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The Effects of Transglutaminase 2 Modulation on Endothelial Cell Biology

Focus on celiac disease pathogenesis

ACADEMIC DISSERTATION
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of the Arvo Building, Lääkärinkatu 1, Tampere,
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“Nothing in life is to be feared. It is only to be understood.”

Maria Skłodowska-Curie

To my family

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, referred to in the text by their Roman numerals I-III.

I. Nadalutti C, Viiri KM, Kaukinen K, Mäki M, Lindfors K (2011) Extracellular transglutaminase 2 has a role in cell adhesion, whereas intracellular transglutaminase 2 is involved in regulation of endothelial cell proliferation and apoptosis. Cell Prolif 44:49-58.

II. Nadalutti C, Korponay-Szabo' IR, Kaukinen K, Griffin M, Mäki M, Lindfors K (2013) Celiac disease patient IgA mediates endothelial adhesion and cell polarization defects via extracellular transglutaminase 2. Cell Mol Life Science, DOI 10.1007/s00018-013-1455-5.

III. Nadalutti C, Korponay-Szabo' IR, Kaukinen K, Wang Z, Griffin M, Mäki M, Lindfors K (2013) Thioredoxin is involved in endothelial cell extracellular transglutaminase 2 activation mediated by celiac disease patient IgA. PlosOne, DOI: 10.1371/journal.pone.0077277.

ABBREVIATIONS

ADP	adenosine diphosphate
ANT-1	ADP/ATP transporter adenine nucleotide translocator
AP-2	activator protein-2
ATP	adenosine triphosphate
β-COP	coatomer complex β
BSA	bovine serum albumin
Ca^{2+}	free calcium ions
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cells
DH	dermatitis herpetiformis
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylene diamine N, N, N', N' tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EmA	endomysial antibody
FACS	fluorescence-activated cell sorting
FAK	focal adhesion kinase
FAs	focal adhesion site
FBS	fetal bovine serum
FN	fibronectin
FXIII	factor XIII
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HBSS	Hank's balanced salt solution
HDACs	histone deacetylases
HLA	human leukocyte antigen
HS	heparan sulfate
HRE	hypoxia response element
HUVEC	human umbilical vein endothelial cells
IEL	intraepithelial lymphocyte
IFN-γ	interferon γ
Ig	immunoglobulin, members A and G represent classes
IGF-1	insulin-like growth factor 1
IGFBP-3	insulin-like growth factor-binding protein 3
kDa	kilodalton
IL	interleukin
LTBP-1	latent TGFB-binding protein-1
MBD	metabolic bone disease
MDC	monodansylcadaverine
Mg^{2+}	magnesium ions
MMP	matrix metalloproteinase
mRNA	messenger RNA
MT-1 MMP	membrane type 1- metalloproteinase
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NF-1	nuclear factor-1
NO	nitric oxide

PBS	phosphate-buffered saline
PDI	protein disulphide isomerase
PECAM	platelet endothelial cell adhesion molecule
PFA	paraformaldehyde
PMA	phorbol 12-myristate 13-acetate
PLC	phospholipase C
PX12	2-[(1-Methylpropyl)-dithio]-1*H*-imidazole
RA	retinoic acid
RAR	retinoic acid receptor
R281	*N*-benzyloxycarbonyl-L-phenylalanyldimethylsulfonium-L-orleucine
RhoA	ras homology gene family, member A
RREs	retinoid-responsive elements
RXR	retinoid X receptor
SDS-PAGE	sodium dodecyl sulphate polyacrylamide-gel electrophoresis
SE	serum-free
SEM	serum enriched medium
siRNA	small interfering RNA
TGs	transglutaminases
TG2	transglutaminase 2, tissue transglutaminase
TGF-β	transforming growth factor β
TMB	3, 3', 5, 5'-tetramethylbenzidine
TNF-α	tumour necrosis factor α
TLR-4	toll-like receptor 4
TRX	thioredoxin

ABSTRACT

Background: In the small intestine, an organized array of finger-like villi offers an extensive epithelial surface area for absorptive function, and the integrity of the villi relies on an appropriate vascular network. In celiac disease (CD), an autoimmune disorder triggered by ingested dietary gluten in susceptible individuals carrying the human leukocyte antigen (HLA) DQ2 or DQ8 genotype, the overall mucosal vascular architecture is completely altered. CD is predominately characterized by a strong antibody response targeted against dietary gluten-derived deamidated gliadin peptides and the self-antigen, transglutaminase 2 (TG2). TG2 is a complex multifunctional protein involved in a variety of basic biological processes, including angiogenesis, where its role is still not fully understood.

Aim: The purpose of this study was to deepen our knowledge of TG2 in endothelial cell biology and to better characterize the role of CD antibodies in the celiac pathogenesis with regard to the endothelium.

Results: It has been demonstrated that down-regulation of TG2 by small interfering RNA (siRNA) deeply affects the endothelial cell biology. The intracellular pool of TG2 has been shown to be involved in the regulation of endothelial cell proliferation by means of its role in cell cycle progression from the G1 to the S phase (original article **I**). In addition, it has been found that exogenous extracellular TG2 efficiently corrects the adhesion defects in endothelial cells with siRNA-depleted endogenous TG2, but does not overcome their deficiency in cell growth and elevated apoptosis. As the phenotype described for siRNA-TG2 endothelial cells did not explain the anti-angiogenic effects exerted by CD autoantibodies it was sought to better understand the molecular mechanism underlying this modulation by means of multiple basic molecular research approaches. It was observed that CD IgA reduced endothelial cell numbers by affecting cell adhesion and protein cross-links without increasing apoptosis, and led to the secretion of active TG2 in culture supernatants (original article **II**). Additionally, cell surface TG2-mediated integrin

clustering in the presence of CD IgA coincided with augmented expression of β1-integrin. CD IgA-treated HUVECs were found to have migratory defects and a less polarized phenotype when compared to control groups associated with the RhoA signaling pathway. The biological effects on endothelial cells mediated by CD IgA were partially overcome, but not completely abolished, by peptide R281, an irreversible extracellular TG2 enzymatic activity inhibitor (original article **II**). Since the results gained from the previous study led unclarified the mechanism by which CD IgA antibodies mediated TG2 extracellular activation and because the redox sensor protein, thioredoxin (TRX) has recently emerged as a novel regulator of extracellular TG2, it was hypothesized that TRX might have had a role in this modulation. It was observed that endothelial cells cultured in the presence of CD IgA evinced decreased TRX surface expression, coupled with increased secretion of the protein into the culture medium. Intriguingly, inhibition of TRX after CD IgA treatment was able to overcome most of the CD IgA-mediated effects, including the TG2 extracellular transamidase activity, providing evidence that in endothelial cells, the celiac patient's antibodies mediate the constitutive activation of extracellular TG2 by a mechanism involving TRX (original article **III**).

Conclusions: Altogether our findings shed new light on the role of TG2 in the endothelium where the extracellular pool of TG2 is involved in regulating cell-matrix adhesion while the cytoplasmic TG2 is important for cell cycle progression and survival (original article **I**). Furthermore, it was shown that CD IgA antibodies disturb the extracellular function of TG2, thus altering endothelial cell-extracellular matrix (ECM) interactions and thereby affecting endothelial cell adhesion, polarization and motility (original article **II**). Intriguingly, CD IgA promoted the secretion of the redox sensor protein TRX, which probably is needed to keep TG2 in a conformation suitable for the constitutive antibody binding and activation (original article **III**). This work set up a possible molecular mechanism by which CD antibodies exert their anti-angiogenic functions, which in turn

might help to explain the altered small-bowel mucosal microvasculature observed in untreated CD patients.

INTRODUCTION

Among the extensive group of inflammatory disorders affecting the intestine, CD is one of the most widespread food-related lifelong conditions, involving alterations in mucosal immunity and gastrointestinal physiology during both initiation and progressive phases of the disease. At the core of these alterations endothelial cells, whose continual adjustments in structure and function coordinate vascular supply, immune cell migration, and regulation of the tissue environment (Cromer et al. 2011), might have a central role.

The villi are the functional absorptive units of the small intestine. Their overall epithelial surface provides an extensive area for nutrient absorption, and their cores contain vascular, muscular, nervous as well as immune components essential for intestinal function. The integrity of the villi depends on appropriate blood vessel formation, which in turn provides them with mechanical support.

Interestingly, it has been shown that in untreated CD patients the small-bowel mucosal vasculature is disorganized (Cooke et al. 1984, Myrsky et al. 2009).

Although the implications of deranged vasculature in CD pathogenesis remain unclarified studies in the 1970s on celiac children in remission after intraduodenal instillation of gluten showed the first discernible changes to consist in infiltration by lymphocytes, plasma cells and mast cells in the lamina propria, with thickening, widening and coarsening of the subepithelial and vascular basement membranes occurring after 48 to 96 hours, and before degeneration and shedding of villous epithelial cells. These changes suggested that the effect of gluten was not directly on the epithelium but might involve the intestinal vasculature (Shiner et al. 1972, Shiner 1973).

Besides being present in the serum of untreated CD patients, the antibodies are produced in the small-intestinal mucosa, where they are found deposited below the epithelial basement membrane and around mucosal blood vessels (Mysrky et al. 2008). In 1997, Dieterich and coworkers showed transglutaminase 2 (TG2) to be the main autoantigen of CD autoantibodies and interestingly, TG2 is

expressed, active and directly involved during wound healing and angiogenesis (Haroon et al. 1999a). In this context it has recently been shown that CD patient autoantibodies specifically targeted against TG2 inhibit several steps of angiogenesis (Myrsky et al. 2008) and increase blood vessel permeability to both macromolecules and lymphocytes (Myrsky et al. 2009) in a mechanism probably involving TG2 enzymatic activity (Myrsky et al. 2009; Caja et al. 2010).

Thus, the present work aimed to find new insights into the molecular aspects of TG2 in endothelial cell biology and to characterize the anti-angiogenic effects exerted by CD autoantibodies in this context.

REVIEW OF THE LITERATURE

1. Transglutaminase 2 (TG2)

1.1 The transglutaminase family

The transglutaminases (TGs; EC 2.3.2.13) are a family of structurally and functionally related enzymes which catalyze Ca^{2+}-dependent post-translational modification of proteins by introducing a protein-protein crosslink (between a specific glutamine and a lysine residue), amine incorporation and site-specific deamidation (Klöck et al. 2012) (Figure 1).

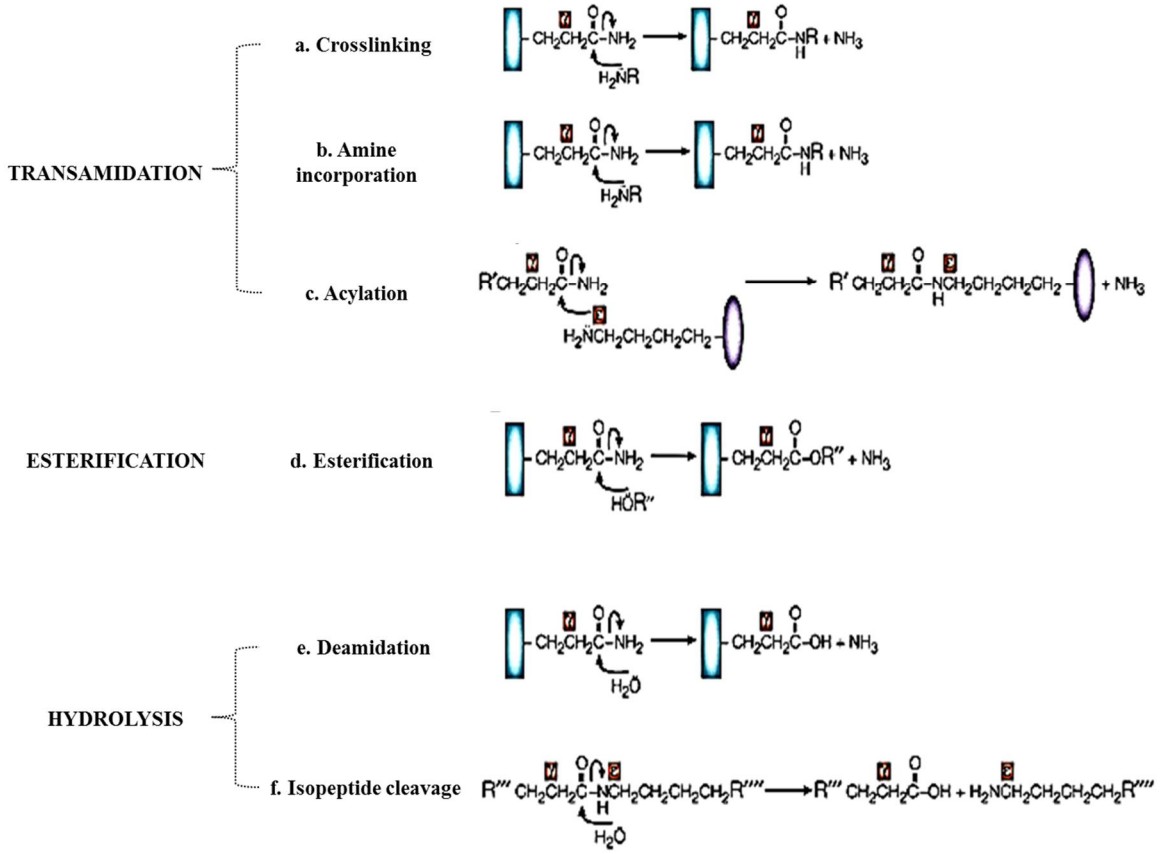

Figure 1. **Transglutaminase (TGs) enzymatic activities**. TGs can catalyze different post-translational reactions. Transamidation causes **a** protein crosslinking by forming an Nε(γ-glutamyl)lysine isopeptide bridge between the lysine (Lys) donor residue of one protein (purple ellipse) and the acceptor glutamine (Gln) residue of another (blue rectangle), **b** the incorporation of an amine (H_2NR) into the Gln residue of the acceptor protein and **c** the acylation of a Lys side chain of the donor protein. Reactions **b** and **c** compete against the crosslinking **a.** Esterification, deamidation and isopeptide cleavage are shown respectively in **d, e** and **f. "**R" represents the side-chain in a primary amine; R', a Gln-containing peptide; R", a ceramide; R"' and R"", the side-chains in branched isopeptides (modified from Lorand et al. 2003).

The term TGs was first introduced respectively by Clarke in 1957 to describe the transamidating activity observed in the guinea-pig liver. TGs are widely distributed enzymes identified in unicellular organisms, mammals and plants. Nine TG genes have been described in Homo sapiens (human), of which eight code catalytically active enzymes (Table 1). These nine evolutionarily related genes, clustered on five different chromosomes, are the products of sequential duplication and rearrangement. They have a structural homology and their expression is highly regulated at transcriptional level. While the overall primary structures of TG enzymes appear to be different, all share the same amino acid sequence at the active site (Figure 2).

Figure 2. **Alignment of the catalytic site region of the transglutaminase (TG) proteins**. In bold text are shown the amino acids forming the core fold consisting of the catalytic cysteine residue.

The catalytic mechanism of TGs resembles that of cysteine proteases. In virtually every case, their enzymatic activity is modulated by elaborate strategies including controlled gene expression, allostery, covalent modification and proteolysis (Lorand et al. 2003).

Only one of them lacks enzymatic functions (Band 4.2), while all the others require Ca^{2+} for their catalytic activity and four of them (TG2, TG3, TG4 and TG5) are inhibited by guanosine

triphosphate (GTP). An overview of the TG family members' function, distribution and disease involvement is presented in Table 1.

Table 1: Overview of mammalian transglutaminases

Protein	Main functions	Distribution	Disease	Alternate names
TG1	Cell envelope formation during keratinocytes differentiation	Membrane-bound and soluble forms in keratinocytes	Lamellar ichthyosis	TG1, transglutaminase of keratinocyte (TGK), keratinocyte TG, particulate TG
TG2	Apoptosis, cell adhesion, matrix stabilization, cell-survival signaling	Widely, ubiquitously distributed in many tissues and cells	Autoantigen in celiac disease	Tissue TG, TG, liver TG, endothelial TG, erythrocyte TG, Ghα
TG3	Cell envelope formation during keratinocyte differentiation	Hair follicle, epidermis, brain	Down-regulated in head and neck squamous and laryngeal cell carcinomas	Epidermal transglutaminase (TGE), callus TG, hair follicle TG, bovine snout TG
TG4	Reproduction	Prostate gland	Unknown	Prostate-TG (TGp), TGp-androgen-regulated-major–secretory-protein, vesiculase, dorsal prostate-protein-1 (DP1)
TG5	Cornified cell envelope formation during keratinocytes differentiation	Foreskin keratinocytes, epithelial barrier lining and skeletal muscular striatum	Secondary effect to the hyperkeratotic phenotype in ichthyosis and in psoriasis	TGx
TG6	Unknown	Testis and lung	Gluten ataxia	TGy
TG7	Unknown	Ubiquitous but mainly in testis and lung	Unknown	TGz
FXIIIa	Blood clotting, wound healing, bone growth	Platelets, placenta, synovial fluid, chondrocytes, astrocytes, macrophages	Factor XIIIa deficiency	Fibrin-stabilizing factor, fibrinoligase, plasma TG, Laki-Lorand factor
Band 4.2	Major component in erythrocyte skeletal network	Erythrocite membranes, bone marrow, spleen	Spherocytic elliptocytosis	B4.2, ATP-binding erythrocyte membrane protein band 4.2

1.2 Introduction to TG2

TG2 is the best characterized member among the transglutaminase family. It is found throughout the body due to its constitutive expression in endothelial cells, smooth muscle cells and fibroblasts as well as in a number of organ-specific cell types (Fesus et al. 1986, Thomazy et al. 1989, Aeschlimann et al. 1993, Akimov et al. 2001a, Akimov et al. 2003). TG2 is a structurally and functionally complex protein, with both intracellular and extracellular functions (Lorand et al. 2003). It is involved in several important biological processes and its expression depends on the cell type in question (Nurminskaya et al. 2012). Although the protein is mainly localized in the cytosol, it can also be found extracellularly, bound to the cell surface or intracellularly in the the mitochondria and in the nucleus (Belkin 2011).

1.2.1 TG2 gene

The human TG2 gene (TGM2) has been mapped on chromosome 20q11-12 and is 32.5kb (Gentile et al. 1994). The TG2 promoter is characterized by a retinoic acid (RA) response element (RRE) located 1.7 kb upstream from the initiation site, an interleukin-6 specific cis-regulatory element located within first the 4.0 kb of the promoter, a TGF-1 response element (GAGTTGGTGC) located 868 base pairs upstream, and two activator protein (AP)-2-like response elements located approximately 634 and 183 base pairs upstream from the transcription initiation site (Figure 3).

1.2.2 TG2 gene expression and regulation

The expression of TG2 is thought to be controlled mainly at the transcription level (Griffin et al. 2002) and it can be regulated by several factors, including cytokines and hormones. The human TG2 promoter is extremely responsive to various activators, as shown in Figure 3.

RA treatment can induce TG2 expression both at the mRNA and at the protein level *in vitro* and *in vivo* (Verma et al. 1992, Defacque et al. 1995). It has been reported that the TG2 gene contains regulatory elements for several transcriptional factors, as for example two tandem RREs, which bind the retinoid receptors RAR and RXR. RA promotes the expression of TG2 via its interaction

with its receptors to promote the formation of RAR/RXR heterodimers or RXR/RXR homodimers, which triggers the transcription of the gene (Chiocca et al. 1988, Mehta et al. 1996).

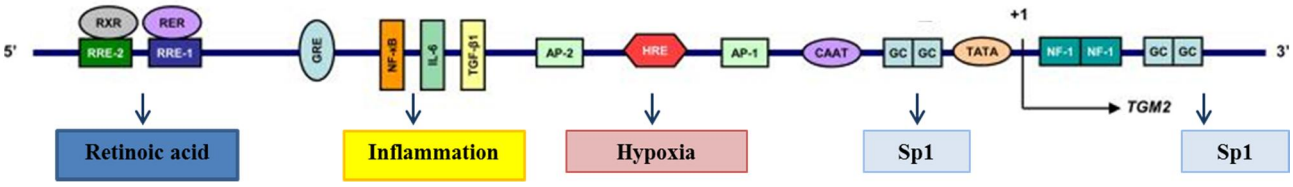

Figure 3. **The human transglutaminase 2 gene (*TGM2)* expression regulatory elements**. TG2 expression regulatory elements are highlighted: retinoic acid response elements (RRE-1 and RRE-2), glucocorticoid response element (GRE). nuclear factor κB response element (NF-κB), interleukin-6 response element (IL-6), tumor growth factor-β1 (TGF-β1), activator protein-2 (AP-2), hypoxia response element (HRE), activator protein-1 (AP-1), CAAT box, GC box (Sp1 binding motifs), TATA box, nuclear factor-1 (NF-1).

Furthermore, it has been shown that TG2 expression can be regulated by several cytokines such as interleukins (e.g., IL-1 and IL-6), the transforming growth factor β1 (TGFβ1), and morphogenic protein 4.

In this regard, the inflammatory cytokine tumor necrosis factor (TNF) α can increase the expression of TG2 in liver cells by the NF-κB (nuclear factor kappa-light-chain enhancer of activated B cells)-signaling pathway (Lu et al. 1995, Nagy et al. 1996, Mirza et al. 1997, Ritter et al. 1998, Shimada et al. 2001) and TGFβ1 can increase the expression of cell surface TG2, which results in enhanced cell adhesion (Priglinger et al. 2004). It is well known that macrophage activation triggers TGFβ expression and increases TG2 levels, hallmarks of phagocytotic capacity (Griffin et al. 2002, Telci and Griffin 2006). Furthermore, TG2 is able to activate the matrix-bound TGFβ via cross-linking of the latent TGFβ-binding protein-1 (LTBP-1). Taken together these findings rule out a positive feedback mechanism between TG2 and TGFβ1 at cellular level.

Some results also suggest that DNA methylation, together with alterations in the chromatin structure, may play a role in TG2 expression (Lu and Davies 1997, Cacciamani et al. 2002). In fact,

the TG2 promoter can be highly methylated at CpG islands, thus silencing the gene in relation to tissue differentiation, resulting in the rather selective expression of the enzyme in endothelial and smooth muscle cells (Lu and Davies 1997). Consequently, the TG2 promoter not only serves as a site for induction, but also plays a role in silencing TG2 expression. The appearance of TG2 largely shows a relationship with aggressiveness in cancer cells (Mehta et al. 2006). In a breast cancer cell model it has been shown that CpG islands in the TG2 promoter are hypermethylated, TG2 expression being thus epigenetically silenced in less aggressive cell types (Ai et al. 2008). Very similar observations were made in non-small cell lung cancer (Park et al. 2010) and in glioma (Dyer et al. 2011). These studies provide strong evidence for the utilization of epigenetic methylation as a means to silence TG2 expression in various cancers. Another mechanism for down-regulating TG2 promoter activity involves histone deacetylases (HDACs). In a neuroblastoma model it has been shown that N-myc protein trans-repressed the activity of the TG2 promoter and hence TG2 expression, by recruiting HDAC1 to a Sp1 binding site on the TG2 promoter (Liu et al. 2007). The studies in question revealed the importance of TG2 expression to the cell, demonstrating how actively the TG2 promoter is regulated by trans-activation, trans-repression and epigenetics. Overall, TG2 is generally up-regulated under stress conditions and can be categorized as a stress response protein (Ientile et al. 2007, Luciani et al. 2009, Caccamo et al. 2012, Ai et al. 2012, Campisi et al. 2012). This up-regulation in TG2 levels is probably part of a cellular protective response (Filiano et al. 2008, Basso et al. 2010, Cho et al. 2012). However, if the insult is excessive and cellular demise is inevitable, TG2 may also facilitate cell death (Luciani et al. 2009, Caccamo et al. 2012).

1.2.3 TG2 architecture

The TG2 gene product is a multi-structural protein of 686 amino acids with a molecular weight of 80 kDa (Lorand and Graham 2003). TG2 is composed of 13 exons separated by 12 introns. The three-dimensional structure of TG2 has been deduced based on that of Factor XIIIa and it contains

four main domains: the N- terminal β- sandwich domain, the α/β catalytic core, and two C-terminal β-barrel domains (Chen and Mehta 1999, Han et al. 2010), as shown in Figure 4. In the N-terminus of the TG2 molecule, there is the fibronectin (FN) binding site mediating the interaction between TG2 and FN with high affinity, reported to be involved in cell adhesion (Akimov et al. 2000, Akimov et al. 2001b), matrix assembly (Telci and Griffin 2006), and TG2 externalization (Balklava et al. 2002) and thought to play a crucial role for the transamidating activity of TG2 (Iismaa et al. 1997).

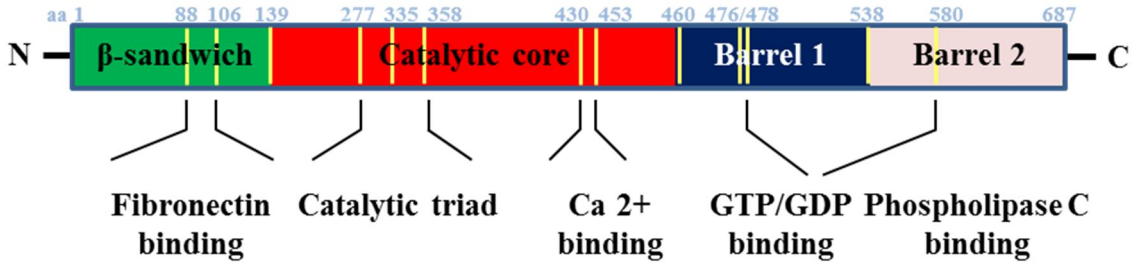

Figure 4. **Transglutaminase 2 (TG2) architecture**. The TG2 protein consists of four distinct domains, each of which has a unique function. The N-terminal β-sandwich domain contains a high-affinity binding site for fibronectin and is responsible for adhesion to the extracellular matrix. The catalytic core domain of TG2 is responsible for its enzymatic activity induced and stabilized by Ca^{2+}. The Barrel 1 domain contains a guanosine-tri/di-phosphate (GTP/GDP) -binding site which plays an important role in TG2-mediated signaling pathways. The C-terminal Barrel 2 domain under certain conditions (e.g., in GTP-bound form) can recruit and activate phospholipase C and contributes to the pro-inflammatory functions of TG2.

In contrast, the α/β catalytic domain of TG2 is involved in GTPase/ATPase and transamidase activities. The GTP-binding site is localized between amino acids 159-173 in the core domain of TG2 even though it has also been reported that Trp332, Arg580 in human and Arg579 in the rat are essential (Murthy et al. 2002). Moreover, in 2010 Han and coworkers, studying the TG2 crystal structure, highlighted the relevance of four residues Arg476, Arg478, Val479 and Tyr583 which might play important roles in the stabilization of TG2 by ATP or GDP. In contrast, Ser482 and Arg580, which are involved in GDP binding, do not form a hydrogen bond with ATP (Han et al.

2010). The GTPase activity has been linked to the function of TG2 as a G protein (Gαh) involved in signaling from α1B/D adrenergic receptors to downstream effectors such as phospholipase Cδ1. In this regard, close to the TG2 C-terminal domain, eighteen-amino acid residues, Val655- Lys672, are essential for the protein to recognize and activate the phospholipase C (PLC), which is important in transmitting the α1B- adrenoceptor (AR) signal (Feng et al. 1999).

As a Ca^{2+}-dependent enzyme TG2 enzymatic activity is regulated by Ca^{2+}-binding within amino acids 430-453 of the core domain (Ikura et al. 1988). In particular, mutagenesis TG2 studies have demonstrated that there are five possible Ca^{2+} binding sites around amino acids 228, 395, 305, 149 and 432 (Kiraly et al. 2009). The Tyr241 in the catalytic domain of TG2 is essential for TG2 activity, while the Cys277 forms the thioester bond involved in transamidation (Chen and Mehta 1999).

1.2.4 Regulation of TG2 protein functions

Under normal conditions, TG2 exists as a latent enzyme (Siegel et al. 2008). This feature is valid in particular for the intracellular pool of the protein, where the low Ca^{2+} levels and the inhibitory effect of GTP/GDP keep it enzymatically inactive (Park et al. 2001). The regulatory effect of GTP on TG2 activity is well known especially from *in vitro* studies (Im et al. 1997, Mhaouty-Kodja 2004). In fact, the binding of GTP to TG2 leads to conformational changes and reduces the affinity for Ca^{2+} (Smethurst and Griffin 1996, Begg et al. 2006, Kiraly et al. 2009). The capability of TG2 to bind and hydrolyze GTP links intracellular TG2 to a major signaling pathway, in which TG2 behaves as a signal transduction GTP-binding protein, Gαh (Feng et al. 1999, Baek et al. 2001, Kang et al. 2002, Kang et al. 2004). Extracellular TG2 transmits outside signals from the membrane (e.g. α1B and α1D adrenoreceptor) to downstream cytoplasmic targets such as PLCδ in order to regulate cellular processes, e.g. cell migration (Murthy et al. 1999, Feng et al. 1999, Kang et al. 2004). Magnesium ions (Mg^{2+}) are also essential for the hydrolysis of both GTP and ATP. Mg^{2+}-ATP induces a conformational change in TG2 which inhibits GTPase but does not interfere with the

cross-linking activity, while Mg^{2+}-GTP binding induces a different conformation which inhibits cross-linking activity without affecting the ATPase activity of TG2 (Lai et al. 1998). Similarly, nitric oxide (NO) is a potent inhibitor of TG2 activity (Bernassola et al. 1999, Santhanam et al. 2010), through a Ca^{2+}-sensitive nitrosylation of multiple cysteine residues (Lai et al. 2001, Telci et al. 2009).

Nevertheless, under extreme conditions of cell stress or trauma after the disturbance or loss of Ca^{2+} homeostasis, TG2 may be activated and cause cross-linking of intracellular proteins, as is observed during apoptosis or necrosis (Griffin et al. 2002, Fesus et al. 2005, Gundemir et al. 2009).

TG2 also functions as protein disulfide isomerase (PDI) (Hasegawa et al. 2003, Mastroberardino et al. 2006), which can mediate post-translational protein modifications. The TG2-PDI activity depends neither on Ca^{2+} nor on GTP (Hasegawa et al. 2003). In this context, it is possible that TG2 can exhibit PDI activity in a fairly reducing environment such as cytosol, where it is most abundant. It is quite obvious from the following observations that the PDI activity of TG2 is catalyzed by a domain different from that used for the enzymatic reaction. In fact, although the alkylation of Cys residues in TG2 completely abolished the transglutaminase activity, as was expected, it did not affect the PDI activity at all (Hasegawa et al. 2003). Recently, it has also been shown that TG2-PDI is important in maintaining the correct assembly of the mitochondrial ADP/ATP transporter adenine nucleotide translocator 1 (ANT1), over and above its proposed potential involvement in mitochondria- related apoptosis (Malorni et al. 2009). Mishra and Murphy also discovered an intrinsic kinase activity for TG2 (Mishra and Murphy 2004). Furthermore, as a PDI, TG2 can phosphorylate the insulin-like growth factor-binding protein-3 (IGFBP-3) and enhance its binding affinity to IGF-I to modulate the pro-apoptotic anti-proliferation effect of IGFBP-3 in breast cancer cells. This IGFBP-3 kinase activity of TG2 can be inhibited by an increase in Ca^{2+} concentration (Mishra and Murphy 2004).

1.3 TG2 secretion

TG2 has essential functions in the extracellular environment, making an understanding of its levels and regulation on the cell surface and in the extracellular matrix (ECM) essential. There are no secretory signal sequences and hydrophobic or transmembrane domains in TG2, unlike the majority of other secretory proteins, and for this reason TG2 cannot be secreted by the classical ER/Golgi-dependent mechanism. In contrast, the latest studies have elegantly shown that TG2 is externalized by a non-classical mechanism which involves the endosomal recycling pathway (Figure 5) (Zemskov et al. 2011, Belkin 2011).

