead>
<tr>
<th>IgG1</th>
<th>Secondary Antibody Dilution</th>
<th>IgG2</th>
<th>Secondary Antibody Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary 1:100</td>
<td>1:500</td>
<td>1:1000</td>
</tr>
<tr>
<td>Antibody Dilution</td>
<td>+ve 1</td>
<td>+ve 1</td>
<td></td>
</tr>
<tr>
<td>1:100</td>
<td>1.063</td>
<td>0.411</td>
<td>0.275</td>
</tr>
<tr>
<td>1:500</td>
<td>0.791</td>
<td>0.321</td>
<td>0.066</td>
</tr>
<tr>
<td>+ve 2</td>
<td>+ve 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100</td>
<td>1.366</td>
<td>0.912</td>
<td>0.641</td>
</tr>
<tr>
<td>1:500</td>
<td>0.866</td>
<td>0.363</td>
<td>0.226</td>
</tr>
<tr>
<td>+ve 3</td>
<td>+ve 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100</td>
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<td>0.464</td>
<td>0.302</td>
</tr>
<tr>
<td>1:500</td>
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</tr>
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<table>
<thead>
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<th>IgG3</th>
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<tr>
<td></td>
<td>Primary 1:100</td>
<td>1:500</td>
<td>1:1000</td>
</tr>
<tr>
<td>Antibody Dilution</td>
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<td>+ve 1</td>
<td></td>
</tr>
<tr>
<td>1:100</td>
<td>0.600</td>
<td>0.378</td>
<td>0.260</td>
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<tr>
<td>1:500</td>
<td>0.157</td>
<td>0.108</td>
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<tr>
<td>+ve 2</td>
<td>+ve 2</td>
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<td></td>
</tr>
<tr>
<td>1:100</td>
<td>0.292</td>
<td>0.193</td>
<td>0.133</td>
</tr>
<tr>
<td>1:500</td>
<td>0.080</td>
<td>0.052</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1:100</td>
<td>0.367</td>
<td>0.217</td>
<td>0.158</td>
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<tr>
<td>1:500</td>
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</tr>
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<td></td>
</tr>
<tr>
<td>0.012</td>
<td>0.006</td>
<td>0.006</td>
<td>0.019</td>
</tr>
</tbody>
</table>

The values in bold represent primary antibody dilutions selected for further optimisation experiments. +ve 1, 2, and 3 represent IgG anti-tTG positive CD sera.

From these experiments, a serum dilution of 1:100 was deemed optimal for all four IgG subtypes. In a second series of checkerboard experiments, serum from the three IgG anti-tTG positive CD patients, and three control individuals with normal duodenal histology, negative IgA anti-tTG/EMA, and negative IgG anti-tTG results were diluted
1:100, followed by a comparison of differing subclass-specific secondary antibody dilutions (Table 4.4).

Table 4.4: IgG anti-tTG subclass ELISA optimisation 2.

<table>
<thead>
<tr>
<th></th>
<th>IgG1</th>
<th></th>
<th>IgG2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG1</td>
<td></td>
<td>IgG2</td>
</tr>
<tr>
<td></td>
<td>Secondary Antibody Dilution</td>
<td>Secondary Antibody Dilution</td>
<td></td>
</tr>
<tr>
<td>1:100</td>
<td>1:250</td>
<td>+ve 1</td>
<td>0.659</td>
</tr>
<tr>
<td>1:100</td>
<td>1:250</td>
<td>+ve 1</td>
<td>0.143</td>
</tr>
<tr>
<td>1:100</td>
<td>1:250</td>
<td>+ve 2</td>
<td>1.109</td>
</tr>
<tr>
<td>1:100</td>
<td>1:250</td>
<td>+ve 2</td>
<td>0.501</td>
</tr>
<tr>
<td>1:100</td>
<td>1:250</td>
<td>+ve 3</td>
<td>0.962</td>
</tr>
<tr>
<td>1:100</td>
<td>1:250</td>
<td>+ve 3</td>
<td>0.214</td>
</tr>
<tr>
<td>-ve 1</td>
<td>0.208</td>
<td>-ve 1</td>
<td>0.208</td>
</tr>
<tr>
<td>-ve 1</td>
<td>0.125</td>
<td>-ve 2</td>
<td>0.039</td>
</tr>
<tr>
<td>-ve 1</td>
<td>0.125</td>
<td>-ve 2</td>
<td>0.093</td>
</tr>
<tr>
<td>-ve 1</td>
<td>0.125</td>
<td>-ve 3</td>
<td>0.105</td>
</tr>
<tr>
<td>-ve 1</td>
<td>0.125</td>
<td>-ve 3</td>
<td>0.083</td>
</tr>
<tr>
<td>-ve 1</td>
<td>0.125</td>
<td>-ve 3</td>
<td>0.069</td>
</tr>
<tr>
<td>-ve 1</td>
<td>0.125</td>
<td>-ve 3</td>
<td>0.053</td>
</tr>
</tbody>
</table>

The values in red represent secondary antibody dilutions used in the final protocol. Primary antibody dilution was 1:100 for each IgG subclass. +ve 1, 2, and 3 represent IgG anti-tTG positive CD sera; -ve 1, 2, and 3 represent EMA/tTG/duodenal biopsy negative control individuals.

The results generated in the second series of optimisation experiments indicated that the optimal secondary antibody dilution was 1:250 for IgG1, and 1:100 for IgG2, IgG3, and IgG4, as these dilutions provided clear discrimination in each assay values between at least two of the three CD patients and controls. The development times were five minutes for the IgG1 and IgG2 ELISAs, and ten minutes for the IgG3 and IgG4 ELISAs.
4.3.8 IgG anti-tTG subclass ELISA

Individuals with a positive IgG tTG result, i.e. the CD patients from Chapter 3.3.9, and the IgG anti-tTG positive autoimmune disease patients detected in Section 4.3.4 were further tested in ELISAs measuring subclass-specific IgG autoantibodies to tTG. Positivity for total IgG anti-tTG was accompanied by elevated levels of autoantibodies to tTG of at least one of the four IgG subclasses for all patients tested. Statistical differences between the various groups were calculated using the Mann-Whitney test.

Coeliac disease

Of the 22 paediatric CD patients positive for total IgG anti-tTG, 13 were positive for one IgG anti-tTG subclass, 4 were positive for two subclasses, 2 were positive for three subclasses, and 3 were positive for all four subclasses. In adults with CD, 14 were positive for one IgG anti-tTG subclass, 3 were positive for two subclasses, 3 were positive for three subclasses, and none were positive for all four subclasses. Positivity for IgG1 autoantibodies to tTG was observed in 86% of children and 40% of adults with CD who were positive for total IgG anti-tTG. Mean levels of IgG1 anti-tTG in CD were significantly increased when compared to that of age-matched controls (p<0.0001 for paediatric CD, p=0.0002 for adult CD) (Fig 4.12a). IgG1 anti-tTG responsiveness to tTG was greater in children than in adults with CD (p=0.0010) (Fig 4.12a). The frequency of IgG2 anti-tTG positivity was similar in both IgG anti-tTG positive children, and adults with CD, being 37%, and 35% respectively. Increased recognition of tTG by IgG2 autoantibodies occurred in both paediatric (p=0.0005), and adult CD (p=0.0051), when compared to their respective control populations (Fig 4.12b). In keeping with the equal proportions of IgG2 positivity in paediatric and adult CD, no significant difference in mean IgG2 levels between these two groups were observed.
In CD patients positive for total IgG anti-tTG, IgG3 autoantibodies were present in 14% of the paediatric group, and 30% of the adult group. Although a low number of CD patients displayed IgG3 anti-tTG positivity, levels of these autoantibodies were significantly increased, compared to controls (p=0.0206 (children), p=0.0016 (adults) (Fig 4.12c). IgG4 anti-tTG positivity was observed in 32%, and 15% of IgG anti-tTG positive coeliac children and adults, respectively. IgG4 anti-tTG levels were significantly increased in both CD study populations, compared to that of controls, with p values of 0.0414 (paediatric CD) and 0.0059 (adult CD) (Fig 4.12d).

Figure 4.12: IgG anti-tTG subclass responses in CD. The dashed line indicates the cut-off, the black line represents the mean of each group. PCD=paediatric CD (n=22), PCTRL=paediatric controls (n=30), ACD=adult CD (n=20), ACTRL=adult controls (n=30). Statistics were calculated using the Mann-Whitney test, with levels of significance referred to in the text.
Of the 22 total IgG anti-tTG positive paediatric CD patients, 14 were untreated, 3 were partially treated, and 5 were treated. In the 20 adults with CD positive for total IgG anti-tTG, 7 were untreated, 10 were partially treated, and 3 were treated. The majority of paediatric CD patients positive for one or more IgG anti-tTG subtype were untreated (7 of 9), however of 6 adults positive for one or more IgG anti-tTG subtype half were untreated, and half were partially treated.

Positivity for IgG1 anti-tTG was detected in 13 of 14 untreated, 3 of 3 partially treated, and 3 of 5 treated paediatric coeliacs with elevated total IgG anti-tTG. The IgG1 anti-tTG positive adult coeliacs comprised 4 of 7 untreated, 3 of 10 partially treated, and 1 of 3 treated individuals. IgG1 anti-tTG levels in paediatric CD did not differ between treatment groups, and were significantly elevated compared to controls irrespective of CD status (p= <0.0001, 0.0065, and 0.0037 for untreated, partially treated, and treated CD respectively) (Fig4.13a). Adults with untreated or partially treated CD had levels of IgG1 anti-tTG significantly higher than that of controls (p= 0.0014, and 0.0027, respectively), with again no difference in levels between treatment groups (Fig 4.13a).

In paediatric CD, the majority of the 8 patients positive for IgG2 anti-tTG were untreated (5 untreated, compared to 1 partially treated, and 2 treated), compared to positivity in 3 untreated, 3 partially treated, and 1 treated adults with CD. Mean IgG2 anti-tTG levels were significantly elevated, compared to controls in untreated and treated CD in both adults and children (p=0.0024 in untreated paediatric CD, 0.0133 in treated paediatric CD, 0.0314 in untreated adult CD, and 0.0359 in treated adult CD) (fig 4.13b). A comparison of IgG3 anti-tTG levels did not distinguish the differing treatment groups and controls in paediatric CD, likely reflecting the low frequency of IgG3 anti-tTG positivity in this group. Of the 3 IgG3 anti-tTG positive paediatric CD patients, 2 were untreated, and
1 was treated. In adult CD, 6 individuals were positive for IgG3 anti-tTG, with 1 being untreated, 3 partially treated, and 2 treated. Levels of IgG3 anti-tTG were significantly elevated in untreated (p=0.0437), and partially treated (p=0.0076) adult CD, when compared to that of controls (Fig 4.13c).

IgG4 anti-tTG positivity in paediatric CD was exclusive to those with untreated disease (7 of 7 IgG4 anti-tTG positive individuals). Levels of IgG4 anti-tTG in untreated paediatric CD were significantly raised when compared to those in partially treated (p=0.0233), and treated (p=0.0014) disease, and controls (p<0.0001) (Fig 4.13d).

Figure 4.13: The relationship of treatment and IgG anti-tTG subclass responses in CD. The black line represents the mean of each group. UT=untreated, P=partially treated, T=treated. Statistics were calculated using the Mann-Whitney test).
Positivity for IgG4 anti-tTG in adult CD was detected in 4 patients, 2 of whom were untreated, and 2 were treated. Significantly increased levels, compared to controls, of IgG4 were observed in untreated adult CD only (p=0.0026) (Fig 4.13d).

As an inverse correlation between IgG anti-tTG positivity and age in paediatric CD had been noted in Chapter 3, the relationship of individual IgG anti-tTG subclass levels with age in paediatric coeliacs positive for total IgG anti-tTG was investigated. In agreement with a previous report by Agardth et al (2003), IgG1 anti-tTG levels were the highest in the 0-4 years CD group, and were significantly raised compared to that of CD patients aged 4-8 (p=0.0225), and adult coeliacs (p=0.0009) (Fig 4.14a). Mean levels of the other 3 IgG anti-tTG subclasses were also the highest in the 0-4 years CD group; however, this did not reach statistical significance (Fig 4.14b-d). Of note is significantly increased levels of IgG3 anti-tTG levels compared to that of controls in the 8-12 years CD group (p=0.0288) (Fig 4.14c), and increased IgG4 anti-tTG levels compared to that of controls in the 0-4 years CD group (p=0.0348) (Fig 4.14d).
Figure 4.14: The relationship between age and IgG anti-tTG subclass positivity in paediatric CD. The black line represents the mean value for each group, levels in adult CD are shown for comparison. The level of significance is referred to in the text, and was calculated using the Mann-Whitney test.

Type 1 diabetes

IgG1 was the dominant IgG anti-tTG subclass in the 9 patients with T1DM positive for total IgG anti-tTG antibodies, being found in 7 (78%) of these individuals (Fig 4.15a). An equal frequency of IgG2 and IgG3 anti-tTG positivity was observed, with positivity detected in 5/9 (56%) of patients for both IgG subtypes (Fig 4.15b/c). IgG4 anti-tTG positivity was noted in one patient only (11%), interestingly at levels (1.7 OD<sub>450</sub>) not seen in any other patient group; this patient was also positive for all 4 IgG anti-tTG subclasses (Fig 4.15d). Levels of IgG1, IgG2, and IgG3 anti-tTG were significantly elevated in patients
with T1DM when compared to control levels (p<0.0001, 0.0023, and 0.0010 for IgG1, IgG2, and IgG3, respectively). Positivity for one IgG anti-tTG subclass only was observed in 3 (33%) individuals, 3 individuals were positive for two subtypes, 2 (22%) were positive for three subtypes, whilst 1 individual (11%) was positive for all four IgG anti-tTG subtypes.

**Crohn’s disease**

The IgG anti-tTG response in Crohn’s disease was dominated by the IgG3 and IgG4 subclasses. Of the 10 Crohn’s disease patients positive for total IgG anti-tTG, IgG3 anti-tTG was detected in 6 (60%) individuals, with a similar frequency (60%) of IgG4 anti-tTG positivity observed (Fig 4.15c/d). IgG1 and IgG2 anti-tTG were infrequently found, being present in 3 (30%), and 2 (20%) of IgG anti-tTG positive Crohn’s disease patients, respectively (Fig 4.15a/b). Elevation of IgG1 (p=0.0148), IgG3 (p=0.0026), and IgG4 (p=0.0012) anti-tTG levels, compared to that of control individuals was observed. In regard to the number of IgG anti-tTG subtypes positive per patient, 4 individuals (40%) were positive for one subtype only, 3 (30%) were positive for two subtypes, 2 (20%) were positive for three subtypes, whilst one patient was positive for all four IgG anti-tTG subtypes.

**Wegener’s granulomatosis**

Five Wegener’s granulomatosis patients were positive for total IgG anti-tTG, and were analysed for IgG anti-tTG subclass usage. Interestingly, IgG1 anti-tTG positivity was not detected in any of these 5 patients, who were all (100%) positive for IgG2 and IgG3 anti-tTG (Fig 4.15a/b/c). IgG4 anti-tTG reactivity was present in 3 (60%) of the total IgG anti-tTG positive WG patients (Fig 4.15d). Positivity to two or more IgG anti-tTG subclasses was observed for all WG IgG anti-tTG positive sera.
Rheumatoid arthritis and autoimmune liver disease

Of the two IgG anti-tTG positive individuals with RA, both were positive for three IgG anti-tTG subclasses. The first patient was positive for IgG1, IgG2, and IgG3 anti-tTG, the second was positive for IgG2, IgG3, and IgG4 anti-tTG (Fig 4.15). In autoimmune liver disease, one IgG anti-tTG positive individual was positive for IgG1, IgG3, and IgG4 anti-tTG, the other was positive for IgG3 anti-tTG only (Fig 4.15). Interestingly, all four of these patients were positive for IgG3 anti-tTG, with massively elevated levels found in one RA patient (Fig 4.15c).

Figure 4.15: IgG anti-tTG subclass responses in patients with autoimmune disease. The IgG subclass usage of IgG anti-tTG positive non-CD patients was investigated. Levels in CD are shown for comparison. The dashed line indicates the cut-off.
4.4 Discussion

4.4.1 The IgG anti-tTG response

Whilst tTG autoantibodies will always be inherently linked with CD, the description of increasing numbers of disorders in which a humoral IgG response to tTG is present implicates an inappropriate targeting of the enzyme during non-specific autoimmune or inflammatory processes. Inflammation is a common feature of the diverse conditions in which IgG anti-tTG antibodies have been demonstrated, such as T1DM, HIV, and end-stage heart failure, this is a process in which tTG is intimately involved, through the formation of protein cross-links and organisation of the extracellular matrix (Griffin, Casadio et al. 2002). It may be that increased expression of tTG during inflammation (Iismaa, Mearns et al. 2009), or inappropriate tTG release due to a defect of apoptosis (Farrace, Picarelli et al. 2001) is a factor in the breaking of immunological tolerance, allowing for the release of cryptic tTG epitopes, neo-epitope formation, or haptenisation.

The detection of IgG anti-tTG in individuals with autoimmune diseases other than CD was initially attributed to impurities in the guinea-pig tTG used as antigen; however, observations made using guinea-pig tTG have largely been validated in systems employing recombinant human tTG antigen. The finding of false-positive anti-tTG of both the IgA and IgG classes using guinea-pig tTG may be especially relevant to those with autoimmune liver disease, as guinea-pig tTG is extracted from the animal’s liver and may contain cross-reactive hepatic antigens (Clemente, Musu et al. 2002).

In order to generate cohorts of non-CD, IgG anti-tTG positive individuals for downstream analysis of epitope specificity and IgG subclass usage, sera from groups of patients suffering from varying autoimmune diseases were screened using the recombinant
human tTG assays developed in Chapter 3. IgG anti-tTG positivity was detected in 28% of patients with T1DM, 19% of patients with Crohn’s disease, and 14% of patients with Wegener’s granulomatosis, at levels comparable to that found in adult CD (Fig 4.6). Two IgA anti-tTG negative, but IgG anti-tTG positive patients with rheumatoid arthritis (RA), and two with autoimmune liver disease, all discovered during screening for an adult control population in Chapter 3 were also included for analysis of their IgG anti-tTG responses.

The frequency (28%) of IgG anti-tTG positivity in the T1DM group was similar to the 34%, previously observed by Lampasona et al. (Lampasona, Bonfanti et al. 1999). During selection of the T1DM patients, care was taken to ensure that a history of CD was not present, due to the co-occurrence of both disorders (Bashiri, Keshavarz et al. 2011). All of the T1DM patients in which IgG anti-tTG was detected, tested negative for IgA anti-tTG antibodies. As with CD, the steps leading to the production of these antibodies are unclear, however a number of plausible theories have been proposed as to the origin of diabetic transglutaminase autoimmunity. The first relates to the increased intestinal permeability (similar to that seen in CD) that has been described in T1DM (Bosi, Molteni et al. 2006; Vaarala 2008), which may allow for generation of a mucosal immune response to tTG. The concept of a mucosal origin of transglutaminase immunity in T1DM and CD is further strengthened by the observation of intestinal IgA anti-tTG deposits in the majority of children with T1DM, regardless of serum IgA anti-tTG positivity (Maglio, Florian et al. 2009), and the finding of ‘coeliac-like’ gliadin immunity such as diabetes-specific HLA-DR-restricted gliadin-specific T cells in IgA anti-tTG negative T1DM adults and children (Mojibian, Chakir et al. 2009). The second theory is centred upon the fact that numerous tTG substrates are autoantigens (Kim, Jeitner et al. 2002), and that tTG
may act as a hapten, cross-linking the prototypic diabetic antigens glutamic acid decarboxylase, insulin, and insulinoma antigen 2 (IA-2) to itself, with resultant intramolecular epitope spreading (Bach 1994; Kim, Jeitner et al. 2002). The third hypothesis, is that pancreatic β cell destruction during the early phase of T1DM releases tTG as a neo-epitope, a notion supported by the reduction of IgG anti-tTG titres upon management of the diabetes and subsequent attenuated β cell destruction (Lampasona, Bonfanti et al. 1999).

In the group of Crohn’s disease patients, approximately one-fifth (10/53) were positive for IgG anti-tTG (Fig 4.6). Similar to previous reports (Farrace, Picarelli et al. 2001; Sárdy, Csikós et al. 2007; Ribeiro-Cabral, da-Silva-Patricio et al. 2011), low-level IgA anti-tTG positivity was detected in five of the ten IgG anti-tTG positive Crohn’s disease patients. The production of anti-tTG in Crohn’s disease may reflect a by-product of the intestinal inflammatory process; in 2004, Di Tola and colleagues reported a correlation between IgA anti-tTG (albeit at low levels), and Crohn’s disease activity index (Di Tola, Sabbatella et al. 2004).