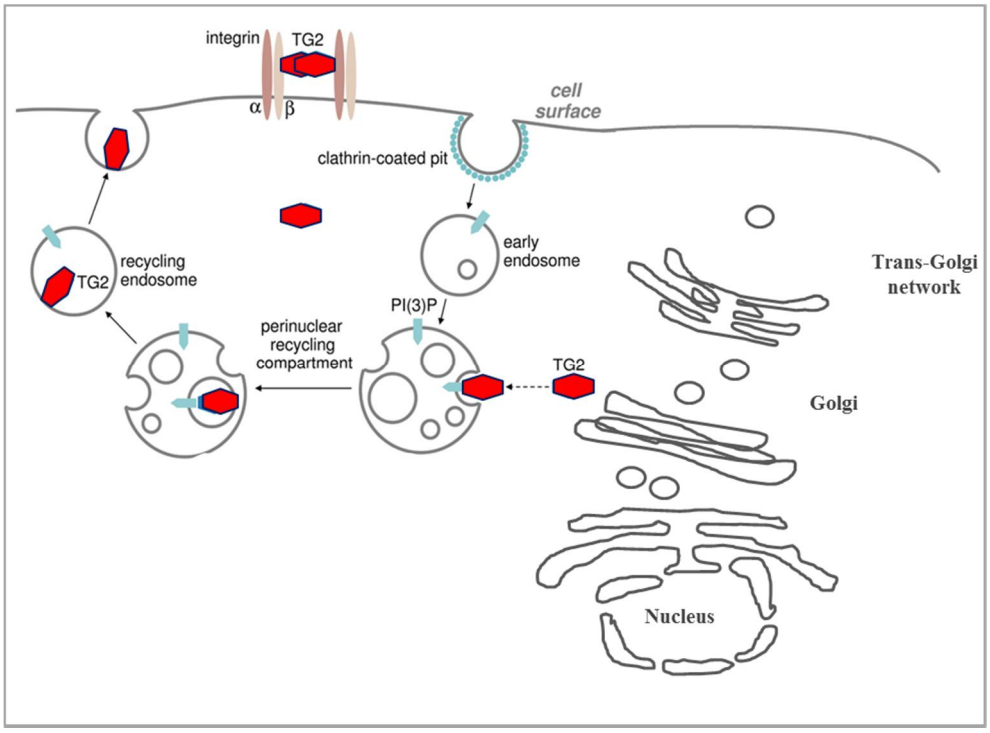

Figure 5. **Non-classical secretion of transglutaminase 2 (TG2).** The unconventional pathway of TG2 cell secretion requires phospholipid-dependent delivery into recycling endosomes. The major endosomal recycling pathway is shown with solid lines. This pathway operates through the perinuclear recycling endosomal compartment. The PI(3)P-dependent recruitment of TG2 from cytoplasma to the membranes of the perinuclear recycling compartment is indicated with dashed lines (modified from Belkin 2011).

Many cell types, including fibroblasts, osteoblasts, endothelial cells, smooth muscle cells, monocytes and macrophages constitutively externalize TG2 on their surface and in the ECM (Zemskov et al. 2006, Belkin 2011).

It has been reported that FN and heparan sulfate proteoglycans, two well-known extracellular TG2 binding partners, influence its exportation in a process which requires, at least in part, TG2 transamidation activity (Upchurch et al. 1991, Gaudry et al. 1999a, Balklava et al 2002, Nicholas et al. 2003, Johnson et al. 2005, Scarpellini et al. 2009). These findings suggest that TG2 is secreted by unconventional mechanisms. In this regard, Zemskov and co-workers (2006) began to delineate the secretion pathway of cytoplasmic TG2 by focusing on its intracellular trafficking routes. They revealed that instead of being directed to the classical ER⁄ Golgi-dependent secretion pathway, de novo synthesized cytoplasmic TG2 is targeted to perinuclear recycling endosomes, and it is delivered inside these transport vesicles prior to exportation. The authors also showed that inactivation of recycling endosomes by blockage of endosome fusion with the plasma membrane, or by down-regulation of Rab11 GTPase, which controls the outbound trafficking of perinuclear recycling endosomes, inhibit TG2 exportation.

The interaction of TG2 with intracellular transport vesicles consists in a two-step process with its initial binding to endosomal phosphoinositides and subsequent tight interaction with as yet unknown endosomal membrane protein(s). Research is currently ongoing in order to identify such TG2-interactors on the recycling endosomes.

1.4 TG2 biological functions

As a widely-expressed multi-functional protein, the precise physiological functions of TG2 are still not clear. Accumulating evidence demonstrates that TG2 is involved in several biological and physiological processes, including cell adhesion (Akimov et al. 2000; Akimov et al. 2001; Balklava et al. 2002; Verderio et al. 2003; Zemskov et al. 2006), proliferation (Mian et al. 1995, Mangala et al. 2007, Kotsakis et al. 2011), differentiation (Gentile et al. 1992, Van Strien et al. 2011), apoptosis

(Fesus et al. 1987, Yoo et al. 2012, Garabuczi et al. 2013) and matrix stabilization (Verderio et al. 1998, Quan et al. 2005, Belkin 2011).

A large body of evidence again suggests that the distribution of TG2 in distinct cell compartments is very likely linked to different functional activities (Park et al. 2010, Nurminskaya et al. 2012).

TG2 is deposited into the matrix in a controlled fashion and its transamidating activity is dormant (Siegel et al. 2008). In fact, TG2 seems to be activated only after disruption of the ECM, rather than released from damaged cells. This effect has been ascribed to the anchorage of TG2 to integrins and to ECM-associated FN, even though it cannot be excluded that additional factors (e.g., enzyme nitrosylation and/or oxidation) may have a role (Melino et al. 1997, Bernassola et al. 1999, Telci et al. 2009, Santhanam et al. 2010). It is thus tempting to speculate that a transient release of reducing agents such as thioredoxin or glutathione from damaged cells might trigger TG2 reduction and/or relieve its inhibition from nitrosylation or oxidation of essential cysteines (Kuo et al. 2002, Jin et al. 2011).

Regardless of the mode of secretion into the ECM, TG2 is thought to be retained on the cell surface by virtue of its interaction with FN (Gaudry et al. 1999b, Akimov et al. 2000). Interestingly, FN-cross-linking mediated by TG2 offers FN protection from degradation (Mosher 1978, Kinsella et al. 1990). Moreover, FN and TG2 secreted into the ECM can interact with each other independently of the enzyme's cross-linking activity (Verderio et al. 2003). In this regard, it has been shown that TG2 secreted to the ECM could bind to the N-terminal portion of FN, interacting either with the type I4-I5 motif (LeMosy et al. 1992) or with a sequence within the gelatin-binding domain of FN (I6-II1-II2-I7-I8-I9) (Radek et al. 1993). Extracellular TG2 is also associated with integrin receptors in a number of different cell types via binding to the extracellular domains of the β1, β3 and β5 integrin subunits (Gaudry et al. 1999, Akimov et al. 2000, Akimov and Belkin 2001b).

Due to the fact that TG2 has an FN binding site (the 42kD gelatin-binding domain) and an integrin binding site (RGD cell-binding site) located in different parts of the FN molecule, there is a

possibility that TG2 and integrin collaborate rather than compete with each other in the cell adhesion process. Unlike FN, integrins do not appear to serve as enzymatic substrates of TG2 or other transglutaminases, and the formation of stable non-covalent integrin-TG2 complexes is independent of the transamidating activity of TG2 (Akimov et al. 2000, Akimov and Belkin 2001). Although the binding sites for TG2 within integrins are still unknown, it has been demonstrated that integrin-TG2 complexes have a 1:1 stoichiometry and all the TG2 on the cell surface is bound to integrin receptors in an FN-independent manner (Akimov et al. 2000, Akimov and Belkin 2001). Besides the evidence of structural interaction, a series of studies led by Mehta and colleagues (2006) demonstrated the connection between TG2 and the integrin-relevant downstream signaling molecule focal adhesion kinase (FAK). Culture of TG2-positive breast cancer cells and TG2-transfected fibroblasts on FN-coated surfaces led to the activation of FAK (Mehta et al. 2006). Conversely, down-regulation of TG2 by small interfering RNA attenuates FN-mediated cell attachment (Herman et al. 2006) and cell survival and FAK phosphorylation (Verma et al. 2006).

TG2 also exists in the ECM as a structural protein (Verderio et al. 1998). The importance of TG2 as an ECM protein in RGD-independent cell adhesion was first indicated by Verderio and coworkers in 2003 and supported by later studies. In this context it was shown that TG2 bound to FN matrix compensated anoikis induced by RGD synthetic peptides. This process was PKC α and FAK-dependent. In addition, a recent study has shown that GTP-bound TG2, when present in the ECM, can induce hypertrophic differentiation of chondrocytes through a α5β1 integrin-dependent and FAK-associated cell adhesion process (Tanaka et al. 2007).

Cell surface TG2 is highly sensitive to the proteolytic degradation mediated by membrane type-matrix metalloproteinases (MT-MMPs) (Zemskov et al. 2006, Nakano et al. 2010), thought to be primarily involved in ECM degradation (Kessenbrock et al. 2010). In particular, proteolysis of TG2 by MT1-MMP specifically suppresses cell adhesion and migration to fibronectin (Belkin et al. 2001).

In contrast, in the intracellular compartment TG2 functions as a transamidating protein as well as a signaling molecule, which alternatively predominate over each other when the enzyme is present respectively in the cytosol or is associated with the membrane internal surface (Park et al. 2010). Of note are also reports that TG2 may influence transcriptional or signaling events by acting as a scaffolding protein in virtue of its interaction with several intracellular proteins involved in important physiological processes (Kuo et al. 2011, Basso et al. 2012, Kumar et al. 2012).

In addition, TG2 has a well-established role in cell death, though its participation needs to be further elucidated, since it has been suggested that TG2 can be either involved in apoptotic cell death or associated with cell survival, in response to different stimuli (Ientile et al. 2007). This ambiguity derives from studies on TG2 -/- mice, which display no specific phenotype attributable to altered apoptosis, where the presence of a compensating mechanism activated by other members of TGase family might play a key role (De Laurenzi et al. 2001, Deasey et al. 2013).

A truncated isoform of TG2 as a consequence of alternate mRNA splicing has been observed in cytokine-induced cells (Fraij et al. 1992, Monsonego et al. 1999, Citron et al. 2002, Festoff et al. 2002, Citron et al. 2005). This TG2 isoform lacks the C-terminal region, which is involved in GTP-binding, thus reducing its affinity for GTP and inducing its activation intracellularly by lower Ca^{2+} concentrations than the full length protein. In this regard, the pro-apoptotic effects are exerted by the formation of protein aggregates in the cytosol of the cells which are themselves cytotoxic (Antonyak et al. 2006).

There are moreover other factors which might contribute to the regulation of intracellular TG2, for example pH variations (Chae et al. 2005), endogenous nitric oxide (Jandu et al. 2013), protein nitrosylation levels (Lai et al. 2001) or monoaminylation (Paulmann et al. 2009, Walther et al. 2011, Hummerich et al. 2012).

1.4.1 TG2 and angiogenesis

TG2 is an important tissue-stabilizing enzyme which is active during wound healing and can function to promote angiogenesis (Haroon et al. 1999a). TG2 is widely expressed by cardiac and vascular cells, including endothelial and smooth muscle cells, which constitutively express it at high levels (Thomazy et al. 1989).

Changes in the behavioral characteristics of endothelial cells, in particular regarding their proliferation, migration, and differentiation into tubular structures, which is influenced by changes in the ECM, lead to the formation of new blood vessels.

Many ECM proteins are known substrates of TG2 and the crosslinking of these proteins by endothelial cell TG2 is thought to play a role in the stabilization of the basement membrane (Martinez et al. 1994). Furthermore, cell surface TG2 is also thought to be involved in cell migration and cell adhesion, by a mechanism independent of its transamidating activity. While some of these TG2 functions are similar to the critical steps involved during angiogenesis, the role of TG2 in this process is poorly understood. TG2 knockout mice do not present vascular abnormalities and down-regulation of TG2 has been reported in endothelial cells undergoing capillary morphogenesis (Bell et al. 2001). These findings fit with the proposed extracellular role of the enzyme in ECM deposition and stabilization, since angiogenesis requires localized destabilization of the matrix by its increased turnover (Ingber 2002). In contrast, conflicting *in vivo* studies have proposed that the enzyme both stimulates and inhibits angiogenesis (Haroon et al. 1999a and Haroon et al. 1999b).

2. TG2 and celiac disease (CD)

2.1 CD history and epidemiology

The first description of CD dates back to the Greek physician Aretaeus the Cappadocian, between the first and second centuries AD, and described as an intestinal disorder associated with diarrhea and malabsorption. The idea that the disease was linked to food ingestion was brought forward in

1888 by Gee but the first CD clinical manifestation was reported only in 1954 by Paulley. Later in 1992, Marsh established a histological classification of celiac lesions, which ranges from hyperproliferative crypts with intraepithelial lymphocytosis to total villous atrophy:

- Marsh 0: normal mucosa
- Marsh 1: increased number of intra-epithelial lymphocytes
- Marsh 2: crypt hyperplasia
- Marsh 3: partial or complete villous atrophy
- Marsh 4: incomplete development (hypoplasia) of the small bowel (Marsh 1992).

Endoscopy with biopsy of the small intestine coupled to positive serology provides a gold standard for CD diagnosis. The serologic studies for CD can be divided into two groups, based on target antigens:

- anti-TG2 antibody test
- anti-gliadin antibody test

The clinical classification of CD is:

- classical, associated with gastrointestinal symptoms;
- atypical, usually characterized by non-gastrointestinal symptoms;
- silent, with no symptoms but with a characteristic intestinal lesion present.

The CD epidemiology has iceberg characteristics, since there are more undiagnosed than diagnosed cases. The risks are higher in first degree relatives and in people with Down's and Turner's syndrome, diabetes or other autoimmune disorders (Bai et al. 2007). In CD, fertility is affected and pregnancy may proceed unfavorably in undiagnosed women (Ferguson et al. 1982).

CD is of global relevance since it affects 0.2-2.5% of the world population (Table 2) (Tack et al. 2010, Abadie et al. 2011).

Table 2: Worlwide prevalence of celiac disease in adults

Geographic area	Prevalence %
Finland	0.55-2.0
Germany	0.19
Great Britain	1.2
Italy	0.18
Russia	0.20
Spain	0.26
Sweden	0.46-0.53
Mexico	2.6
USA	0.4-0.95
Iran	0.6
Syria	1.6
Turkey	1.3
Tunisia	0.28
New Zealand	1.2

2.2 CD pathogenesis

The pathogenesis of CD is multifactorial and involves both innate and adaptive immune responses (Figure 6). Patients have dysfunctional immune regulation, accompanied by a massive intestinal autoantibody response, mainly of IgA class, against gluten-derived deamidated gliadin peptides and the self antigen, TG2, paralleled by the development of a small-bowel mucosal lesion. In addition to circulating in the blood, both conventional anti-gliadin antibodies and anti-TG2-autoantibodies are found in intestinal secretions. Interestingly, in untreated CD patients anti-TG2-autoantibodies are found deposited in the small-bowel mucosa below the epithelial basement membrane and around capillary vessels before the development of villous atrophy and crypt hyperplasia, which represent typical features of the disease.

The intestinal immunological response is triggered by specific gliadin peptide populations resulting from their incomplete digestion by gastric and pancreatic enzymes. In the small intestine of CD patients, specific glutamine residues within gluten are deamidated by TG2, thus increasing their binding affinity for HLA-DQ2 or DQ8 and enabling T cell activation and inflammation (Molberg et

al. 1998, Schuppan and Hahn 2002). Toxic gliadin peptides such as p31–43 induce an innate immunity response probably mediated by the cytokine interleukin-15 (IL-15) (Maiuri et al. 2003), which ultimately leads to epithelial cell damage. Furthermore, the predominant cytokine secreted when DQ2-restricted, gluten-responsive T cells derived from the celiac intestine are activated (Sollid 2002) is interferon-γ (IFN- γ), which in turn triggers TRX-mediated activation of extracellular TG2, establishing an auto-amplificatory loop for gluten-mediated inflammation (Jin et al. 2011). The upregulation of IL-15 and IFN- γ in the lamina propria induces dendritic cells to acquire a proinflammatory phenotype (Abadie et al. 2012).

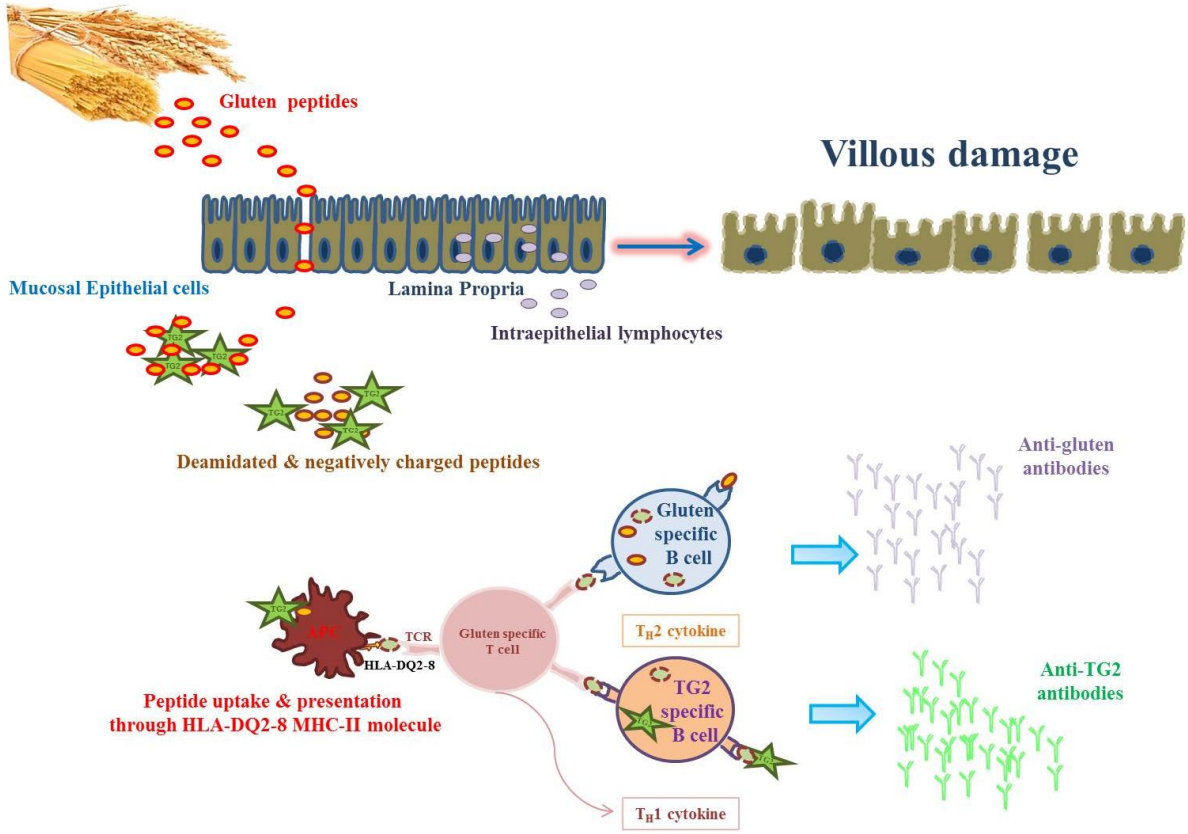

Figure 6. **Celiac disease pathogenesis overview.** Mature dendritic cells (DCs) present gluten in order to induce the activation of gluten-specific HLA-DQ2- or HLA-DQ8-restricted $CD4^+$ T cells. TG2 and gluten form complexes, internalized by TG2-specific autoreactive B cells and then presented through HLA-DQ2 or HLA-DQ8 at their surface. Stressed epithelial cells produce interleukin-15 (IL-15) and type I interferon (IFN), thus acquiring pro-inflammatory properties. The gluten-specific $CD4^+$ T cells provide help to both autoreactive TG2-specific and gluten-specific B cells, which mature into antibody-producing plasma cells. Activated gluten-specific $CD4^+$ T cells also provide signals (which remain to be defined) to pre-activated epithelial cells, which upregulate the expression of IL-15.

However, the transport of immunogenic gliadin peptides such as 33-mer and its shorter fragment p57–68 into the lamina propria, initiates an adaptive immunity response. Moreover, the formation of gliadin-TG2 complexes supports the production of anti-TG2 antibody, which can in turn modify the activity of the enzyme and some enzymatic-independent functions of the protein at the cell surface (Molberg et al. 2000, Caputo et al. 2004).

2.2.1. Role of autoantibodies in the pathogenesis of CD

The presence of autoantibodies was formerly thought to be only an epiphenomenon and later they were generally regarded merely as bystanders in CD pathogenesis (Sollid et al. 2005, Green et al. 2006).

In contrast, current studies have for the most part shown patient-derived anti-TG2 autoantibodies to have biological functions. In 1999 a group under Mäki first provided evidence for this by showing that CD-associated IgA class antibodies disturb TGF-β-mediated fibroblast-epithelial cell cross-talk in an *in vitro* crypt-villus axis model, with a simultaneous increase in proliferation rate (Halttunen et al. 1999). This primary finding indicated that CD-specific autoantibodies might also have contributed to the formation of the gluten-triggered jejunal mucosal lesion in CD. Later, in 2004, Freitag and coworkers demonstrated that immunized wildtype, but not TGase 2 (-/-) mice, with human or guinea pig TG2, developed periductal lymphocytic infiltrates in lacrimal glands. Although no intestinal lesions were found, this observation provided support for the conception that the development of autoimmunity against TG2 was a pathological event which might ultimately lead to organ damage (Freitag et al. 2004). Zanoni and coworkers again showed that in active CD, a subset of anti-transglutaminase IgA antibodies recognized the viral protein VP-7, suggesting a possible involvement of rotavirus infection in the pathogenesis of the disease, through a mechanism of molecular mimicry. Moreover, such antibodies recognized self-antigens such as TG2, and were functionally active, able to increase intestinal permeability and to induce monocyte activation. *In vivo,* activation of monocytes was associated with increased levels of TG2 enzymatic activity

(Fesus et al. 1981). Thus evidence occurred for the involvement of innate immunity in the pathogenesis of CD through a previously unknown mechanism of engagement of Toll-like receptor 4 (Zanoni et al. 2006). In 2007, another group reported that TG2 autoantibodies per se, by interacting with the extracellular membrane-bound TG2, had an important role in epithelial cell proliferation in CD (Barone et al. 2007).

More recently, our group has provided a large body of evidence for the anti-angiogenic effects exerted by CD-specific autoantibodies recognizing TG2. In 2008, Myrsky and coworkers for the first time validated the conception that in CD, ingestion of gluten leads to an altered appearance of small-bowel mucosal microvasculature. It was thus conceivable that the small-bowel mucosal vascular biology might have been involved in the pathogenesis of CD. Successive *in vitro* studies confirmed this observation in demostrating that CD-specific autoantibodies disturbed several steps of angiogenesis by inhibiting the sprouting of endothelial cells and by preventing migration of endothelial cells along the newly formed branch (Myrsky et al. 2008). It was found, furthermore that disease-specific autoantibodies targeted against TG2 contributed to the pathogenic cascade of CD by increasing blood vessel permeability by augmented activities of TG2 and RhoA (Myrsky et al. 2009). In 2010 Caputo and co-workers reported that interaction between anti-TG2 antibodies and extracellular membrane-bound TG2 inhibits peptide 31-43 (but not peptide 57-68) uptake by cells, thereby impairing the ability of p31-43 to drive Caco-2 cells into the S-phase of the cell cycle. This effect did not involve TG2 enzymatic activity.

Teesalu and coworkers in 2012 provided evidence for a role of CD antibodies in cell adhesion. In fact, sera and purified anti-TG2 antibodies from CD patients with high anti-TG2 IgA levels reduced TG2 binding to heparin/HS as compared with those with low anti-TG2 IgA or controls. Treatment of FN-TG2-coated wells with CD patients' sera or purified anti-TG2 antibodies reduced attachment of Caco-2 cells onto the plate as compared with the control samples. The effect of CD patient antibodies on Caco-2 cell attachment to the FN-TG2 matrix was shown to occur independently of

the inhibition of cell adhesion by Arg-Gly-Asp sequence containing peptides. Thus, the authors speculated that modulation of the adhesion function of TG2 by autoantibodies from patients with CD could be related to the inhibition of TG2 binding to HS residues of cell surface proteoglycans and could have possible implications for CD pathogenesis (Teesalu et al. 2012). Indeed, blistering of epithelial cells in the small-intestinal lesion of CD patients has previously been reported (Kainulainen et al. 2002), and the finding that IgG purified from CD patients' sera impaired the invasiveness of trophoblastic cells (Di Simone et al. 2010) also indirectly supported the role of anti-TG2 antibodies in modifying cellular adhesion (Teesalu et al. 2012).

2.2.2 Extraintestinal manifestations of CD

CD may also manifest itself with extraintestinal symptom, such as neurological problems, dermatitis herpetiformis (DH), metabolic bone disease (MBD), malignant disorders and other autoimmune conditions. In none of these cases has a clear relationship between the extraintestinal manifestations and the specific autoantibodies produced in CD been defined.

In patients with gluten ataxia, CD autoantibodies are present and deposited in the brain vasculature (Hadjivassiliou et al. 2006, Nanri et al. 2011, Hadjivassiliou 2012). It is not clear whether or not these autoantibodies possess biological functions, but Cervio and coworkers (2007) showed that anti-neuronal antibodies in the sera of CD patients coupled with neurologic disorders evoked apoptosis in a human neuroblastoma cell line. This neuronal damage was caused by the presence of IgG-class anti-neuronal antibodies, whose deprivation from the sera was associated with an apoptotic degree similar to controls. In contrast, when the authors examined CD sera without neurologic disorders and anti-neuronal antibodies, a certain degree of apoptosis, albeit to a lesser extent, was observed, supporting a neurotoxic potential intrinsic to CD. This pro-apoptotic mechanism, involving the mitochondria impairment pathway, was very likely dependent on the combination of anti-gliadin and anti-TG2 antibodies. Thus, the findings supported the conception that evident neurologic manifestations in patients with CD derive from a combination of sub-

threshold neurotoxic effects due to humoral markers of CD with those triggered predominantly by anti-neuronal antibodies (Cervio et al. 2007).

A cutaneous manifestation of CD is DH, diagnosed by skin biopsy. In patients with DH, TG3 appears to be the dominant autoantigen and it is expressed primarily in the epidermis and small intestine (Sardy et al. 2002, Jaskowski et al. 2009).

Again, MBD is a less well recognized manifestation of CD associated with a wide spectrum of musculoskeletal signs and symptoms such as bone pains, proximal muscle weakness, osteopenia, osteoporosis and fracture (Rastogi et al. 2012). In addition, it has been reported that small-bowel adenocarcinoma, esophageal and oropharyngeal squamous-cell carcinoma and non-Hodgkin's lymphoma occur more frequently in CD patients in comparison to the general population (Bai et al. 2007). Furthermore, in CD patients other autoimmune disorders, including type 1 diabetes, thyroid disease, cardiomyopathy and autoimmune liver disease, occur ten times more frequently than in healthy subjects (Bai et al. 2007, Naiyer et al. 2008).

CD is also associated with poor pregnancy outcome, indicating a direct immune effect on placenta function in untreated women (Anjum et al. 2009).

2.3 Effects of CD on TG2 enzymatic activity

CD autoantibodies recognize TG2 *in vivo* in many organs (Dieterich et al. 1997, Korponay-Szabo' et al. 2004, Salmi et al. 2006) and this binding with regard to TG2 biology has a central role in CD pathogenesis.

TG2 has complex biochemical and cellular functions: under normal conditions its enzymatic activity is inhibited within cells, while it is latent in the extracellular compartment, where TG2 functions mainly in a FN-bound form (Akimov et al. 2000, Verderio et al. 2003).

In CD, TG2 increases the antigenicity of gliadin peptides by deamidation of glutamine residues, which then interact with the enzyme in a higher selective manner. These complexes are crucial for the T-cell recognition process (Molberg et al. 1998, Arentz-Hansen et al. 2000, Shan et al. 2002).

No clear relation between TG2 enzymatic activity and CD autoantibodies has yet been established. In previous studies, it has been shown that total IgA and/or IgG fractions isolated from celiac sera reduce TG2 enzymatic activity in cell extracts (Esposito et al. 2002). Some studies have not highlighted any change and others have described mild inhibitory effects, which, nevertheless, were not sufficient to block protein crosslinking (Roth et al. 2003, Dieterich et al. 2003). In contrast, elevated TG2 expression and enzymatic activity have been reported in the subepithelial celiac jejunal mucosa (D'Argenio et al. 1989, Esposito et al. 2003), the preferred site for anti-TG2 antibody deposition *in vivo* (Korponay-Szabo' et al. 2004).

Accordingly, it is of research interest to establish how anti-TG2 autoantibody from celiac patients modulates the enzymatic activities of this fascinating protein and in turn, how these biological effects are correlated to the clinical manifestations of the disease.

3. AIMS OF THE STUDY

The aims of the present study were:

1. To characterize the biological effects of CD IgA class autoantibodies by a molecular approach (**I, II, III**).
2. To study down-regulation of TG2 by small interfering RNA as a possible mechanism of CD IgA action (**I**).
3. To establish how CD IgA, by influencing TG2 physiology, disturbs endothelial cell biology (**II**).
4. To find new insights into the TG2 activity modulation mediated by CD IgA (**III**).

4. MATERIALS AND METHODS

4.1 Cell lines and cell culture (I-III)

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Cambrex Bio Science, Walkersville, MD, USA) and maintained in EGM-1 or EGM-2 medium (Clonetics ®, San Diego, CA, USA). The cell cultured endothelial medium (EBM, Clonetics) was supplemented with 2% fetal bovine serum (FBS) (Gibco Invitrogen, Paisley, Scotland, UK), and 15 μg/mL endothelial cell growth supplements (Clonetics). In all experiments, HUVECs were used between passages 2 and 6 and sustained in a humidified 37°C incubator with a 5% CO_2 atmosphere.

4.2 Small interfering RNA (I)

In order to knock down TG2 expression in endothelial cells by siRNA, a specific oligonucleotide for siRNA-TG2 (5'-AAGGGCGAACCACCTGAACAA-3') was purchased from Invitrogen (Invitrogen Life Technologies, Carlsbad, CA, USA). The general toxicity of siRNA was examined using a negative oligonucleotide control sequence, not homologous to any human mRNA (5'-AATTCTCCGAACGTGTCACGT-3') (Invitrogen Life Technologies). Transfections of siRNA oligos by the lipofectamine-RNAi system (Invitrogen) were carried out according to manufacturer's instructions.

4.3 Serum IgA class antibodies (II, III)

Serum samples from twenty-seven biopsy-proven celiac disease patients on a gluten-containing diet and fifteen non-celiac healthy controls were used. All celiac sera had anti-TG2 antibody titers above 100 U/mL (Celikey®; Pharmacia Diagnostic GmbH, Freiburg, Germany) and endomysial antibodies titers 1≥1000, while all control sera were negative. Total serum was purified with an anti-human IgA agarose (Sigma Aldrich, St Louis, Missouri) column and washed prior to elution of the IgA with 0.1 M glycine-HCl in 0.5 M NaCl, pH 2.5. The collected IgA fractions were neutralized with 1 M Tris-HCl pH 8.0 before removal of glycine by passing the samples through PD-10 columns (Pharmacia Upjohn, Lund, Sweden). The concentrations were determined by

ELISA using affinity-purified human IgA (Dako, Glostrup, Denmark) as standard. The IgA samples were lyophilized and resolubilized in phosphate buffered saline (PBS) to a final concentration of 100 μg/ml. Immunoglobulins class A derived from celiac disease patient or healthy control sera, as described above, were used in all experiments at a concentration of 1 μg/mL with the exception of the dose-dependent assay, where they were administered to the cells between 0.5 μg/mL to 25 μg/mL.

4.4 Other chemicals (II, III)

The extracellular TG2 inhibitor R281 was a kind gift from Professor Martin Griffin; it was supplemented to the culture medium at a final concentration of 200 μM, prior to the addition of other study compounds. The cell-permeable Rho inhibitor C3 transferase (Cytoskeleton, Denver, CO, USA) was used at a final concentration of 1.0 μg/mL before the addition of class A immunoglobulins. The thioredoxin inhibitor, 2-[(1-Methylpropyl)dithio]-1*H*-imidazole (PX12), (TOCRIS, Bioscience, Ellisville, MI) was employed in all experiments at a final concentration of 1 μM.

4.5 Cell number assay (I, II)

The cell viability assay was performed on 96-well plates (Nunclon, Thermo Fisher Scientific, Roskilde, Denmark), pre-coated with 5 μg of human plasma FN (Sigma Aldrich). HUVECs were plated on FN pre-coated plates until confluent and starved overnight (O/N) in 1% FBS. Thereafter, endothelial cells were treated for 24h under different experimental conditions. Cell proliferation was measured following CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) manufacturer's instructions (Promega, Madison, Wisconsin, USA).

4.6 Cell adhesion assay (I, II)

The cell adhesion assay was performed on 96-well plates (Nunclon, Thermo Fisher Scientific), following the manufacturer's instructions (Promega). Briefly, after cell detachment using trypsin,

HUVECs under different experimental conditions were seeded on 96-well plates and allowed to attach for 1 h **(I).** In addition, to investigate the predisposition of endothelial cells to detach in the presence of different study compounds, HUVECs were trypsinized (Sigma Aldrich) for 5 minutes and the number of remaining adherent cells assessed by formazan reduction as above (Promega) **(II).** Further, to distinguish weak and strong adhesion, confluent HUVEC monolayers under different experimental conditions were detached with 5mM EDTA in PBS (pH 7.4) and allowed to adhere for 1h on FN-precoated chamber slides (BD-Falcon TM, Bedford, Massachusetts) in serum-free culture media. The cells were then fixed with 4% paraformaldehyde (4% PFA) and images taken at 20x magnification using the Axiovision 3.0 program (Carl Zeiss Vision GmbH, Munich, Germany). Weakly adhering cells (round cells) were counted manually on captured images **(II).**

4.7 Flow cytometry studies (I, II)

For cell cycle analysis, endothelial cells under different experimental conditions were washed with PBS and incubated with FACS hypotonic staining buffer, according to the Sigma-Aldrich manufacturer's instructions, followed by flow cytometery (Expo32 ADC, Epics_XL-MCL; Beckman Coulter, Harbor Boulevard, CA, USA) analysis **(I).**

Similarly, the number of apoptotic cells was quantified using Annexin V-FITC and propidium iodine (Calbiochem, Beckman Coulter, Harbor Boulevard, CA, USA) and analysed by flow cytometery (Expo32 ADC) according to the manufacturer's instructions **(I, II).**

4.8 TG2 knock-down rescue assays (I)

Rescue assays for cell number, adhesion and apoptosis were specifically designed for silenced TG2 endothelial cells on 96-well plates precoated with 5 µg of human plasma FN and 20 µg of purified guinea pig TG2 (Sigma-Aldrich) (FN-TG2) or human recombinant TG2 (His-rhTG2) (Zedira GmbH, Darmstadt, Germany). The cells were then analysed for different cell parameters as described above according to the manufacturer's instructions **(I).**

4.9 Protein functional studies (I-III)

4.9.1 Protein detection: immunoblotting and immunofluorescence (I-III)

For SDS-PAGE, cell lysates or cell supernatants were boiled in Laemmli buffer and resolved on SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) and blotted with the primary antibodies and HRP-conjugated secondary antibodies (as indicated in original publications **I-III**). Proteins were detected with the ECL Plus Western Blotting Detection System (Amersham Biosciences). Band intensities were quantified using 1D image analysis software (Kodak, New Haven, CT, USA).

HUVECs were fixed with 4% PFA for 10 minutes and then washed with PBS, followed by permeabilization with 0.1% Triton X-100. Unspecific binding of the antibodies was blocked by 5% BSA for 1 hour before incubation of the cells with primary antibody usually diluited 1:200 for 1 hour at 37 ^{0}C. After washes with PBS, HUVECs under different experimental conditions were incubated with specific secondary fluorophor-conjugated antibody as described in original publications **I-III**. All stainings were visualized by fluorescent microscopy analysis (BX60F5, Olympus Optical CO.Ltd, Tokyo, Japan).

4.9.2 Enzyme-linked immunosorbent assay (II, III)

An enzyme-linked immunosorbent assay (ELISA) was used to investigate the expression of several proteins in HUVECs under different experimental conditions, as indicated in original articles **II** and **III**. Endothelial cells were grown to confluence on commercial FN-pre-coated 96-well plates (BD Biosciences Discovery Labware), starved O/N in EGM-2 with 1% of FBS, and incubated for 24h in the presence of different study compounds. After washings with HBSS (Sigma Aldrich), the cells were fixed with 4% PFA (Sigma Aldrich) and blocked with 5% BSA (Sigma Aldrich). Thereafter, the plates were incubated for 1 hour at 37^{0}C with specific primary antibodies, as described in original articles **II** and **III**. Subsequently, the wells were extensively washed and an appropriate secondary HRP-conjugated antibody (1:2000; Dako) was added for an additional 30 minutes at

37^0C. Finally, the peroxidase substrate TMB (Sigma Aldrich) was used and the enzymatic reaction was stopped with 2.5 M H_2SO_4. The absorbance was measured at 450 nm by spectrophotometer (Multiskan Ascent; Thermo Labsystems).

4.9.3 Golgi reorientation assay (II)

Cell polarity was studied by Golgi apparatus reorientation after 4 hours of scratch wound healing. HUVECs were grown onto FN-coated (10 μg/ml) chamber slides (BD-Falcon™, Bedford, MA, USA) until confluent, starved O/N in EGM-2 with 1% FBS, and subjected to different experimental conditions for 24 hours. Endothelial cell monolayers were wounded with a pipette tip, washed with PBS, incubated in serum-free media for 4 h with the monoclonal β-COP antibody clone M3A5, (Sigma Aldrich) and after extensive washings, the cells were fixed with PFA 4%. After permeabilization, HUVECs under different experimental conditions were blocked with 0.05% BSA for 1h at RT. Localization of the Golgi apparatus was detected by Alexa Fluor® 488 conjugated secondary anti-mouse (1:3000) (Invitrogen) and actin cytoskeleton changes were visualized using FITC-conjugated phalloidin (Invitrogen). Images were captured at 60× using an inverted microscope (IX51; Olympus). Golgi reorientation at the wound edges was analysed to quantify endothelial cell polarization. Thus, a square was drawn over the nucleus and divided into quadrants. Quadrant A was assigned to the area of the cell between the nucleus and the leading edge. A properly reoriented Golgi was indicated by β-COP staining completely within quadrant A. The stainings were visualized by fluorescent microscopy analysis (BX60F5, Olympus Optical CO.Ltd, Tokyo, Japan).

4.9.4 Integrin clustering assay (II)

β-1 integrin clustering was studied in HUVECs under different experimental conditions, seeding the cells onto FN-coated (10 μg/ml) chamber slides (BD-Falcon™) in serum-free (SE) or in 1% serum-enriched medium (SEM). Cells were left to attach for 4 h in SE and for 24 h in SEM. Live cells were in incubated with β1-integrin primary antibody (MAB 1965 Chemicon) for 1h and washed

with PBS. After fixation with 4% PFA and permeabilization with 0.25% Triton X-100, the cells were blocked with 0.1% BSA in PBS for 25 min, followed by incubation for 1h with the specific conjugated secondary antibody Alexa Fluor® 488 (Invitrogen). Images were examined by fluorescent microscopy analysis (BX60F5, Olympus Optical CO.Ltd, Tokyo, Japan).

4.9.5 Human IFN-γ assay (III)

Quantitative measurement of human IFN-γ in culture supernatants of endothelial cells under different experimental conditions was implemented by an in vitro enzyme-linked immunosorbent assay according to the manufacturer's instructions (Fisher Scientific, Waltham, MA).

4.9.6 TG2 activity assays (III)

Extracellular transamidating TG2 activity was studied in HUVECs under different experimental conditions by live-cell ELISA with monodansylcadaverine (MDC) (0.2mM; Sigma Aldrich) for 2h at 37^0C. Endothelial cells or ECM-HUVECs were extensively washed and fixed with 4% paraformaldehyde (PFA) (Sigma Aldrich). TG2-mediated MDC incorporation was detected using rabbit anti-dansyl antibody for 30 min at 37^0C (1:1000; Invitrogen), followed by incubation for 30 minutes at 37^0C with specific secondary antibody, horseradish peroxidase-conjugated polyclonal swine anti-rabbit immunoglobulin (1:2000; Dako, Copenhagen, Denmark). Thereafter, the peroxidase substrate TMB (Slow Kinetic Form for ELISA; Sigma Aldrich) was used and the enzymatic reaction stopped with 2.5 M H_2SO_4. Absorbance was measured at 450 nm by spectrophotometer (Multiskan Ascent; Thermo Labsystems, Waltham, MA).

4.9.7 In vitro redox modulation of TG2 bound FN (III)

In order to study the *in vitro* redox regulation of TG2 bound to FN mediated by CD IgA, Human FN 96-well Multiwell ^{TM} plates (BD Bioscences, Bedford, Massachussets, USA) were coated with 5μg/mL His-rhTG2 (Zedira) in Tris-buffered saline for 1h at 37^0C. Enzymatic TG2 activity was measured under different experimental conditions, as indicated in original article **III**, by addition of

40 µM 5-biotinamidopentylamine (5-BP) (Life Science Research Pierce Biotechnology, Rockford, IL). After extensive washings, incorporated 5-BP to TG2 bound to FN was detected with streptavidin HRP-conjugated secondary antibody (Life Science Research Pierce Biotechnology). Lastly, the peroxidase substrate TMB (Sigma Aldrich) was used and the enzymatic reaction stopped with 2.5 M H_2SO_4. Absorbance was measured at 405 nm by spectrophotometer (Multiskan Ascent; Thermo Labsystems).

4.9.8 Binding of celiac autoantibodies to TG2 after redox modulation (III)

Microtiter plates (Maxisorp, Nunc A/S, Roskilde, Denmark) were coated with TG2 purified from guinea pig liver (5 µg/mL) (Sigma Aldrich) for 1h at RT. Thereafter, the plates were extensively washed and incubated with 100 µM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) for 20 minutes. Serum samples from six celiac patients diluted 1:200 or CUB7402 (1:16000) (NeoMarkers) were added in triplicate for 30 minutes prior to the addition of 50 mM DTT for another 20 minutes. Celiac autoantibodies bound to TG2 were analysed by ELISA as indicated in original article **III**.

4.10 Statistical analysis (I- III)

Statistical analysis was performed using the non-parameric Mann–Whitney U-Test and data were presented as mean values. A p-value <0.05 was considered statistically significant.

4.11 Ethical considerations

The study protocol was approved by the Ethics Committee of Tampere University Hospital, Tampere, Finland and Heim Pal Children's Hospital, Budapest, Hungary. All patients gave written informed approval.

5. RESULTS

5.1 Biological effects of siRNA-TG2 in endothelial cells

Endogenous down-regulation of TG2 by siRNA caused severe morphological changes (Figure 7) in endothelial cells (HUVECs) in a dose- and time-dependent manner (Figure 1 in **I**). The most evident effect was a significant decrease in cell number and increased apoptosis (Figure 2-5 in **I**) already after 1 day of transfection.

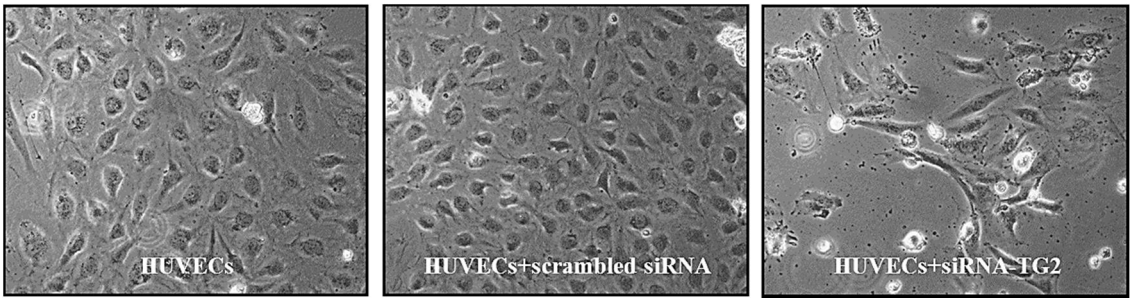

Figure 7. **Phenotype of endothelial cells after transglutaminase 2 (TG2) knock-down.** HUVECs (Human Umbilical Vein Endothelial Cells) alone or transfected with scrambled RNA have normal small round morphology when compared to TG2-depleted HUVECs characterized by decreased cell number and increased apoptosis.

In addition, down-regulation of TG2 by siRNA caused a marked loss of cell adhesion on FN (Figure 6 in **I**), cells appeared abnormally elongated and rounded, and showed shortcomings in spreading and detached easily. This was coupled with a reduced FAK protein level and a subsequent decrease in pFAK (Figure 3 in **I**), known key players in cell adhesion. To clarify whether reduced number of TG2-siRNA cells was as a result of deficiencies in cell cycle progression, cell cycle analysis was carried out by flow cytometry. These experiments revealed a statistically higher fraction of siRNA-TG2 cells in the G0/G1 phase (Figure 4 in **I**), revealing the importance of the intracellular TG2 pool for endothelial cell survival. Interestingly, the arrest of TG2-siRNA-HUVECs in the G0/G1 phase was sustained by progressive up-regulation of cyclin E and downregulation of cyclin B. It is of note that in HUVECs where TG2 expression was silenced by RNAi up-regulation of p53 and its downstream target gene p21 was observed (Figure 5 in **I**), these being involved in cell cycle arrest preceding cell death. In this regard, increased apoptosis in

siRNA-TG2 cells was further confirmed by cell cycle analysis and staining with annexin V-FITC (Figure 4a and Figure 5a in **I**). The main features of siRNA-TG2 on endothelial cells after five days of silencing are schematically summarized in Table 3.

Table 3: Biological effects of transglutaminase 2 (TG2) small interfering RNA (siRNA) on endothelial cells after 5 days of transfection

	CONTROL	**SCRAMBLED siRNA**	**siRNA-TG2**
cell number	100%	75%	35%
adherent cell number	100%	95%	25%
number of cells in G0/G1 phase	61%	59%	70%
apoptotic cell number	2%	3%	18%

5.2 Biological relevance of extracellular TG2 for endothelial cell biology

It was next sought to redeem the defects in siRNA-TG2 cell adhesion, proliferation, and apoptosis by the addition of exogenous TG2, which also allowed better characterization of the biological functions of TG2 in relation to the different endothelial cell compartments. Outstandingly, it was found that extracellularly added TG2 efficiently corrected the adhesion defects in HUVECs with siRNA-depleted endogenous TG2, but it did not overcome cell growth deficiency and elevated apoptosis (Figure 8). Altogether these findings implied that in the endothelium, the extracellular TG2 is involved in the regulation of cell-matrix adhesion, while the cytoplasmic TG2 is important for endothelial cell cycle progression and survival.

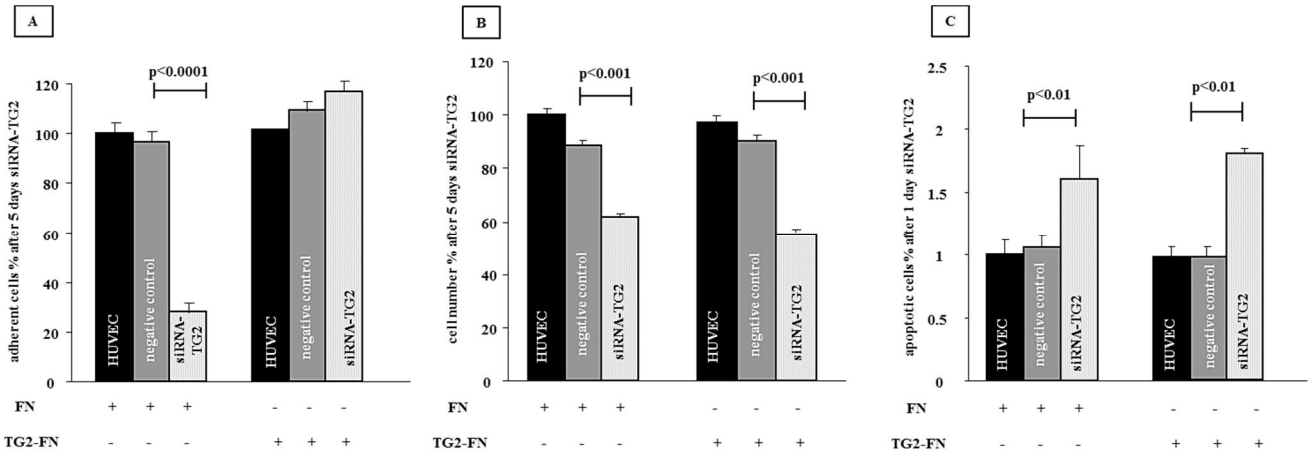

Figure 8. **Effects of extracellular transglutaminase 2 (TG2) on endothelial cell biology.** The addition of exogenous TG2 is able to reverse the adhesion defects (A) but does not overcome proliferation failure (B) or increased apoptosis (C) observed in cells TG2-depleted by small interfering RNA.

5.3 Identification of the regulatory mechanism of CD antibodies (II)

5.3.1 CD IgA inhibits cell proliferation by disturbing cell adhesion

In vitro studies on endothelial cells with CD IgA antibodies showed important morphological changes when compared to control groups (Figure 9). The cells appeared enlarged, atypically elongated and prone to detach.

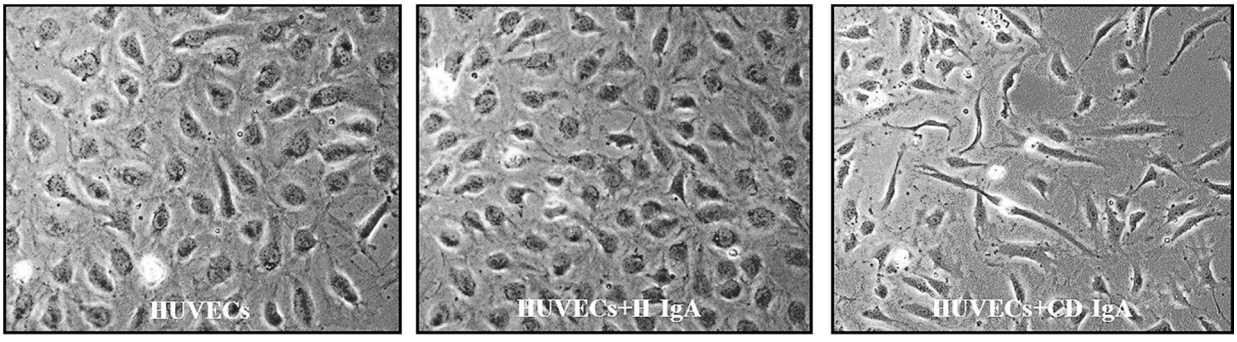

Figure 9. **Phenotype of endothelial cells treated with celiac IgA antibodies (CD IgA).** HUVECs (Human Umbilical Vein Endothelial Cells) alone or in the presence of control IgA (H IgA) have normal small round morphology when compared to HUVECs incubated with CD IgA which are characterized by decreased cell number and altered cell shape.

HUVECs in the presence of CD IgA did not proliferate as control groups (Figure 1a in **II**).

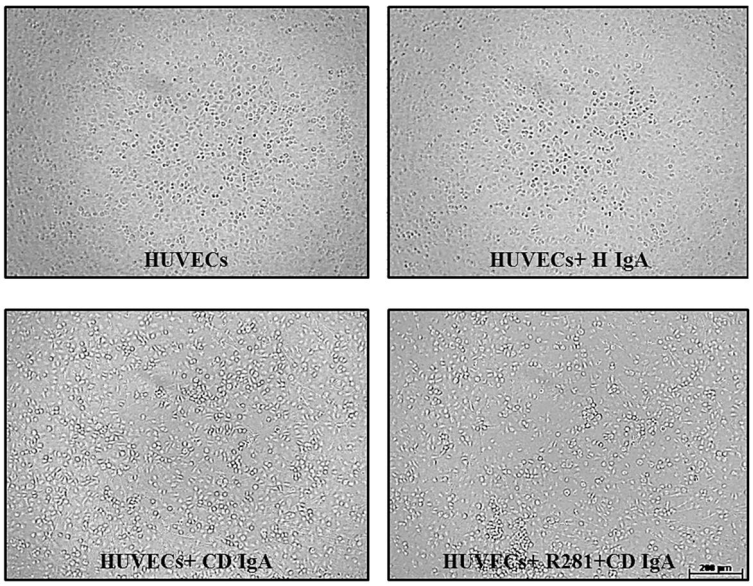

Figure 10. **Celiac patient immunoglobulin A inhibits endothelial cell number by affecting cell adhesion.** Images of endothelial cells alone or treated with IgA from healthy (H IgA) and celiac patients (CD IgA) for 24h and plated on fibronectin-precoated chambers. Round cells indicate weak adhesion. Inhibition of extracellular TG2 activity by administering a site directed, non-permeable inhibitor, R281, did not prevent the adhesion defects observed in the presence of CD IgA.

Remarkably, it was observed that HUVECs alone or incubated in the presence of H IgA showed extended and flattened morphology after attachment on FN-precoated chambers for 1h (Figure 10), this reflecting increased adhesion of the cells to the substratum, while CD-HUVECs had rounded shape revealing weak adhesion (Figure 10). The phenotype observed in HUVECs incubated with CD IgA was not dependent on increased TG2 enzymatic activity, since R281, a non-reversible extracellular TG2 inhibitor, was not able to overcome the adhesion defects.

The effects mediated by CD IgA antibodies on endothelial cell biology are summarized in Table 4.

Table 4: Effects of celiac IgA on endothelial cell biology

	HUVECs	**HUVECs+H IgA**	**HUVECs+CD IgA**	**HUVECs+R281+CD IgA**
cell number	100%	100%	97%	97%
adherent cell number	100%	99%	75%	77%
round cell number	1%	1%	38%	45%
apoptotic cell number	1%	1.1%	1.3%	1.2%

As the integrity and the composition of the vascular ECM are essential to promote cell adhesion, HUVECs under different experimental conditions were next examined for these cell parameters. Unexpectedly, in endothelial cells protein cross-links were decreased in the presence of CD IgA autoantibodies when compared to control groups (Figure 2a and 2b in **II**), and this was coupled with altered distribution of FN, a TG2 co-receptor, on the endothelial cell surface (Figure 11). These features were not reversed when R281 was used, indicating that TG2 extracellular enzymatic activity was not involved. In addition, CD IgA induced decreased expression of vinculin (Figure 4a, 4b and 4c in **II**) which deficit was ameliorated by preincubation of HUVECs with R281 prior to addition of CD IgA.

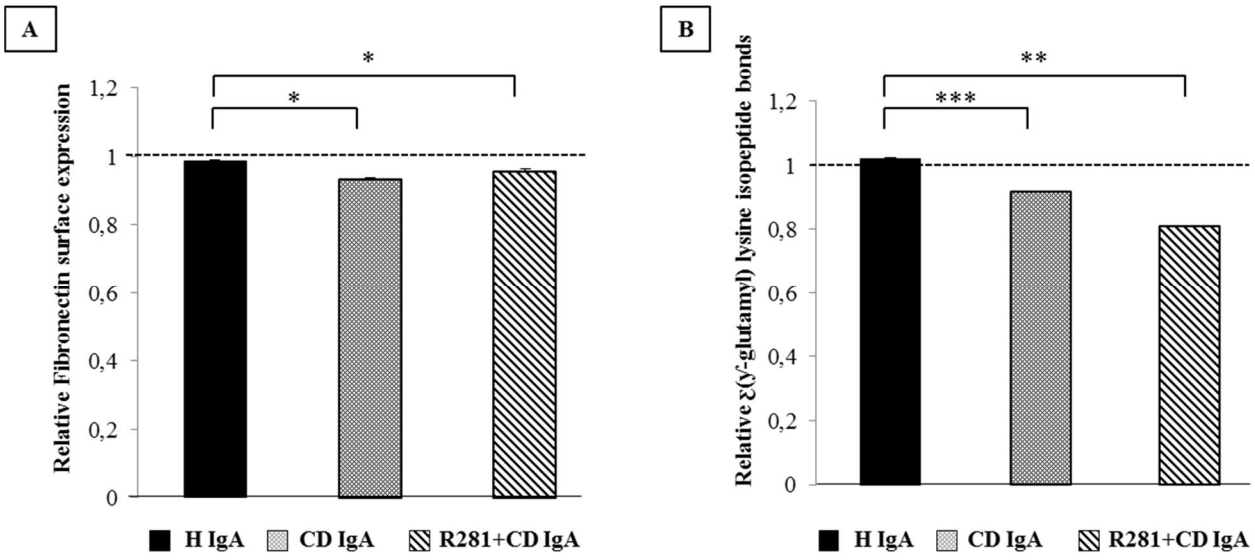

Figure 11. **Celiac patient immunoglobulin A (CD IgA) influences extracellular fibronectin and protein cross-links in endothelial cells.** The relative amount of fibronectin (A) and (B) the extent of Nε(γ-L-glutamyl)-L-lysine cross-links are shown in HUVECs with no supplementation or after 24-hour treatment in the presence of CD IgA and healthy IgA (H IgA). A P-value <0.05 was considered significant (*p<0.05, **p< 0.01 and ***p<0.001).

5.3.2 Molecular characterization of the anti-angiogenic effects mediated by celiac IgA

Since CD IgA affected attachment of endothelial cells in virtue of altered FN architecture and reduced cross-links where TG2 has a fundamental role, it was next sought to establish whether altered expression of TG2 might have account for these abnormalities. Increased secretion and

surface expression of TG2 were observed (Figure 3a and 3b in **III**) when CD IgA were administered to the cells.

Interestingly, TG2 secreted from endothelial cells cultured with CD IgA was enzymatically active. Further investigations showed secreted TG2 to be expressed intact or in high molecular form, this excluding the involvement of MT1-MMP in the proteolytic degradation of surface TG2 to explain the augmented release observed in the presence of CD IgA antibodies (Figure 5b in **II**). Scratch wound healing assay and Golgi reorientation analyses showed that CD IgA have anti-migratory effects on endothelial cells. In this regard, upon creating a wound edge and allowing for initial cell movement within the denuded space, CD-HUVECs were not competent to establish an axis of polarity with lamellipodia and Golgi markers facing the wound edge. Extracellular TG2 activation mediated by CD IgA was not involved in the process, since the employment of R281 had no preventive effect. In contrast, pre-administration of the C3 transferase inhibitor corrected the polarity defects, supporting an involvement of the RhoA signaling pathway in the anti-migratory phenotype mediated by CD IgA (Figure 6a and 6b in **II**). Additionally, it was found that CD IgA antibodies induced surface TG2 and β-1 integrin clustering on the endothelial cell surface when compared to control groups (Figure 7 in **II**). This suggested that the activation of the small GTPase RhoA previously described requires a non-enzymatic mechanism.

5.3.3 Biological redox relevance for CD IgA-mediated-TG2 extracellular activation (III)

The mechanism underlying CD IgA-mediated TG2enzymatic activation in endothelial cells (Myrsky et al. 2009) remained unclear in our *in vitro* system.

In this context, the latest research has discovered an unexpected role for human thioredoxin (TRX) in the modulation of TG2 biology. In fact, Stamnes and coworkers (2010) have provided evidence that TRX can activate extracellular TG2 in cryosections of human and mouse small-intestinal biopsies, revealing a new strategy for inhibiting the undesirable consequences of TG2 activity in this widespread, lifelong disease.

With this in mind the present study get out to elucidate the mechanism by which CD IgA mediates TG2 enzymatic activity and the potential role of TRX in this process.

First, it was observed that CD IgA promoted the constitutive activation of extracellular TG2 on endothelial cells monolayers, in a time-independent but dose-dependent manner (Figure 12). Further analyses localized this rise in the ECM of the cells treated with CD IgA, coupled with an increase in the signal of low molecular mass proteins in CD-HUVECs when compared to control groups (Figure 2b in **III**). In samples derived from endothelial cultures with R281 and CD IgA the intensity of the 45 kDa band was clearly reduced and similar to the controls.

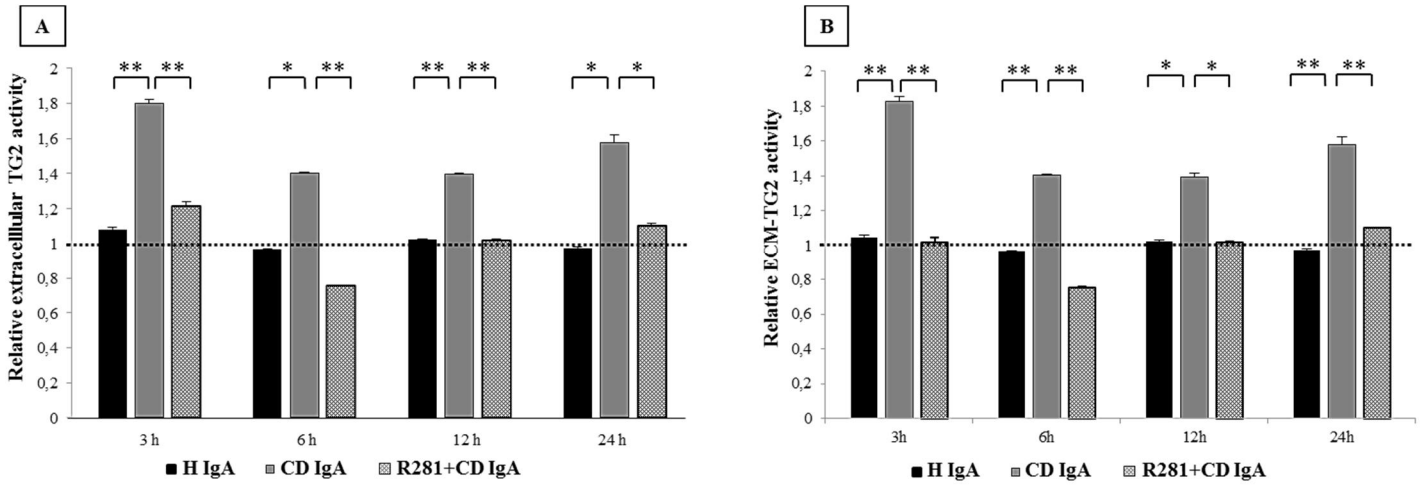

Figure 12. **Celiac patient immunoglobulins A increase transglutaminase 2 (TG2) activation on endothelial cells.** The relative extracellular TG2 activity was measured by monodansylcadaverine incorporation on the endothelial cell surface (A) and on the endothelial extracellular matrix (B) at different time-points. Extracellular TG2 activity was inhibited by administering a site directed, non-permeable inhibitor, R281.

Since earlier studies had ascertained that extracellular TG2 activity is sensitive to redox changes, we next investigated whether the redox regulation of TG2 might also have an effect on the enzymatic modulation mediated by CD IgA. Interestingly, in the absence of the reducing agent DTT and exogenous Ca^{2+}, TG2 was enzymatically inactive and both agents were required for maximal activity (Figure 4a in **III**). The addition of CD IgA or H IgA did not affect the transamidating TG2

activity either in the presence or in the absence of Ca^{2+} and DTT together. Notably, in the presence of DTT but without exogenous Ca^{2+}, CD IgA autoantibodies were able to induce enzymatic activity of TG2 in contrast to H IgA. This finding was supported by the observation that under oxidizing conditions induced by DTNB, reduced amounts of CD IgA were able to bind TG2, while the process could be reversed by treating the enzyme with DTT after DNTB and before adding the antibodies. Furthermore, neither DTNB nor DTT had any effect on the binding of the anti-TG2 monoclonal antibody CUB7402, which recognizes a linear epitope (Figure 4b in **III**).