In a 2007 study by Sardy et al., involving screening large groups of patients with autoimmune diseases for IgA anti-tTG against a recombinant human antigen, the median level of IgA anti-tTG in patients with Wegener’s granulomatosis was significantly greater than that of controls, being one of the highest of all the disease groups investigated (Sárdy, Csikós et al. 2007). Although the WG IgA anti-tTG positive sera were subsequently negative for IgA EMA, upon immunoblotting with purified tTG specific binding occurred at a band corresponding to the molecular weight of tTG (Sárdy, Csikós et al. 2007). Investigation into the presence of IgG anti-tTG in WG has not been previously reported;
section 4.3.4 identifies positivity for this autoantibody in 5/37 (14%) of patients with WG (Fig 4.6). IgA anti-tTG was not present in any of these five individuals.

The results of this chapter, in consensus with previous literature, identify IgG anti-tTG positivity in patients with T1DM, Crohn’s disease, rheumatoid arthritis, autoimmune liver disease, and additionally, Wegener’s granulomatosis. This is in agreement with the observation that autoimmune diseases - usually associated with a ‘signature’ antigen which may be intimately linked with disease pathogenesis or detection, generally display autoantibody responses to a spectrum of self-antigens, some which may be shared by multiple diseases (Shoenfeld, Gershwin et al. 2007). In CD, the ever increasing list of autoantigens includes the tTG substrates collagen (Bowness, Folk et al. 1987; Dieterich, Esslinger et al. 2006) and actin (Nemes, Adany et al. 1997; Clemente 2000), the tTG-associated Ghp subunit (calreticulin) (Sánchez, Tučková et al. 2000), the mitochondrial ATP synthase β chain (Stulík, Hernychová et al. 2003), zonulin, a protein which regulates the permeability of intestinal tight junctions (Fasano, Not et al. 2000), and the glycolytic enzyme enolase α (Stulík, Hernychová et al. 2003). That autoantibodies of the IgA class are always present to the antigens listed above in CD infers a mucosal origin of the humoral response.

The finding of anti-tTG in Crohn’s disease is part of the appearance of a diverse, non tissue-specific range of autoantibodies such as IgA and IgG perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) (Gigase, De Clerck et al. 1997; Mendoza and Abreu 2009), IgA and IgG anti-pancreatic antibodies (Seibold, Mork et al. 1997; Bogdanos, Rigopoulou et al. 2011), and IgG antibodies directed against glycolytic enzymes, including enolase α (Vermeulen, Vermeire et al. 2011). The autoantigens of T1DM are largely of
pancreatic origin, including GAD, insulin, and IA-2 (Knip and Siljander 2008), zinc transporter 8 (Yang, Luo et al. 2010), but also include proteins such as CCL3 (Huang, Mo et al. 2012) and heat shock protein 10 (Takizawa, Endo et al. 2009), with autoantibodies being of the IgG class. Wegener’s granulomatosis is characterised by antibodies to proteinase 3 (cytoplasmic anti-neutrophil cytoplasmic antibody) (Bruner, Vista et al. 2010), but endothelial cell antibodies also occur (Holmen, Elsheikh et al. 2007). The ubiquitous expression of tTG (Badarau, Collighan et al. 2011) may partially explain its serial appearances as an antigen in a variety of autoimmune disorders. Proteins that are also the target of antibodies in multiple autoimmune diseases such as actin (Ishigatsubo, Sakamoto et al. 1989; Musante, Candiano et al. 2005; Gueguen, Dalekos et al. 2006) and RA33 (Isenberg, Steiner et al. 1994; Steiner, Skriner et al. 1996; El-Kased, Koy et al. 2009) tend to share a pattern of ubiquitous tissue distribution (Fritsch, Eselbock et al. 2002; Bertola, Ott et al. 2008), implying that immunity to these self-components may be secondary to an already established immune response against a primary, tissue-specific antigen.

4.4.2 Epitope specificity of IgG anti-tTG

Autoimmune diseases are frequently associated with disease-specific epitopes which may be useful in a diagnostic setting (Herkel and Lohse 2008), but may also provide mechanistic insights into the pathogenesis and origin of such responses (Brito, Biamonti et al. 1994), information on which remains elusive. The epitope specificity of antibodies to the diabetic autoantigen GAD65 have been extensively studied, with the pattern of epitope recognition of anti-GAD65 distinguishing between patients with T1DM and Stiff-Person syndrome (Kim, Namchuk et al. 1994; Powers, Bavik et al. 1999). A change in the pattern of GAD epitope recognition from epitopes located in the middle and the C-
terminus of GAD65 to epitopes in the N-terminus and the middle region has been associated with progression to overt diabetes in at-risk individuals (Schlosser, Banga et al. 2005). The results of this chapter suggest a difference in epitopes recognised by IgG antibodies to tTG in CD, when compared to that of T1DM, Crohn’s disease, Wegener’s granulomatosis, rheumatoid arthritis, and autoimmune liver disease. In epitope masking experiments, blocking of the tTG active-site CD IgA epitope caused a reduction in IgG anti-tTG binding for CD patients only (Fig 4.8), confirming the presence of shared CD IgA/IgG anti-tTG epitopes noted in Chapter 3. Masking of this epitope did not affect IgG anti-tTG binding in non-coeliac autoimmune patients (Fig 4.9), excluding it as the target of humoral tTG immunity in these individuals. This observation was further strengthened by the equal binding pattern of IgG anti-tTG displayed to both the wild-type tTG protein and a mutant tTG lacking the entire catalytic triad of amino acids in patients with autoimmunity (Fig 4.11). This in contrast to the almost complete abrogation of CD IgG anti-tTG binding to the mutant protein seen in CD, and serves as further evidence as to the antigenicity and correct protein assembly of the mutant tTGs generated in Chapter 2. Given the fine specificity of the anti-tTG response in CD, possibly due to gliadin/tTG interaction at the tTG active-site as discussed in section 3.4.3, a difference in the epitope specificity of non-CD IgG anti-tTG is not surprising and provides further evidence as to the unusual circumstances in which tTG may become antigenic in CD. The difference in epitope specificity of CD and non-CD IgG may reflect the circumstances in which tTG becomes antigenic in non-coeliac pathologies, which may be related to an apoptotic defect, or an epitope spread from a primary autoantigen to tTG. The effect of a gluten-free diet on tTG autoantibody levels in non-CD IgG anti-tTG positive individuals would prove informative as to any influence exerted by gliadin on anti-tTG production outside of CD. IgG autoantibody responses are generally polyclonal with multiple epitope
recognition sites noted (Huang, Scofield et al. 1997; Chimanovitch, Schmidt et al. 1999; Silva, Hummel et al. 2010). Further investigation is needed to discover regions of the tTG molecule targeted by non-CD IgG anti-tTG and identify potential disease-specific epitopes. The site at which the autoantibody response is initiated may also influence the difference in epitope specificity of IgG anti-tTG. In CD, the anti-tTG response is of mucosal origin - the CD lesion is characterised by abundant IgA anti-tTG secreting plasma cells, with IgG and IgM anti-tTG secreting cells also detected (Di Niro, Mesin et al. 2012). It is tempting to speculate that the absence of an IgA anti-tTG response in IgG anti-tTG positive individuals with autoimmune disease excludes the intestinal mucosa as the source of these autoantibodies. However, it is also plausible that the immune response to tTG may be initiated in the intestine in certain pathologies, such as T1DM and Crohn’s disease. In Crohn’s disease, the intestine is the major disease site, and low-level IgA anti-tTG positivity may be detected in some Crohn’s patients. Further studies in this field involving immunostaining or flow cytometric analysis of duodenal biopsies from non-CD IgG anti-tTG positive individuals for tTG-secreting plasma cells could provide valuable information as to the source of anti-tTG in these patients.

4.4.3 IgG anti-tTG subclass usage

ELISAs measuring the IgG anti-tTG subclasses allowed a profile of subclass usage between the differing autoimmune disease groups to be developed. In CD, the IgG anti-tTG subclass profile was dominated by IgG1, regardless of treatment status (Fig 4.12a, Fig 4.13a) with the highest levels observed in children <4 years of age (Fig 4.14a), both in concurrence with previous reports (Agardh, Borulf et al. 2003; Schilling, Spiekerkoetter et al. 2005). Positivity to the other three IgG subclasses was also detected, with IgG2 being positive in approximately 35% of CD patients (Fig 4.12b). IgG4 positivity was
exclusively associated with untreated disease in paediatric CD (Fig 4.13d). The subclass usage of IgG anti-tTG in T1DM displayed a similar pattern to that found in CD, with IgG1 again prominent (Fig 4.15a). In the two patients with rheumatoid arthritis, both were positive for IgG2 and IgG3 anti-tTG, with one each positive for IgG1 and IgG4 anti-tTG, whilst the two autoimmune liver disease patients were positive for IgG3 (Fig 4.15), although the numbers of these patients are too low to make global conclusions. The IgG anti-tTG response in Crohn’s disease was characterised by positivity to IgG3 and IgG4 anti-tTG in 60\% of patients, whereas all of the Wegener’s granulomatosis patients were positive for IgG2 and IgG3 anti-tTG, with 3/5 positive for IgG4 (Fig 4.15), and negative for IgG1.

A number of factors may determine the IgG subclasses used in response to antigen, such as the presentation of the antigen to T helper cells, the biochemical nature of the antigen, and the local cytokine environment (Shoenfeld, Gershwin et al. 2007). In mice, Th1 and Th2 responses are correlated with the appearance of the IgG2a and IgG1 subclasses, respectively (Lewkowich, Rempel et al. 2004). In humans the picture is not so clear, but a Th1 response is thought to lead to production of IgG1, IgG2, and IgG3 (Holdsworth, Kitching et al. 1999; Wu, Oka et al. 2005), whereas Th2 responses, controlled by IL-4, result in the production of IgG4 and IgE (Ohtani, Wakui et al. 2004). The IgG subclasses in humans differ in their ability to activate complement, with IgG3 being the strongest activator, followed by IgG1 and IgG2, whereas IgG4 cannot activate complement (Sitaru, Mihai et al. 2007). The rank of affinity for Fcγ receptors on phagocytic cells is IgG3>IgG1>IgG4>IgG2 (Goh, Grant et al. 2011).
An interesting observation is the similarity in the IgG anti-tTG subclass usage profile between CD and T1DM, two diseases thought to share common pathogenic features such as impaired intestinal mucosal integrity (de Kort, Keszhelyi et al. 2011), and a shared genetic background (Vorobjova, Uibo et al. 2011). Certainly the signature antibody responses in both conditions are predominantly IgG1; anti-GAD and IA-2 in T1DM (Bonifacio, Scirpoli et al. 1999; Hawa, Fava et al. 2000), and anti-tTG and gliadin in CD (Hvatum, Scott et al. 1992), however as per the results in this chapter, positivity to all subclasses occurs. In the study by Bonifacio and colleagues, IgG2, IgG3, and IgG4 antibodies to GAD and IA-2 were usually only detected during peak IgG1 responses (Bonifacio, Scirpoli et al. 1999).

WG is considered a ‘Th1-driven’ disease, as the granulomatous inflammation found in nasal and mucosal tissue is dominated by IFN-γ (Csernok, Trabandt et al. 1999). Interestingly T helper cells specific for the signature autoantigen of WG, proteinase 3, display a pattern of skewing towards Th2 and Th17 cell populations including Th17/Th1, Th17/Th2 and Th22 (Fagin, Csernok et al. 2011), which may account for the mixed IgG1, IgG3, and IgG4 responsiveness found to this antigen in WG (Fagin, Csernok et al. 2011). The mixed IgG anti-tTG subclass response observed in WG patients may represent a similar scenario of T cell help in skewing the anti-tTG response. The complete absence of IgG1 anti-tTG production in WG patients is intriguing, and may possibly reflect a difference in the Th1 responsiveness to tTG, represented by IgG2 and IgG3 anti-tTG. In Crohn’s disease, which is a classical ‘Th1-driven’ condition, the IgG autoantibody responses to antigens such as p-ANCA and pancreas are principally of the IgG1 subclass (Seibold, Mork et al. 1997; Vasiliauskas, Kam et al. 2000). In this study, the majority of Crohn’s disease patients had IgG3 or IgG4 anti-tTG, with only a minority (30%) positive
for IgG1 anti-tTG. The relative lack of IgG1, and presence of IgG3 and IgG4 responsiveness to tTG in WG and Crohn’s may be explained by commonalities in the immune response to tTG. Of note is that both of these diseases are characterised by granuloma formation (Freeman 2007; Schilder 2010), and may display ANCA reactivity, albeit to different ANCA antigens (Mendoza and Abreu 2009; Kallenberg 2011). IgE anti-tTG may be occasionally found in coeliac individuals (Schilling, Spiekerkoetter et al. 2005); it would be useful to determine if the IgG4 response to tTG (in any of the patient groups studied in this chapter) is accompanied by one of the IgE class.

4.4.4 Conclusions

To summarise, the work in this chapter was carried out in order to compare the IgG anti-tTG response found in CD to that found in non-coeliac autoimmune diseases. By comparing the epitope specificity and subclass usage of the IgG anti-tTG response, clues as to the mechanisms of tTG autoimmunity in CD and beyond may be gleaned.

Using the recombinant human wild-type tTG purified in Chapter 2, IgA anti-tTG was affinity-purified from the pooled serum of CD patients and used to silence epitopes on the tTG molecule. The masking of these epitopes caused a reduction in the binding of IgG anti-tTG in CD patients, confirming the observation made in Chapter 3 that the IgA and IgG anti-tTG responses in CD appear to share similar epitopes. In contrast to this, the binding of IgG anti-tTG from patients with autoimmune disease was not affected by the silencing of this epitope, indicating a difference in tTG epitopes in these diseases. The equal binding of IgG anti-tTG from non-CD patients to both the wild-type and CHD ΔtTG serves as further evidence for this conclusion.
ELISAs measuring the levels of anti-tTG of all four IgG subtypes were established, and used to determine the usage profile of these antibodies in IgG anti-tTG positive individuals. The rank order of IgG subclass usage of anti-tTG in CD was found to be IgG1>IgG2>IgG4>IgG3 for paediatric CD, and IgG1>IgG2>IgG3>IgG4 for adult CD. A comparison of the IgG subclass of anti-tTG revealed similarities in subclass usage in CD and T1DM, which were both dominated by IgG1. In the other autoimmune disease groups studied, IgG3 was the most frequent anti-tTG subclass observed, with IgG1 anti-tTG reactivity being almost always absent in Crohn's disease and Wegener's granulomatosis.

The differences in epitope specificity and subclass usage of IgG anti-tTG observed between CD and non-CD individuals may reflect a combination of differing mechanisms underlying tTG autoimmunity, the first being the unique gliadin/tTG interaction that occurs in CD, and the second being the location where the loss of immunological tolerance to tTG occurs. Further characterisation of tTG epitopes recognised by non-CD individuals, and inter-disease variations thereof, could further expand these observations.
Chapter 5
Investigation of T cell responses to tissue transglutaminase
5.1 Introduction

5.1.1 T lymphocytes

T lymphocytes (T cells) are the facilitators of cell-mediated immunity (Whitacre, Lin et al. 2012), and have long been referred to as the orchestrators of the adaptive immune response. The presence of a membrane-bound structure, the T cell receptor (TCR) on their surface characterises T cells (Smeets, Fleuren et al. 2012), and functions to recognise peptide antigen presented in the context of an MHC molecule (Day, Ramsland et al. 2009). The TCR is composed of a heterodimer of chains containing immunoglobulin superfamily member motifs (Williams and Barclay 1988), with the majority of T cells (~95%) expressing α and β chains, and a minority (~5%) expressing γ and δ chains (Dunne, Mangan et al. 2010). Activation of T cells is initiated via the TCR (van der Merwe and Dushek 2011), (ensuring the antigen-specific nature of the T cell response), with the signal then transduced by CD3 and the ζ chains (Minguet, Swamy et al. 2008). Further activation requires subsequent co-stimulation of accessory molecules on the surface of the T cell such as CD28 and inducible costimulator (ICOS, CD278), with their corresponding ligands CD80/CD86 and ICOS ligand (ICOS-L, CD275), respectively, expressed on the surface of the APC (Nurieva, Liu et al. 2009; Sharpe 2009). Cytokines produced by activated APCs such as IL-12 also support the activation and differentiation of naive T cells into effector cells (Foster, Leen et al. 2007).

T cells may be divided into distinct subsets based upon their function, coupled with the expression of specific cell-surface markers for each population. The best characterised of these subsets are the helper T cells (Th cells), which express the CD4 glycoprotein on their cell surface and secrete cytokines which direct many of the cellular responses of the innate and adaptive immune systems, and cytotoxic T cells, which express the CD8
molecule and function to kill virally infected or cancerous cells (Abbas, Lichtman et al. 2012). Additional T cell populations such as regulatory T cells (also CD4+) function to inhibit immune responses (Sakaguchi, Miyara et al. 2010), whereas natural killer T cells share properties of both T cells and natural killer cells and can enhance or suppress immune responses (O’Reilly, Zeng et al. 2011). T cells expressing the γδ TCR are also considered a distinct T cell subset with both helper and cytotoxic roles, that bridge innate and adaptive immunity (Liu and Huber 2011; Romi, Soldaini et al. 2011; Bansal, Mackay et al. 2012).

Whereas naive CD4+ T cells secrete IL-2 when activated (Bucy, Karr et al. 1995), effector CD4+ T cells may be sub-divided into distinct subsets defined by the cytokines they release upon activation (Wan 2010). For twenty or so years, the Th1/Th2 paradigm classified Th cells into two differing groups, Th1 or Th2 (Romagnani 1991; Allen and Maizels 1997). Th1 cells are characterised by the expression of the transcription factor T-bet, production of high levels of IFN-γ, phagocyte activation capability, and the ability to direct an antibody response of complement-fixing and opsonising antibodies (Nakamura, Kamogawa et al. 1997; Szabo, Kim et al. 2000; Annunziato and Romagnani 2009). Th2 cells are under the control of the transcription factor GATA-3, secrete IL-4 and IL-13 which ultimately results in an IgE-dominated antibody response; evolutionary advantageous in the protection from parasitic infection, but also responsible for atopy (Hartung, Bohnert et al. 2003; Mantel, Kuipers et al. 2007; Allen and Maizels 2011).

The Th1/Th2 paradigm has been revised in recent years following the discovery of new classes of Th cells such as regulatory T cells, Th9 cells, Th17 cells and follicular helper T cells (Tfh cells) (Wan 2010). Regulatory T cells, which dampen immune responses and are important in maintaining self-tolerance are characterised by the production of anti-inflammatory cytokines such as IL-10 and TGF-β (Schwartz 2005; Pyzik and Piccirillo
2007). Th17 cells, protective in instances of certain bacterial and fungal infections (Jin, Zhang et al. 2008), have emerged as pathogenic in a multitude of human autoimmune diseases. The ever-growing list of autoimmune conditions in which involvement of Th17 cells and their signature cytokine, IL-17A have been identified includes systemic lupus erythematosis (Wong, Lit et al. 2008), rheumatoid arthritis (Lubberts 2010), Crohn’s disease (Seiderer, Elben et al. 2008), multiple sclerosis (Waite and Skokos 2012), and coeliac disease (CD) (Castellanos-Rubio, Santin et al. 2009; Harris, Fasano et al. 2010; Monteleone, Sarra et al. 2010).

Follicular T helper cells (Tfh), named due to their location in the follicle of secondary lymphoid tissues, have emerged as a Th cell specialised in promoting immunoglobulin class-switching and production by B cells (Kuchen, Robbins et al. 2007; Spolski and Leonard 2010). Receptors for the signature cytokine of the Tfh cell, IL-21, are found on a variety of cell types such as T cells, B cells, NK cells, NKT cells, fibroblasts, and epithelial cells, with the highest levels being expressed on activated B cells (King 2009). IL-21 also acts in an autocrine fashion to promote the expansion of Tfh cells themselves (Vogelzang, McGuire et al. 2008). Dysregulation of Tfh cells has been implicated in human autoimmune disease; expansion of Tfh cells, and increased serum IL-21 has been noted in SLE (Simpson, Gatenby et al. 2010), with IL-21R overexpressed in the synovium of RA patients (Li, Shen et al. 2006).