Since it was found that CD IgA-mediated TG2 activation occurred solely under reducing conditions, it was sought to establish whether TRX, the novel redox TG2 regulator, was involved in the process as a physiological reducing agent. Interestingly, it was found that the overall TRX protein expression level was comparable in all treatment groups. Conversely, endothelial cells incubated with CD IgA secreted TRX into the cell cultured media, whereas control cells did not. This was coupled with its decreased surface expression in CD IgA-treated HUVECs (Figure 5b in **III**). It is worth mentioning that co-administration of R281, the inhibitor for extracellular TG2 activity, was not capable of circumventing these effects exerted by CD IgA (Figure 5a and 5b in **III**). Bearing these findings in mind, it was further investigated whether inhibition of extracellular TRX by PX12 might have by-passed the effects of CD IgA autoantibodies observed on HUVECs. Indeed this was the case, as pretreatment of endothelial cultures with PX12 prevented the extracellular TG2 enzymatic activation mediated by CD IgA (Figure 6c in **III**).

Furthermore, inhibition of extracellular TRX with PX12 was able to block the effects mediated by CD IgA on TG2 surface expression and secretion of TRX into the culture supernatants (Figure 6a-6b in **III**).

6. DISCUSSION

CD is a disorder with strong genetic component in which the body's immune system responds to gluten by attacking the small-intestinal mucosa, damaging it to the point whether it can no longer absorb nutrients effectively. This is why many of the consequences of the disease are similar to malnutrition, especially in the classical malabsorptive form of the disease (Williamson et al. 2002). Today, however CD is often clinically silent: patients with a gluten-induced manifest small-intestinal mucosal lesion may have no gastrointestinal symptoms and no sign of malabsorption, and adult patients are often diagnosed on the basis of extraintestinal symptoms or signs of CD. The disease results from an interaction of genetic and environmental factors. Among the environmental influences which may play a role are gastrointestinal infections, but the main driving force behind the disease is the extent and timing of gluten exposure.

The disease causes damage to an organized arrangement of finger-like structures in the small intestine, called villi, which provide an enormous epithelial surface area for absorptive function. The gluten-induced villous atrophy with crypt hyperplasia is thought to be T-cell driven (Abadie et al. 2011). The mechanical support and the integrity of the villi depend on proper blood vessel formation. Our group has recently provided a large body of evidence that the small-intestinal mucosal vasculature is altered in untreated celiac patients (Myrsky et al. 2009). One of the most intriguing findings is that CD-specific autoantibodies inhibit angiogenesis (Myrsky et al. 2008), which again suggests that different molecular mechanisms might be involved in the development of the CD enteropathy.

It has previously been suggested that TG2-targeted autoantibodies from CD patients are functional (Halttunen et al. 1999). These autoantibodies bind *in vivo* the self-antigen, TG2 (Korponay-Szabo et al. 2004, Salmi et al. 2006) and, additionally, TG2 autoantibody deposits have also been detected in disease-affected organs different from the site of gluten exposure (Korponay-Szabo’ et al. 2004, Hadjivassiliou et al. 2006).

Since TG2, a multifunctional protein found to be the main, if not the only autoantigen for CD autoantibodies, is expressed, active, and directly involved in angiogenesis (Haroon et al. 1999), our objective was to gain new insights into the CD pathogenesis with regard to TG2 physiology, autoantibodies and endothelium (**I, II, III**).

6.1 Biological effects of down-regulation of TG2 by siRNA in endothelial cells (I)

It was first hypothesized that down-regulation of TG2 could explain the anti-angiogenic effects exerted by CD autoantibodies on the vasculature and thus small interfering RNA (siRNA) to knock-down TG2 in endothelial cells was used.

The assumption was not however confirmed. Down-regulation of TG2 in endothelial cells affected cell number by loss of adhesion, inhibition of proliferation and increasing apoptosis, in a time- and dose-dependent manner. The adhesion problems described are in line with earlier reports describing the involvement of TG2 in the process independently of its enzymatic activity but associated with diminished phosphorylation of FAK at tyrosine 397 (pY397), which is often used as a measure of FAK activity. Notably, our findings showed in HUVECs-siRNATG2 greater p53⁄p21 levels related to cell cycle arrest and enhancement apoptosis (Figure 13). In order to clarify the role of TG2 in the different cellular compartments, exogenous TG2 was added to overcome the defects in siRNA-TG2 so far characterized. Although adhesion defects could have explained the initial result of reduced cell number after TG2 down-regulation, it was seen that exogenous TG2 was able to reverse only the adhesion defects without overcoming the proliferation failure. These findings implied that in the endothelium, the extracellular pool of TG2 is involved in the regulation of cell-matrix adhesion processes, while the cytoplasmic pool of TG2 is important for endothelial cell cycle progression and survival.

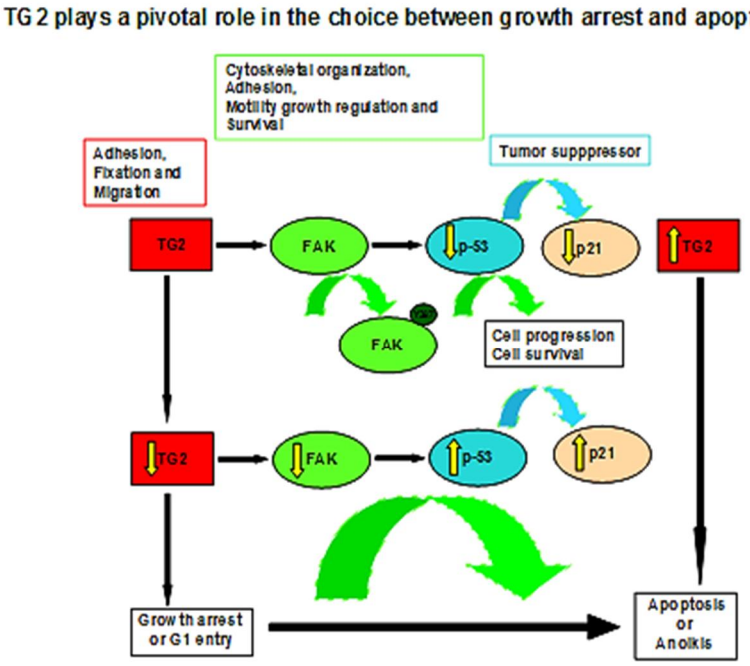

Figure 13. **Effects of down-regulation of transglutaminase 2 (TG2) in endothelial cells.** Depletion of endogenous TG2 by small interfering RNA causes apoptosis by arresting the cell cycle progression in the G0/G1 phase and by up-regulating p53 and its downstream target gene, p21.

6.2 Anti-angiogenic effects mediated by CD autoantibodies via extracellular TG2 (II)

Bearing in mind that CD IgA antibodies were found to increase the enzymatic activity of TG2 and influence the small GTPase RhoA, thus altering vascular permeability (Myrsky et al. 2009), it was sought to better understand the molecular mechanism beside this modulation.

The vascular endothelium functions as a semi-selective barrier for macromolecule transport across the vessel wall. ECM responds to external stimuli by cytoskeletal rearrangements and activation or inhibition of contractile machinery, which determine the barrier-enhancing or barrier-disruptive ECM response (Dudek et al. 2001, Mehta et al. 2006). In this regard, it has been shown that the activation of Rho GTPase is a key mechanism in endothelial ECM permeability dysfunction (Birukova et al. 2004). Furthermore, there is evidence for novel roles of factor XIIIA and TG2 in the cardiovascular biology, including the modulation of platelet activity, the regulation of vascular permeability and angiogenesis (Sane et al. 2007).

Firtsly, it was observed that endothelial cells incubated in the presence of CD IgA underwent important morphological changes and the cells did not proliferate as in control groups (**II**). The proliferation defects so far highlighted were due to impaired adhesion, which was independent of TG2 extracellular activation, since R281 was not able to correct this deficiency. Remarkably, the overall architecture of extracellular endothelial cells was altered in the presence of CD IgA autoantibodies due to a disorganized actin cytoskeleton, decreased protein cross-link levels and altered appearance of the FN network in comparison to control groups. This might suggest that the FN polymerization process catalyzed by extracellular TG2 cross-linking activity is disturbed when CD IgA are present. Furthermore, no change in FN protein expression was detected among all our study groups and the morphological changes described were not associated with increased extracellular TG2 activity mediated by CD IgA, since the administration of R281 was not able to overcome the CD IgA biological effects.

Importantly, TG2 is localized on the surface of various cell types as well as in the ECM (Upchurch et al. 1991, Martinez et al. 1994). Early studies have suggested that the presence of TG2 outside the cell is due to release of the protein from damaged cells (Upchurch et al. 1991). However, later findings convincingly showed that TG2 is constitutively externalized from undamaged cells and is deposited on the cell surface and in the ECM (Barsigian et al. 1991, Gaudry et al. 1999). It was thus now sought to establish whether the release of TG2 was augmented in relation to the increased extracellular TG2 enzymatic activity mediated by CD IgA previously reported. Interestingly, it emerged that HUVECs in the presence of CD IgA autoantibodies promoted the secretion of TG2 in the extracellular compartment when compared to control groups independently of TG2 extracellular activation since R281 did not prevent its release. Interestingly, the secreted TG2 was proved to possess transamidase activity. In contrast, this feature was not coupled with the parallel extracellular TG2 deposition on endothelial cell monolayers, since only a moderate increase in TG2 expression was observed in the CD IgA group.

In addition, the augmented expression of MT1-MMP highlighted in HUVECs treated with CD IgA might contribute to the degradation of endothelial ECM, thus contributing to the loss of adhesive properties of the cells and decreasing cell–ECM interactions.

The important changes in endothelial cell morphology and ECM dynamics induced by CD IgA suggested that it would affect cell migration and polarization. Since RhoA affects polarity in migrating cells (Tomar et al. 2009), attention now turned to directional cell motility in HUVEC under different experimental conditions. It was found that endothelial cells when treated with CD IgA autoantibodies lose the polarized phenotype when compared to control groups, with the Golgi not facing the edge of the wounded area and the pattern of F-actin more disrupted with extension of lamellipodia not only in the direction of the wound but also at right angles to this. It is worthy of note that the effects exerted by CD IgA were reversed prior to incubation of the cells with C3 transferase inhibitor and not by R281, suggesting an involvement of the RhoA signaling pathway in the polarized defect described. Furthermore, it was observed that CD IgA autoantibodies induced surface TG2 and β-1 integrin clustering, suggesting that surface TG2 increases the integrin-mediated activation of the small GTPase RhoA by a TG2-independent enzymatic mechanism, as reported by Janiak and coworkers (2006).

Altogether, these findings reveal that CD IgA autoantibodies disturb the protein cross-linking function of TG2, thus altering endothelial cell-ECM interactions and in turn affecting endothelial cell motility. The adhesion defects observed in HUVECs cultured in the presence of CD IgA have been shown to be independent of TG2 transamidase activity, as established in previous works (Akimov et al. 2000, Verderio et al. 2003). Moreover, these results strongly suggest that the anti-migratory effects exerted by CD IgA occur via activation of the RhoA signaling pathway, which requires constitutive integrin activation.

If CD IgA autoantibodies act on vascular function *in vivo* similarly to those described in the present study *in vitro*, this could explain the altered small-bowel mucosal microvasculature found in untreated CD patients (Myrsky et al. 2009).

6.3 Role of TRX in CD IgA-mediated-TG2 extracellular activation and antibody binding (III)

The endothelial extracellular TG2 enzymatic activation of TG2 mediated by CD autoantibodies is still poorly understood (**II**). Hence, in this work it was sought to deepen our understanding of this issue taking advantage of the latest discoveries.

It has recently been shown that TRX, a redox sensor protein within cells which catalyzes the reduction of disulfide bonds, is a regulator of extracellular TG2 activity (Jin et al. 2011).

Interestingly, regulation of the cellular redox status has emerged as a promising therapeutic strategy to prevent uncontrolled inflammatory response. Human TRX, originally identified as a secretory protein adult T cell leukemia-derived factor, has been implicated in a wide variety of redox regulations in both intracellular and extracellular compartments. It was thus reasonable to envisage a potential role of TRX in the extracellular TG2 activation mediated by CD autoantibodies.

In the present study it was observed that CD IgA lead to an early and constitutive activation of extracellular TG2 in culture endothelial cells in a time-independent but dose-dependent manner.

In the literature, a correlation between the amount of disease-specific autoantibodies and TG2 enzymatic activity has also been documented by other groups (Esposito et al. 2002, Dietrich et al. 2003), but with contrasting outcomes as regards the activation of the protein, attributable to different experimental conditions adopted (Esposito et al. 2002, Dietrich et al. 2003) or by the structural TG2 variant used, which is reported to be an artifact (Kanchan et al. 2013). Bearing in mind that TG2 is subject to redox-regulation in the extracellular environment, interest now focused on how the reducing or oxidizing conditions might have affected CD IgA-mediated modulation of TG2. Interestingly, in an *in vitro* system, CD IgA autoantibodies induced TG2 activation only in the presence of a reducing agent without the addition of exogenous Ca^{2+}, supporting the conception that

in order to exert their biological effects they required a reducing environment. It is of note that oxidation altered the protein's conformation in such ways that the antigenic epitopes were less accessible and reversed by reducing conditions. In this regard, the finding that in CD IgA-treated HUVECs there was an increase in the secretion of TRX into the extracellular space provides evidence that this redox protein might have had a role in CD IgA-mediated TG2 activation. In line with this observation, it was found that PX12, a TRX inhibitor, prevented the extracellular TG2 activation mediated by CD IgA. In addition, the inhibition of extracellular TRX by PX12 was able to prevent secretion of the protein and to normalize TG2 surface expression in endothelial cells treated with CD IgA, but did not prevent its decrease on the surface. Previous works have shown that the pro-inflammatory cytokine IFN-γ can induce secretion of TRX at levels capable of activating TG2 (Diraimondo et al. 2012). In contrast, the findings here exclude a potential role for IFN-γ in the constitutive activation of extracellular TG2 mediated by CD IgA, since this was not detectable in the experimental system.

Intriguingly, the decreased surface expression of TRX and the augmented TG2 expression on HUVECs may led to speculation that at the cell surface TG2 might be present in a conformation not amenable to the enzymatic activation mediated by CD IgA.

7. SUMMARY & CONCLUSIONS

In summary, this work provided novel insights into molecular mechanisms that regulate angiogenesis by underscoring the significance of TG2 in different endothelial cell compartments. It was shown that TG2 depletion in endothelial cells resulted in cell-cycle arrest and apoptosis highlighting a central role for TG2 in cell-cycle progression and survival, at least *in vitro*.

Intriguingly, the anti-angiogenic effects exerted by CD patient derived antibodies might be explained by their capability to influence the extracellular enzymatic functions of TG2 rather than causing down-regulation of the protein in the endothelium. In this context, it was found that CD IgA induced the constitutive activation of TG2 in the ECM of endothelial cells by a mechanism involving the redox sensor protein TRX, needed to keep TG2 in a reduced form and to allow its binding to CD IgA antibodies. Interestingly, while the transamidase activity of TG2 was enhanced in the presence of CD IgA antibodies, the amount of extracellular protein cross-links was diminished. This suggests that CD IgA antibodies disturb the extracellular protein cross-linking function of TG2, thus altering endothelial cell–ECM interactions and by this means affecting endothelial cell adhesion, polarization, and motility (Figure 14). These alterations were coupled to increased expression and constitutive activation of β1-integrin which, in contrast, might represent a defense mechanism adopted by CD IgA treated cells to overcome the abnormal ECM organization.

Moreover, the results accrued strongly imply the RhoA signaling pathway involvement in the motility defects so far described in the presence of CD IgA, which activation most likely depends on integrin clustering.

Noteworthy, endothelial cells secreted enzymatically active TG2 when cultured in the presence of CD IgA. Thus, since the specific role of TG2 in the extracellular compartment is still not well characterized in the context of inflammation, it would be important to further investigate this issue and envisage a possible immune-modulatory function of TG2 in this process.

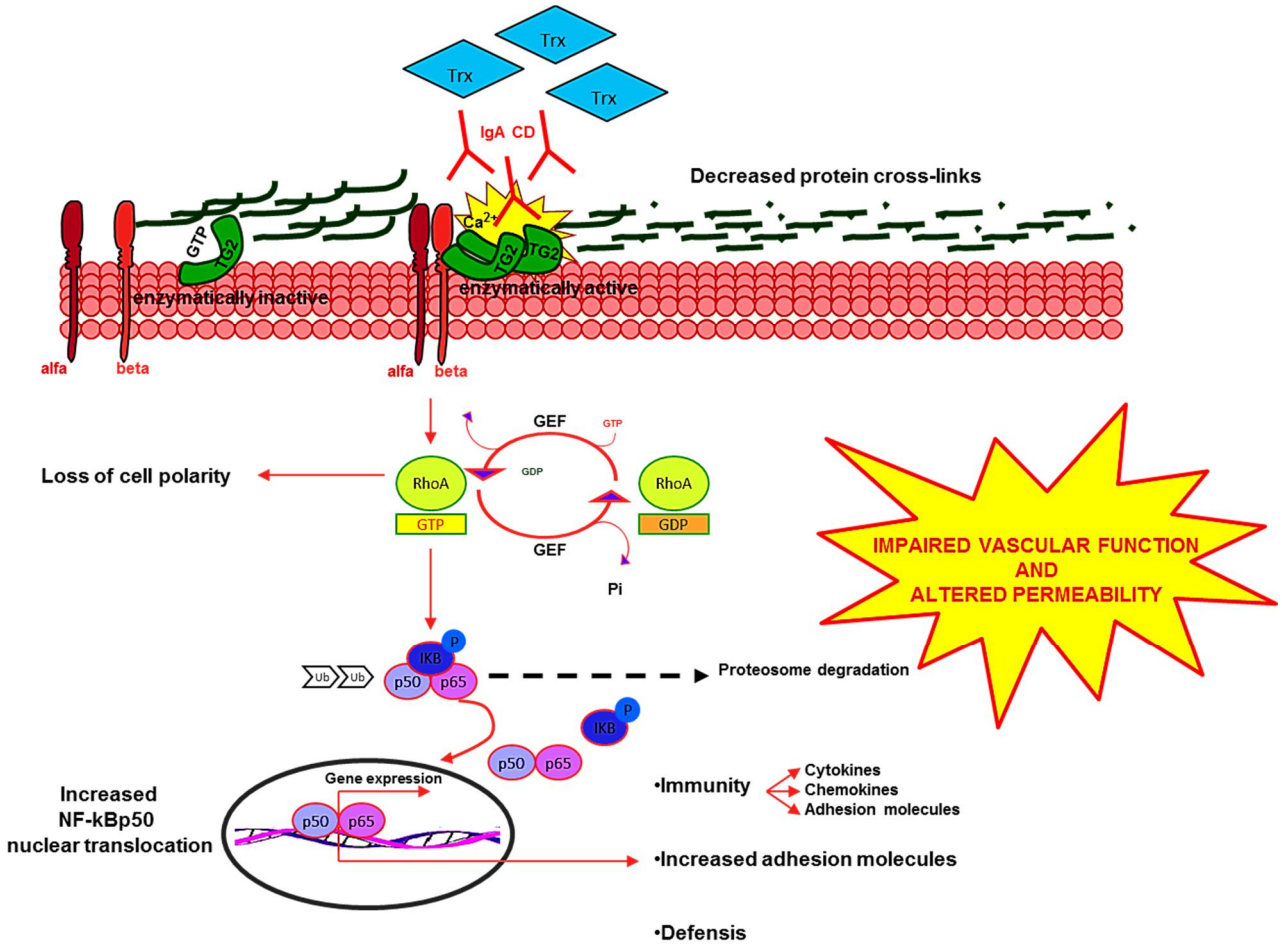

Figure 14. **The proposed mechanism of action of celiac disease autoantibodies.** In endothelial cells, celiac patient immunoglobulins IgA (CD IgA) induce extracellular TG2 constitutive activation by the secretion of thioredoxin. This in turn inhibits the cross-linking activity of TG2 necessary to maintain an organized extracellular matrix and to support cell adhesion. Moreover, TG2 surface clustering mediated by CD IgA is coupled to integrin activation, very likely responsible for triggering the "out-in" signal involving the RhoA signaling pathway. Altogether these events might lead to impaired vascular function and altered permeability in the small-bowel mucosal microvasculature in untreated CD patients.

To conclude, since angiogenesis sustains inflammation and because this work provides a large body of evidence for the anti-angiogenic effects exerted by CD patient derived antibodies by a mechanism that involves the modulation of extracellular TG2 it is possible to speculate a defense mechanism induced by CD antibodies to limit the deleterious effects of gluten in the patients, inhibiting angiogenesis.

Thus, the understanding of the main roles of TG2 in angiogenesis and inflammation can lead to therapeutic approaches in CD and its related disorders.

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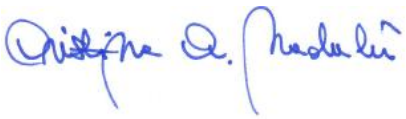

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Extracellular transglutaminase 2 has a role in cell adhesion, whereas intracellular transglutaminase 2 is involved in regulation of endothelial cell proliferation and apoptosis

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Abstract

Objective: Transglutaminase 2 (TG2) is a multifunctional protein with an important role in vascular biology, where it is involved in cell–matrix interaction, cell attachment and cell population expansion. In efforts to elucidate the role of TG2 in endothelial cell biology, in this study, we measured several endothelial cell characteristics in cells where TG2 was specifically knocked down by RNAi.

Materials and methods: The effect of small interfering RNA (siRNA)-TG2 on human umbilical vein endothelial cells was studied. Adhesion and cell viability were assessed by chemical reduction of MTT, and cell proliferation was analysed by flow cytometry. Apoptosis was evaluated by annexin V/PI dual staining and protein expression level was assayed by western blotting.

Results: We found that siRNA-TG2 reduced endothelial cell number, lead to cell adhesion deficiency, cell cycle arrest in G_1 phase and induction of apoptosis. Our results show that exogenously added TG2 could reverse loss of adhesion but did not overcome the defect in cell proliferation, nor could it inhibit siRNA-TG2-induced apoptosis.

Conclusion: We conclude that TG2 loss in endothelial cells causes reduction in cell number as a result of cell cycle arrest, flaws in adhesion and induction of apoptosis. Our results imply that reduction in cell number and increased apoptosis in response to TG2 silencing is independent of the cell adhesion process. Altogether, our findings underline the significance of TG2 in endothelial cell cycle progression and cell survival, *in vitro*.

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Introduction

Transglutaminase 2 (TG2) is a member of the transglutaminase (TG) enzyme family (EC2.3.2.13), involved in a plethora of biological functions. TG2 is ubiquitously expressed in most mammalian tissues and is involved in a number of cell processes such as angiogenesis (1), apoptosis (2), cell differentiation (3) and wound healing (4,5).

TG2 is predominantly an intracellular protein and functions inside the cell as a protein disulphide isomerase, an ATPase or a protein kinase (6–9), however, its best characterized feature is its G-protein function (10,11). In addition, TG2 can be found inside the nuclear compartment in association with histones, and histone phosphorylation activity has also been reported there (12). Moreover, TG2 can be secreted to the outside of cells, where it stabilizes the extracellular matrix, making it resistant to mechanical and proteolytic degradation (13,14). Furthermore, TG2 is present on the cell membrane, where it can associate with integrins and provide a binding site for fibronectin (FN) by simultaneously binding to β-integrins and FN. TG2-mediated adhesion also involves focal adhesion kinase (FAK), whose expression, maturation and activity TG2 is known to regulate (15).

Altogether, TG2 plays a key role in a number of physiological processes and a cell survival function for TG2 is thus strongly implicated. As TG2 has an established role in angiogenesis (1,4), the molecular mechanism by which it affects proliferation, adhesion and apoptosis in endothelial cells has been examined here, using small interfering RNA (siRNA) knock-down. We have shown a central role for TG2 in cell survival in living cells, where it is needed for cell cycle progression from G_1 to S phase. In addition to this, our data demonstrate that the roles of TG2 in

proliferation and apoptosis are dependent on the intracellular pool of the protein, whereas its role in adhesion is mediated by extracellular TG2 independently of its transamidase activity.

Materials and methods

Cell lines and cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Cambrex Bio Science, Walkersville, MD, USA) and maintained in HuMedia-EGM™ (EGM-1) (Clonetics®, San Diego, CA, USA). In all experiments performed, cells used were between passages 2 and 6.

TG2 knock-down by siRNA

Specific oligonucleotide for siRNA-TG2 (5′-AAGGGC GAACCACCTGAACAA-3′) was purchased from Invitrogen (Invitrogen Life Technologies, Carlsbad, CA, USA). To control general toxicity of siRNA, a negative oligonucleotide control sequence, not homologous to any human mRNA (5′-AATTCTCCGAACGTGTCACGT-3′) (Invitrogen Life Technologies), was used. Transfections of siRNA oligos were carried out using Lipofectamine™ RNAi MAX Transfection Reagent (Invitrogen), according to manufacturer's instructions. Silencing efficiency of TG2 was determined by western blot analysis.

SDS–PAGE and immunoblotting

For western blotting, HUVECs were resuspended in Laemmli loading buffer [63 mM Tris–HCl, 10% glycerol, 2% SDS, 0.1% (w/v) bromophenol blue, 2.5–5.0% β-mercaptoethanol] and 10 μl of total lysates was separated by 10% SDS–PAGE electrophoresis under reducing conditions. After gel electrophoresis and transfer of proteins to a nitrocellulose membrane, nitrocellulose sheets were blocked at room temperature (RT) for 1 h in 5% non-fat dry milk, and incubated overnight at +4 °C using specific primary antibodies. These included mouse monoclonal antibodies against TG2, CUB7402 (NeoMarkers, Fremont, CA, USA), phospho-FAK (Chemicon, Lake Placid, NY, USA), active caspase-3 (R&D System, Minneapolis, MN, USA), α- actin (1A4; Sigma-Aldrich, St Louis, MO, USA), β-actin (AC-74, Sigma-Aldrich), γ-tubulin (GTU-88, Sigma-Aldrich), cyclin E (sc-247) (Santa Cruz Biotechnology, Delaware Avenue, CA, USA), cyclin B (sc-245) (Santa Cruz Biotechnology) and p-21 (sc-817) (Santa Cruz Biotechnology), as well as rabbit polyclonal antibody against human FAK (Upstate Cell Signaling, Danvers, MA, USA). Immunoreactivity was detected by sequential incubation of membranes with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, Glostrup, Denmark) for 1 h at RT, and visualized using a chemiluminescence detection system (Amersham, ECL Plus Western Blotting Detection System, Buckinghamshire, UK). Autoradiographical films were scanned for densitometry analysis using 1D image analysis software (Kodak, New Haven, CT, USA). Protein expression was normalized to β-actin or γ-tubulin, and HUVECs without any treatment were considered as 100% standard.

Cell number assay

Cell viability assay was performed in 96-well plates, precoated with 5 μg of human plasma FN (Sigma-Aldrich). Briefly, 1×10^4 cells collected after siRNA-TG2 on days 1, 3 and 5 were plated on FN precoated plates and grown for 24 h. Cell viability was measured by addition of Cell Titer 96 solution (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Adhesion assay

The cell adhesion assay was performed on FN-precoated plates. Briefly, after cell detachment using trypsin, HUVECs were washed in Hank's balanced salt solution (HBSS) and 1×10^4 cells were seeded in 96-well plates. Cells were allowed to attach for 1 h and attachment was quantified using Cell Titer 96 solution (Promega), according to the manufacturer's instructions.

Immunofluorescent staining

HUVEC cells transfected by siRNA oligos were cultured on FN-precoated chamber slides (BD-Falcon™, Bedford, MA, USA) for 1, 3 and 5 days. HUVECs were fixed in 4% paraformaldehyde, washed with phosphate-buffered saline (PBS) and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich); cells were then blocked with 1% BSA. Immunolabelling was carried out using anti-FAK antibody (diluted 1:200), which was detected by Alexa Fluor® 488-conjugated secondary anti-rabbit antibody (1:3000) (Invitrogen, Molecular Probes™, Leiden, the Netherlands). Actin stress fibres were visualized using fluorescein isothiocyanate (FITC)-labelled phalloidin (Invitrogen, Molecular Probes™). All staining was visualized by confocal fluorescent microscopy (Axion Vision, Olympus IX70, Wallac, Waltham, MA, USA).

Cell cycle analysis by flow cytometry

For cell cycle analyses, HUVECs were grown to 60–70% confluence, detached using 0.25% (w/v) trypsin in

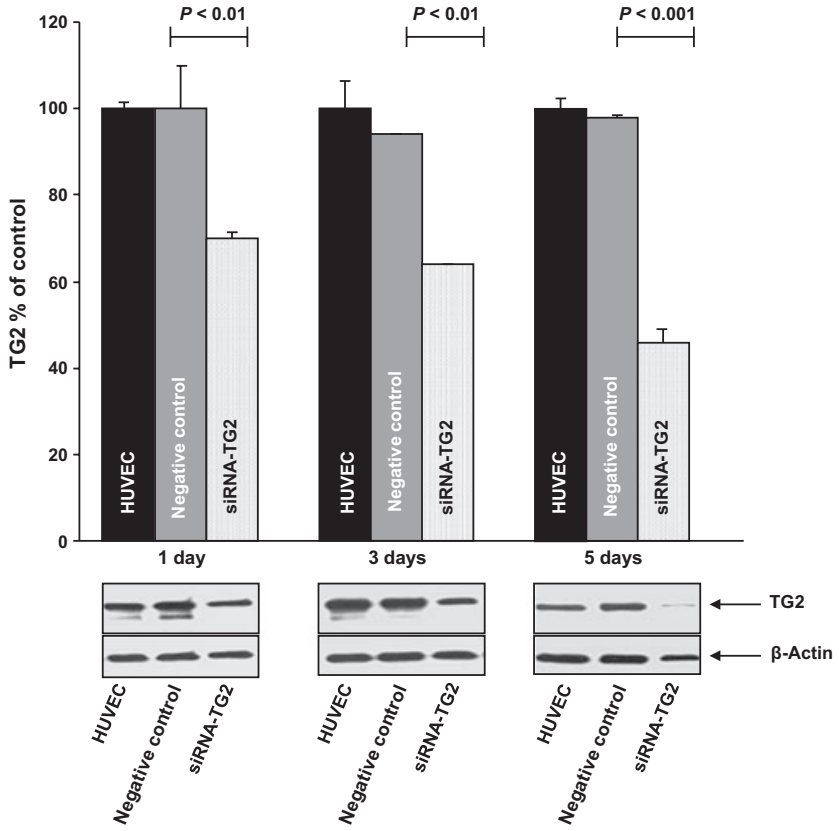

Figure 1. Evaluation of transglutaminase 2 (TG2) knock-down efficiency in endothelial cells. Relative amount of TG2 expression in untransfected endothelial cells (HUVEC) as well as those transfected with control siRNA (negative control) or siRNA-TG2 1, 3 and 5 days post-transfection. Bars represent mean TG2 expression as percentage of control and error bars indicate standard error of the mean. *P*-value < 0.05 was considered significant. Protein expression was normalized to β-actin and control was considered as 100%. Data derived from at least three independent experiments, repeated in duplicate are shown. In the lower part, representative Western blots show amounts of TG2 and β-actin used as loading control for protein extracts.

5 mM EDTA (Invitrogen) on days 1, 3 and 5 after transfection. Cells were washed with PBS and incubated with FACS hypotonic staining buffer (100 mM sodium citrate, 0.2% Triton X-100, 1 mg propidium iodide (PI) and 700 U/ml RNase), as described in Sigma-Aldrich protocols, and analysed by flow cytometery (Expo32 ADC, Epics®XL-MCL; Beckman Coulter, Harbor Boulevard, CA, USA).