Interestingly, increasing experimental evidence suggests that Th cell differentiation is reversible, with Th cells displaying plasticity between differing phenotypes (Locksley 2009), attributable to epigenetic Instability of cytokine and transcription factor gene loci (Mukasa, Balasubramani et al. 2010).
5.1.2 T cells in autoimmunity

The demonstration of autoreactive T cells is a recurring feature of autoimmune disease (Kroemer, Hirsch et al. 1996), however when one considers the role of T cells in regulating the immune response to proteins, and given that most self-antigens are protein in nature (Abbas, Lichtman et al. 2012), this is not surprising. The strong association of many autoimmune diseases with the MHC complex (de Bakker, McVean et al. 2006), the function of which is to present antigen to T cells, provides further evidence for a contribution of T cells to the pathogenesis of these disorders. Adoptive transfer of antigen-specific, self-reactive T cells can cause autoimmune disease in animal models of disease such as experimental autoimmune keratitis (Akpek, Liu et al. 2000), experimental autoimmune encephalomyelitis (Kojima, Wekerle et al. 1997), and the non-obese diabetic mouse model of diabetes (Anderson and Bluestone 2005). The study of autoantigen-specific T cells may be loosely approached in two steps: the first is the actual detection of such cells in a patient sample of peripheral blood or tissue, usually accomplished by measuring the effect of stimulation with the antigen of interest on cellular proliferation and activation. Once autoreactive T cells have been identified, the aim of the second step is to isolate and generate clones or sustainable lines of these cells for further study.

Lymphocyte proliferation assays, based on the clonal expansion of self-reactive lymphocytes when exposed to a given autoantigen, will detect individuals immunised to that antigen (Hay, Westwood et al. 2002). A common approach to analyse lymphocyte proliferation includes the measurement of incorporation of a detectable compound such as tritiated thymidine or 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) into the dividing cells. CFSE incorporation, measured using flow cytometry, has the advantage of increased sensitivity and the capability of measuring the proliferation of different
lineages of cells (Mannering, Wong et al. 2010). Measurement of T cell proliferation and activation in response to autoantigen is frequently performed after six days of stimulation when using freshly isolated peripheral blood mononuclear cells (PBMCs) (Tree, Duinkerken et al. 2004). For T cells specific for ribosomal P2 protein in SLE (Crow, DelGiudice-Asch et al. 1994), heat-shock protein 60 (Abulafia-Lapid, Elias et al. 1999) and GAD (Rharbaoui, Mayer et al. 1999) in T1DM, and myelin basic protein in multiple sclerosis (Kumar, Putzki et al. 2006), optimal proliferation was observed after six days in culture. A similar stimulation time is recommended for the generation of gliadin-specific T cells (O'Keeffe, Mills et al. 1999; Marsh 2000).

Autoantigen-specific T cells from responsive individuals may then be cultured into lines that retain functional capacity, such as activation and cytokine production, and are capable of in-vitro proliferation. Such T-cell lines have been generated in a wide range of autoimmune conditions, reactive to defined autoantigens such as myelin basic protein in multiple sclerosis (Van der Aa, Hellings et al. 2003), GAD and IA-2 in T1DM (Endl, Otto et al. 1997; Hawkes, Schloot et al. 2000), the thyroid stimulating hormone receptor in Grave’s disease (Mullins, Cohen et al. 1995), and multiple autoantigens of RA (Melchers, Jooss-Rudiger et al. 1997; Fritsch, Eselbock et al. 2002). A common feature of studies on autoreactive T cells is that cell lines can be derived from a minority of responsive control individuals, possibly due to their possession of the appropriate HLA alleles (Mullins, Cohen et al. 1995). T-cell lines to IA-2 (Hawkes, Schloot et al. 2000), myelin basic protein (Pender, Csurhes et al. 1996), and the principal autoantigen of pemphigus vulgaris, desmoglein 3 (Hertl, Amagai et al. 1998) have been generated from controls.

The antigens used for T cell proliferation and stimulation may be either the whole protein, or a defined peptide if the epitope has been identified (Sospedra, Pinilla et al. 2003). Due to the peptide nature of T cell epitopes, protein must be efficiently processed
by the antigen-presenting cell (APC) in order to facilitate its presentation to T cells (Singh, Singh et al. 2010), however using whole protein has the advantage of allowing responses to be detected regardless of HLA type, which may be a restricting factor in some peptide-based T cell assays (Mannering, Wong et al. 2010).

5.1.3 T cell responses in coeliac disease

Due to the T lymphocytosis observed in the active CD intestinal lesion (Halstensen, Farstad et al. 1990), and the strong association of the disease with HLA expression (Demarchi, Carbonara et al. 1983), the concept of T cell involvement in the pathogenesis of CD had been long-established prior to the isolation of gliadin-specific T cells from the CD intestinal mucosa (Lundin, Scott et al. 1993). In the time since this groundbreaking observation, the CD4+ gliadin-specific T cell response has been dissected and extensively characterised in relation to antigen recognition, phenotype, and function. The hallmark of gliadin-specific T-cell lines and clones is a mucosal-homing phenotype (Anderson 2005; Ben-Horin, Green et al. 2006), and production of large amounts of IFN-γ; a cytokine that contributes substantially to the intestinal destruction in CD (Nilsen, Lundin et al. 1995; Raki, Fallang et al. 2007; Garrote, Gomez-Gonzalez et al. 2008). Further proinflammatory Th1 cytokines may also be produced by gliadin-specific T cells such as TNF-α (Nilsen, Lundin et al. 1995; Ben-Horin, Green et al. 2006), IL-2 (O’Keeffe, Mills et al. 1999; Gianfrani, Levings et al. 2006), and IL-6 (Nilsen, Lundin et al. 1995; Harris, Fasano et al. 2010). IL-4 production has been demonstrated by Nilsen and co-workers in both mucosal and peripheral blood derived gliadin-reactive T-cell clones (Nilsen, Lundin et al. 1995; Nilsen, Gjertsen et al. 1996), and by Troncone et al. in mucosa-derived gliadin-reactive T-cell clones (Troncone, Gianfrani et al. 1998). However, IL-4 production
by peripheral blood gliadin-specific T cells was not detected in studies by Ben-Horin (Ben-Horin, Green et al. 2006) or O'Keeffe (O'Keeffe, Mills et al. 1999).

Recent research has focused on the involvement of the newly-discovered Th17, and Tfh cell subsets in CD (De Nitto 2009; Monteleone, Sarra et al. 2010). Gliadin-specific Th17 cells have been identified in the mucosa of CD patients, producing the Th17-related cytokines IL-17A, IL-21, IL-22, and TGF-β (Fernandez, Molina et al. 2011). In contrast to this finding, Bodd and colleagues found that HLA-DQ2-restricted gluten-reactive T cells produce IL-21 but not IL-17 or IL-22 (Bodd, Raki et al. 2010), a phenotype typical of Tfh cells (Yu, Batten et al. 2009). Another characteristic of the IL-21-producing gluten-reactive cells in the study by Bodd et al. which may further their identification as bona fide Tfh cells was double positivity for the production of IFN-γ and IL-21; a similar observation was made in Tfh cells isolated from the intestinal lamina propria of patients with inflammatory bowel disease (Sarra, Monteleone et al. 2010).

Production of the anti-inflammatory cytokines IL-10 and TGF-β by gliadin-specific T cells (Nilsen, Lundin et al. 1995; O'Keeffe, Mills et al. 1999), may be indicative of the expected immune response to such a dietary antigen, which strives to maintain tolerance (Battaglia, Gianfrani et al. 2004). In a 2006 study, Gianfrani and colleagues developed gliadin-specific Tr1-cell clones from the intestinal mucosa of CD patients that were hyporesponsive to antigen stimulation, and could suppress the proliferation of pathogenic gliadin-specific T-cell clones derived from the same patients (Gianfrani, Levings et al. 2006). Increased resistance of CD mucosal-derived intraepithelial and lamina propria lymphocytes to the suppressive effects of peripheral-derived regulatory T cells, compared to that in controls has been observed (Hmida, Ahmed et al. 2012), further evidence that a defect of Treg cell function may contribute to the development of CD.
Interestingly, gliadin-specific T-cell lines and clones may also be generated from PBMC from control individuals, and produce large amounts of IL-10 and IFN-γ (Nilsen, Gjertsen et al. 1996; O'Keeffe, Mills et al. 1999). In one such study, gliadin-specific T cell responses were detected in the periphery of 4 of 20 control individuals, with 2 of the 4 being HLA-DQ restricted (Jensen, Sollid et al. 1995). The isolation of gliadin-specific T cells from the small intestine is a CD-specific phenomenon (Lundin, Scott et al. 1993), possibly reflecting the homing, influx, and proliferation of these cells in response to pro-inflammatory innate immune signals induced by dietary gliadin peptides.

Apart from the apparent control of the immune reaction that leads to formation of the CD lesion (Bodd, Raki et al. 2010), another important role in CD pathology has been assigned to gliadin-specific T cells- the provision of T cell help to tTG-specific B cells, ultimately resulting in the anti-tTG response characteristic of CD (Schuppan 2000; Barker and Liu 2008). The ‘hapten-carrier’ theory proposed by Sollid (Sollid, Molberg et al. 1997), as described section 1.2.9, was based upon the fact that T cells specific for tTG had never been isolated. However, in a landmark paper, Ciccocioppo et al. (2010) have recently described the presence of tTG-specific T cells in the periphery of untreated, HLA-DQ2 positive CD patients (Ciccocioppo, Finamore et al. 2010). tTG-specific T-cell lines and clones were developed, being mostly (85%) CD4+, and had cytokine secretion profiles dominated by IFN-γ, with additional IL-1β, IL-6, IL-10, IL-12, TGF-β, and TNF-α production. As well as the classical Th1 cytokine production by the tTG-specific T cells observed by Ciccocioppo, an immunomodulatory function is also suggested by their production of IL-10 and TGF-β (Sanjabi, Zenewicz et al. 2009), as is a profile with possible Th-17 polarising capabilities (IL-1β, IL-6, and TGF-β) (Abbas, Lichtman et al. 2012). Proliferation of the tTG-specific T cells in response to tTG could be blocked with anti-HLA-DQ2, and culture supernatants from these cells were able to induce the histological
changes characteristic of CD in specimens of duodenal mucosa from treated CD patients (Ciccocioppo, Finamore et al. 2010).

A limitation of the study by Ciccocioppo et al. was that the effect of antigen stimulation on the activation state of the tTG-specific T cells was not measured. The expression of molecules such as HLA-DR and CD25 on the surface of T cells can provide evidence for specific stimulation in response to antigen (Caruso, Licenziati et al. 1997). HLA-DR and CD25 upregulation by gliadin-specific T cells upon gliadin stimulation has been noted in previous studies (O'Keeffe, Mills et al. 1999; Raki, Fallang et al. 2007). Further characterisation of the tTG-specific T cell, and the involvement of non-classical T-helper cytokines such as IL-17 and IL-21 may provide more information on the role of these cytokines in CD, especially given the conflicting reports as to the antigen-specific T cell source of IL-17, and the importance of IL-21 in autoantibody production. The demonstration of the existence of a cellular immune response to tTG also offers the opportunity to make a comparison of T and B cell epitopes or regions of the tTG protein that are important in both humoral, and cellular responses to tTG. As the active-site of tTG has been shown to be important for B-cell immunity in CD, an evaluation of the importance of this region of the protein in T cell responses to, and the antigenic processing of tTG may prove informative.

5.1.4 Flow Cytometry

Developed in the 1960’s and 1970’s, flow cytometry allows for the analysis of various properties of individual cells and particles suspended in a fluid (Melamed, Lindmo et al. 1990). A single cell suspension is required for flow cytometric analysis, which is aspirated into a flow cell. The fluid flows past a detector point, where the cells pass one or more focussed laser beams and are illuminated one at a time. As the laser beam strikes the
cells, the light is either absorbed or scattered in a forward and a side direction, depending on the internal structure of the cell and its size and shape (Shapiro 2003). The cells are usually labelled with fluorescence-conjugated probes (fluorochromes) which absorb light at appropriate wavelengths and subsequently fluoresce. Emitted light and scatter is captured, filtered based on wavelength, and subsequently converted into electronic signals proportional to the amount of light detected. These signals are converted to digital data, resulting in quantitative information about each cell analyzed. The forward light scatter provides information on cell size, while side scatter provides information on granularity. Marker expression may be determined by analysis of fluorescence intensity.

5.1.5 Chapter aims

The aim of this chapter was to examine the presence of tTG-specific T cells in patients with treated CD, and in control individuals. Proliferation of peripheral blood T cells to two commercial tTG preparations was assessed by tritiated thymidine incorporation in order to detect responsive individuals for tTG-specific T-cell line generation. The establishment of tTG-specific T-cell lines, which proliferate specifically in response to tTG allowed the assessment of the response to antigen stimulation in terms of the T cell activation markers CD25 and HLA-DR. Cytokine production by these T-cell lines was monitored, and intracellular cytokine staining was assessed as a possible measure of response to antigen stimulation. A further aim was the investigation of IL-17 and IL-21 production by tTG-specific T cells. In an attempt to compare T and B cell tTG epitopes, the ability of tTG-specific T cells to expand in response to the wild-type and mutant recombinant tTG proteins produced in Chapter 2 was compared.
5.2 Materials and methods

5.2.1 Patients

CD patients and controls were recruited from the Dept. of Immunology, St James’ Hospital Dublin. For proliferation assays, 26 CD patients, of whom 25 were treated, were used to measure PBMC reactivity to tTG. This patient group had a mean age of 58 years (range 29-83), and contained 16 females and 10 males. Proliferation in response to tTG was also assessed in a control group of EMA/tTG negative healthy laboratory workers (n=15), which had a mean age of 35 years (range 25-65), and contained 5 females and 10 males.

For short-term activation marker analysis, a group of ten CD patients, of whom nine were treated, was investigated. This group had a mean age of 57 years (range 25-83), and consisted of 9 females and one male. Six control individuals were analysed for short-term activation marker analysis in response to tTG; this EMA/tTG negative group consisted of 4 females and 2 males, and had a mean age of 30 years (range 23-39).

Ethical approval for this study was obtained from the ethics committee, St. James’ Hospital.

5.2.2 Antigen preparations

For proliferation assays, two differing commercial sources of tTG were used; a tTG purified from human erythrocytes, and a His-tagged recombinant human tTG produced in insect cells (Zedira). The erythrocyte tTG (RBC tTG) was a kind gift from Dr. Walter Binder of Inova Diagnostics Incorporated, and had been used in the study by Ciccocioppo and colleagues in the study identifying tTG-specific T cells in the periphery of CD patients (Ciccocioppo, Finamore et al. 2010). The recombinant human wt and CHDA tTGs
generated in Chapter 2 were also used in a small number of proliferation assays. All tTGs were used at a concentration of 10 μg/ml for each stimulation described in this chapter, as per Ciccocioppo et al (2010).

5.2.3 PBMC Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from patients and controls by density gradient centrifugation. Venous blood was collected into heparinised tubes, mixed 1:1 with PBS and layered onto a density gradient solution (Lymphoprep, Axis- Shield) at a ratio of 17mls blood to 7mls Lymphoprep. This mixture was then centrifuged at 400xg for 25 minutes at 4°C, with no brake. The resulting buffy coat was removed and washed twice in T cell medium (TCM, Appendix i) at 800xg for 7 minutes. After the second wash, the pelleted PBMCs were resuspended in 1ml TCM containing 5% autologous serum, with cellular numbers and viability subsequently assessed using ethidium bromide/acridine orange staining. The concentration of the PBMC-containing solution was then adjusted to $1 \times 10^6$/ml in TCM containing 5% autologous serum, before immediate use in culturing experiments. All work was conducted under sterile conditions.

5.2.4 Measurement of proliferation by $^3$H-thymidine incorporation

PBMCs from CD patients or control individuals were adjusted to a concentration of $5 \times 10^5$/ml in TCM containing 5% autologous serum. 200μl per well of this suspension was then cultured with the appropriate antigen in a round-bottomed 96-well microtitre plate, with cultures performed in triplicate. Wells containing medium and cells only were added in order to measure background proliferation, and calculate the stimulation index (SI). The tTG antigens used were RBC tTG (Inova), rh tTG (Zedeira), wt and CHD ΔtTGs.
synthesised in Chapter 2, all at a concentration of 10μg/ml. Phytohaemagglutinin (PHA) and purified protein derivative (PPD) of *Mycobacterium tuberculosis* at concentrations of 2 and 10 μg/ml, respectively, were used as positive proliferative control antigens. Cultures were then incubated at 37°C, in the presence of 5% CO₂ and a humidified atmosphere, for 6 days. 0.5μCi of ³H-thymidine (Perkin-Elmer) was added to each well for the last 18 hours of culture, with cells harvested on day 6. Proliferation was assessed by measuring the thymidine incorporation using a Microbeta Trilux scintillator (Perkin-Elmer). The SI was calculated by dividing the mean of the three cpm values for each antigen by that of the unstimulated wells, with an SI >2 being considered positive. In experiments measuring the proliferation of tTG-sensitised cell lines, the incubation time was shortened to 72 hours.

### 5.2.5 Flow cytometry

Flow cytometry was used to investigate the effect of tTG on the activation marker status of T cells. Following culture with the appropriate antigen, the cellular concentration was adjusted to 5x10⁵/ml, with 1ml of this solution transferred to fluorescence-associated cell sorter (FACS) tubes. The tube was then topped up with 1ml phosphate buffered saline containing 0.5% bovine serum albumin (PBA), and centrifuged at 1500rpm for 8 minutes. For exclusion of dead cells, 5μl of a 1:10 working solution of aqua fluorescent reactive dye (Invitrogen) was added to each tube, incubated at room temperature for 30 minutes, and washed with 2mls PBA at 1500rpm for 8 minutes. The pellet was then resuspended in 50μl PBA, the cell-surface antibodies (Table 5.1) were added, with the tubes then incubated at 4°C in the dark, for 10 minutes. Following this incubation, unbound antibody was washed away by the addition of 2mls PBA and centrifugation at 1500rpm for 8 minutes. In order to fix the cells, and prevent internalisation of cell-
surface markers, each sample was resuspended in 500µl of 4% paraformaldehyde (PFA, Sigma), and incubated in the dark for 10 minutes at room temperature. The cells were then washed by the addition of 2mls PBA and centrifugation at 1500rpm for 8 minutes. The supernatant was discarded, the cells resuspended in 500µl PBA, and immediately analysed using a Facscyan flow cytometer and Summit software. Gates were set using the strategy outlined in Appendix ii.

Table 5.1: The antibody panel used for T cell phenotype/activation marker staining.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Marker/Fluorochrome</th>
<th>Manufacturer</th>
<th>Amount used per tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL1 – FITC</td>
<td>HLA-DR FITC</td>
<td>Immunotools</td>
<td>5µl</td>
</tr>
<tr>
<td>FL2 – PE</td>
<td>CD25 PE</td>
<td>Immunotools</td>
<td>5µl</td>
</tr>
<tr>
<td>FL4 - PECy^5/PerCP</td>
<td>CD8 PECy^5</td>
<td>Biolegend</td>
<td>5µl</td>
</tr>
<tr>
<td>FL7 – Violet 2</td>
<td>AQUA 405 (Dead cell)</td>
<td>Invitrogen</td>
<td>5µl</td>
</tr>
<tr>
<td>FL8 - APC</td>
<td>CD4 APC</td>
<td>Biolegend</td>
<td>5µl</td>
</tr>
<tr>
<td>FL9 – APC/Cy^7</td>
<td>CD3 APC efluor780</td>
<td>eBioscience</td>
<td>5µl</td>
</tr>
</tbody>
</table>

5.2.6 Intracellular cytokine staining

Cytokine production by the tTG-specific T cells in response to antigenic stimulation was assessed by intracellular cytokine staining. In order to amplify cytokine production by the autoreactive T cells, 10ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) and 1µg/ml ionomycin (Sigma) were added for the last 10 hours of culture. Brefeldin A (BFA, Sigma), a fungal derived protein transport inhibitor that blocks cytokine release from the endoplasmic reticulum, was also added for the final 10 hours of culture, at a concentration of 10µg/ml. After this incubation, 5x10^5 cells were transferred to FACS tubes, topped up to a volume of 2mls with PBA, and centrifuged at 1500rpm for 8 minutes. The pellet was then resuspended in 50µl PBA, with dead cell, and cell-surface
staining for CD3 performed as per Section 5.2.5. Following fixation with 4% PFA, the cells were permeabilised by the addition of 1ml of 0.2% saponin (Sigma) for 10 minutes in the dark, topped up with 1ml PBA, and pelleted at 1500rpm for 8 minutes. The supernatant was discarded, the cells were resuspended in the residual saponin (approximately 50 μl) and incubated with the appropriate anti-cytokine monoclonal antibody (Table 5.2) for 25 minutes at 4°C, in the dark. Following this incubation, the cells were washed by the addition of 2mls PBA and centrifugation at 1500rpm for 8 minutes. The supernatant was discarded, the cells resuspended in 500μl PBA, and then immediately analysed using a Facscyan flow cytometer and Summit software. Gates were set using the strategy outlined in Appendix ii.