Detection of cell death and apoptosis

The number of cells undergoing apoptosis was quantified using an Annexin V-FITC kit (Calbiochem, Beckman Coulter, Harbor Boulevard, CA, USA), according to the manufacturer's instructions. Briefly, 1×10^4 cells in different culture conditions were collected, washed, resuspended in binding buffer, and mixed with Annexin V-FITC and PI. After 5 min dark incubation at room temperature, cells were analysed using the flow cytometer (Expo32 ADC, Epics®XL-MCL).

Rescue siRNA-TG2 phenotype assays

To establish whether proliferation defects observed in siRNA-TG2 HUVECs were due to extracellular TG2, we designed rescue assays for all cell parameters analysed for silenced TG2 cells. Cell number and adhesion rescue assays were performed in 96-well plates precoated with 5 μg of human plasma FN and with 20 μg purified guinea pig TG2 (Sigma-Aldrich) (FN-TG2), as described elsewhere (16). Further experiments were performed using human recombinant TG2. Briefly, for cell number assay, 1×10^4 cells collected after siRNA-TG2 on days 1, 3 and 5 were plated on FN-TG2-precoated plates and grown for 24 h. Cell viability was measured by addition of Cell Titer 96 solution (Promega), according to the manufacturer's instructions. Cell adhesion rescue assay was performed as described previously (16) on FN-TG2-precoated plates. Briefly, after cell detachment with trypsin, HUVECs were washed in HBSS then 1×10^4 cells were seeded on 96-well FN-TG2-precoated plates. Cells were allowed to attach for 1 h and attachment was quantified using the same kit used as for the cell viability assay, according to the manufacturer's instructions. Apoptosis rescue assay was performed on FN-TG2-precoated plates. Briefly, cells with siRNA-TG2 were seeded in six-well FN-TG2-precoated plates. HUVECs were allowed to expand in number for 24 h and proportion of apoptotic cells was quantified as described above, according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using the non-parametric Mann–Whitney *U*-test and data are presented as mean values. *P*-value <0.05 was considered statistically significant.

Results

Down-regulation of TG2 by siRNA led to decrease in number of endothelial cells

To gain new insights into the role of TG2 in endothelial cell biology, effects of its down-regulation by siRNA on HUVECs were investigated. Conventional western blotting showed that expression of TG2 after siRNA decreased in a time-dependent manner. Maximum down-regulation of TG2 was observed after 5 days silencing, when expression level of the protein was reduced to 40% of that in cells transfected with control siRNA (Fig. 1). The immediate early effect of TG2 knock-down was significant reduction in total number of cells already after only 1 day of siRNA-TG2 (Fig. 2). Decrease in cell number was progressive and subsequently, after 5 days, number of cells had declined to 35% when compared with that of the non-transfected control group (Fig. 2).

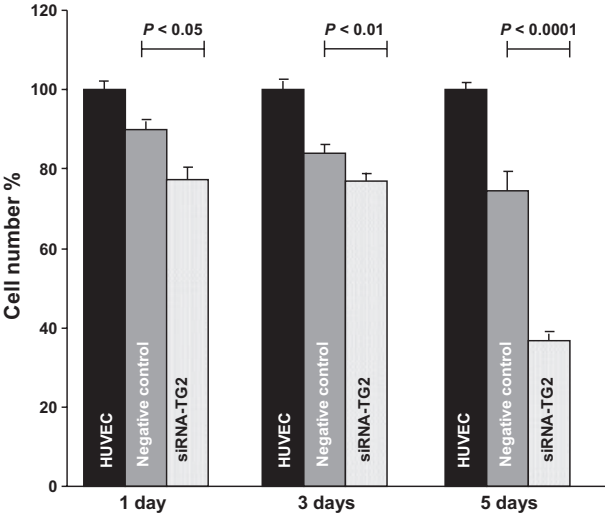

Figure 2. Assessment of endothelial cell number after transglutaminase 2 (TG2) silencing at different time-points. HUVECs were transfected with Scrambled siRNA (100 nM) and siRNA-TG2 (100 nM), as described in the Materials and methods section and cell viability was assessed by chemical reduction of MTT after different time points, as indicated. Data are expressed as percentage of control values on fibronectin, which represents 100%. Error bars indicate standard error of mean. 'HUVEC' indicates cells without any treatment; negative control represents cells transfected with non-silencing and siRNA-TG2 with TG2-specific siRNA-oligonucleotides respectively. Data are derived from at least three independent experiments, repeated in duplicate with initial cell number of 1×10^4. A *P*-value <0.05 was considered significant.

As reduction in cell number could be due to adhesion or proliferation defects or increased apoptosis, we next analysed these cell parameters after transient post-transcriptional silencing of TG2. Knocking down TG2 by siRNA caused marked loss of cell adhesion on FN already after 1 day silencing of TG2 (Fig. 3a). In addition to this, cells appeared rounded, showed shortcomings in spreading and detached easily (data not shown). As it is generally accepted that FAK and its activity promote cell adhesion (17), we investigated whether siRNA-TG2 led to changes in FAK total protein and pFAK (pY397) expression. Interestingly, down-regulation of TG2 by silencing resulted in reduced amount of FAK protein level and subsequent decrease in pFAK (pY397) levels after 3 and 5 days siRNA-TG2, in comparison with control groups (Fig. 3b).

Further to this, immunofluorescence analyses of TG2-silenced cells revealed that colocalization of FAK and F-actin was disrupted (Fig. 3c, white arrows) and focal adhesions were disorganized after 5 days of siRNA-TG2, compared with control groups (Fig. 3c, white arrowheads).

Furthermore, down-regulation of endogenous TG2 caused extensive actin reorganization and reduced stress fibre formation (Fig. 3c, white arrows); presumably as a consequence of this, cells adopted an aberrant shape when compared to both control groups. Moreover, we also observed that in siRNA-TG2 cells, there was marked decrease in α-smooth muscle actin expression (data not shown).

Down-regulation of TG2 arrested cell cycle progression at G_1 phase and induced apoptosis

To address the question of whether reduced number of TG2-siRNA cells was due to defects in cell cycle progression, cell cycle analysis was carried out by flow cytometry. These experiments revealed a statistically higher fraction of siRNA-TG2 cells in G_0/G_1 already at day 3, compared to control groups, as shown in Fig. 4a. As the cell cycle is tightly controlled by regulatory molecules such as cyclins, we investigated protein expression level of cyclin E and cyclin B, which are known to be essential for control of cell cycle progression in G_1/S and G_2/M transition phases respectively. Our results showed that the G_0/G_1 block observed in cells with down-regulation of endogenous TG2 was accompanied by significant and progressive up-regulation of cyclin E (Fig. 4b) and down-regulation of cyclin B (Fig. 4c). Moreover, cell cycle analysis revealed an increased number of apoptotic cells,

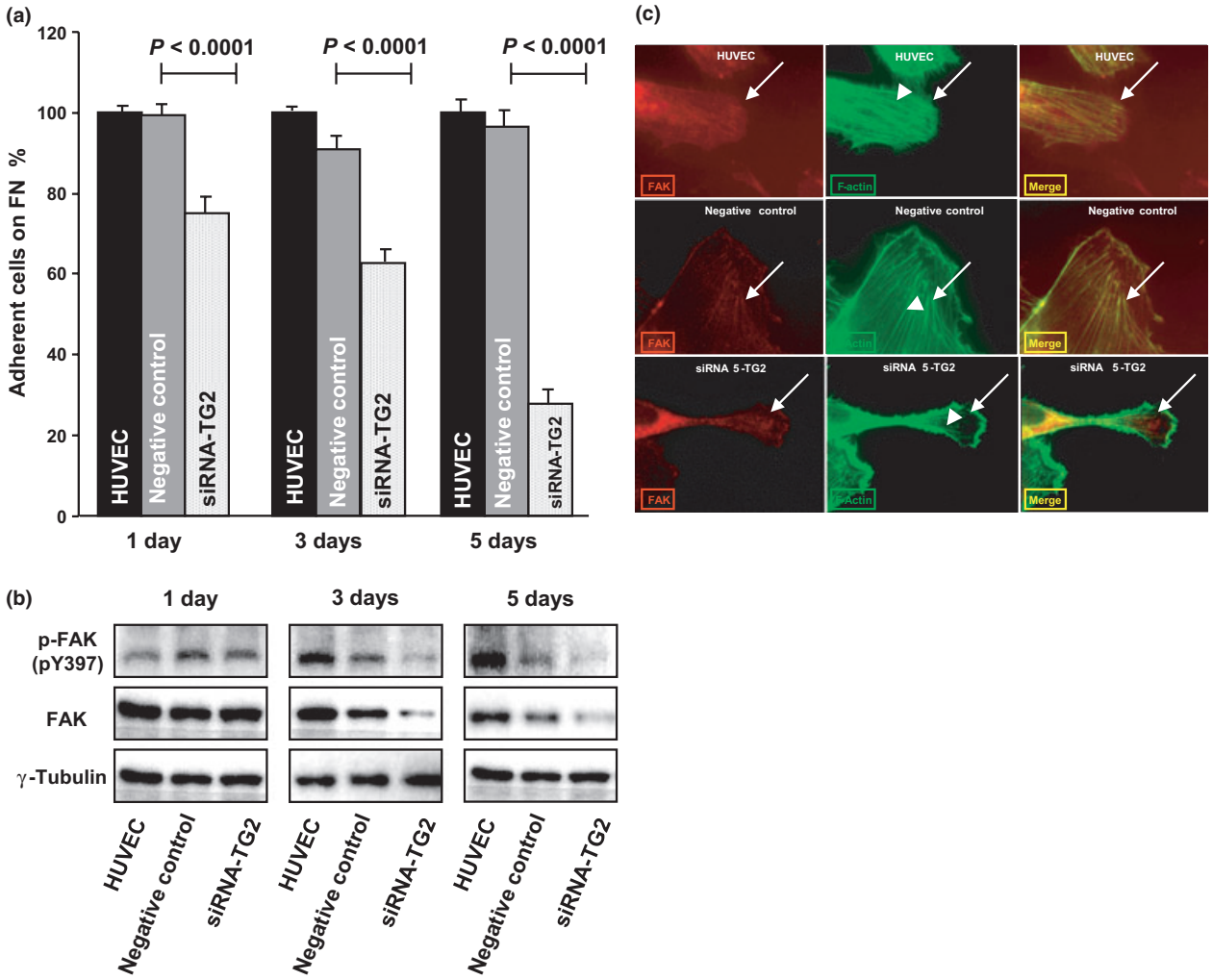

Figure 3. Analyses of adhesion and focal adhesion kinase (FAK) expression after transglutaminase 2 (TG2) silencing. (a) Endothelial cells were transfected with scrambled siRNA (100 nM) and siRNA-TG2 (100 nM), as described in the Materials and methods section and cell adhesion was determined by chemical reduction of MTT. Data are expressed as percentage of control values, which represented 100%. Experiments were performed with 1×10^4 of cells. Error bars indicate standard error of the mean. All data are derived from at least three different experiments, repeated in duplicate. *P*-value <0.05 was considered significant. (b) Western blot analysis was performed to determine constitutive level of total FAK and pFAK (pY397) expression after siRNA-TG2 at different time points, as indicated. Equal protein loading was confirmed by γ-tubulin. (c) Immunofluorescence microscopy analysis detecting disruption of focal adhesions (white arrows), after down-regulation of TG2 at day 5, and loss of FAK colocalization with stress fibres (white arrowheads) in comparison with control cells. 'HUVEC' indicates cells without any treatment; negative control represents cells transfected with control non-silencing siRNA and siRNA-TG2 cells treated with specific siRNA-TG2 oligonucleotides.

clearly identifiable after 5 days silencing (Fig. 4a). This was confirmed by staining with annexin V-FITC. Analyses of annexin V binding showed that the down-regulation of TG2 induced apoptosis dramatically in a time-dependent manner (Fig. 5a). It is well known that p53 and its downstream target gene *p21* are involved in cell cycle arrest preceding apoptosis (18,19). We therefore investigated whether increased apoptosis of TG2-siRNA cells could be coupled to increased expression of p53, p21 and downstream effector caspase 3, and found increased apoptosis to coincide with activation of caspase 3 and up-regulation of p53 along with its downstream target gene *p21*, as shown by western blotting (Fig. 5b).

FN-TG2 restored adhesion in siRNA-TG2 endothelial cells but did not reverse proliferation and apoptosis

To establish whether lower proliferation and higher apoptosis of siRNA-TG2 cells were solely due to adhesion defects, we applied rescue assays adopted from Verderio

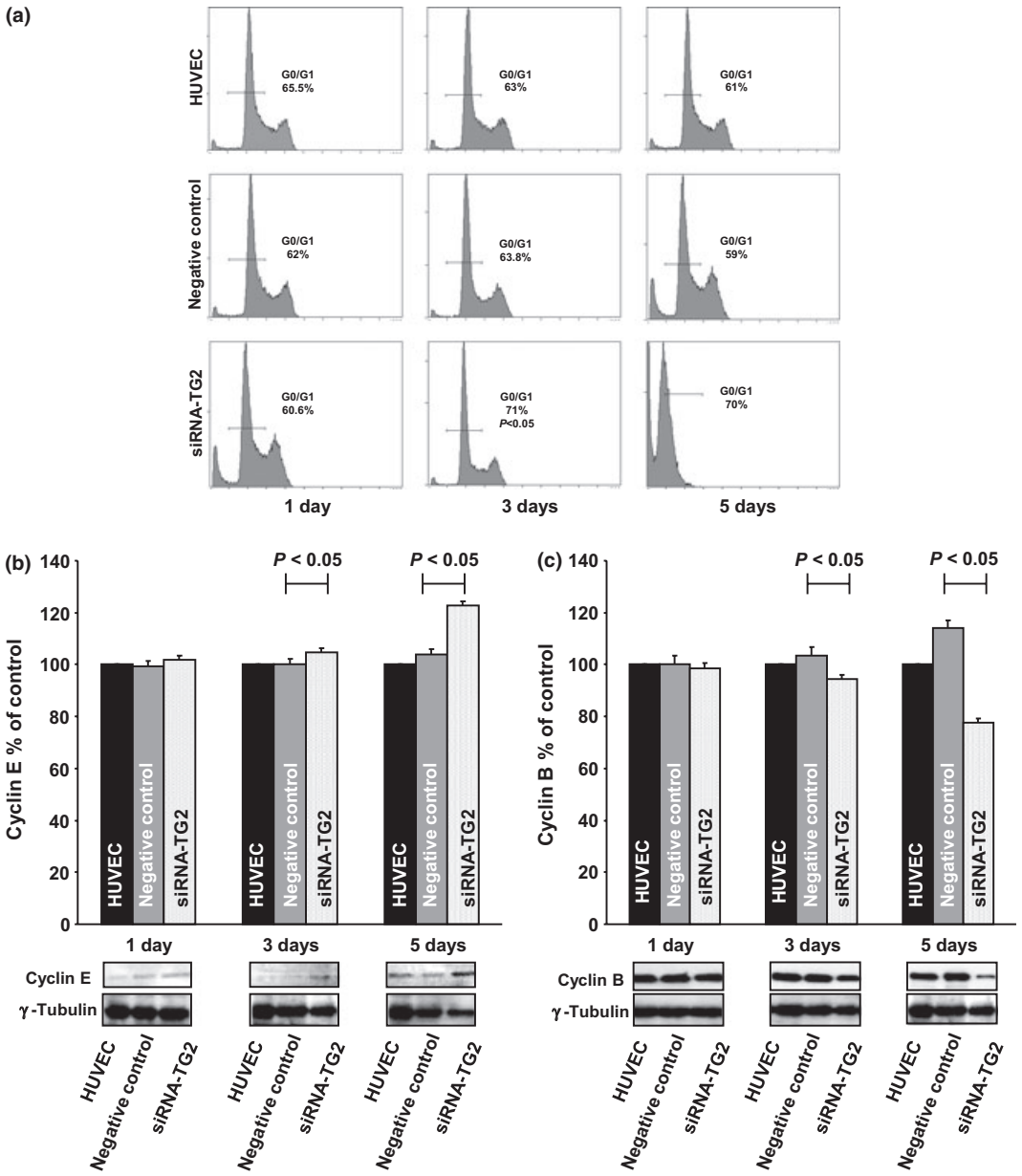

Figure 4. Effect of transglutaminase 2 (TG2) knock-down on cell cycle. (a) Cells were transfected with scrambled siRNA (100 nM) and siRNA-TG2 (100 nM), as described in the Materials and methods section and cell cycle analysis was studied by flow cytometry, showing G_1 cell cycle arrest with down-regulation of TG2. Expression and quantification of cyclin E (b) and cyclin B (c) after siRNA-TG2 by western blot analyses, at different time-points; γ-tubulin was used as a loading control for protein extracts. Bars represent cyclin E and cyclin B as percentage of the control with error bars indicating standard error of the mean. Data derived from at least three different experiments, repeated in duplicate are shown. *P*-value <0.05 was considered significant. 'HUVEC' indicates cells without any treatment, negative control represents cells transfected with control non-silencing siRNA and siRNA-TG2 cells treated with specific siRNA-TG2 oligonucleotides.

et al. (16) with minor modifications, to see if addition of exogenous TG2 could rescue phenotypes observed after down-regulation of TG2 by small interfering RNAs. Adhesion defects were completely abolished in siRNA-TG2-treated cells, plated in 96-well plates precoated with FN and commercial purified guinea pig TG2 (Fig. 6a). On the other hand, exogenously added guinea pig TG2 bound to FN was not able to circumvent reduction in cell number of siRNA-TG2 cells (Fig. 6b). Comparable results were achieved by human recombinant TG2 concerning proliferation (data not shown). Similarly, exogenous addition of purified guinea pig TG2 was not

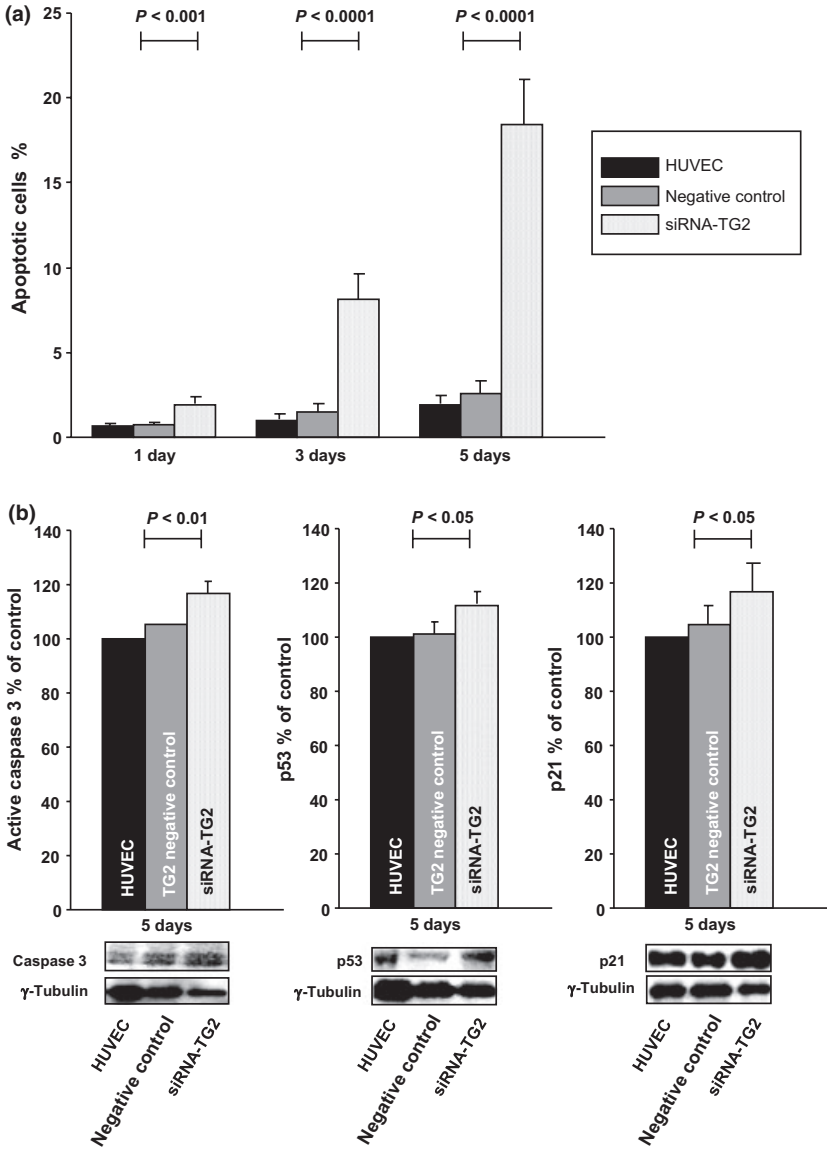

Figure 5. Effect of transglutaminase 2 (TG2) silencing on apoptosis. (a) HUVECs were transfected with Scrambled siRNA (100 nM) and siRNA-TG2 (100 nM), as described in the Materials and methods section, and apoptosis was assessed by AnnexinV-FITC labelling at different time points. Bar represent proportion of apoptotic cells expressed as percentage of total cells. In each experiment and time point, 1×10^4 cells were analysed. (b) Expression of pro-apoptotic protein caspase 3 as well as p53 and p21 on day 5 as shown by western blot analyses. Quantification of bands was performed using Kodak 1D image analysis software (Kodak, New Haven, CT, USA). Bars represent relative amounts of each protein indicated as percentage of control. Inserts below show representative western blots from experiments where γ-tubulin was used as a loading control for protein extracts. Error bars indicate standard error of mean. Data derived from at least three different experiments repeated in duplicate are shown. *P*-value <0.05 was considered significant. 'HUVEC' indicates cells without any treatment; negative control represents cells transfected with control non-silencing siRNA and siRNA-TG2 cells treated with specific siRNA-TG2 oligonucleotides.

able to normalize proportion of apoptotic cells where endogenous TG2 had been down-regulated, after 1 day of silencing (Fig. 6c). In this regard, we were not able to perform a rescue apoptosis assay with siRNA-TG2 at days 3 and 5 after transfection, as cells were non-viable for completion of detection of cell death by Annexin V-FITC and PI staining.

Discussion

In the present study we found that down-regulation of endogenous TG2 in living HUVECs by siRNA, reduced cell number, by loss of adhesion, inhibition of proliferation and increasing apoptosis, in a time-dependent manner. Furthermore, our results show that exogenous addition of TG2 could reverse loss of adhesion, whereas it did not overcome loss of cell proliferation nor could it inhibit TG2-siRNA-induced apoptosis.

Our findings indicating that TG2 is needed for complete adhesion are in line with earlier reports describing involvement of TG2 in adhesion of various cell types (20–26) in a manner independent of its transamidase activity (data not shown), (16,27). Moreover, our study is in agreement with previous studies showing diminished activation and autophosphorylation of FAK (pY397) following TG2 silencing, in pancreatic cancer cells (28). Notably, it has been recently shown by Lim *et al.* (29), that in primary human cells, FAK knock-down raised p53/p21 levels. In line with this, we have observed that endogenous down-regulation of TG2 induced decrease in FAK and

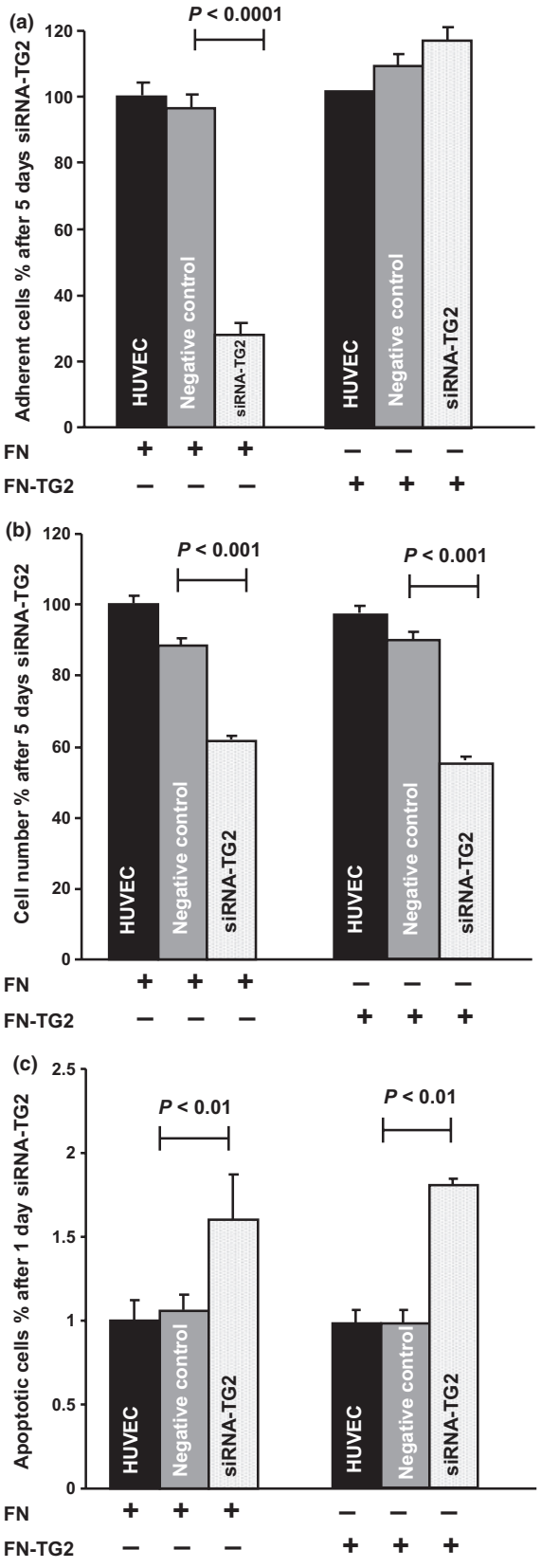

Figure 6. Exogenous addition of transglutaminase 2 (TG2) to rescue siRNA-TG2 endothelial cell phenotype. Endothelial cells were transfected with Scrambled siRNA (100 nM) and siRNA-TG2 (100 nM), as described in the Materials and methods section, and (a) adhesion, (b) cell number and (c) apoptosis of untransfected HUVECs as well as those transfected with control non-silencing siRNA (negative control) and siRNA targeting TG2 (siRNA-TG2) on fibronectin (FN) and to FN-bound TG2, were evaluated. Data expressed as percentage of control values, of 100%. Experiments were performed with 1×10^4 of cells. In (a) and (b), bars represent percentage of cells compared with control. In (c), bar represents proportion of apoptotic cells expressed as percentage of the total cells. Error bars indicate standard error of mean. Data are derived from at least three different experiments, repeated in duplicate. *P*-value <0.05 was considered significant.

pFAK expression was coupled with increase in p53/p21 and enhancement of apoptosis.

Although adhesion defects in the siRNA-TG2 of our cells could have explained the initial result of reduced cell number after TG2 down-regulation, we have shown that addition of exogenous TG2 was able to reverse the adhesion defect only, as also reported previously (16), without overcoming proliferation failure. This would suggest that reduction in cell number in response to TG2 silencing is independent of the adhesion process.

To our knowledge, the present study is the first to demonstrate that down-regulation of TG2 expression in endothelial cells leads to cell cycle arrest and this is coupled with elevated expression of cyclin E and decreased expression of cyclin B, known to play an essential role in cell cycle progression through G_1 to S and from G_2 to M phase respectively. It is important to note that Mangala *et al.* (30) have shown that knock-down of TG2 in breast cancer cells with siRNA markedly reduced their viability, even when they were cultured on FN-coated surfaces. Our results are in line with these findings but in our system, the proliferation rescue assay performed on FN-bound TG2 provided additional insight that any survival function of TG2 was due to its presence in an intracellular pool.

Noteworthy, earlier reports have shown that overexpression of TG2 in malignant hamster fibrosarcoma cells resulted in impairment of the cell cycle (31). However, Mian *et al.* (31) have demonstrated that increased expression of TG2 protein can affect progression through the cell cycle from S phase to G_2/M, and this depends on GTP-binding activity of TG2. Hence, it is interesting that both overexpression and down-regulation of TG2 induce impairment of cell cycle progression, thus supporting a direct involvement of TG2 in regulation of cell survival and cell division.

It is well established that growth arrest may lead to either cell survival but permanently arrested, or to death (32). Apoptotic cell death is activated if cells are not able to overcome the injury causing cell cycle arrest.

According to our results, this would seem to be the case for our endothelial cells, which, due to loss of intracellular TG2, pursue G_1 cell cycle arrest and subsequently commit apoptosis *via* activation of the caspase cascade. Furthermore, we found that increased apoptosis coupled with increased p53/p21 levels could not be prevented by addition of exogenous TG2, thus implying that in our experimental settings, the crucial cell survival role is played by intracellular TG2.

Notably, it has been reported that depending on cell context, TG2 can either promote or inhibit cell death. Yet, increased expression of TG2 in several types of cancer cell has been associated with augmented cell invasiveness and cell survival (28–31,33,34). Conversely, down-regulation of TG2 by siRNA or TG2 inhibition by small molecule inhibitors has been shown to significantly sensitize cancer cells to apoptosis (28,30,33). Additionally, TG2-regulated pathways are involved in promoting or protecting normal and tumour cells from death-induced signalling (33,34). Consequently, different outcomes related to level of TG2 expression and apoptosis, might be explained by different cell lines being used in investigations or that primary cells such as HUVECs are more vulnerable to loss of TG2 than cancer cells, where expression level of TG2 is increased. Even if TG2 knock-out mice did not exhibit growth defects *in vivo,* this may be due to different regulatory signals or compensatory survival mechanisms by other transglutaminase family members.

In conclusion, our results demonstrate that knocking down TG2 in endothelial cells leads to cell cycle arrest at G_1 phase, to adhesion defects and to increased apoptosis. Our findings indicate that the extra- and intracellular functions of TG2 are clearly distinct from each other with regard to cell adhesion and regulation of cell cycle and apoptosis respectively.

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Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Celiac disease patient IgA antibodies induce endothelial adhesion and cell polarization defects via extracellular transglutaminase 2

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Abstract We have recently found that celiac disease patient serum-derived autoantibodies targeted against transglutaminase 2 interfere with several steps of angiogenesis, including endothelial sprouting and migration, though the mechanism involved remained to be fully characterized. This study now investigated the processes underlying the antiangiogenic effects exerted by celiac disease patient antibodies on endothelial cells, with particular regard to the adhesion, migration, and polarization signaling pathway. We observed that celiac IgA reduced endothelial cell numbers by affecting adhesion without increasing apoptosis. Endothelial cells in the presence of celiac IgA showed weak attachment, a high susceptibility to detach from fibronectin, and a disorganized extracellular matrix due to a reduction of protein cross-links. Furthermore, celiac patient IgA led to secretion of active transglutaminase 2 from endothelial cells into the culture supernatants. Additionally, cell surface transglutaminase 2 mediated integrin clustering in the presence of celiac IgA was coupled to augmented expression of β1-integrin. We also observed that celiac patient IgA-treated endothelial cells had migratory defects and a less polarized phenotype when compared to control groups, and this was associated with the RhoA signaling pathway. These biological effects mediated by celiac IgA on endothelial cells were partially influenced but not completely abolished by R281, an irreversible extracellular transglutaminase 2 enzymatic activity inhibitor. Taken together, our results imply that celiac patient IgA antibodies disturb the extracellular protein cross-linking function of transglutaminase 2, thus altering cell-extracellular matrix interactions and thereby affecting endothelial cell adhesion, polarization, and motility.

Keywords Celiac disease antibodies · Endothelial cells · Transglutaminase 2 · Integrins · Adhesion · Polarization

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Introduction

In the small intestine, an organized array of finger-like villi provides an extensive epithelial surface area for absorptive function. The integrity of the villi relies on an appropriate vascular network providing mechanical support and thus helping to keep the villi tall [1]. In celiac disease, an autoimmune disorder triggered by ingested dietary gluten in susceptible individuals carrying human leukocyte antigen

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(HLA) DQ2 or DQ8 genotype, the overall architecture of the mucosal vasculature is abnormal [2, 3]. The blood vessel alterations occur in the context of small bowel mucosal villous atrophy, crypt hyperplasia, and inflammation, these constituting typical features of the disease.