### Table 5.2: The antibody panel used for intracellular cytokine staining.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Marker/Fluorochrome</th>
<th>Manufacturer</th>
<th>Amount used per tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL1 – FITC</td>
<td>IFN-γ FITC</td>
<td>eBioscience</td>
<td>5μl</td>
</tr>
<tr>
<td>FL2 – PE</td>
<td>IL-21 PE</td>
<td>eBioscience</td>
<td>5μl</td>
</tr>
<tr>
<td>FL4 - PECy5/PerCP</td>
<td>IL-17A PerCPCy5.5</td>
<td>eBioscience</td>
<td>5μl</td>
</tr>
<tr>
<td>FL7 – Violet 2</td>
<td>AQUA 405 (Dead cell)</td>
<td>Invitrogen</td>
<td>5μl</td>
</tr>
<tr>
<td>FL8 – APC</td>
<td>IL-10 APC</td>
<td>Becton Dickinson</td>
<td>5μl</td>
</tr>
<tr>
<td>FL9 – APC/Cy7</td>
<td>CD3 APC efluor780</td>
<td>eBioscience</td>
<td>5μl</td>
</tr>
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</table>

#### 5.2.7 The effect of tTG on T cell activation

The *in vitro* effect of tTG on the activation of T cells in short-term PBMC cultures isolated from both CD patients and controls was investigated by comparing the levels the activation markers CD25 and HLA-DR between stimulated and stimulated and unstimulated CD3 positive cells. $1 \times 10^5$ PBMC from individuals with CD (n=10), or controls
(n=6) were cultured in a volume of 200µl in a 96-well round-bottomed microtitre plate. The antigens used were: RBC tTG (Inova), rh tTG (Zedeira), wt and CHD ΔtTGs synthesised in Chapter 2, all at a concentration of 10µg/ml, and 2 µg/ml PHA. Unstimulated cells were also plated in similar numbers to provide background levels of activation marker expression. Four wells of each antigen were plated, incubated at 37°C in the presence of 5% CO₂ and a humidified atmosphere for 72 hours, pooled and stained for flow cytometry as per Section 5.2.5.

5.2.8 Cell line generation

For the generation of tTG-specific cell lines, after initial experiments using smaller numbers of cells (200,000 per well in a 96 well round-bottomed plate due to sample constraints), it was decided to move to using a larger amount of cells in a 24 well plate in order to generate sufficient numbers of cells for downstream analysis. 1 ml of PBMCs at a concentration of 1x10⁶/ml in TCM containing 5% autologous serum, isolated from CD patients or control individuals, was incubated with RBC tTG at 10µg/ml at 37°C, in the presence of 5% CO₂ and a humidified atmosphere. Wells containing 1x10⁶ PBMC only were cultured as a negative control. After 5 days in culture, 20 IU/ml of recombinant human IL-2 (Sigma) was added to each well. On day 7, wells were visually examined for growth by comparing cellular numbers in stimulated and unstimulated wells using an inverted microscope, and observing a colour change from pink to yellow in the culture media. Where cellular growth had occurred, 500µl of supernatant was removed and frozen at -20°C, being replaced with fresh media. The cells were then cultured for a further 7 days, receiving 20 IU/ml IL-2 on day 9, examined on the inverted microscope, and counted.
For further expansion of cell lines, further stimulation with RBC tTG (Table 5.2.3) was performed on days 14 and 28 of culture, using irradiated autologous PBMCs as antigen-presenting cells. PBMCs were isolated as per Section 5.2.3, and adjusted to a concentration of 1x10^6/ml in TCM containing 5% autologus serum. RBC tTG was added to this solution at a concentration of 10μg/ml, and the cells were incubated for 4 hours at 37°C in the presence of 5% CO₂ and a humidified atmosphere. Following this incubation, the cells were washed by centrifugation at 1500rpm for 8 minutes to remove unprocessed antigen, resuspended in PBS, and irradiated at 40Gy. The cells were centrifuged at 1500rpm for 8 minutes, resuspended in TCM containing 5% autologus serum, counted, added to the tTG-stimulated cell line at a ratio of 1:1, and returned to culture. 20 IU/ml IL-2 was added to each culture on day 14, and weekly thereafter. At weekly intervals, 500μl of the culture supernatant was removed and stored at -20°C, being replaced with 500μl of TCM containing 5% autologus serum. The cells were counted again on day 28, with the concentration adjusted to 1x10^6/ml, and re-plated in a 24-well plate prior to re-stimulation.

In order to demonstrate the antigen-specificity of the cell lines generated, the proliferative, activation, and cytokine responses to stimulation with differing sources of tTG, and the control antigens PHA and PPD, were measured. Cellular proliferation was assessed by both ^3H-thymidine and Celltrace™ Violet (Invitrogen) incorporation, with cytokine responses measured by intracellular cytokine staining. These measurements were made on days 6, 17, and 31 of culture, the latter two being in response to the antigen-stimulation on days 14, and 28, respectively. The antigens used in the extended proliferation/stimulation assays are described in Table 5.3. For the measurement made on day 6 (i.e. the initial stimulation) the procedures were identical to those described in Sections 5.2.4/5/6. The re-stimulation performed on days 14 and 28 used cells from the
tTG-sensitised cell line. Five days before re-stimulation, the target cells were removed from the cell line, washed twice in TCM, and plated at 1x10^6/ml in a 24-well flat-bottomed plate to a 1ml volume, in order to starve them of IL-2 prior to their re-exposure to antigen. Irradiated autologus feeder cells pulsed with each test antigen were prepared, with 1x10^5 APCs added to 1x10^5 of the IL-2-starved cells from the cell line in a volume of 200μl in a 96-well round-bottomed microtitre plate. These suspensions were then returned to culture for a further 72 hours, and harvested as per Sections 5.2.4/5/6. In experiments measuring Celltrace™ proliferation, an extra step was performed prior to the addition of the APCs. 1μl of 5mM Celltrace™ Violet was added to each ml of the culture medium containing 1x10^6 cells, with the mixture incubated at 37°C for 20 minutes in the dark. Unbound dye was quenched by the addition of 5mls TCM and subsequent 5 minute incubation at 37°C in the dark. The cells were pelleted at 1500 rpm for 8 minutes, before plating for the proliferation/activation marker expression assay. For the ^3H-thymidine proliferation assays, measurements were made in triplicate; for the activation/Celltrace™ proliferation and intracellular cytokine staining four wells each were plated. The wells containing cells from the T-cell line and feeder cells only were used to calculate the background level of proliferation, activation marker, and cytokine expression.
Table 5.3: The extended proliferation assay performed on samples of cells from the tTG-specific T-cell lines.

- Unstimulated cells only
- Unstimulated cells + Feeders only (no Ag)
- tTG stimulated cells + Feeders Only (no Ag)
- tTG stimulated cells + Feeders (RBC tTG)
- tTG stimulated cells + Feeders (rh tTG)
- tTG stimulated cells + Feeders (wt tTG)
- tTG stimulated cells + Feeders (CHDA tTG)
- tTG stimulated cells + Feeders (PPD)
- tTG stimulated cells + Feeders (PHA)

A sample of cells from each cell line was tested for proliferation in response to varying tTG antigens, PHA, and PPD. Cultures were incubated for 72 hours, before being harvested, with proliferation measured by both $^3$H-thymidine and Celltrace™ Violet incorporation.

5.2.9 Measurement of cytokines by ELISA

The cytokines IFN-γ, IL-4, IL-10, IL-17A, and IL-21 were measured using Biolegend ELISA kits, as per the manufacturer's protocols.
5.3 Results

5.3.1 Proliferative responses to tTG detected by $^3$H-thymidine incorporation

The capacity of PBMCs from 26 CD patients and 15 controls to proliferate in response to various tTG preparations was assessed by $^3$H-thymidine incorporation. Table 5.4 details the PBMC responses of the CD group to RBC tTG, human recombinant tTG (hr tTG), wt tTG, CHDA tTG, and PHA, which was used as a positive control in all proliferation assays, whilst table 5.5 summarises this information for control individuals.

Table 5.4: Proliferative responses to the different tTG preparations in CD patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age</th>
<th>tTG (EMA)</th>
<th>RBC tTG (SI)</th>
<th>rh tTG (SI)</th>
<th>wt tTG (SI)</th>
<th>CHDA tTG (SI)</th>
<th>PHA (SI)</th>
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</thead>
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<td>564.2</td>
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<td>-</td>
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<td>-</td>
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<td>74.8</td>
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</table>

Positive proliferation in response to any of the tTG preparations is indicated in red. SI represents stimulation index, values ≥ 2 were considered positive. – indicates not tested.
For the RBC tTG antigen, proliferation was detected in 7/25 (28%) CD patients, and 2/15 (13%) of controls tested (Fig 5.1). The mean SI induced by RBC tTG was 1.7 units in CD patients, and 1.4 units in controls, with no statistically significant difference in reactivity observed between the two groups (p= 0.4582, Mann-Whitney test). A similar response to the other commercial tTG preparation, rh tTG, was noted, with positivity detected in 5/15 (30%) of CD patients, and 1/10 (10%) of controls tested (Fig 5.3.1). The mean SI response to rh tTG was 1.5, and 1.0 units for CD patients and controls, respectively, a difference that did not reach statistical significance (p= 0.1427, Mann-Whitney test).

Table 5.5: Proliferative responses to the different tTG preparations in controls

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age</th>
<th>tTG (EMA)</th>
<th>RBC tTG (SI)</th>
<th>rh tTG (SI)</th>
<th>wt tTG (SI)</th>
<th>CHDA tTG (SI)</th>
<th>PHA (SI)</th>
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<td>0.9</td>
<td>0.8</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Positive proliferation in response to any of the tTG preparations is indicated in red. SI represents stimulation index, values > 2 were considered positive. – indicates not tested.

Of the 15 CD patients tested with both commercial tTG antigens, 2 proliferated to RBC tTG only, 1 to rh tTG only, and 4 proliferated to both preparations. Ten control individuals were tested with both commercial tTG antigens, 2 were positive for RBC tTG.
only, while 1 was positive for rh tTG only, with no individual proliferating to both antigens.

![Figure 5.1: Proliferative responses to RBC tTG and rh tTG.](image)

The ability of the recombinant tTG proteins generated in Chapter 2 to stimulate proliferation of PBMCs from CD patients and controls was also measured by $^3$H-thymidine incorporation. A proliferative response to wt tTG was detected in 6/10 (60%) of CD patients, and 1/5 (20%) of controls (Fig 5.2). Of the 6 wt tTG positive CD patients, only 2 were positive for RBC or rh tTGs, whilst the 1 positive control individual also proliferated to RBC tTG.
Figure 5.2: Proliferative responses to RBC and wt tTG. Proliferation was measured by $^3$H-thymidine incorporation in CD patients (a) and controls (b). The cut-off, an SI ≥ 2, is indicated by the dashed line.

As the experimental work described in Chapter 3 highlighted the importance of the tTG active-site as a B cell epitope, it was decided to compare the PBMC stimulatory capacity of the triple active-site mutant CHDA tTG with that of the wild-type protein in order to assess the tTG core region as a potential T cell epitope. This comparison was made in 5 CD patients, 4 of whom were positive for proliferation to wt tTG, and in 5 controls, of which 1 was positive for wt tTG. Figure 5.3 illustrates the results of these experiments, in which proliferation in response to CHDA tTG was maintained, or in some individuals enhanced, despite the amino acid substitutions at the tTG active-site. Of the four wt tTG responsive CD patients, SI positivity to CHDA tTG was maintained in 3, whilst the fourth was just below the cut-off (SI 1.9 units, Fig 5.3a). A proliferative response to CHDA tTG was also observed in a control individual who did not proliferate to the RBC, rh, or wt tTG antigens (Fig 5.3b).
Figure 5.3: Proliferative responses to wt and CHDA tTG. Proliferation was measured by $^3$H-thymidine incorporation in CD patients (a) and controls (b). The cut-off, an SI ≥2, is indicated by the dashed line.
5.3.2 Activation of T cells by tTG

The effect of the differing tTG preparations on the activation state of PBMC in short-term (72 hours) cultures was investigated in both CD patients (n=10) and controls (n=6). For these experiments PHA was used as a positive control, causing a significant increase in the percentage of CD3+ cells expressing either CD25 (p <0.0001 for patients, p=0.0198 for controls, paired t test) or HLA-DR (p=0.0046 for patients, 0.0306 for controls, paired t test) (Fig 5.4), when compared to values for unstimulated cells.

Fig 5.4: The effect of PHA on CD25 and HLA expression by T cells from both CD patients and controls. PHA was used as a positive control for CD25 (a) and HLA-DR (b) expression in 72 hour cultures to investigate activation marker expression.

In general there was a trend towards increased CD25 expression by T cells from the PBMC of the CD patients (Fig 5.5a), who with the exception of patient 10, were all treated; however due to the variability of response a significant increase was caused by rh tTG only (p=0.0034, paired t test). This effect was not as pronounced in the control population (Fig 5.5b), however wt tTG caused a significant increase in the percentage of CD3/CD25 positive cells (p=0.0022, paired t test).
Figure 5.5: The effect of short-term culture on CD25 expression by CD and control T cells. PBMC were cultured with the differing tTG sources for 72h. (a) represents the results for CD patients; (b) represents the results for control individuals. A typical plot generated from such an experiment is detailed in (c), which represents CD patient 5.

HLA-DR expression by CD3+ cells, in response to tTG was more varied, with increases seen in fewer patients, such as patients 3 and 5 (Fig 5.6a). A significantly increased...
percentage of CD3/HLA-DR positive cells in CD patients was seen in response to rh tTG in both patients and controls (p=0.0479, 0.0204, respectively, paired t test).

![Graph showing HLA-DR expression by CD and control T cells.](image)

**Figure 5.6:** The effect of short-term culture on HLA-DR expression by CD and control T cells. PBMC were cultured with the differing tTG sources for 72h. (a) represents the results for CD patients; (b) represents the results for control individuals. A typical plot generated from such an experiment is detailed in (c), which represents CD patient 3.
5.3.3 Generation of tTG-specific T-cell lines

By stimulation with RBC tTG, three T-cell lines were derived from the peripheral blood of a treated CD patient, and two responsive controls. The three individuals were identified as tTG-responsive in \(^3\text{H}\)-thymidine incorporation assays, where a PBMC proliferative response to RBC tTG was detected.

5.3.3a CD01 T-cell line

Cell line CD01 was generated from a patient with treated CD who was identified via reactivity to all tTGs tested (RBC tTG, rh tTG, wt tTG, and CHDΔ tTG) in initial 6 day \(^3\text{H}\)-thymidine incorporation assays. In short-term culture also, RBC and wt tTGs increased HLA DR expression after 3 days in this patient, whereas rh tTG caused CD25 up-regulation (Fig 5.5a/6a, patient number 6). The patient, a male of 51 years, had biopsy-confirmed CD (1999), and was on a long-term gluten-free diet, as evidenced by serial tTG/EMA negativity since 2003.

The cell line was established through stimulation with the RBC tTG antigen, as per Section 5.2.8. Upon weekly inspection under the inverted microscope, the cells had a lymphoid morphology, with increasing ‘clustered’ appearance and blast formation over the length of culture, and post re-stimulation with RBC tTG. Cell counts were performed on days 14 and 28, prior to re-stimulation with irradiated autologus APCs that had been pre-pulsed with RBC tTG. The results of the cell counts can be seen in Table 5.6, which details the expansion of cellular numbers in response to RBC tTG stimulation.
Table 5.6: Cell counts of the CD01 T-cell line.

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<th>Well</th>
<th>Day 14 count (cells/ml)</th>
<th>Day 28 count (cells/ml)</th>
</tr>
</thead>
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<td>$0.80 \times 10^6$</td>
</tr>
<tr>
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<td>$2.71 \times 10^6$</td>
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<tr>
<td>2</td>
<td>$1.97 \times 10^6$</td>
<td>$2.25 \times 10^6$</td>
</tr>
<tr>
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<td>$1.89 \times 10^6$</td>
<td>$3.35 \times 10^6$</td>
</tr>
<tr>
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</tr>
<tr>
<td>5</td>
<td>$2.64 \times 10^6$</td>
<td>$3.15 \times 10^6$</td>
</tr>
<tr>
<td>6</td>
<td>$1.99 \times 10^6$</td>
<td>$2.44 \times 10^6$</td>
</tr>
<tr>
<td>7</td>
<td>$2.12 \times 10^6$</td>
<td>$2.62 \times 10^6$</td>
</tr>
<tr>
<td>8</td>
<td>$2.37 \times 10^6$</td>
<td>$3.01 \times 10^6$</td>
</tr>
</tbody>
</table>

Upon flow cytometric analysis, the cell line was predominantly CD4+, with the percentage of CD4+ cells increasing with time in culture (Fig 5.7). Whilst the percentage of CD8+ cells diminished over the culture period, that of CD4/CD8 double positive cells also increased (Fig 5.7).

Figure 5.7: The phenotype of T cells from the CD01 T-cell line over time. Samples of the cell line were stained by flow cytometry on day 6, and prior to the re-stimulations on days 14 and 28.
Proliferative responses of the T-cell line CDO1

In order to confirm the antigen-specificity of the cell line, re-stimulation with the antigen used to establish the cell line (RBC tTG), and an extended panel of tTG antigens was performed, with responsiveness measured by $^3$H-thymidine incorporation. At each re-stimulation (days 14 and 28), a proliferative response to all tTG proteins was detected, with the strongest response being re-stimulation 2 (day 14, Fig. 5.8). Although the cell line had been established using RBC tTG, it had the ability to proliferate in response to all tTG antigens tested, including CHDΔ tTG. After the initial stimulation, CD01 lost responsiveness to the protein antigen PPD, indicating a positive selection of tTG-specific cells during culture (Fig. 5.8).

![Graph](image)

**Figure 5.8:** The proliferation of cells from CD01 as measured by $^3$H-thymidine incorporation. Responses were measured after 6 days in culture for stimulation 1, and after 3 days post stimulations 2 (day 14) and 3 (day 28). SI indicates the stimulation index which was calculated using unstimulated PBMC for stimulation 1, and samples of CD01 and irradiated autologus feeder cells only for stimulations 2 and 3. The dashed line indicates the cut-off for positivity, an SI >2.
PHA was used as a positive control for each stimulation; however by stimulation 3 (day 28) the level of proliferation of cells from CD01 induced by PHA had diminished (Fig. 5.8). In conjunction with activation marker analysis, cellular proliferation of CD01 to RBC tTG and PHA was also measured by Celltrace™ Violet incorporation. At each round of stimulation, there was a decrease in mean fluorescence intensity (MFI) of staining of CD3+ T cells in the RBC tTG-stimulated samples when compared to that of unstimulated values, corresponding to cellular proliferation (Table 5.7, Fig 5.9). PHA, used as a positive control, induced the greatest decrease in MFI for all three stimulations.

**Table 5.7: Results of Celltrace™ Violet proliferation assays for the CD01 T-cell line**

<table>
<thead>
<tr>
<th>Sample</th>
<th>MFI Stimulation 1 (day 0)</th>
<th>MFI Stimulation 2 (day 14)</th>
<th>MFI Stimulation 3 (day 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>783</td>
<td>130</td>
<td>1942</td>
</tr>
<tr>
<td>RBC tTG</td>
<td>747</td>
<td>83</td>
<td>671</td>
</tr>
<tr>
<td>PHA</td>
<td>190</td>
<td>52</td>
<td>552</td>
</tr>
</tbody>
</table>

MFI indicates mean fluorescence intensity, which has been rounded to the nearest whole number. Stimulation 1 was performed for 6 days; stimulations 2 and 3 were performed for 3 days. Unstimulated values in stimulations 2 and 3 were calculated using cells from CD01 and irradiated autologus feeder cells only. Cells were gated on CD3 positivity.

**Figure 5.9: Overlay histograms generated in Celltrace™ Violet proliferation experiments using CD01.** The dashed line represents the unstimulated values, whilst the continuous line represents
RBC tTG, and the grey shaded area PHA. Stimulations 1, 2, and 3 are represented by a, b, and c, respectively. Cells were gated on CD3 positivity.

The effect of re-stimulation on CD25 and HLA-DR expression by CD01

The effect of each round of antigenic stimulation on CD25 and HLA-DR expression by the T-cell line CD01 was investigated. The baseline level of both of these T cell activation markers was established after the first stimulation (day 6, Fig 5.10a), where there was no difference observed between the unstimulated and RBC tTG stimulated CD3/CD25+ positive cells. Increased CD25 expression by CD3+ T cells in response to rh and wt tTGs was noted on day 6 (Fig 5.10a). In the re-stimulation experiment performed on day 14, all of the tTG antigens tested induced CD25 expression by CD3+ cells, whereas the negative control antigen PPD had no effect (Fig 5.10a). The third and final re-stimulation of CD01 was accompanied by increased numbers of CD3/CD25+ cells in response to RBC, wt, and CHDA tTGs, an effect that was not observed in response to rh tTG or PPD (Fig 5.10a). At each round of stimulation, PHA induced high levels of CD25 expression on CD3+ cells from the CD01 cell line. From Fig 5.10, it can be clearly seen that the level of CD25 increased with time in culture, possibly due to the combined effects of IL-2 in the culture media and further selection of the tTG-specific T cells. Further analysis of the CD25-expressing T cells from CD01 showed them to be almost exclusively CD3/CD4+ (Fig 5.10c).
Figure 5.10: The effect of tTG stimulation on CD25 expression by T cells from CD01. (a): Stimulation with the differing tTG preparations, PHA, and PPD were performed on days 0, 14, and 28. For stimulation 1, unstimulated PBMC were used to calculate background levels of CD25; for stimulations 2 and 3, cells from the CD01 cell line and feeder cells only were used. (b) represents a typical plot generated, in this case CD01 stimulation 3, whereas (c) from the same experiment demonstrates the up-regulation of CD25 by CD4+ cells, which had been gated on CD3.
HLA-DR expression by T cells from the periphery of the treated CD patient was upregulated in response to RBC, rh, and CHDΔ tTG stimulation after day 6, and in each round of re-stimulation with RBC tTG (Fig 5.11a). Increased numbers CD3/HLA-DR positive cells were observed in response to wt tTG stimulation on days 14 and 28, with CHDΔ tTG causing an increase on the day 14 stimulation (Fig 5.11a). PHA was used as a positive control for each round of stimulation, and in each case induced CD25 expression by T cells from CD01, whilst responsiveness to PPD was lost by the second stimulation (Fig 5.11a). A typical flow plot generated in re-stimulation experiments is detailed in Fig 5.11b. The T cells up-regulating HLA-DR in response to tTG stimulation were predominantly CD4+, an example of which is contained in Fig 5.11c. As with CD25, increasing background levels of HLA-DR expression were observed over the duration of the culture.
Figure 5.11: The effect of tTG stimulation on HLA-DR expression by T cells from CD01. (a): Stimulation was performed on days 0, 14, and 28. For stimulation 1, unstimulated PBMC were used to calculate background levels; for stimulations 2 and 3, cells from the CD01 cell line and feeder cells only were used. (b) represents a typical plot generated, in this case CD01 stimulation 2, (c) generated in the same experiment demonstrates the up-regulation of HLA-DR by CD4+ cells, which had been gated on CD3.
Cytokine production by CD01

The culture supernatants of the CD01 T-cell line were sampled weekly (immediately prior to re-stimulation on days 14 and 28) and analysed for secreted cytokines by ELISA. Wells containing PBMC in medium only were used to establish levels of spontaneous cytokine production. These cells were then treated in an identical fashion to the cell line with regard to feeding and the addition of IL-2 to the culture medium, and at each round of stimulation received irradiated autologous feeder cells, without antigen.

The hallmark of CD01 was the production of large amounts of IFN-γ, which reached levels of 4,000 pg/ml after the second round of stimulation with RBC tTG (Fig 5.12a). IFN-γ production peaked in the sample taken on day 21, reflecting the week following the first re-stimulation of the cell line with RBC tTG, but remained present at high levels for the duration of the culture. IL-10 was produced after the initial stimulation only, and did not appear in the culture medium until at least 7 days after this stimulation (Fig 5.12b). There was a consistent absence of IL-4 in the culture supernatants throughout the culture period, as detailed in Fig 5.12c. Low levels (200-500 pg/ml) of IL-17A were produced by CD01 from the initial stimulation onwards, an effect not seen in the antigen-free cultures (Fig 5.12d).

The initial stimulation with RBC induced the production of high levels of IL-21 by CD01, compared to trace amounts of spontaneous production (Fig 5.12e). There was a lull in IL-21 production by the cell line for the following two weeks of culture, with levels again rising following stimulations 2 and 3.
Figure 5.12: Cytokine production by the T-cell line CD01. The culture supernatants of CD01 were sampled weekly, and the levels of cytokines measured by ELISA. Medium indicates an equivalent amount of PBMC cultured in an identical fashion, but without antigen.
The effect of re-stimulation on intracellular cytokine production by CD01

The production of the cytokines IFN-γ, IL-10, IL-17A, and IL-21 by T cells from the CD01 cell line in response to stimulation with the various tTG preparations, PHA, and PPD was further analysed by intracellular cytokine staining. Increases in the amount of IFN-γ production were seen at each round of stimulation, for all tTG antigens tested (Fig 5.13a), with the highest levels usually induced by RBC tTG, the preparation from which the cell line was established.

**Figure 5.13: Intracellular IFN-γ production by the T-cell line CD01 as a response to stimulation.**
(a): Stimulation with the differing tTG preparations, PHA, and PPD were performed on days 0, 14, and 28. For stimulation 1, unstimulated PBMC were used to calculate background levels of IFN-γ production; for stimulations 2 and 3, cells from the CD01 cell line and feeder cells only were used to calculate background. A typical flow cytometry plot generated in such an experiment is detailed in (b), which represents stimulation 3.
PHA or PPD failed to induce levels of IFN-γ above that of the cell line and feeders only culture which was used to establish baseline levels of cytokine secretion for comparison with antigen-stimulated cultures (Fig 5.13a/b). Stimulation 3 (day 28) with RBC tTG induced the production of IL-10 and IL-17A by T cells from the CD01 cell line (Fig 5.14a/b). PHA had mixed effects as a positive control, inducing IL-10 production at stimulation 2, and IL-17A production at stimulation 1, whereas PPD had no effect on the production of either of these cytokines by CD01 (Fig 5.14a/b).

An increase in the percentage of IL-21 producing T cells was seen in response to stimulation 1 (Fig 5.14c/d), which correlated with the detection of this cytokine by ELISA in the day 7 culture media (Fig 5.12e). By stimulation 3, the background level of IL-21 positivity by T cells from the CD01 cell line had reached levels of 23%, increasing to 25% for the RBC tTG stimulated sample, again reflecting the presence of this cytokine in the latter stages of culture. PHA was effective in increasing the amount of CD3/IL-21 positive cells seen after stimulations 1, 2 and 3, whilst PPD had no effect (Fig 14.xc/d).
Figure 5.14: Intracellular IL-10, IL-17, and IL-21 production by the T-cell line CD01 as a response to stimulation. Levels of IL-10 (a), IL-17 (b), and IL-21 (c) were assessed in response to stimulations performed on days 0, 14, and 28. For stimulation 1, unstimulated PBMC were used to calculate background levels of IFN-γ production; for stimulations 2 and 3, cells from the CD01 cell line and feeder cells only were used to calculate background. A representative flow plot, in this case IL-21, stimulation 1, is shown in (d).
5.3.3b CT01 T-cell line

Cell line CT01 was generated from a non-coeliac control individual, a male of 32 years of age whose cells initially proliferated to RBC, wt, and CHDΔ tTGs (Stimulation 1, Fig 5.16). The RBC tTG antigen was again used to generate the T-cell line. During culture, the cells had a lymphoid morphology, with blast formation observed after re-stimulation with RBC tTG. The cell numbers expanded during culture, the results of the cell counts performed on days 14 and 28, prior to re-stimulation, can be seen in Table 5.7. PBMC cultured in medium alone, receiving feeder cells only at stimulations 2 and 3 were used to assess spontaneous cellular growth.

<table>
<thead>
<tr>
<th>Well</th>
<th>Day 14 count (cells/ml)</th>
<th>Day 28 count (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>0.56x10^6</td>
<td>0.88x10^6</td>
</tr>
<tr>
<td>1</td>
<td>2.12x10^6</td>
<td>2.26x10^6</td>
</tr>
<tr>
<td>2</td>
<td>3.21x10^6</td>
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<td>2.75x10^6</td>
<td>3.16x10^6</td>
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<td>7</td>
<td>2.52x10^6</td>
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</tr>
<tr>
<td>8</td>
<td>2.80x10^6</td>
<td>2.88x10^6</td>
</tr>
</tbody>
</table>

The T cell line expressed CD4 at increasing percentages of CD3+ cells over the duration of the culture, rising from 60% to 81% from day 6 to day 28, with a reciprocal decrease in the percentage of CD8+ T cells from 35% to 20% (Fig 5.15). The percentage of CD4/CD8
double-positive T cells increased from 0.35 on day 6, to 1.09 on day 14, and 2.54 on day 28 (Fig. 5.15).

![Figure 5.15: The phenotype of T cells from the CT01 cell line over time. Samples of the cell line were stained for flow cytometry on day 6, and prior to the re-stimulations on days 14 and 28.](image)

**Proliferative responses of the T-cell line CT01**

At each re-stimulation (days 14 and 28), a proliferative response to the RBC, rh, and wt tTG proteins was detected, as measured by $^3$H-thymidine incorporation, with the strongest response again detected at re-stimulation 2 (day 14, Fig. 5.16). Although the cell line had been established using RBC tTG, by stimulation 2 it had the ability to proliferate in response to all tTG antigens tested, including CHDΔ tTG. After the initial stimulation, PPD responsiveness was lost, again indicating a positive selection of tTG-specific cells during culture (Fig. 5.16). PHA was used as a positive control for each stimulation, with the strongest response seen at stimulation 2; however in a similar fashion to the CD01 cell line, by stimulation 3 (day 28) the level of proliferation of cells from CT01 induced by PHA was reduced (Fig. 5.16).
Figure 5.16: The proliferation of cells from CT01 as measured by $^3$H-thymidine incorporation. Responses were measured after 6 days in culture for stimulation 1, and after 3 days post stimulations 2 (day 14) and 3 (day 28). SI indicates the stimulation index which was calculated using unstimulated PBMC for stimulation 1, and samples of CD01 and irradiated autologus feeder cells only for stimulations 2 and 3. The dashed line indicates the cut-off for positivity, an SI >2.

The proliferation of CD3+ cells from CT01 in response to RBC tTG and PHA was also demonstrated by Celltrace™ Violet incorporation. At all three rounds of stimulation, there was a decrease in mean fluorescence intensity (MFI) in the RBC tTG-stimulated samples when compared to that of unstimulated values, corresponding to a positive proliferative response (Table 5.8, Fig 5.17). In these experiments, PHA was the strongest stimulus for proliferation.
Table 5.8: Results of Celltrace™ Violet proliferation assays for the CTO1 T-cell line

<table>
<thead>
<tr>
<th>Sample</th>
<th>MFI Stimulation 1 (day 0)</th>
<th>MFI Stimulation 2 (day 14)</th>
<th>MFI Stimulation 3 (day 28)</th>
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</thead>
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<tr>
<td>Unstimulated</td>
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<td>416</td>
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<tr>
<td>RBC tTG</td>
<td>937</td>
<td>270</td>
<td>145</td>
</tr>
<tr>
<td>PHA</td>
<td>478</td>
<td>180</td>
<td>87</td>
</tr>
</tbody>
</table>

MFI indicates mean fluorescence intensity, which has been rounded to the nearest whole number. Stimulation 1 was performed for 6 days, whilst stimulations 2 and 3 were performed for 3 days. Unstimulated values in stimulations 2 and 3 were calculated using cells from CTO1 and irradiated autologous feeder cells only. Cells were gated on CD3 positivity.

Figure 5.17: Overlay histograms generated in Celltrace™ Violet proliferation experiments using CTO1. The dashed line represents the unstimulated values, whilst the continuous line represents RBC tTG, and the grey shaded area PHA. Stimulations 1, 2, and 3 are represented by a, b, and c, respectively. Cells were gated on CD3 positivity.
The effect of re-stimulation on CD25 and HLA-DR expression by CT01

No difference in the level of CD3+ CD25 or HLA-DR expressing cells between RBC tTG-stimulated and unstimulated cells was observed after the first stimulation (day 6, Fig 5.18a/19a). All of the tTG antigens tested in the re-stimulation experiment performed on day 14 induced CD25 expression by CD3+ cells, whereas the negative control antigen PPD had no effect (Fig 5.18a). The third re-stimulation of CT01 was accompanied by increased numbers of CD3/CD25+ cells in response to wt, and CHDΔ tTGs only (Fig 5.18a). At each round of stimulation, PHA induced high levels (25-55%) of CD25 expression on CD3+ cells from the CD01 cell line. As with the cell line CD01, CD25 up-regulation in response to tTG stimulation occurred on CD4+ cells, and baseline expression levels of CD25 increased with time in culture.
RBC tTG caused an increase in the percentage of HLA-DR expressing T cells in both re-stimulation experiments (Stimulations 2 and 3, Fig 5.19a). At stimulation 2, all of the tTG antigens tested caused an increase in HLA-DR expressing T cells, whilst this effect was replicated at the third stimulation by RBC and CHDΔ tTGs only. HLA-DR up-regulation on cells from CT01 was a CD4+ T cell specific phenomenon. Of the control stimuli, PHA induced HLA-DR expression at each round of stimulation, whilst PPD was effective in the
initial stimulation only (Fig 5.19a). The data generated from a typical re-stimulation experiment is shown in Fig 5.19b, in this case stimulation 2.

Figure 5.19: The effect of tTG stimulation on HLA-DR expression by T cells from CT01. (a): Stimulation was performed on days 0, 14, and 28. For stimulation 1, unstimulated PBMC were used to calculate background levels; for stimulations 2 and 3, cells from the CT01 cell line and feeder cells only were used. (b) represents a typical plot generated, in this case CT01 stimulation 2.
Cytokine production by CT01

During the first two weeks of culture, there was spontaneous production of IL-10 by both the unstimulated and CT01 cells (Fig 5.20b), and production of low levels of IL-21 in response to the initial stimulation with RBC tTG (Fig 5.20e). In the week following the second stimulation of the cell line with RBC tTG, moderate levels (1500 pg/ml) of IFN-γ were produced, which remained a feature of the cell line for the rest of the culture (Fig 5.20a). Large amounts of IL-17A were produced from the second stimulation onwards, as were smaller amounts of IL-21 (Fig 5.20 d/e). IL-4 was generally not detected in the culture media of CT01, with the exception of trace amounts contained in the supernatant sampled on day 21 (Fig 5.20c). Whilst the levels of IFN-γ decreased in the final 2 weeks of culture, high levels of IL-10 were detected in this period (Fig 5.20a/b).
Figure 5.20: Cytokine production by the T-cell line CT01. The culture supernatants of CD01 were sampled weekly, and the levels of cytokines measured by ELISA. Medium indicates an equivalent amount of PBMC cultured in an identical fashion, but without antigen.
The effect of re-stimulation on intracellular cytokine production by CT01

The production of IFN-γ was an effective marker for stimulations 2 and 3 of the CT01 T-cell line, with all tTGS tested in stimulation 2 inducing IFN-γ production by CD3+ cells, and RBC and rh tTGS replicating this effect in stimulation 3 (Fig 5.21a/b). The timeline appearance of IFN-γ in the culture supernatants of the cell line correlated with the absence of an intracellular IFN-γ response to stimulation 1.

Figure 5.21: Intracellular IFN-γ production by the T-cell line CT01 as a response to stimulation. (a): Stimulation with the differing tTG preparations, PHA, and PPD was performed on days 0, 14, and 28. For stimulation 1, unstimulated PBMC were used to calculate background levels of IFN-γ production; for stimulations 2 and 3, cells from the CT01 cell line and feeder cells only were used to calculate background. A typical flow cytometry plot generated in such an experiment is detailed in (b), which represents stimulation 2.
PHA increased the percentage of CD3/IFN-γ positive cells at stimulations 1 and 3, whilst PPD caused a mild increase in the same rounds of stimulation (Fig 5.21a/b). Increases in CD3/IL-10 positive cells were seen in response to stimulation 2 with RBC and rh tTGs, and stimulation 3 with RBC tTG (Fig 5.22a). The percentage of IL-10 positive T cells increased with the culture length, reflecting the appearance of this cytokine in the cell line culture medium. PHA was effective in inducing intracellular IL-10 production by T cells from CT01 at increasing levels per each round of stimulation, whilst PPD induced IL-10 production at stimulation 2 only (Fig 5.22a).

Intracellular IL-17A production by cells from CT01 occurred in response to stimulation 1 with RBC tTG and PHA, and stimulation 3 with RBC, rh, and wt tTGs (Fig 5.22b). The background level of CD3/IL-17A positive cells from CT01 reached 6% by stimulation 3, mirroring the high IL-17A levels detected in supernatants from the cell line culture medium (Figs 5.20d, 5.22b). Stimulation 3 induced intracellular IL-21 production, in response to RBC, rh, and wt tTGs (Fig 5.22c/d), with the high level of CD3/IL-21 positivity in samples from the cell line CT01 at this stimulation reflected by the detection of this cytokine in the cell line supernatants on day 35 (Fig 5.20e). At each round of stimulation, PHA was effective at inducing intracellular IL-21 production by CD3+ cells from CT01, whilst PPD did not induce IL-21 production above background levels (Fig 5.22c/d).
Figure 5.22: Intracellular IL-10, IL-17, and IL-21 production by the T-cell line CT01 as a response to stimulation. Levels of IL-10 (a), IL-17 (b), and IL-21 (c) were assessed in response to stimulations performed on days 0, 14, and 28. For stimulation 1, unstimulated PBMC were used to calculate background levels of IFN-γ production; for stimulations 2 and 3, cells from the CT01 cell line and feeder cells only were used to calculate background. A representative flow plot, in this case IL-21, stimulation 3, is shown in (d).
5.3.3c CT02 T-cell line

Cell line CT02 was also generated from a non-coeliac control individual, a male of 56 years of age who initially proliferated to RBC tTG only (Stimulation 1, Fig 5.24a), with this antigen again used to generate the T-cell line. The cells of CT02 had the typical lymphoid morphology, with blast formation, that was also observed for the other T-cell lines during culture. The results of the cell counts performed on days 14 and 28 prior to re-stimulation, detail the expansion of CT02, and can be seen in Table 5.9. PBMC cultured in medium alone, receiving feeder cells only at stimulations 2 and 3 were used to assess spontaneous cellular growth.

Table 5.9: Cell counts of the CT02 T-cell line.

<table>
<thead>
<tr>
<th>Well</th>
<th>Day 14 count (cells/ml)</th>
<th>Day 28 count (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
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<td>0.34x10^6</td>
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</tr>
<tr>
<td>7</td>
<td>2.85x10^6</td>
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</tr>
<tr>
<td>8</td>
<td>1.88x10^6</td>
<td>3.08x10^6</td>
</tr>
</tbody>
</table>

Upon flow cytometric analysis on days 6, 14, and 28, the T cells from CT02 were found to be predominantly CD4+, remaining at around 60% for the duration of the culture period (Fig 5.23). The percentage of CD8+ T cells expanded slightly from 30-40% over the
duration of the culture (Fig 5.23). This was unlike the previous two T-cell lines, CD01 and CT01, which displayed increasing percentages of CD4+ cells with culture length. However, in a similar fashion to the other two cell lines, the percentages of CD4/CD8 double-positive T cells in CT02 rose from 0.89% on day 6 to 3.44% on day 14, and 4.04 on day 28 (Fig 23.X).