Celiac disease is also hallmarked by a strong antibody response targeted against dietary gluten-derived deamidated gliadin peptides and an autoantigen, transglutaminase 2 (TG2) [4–6]. The target of the disease-specific IgA class autoantibodies, TG2, is a highly complex multifunctional protein with both enzymatic and non-enzymatic functions. As an enzyme, TG2 is able to catalyze post-translational modifications of glutamine residues on proteins and peptides through transamidation or deamidation reactions [7]. The TG2-mediated deamidation reaction is of particular importance during the pathogenesis of celiac disease, as distinct gliadin peptides can be deamidated by TG2, resulting in their increased affinity for HLA DQ2 and DQ8, thereby enabling a stronger antigen presentation and inflammatory response [8]. As a transamidase, again, TG2 catalyzes the formation of ε(γ-glutamyl)-lysine crosslinks between various proteins, including fibronectin (FN) and collagen, and this activity plays an important role in the organization of the extracellular matrix [9]. On the other hand, the non-enzymatic functions of TG2 include its role in cell adhesion via action as an FN co-receptor for β integrins [10] and syndecans [11].

The various functions of TG2 are involved in a variety of basic biological processes, including angiogenesis [12, 13]. The modulation of angiogenesis by TG2 is apparently complex, as there are data showing that both increased and decreased TG2 enzymatic activity leads to altered angiogenic response [14–17]. Our group has previously shown that celiac disease autoantibodies targeted against TG2 inhibit angiogenesis [18] and increase vascular permeability [2]. It appears that these outcomes involve increased enzymatic activity of TG2 [2, 19] and the Rho family of small GTPase proteins [2, 20], although the detailed molecular mechanism whereby this occurs is not yet fully understood. This present study sought to provide new insights into the molecular mechanism behind the altered endothelial cell biology and inhibition of angiogenesis mediated by celiac disease patient antibodies.

Materials and methods

Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) (Lonza Cambrex Bio Science, Walkersville, MD, USA) were maintained in EGM-2 medium (Clonetics, San Diego, CA, USA) with 2 % fetal bovine serum (FBS) (Gibco Invitrogen, Paisley, Scotland) and 15 μg/ml endothelial cell growth supplements (Clonetics). Cells between passages two and six were used in the experiments. In the experiments, the following chemical compounds administered 1 h before the addition of serum IgA were used: a non-reversible and cell-impermeable inhibitor for extracellular TG2 activity, R281 [21], at a final concentration of 200 μM, and a cell-permeable Rho inhibitor C3 transferase (Cytoskeleton, Denver, CO, USA) at 1 μg/ml.

Purification of serum IgA class antibodies

Serum samples from nine biopsy-proven celiac disease patients on a gluten-containing diet and five non-celiac healthy controls were included in the present study. All celiac sera had anti-TG2 antibody titers above 100 U/ml (Celikey; Pharmacia Diagnostic GmbH, Freiburg, Germany) and endomysial antibodies titers 1:≥1,000, while all control sera were negative. Total serum IgA were purified as previously described with anti-Human IgA agarose (Sigma-Aldrich, St. Louis, MO, USA) [18] and diluted in phosphate-buffered saline (PBS) to a final concentration of 100 μg/ml. Purified IgA fractions were used in all experiments at a concentration of 1 μg/ml. The study protocol was approved by the Ethics Committee of Tampere University Hospital, Tampere, Finland. All patients gave written informed consent.

Cell number assay, detachment, and adhesion assay

For the determination of endothelial cell number, 1×10^4 HUVECs were plated on 96-well plates (Nunclon, Thermo Fisher Scientific, Rockford, IL, USA), pre-coated with 5 μg of human plasma FN (Sigma-Aldrich) and grown to confluence. Thereafter, the cells were starved overnight (O/N) in 1 % FBS before a 24-h treatment with IgA. Subsequently, the number of HUVECS was determined by [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl] tetrazolium bromide CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI, USA) following the manufacturer's instructions. Experiments were performed in quadruplicate and repeated three times. The results are expressed as percentage of HUVECs without any treatment.

In order to study the susceptibility of cells to detach, HUVECs grown and treated as above were trypsinized (Sigma-Aldrich) for 5 min and carefully washed with PBS. The number of adherent cells remaining on the plate was determined by MTS assay as above (Promega). Experiments were performed in quadruplicate and repeated three times. The results are expressed as percentage of HUVECs without any treatment.

Further, to investigate weak versus strong adhesion, confluent HUVEC monolayers grown on six-well plates

(Sigma-Aldrich) were starved O/N in 1 % FBS, treated with IgA for 24 h, and detached with 5 mM EDTA in PBS (pH 7.4). Subsequently, 1×10^4 cells in serum-free culture media were allowed to adhere for 1 h on FN-precoated chamber slides (BD-Falcon, Bedford, MA, USA). The cells were carefully washed with PBS (pH 7.4) and then fixed with 4 % paraformaldehyde (4 % PFA). Images of cells from non-overlapping fields were taken at 20× magnification using the Axiovision 3.0 program (Carl Zeiss Vision GmbH, Munich, Germany). Weakly adhering cells (round cells) were counted manually on captured images. The results are given as the percentage of weakly adhering cells among all cells.

Quantification of apoptotic cells

The number of cells undergoing apoptosis was quantified using an Annexin V-FITC kit (Calbiochem, Beckman Coulter, Brea, CA, USA), according to the manufacturer's instructions. Briefly, 1×10^4 cells in different culture conditions were collected, washed, resuspended in binding buffer, and mixed with Annexin V-FITC and propidium iodine. After 5-min dark incubation at room temperature (RT), cells were analyzed using a flow cytometer (Expo32 ADC, Epics_XL-MCL, Beckman Coulter).

Microtiter plate assay for quantification of surface and matrix expression of proteins

HUVECs were grown to confluence on commercial 96-well plates (Nunclon). After O/N starvation in EGM-2 with 1 % of FBS, they were incubated for 24 h in the presence of purified serum IgA. The cells were then washed with Hanks' balanced salt solution (HBSS) (Sigma-Aldrich), fixed with 4 % paraformaldehyde (PFA) (Sigma-Aldrich) and blocked with 5 % bovine serum albumin (BSA) (Sigma-Aldrich). The plates were then incubated with primary antibodies against human FN (1:200) (F3648, Sigma-Aldrich), *N*-ε-(γ-L-glutamyl)-L-lysine isopeptide (1:200) (mab0012, Covalab, Villeurbanne, France), MT1-MMP (1:200) (MAB3328, Millipore, Billerica, MA, USA), β1-integrin (1:200) (MAB1965, Millipore) or vinculin (1:200) (7F9, Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h at 37 °C. After incubation with primary antibodies, the cells were extensively washed and incubated in the presence of secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins (1:2,000) (Dako, Glostrup, Denmark) at 37 °C for 1 h. Finally, the peroxidase substrate TMB (Sigma-Aldrich) was used and the enzymatic reaction was stopped with 2.5 M H_2SO_4. Absorbance was measured at 450 nm by spectrophotometer (Multiskan Ascent, Thermo Labsystems, Santa Clara, CA, USA).

Immunofluorescent stainings

HUVECs were grown to confluence on FN-precoated chamber slides (BD-Falcon) and subjected to different experimental conditions. For visualization of cross-links, the cells were washed with PBS, fixed in 4 % PFA, and then blocked with 1 % BSA. Immunolabeling was carried out using anti-*N*-ε-(γ-L-glutamyl)-L-lysine isopeptide antibody (1:200) (Covalab), which was detected by Alexa Fluor 488 conjugated secondary anti-mouse antibody (1:3,000) (Invitrogen, Molecular Probes, Leiden, Netherlands). FN staining was performed by incubating live cells grown on chamber slides (BD-Falcon) with anti-human FN (Sigma-Aldrich) diluted 1:200 for 1 h at 37 °C. Thereafter, unbound antibodies were carefully washed with PBS and the cells were fixed with 4 % PFA. Subsequently, non-specific binding was blocked with 1 % BSA and the bound antibodies detected with Alexa Fluor 488 or Alexa Fluor 568 conjugated secondary anti-mouse or anti-rabbit antibodies (1:3000) (Invitrogen). All stainings were visualized by fluorescent microscopy analysis (Olympus Optical Co. Ltd, Tokyo, Japan).

SDS-PAGE and immunoblotting

For Western-blot analysis, confluent HUVECs under different experimental conditions were resuspended in Laemmli loading buffer (63 mM Tris–HCl, 10 % glycerol, 2 % SDS, 0.1 % (w/v) Bromophenol Blue, 2.5–5.0 % beta-mercaptoethanol). Total lysates (10 μl) or cell supernatants were separated by 12 % SDS-PAGE electrophoresis under reducing conditions. After gel electrophoresis and transfer of the proteins to a nitrocellulose membrane (Hybond C-extra, Amersham Biosciences Ltd, Little Chalfont, UK), the nitrocellulose sheets were blocked at RT for 1 h in 5 % non-fat dry milk and incubated O/N at +4 °C using specific primary antibodies. The primary antibodies used here included mouse monoclonal antibodies against TG2, CUB7402 (NeoMarkers, Fremont, CA, USA), β1-integrin (MAB 1965, Chemicon), vinculin (7F9, clone sc-73614, Santa Cruz Biotechnology, Wembley, UK). Monoclonal antibody against γ-tubulin (GTU-88, Sigma-Aldrich) was chosen as the internal loading control. Blots were then washed and immunoreactivity detected by sequential incubation of the membranes with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Dako) for 1 h at RT. Thereafter, the membranes were analyzed using the chemiluminescence detection system (Amersham, ECL plus Western blotting detection system). Exposed autoradiography films (Kodak, New Haven, CT, USA) were subjected to densitometry investigation using Kodak 1D image analysis software (Kodak). The protein expression was normalized to γ-tubulin and control taken

as 100 %. The calculated values are derived from three independent experiments performed in duplicate.

Determination of TG2 activity in culture supernatants

Secreted TG2 enzymatic activity in culture supernatants of endothelial cells was measured by 5-biotinamidopentylamine (5-BP) (Thermo Fisher Scientific) incorporation into FN (Human FN 96-well Multiwell) (BD Biosciences). Supernatants of HUVECs under different experimental conditions were collected after 24 h and subsequently incubated with 40 μM 5-BP for 2 h at 37 °C on FN-precoated plates. After extensive washing with Tris HCl pH 7.4, incorporated 5-BP was detected with a streptavidin biotinylated HRP complex (1:5,000) (Life Science Research Pierce Biotechnology). Lastly, the peroxidase substrate TMB (Sigma-Aldrich) was used and the enzymatic reaction stopped with 2.5 M H_2SO_4. Absorbance was measured at 405 nM by spectrophotometer (Multiskan Ascent, Thermo Labsystems). In order to verify the presence of TG2 during the procedure, some wells were incubated with CUB7402 (1:200) (NeoMarkers, Fremont, CA, USA) instead of 5-BP. Data are derived from two independent experiments, repeated in quadruplicate.

Integrin clustering assay

Integrin clustering was studied as previously reported [22] with minor modifications. Briefly, 2×10^5 HUVECs cells cultured under different experimental conditions were seeded onto FN-coated (10 μg/ml) chamber slides (BD-Falcon) in serum-free (SE) or in 1 % serum-containing medium (SEM). Cells were left to adhere and grow for 4 h in SE and 24 h in SEM. Live cells were incubated with β1-integrin primary antibody (Chemicon) for 1 h, washed with PBS, fixed with 4 % PFA and permeabilized with 0.25 % Triton X-100. Thereafter, the cells were blocked with 0.1 % BSA in PBS for 25 min, followed by incubation for 1 h with the conjugated secondary antibody Alexa Fluor 568 (Invitrogen). Slides were visualized by fluorescent microscopy analysis (Olympus Optical Co. Ltd).

Golgi reorientation assay

Cell polarity assay was performed as previously described [23], with minor modifications. Briefly, 2×10^5 HUVECs were grown onto FN-coated (10 μg/ml) chamber slides (BD-Falcon) until confluent, starved O/N in culture media 1 % FBS, and subjected to different experimental conditions. After 24 h, endothelial cell monolayers were wounded with a pipette tip, washed with PBS and incubated in serum-free media for 4 h with the monoclonal β-COP antibody (clone M3A5, Sigma-Aldrich). After extensive washings, HUVECs were fixed with 4 % PFA, permeabilized, and then blocked with 0.05 % BSA for 1 h at RT. Golgi apparatus was detected by Alexa Fluor 568 conjugated secondary anti-mouse (1:3,000) (Invitrogen). Actin cytoskeleton changes were visualized using FITC-conjugated phalloidin (Invitrogen). Images were captured at 60× using an inverted microscope (IX51; Olympus Optical Co. Ltd). To quantify endothelial cell polarization, Golgi reorientation at the wounded edges was analyzed. A square was drawn over the nucleus and divided into quadrants. Quadrant A was assigned to the area of the cell between the nucleus and the leading edge. A properly reoriented Golgi was indicated by β-Cop staining entirely within quadrant A. Analyses were performed on 100 cells per experiment to determine the percentage of cells with reoriented Golgi. All stainings were visualized by fluorescent microscopy analysis (Olympus Optical Co. Ltd).

Statistical analysis

Statistical analysis was performed using the non-parametric two-tailed Mann–Whitney *U* test. Data are presented as means + standard error of means. A p value <0.05 was considered significant.

Results

Celiac disease IgA affects endothelial cell number, adhesion, and detachment

We first addressed the anti-angiogenic properties of celiac disease patient IgA (CD IgA) by investigating their effects on endothelial cell number. We noted that there was a small but statistically significant reduction in the number of HUVECs cultured on FN in the presence of CD IgA for 24 h when compared to cells without treatment or incubated with control IgA (H IgA) (Fig. 1a). As the reduction in cell number might be due to adhesion defects or increased apoptosis, we next investigated these cell parameters in our experimental settings. Interestingly, we observed that in HUVEC cultures supplemented with CD IgA there was a significantly lower number of cells left on the plates after detachment by trypsinization when compared to control groups (Fig. 1b). Additionally, the percentage of round, weakly adherent cells on the FN-coated surface was significantly greater among HUVECs treated with CD IgA than in control cultures (Fig. 1c). Pre-incubation of the cells with the extracellular TG2 enzymatic activity inhibitor R281 was not able to normalize any of the above-mentioned effects (Fig. 1a–c). The numbers of apoptotic cells in control conditions and after treatment with H IgA or CD IgA were comparable (30, 33, and 38 %, respectively).

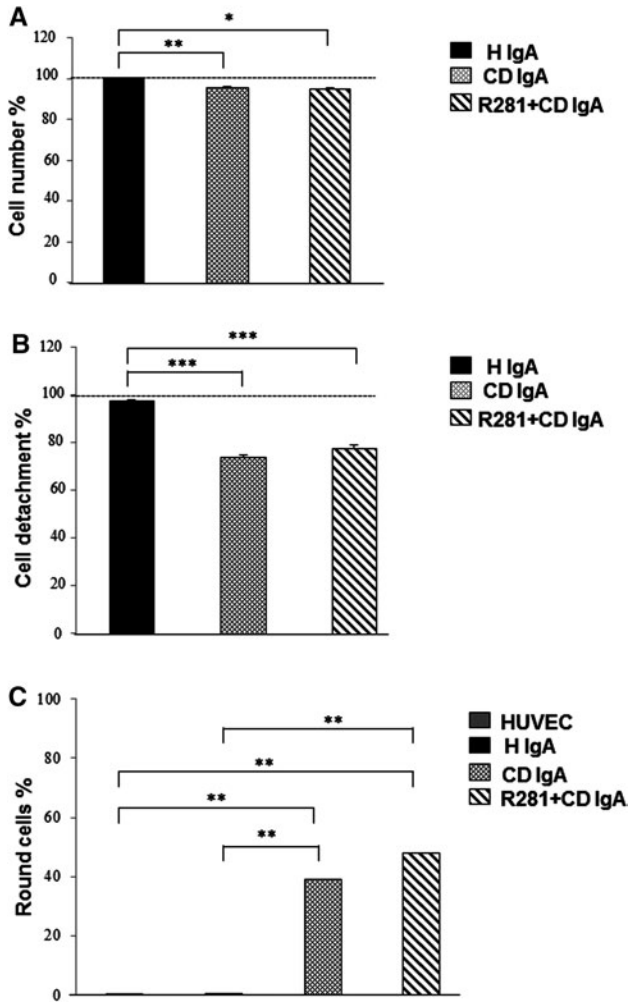


Fig. 1 Celiac patient immunoglobulins A (CD IgA) reduces endothelial cell number and affects cell adhesion. **a** HUVEC cell number in cultures without any supplementation and after 24-h treatment with CD or healthy IgA (H IgA) analyzed by chemical reduction of MTS. **b** The same experimental setting was used to investigate the number of adherent cells on the plate after 5 min of trypsinization. For **a** and **b**, the results were normalized to HUVECs without any treatment and indicated by a *dashed line*. Data are derived from three independent experiments repeated in quadruplicate. **c** Quantification of weakly adhering cells to fibronectin-precoated chambers as percentage of total cell number. Before adhesion, the cells were grown for 24 h without any supplementation or with H IgA or CD IgA. Quantification was performed by counting the number of round cells (weak adhesion) in randomly selected microscopic pictures of three independent experiments. In all panels, the *bars* represent the mean and *error bars* standard error of mean. A p value <0.05 was considered significant (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) and only statistically significant results are reported. The extracellular transglutaminase 2 activity was inhibited by administering a site-directed, non-permeable inhibitor, R281

The extracellular environment of endothelial cells is altered in the presence of celiac IgA

As we found the attachment of HUVECs to FN to be abnormal in the presence of CD IgA, we next investigated whether the altered expression of FN, a matrix ligand of cell surface TG2 [10], might account for these effects. Interestingly, we found that even though the overall FN expression in total cell lysates was not affected by CD IgA when analyzed by Western blotting (data not shown), there was a slight decrease in its surface expression measured by the microtiter plate assay (Fig. 2a). Moreover, immunofluorescent staining showed differences in the architecture of the extracellular FN network in the presence of CD IgA (Fig. 2b). These effects on FN were not reversed by inhibiting the extracellular TG2 enzymatic activity with R281 (Fig. 2a, b). As FN, among other common extracellular matrix proteins, is polymerized by TG2 cross-linking activity [22, 24, 25], we next determined the relative numbers of stable cross-links in our cell cultures. Using a microtiter plate assay, we observed a statistically significant decrease in *N*-ε-(γ-L-glutamyl)-L-lysine isopeptide bonds in HUVECs incubated with CD IgA autoantibodies when compared to control groups (Fig. 2c). Similarly, immunostaining of extracellular cross-links supported this finding (Fig. 2d).

As cell surface TG2 is susceptible to degradation by membrane type 1 matrix metalloproteinase (MT1-MMP) [26, 27], we next studied whether increased MT1-MMP expression could elucidate the adhesion defects observed in endothelial cells grown in the presence of CD IgA. Results obtained by the microtiter plate method showed that in the presence of CD IgA, the expression of MT1-MMP was moderately but significantly increased. This increase could not be prevented by the non-permeable TG2 enzymatic inhibitor, R281 (Fig. 3a). Since increased expression of MT1-MMP is related to extensive proteolysis of cell surface TG2, resulting in the appearance of three major cleavage products of molecular masses of ~75, ~66, and ~56 kDa in culture media [22], we next collected supernatants of HUVECs under different experimental conditions to further investigate this issue by Western blotting. Interestingly, we found two main bands, one of which corresponded to intact TG2 (molecular mass of ~75 kDa) and the other to higher molecular weight TG2 (molecular mass of ~200 kDa) highly expressed in the presence of CD IgA (Fig. 3b). Of note, we also found that TG2 secreted from endothelial cells cultured in the presence of CD IgA was enzymatically active (Fig. 3c). The secretion of TG2 into the supernatants was not prevented by R281, whereas its enzymatic activity was abolished (Fig. 3b, c).

Celiac IgA has an effect on integrin β1 and vinculin expression

Since TG2 has a prominent role in cell adhesion as a β1-integrin co-receptor for FN in a non-enzymatic fashion [10], we next considered β1-integrin expression in

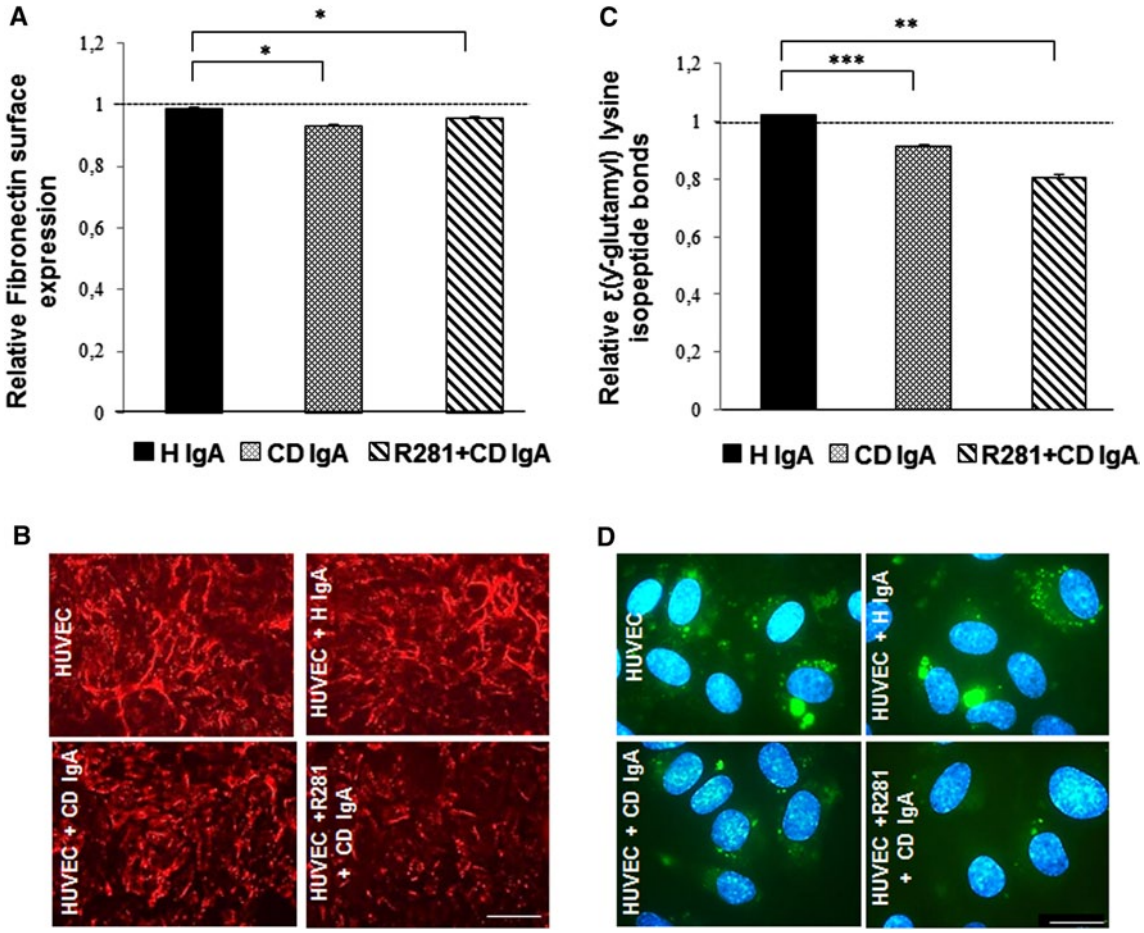


Fig. 2 In endothelial cells, celiac patient immunoglobulin A (CD IgA) affects extracellular fibronectin and cross-links. The relative amount of **a** fibronectin as well as **c** the extent of *N*-ε(γ-L-glutamyl)-L-lysine cross-links were analyzed by a microtiter plate assay in HUVECs with no supplementation or after 24-h treatment in the presence of CD IgA and healthy IgA (H IgA). The results were normalized to HUVECs without any treatment, indicated by the *dashed line*. *Bars* represent the mean and *error bars* indicate standard error of the mean. A p value <0.05 was considered significant (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) and only statistically significant results are reported. Data derived from three independent experiments repeated in quadruplicate are shown. **b** Representative immunofluorescence stainings of fibronectin in live HUVECs alone or incubated for 24 h with H IgA and CD IgA. *Scale bar* 100 μM. **d** Representative immunofluorescence stainings of protein cross-links in control HUVECs with no treatment or incubated for 24 h with H IgA and CD IgA. Cross-links are shown in *green* and DAPI was used as nuclear counterstain (*blue*). *Scale bar* 50 μM. The extracellular TG2 activity was inhibited by administering a site-directed, non-permeable inhibitor, R281

HUVECs under different experimental conditions. Immunostaining for β1-integrin revealed that in all groups after 4-h adhesion the cells form similar, punctuate β1-integrin patterns, mainly localized on the perinuclear region in those receiving CD IgA (Fig. 4a). After 24 h, in control cultures β1-integrin was mostly found as punctuate structures along the entire cell body, especially on the periphery of the cells, very likely at focal adhesion sites (Fig. 4b). In contrast, endothelial cells treated with CD IgA were characterized by a prominent β1-immunostaining at the periphery of the cell. In addition, we observed that the contact area of the cells appeared filamentous and lacked a bright β1-integrin signal (Fig. 4b). We also assessed the expression of β1-integrin by Western blotting and by a microtiter plate method after 24-h treatment with serum IgA and found it to be increased in HUVECs cultured in the presence of CD IgA (Fig. 5a–c). Preincubation with the extracellular TG2 enzymatic inhibitor R281 did not affect this increase (Fig. 5c).

Adhesion is mediated by the formation of proper focal adhesion sites which include the recruitment of a cytoplasmic protein, vinculin [28] and we thus next investigated its expression. When measured by both the microtiter plate method and Western blotting, we noted a reduction in vinculin expression in cells treated with CD IgA when compared to controls (Fig. 6a–c). As mentioned previously, this decrease was overcome by pre-incubating the cells with R281 (Fig. 6b, c).

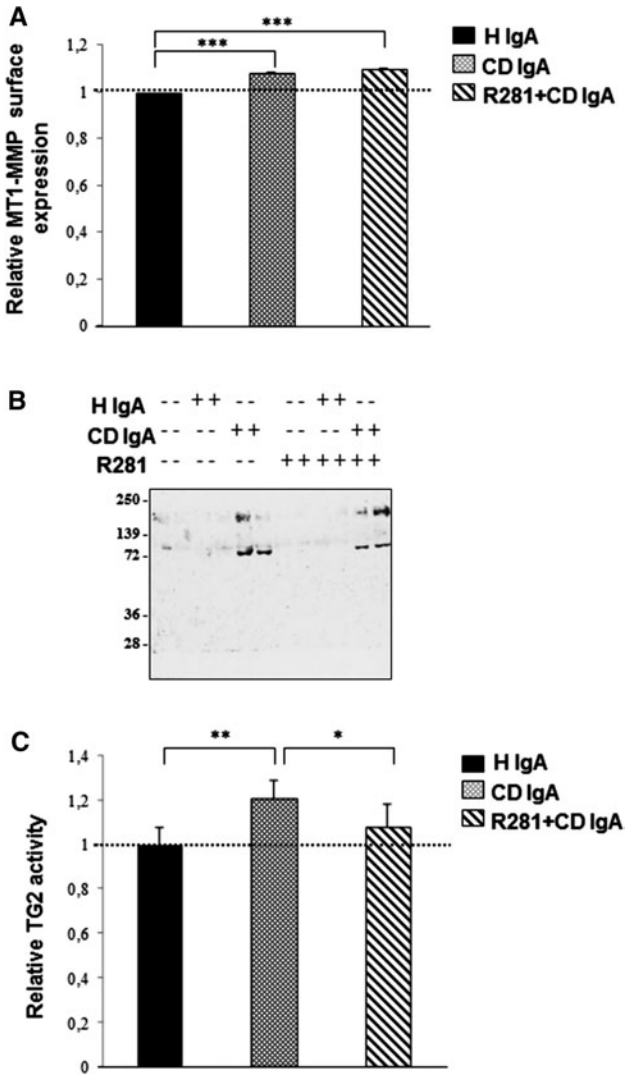


Fig. 3 Celiac patient immunoglobulins A (CD IgA) increase surface expression of membrane type 1 matrix metalloproteinase (MT1-MMP) and promote transglutaminase 2 (TG2) secretion from endothelial cells. **a** The relative surface expression of MT1-MMP in endothelial cells without any treatment or in the presence of CD IgA or control non-celiac subject immunoglobulin-A (H IgA) was studied by a microtiter plate method. *Bars* represent mean MT1-MMP expression as fold of control (*dashed line*) and *error bars* indicate standard error of the mean. A p value <0.05 was considered significant (***$p < 0.001$) and only statistically significant results are reported. Data are derived from three independent experiments repeated in quadruplicate. **b** A representative Western blot of endothelial cell culture supernatants under different experimental conditions performed with CUB7402 to detect TG2. **c** The relative activity of secreted TG2 was analyzed by microtiter plate assay. *Bars* represent mean 5-BP incorporation into fibronectin as fold of control and *error bars* indicate standard error of the mean. A p value <0.05 was considered significant (*$p < 0.05$, **$p < 0.01$) and only statistically significant results are reported. Data are derived from two independent experiments repeated in quadruplicate. R281 indicates a non-permeable, site-directed TG2 enzymatic activity inhibitor

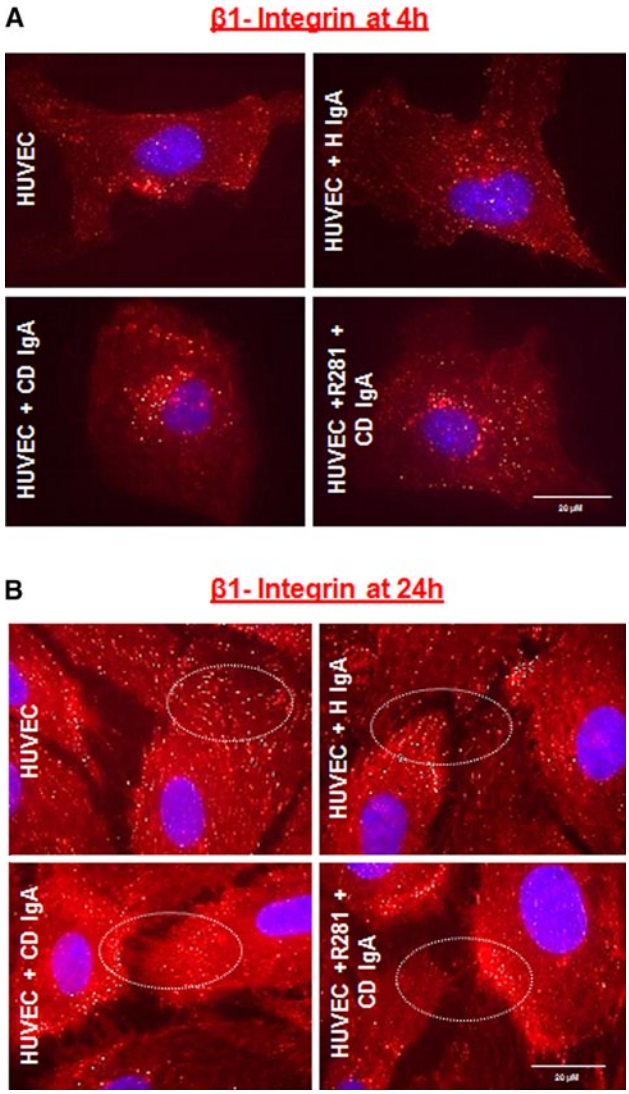
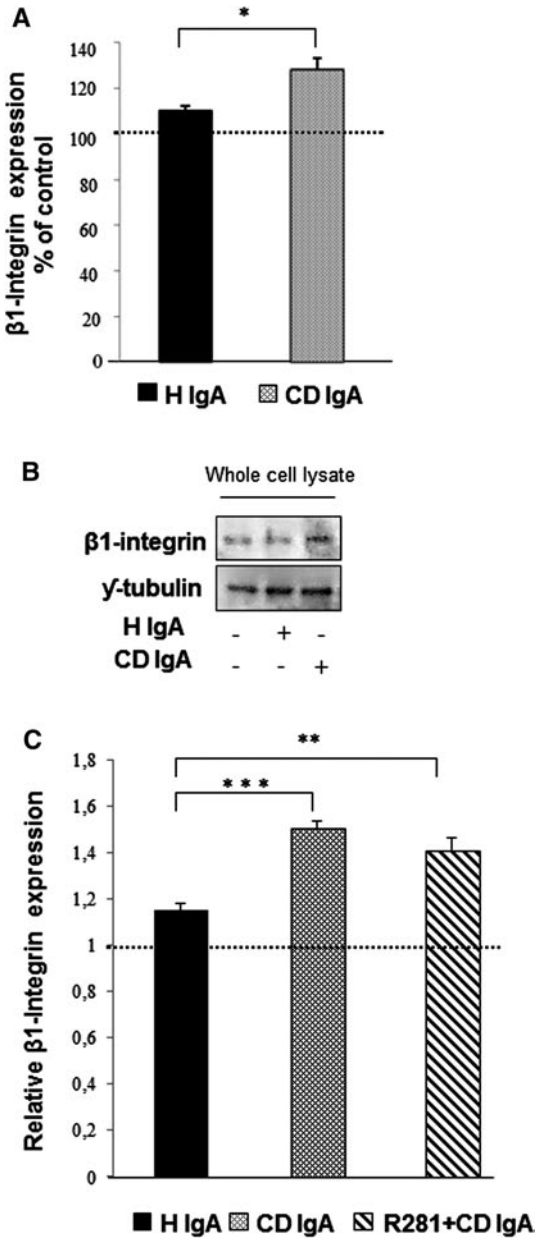


Fig. 4 Celiac patient immunoglobulins A (CD IgA) affect β1-integrin clustering in endothelial cells. Representative immunofluorescence stainings of serum-starved endothelial cells (HUVECs) allowed to adhere on fibronectin-precoated chambers for 4 h (in 0 % FBS) (**a**) and for 24 h (in 1 % FBS) (**b**) and immunostained with of β1-integrin primary antibody. Differences in β1-integrin (in *red*) are indicated by *white circles* and DAPI was used as nuclear counterstaining (*blue*). Images were selected from three independent experiments. *Scale bar* 20 μM. The extracellular TG2 activity was inhibited by preincubation of the cells with the non-permeable site-directed inhibitor, R281

Endothelial cell polarization is affected in the presence of celiac disease IgA

Since transient integrin clustering and the establishment of mature focal adhesion sites are necessary for cells to acquire a polarized phenotype [23], we next sought to



◀**Fig. 5** Celiac patient immunoglobulins A (CD IgA) increase β1-integrin expression in endothelial cells after 24-h treatment. **a** Quantification of Western-blot analyses of β1-integrin in whole endothelial cell lysates under different experimental conditions. Protein expression was normalized to γ-tubulin and control was taken as 100 %. The *dashed line* indicates HUVECs without any treatment. *Bars* represent mean of data derived from four independent experiments repeated in duplicate and *error bars* indicate standard error of the mean. A p value <0.05 was considered significant ($*p < 0.05$) and only statistically significant results are reported. **b** Representative Western blot of β1-integrin in whole endothelial cell lysates under different experimental conditions. **c** The relative amount of β1-integrin was analyzed by microtiter plate method in unpermeabilized HUVECs alone or incubated with CD IgA and healthy (H IgA) subject's immunoglobulins. The *dashed line* indicates HUVECs without any treatment. *Bars* signify mean and *error bars* standard error of the mean. A p value <0.05 was considered significant ($**p < 0.01$, $***p < 0.001$) and only statistically significant results are reported. Data are derived from three independent experiments repeated in quadruplicate. The extracellular transglutaminase 2 activity was inhibited by administering a site-directed, non-permeable inhibitor, R281

evaluate this cell parameter by wound healing and Golgi reorientation analysis.