Figure 5.23: The phenotype of T cells from the CT02 cell line. Samples of the cell line were stained for flow cytometry on day 6, and prior to the re-stimulations on days 14 and 28.
Proliferative responses of the T-cell line CT02

At each re-stimulation (days 14 and 28), a proliferative response to all of the tTG proteins tested was detected, in $^3$H-thymidine incorporation assays, with similar levels of responsiveness noted at re-stimulations 2 and 3 (Fig. 5.24.X). Given that the cell line had been established using RBC tTG, and that PBMC from this individual proliferated to this antigen only at stimulation 1, it is interesting that by stimulation 2 the T-cell line had the ability to proliferate in response to all tTG antigens tested, including CHDΔ tTG. The proliferation of cells from the cell line to all of the tTG antigens also replicated at stimulation 3. High levels of proliferation in response to PHA seen at each round of stimulation, whilst following the initial stimulation, PPD responsiveness was lost (Fig. 5.24).

![Figure 5.24](image)

**Figure 5.24:** The proliferation of cells from CT02 as measured by $^3$H-thymidine incorporation. Responses were measured after 6 days in culture for stimulation 1, and after 3 days post stimulations 2 (day 14) and 3 (day 28). SI indicates the stimulation index which was calculated using unstimulated PBMC for stimulation 1, and samples of CD02 and irradiated autologus feeder cells only for stimulations 2 and 3. The dashed line indicates the cut-off for positivity, an SI >2.
Proliferation by T cells from the CT02 cell line in response to RBC tTG, as measured by Celltrace™ Violet incorporation, occurred at the initial stimulation, and the two subsequent re-stimulations, where the decrease in MFI was more pronounced (Table 5.10, Fig 5.25). In a similar fashion to experiments using the other two T-cell lines CD01 and CT01, PHA induced the strongest proliferative response by CD3+ cells in this assay.

Table 5.10: Results of Celltrace™ Violet proliferation assays for the CT02 T-cell line

<table>
<thead>
<tr>
<th>Sample</th>
<th>MFI Stimulation 1 (day 0)</th>
<th>MFI Stimulation 2 (day 14)</th>
<th>MFI Stimulation 3 (day 28)</th>
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<tr>
<td>Unstimulated</td>
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</tbody>
</table>

MFI indicates mean fluorescence intensity, which has been rounded to the nearest whole number. Stimulation 1 was performed for 6 days, whilst stimulations 2 and 3 were performed for 3 days. Unstimulated values in stimulations 2 and 3 were calculated using cells from CT02 and irradiated autologus feeder cells only. Cells were gated on CD3 positivity.

Figure 5.25: Overlay histograms generated in Celltrace™ Violet proliferation experiments using CT02. The dashed line represents the unstimulated values, the continuous line represents RBC tTG, and the grey shaded area PHA. Stimulations 1, 2, and 3 are represented by a, b, and c, respectively. Cells were gated on CD3 positivity.
The effect of re-stimulation on CD25 and HLA-DR expression by CT02

Although the initial stimulation with each tTG preparation had no effect on CD25 expression on T cells from CT02, by each re-stimulation (days 14 and 28), increased CD25 expression was seen in response to all tTGs tested (Fig 5.26a). The most consistent increase in CD25 expression in response to the differing tTGs was generally seen at stimulation 2, although wt and CHDΔ tTG induced relatively high levels (10 and 7%, respectively) of CD25 expression at stimulation 3. At the first stimulation, both PPD and PHA induced large percentages of CD3/CD25 positive cells, an ability maintained by PHA but lost by PPD in subsequent re-stimulations (Fig 5.26a). The cells from CT02 up-regulating CD25 expression in response to all test antigens and mitogens were found to be CD4+ when further analysed.
Figure 5.26: The effect of tTG stimulation on CD25 expression by T cells from CT02. (a): Stimulation was performed on days 0, 14, and 28. For stimulation 1, unstimulated PBMC were used to calculate background levels; for stimulations 2 and 3, cells from the CT02 cell line and feeder cells only were used. (b) represents a typical plot generated, in this case CT02 stimulation 2.

HLA-DR expression by T cells from the CT02 cell line was also an effective marker of responsiveness of these cells to tTG, the results of which are detailed in Fig 5.27a. At the initial stimulation using PBMC, an increase in the percentage of CD3/HLA DR positive cells was seen in response to RBC, rh, and CHDΔ tTG (Fig 5.27a). Stimulation 2 was accompanied by HLA-DR up-regulation in response to all tTG antigens tested, with rh and wt tTGs displaying the greatest stimulatory capacity. By stimulation 3, a weak increase in
HLA-DR expression compared to cells from CT02 and irradiated feeder cells without antigen was seen for wt tTG only. PHA was an effective positive control for each stimulation, inducing large percentages of HLA-DR+ T cells, whilst in contrast, the ability of PPD to up-regulate HLA-DR expression was not seen following stimulation 1, where it induced a level comparable to that of PHA (Fig 5.27a). A representative flow plot used to calculate HLA-DR expression data is shown in Fig 5.27b. In a similar fashion to the two other T-cell lines, HLA-DR expression in response to stimulation with the various test antigens and mitogens was generally restricted to CD4+ cells.
Figure 5.27: The effect of tTG stimulation on HLA-DR expression by T cells from CT02. (a): Stimulation was performed on days 0, 14, and 28. For stimulation 1, unstimulated PBMC were used to calculate background levels, whereas for stimulations 2 and 3, cells from the CT02 cell line and feeder cells only were used. (b) represents a typical plot generated, in this case CT02 stimulation 2.
Cytokine production by CT02

IL-10 was the only cytokine detected in the day 7 culture supernatant from the CT02 T-cell line, and the production of this cytokine was maintained throughout the duration of the culture period (Fig 5.28b). Spontaneous IL-10 production by the unstimulated control cells was seen at days 14 and 21. From day 14 onwards, large amounts (2,000 pg/ml) of IFN-γ were found in the culture media, which receded to moderate levels (1,000 pg/ml) in the final two weeks of culture (Fig 5.28a). IL-4 and IL-17A were generally not produced by CD02, with the exception of trace amounts (10-30 pg/ml) of IL-4 detected on days 14 and 21 (Fig 5.28c), and low amounts (130 pg/ml) of IL-17A measured on day 14 (Fig 5.28d). IL-21 production by CT02 occurred in the latter stages of culture, at comparable levels to that produced by the CD patient-derived cell line, CD01 (Fig 5.28e).
Figure 5.28: Cytokine production by the T-cell line CT02. The culture supernatants of CD01 were sampled weekly, and the levels of cytokines measured by ELISA. Medium indicates an equivalent amount of PBMC cultured in an identical fashion, but without antigen.
The effect of re-stimulation on intracellular cytokine production by CT02

Intracellular IFN-γ production by T cells from CT02 in response to all tTGs tested was observed for stimulations 2 and 3 (Fig 5.29a). Stimulation 2 was associated with a massive increase in the percentage of CD3/IFN-γ positive cells (Fig 5.29b), whilst the increase observed in response to stimulation 3 was not as pronounced due to the high background IFN-γ positivity of T cells from CT02. In the initial stimulation, PPD raised the percentage of CD3/IFN-γ positive cells above that of the background level, whereas PHA did not (Fig 5.29a). This situation was reversed at stimulation 2, with PHA causing an increase in CD3/IFN-γ positivity of 25%, whilst at stimulation 3 PHA caused a large increase in CD3/IFN-γ positivity, compared to a mild (1%) increase induced by PPD.

Intracellular IL-10 production in response to tTG was seen at stimulation 3 (day 28) only, where RBC, wt, and CHDA tTGs increased the percentage of CD3/IL-10 positive cells (Fig 5.30a). PHA induced a positive response at each stimulation, whereas PPD did not. The background level of IL-10 positivity of T cells from CT02 increased with time in culture. No intracellular IL-17A production by T cells from CT01 was detected in response to stimulation with any of the tTG preparations, in keeping with the absence of this cytokine in culture supernatants (Fig 5.30b, 5.28d).
Figure 5.29: Intracellular IFN-γ production by the T-cell line CT02 as a response to stimulation. (a): Stimulation with the differing tTG preparations, PHA, and PPD were performed on days 0, 14, and 28. For stimulation 1, unstimulated PBMC were used to calculate background levels of IFN-γ production, whilst for stimulations 2 and 3, cells from the CT02 cell line and feeder cells only were used to calculate background. A typical flow cytometry plot generated in such an experiment is detailed in (b), which represents stimulation 2.

A positive effect on intracellular IL-21 production was induced by rh and wt tTG at stimulation 2, whereas RBC and rh tTG increased CD3/IL-21 positivity at stimulation 3 (Fig 5.30c/d). At each round of stimulation, PHA caused an increase in the percentage of CD3/IL-21 cells, whilst PPD had no effect at any of the three stimulations (Fig 5.30c/d).
Figure 5.30: Intracellular IL-10, IL-17, and IL-21 production by the T-cell line CT02 as a response to stimulation. Levels of IL-10 (a), IL-17 (b), and IL-21 (c) were assessed in response to stimulations performed on days 0, 14, and 28. For stimulation 1, unstimulated PBMC were used to calculate background levels of IFN-y production; for stimulations 2 and 3, cells from the CT02 cell line and feeder cells only were used to calculate background. A representative flow plot, in this case IL-21, stimulation 3, is shown in (d).
5.4 Discussion

5.4.1 tTG-specific T cells

The demonstration of T cells specific for tTG by Ciccocioppo and co-workers in 2010 amounts to one of the most significant contributions in recent years to the understanding of anti-tTG immunity in CD. With the identification of tTG as the EMA autoantigen in 1997, logic pointed to an immune response driven by tTG-specific T cells, as expected for such an autoantibody response. Within six months, an alternative hypothesis as to the source of T cell help needed for anti-tTG production, the ‘Hapten-carrier theory’ was proposed by Sollid et al. (Sollid, Molberg et al. 1997). The rationale of this theory is that T cell help (in a global context) can be provided by linking self-antigens with T cell epitopes of foreign antigens (in a hapten-carrier fashion), and that this was applicable to CD “based on the observation that tTG and gliadin form complexes” (Sollid, Molberg et al. 1997).

The experimental work described in this chapter confirms the existence of T lymphocytes with a specificity for tTG, as demonstrated by Ciccocioppo, and that the presence of these cells in the periphery is not restricted to individuals with untreated CD. Cells responsive to tTG-stimulation were identified in patients with treated CD and some control individuals through the use of proliferation assays measuring $^3$H-thymidine incorporation into tTG-stimulated cells. Three T-cell lines were generated by the stimulation of PBMCs with RBC tTG: one from an individual with treated CD, and two from responsive control individuals. In order to identify these T-cell lines as true tTG antigen-specific T cells, extensive stimulation experiments were performed in which the response to tTG was measured using differing read-outs such as cellular proliferation, up-regulation of activation markers, and intracellular cytokine production.
The development of a $^3$H-thymidine incorporation proliferation assay allowed the screening of groups of CD patients and controls for PBMC responsiveness to tTG, and served to identify individuals for further study. Such proliferation assays are widely used in the study of T cell responses to autoantigens, and have also been used in the detection of gliadin-specific T cells in both CD (Lundin, Scott et al. 1993) and dermatitis herpetiformis (Baker, Garioch et al. 1995). In response to the two commercial tTG preparations, RBC and recombinant human (rh) tTG, proliferation of PBMC occurred in approximately 30% of CD patients, all of whom were treated, and 10% of control individuals (Fig 5.1). The stimulation index used as a cut-off for positivity in $^3$H-thymidine incorporation assays varies from 1.5 (Greidinger, Zang et al. 2008) to 3 (O'Keeffe, Mills et al. 1999). In this study, a value of 2 SI was used as the cut-off, in a similar fashion to studies investigating T cell proliferative responses to topoisomerase I in patients with systemic sclerosis (Veeraraghavan, Renzoni et al. 2004), and small nuclear ribonucleoproteins in SLE (Riemekasten, Weiss et al. 2002).

T-cell lines were generated using the RBC tTG antigen, a kind gift from Dr. Walter Binder of Inova Diagnostics Incorporated. This antigen had been used in the study by Ciccocioppo et al., where its reactivity was abolished by proteinase K digestion, and endotoxin activity was determined as being <10 EU/ml (Ciccocioppo, Finamore et al. 2010).

The proliferative specificity of the T-cell lines was established by measuring $^3$H-thymidine incorporation in response to their 'parent' antigen RBC tTG, as well as to an extended panel of differing tTG preparations. By the second round of antigenic stimulation, reactivity to all of the tTG antigens by samples from each cell line was generally observed, a feature also noted in the study by Ciccocioppo, where tTG-specific T-cell clones could proliferate to either RBC tTG, or to a recombinant human tTG expressed in
*E. coli*, in a similar fashion to the wt tTG protein generated in Chapter 2 of this study. Further specificity of each T-cell line was demonstrated by the loss of reactivity to the control antigen PPD after the first round of stimulation. Using the flow cytometric method of measuring proliferation, CellTrace™ Violet incorporation, the proliferation of CD3+ cells from each cell line in response to RBC tTG at each stimulation was confirmed.

The up-regulation of the activation markers CD25 and HLA-DR by tTG-stimulated CD4+ T cells was a feature of each cell line. Increases in CD25 expression were seen at the first re-stimulation (day 14) for each cell line, and at the second (day 28) for cell lines CD01 and CT02. HLA-DR up-regulation was noted at each round of stimulation for CD01, stimulations 2 and 3 for CT01, and stimulations 1 and 2 for CT02.

The hallmark of the tTG-specific T-cell lines was the secretion of large amounts of IFN-γ, maximal amounts of which were detected following the first re-stimulation of each cell line. The CD patient-derived T-cell line CD01 produced the highest levels of IFN-γ (2,000-4,000 pg/ml,) which persisted for the culture period following the first re-stimulation of the cell line with RBC tTG, in contrast to the reduced levels (1,000 pg/ml) seen towards the end of the culture of the two control-derived cell lines. A decrease in IFN-γ production by the two control T-cell lines for the final two weeks in culture corresponded with the appearance of IL-10 in the culture medium, particularly for CT01, whereas IL-10 production by the CD01 cell line occurred during the first two weeks of culture only.

Measurement of intracellular IFN-γ production by CD3+ cells from each T-cell line proved a specific response to re-stimulation with tTG. This response was evident in the CD01 cell line from the initial stimulation of PBMC, reflecting IFN-γ appearance in the cell line supernatants during the first week of culture. In both of the control T-cell lines, intracellular IFN-γ production by CD3+ cells in response to tTG stimulation was induced at the first and second re-stimulations, possibly reflecting the positive selection and
expansion of tTG antigen-specific T cells during the first two weeks of culture. For each cell line, the greatest increase in intracellular IFN-γ production by CD3+ cells was seen at the first re-stimulation, with the amount of IFN-γ detected in the culture supernatants following a similar pattern of peaking during this period of culture.

IL-17A was produced at low levels (300 pg/ml) by cell line CD01 throughout the culture period, and at high levels (1,500 pg/ml) by cell line CT01 for the culture period following the first-restimulation. The propensity of tTG-specific T cells from these individuals to produce IL-17A was highlighted by an increase in IL-17A-producing CD3+ cells as measured by intracellular cytokine staining in response to stimulation three for CD01, and stimulations one and three for CT01. By stimulation three, the background level of IL-17A positive T cells in samples from the CT01 cell line had reached 6%, a level not seen for the other two T cell lines, which correlated with the secretion of this cytokine by the CT01 cell line.

Another feature of the tTG-specific T-cell lines was the production of IL-21, a cytokine which in recent years has been identified as implicated in CD (De Nitto 2009; Bodd, Raki et al. 2010). The highest levels of IL-21 were produced by the CD patient-derived CD01 cell line, which consistently produced IL-21 during the culture period, and had a positive intracellular IL-21 response to stimulations one and three. CT01 produced low levels (25-50 pg/ml) throughout the 35 day culture period, with an increase in the percentage of CD3/IL-21 positive cells measured by intracellular cytokine staining occurring in response to stimulation three. IL-21 production by CT02 occurred at relatively high levels (200 pg/ml) during the last two weeks of culture, where stimulation three was accompanied by an increase in CD3/IL-21 positive cells. For all of the cell lines, the background percentage of CD3/IL-21 positive cells increased over the culture period indicating the expansion of this cellular population in response to the RBC tTG antigen.
The dominance of IFN-γ, both in the culture supernatants and intracellular cytokine responses of each cell line would appear to place tTG-specific T cells into the Th1 category, a conclusion also reached by Ciccocioppo et al. (Ciccocioppo, Finamore et al. 2010), and supported by the absence of secreted IL-4 in the culture media. As the cells used in this study were polyclonal T-cell lines, as opposed to T cell clones, the expansion of other T helper cell types, namely regulatory T cells, Th17 cells, and Tfh cells may have occurred, and is implicated by the detection of the cytokines IL-10, IL-17A, and IL-21 in culture supernatants and positive intracellular cytokine responses to tTG stimulation. The production of IFN-γ and IL-10 was also a feature of the T-cell clones generated by Ciccocioppo, and suggests a potential immunomodulatory function for tTG-specific T cells. Production of IFN-γ and IL-10 is a feature of islet antigen-specific T cells in type 1 diabetes mellitus (Petrich de Marquesini, Fu et al. 2010), and has been also described for gliadin-specific T cells in CD (Nilsen, Gjertsen et al. 1996; O’Keeffe, Mills et al. 1999). Interestingly, IL-10 was more frequently detected in the culture supernatants of the control-derived cell lines, which is perhaps more indicative of the normal tolerogenic response to such a self-protein.

The production of IL-17A by gliadin-specific T cells is currently unresolved, due to recently published conflicting reports (Bodd, Raki et al. 2010; Fernandez, Molina et al. 2011). In this study, IL-17A was a product of tTG-specific T cells in two of the cell lines generated (CD01, CT01), identifying these cells as an alternative source of this cytokine in CD. IL-21 production was a feature of tTG specific T cells from all three individuals, at levels similar to that produced by gliadin-specific T cells in a report by Bodd et al. (Bodd, Raki et al. 2010). IL-21 can be a product of either Th17 or Tfh cells (Abbas, Lichtman et al. 2012). However, the source identified in this study may putatively be identified as Tfh cells, as double IL-17A/IL-21 positivity was not observed upon intracellular flow
cytometry in response to tTG stimulation. The observation that cell line CT02 produced IL-21 but not IL-17A lends further weight to this hypothesis.

In proliferation, activation marker, and intracellular cytokine staining experiments, there was no observed difference in reactivity of T cells to the wt or active-site mutant tTG proteins, indicating that this region of the protein is not required for antigen processing and presentation to T cells. Further experimental work to identify T cell epitopes of tTG may prove fruitful; T cell proliferation could be blocked by an antibody to HLA-DQ2 in the study by Ciccocioppo and colleagues, whilst unpublished data from our group demonstrates that a similar effect is attained by blocking HLA-DR (Christian Coates, manuscript in preparation), suggesting the possible use of computer-based algorithms to predict tTG peptides that may be presented by either MHC class II molecule. Identification of tTG T cell epitopes and the range thereof recognised by both CD patients and responsive control individuals may help to further dissect the T cell response to tTG, and allow for the synthesis of T cell reactive peptides of tTG for use in the generation of T cell lines and clones. The MHC class II restricted nature of the T cell anti-tTG response may explain the failure of CD8+ T cell expansion in response to tTG stimulation observed in this study, and elsewhere (Ciccocioppo, Finamore et al. 2010).

A feature of this study was the identification of proliferative responses to tTG in a small number of control individuals, and the subsequent generation of autoreactive T-cell lines from cells isolated from these individuals. The generation of autoreactive T cells in vivo is maintained by a number of regulatory mechanisms, including thymic central tolerance, and peripheral tolerogenic mechanisms such as the deletion, re-programming, or rendering anergic of self-reactive T cells (Lohr, Knoechel et al. 2005). Such autoreactive T cells have been classically linked with autoimmunity; however, self antigen-specific T
cells are frequently detected in otherwise healthy individuals (Danke, Koelle et al. 2004). T cells specific for β2-glycoprotein (Hattori, Kuwana et al. 2000), GAD65 (Danke, Yang et al. 2005), and myelin basic protein (Pender, Csurhes et al. 1996) have been detected in normal controls, whilst in a study using peptides of the Goodpasture’s disease autoantigen (the α3 chain of type IV collagen), all of 11 controls used had T cell reactivity to at least one peptide (Zou, Hannier et al. 2008). That such autoreactive T cells can exist in healthy individuals without causing autoimmunity emphasises that antigen alone is not sufficient for T cell activation. Further stimuli such as accessory molecule co-stimulation, and engagement with the APC are required. This may be especially pertinent for an antigen such as tTG, given its ubiquitous tissue expression (Griffin, Casadio et al. 2002). Whilst the study by Ciccocioppo et al. did not generate tTG-reactive T-cell lines or clones from control individuals, or individuals with treated CD, this may be due to methodological differences between the two studies. In the study by Ciccocioppo et al., the generation of tTG-specific T-cell lines and clones was attempted in ten CD patients, of whom four were untreated, and four controls, with successful T-cell lines and clones generated from three untreated CD patients only. In our study, 25 patients, the majority of whom were treated, and 15 controls were screened for PBMC reactivity to tTG, with subsequent selection of responsive individuals for cell line generation. The response of proliferation to either commercial source of tTG used in approximately 30% of the CD patients is similar to the success rate of cell line generation by Ciccocioppo et al. (Ciccocioppo, Finamore et al. 2010).

Apart from the contribution of tTG-specific T cells (and their principal product, IFN-γ) to the generation and maintenance of the CD intestinal lesion, an obvious function of these cells is the provision of help to tTG-specific B cells. IFN-γ may also skew class-switching of
anti-tTG IgG in CD to the Th1-like pattern of IgG1 observed in Chapter 4 of this thesis, whilst IL-21 is emerging as an important cytokine for IgA class-switching in Peyer's patches (Spolski and Leonard 2010). Th17 cells have previously been demonstrated to impact on neutrophil recruitment in an IL-17A-independant manner (Pelletier, Maggi et al. 2010). It is possible that tTG-specific Th17 cells may participate in the recruitment of the polymorphonuclear cell infiltrate seen in the CD intestinal lesion (Ensari 2010). Further isolation of Th-polarised tTG-specific T-cell clones, and their interaction with tTG-specific B cells with regard to activation and autoantibody isotype/subtype profile may prove informative. The isolation of tTG-specific T cells from intestinal biopsies of CD patients or controls could be seen as the next logical step in the study of these cells; if only to investigate the presence of these cells in the intestine as a CD-specific phenomenon in a similar fashion to that of gliadin-specific T cells, which can be isolated from the intestine of CD patients, but not from control individuals (Lundin, Scott et al. 1993).

5.4.2 Conclusions

The work described in this chapter was performed in order to investigate the presence of tTG-specific T cells in individuals with treated CD, and normal control individuals. Groups of treated CD patients and controls were screened for PBMC reactivity to two commercial tTG preparations by the measurement of ³H-thymidine incorporation, with proliferation detected in 30% of CD patients and 12% of controls. T-cell lines were generated by fortnightly stimulation with tTG from one treated CD patient and two control individuals, all of whom had tested positive in tTG proliferation assays. The antigen specificity of the T-cell lines was demonstrated by increased proliferation and up-regulation of the activation markers CD25 and HLA-DR in response to re-stimulation.
with tTG. Long-term culture generated T-cell lines that were predominantly CD4+ and secreted large amounts of IFN-γ. A T cell stimulation assay measuring intracellular cytokine production by tTG-specific T cells was developed, with IFN-γ production by these cells proving a robust marker of stimulation with the tTG antigen. tTG-specific T cells were also found to produce IL-10, in keeping with previous reports, and are reported here to also produce IL-17A, and IL-21, two cytokines of emerging interest in the field of CD research.
Chapter 6

General Discussion
6.1 Review of results

Tissue transglutaminase (tTG) and coeliac disease (CD) will forever be associated, principally due to the role of the latter in disease pathogenesis, where it serves to heighten the immunogenicity of gliadin fragments by deamidation, inducing negative charges that favour binding to HLA-DQ2 or DQ8 molecules (Sjostrom, Lundin et al. 1998; Vader, de Ru et al. 2002). tTG was also identified as the target of anti-endomysial antibodies in 1997 (Dieterich, Ehnis et al. 1997), with the detection of IgA anti-tTG autoantibodies now serving as a specific and sensitive indicator of CD (Schuppan and Hahn 2001; Sakly, Bienvenu et al. 2005). Despite this, there is little known as to the origin of the immune response directed against tTG seen in CD, and indeed in non-CD pathologies. The aim of this thesis was to investigate various facets of tTG immunity in CD and beyond, including a further dissection of IgA and IgG anti-tTG responses and their epitopes, in both paediatric and adult CD, an investigation of differences in IgG anti-tTG epitope specificity and subclass usage between individuals with CD and those with other autoimmune diseases, and the development of T cells with a specificity for tTG.

In Chapter 2, five recombinant GST-tagged tTG proteins were generated in E. coli using a modified protocol to that developed by Byrne and his co-workers (Byrne, Ryan et al. 2007). The recombinant tTG proteins consisted of the wild-type protein, and four differing active-site mutants of tTG generated by site-directed mutagenesis for use in epitope mapping studies using CD sera. The inclusion of a bacterial protease inhibitor cocktail increased yields from those described by Byrne, and allowed the recombinant proteins to be quantified using the Bradford assay.

Chapter 3 describes the use of the five recombinant tTG proteins in ELISA systems measuring IgA and IgG anti-tTG antibodies from the sera of CD patients in an effort to compare the antigenicity of the wild-type protein with that of each mutant tTG. In these
experiments, all of the mutant tTG proteins showed diminished reactivity when compared to the wild-type protein for both IgA and IgG anti-tTG antibodies from CD patients. The autoantibody response to the mutant tTG preparations in both adults and children with CD was compared, identifying the core region of tTG as a target from early in disease. Increased levels of IgG anti-tTG were found in children <4 years of age, which may reflect a developing immune system that is not completely proficient at class-switching to IgA. As a control for the various active-site mutations, the binding of commercially available anti-tTG antibodies to bind to each protein was compared, with no differences observed.

Chapter 4 further explores the IgG anti-tTG response, determining the IgG anti-tTG subclass usage in CD and a number of autoimmune conditions, and also further investigates the epitope specificity of IgG anti-tTG in CD, with comparisons again made to other autoimmune diseases. Groups of non-CD patients were screened for IgG anti-tTG antibodies, with positivity detected in 28% of patients with T1DM, 19% of patients with Crohn’s disease, and 14% of patients with Wegener’s granulomatosis, at levels comparable to that found in adult CD. A potential difference in epitope specificity of IgG anti-tTG responses in these patients compared with that in CD was identified by the use of an epitope-masking experiment, in which affinity-purified CD IgA anti-tTG pre-incubation of a tTG-containing ELISA well resulted in a reduction in binding of CD IgG anti-tTG only. More evidence for a difference in the epitope specificity of CD and non-CD IgG anti-tTG was provided by the equal binding of these antibodies to the wild-type and CHDA tTG mutant proteins in non-coeliac IgG positive individuals. The IgG subclass profile of anti-tTG in CD was found to be IgG1>IgG2>IgG4>IgG3 in paediatric CD, and IgG1>IgG2>IgG3>IgG4 in adult CD. A similarity in subclass usage was found in T1DM, which was also dominated by IgG1. IgG1 anti-tTG reactivity was almost always absent in
Crohn’s disease and Wegener’s granulomatosis, with 100% of the latter positive for IgG2 and IgG3 anti-tTG.

T cell responses to tTG were studied in Chapter 5, with proliferation of PBMC in response to differing tTG preparations observed in approximately 30% of treated CD patients, and 12% of controls tested. Three T-cell lines responsive to tTG were generated from one treated CD patient and two controls, with IFN-γ being the principal cytokine product of each cell line. The production of IL-10, IL-17A, and IL-21 was also a feature of the T-cell lines. The antigen specificity of each cell line was ascertained by the measurement of proliferation to different tTG sources by \(^3\)H-thymidine and Celltrace™ Violet incorporation, and by the induction of the activation markers CD25 and HLA-DR on CD4+ T cells from each cell line. Intracellular IFN-γ production by T cells from the cell lines was measured as a response to tTG stimulation, and proved a reliable read-out for each round of antigenic re-stimulation. Mutagenesis of the catalytic triad of tTG had no effect on T cell responses to tTG, as measured by \(^3\)H-thymidine incorporation, activation marker analysis, and intracellular IFN-γ production from each T-cell line. PBMC proliferation to the mutant tTG in CD patients and controls occurred in a similar fashion to that directed against the wild-type protein, indicating this region of the protein may not be required for tTG processing by APCs, and subsequent tTG-specific T cell activation.

6.2 The origins of tTG autoimmunity in coeliac disease

In comparison to the immune response directed against gliadin in CD which has been extensively characterised in terms of the steps leading to T cell activation, the identification and prediction of T cell stimulatory peptides, and ultimate antibody formation, little experimental evidence exists as to the corresponding data regarding tTG. A crucial factor may be the determination of the exact molecular mechanism...
underlying the relationship between tTG and gliadin in respect to tTG immunity. The interaction of gliadin and tTG, and subsequent immune complex formation (Fleckenstein 2004) may be responsible for the B cell epitope spread from gliadin to tTG postulated to occur in CD, as supported by the detection of antibodies to gliadin/tTG neo-epitopes in the serum of CD patients (Matthias, Pfeiffer et al. 2010).

The uptake, and ultimate presentation of tTG to the immune system by APCs in CD also remains a mystery; uptake of tTG/gliadin complexes, or of tTG alone (due to exposure of cryptic antigens) by mucosal DCs may directly initiate the immune response to tTG. The identification of T cells specific for tTG supports this hypothesis, and provides a target for the study of APC/T cell interactions in CD. A recent report by Beitnes and co-workers suggest that CD14/CD11c+ DCs are the principal APC subset responding to gluten challenge in the CD intestine (Beitnes, Raki et al. 2012), an investigation of the capability of these cells to present tTG or tTG/gliadin complexes may prove fruitful. Due to the nature of gliadin/tTG interaction, it may be that the T cell response to tTG in CD may also be a result of intermolecular epitope spreading early in disease development, a phenomenon observed for islet autoantigens in the pre-clinical phase of T1DM (Brooks-Worrell, Gersuk et al. 2001).

tTG-specific T cells could provide help for the production of anti-tTG autoantibodies; with an IgG anti-tTG bias towards IgG1 reflecting the Th1 nature of these cells, whilst IgA anti-tTG production is possibly influenced by cytokines such as IL-21, identified in this study as a tTG-specific T cell product. Help from gliadin-specific T cells, as per the hapten-carrier theory, cannot be excluded given an interesting recent report that B cells engineered to express a tTG-specific BCR were able to process and present TG2-gliadin complexes, and activate gluten-specific T cells isolated from CD patients (Di Niro, Mesin
et al. 2012). It may be that help from gliadin-specific T cells for tTG autoantibody production occurs downstream to the breaking of immunological tolerance to tTG and activation of tTG-specific T cells, as a later pathogenic event correlating with the influx of tTG-secreting plasma cells to the CD lesion, and appearance of serum anti-tTG.

In this study, CD-dependant differences in IgG anti-tTG responsiveness such as epitope recognition and subclass usage were observed between IgG positive individuals, implicating differing mechanisms underlying the development of tTG immunity outside of CD. A possible cause of this is that the inappropriate immune responses to gliadin (at both an innate and adaptive immune levels) is facilitating tTG autoimmunity in CD but not in other autoimmune diseases, with the postulated T cell epitope spread from gliadin to tTG a major factor in determining the characteristics of the CD anti-tTG response. Whilst IgA anti-tTG antibodies are almost completely specific for CD, it is becoming increasingly apparent that tTG immunity in general is not a CD-specific phenomenon, a theme central to this study. Apart from the aforementioned IgG anti-tTG positivity observed in autoimmune, infectious, and inflammatory diseases, the identification of intestinal anti-tTG deposits are providing an even more sensitive indicator of an immune response to tTG in individuals seronegative for anti-tTG autoantibodies. Deposits of IgA anti-tTG were demonstrated in 11/19 IgA anti-tTG seronegative T1DM children, interestingly with a different VH gene usage when compared to that of IgA anti-tTG seropositive T1DM children (VH1 and VH3 vs. VH5) (Maglio, Florian et al. 2009). In relatives of CD patients who are genetically predisposed to CD but negative for serum IgA anti-tTG, Not and colleagues have demonstrated intestinal IgA anti-tTG deposits, the presence of which was related to non-CD, gluten-independent extraintestinal symptoms which resolved on a gluten-free diet (Not, Ziberna et al. 2011).
The demonstration of tTG-specific T cells in a proportion of non-CD control individuals may reflect the occasional escape of autoreactive T cells from thymic negative selection, or unseen regulatory networks such as that involved in the generation of anti-idiotypic antibodies. For antigens such as GAD65 in T1DM, antigen-specific T cells may be found in control individuals (Danke, Yang et al. 2005), as may anti-idiotypic antibodies, the lack of which has been proposed to define T1DM (Oak, Gilliam et al. 2008). Anti-idiotype antibodies have been described for the autoantigens PDC-E2 (associated with PBC) (Chen, Rowley et al. 1999), ribosomal P (associated with SLE) (Stafford, Anderson et al. 1995), and thyroglobulin (Hara, Sridama et al. 1988). In 2007, Ferrara and co-workers discovered anti-tTG idiotypes in the intestine of subjects genetically predisposed to CD but with a normal intestinal mucosa, and in CD patients (Ferrara, Dal Bo et al. 2007). The ratio of these anti-anti-tTG antibodies was higher in the asymptomatic individuals than the CD patients, highlighting another possible mechanism in the regulation of immune responses to tTG. A similar observation was made in mice in which anti-tTG production was artificially induced. Further evidence of cryptic anti-tTG reactivity was demonstrated by Zoller-Utz et al. with the observation that anti-tTG in normal subjects may be detected by heating their serum to 56°C prior to antibody measurement (Zölle-Utz, Esslinger et al. 2009). The authors identified fibrinogen as the binding partner for anti-tTG in normal individuals, with the complex disassociated upon heating, however it may be that an antibody/anti-idiotype complex was also liberated, giving rise to the increased anti-tTG positivity seen. A further investigation of tTG anti-idiotype antibodies in CD may prove informative, particularly a determination of the IgG subclass usage of tTG IgG anti-idiotype antibodies, which has been shown to be different for T1DM patients and controls for anti-idiotypic responses to GAD65 (Oak, Radtke et al. 2011). The epitope
specificity of the liberated anti-tTG antibodies in both CD and non-CD individuals may also provide an interesting comparison.

6.3 The consequences of anti-tTG immunity in coeliac disease

Since the discovery of tTG as the autoantigen contained in the endomysium, the function (if any) of anti-tTG antibodies in CD pathogenesis has been vigorously debated (Caputo, Barone et al. 2009; Lindfors, Mäki et al. 2010; Caja, Mäki et al. 2011). Debate supporting a role for these antibodies has always been conducted with ‘an elephant in the room’, that is the high frequency of CD occurring in IgA-deficient patients (Jacob, Pastorino et al. 2008). However, recent reports indicate that intestinal IgA anti-tTG deposits may be detected in ‘IgA-deficient’ CD patients (Romano 2011), which demonstrates mucosal production of IgA, despite low or undetectable serum levels. Other immunoglobulin isotypes such as IgM and IgG may be contributory to the intestinal inflammation in CD, with the potential for complement activation by deposits of anti-tTG of these isotypes (Borrelli, Maglio et al. 2010).

Apart from a contribution to the intestinal pro-inflammatory environment, anti-tTG deposits may contribute to extraintestinal symptoms in CD (Caputo, Barone et al. 2009), whilst the effects of IgA anti-tTG on tTG enzymatic function are debated (Esposito, Paparo et al. 2002; Dieterich 2003; Kiraly, Vecsei et al. 2006). A recent report by our group suggests a dose-dependent inhibition of tTG crosslinking function by affinity-purified CD IgA anti-tTG (Byrne, Feighery et al. 2010). Anti-tTG has also been shown to disturb angiogenesis (Myrsky, Kaukinen et al. 2008), and activate TLR4 (Zanoni, Navone et al. 2006). Given these observations, tTG may represent a therapeutic target in CD, either by blocking enzymatic activity (and therefore gliadin deamidation), or by blocking the interaction of anti-tTG with the enzyme, and thus preventing anti-tTG deposition.
However, caution must be exercised, due to the possible effects of interfering with the physiology of such a ubiquitously expressed and multi-functional enzyme.
References


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Appendix I

All reagents and chemicals are from Sigma-Aldrich unless otherwise stated.

**PCR reagents**

**PCR 6x loading buffer**

30% glycerol, 0.25% bromophenol blue

### 12% SDS-PAGE reagents

<table>
<thead>
<tr>
<th></th>
<th>12% Resolving Gel</th>
<th>5% Stacking Gel</th>
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</thead>
<tbody>
<tr>
<td>Distilled H₂O</td>
<td>3.3 mls</td>
<td>2.1 mls</td>
</tr>
<tr>
<td>30% Acrylamide (Bio-Rad)</td>
<td>4 mls</td>
<td>0.5 mls</td>
</tr>
<tr>
<td>Tris</td>
<td>2.5 mls (1.5M)</td>
<td>0.37 mls (0.5M)</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100µl</td>
<td>30µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>100µl</td>
<td>30µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5µl</td>
<td>3µl</td>
</tr>
</tbody>
</table>

**Running buffer**

25mM Tris-HCl

200mM Glycine

0.1% (w/v) SDS

**Destain solution**

12.5% (v/v) isopropanol

10% (v/v) glacial acetic acid
Cell culture reagents

T cell Medium

500ml RPMI (Invitrogen)
5.5ml HEPES
5.5ml sodium pyruvate
5.5ml non-essential amino acids
5.5ml essential amino acids
5.5ml penicillin/streptomycin
5.5ml L-gutathione
5.5ml fungizone
550µl β-mercaptoethanol
Gating strategy used in flow cytometry experiments. Cells were gated from the lymphocyte gate, with exclusion of dead cells (a). Quadrants were assigned using fluorescence minus one (FMO), initially for CD3 (b), and then for all other parameters (c). Cells were then further gated on CD3 positivity, which was assigned using the CD3 FMO (d), resulting in the generation of plots such as those shown in (e).
The Binding of Autoantibodies to the Core Region of Tissue Transglutaminase Is a Feature of Paediatric Coeliac Disease

Ross Comerford, Greg Byrne, Conleth Feighery, and Jacinta Kelly

ABSTRACT

Objectives: Production of autoantibodies to the enzyme tissue transglutaminase (tTG) is a hallmark of coeliac disease (CD). We have previously demonstrated that the immunoglobulin (Ig) A response to tTG in adult CD specifically targets its catalytic domain, containing the active site triad of amino acids. The aim of this study was to investigate this phenomenon in paediatric patients with CD, and to elucidate the contribution of each active site residue to epitope recognition. The specificity of the IgG anti-tTG response was also investigated, and compared with that of the IgA anti-tTG response, in both paediatric and adult patients with CD.

Patients and Methods: Wild-type and novel variants of tTG were generated via site-directed mutagenesis, and expressed as glutathione S-transferase fusion proteins in Escherichia coli BL21. The mutant variants of tTG had substitutions of L, T, or all of the 3 of the catalytic triad amino acids. All of the recombinant tTGS were tested for their antigenicity in IgA and IgG enzyme-linked immunosorbent assays with cohorts of paediatric (n = 63) and adult (n = 30) CD sera.

Results: Substitution of even 1 amino acid in the catalytic triad resulted in a significant reduction of CD IgA and IgG anti-tTG binding, with all of the mutant proteins displaying diminished antigenicity compared with the wild-type protein.

Conclusions: The core region of tTG is specifically targeted from early on in disease course by CD patient autoantibodies of both the IgA and IgG class.

Key Words: autoantibodies, coeliac disease, epitope mapping, tissue transglutaminase

CD is a chronic inflammatory disorder of the small intestine characterised by an immune response to ingested gluten and related cereal proteins, affecting approximately 1 in 100 white individuals (1). Traditionally, CD was considered a disease of childhood, presenting with the classical symptoms of diarrhoea, malabsorption, and failure to thrive (2); however, the mean age of diagnosis has risen, with CD often presenting with more subtle and variable clinical symptoms (3).

In CD, autoreactivity arises via a complex interplay of environmental (gluten) and genetic factors, most prominently the HLA association with the human leukocyte antigen (HLA) genes (4). The majority of patients express the HLA-DQ2 molecule (5), with a minority expressing the HLA-DQ8 molecule (6). Continued gluten ingestion results in the characteristic small intestinal histological profile of villous atrophy, crypt hyperplasia, and infiltration of mononuclear cells in the lamina propria (7). Serum immunoglobulin (Ig) G antibodies to tissue transglutaminase (tTG), identified as the major autoantigen in CD by Dieterich et al. (8), are a sensitive and specific serological test for diagnosis of the disease (9).

tTG is a member of a family of related, calcium-dependent transglutaminase enzymes responsible for the posttranslational modification of proteins. Proteins can be cross-linked by tTG through the formation of an isopeptide bond between glutamine and lysine residues on each target protein (10). Under certain conditions, tTG will deamidate the target glutamine in the substrate protein, with the neutral glutamine being changed to a negatively charged aspartic acid residue (11). The deamidation reaction, catalysed by tTG, is of paramount importance in CD pathogenesis. The high proline and glutamine content of gluten makes it an excellent substrate for deamidation by tTG, generating negative charges that strengthen binding at crucial anchor positions in the HLA-DQ2/DQ8 molecule (12). This facilitates presentation of deamidated gluten peptides to CD4+ T cells in the intestinal lamina propria, and amplifies the immune response seen in CD.