In unwounded endothelial cells monolayers, the Golgi apparatus is randomly oriented [23]. Upon creating a scratch wound edge and allowing for initial cell movement within the denuded space, cells will establish an axis of polarity with lamellipodia and Golgi markers facing the wound edge. Whereas over 90 % of HUVECs incubated with H IgA exhibited Golgi orientation towards the wound edge, only 50 % of HUVECs treated with CD IgA displayed such behavior, with a more disrupted pattern of F-actin and lamellipodia not projected towards the direction of the wound (Fig. 7a–c). Incubation of the cells with R281 prior to CD IgA treatment seemed to ameliorate the defective polarization phenotype even though it did not reverse it totally. In contrast, pre-administration of the C3 transferase inhibitor reversed the polarity defects observed in HUVECs with CD IgA, albeit not to control levels (Fig. 7a–c).

Discussion

The aim of this study was to find new insights into the mechanism of action underlying the antiangiogenic effects exerted by CD IgA in the endothelium, in vitro, without considering the mesenchymal compartment. We observed that the number of endothelial cells was decreased in the presence of CD IgA. This was presumably caused by enhanced susceptibility to detach from FN and an ability to adhere only weakly. Proper cell adhesion relies on the integrity and stabilization of the ECM of the cell [29]. Interestingly, we found that the presence of CD IgA leads to an irregular appearance of the FN network and a reduction in the number of protein cross-links, suggesting that the FN polymerization process catalyzed by extracellular TG2 cross-linking activity is disturbed, this accounting for the abnormal ECM, altered adhesion properties, and reduction in endothelial cell numbers. Of note, inhibition of extracellular TG2 by R281 did not modify the CD IgA-mediated effects. The finding of a decreased ECM cross-links was unexpected, since our previous works [2, 19] have shown that celiac disease autoantibodies increase endothelial TG2 enzymatic activity when measured by incorporation of primary amines. However, this discrepancy might be explained by the fact that transamidation (using a small primary amine substrate) and protein crosslinking by TG2 take place under different conditions. Thus, bearing

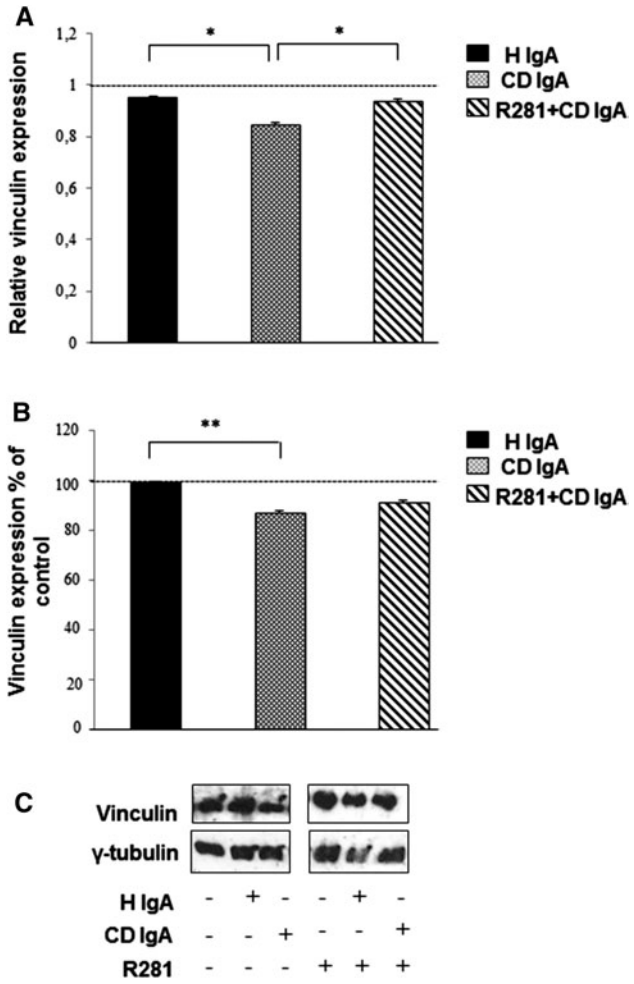


Fig. 6 Celiac patient immunoglobulins A (CD IgA) reduce vinculin protein expression in endothelial cells. **a** The relative amount of vinculin in HUVECs alone or incubated with CD IgA and healthy (H IgA) subject's immunoglobulins was studied by microtiter plate assay after 24-h treatment. **b** Quantification of Western-blot analyses of vinculin in whole endothelial cell lysates under different experimental conditions. Protein expression was normalized to γ-tubulin and control was taken as 100 %. The *dashed line* indicates HUVECs without any treatment. In **a**, **b**, *bars* represent the mean and *error bars* indicate standard error of the mean. A p value <0.05 was considered significant ($*p < 0.05$, $**p < 0.01$) and only statistically significant results are reported. Data are derived from three independent experiments repeated in quadruplicate. **c** Representative Western blot of vinculin in whole endothelial cell lysates under different experimental conditions is shown. The extracellular transglutaminase 2 activity was inhibited by administering a site-directed non-permeable inhibitor, R281

in mind that the cross-linking activity of TG2 is required for FN assembly and deposition [22, 30], we may speculate that this function of TG2 is inhibited in the presence of CD IgA and leads to decreased expression and abnormal appearance of extracellular FN. A recent work, again, reporting that major auto antigenic epitopes in TG2 recognized by celiac patient autoantibodies are clustered in the N-terminal half of the enzyme [31], in part corresponding to the FN binding site of TG2, allows us to alternatively explain our findings by the inability of anti-human-FN antibody to recognize FN when it is complexed to TG2 and CD IgA. In this regard, our preliminary unpublished data would imply that this indeed might be the case, since in the presence of CD IgA the detection of FN associated with TG2 is reduced.

It has also been reported that treatment of FN-TG2 matrix with TG2 autoantibodies from celiac disease patients reduces cell attachment [32]. Mesin and coworkers have hypothesized that anti-TG2 IgA could displace TG2 from the FN meshwork, and such a mechanism could explain the altered cell adhesion properties [31, 33]. In contrast to this claim, we observed in our in vitro experimental settings that when TG2 is immobilized on FN, it is not released in the presence of CD IgA (unpublished results) nor could any interference be measured between the celiac autoantibodies and FN binding in the presence of Ca^{2+} [31], which is abundantly available for TG2 in extracellular conditions. On the other hand, we saw augmented secretion of TG2 in endothelial cell culture supernatants treated with CD IgA, independent of the TG2 extracellular activation, since administration of R281 was not capable of preventing this effect. However, we can exclude the involvement of MT1-MMP in this process because only a moderate increase in its expression was observed on the endothelial cell surface of HUVECs-CD IgA which did not promote the proteolytic degradation of TG2. Interestingly, the secreted TG2 was proved to possess transamidase activity. In this regard, a recent work has suggested that the release of TG2 could possibly lead to increased enzymatic activity as the enzyme is brought into solution [31], which would explain our previously reported findings of increased TG2 activity in the presence of celiac IgA. Nevertheless, the increased TG2 amino incorporation activity observed in the presence of celiac patient autoantibodies was measured in an endothelial culture system after supernatants were removed, this pointing towards another mechanism. In addition, externalized TG2 is rapidly incorporated into the ECM [27], as could have occurred in our experimental settings, this explaining the increased TG2 transamidase activity so far observed in the presence of celiac autoantibodies. However, our view is that this might not be the case, since TG2 secretion induced from endothelial cells by CD IgA was not accompanied by a higher expression of extracellular TG2.

Cell-secreted and surface TG2 have different but complementary roles in promoting cell adhesion. At the cell surface, in a non-enzymatic fashion, TG2-FN-mediated integrin clustering potentiates outside-in signaling, while TG2-FN-mediated syndecan-4 binding leads to inside-out integrin signaling to promote cell adhesion and spreading. Functional collaboration between integrin, syndecans,

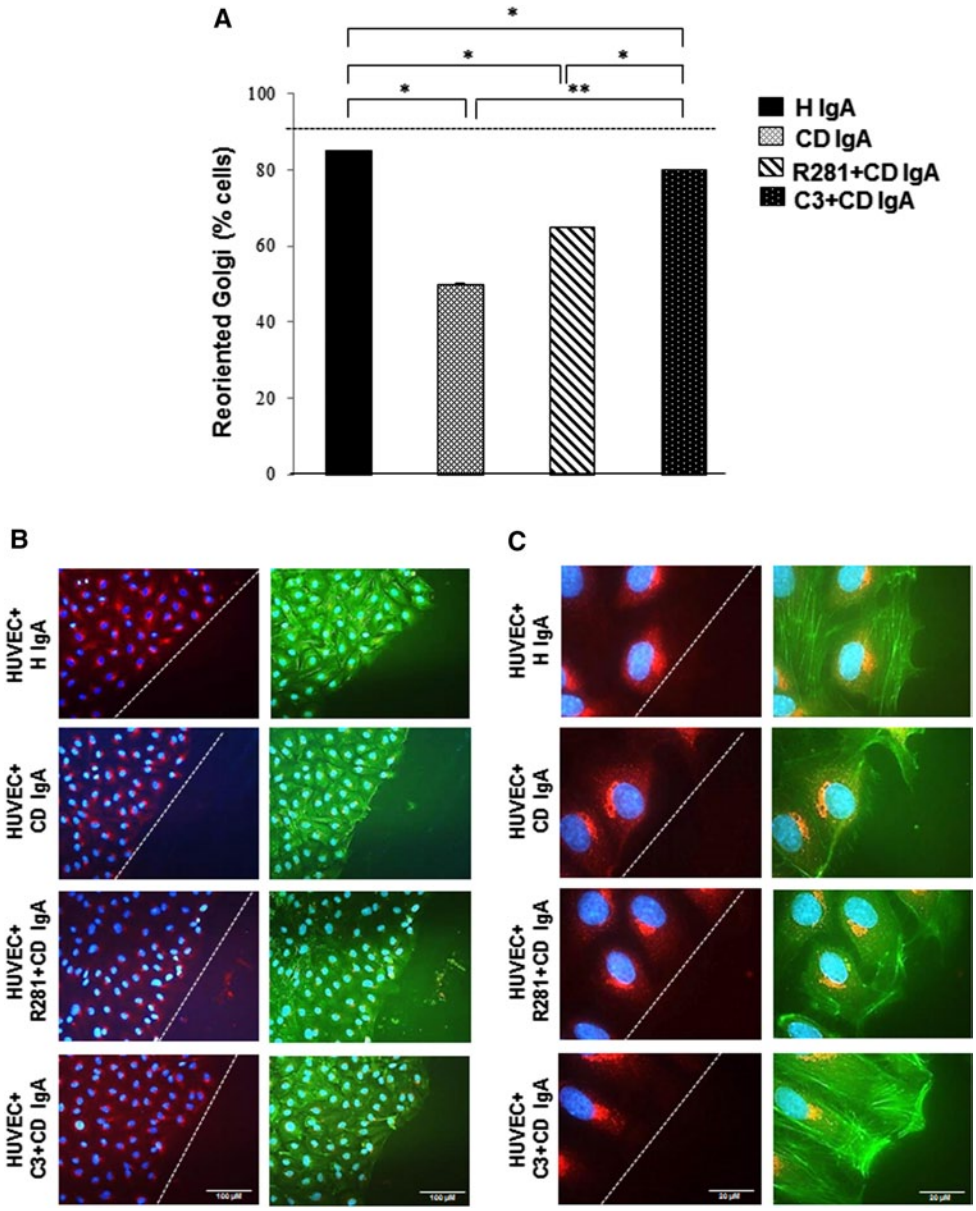


Fig. 7 Celiac patient immunoglobulins A (CD IgA) inhibit endothelial cell polarization and migration. **a** Quantification of cell polarization in HUVECs on fibronectin incubated with CD IgA and healthy (H IgA) subject's immunoglobulins after 24-h treatment by Golgi-reorientation assay. *Bars* signify the percentage of HUVECs which had reoriented Golgi towards the wound edge and *error bars* indicate standard error of the mean. A p value <0.05 was considered significant ($*p < 0.01$, $**p < 0.001$) and only statistically significant results are reported. More than 500 cells were selected over three independent experiments. **b**, **c** Representative immunofluorescence stainings of Golgi by B-COP antibody (*red*) in HUVECs under different experimental conditions. Actin cytoskeleton was visualized by phalloidin (*green*) and DAPI (*blue*) was used as nuclear counterstaining. Images were selected over three independent experiments. The *white dashed lines* represent the edge of the wound. **b** *Scale bar* 100 μM and **c** *scale bar* 20 μM. The extracellular TG2 activity was inhibited by administering a site-directed non-permeable inhibitor, R281. Similarly, C3 transferase was administered to inhibit small Rho GTPases in the phenotype

and TG2 is reflected by the alteration of integrin clustering and focal adhesion assembly [34, 35]. We found that in endothelial cells the presence of CD IgA induced the formation of punctate β1-integrin structures after 4 h of adhesion mostly localized around the perinuclear area. After 24 h, a more pronounced alteration in integrin distribution was observed. In fact, cells incubated with CD IgA displayed distinct filaments between the cells coupled to marked β1-integrin immunostaining at the periphery of the cells, in contrast to controls, where more homogeneous punctate arrangements were observed, this probably corresponding to focal adhesion sites. This would suggest that CD IgA induces constitutive clustering and activation of integrins different from the transient integrin clustering required for cell adhesion [34]. This activation was related to increased expression of β1-integrin in the presence of CD IgA.

It has been shown that integrin clustering promotes RhoA activation [34], and this activation is involved in increased vascular permeability in the presence of CD IgA, at least in vitro [2]. Since RhoA affects polarity in migrating cells [23], we next studied directional cell motility in HUVECs under different experimental conditions. We found that endothelial cells when treated with CD IgA lose polarization when compared to control groups. It is noteworthy that the effects exerted by CD IgA were reversed with prior incubation of the cells with C3 transferase inhibitor but not completely by R281, suggesting an important involvement of the RhoA signaling pathway in the polarization defects described above. We however cannot entirely

exclude the collaboration of other members of the Ras family, since C3 transferase inhibits the activity of all small GTPases. Moreover, we observed that endothelial cells treated with CD IgA had rounded and less organized focal adhesion sites when compared to controls (unpublished data) together with decreased expression of the late adhesion molecule vinculin. It is of note that R281 was able to ameliorate the expression level of vinculin, suggesting a role for extracellular TG2 enzymatic activity on vinculin expression.

Although a limitation of our study was that we used untreated celiac disease patient's total serum IgA that likely includes different pools of antibodies in addition to anti-TG2, such as anti-actin, anti-desmin, anti-reticulin autoantibodies [36] as well as anti-deamidated gliadin peptide antibodies, our net results would imply that CD IgA antibodies disturb the extracellular protein cross-linking function of TG2, thus altering endothelial cell–ECM interactions and thereby affecting endothelial cell adhesion, polarization, and motility. These alterations are possibly attributable to altered focal adhesion sites triggered by abnormal ECM organization, the cells seeking to overcome the defect by modulating the expression of β1-integrin, and increased RhoA activity.

Finally, we were intrigued to find that CD IgA promoted the secretion of TG2 into the culture medium from endothelial cells and this secreted TG2 was enzymatically active. Even though the role of TG2 in the extracellular compartment implies its involvement mainly in the stabilization of the ECM and in cell adhesion, we cannot exclude the existence of other undefined functions. In this regard, we might hypothesize that in pathological conditions such as celiac disease, the secretion of TG2 might be related to immune-modulatory functions linked to its active state.

If CD IgA antibodies act on vascular function in vivo similarly to what we have described in the present study in vitro, we could explain the altered small bowel mucosal microvasculature in untreated celiac disease patients [37]. Further studies are thus clearly called for in order to better clarify the mechanism herein proposed, this possibly helping to elucidate the anti-angiogenic effects exerted by CD IgA in the pathogenesis of the disease.

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Thioredoxin Is Involved in Endothelial Cell Extracellular Transglutaminase 2 Activation Mediated by Celiac Disease Patient IgA

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Abstract

Purpose: To investigate the role of thioredoxin (TRX), a novel regulator of extracellular transglutaminase 2 (TG2), in celiac patients IgA (CD IgA) mediated TG2 enzymatic activation.

Methods: TG2 enzymatic activity was evaluated in endothelial cells (HUVECs) under different experimental conditions by ELISA and Western blotting. Extracellular TG2 expression was studied by ELISA and immunofluorescence. TRX was analysed by Western blotting and ELISA. Serum immunoglobulins class A from healthy subjects (H IgA) were used as controls. Extracellular TG2 enzymatic activity was inhibited by R281. PX12, a TRX inhibitor, was also employed in the present study.

Results: We have found that in HUVECs CD IgA is able to induce the activation of extracellular TG2 in a dose-dependent manner. Particularly, we noted that the extracellular modulation of TG2 activity mediated by CD IgA occurred only under reducing conditions, also needed to maintain antibody binding. Furthermore, CD IgA-treated HUVECs were characterized by a slightly augmented TG2 surface expression which was independent from extracellular TG2 activation. We also observed that HUVECs cultured in the presence of CD IgA evinced decreased TRX surface expression, coupled with increased secretion of the protein into the culture medium. Intriguingly, inhibition of TRX after CD IgA treatment was able to overcome most of the CD IgA-mediated effects including the TG2 extracellular transamidase activity.

Conclusions: Altogether our findings suggest that in endothelial cells CD IgA mediate the constitutive activation of extracellular TG2 by a mechanism involving the redox sensor protein TRX.

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Introduction

Transglutaminase 2 (TG2) is a ubiquitously expressed highly complex multifunctional protein with enzymatic, cell adhesion, cell signaling and G-protein activities [1,2]. These functions of TG2 are regulated by other proteins and cofactors, including Ca^{2+} and GTP, and by the availability of these modulators in different tissue compartments [3,4]. In the extracellular environment, the abundance of Ca^{2+} and the low GTP/GDP nucleotide concentrations may allow TG2 to act as an enzyme modifying proteins at post-translational level by transamidation or deamidation [5] while this activity is probably repressed in the cytoplasm, where TG2 can act as a GTP-ase. Cell surface TG2 is implicated in cell adhesion by protein-protein interactions. As a transamidase, TG2 catalyzes the formation of isopeptide bonds (ε-γ-glutamyl- lysine crosslink) between a

selected glutamine residue on one substrate and a lysine residue on another. In the absence of adequate concentrations of any suitable amine substrate, TG2 deamidates the targeted glutamine side chain, resulting in its conversion to glutamate [6].

The enzymatic activity of TG2 is of research interest in that it is implicated in the pathogenesis of a number of human conditions, including celiac disease, a dietary gluten-induced autoimmune-mediated enteropathy affecting HLA-DQ2 or HLA-DQ8 subjects [7]. In celiac disease, TG2 enzymatic activity is known to lead to the deamidation of gluten peptides, which increases their affinity for HLA-DQ2 or HLA-DQ8 molecules [8]. This triggers an inflammatory T cell response ultimately resulting in the destruction of the small-bowel mucosal architecture [9]. Furthermore, during the disease pathogenesis the patients mount an anti-TG2 autoantibody response [10]. These autoantibodies are present in the serum and in various tissues in the patients, particularly in the small-intestinal mucosa as deposits on the subepithelial basement membrane and around capillary walls [11,12]. Intriguingly, the small-bowel mucosal TG2 located at the sites where the antibodies bind has been suggested to be catalytically active in untreated celiac patients [13]. It has thus been proposed that celiac disease-specific autoantibodies might account for the enzymatic activation of small-bowel mucosal TG2, although contrasting results are also reported [14].

Our previous studies have suggested that celiac IgA increases TG2 enzymatic activity on endothelial cells, but the mechanism involved has not yet been characterized [15,16]. Interestingly, it has recently been shown that thioredoxin (TRX), a redox sensor protein in cells, is a novel regulator of extracellular TG2 activity [17]. Therefore, the present aim was thus to deepen the understanding of CD IgA-mediated TG2 enzymatic activation and to establish whether TRX might be a key molecule in this process.

Materials and Methods

Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Cambrex Bio Science, Walkersville, MD) and maintained in EGM-2 medium (Clonetics®, San Diego, CA) with 2% fetal bovine serum (FBS) (Gibco Invitrogen, Paisley, Scotland, UK) and 25 µg/mL endothelial cell growth supplements (Clonetics). In all the experiments performed, the cells were used between passages 2 and 6. A non-reversible and cell-impermeable inhibitor for extracellular TG2 activity, R281 [18], was used throughout at a final concentration of 200 µM. In our experimental settings we employed also the TRX inhibitor PX-12 (1-methylpropyl 2-imidazolyl disulfide) at a non-toxic concentration of 1 µM (TOCRIS, Bioscience, Ellisville, MI). All the chemical compounds were administered 1h before addition of serum IgA.

Purification of serum IgA class antibodies

Serum samples from nine biopsy-proven celiac disease patients on a gluten-containing diet and five non-celiac healthy controls were included in the present study. All celiac sera were positive for anti-TG2 antibodies (titer ≥100 U/mL) (Celikey®; Pharmacia Diagnostic GmbH, Freiburg, Germany) and endomysial antibodies (titer 1≥1000), while all control sera were negative. Total serum IgA from healthy donors (H IgA) and celiac patient sera (CD IgA) were purified as previously described [11] and diluted in PBS to a final concentration of 100 µg/mL. Purified IgA fractions, if not otherwise stated, were used in all experiments at a concentration of 1 µg/ml.

Ethics Statement

The study protocol was approved by the Ethics Committee of Tampere University Hospital, Tampere, Finland, and all patients gave written informed consent.

TG2 activity assays

In order to determine the extracellular transamidating TG2 activity HUVECs were plated onto commercial fibronectin (FN)-pre-coated 96-well plates (BD Biosciences Discovery Labware, Bedford, MA) at a density of $2x10^5$ cells per well and grown to confluence. Thereafter, the cells were starved overnight (O/N) in EGM-2 containing 1% of FBS followed by incubation in the presence of different study compounds. At indicated time-points the cells were washed with Hanks Balanced Salt Solution (HBSS; Sigma Aldrich, St. Louis, MI), followed by incubation with the TG2 substrate monodansylcadaverine (MDC) (0.2 mM; Sigma Aldrich) for 2h at 37°C. Subsequently, the wells were extensively washed and the layers fixed with 4% paraformaldehyde (PFA) (Sigma Aldrich). TG2-mediated MDC incorporation was detected using rabbit anti-dansyl antibody for 30 min at 37°C (1:1000; Invitrogen, Carlsbad, CA). A secondary antibody, horseradish peroxidase-conjugated polyclonal swine anti-rabbit immunoglobulin (1:2000; Dako, Copenhagen, Denmark), was then added to the plates and incubated for 30 min at 37°C. Finally, the peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Slow Kinetic Form for ELISA; Sigma Aldrich) was used and the enzymatic reaction stopped with 2.5 M H_2SO_4. Absorbance was measured at 450 nm by spectrophotometer (Multiskan Ascent; Thermo Labsystems, Waltham, MA). In order to demonstrate TG2-specific incorporation of MDC, R281, was used.

Due to the fact that TG2 is ubiquitously expressed we further investigated where the enzyme was activated, focusing on the extracellular matrix environment. To this end, HUVECs grown to confluence as described above, were dislodged from their substratum by cell dissociation buffer (PBS, pH 7.4, with 2 mm EDTA), leaving the cell-assembled matrix (ECM). Thereafter, the plates were examined as described.

For Western blotting analyses of extracellular TG2 activity, the ECM fractions, obtained upon EDTA treatment, were directly solubilized by the addition of Laemmli gel loading buffer. The samples were loaded and run on 12% SDS-PAGE gels using the Bio-Rad Mini-Protein Tetra Cell system (Bio-Rad Laboratories, Espoo, Finland). Proteins were transferred to nitrocellulose filters (Hybond C-extra; Amersham Biosciences, Little Chalfont, UK) and non-specific binding was blocked with 5% non-fat powdered milk for 1h at room temperature (RT). The membranes were then incubated overnight (O/N) at +4°C

with rabbit anti-dansyl antibody (1:10000; Invitrogen). Thereafter, immunoreactivity was detected by consecutive incubation of the membranes with polyclonal swine anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, Glostrup, Denmark) for 1h at RT. The bands were visualized using the chemiluminescence detection system (Amersham, ECL Plus Western Blotting Detection System, Buckinghamshire, UK) and quantified by the Kodak 1D Image analyses software (Kodak, New Haven, CT). As loading control, γ-tubulin (GTU-88, Sigma Aldrich) was used. As above, R281 was used to demonstrate TG2 specificity of MDC incorporation.

Additionally, to investigate the relationship between the transamidating extracellular TG2 activity and the amount of CD IgA, we have designed a dose response test based on the microtiter plate assay using live cells. Briefly, HUVECs were plated on FN pre-coated 96-well plates (BD Biosciences Discovery Labware) and cultured to confluence in EGM-2 medium. The day prior to the experiment, the cells were starved O/N in EGM-2 medium (Clonetics) containing 1% FBS (Gibco Invitrogen) and then treated with different CD IgA concentrations (0.5 µg/mL; 1 µg/mL; 5 µg/mL; 25 µg/mL) for 3 h at 37°C, 5% CO_2. Thereafter, in situ-TG2 activity was investigated as described above except that the cells were not removed from the plates using a modification of the in situ assay by Verderio [19].

Quantification of extracellular TG2 expression on endothelial cells

In order to investigate the extracellular surface expression of TG2 by ELISA, HUVECs were grown to confluence on commercial FN-pre-coated 96-well plates (BD Biosciences Discovery Labware). After O/N starvation in EGM-2 with 1% of FBS, the HUVECs were incubated for 24h in the presence of different study compounds. The cells were then washed with HBSS (Sigma Aldrich), fixed with 4% PFA (Sigma Aldrich) and blocked with 5% BSA (Sigma Aldrich). The plates were incubated either with mouse monoclonal antibody CUB7402 TG2 (NeoMarkers, Fremont, CA) or with an anti-TG2 antibody recognizing the FN-binding domain, (clone 4G3, Millipore, MA) for 1h at 37°C. Subsequently, the wells were extensively washed and a specific secondary HRP-conjugated anti-mouse immunoglobulins (1:2000; Dako) was added for 30 minutes at 37°C. Finally, the peroxidase substrate TMB (Sigma Aldrich) was used and the enzymatic reaction was stopped with 2.5 M H_2SO_4. The absorbance was measured at 450 nm by spectrophotometer (Multiskan Ascent; Thermo Labsystems).

In vitro redox modulation of TG2 bound FN

In order to establish whether CD IgA is able to activate oxidized inactive TG2 we used a modified protocol by Stamnaes and coworkers [17]. The assay is based on the knowledge that TG2 is sensitive to oxidative environments and gradually loses activity upon handling and storage due to oxidation [20]. In this system, Human FN 96-well MultiwellTM (BD Bioscences, Bedford, Massachussets, USA) were coated with 5µg/mL human recombinant TG2 (His-rhTG2) (Zedira GmbH, Darmstadt, Germany) in Tris-buffered saline for 1h at 37°C. Enzymatic activity was measured by addition of 40 µM 5-biotinamidopentylamine (5-BP) (Life Science Research Pierce Biotechnology, Rockford, IL) under different sample conditions. In the experiments Ca^{2+} was used at the concentration of 10 mM and dithiotreitol (DTT) at 1 mM. After extensive washings, incorporated 5-BP was detected with streptavidin HRP-conjugated secondary antibody (Life Science Research Pierce Biotechnology). Lastly, the peroxidase substrate TMB (Sigma Aldrich) was used and the enzymatic reaction stopped with 2.5 M H_2SO_4. Absorbance was measured at 450 nm by spectrophotometer (Multiskan Ascent; Thermo Labsystems). In order to verify that TG2 remained immobilized during the procedure, some wells were incubated with CUB7402 (1:200) (NeoMarkers, Fremont, CA) instead of 5-BP.