The transamidation and deamidation reactions of tTG are mediated by a papain-like catalytic triad of amino acids, which is relatively conserved across all of the mammalian transglutaminases (13). In tTG, this comprises cysteine (14), histidine (15), and aspartic acid (16), contained in the catalytic core domain. Previous studies by our group (14) and others (15) have demonstrated that CD IgA anti-tTG autoantibodies target the enzyme’s catalytic core, representing a highly focused and specific autoantibody response. We have shown, using wild type and a site-directed mutagenic tTG lacking the entire Cys-His-Asp catalytic triad, that the antibody binding of adult CD anti-tTG antibodies was focused on this region, and its mutation caused a highly significant drop in antibody binding.

The aim of the present study was to map the evolution of this highly specific autoantibody response by comparing the specificity of paediatric and adult anti-tTG responses. This should shed light on the nature of the antibody response at the onset of disease: that is, does the paediatric anti-tTG response also focus on the catalytic triad of tTG, or is this a phenomenon that evolves during disease progression? The contribution of individual amino acids of the
catalytic triad to CD autoantibody epitopes in both adults and children was also assessed.

In the present study, a series of novel (TG active-site mutants) were generated, containing single and double amino acid substitutions of the catalytic triad, as well as the previously described triple mutant (14). The mutated (TG) proteins were then applied as antigens in an enzyme-linked immunosorbent assay (ELISA) system with serum from adult and paediatric patients with CD, with reactivity of each mutant (TG) then compared to the wild-type (TG) protein. An IgA anti-TG response also occurs in CD, but is less disease specific than the IgA response (16). To further examine whether the targeting of the (TG) active site in CD is an iso-type-specific IgA phenomenon, or whether there are shared IgA/IgG (TG) epitopes, the epitope specificity of the IgA anti-TG response in CD was also assessed.

**PATIENTS AND METHODS**

**Serum Samples**

Serum samples from 93 patients with CD were used to assess (TG) autoantibody binding. The adult group consisted of 30 patients (male:female [MF] 1.2:8; age 22–85 years, median 59), whereas the paediatric group consisted of 63 patients (MF 1:1.7; age 2–15 years, median 8). The diagnosis of CD was based upon positive IgA endomysial antibodies and anti-TG serology, reduction in levels of these autoantibodies with gluten withdrawal, and duodenal histology where available. Both CD study populations were further subdivided by disease activity status. Individuals with partially treated CD were defined as patients with low to medium levels of IgA anti-TG (20–50 arbitrary units) or IgG anti-TG levels posttreatment of a gluten-free diet. In the paediatric CD group, 23 patients had untreated CD, 15 patients had partially treated CD, and 25 patients had treated CD. In the adult CD group, 5 were treated, 13 were partially treated, and 12 were untreated. Cut-off points for IgA and IgG anti-TG ELISA positivity (mean ± 2 standard deviation) were established by assaying serum from groups of normal adult (n = 30, MF 1:2, age 24–82 years, median = 52) and paediatric (n = 57, MF 1:1, age 1–15 years, median = 8) individuals with negative IgA anti-TG serology, as measured by the Celikay ELISA system. All of the adult nonodulocats had normal intestinal histology. All of the serum samples were obtained from the Immunology laboratory, St James’ Hospital. Ethical approval for the present study was granted from St James’ Hospital and Our Lady’s Children’s Hospital, Dublin.

**Polymerase Chain Reaction, Site-directed Mutagenesis and Cloning**

The wild-type (TG) cDNA was cloned into pGEX-4T-1 vector, as described previously (14). Selected residues of the catalytic triad Cys-His-Asp were replaced with alanine residues via site-directed mutagenesis, using the Quickchange system (Stratagene, La Jolla, CA) (Table 1), as per the method of Byrne et al. (14). *Escherichia coli* BL-21 were then transformed with the appropriate sequence-containing plasmid and stored on bacterial cryobeads at −70°C until needed.

**Protein Production and Purification**

 Cultures were grown in nutrient broth to OD₆₀₀ ~0.5 and protein expression was induced by addition of 1 mM IPTG

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mutations performed</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT TG</td>
<td>None</td>
</tr>
<tr>
<td>CA TG</td>
<td>C27TA</td>
</tr>
<tr>
<td>CHA TG</td>
<td>C27TA, H335A</td>
</tr>
<tr>
<td>CHDA TG</td>
<td>C27TA, H335A, D358A</td>
</tr>
<tr>
<td>HDA TG</td>
<td>H335A, D358A</td>
</tr>
</tbody>
</table>

(TG) = tissue transglutaminase.

IPTG followed by incubation overnight at room temperature with 150 rpm agitation. The bacteria were pelleted and resuspended in 50 mM Cellytic reagent (Sigma-Aldrich, St Louis, MO) containing 1 ml protein inhibitor cocktail (Sigma-Aldrich), 10 mg lysozyme (Sigma-Aldrich), and 10 μl benzonase endonuclease (Sigma-Aldrich). Lysozyme was incubated at room temperature for 20 minutes with mixing and subsequently pelleted at 10 000 × g for 15 minutes. Pellets were stored at −80°C before purification. For purification of the recombinant (TGs), pellets were resuspended in 50 mM Cellytic reagent (Sigma-Aldrich, St Louis, MO) containing 1 ml protein inhibitor cocktail (Sigma-Aldrich), 10 mg lysozyme (Sigma-Aldrich), and 10 μl benzonase endonuclease (Sigma-Aldrich). Lysozyme was incubated at room temperature for 20 minutes with mixing and subsequently pelleted at 10 000 × g for 15 minutes. Four milliliters of glutathione sepharose (HiMedia, Little Chalfont, UK) were added to the resulting supernatant, and incubated for 1 hour at 4°C, with mixing. The sepharose was washed 4 times with phosphate buffered saline (PBS), and the glutathione-5-transferase (GST)-fusion protein eluted from the beads with 1 ml elution solution (100 mM Glycine, 200 mM EDTA, 1 mM DTT, 15% glycerol, pH 8.0). Recombinant (TG) was eluted with 50% glycerol to prevent freezing, and stored at −20°C. Purity of protein batches was routinely checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

ELISA

ELISA was performed for recombinant wild-type and mutant (TGs). Certified 96-well Maxisorp plates (Nunc, Roskilde, Denmark) were coated with 0.3 μg recombinant protein per well in coating buffer (50 mM Tris-L, 150 mM NaCl, 5 mM CaCl₂, pH 7.5) overnight at 4°C. Wells were blocked with 5% casein in PBS for 1 hour, and washed 4 times with PBS containing 0.01% Tween between each step. Control antibodies CUB 7402 (Abcam, Cambridge, UK); polyclonal anti- (TG) (Abcam), 5G7G6 mouse monoclonal anti- (TG), and gift from Dr Fernando Chiodo), and anti-GST (Abcam) were used as primary antibodies to characterize each recombinant (TG) protein. CUB 7402 was diluted 1:5 000 in PBS plus 0.01% Tween, followed by HRP-conjugated rabbit anti-mouse (Dako, Copenhagen, Denmark) diluted 1:2500 in PBS plus 0.01% Tween. Polyclonal anti- (TG) was diluted 1:1000 in PBS plus 0.01% Tween, followed by HRP-conjugated swine anti-rabbit (Dako) diluted 1:2500 in PBS plus 0.01% Tween. 5G7G6 was diluted 1:5000 in PBS plus 0.01% Tween, followed by HRP-conjugated rabbit anti-mouse (Dako) diluted 1:2500 in PBS plus 0.01% Tween. Rabbit anti-GST was diluted 1:5000, followed by HRP-conjugated swine anti-rabbit (Dako) diluted 1:2500.

To generate AU for the reporting of the anti- (TG) ELISA results, a standard curve was constructed for both the IgG and IgA assays, using 4 pooled CD serum samples with strong IgA anti- (TG) positivity as measured by the Celikay ELISA system. For the IgA anti- (TG) ELISA, the range of dilutions was 1:320 to 1:20 480, whereas for the IgG anti- (TG) ELISA, the range was 1:10 000–1:1 000 000.
Characterisation of Wild type and Mutant ITGs

The ability of control antibodies to TG to bind to each recombinant protein was assessed by ELISA. As a control for conformational integrity and coating efficiency of the wild-type and mutant proteins, both monoclonal murine (CUB 7402, 5G7G6) anti-TG and polyclonal anti-TG were assayed with each protein. Similar binding patterns were observed for each recombinant protein, with the exception being a decrease in 5G7G6 binding to CA-TG (Fig. 2).

ELISA

CD serum IgA reactivity to wild-type TG correlated strongly with IgA anti-TG results from the Cellexic (TG ELISA system) for all samples tested ($r = 0.785$). There was a significant difference in recognition of the wild-type TG protein between patients with CD and age-matched controls ($P < 0.001$) for both paediatric and adult CD, Mann-Whitney test). The range of IgA anti-TG values was reflective of treatment status, with untreated patients displaying the highest levels in both children and adults.

In paediatric CD, mean IgA anti-TG levels to the wild-type TG were 96.3 AU for untreated patients, 47.5 AU for partially treated patients, and 13.3 AU for treated patients (cut-off 3.9 AU). In adult CD, mean reductions in IgA autoantibody binding compared with the wild-type protein were 89% for CDA-TG ($P < 0.001$), 90% for CDA2-TG ($P < 0.001$), 97% for CHD2-TG ($P < 0.001$), and 91% for HD2-TG ($P < 0.001$) (Wilcoxon signed-rank test).

IgG autoantibodies to wild-type TG were more frequently detectable in untreated CD, with the highest levels being observed in untreated patients younger than 4 years. Mean IgG anti-TG levels to the wild-type TG in paediatric CD were 61.2 AU for untreated patients, 4.4 AU for partially treated patients, and 7.6 AU for treated patients (cut-off 15.3 AU). In paediatric CD, there was a significant difference in IgG anti-TG binding to wild-type TG between paediatric patients with CD and controls for untreated patients only ($P < 0.0003$, Mann-Whitney test). Paediatric patients with CD without untreated disease had significantly higher levels of IgG anti-TG compared with those who were partially treated ($P = 0.0003$, Mann-Whitney test), or treated ($P = 0.0042$, Mann-Whitney test).

The mean reduction in binding to mutant TG proteins, compared with wild-type TG, was 33% in paediatric CD (range $-100\%$ to $+180\%$). The mean reduction in binding was 47% for CDA2-TG ($P = 0.001$), 43% for CHD2-TG ($P = 0.001$), 32% for CHD2-TG ($P = 0.001$), and 32% for HD2-TG ($P = 0.001$) (Wilcoxon signed-rank test). When the paediatric CD group was further subdivided by treatment status, the mean reduction in binding to all mutant proteins was highest in patients with untreated disease. The mean reduction in binding to all 4 mutants, compared with the wild-type protein, was 67% in untreated CD, 47% in partially treated CD, and 8% in treated CD.

In adult CD the pattern of reduced binding to mutant TG proteins was replicated. In the IgA assay, there was a mean reduction in binding to mutant TG proteins of 76% (range $-99\%$ to $+47\%$, $P < 0.001$ for each mutant protein). When IgG anti-TG levels in adult CD were $7.7$ AU for untreated patients, $8.0$ AU for partially treated patients, and $4.9$ AU for treated patients (cut-off $5.3$ AU). In adult CD there was a clear distinction in IgG recognition of the wild-type protein between patients and controls for patients with untreated ($P = 0.0086$, Mann-Whitney test) and partially treated CD ($P = 0.002$, Mann-Whitney test). Reduction of IgG anti-TG
Characterization of wild-type and mutant tissue transglutaminases (tTGs) using the control antibodies, (A) CUB 7402, (B) 5G7G6, (C) polyclonal anti-tTG, and (D) antiglutathione-S-transferase (GST). CA indicates the cysteine residue of the catalytic triad that has been replaced with alanine, CHA indicates the cysteine and histidine residues that have been replaced with alanine, CHDA indicates the cysteine and histidine and aspartic acid residues that have been replaced with alanine, whereas HDA indicates the histidine and aspartic acid residues that have been replaced with alanine. The top of the bar represents the mean of 5 (polyclonal anti-tTG, 5G7G6) and 6 (CUB 7402, anti-GST) experiments, whereas the error bars reflect the range of values.

binding to the mutant tTG proteins was also observed in adult CD, with a mean reduction of 22% for all mutated tTGs (Fig. 4A and B). In adults with CD, the highest level of reduction in IgG anti-tTG binding to the mutant tTG proteins was seen in partially treated disease. The mean reduction in binding to all 4 mutants, compared with the wild-type protein, was 20% in untreated adult CD, 29% in partially treated adult CD, and 11% in treated adult CD.

**DISCUSSION**

In the present study, we have mapped the evolution of the autoantibody response to tTG, demonstrating specific targeting of the core region early in disease course, and through the generation of unique active-site mutants of tTG, investigated the importance of single residues of the tTG catalytic triad for autoantibody binding. In the mean, the active-site mutations in tTG reduce binding of IgA anti-tTG by 82% and IgG anti-tTG binding by 28% for the paediatric and adult CD study groups (Figs. 3 and 4). Replacement of even a single amino acid of the catalytic triad with an alanine residue (CA tTG) caused a marked reduction in CD autoantibody binding. The diminished recognition of all of the mutant tTGs by CD autoantibodies indicates the importance of the core region for CD antigenicity, with an intact catalytic triad of amino acids, cysteine, histidine, and aspartic acid required for autoantibody binding. This is as one would expect for a B-cell epitope, with amino acids distant in the linear protein sequence being in close proximity upon protein folding (17). There is a possibility that the introduction of mutations in the core region of tTG could

![FIGURE 2](image)

**FIGURE 2.** Characterisation of wild-type and mutant tissue transglutaminases (tTGs) using the control antibodies, (A) CUB 7402, (B) 5G7G6, (C) polyclonal anti-tTG, and (D) antiglutathione-S-transferase (GST). CA indicates the cysteine residue of the catalytic triad that has been replaced with alanine, CHA indicates the cysteine and histidine residues that have been replaced with alanine, CHDA indicates the cysteine and histidine and aspartic acid residues that have been replaced with alanine, whereas HDA indicates the histidine and aspartic acid residues that have been replaced with alanine. The top of the bar represents the mean of 5 (polyclonal anti-tTG, 5G7G6) and 6 (CUB 7402, anti-GST) experiments, whereas the error bars reflect the range of values.

![FIGURE 3](image)

**FIGURE 3.** (A) Recognition of weight (wt) and mutant tissue transglutaminases (tTGs) by paediatric coeliac disease IgA anti-tTG, (B) Recognition of wt and mutant tTGs by adult CD IgG anti-tTG. Replacement of even 1 amino acid of the catalytic triad of tTG resulted in a loss of autoantibody binding. The grey bars indicate the cut-off values.

![FIGURE 4](image)

**FIGURE 4.** (A) Recognition of wt and mutant tissue transglutaminases (tTGs) by paediatric CD IgG anti-tTG, (B) Recognition of wt and mutant tTGs by adult coeliac disease IgG anti-tTG. All of the mutant tTGs displayed diminished antigenicity, when compared with the wild-type tTG protein. The grey bars indicate the cut-off values.
have affected protein folding, and hence conformation-dependent autoantibody binding. A similar pattern of recognition by control monoclonal and polyclonal anti-TG antibodies was observed for the wild-type and mutant TG proteins, suggesting correct protein folding. The similar recognition of all of the proteins by the monoclonal anti-TG CUB 7402 may be especially pertinent because the epitope recognized by this antibody (amino acids 447-478) is in the core region of TG.

Production of anti-TG in CD occurs at local level in the mucosal-associated lymphoid tissue, a system that is under constant antigenic challenge and stimulation, and has evolved to preferentially produce IgA to neutralise pathogens and build a tolerance to dietary antigens. Multiple rounds of somatic hypermutation and VDJ recombination occur at mucosal level, the result being affinity matured and highly specific IgA antibodies (18). It is possible that the generation of highly specific IgA anti-TG antibodies is a result of affinity maturation and epitope focusing over a prolonged disease duration (19).

To examine this hypothesis, we have compared the anti-TG specificity of both adult and paediatric patients with CD, with the paediatric population representing an earlier stage of disease evolution and duration. Many subtle immunological differences, which may have relevance in the aetiology and pathogenesis of CD, have been noted between adults and children. In studies investigating the T-cell responses to gliadin peptides, Vader et al (20) demonstrated that T cells from children with CD target a more diverse set of epitopes when compared with adult patients. Furthermore, paediatric T cells reacted to nondeaminated gliadin peptides more frequently than adult T cells, suggesting that the childhood T-cell response changes over time, focusing on immunodominant epitopes. A more rapid response to gluten withdrawal has also been noted in children, with the possibility of regaining tolerance to gluten (21). In the present study, we have demonstrated a highly focused and specific anti-TG response in paediatric CD, indicating that the core region of TG is targeted from early on in disease progression.

The demonstration of autoantibodies directed against enzymes is a recurring feature of autoimmune disease (22-25); however, the targeting of a single region of TG by CD autoantibodies is an interesting immunological phenomenon. Generally speaking, autoimmune responses directed against self-proteins, particularly enzymes, are of the IgG class and involve the recognition of multiple antigenic epitopes. Multiple IgG autoantibody epitopes have been described for proteinase 3 in Wegner granulomatosis (26) and for GAD65 in type 1 diabetes mellitus (27). Interestingly, the antigenic regions on these 2 proteins include their active site(s). Our results indicate that the IgG anti-TG response is specifically dependent on an intact TG active site, similar to the IgG anti-TG response. This would concur with reports by Shlattere et al (15), who have demonstrated a similar binding pattern for both IgG and IgG1 anti-TG to truncated fragments of recombinant human TG. We have also observed higher levels of IgG1 anti-TG in untreated paediatric patients with CD younger than 4 years of age, possibly reflective of an evolving mucosal immune system, which is not yet completely proficient at preferentially class-switching to IgA. Additionally, a significant IgG anti-TG response was detected in 15% of the paediatric control group (data not shown). These individuals were negative for IgA anti-TG, and showed similar recognition of the wild-type and mutant TG proteins, implying structural integrity and correct folding of the mutant TG proteins. It is possible that these children were suffering from an infectious disease because this has been implicated for anti-TG positivity in noncoeliac individuals (28).

There is a possibility that some enzymes, by their very nature, are predisposed to becoming targets of autoimmunity. Their active site may normally be sequestered from the immune system during inactivity, or new intermediates formed during catalysis can induce autoimmunity via neo-epitope formation. Certainly, a strong argument can be made for both of these processes occurring with TG. It is known that under inactive conditions, the active site of TG is buried deep within a narrow cleft between domains 3 and 4 of the protein (10) and that calcium activation of TG causes a conformational change that exposes the active site (29). TG-gliadin complexes occur, with TG capable of cross-linking gliadin to itself at the active site. This could potentially freeze the enzyme in its "open" conformation, with exposure of the active site, and possible neo-epitope formation (30). The recently described increased sensitivity of CD IgA anti-TG detection when TG is in its "open" conformation supports this notion (31).

The pathological role of anti-TG antibodies in CD remains controversial. Conflicting reports exist as to the degree, if any, of inhibition of the enzymatic function of TG by autoantibodies, and of the biological relevance of demonstrable inhibition (32-34). In the present study, we have confirmed the targeting of the TG active site by CD autoantibodies, and one would expect some interference with enzymatic function, if only prevention of substrate access to the active site. We have recently demonstrated significant inhibition of TG function by CD anti-TG IgA (35).

To conclude, we have demonstrated the presence of a highly conformational epitope in the core region of TG that is specifically targeted by CD autoantibodies from early on in disease course. We believe that this is a result of intramolecular epitope spreading from gliadin to TG, possibly as a result of the formation of gliadin-TG complexes at the active site of TG. The potential contribution to CD pathology and the effects on TG function of these autoantibodies requires further elucidation.

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