Binding of celiac autoantibodies to TG2 after redox modulation

TG2 purified from guinea pig liver (5 µg/mL) (Sigma Aldrich) was coated on microtiter plates (Maxisorp, Nunc A/S, Roskilde, Denmark) for 1 hour at RT in Tris-buffered saline with 5 mM $CaCl_2$. After extensive washings with Tris-buffered saline with 10 mM EDTA and 0.1% Tween 20, the plates were treated with 100 µM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) for 20 minutes, followed by incubation with 50 mM DTT for another 20 minutes and further washings. Serum samples from six celiac patients diluted 1:200 or CUB7402 (1:16000) (NeoMarkers) were added in triplicate for 30 minutes. Bound antibodies were measured according to Sulkanen and coworkers [21].

Thioredoxin assay

The expression and release of thioredoxin was evaluated in endothelial cells by ELISA and Western blotting. For ELISA, HUVECs were plated onto commercial FN pre-coated 96-well plates (BD Biosciences Discovery Labware) at a density of $1x10^4$ cells per well. The cells were grown in complete culture media until confluence and starved O/N before the assay. Thereafter, the HUVECs were incubated with purified IgA class antibodies from healthy donors and celiac patients for 24h at 37°C, with or without pre-incubation with different inhibitors. The cells were then washed, fixed with 4% PFA (Sigma Aldrich) and blocked in 2% BSA buffer. Surface expression of thioredoxin on endothelial cells was detected by incubation of the plates using mouse anti-thioredoxin antibody (1:200) (sc-166393, Santa Cruz Biotechnology, Scotts Valley, CA) for 1h at 37°C, followed by incubation with the specific HRP-conjugated secondary antibody (1:2000; Dako) Finally, the peroxidase substrate TMB (Sigma Aldrich) was used and the enzymatic reaction stopped with 2.5 M H_2SO_4. Absorbance was measured at 450 nm by spectrophotometer (Multiskan Ascent; Thermo Labsystems).

For Western blotting analyses, HUVECs were cultured in complete endothelial growth medium in 24-well plate format and starved in EGM-2 medium (Clonetics) with 1% FBS (Gibco Invitrogen) O/N before the assay. The cells were then treated for 24h with H IgA or with CD IgA, preceded by pre-incubation with specific inhibitors, as described above. Thereafter, the culture medium was collected, briefly centrifuged and solubilized by addition of Laemmli buffer. Whole cell lysates

were obtained by direct solubilization and extraction of proteins from cell monolayers in Laemmli buffer. Lastly, cell culture supernatants and whole cell lysates were subjected to Western blotting, using as primary antibodies mouse anti-TRX antibody (1:2000) (Santa Cruz Biotechnology) and γ-tubulin (Sigma Aldrich) as loading control. Immunoreactivity was detected as above by sequential incubation of the membranes with appropriate HRP-conjugated secondary antibody (Dako) for 1h at RT. Visualization was by the chemiluminescence detection system (Amersham), ECL Plus Western Blotting Detection System) and quantification by the Kodak 1D Image analyses software (Kodak).

Human INF-γ assay

Quantitative measurement of human INF-γ in culture supernatants of endothelial cells under different experimental conditions was implemented by an *in vitro* enzyme-linked immunosorbent assay according to the manufacturer's instructions (Fisher Scientific, Waltham, MA).

Immunofluorescent stainings

HUVEC cells were cultured on FN-precoated chamber slides (BD-Falcon™, Bedford, MA) under different experimental conditions. The cells were then fixed in 4% PFA, washed with PBS1x and blocked with 1% BSA. Immunolabelings were carried out using as primary antibody anti-TG2 (diluted 1:50) (clone 4G3, Millipore) detected by Alexa Fluor® 488-conjugated secondary anti-mouse antibody (1:3000) (Invitrogen, Molecular Probes™, Leiden, the Netherlands). Stainings were visualized by fluorescent microscopy analysis (Olympus Optical CO.Ltd BX60F5, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using the non-parametric Mann–Whitney U-Test and the data were presented as mean values. A p-value <0.05 was considered statistically significant.

Results

Effects of celiac IgA on endothelial cell extracellular transglutaminase 2 transamidating activity and amount

In order to establish whether CD IgA increases the endothelial cell extracellular transamidating activity of TG2 in a time- and/or dose-dependent manner we performed an *in situ* TG2 activity assay with confluent HUVECs monolayers starved O/N. We observed that extracellular TG2 activity in HUVECs was only present on addition of CD IgA but not when H IgA was used (Figure 1A). With CD IgA extracellular TG2 enzymatic activity was already detectable after 3h of treatment. Although at this time-point maximal activation of the enzyme was observed, the induction was detectable at all-time points considered. Pretreatment of the non-permeable, site-directed TG2 inhibitor R281 abolished the increased extracellular TG2 transamidating activity induced by CD IgA throughout the experiment. To study the dose-dependent relationship between CD IgA-mediated extracellular TG2 activation, we added different amounts of IgA, ranging from 0.5μg/mL to 25μg/mL, to cultured endothelial cells. Interestingly, there was a dose-dependent rise in extracellular TG2 enzymatic activity in HUVECs incubated with increasing CD IgA concentrations (Figure 1B). The maximal activity of 1.7-fold was reached with 5 μg/mL, after which no further increase was achieved. Inhibitor R281 had no effect on the enzymatic activity when administered (data not shown).

Since endothelial cells express high levels of TG2 on the cell surface but also secrete it into their ECM, we sought further to investigate the precise localization of the TG2 activity stimulated by CD IgA. To this end, we studied TG2-mediated incorporation of MDC into the ECM fractions laid down by HUVECs, under different experimental conditions. We identified a statistically significant increase in relative ECM-TG2 activity when CD IgA was administered to cultured endothelial cells (Figure 2A). The observed effect was comparable to that detected on cell monolayers over time. Further, we collected deoxycolate-insoluble matrix protein under different experimental conditions and subjected them to Western blotting analyses in order to visualize MDC incorporation into ECM (Figure 2B). We detected several bands spread over a wide molecular weight range in all treatment groups. However, in the ECM derived from endothelial cells incubated with CD IgA there was an increase in the signal of low molecular mass proteins when compared to control groups, as indicated by the black circle in Figure 2B. In samples derived from cultures with R281 and CD IgA the intensity of the 45 kDa band was clearly reduced and similar to the controls.

As HUVECs in the presence of CD IgA are characterized by increased extracellular TG2 transamidating activity, we sought to ascertain whether this was associated with augmented extracellular TG2 expression. The relative quantification of cell surface-associated TG2 in non-permeabilized HUVECs with 4G3 demonstrated that in the presence of CD IgA there was a 1.1-fold increase in extracellular TG2 (Figure 3A and 3B). Comparable results were achieved with CUB7402 which recognizes a linear TG2 epitope (data not shown). Co-administration of the non-reversible and cell impermeable inhibitor for extracellular TG2 activity R281 was not able to prevent this effect (Figure 3A and 3B).

Redox regulation of TG2 is important for the enzymatic activation by CD IgA

As previous studies have shown that extracellular TG2 activity is sensitive to redox changes, we next investigated whether the redox regulation of TG2 might also have an effect on the ability of CD IgA to activate the enzyme. We observed that in the absence of a reducing agent DTT and exogenous Ca^{2+}, TG2 was enzymatically inactive and both agents were required for maximal activity (Figure 4A). The addition of CD IgA or H IgA did not affect the transamidating TG2 activity either in the presence or in the absence of both Ca^{2+} and DTT. Interestingly, in the presence of DTT but without exogenous Ca^{2+}, CD IgA autoantibodies were able to induce enzymatic activity of TG2 in contrast to H IgA. We also observed that under oxidizing conditions induced by DTNB, reduced amounts of CD IgA bind to the enzyme and that the process can be reversed by treating the enzyme with DTT after DNTB and

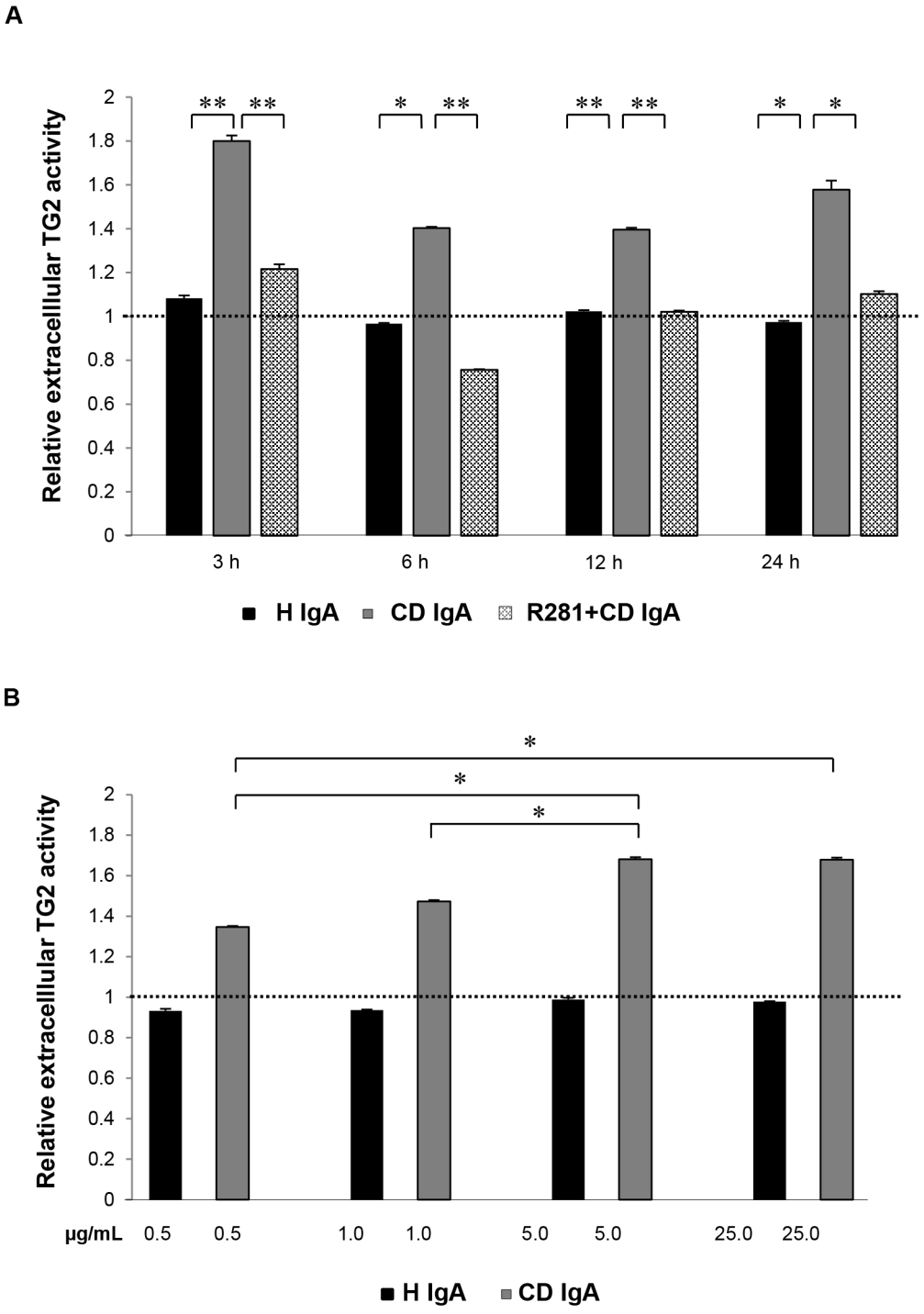

Figure 1. Endothelial extracellular transglutaminase 2 (TG2) activity analyses by monodansylcadaverine (MDC) incorporation. The relative extracellular TG2 activity (**A**) at different time points and with (**B**) increasing concentrations of class A immunoglobulins are shown. The dashed line indicates TG2 activity in untreated endothelial cells (HUVECs). Bars represent mean MDC incorporation as fold of control and error bars indicate standard error of the mean. P-value < 0.05 was considered significant ($^{**}p<0.001$ and $^{*}p<0.01$). Data derived from three independent experiments, repeated in quadruplicate. HUVECs were treated with celiac disease patient (CD IgA) and non-celiac subject's immunoglobulin-A (H IgA). Extracellular TG2 activity was inhibited by administering a non-permeable, site directed TG2 inhibitor, R281.

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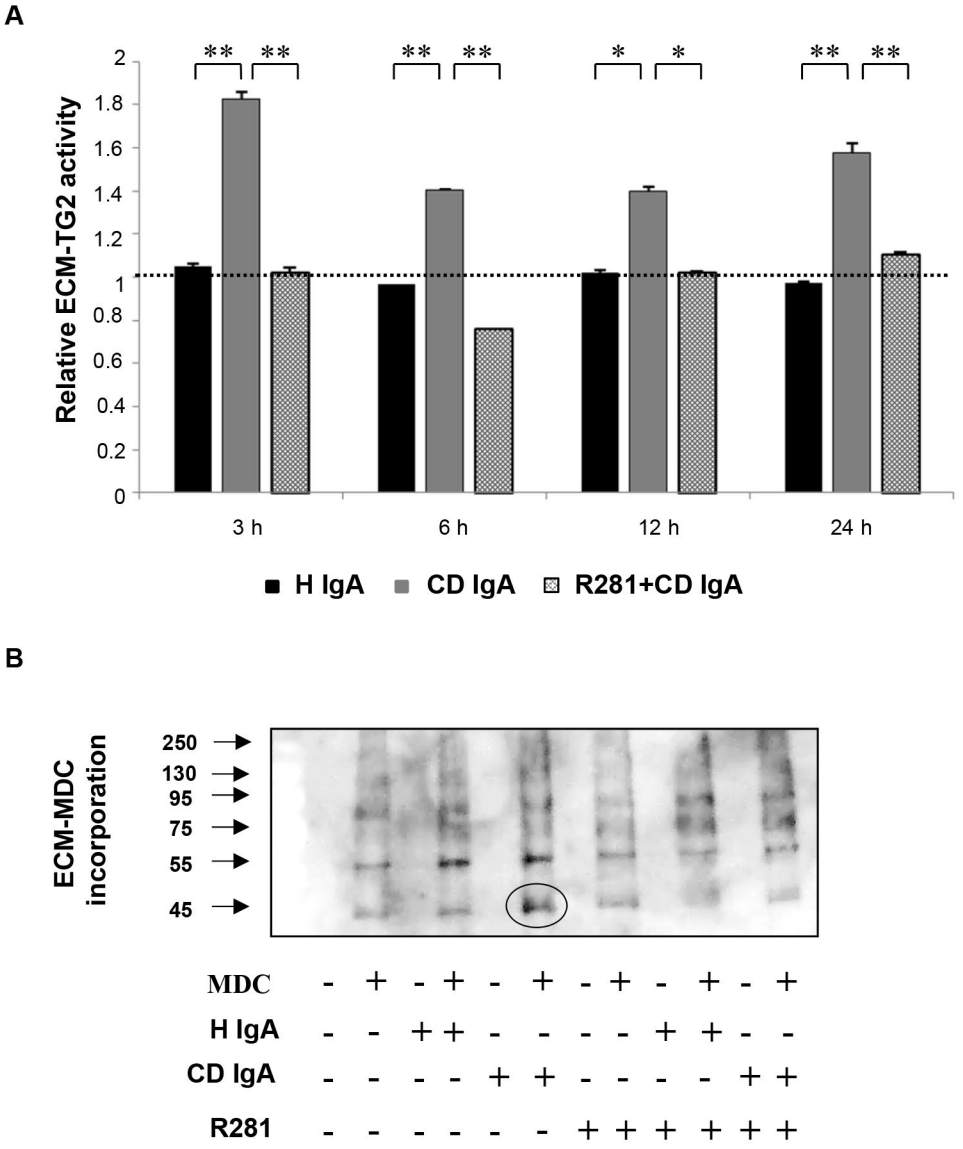

Figure 2. Endothelial extracellular matrix (ECM) transglutaminase 2 (TG2) activity analyses by monodansylcadaverine (MDC) incorporation. (**A**) The relative ECM-TG2 activity at different time points by ELISA. The dashed line indicates TG2 activity in untreated endothelial cells (HUVEC). Bars represent mean MDC incorporation as fold of control and error bars indicate standard error of the mean. P-value < 0.05 was considered significant ($^{**}p<0.001$ and $^{*}p<0.01$). Data derived from three independent experiments, repeated in quadruplicate. (**B**) Western blotting analyses of endothelial ECM-TG2 activity measured by the incorporation of MDC. Endothelial cells were treated with celiac disease patient (CD IgA) and non-celiac subject's immunoglobulin-A (H IgA). Extracellular TG2 activity was inhibited by administering a non-permeable, site directed TG2 inhibitor, R281.

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before adding the antibodies. Neither DTNB nor DTT had any effect on the binding of anti-TG2 monoclonal antibody CUB7402, which recognizes a linear epitope (Figure 4B).

Celiac IgA-mediated activation of extracellular transglutaminase 2 requires thioredoxin

Since we found that CD IgA mediated TG2 activation occurs only under reducing conditions we sought to establish whether the novel redox TG2 regulator TRX was involved in the process as a physiological reducing agent. We found that the overall TRX protein expression level to be comparable in all treatment

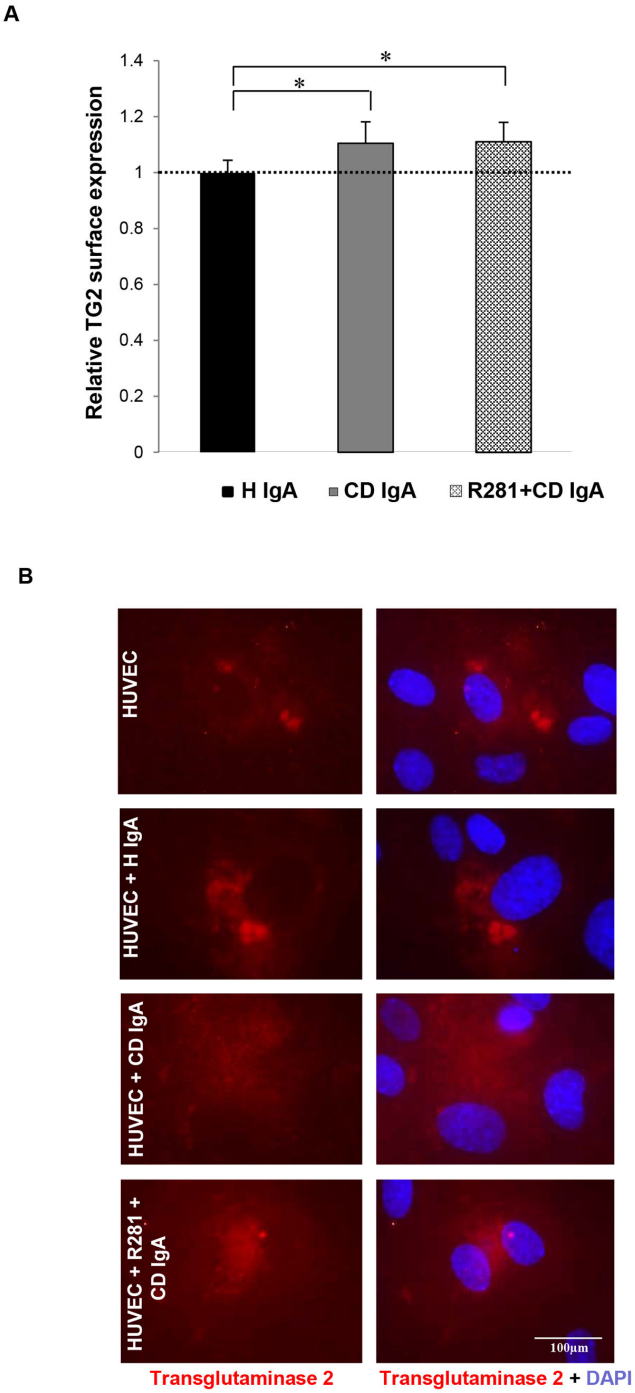

Figure 3. Celiac patient immunoglobulins A (CD IgA) increase transglutaminase 2 (TG2) surface deposition on endothelial cells. (**A**) The relative surface expression of TG2 on non-permeabilized endothelial cells (HUVECs) was studied by ELISA. The dashed line indicates TG2 surface expression in untreated HUVECs. Bars represent mean TG2 expression as fold of control and error bars indicate standard error of the mean. P-value <0.05 was considered significant (*p<0.001). Data derived from three independent experiments, repeated in quadruplicate are shown. (**B**) Surface expression of TG2 on non-permeabilized HUVECs was also investigated by immunofluorescence (IF) with the monoclonal TG2 antibody, 4G3. Representative IF stainings are shown. Control non-celiac subject's immunoglobulin-A (H IgA). Extracellular TG2 activity was inhibited by administering a site directed, non-permeable inhibitor, R281.

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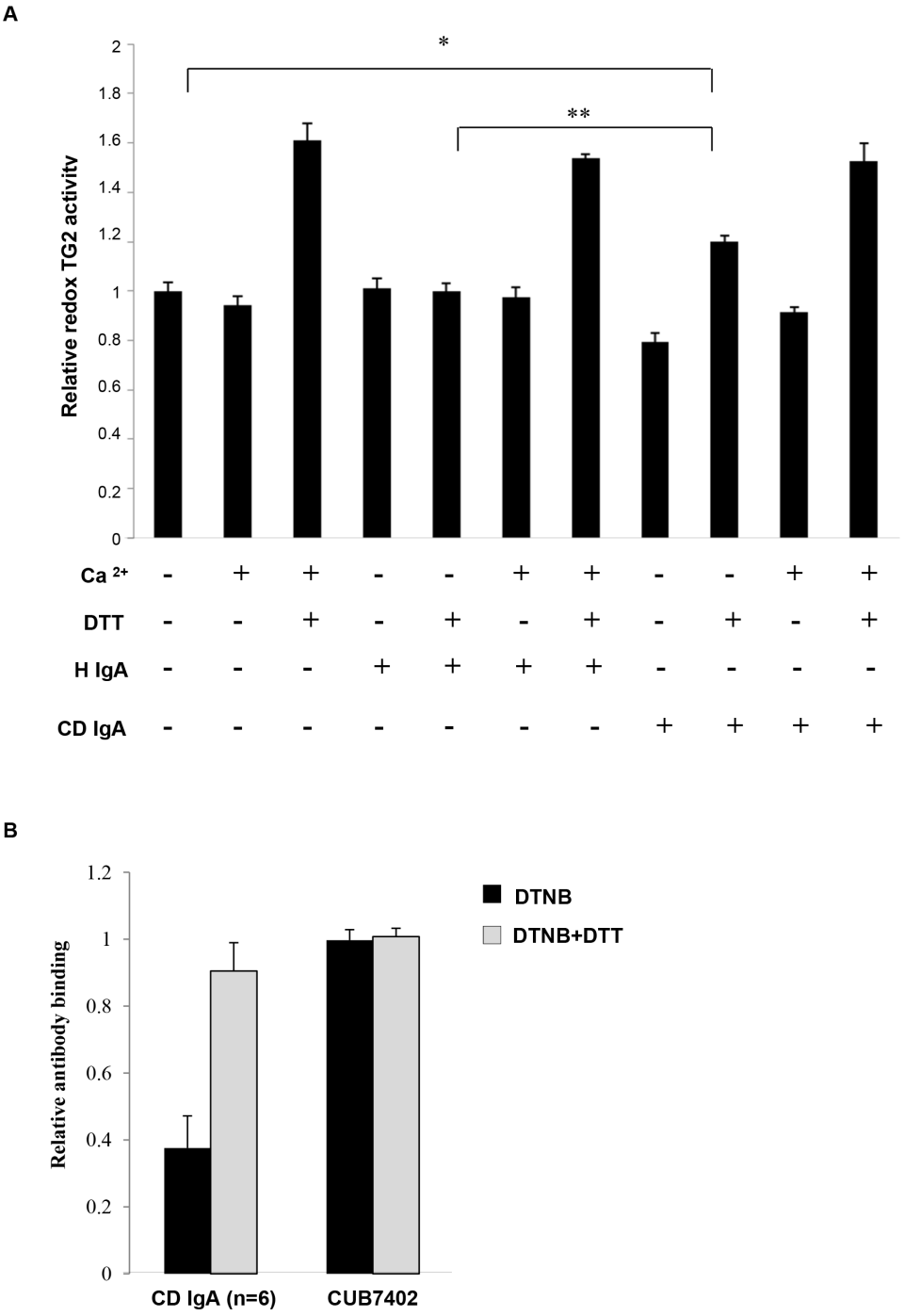

Figure 4. Celiac disease IgA (CD IgA) can activate fibronectin-bound transglutaminase 2 (FN-TG2) under reducing conditions. The effect of CD IgA on TG2 enzymatic activity was assessed by EZ-Link Pentylamine-Biotin (5-BP) incorporation on FN. In the absence of both the reducing agent, dithiothreitol (DTT) and Ca^{2+}, TG2, alone or with any IgA, was enzymatically inactive. Maximal TG2 activity was obtained in the presence of DTT and Ca^{2+} even in the presence of class A immunoglobulins. On the contrary, in the presence of a reducing agent, DTT, but without exogenous Ca^{2+}, CD IgA was able to induce the activation of TG2 in contrast to non-celiac IgA (H IgA). Bars represent mean 5-BP incorporation on FN as fold of TG2 in the absence of DTT and Ca^{2+}. Error bars indicate standard error of the mean; P-value < 0.05 was considered significant (***p<0.001, **p<0.01 and *p<0.05). Data derived from three independent experiments, repeated in quadruplicate are shown.

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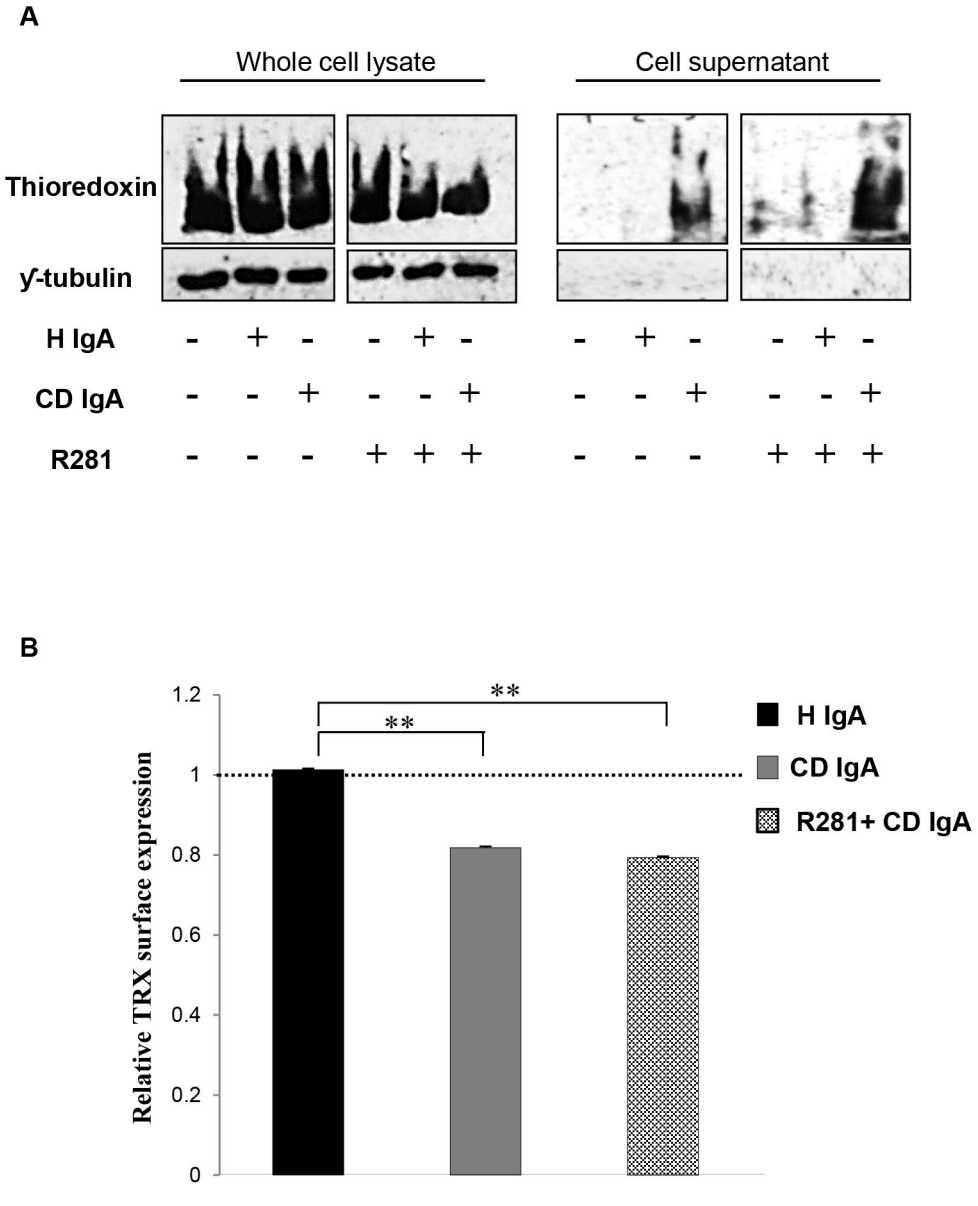

Figure 5. Celiac IgA (CD IgA) promotes secretion of thioredoxin. (TRX) and decreases its endothelial surface expression. (**A**) Representative Western blots from total cell lysates and supernatants show amounts of TRX and γ-tubulin used as loading control for protein extracts. (**B**) Relative surface expression of TRX was studied by ELISA. The dashed line indicates TRX surface expression in untreated HUVECs. Bars represent mean TRX expression as fold of control and error bars indicate standard error of the mean. P-value <0.05 was considered significant (*p<0.05 and **p<0.001). Data derived from three independent experiments, repeated in quadruplicate are shown. Endothelial cells were treated with CD IgA and non-celiac subject's immunoglobulin-A (H IgA). Extracellular TG2 activity was inhibited by administering a site directed, non-permeable inhibitor, R281.

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groups (Figure 5A). On the other hand, endothelial cells incubated with CD IgA secreted TRX into the cell cultured media, whereas control cells did not. TRX secretion was coupled to its decreased surface expression in CD IgA-treated HUVECs (Figure 5B). It was of note that, co-administration of the inhibitor for extracellular TG2 activity, R281, was not able to prevent these effects exerted by CD IgA (Figure 5A and 5B).

In view of these observations, we next sought to establish whether inhibition of extracellular TRX by PX12 might counteract the effects of CD IgA autoantibodies observed on

HUVECs. Such seemed indeed to be the case, as pretreatment of endothelial cultures with PX12 prevented the extracellular TG2 activation mediated by CD IgA (Figure 6A). In addition to this, inhibition of extracellular TRX with PX12 was able to overcome the effects mediated by CD IgA on TG2 surface expression and secretion of TRX into the culture supernatants (Figure 6B and 6C).

Discussion

Our previous early studies indicated that CD IgA disturbs angiogenesis, at least *in vitro*, presumably by increasing extracellular TG2 transamidase activity, but the underlying mechanism here is poorly understood [15,16]. Here we show that the administration of CD IgA to confluent HUVEC monolayers starved O/N, leads to an early and constitutive activation of extracellular TG2 (Figures 1A and 2A). Interestingly, we observed no time dependent correlation with the transamidating activation of TG2 by CD IgA, as the induction was detectable at all-time points examined (Figures 1A and 2A). We did, however, note a marked CD IgA dose-dependent activation of extracellular TG2 (Figure 1B), in line with previous work reporting dose-dependent enhancement of TG2 transamidase activity by celiac autoantibodies [22]. The correlation between the amount of disease-specific autoantibodies and TG2 enzymatic activity has also been documented in other previous articles [23,24], but with contrasting outcomes as regards the activation of the protein. In fact, both groups show that disease specific autoantibodies had an inhibitory effect on TG2 enzymatic activity, this possibly due to different experimental conditions [23,24].

In view of evidence which correlates increased extracellular TG2 enzymatic activity with high TG2 expression levels [19,25-27] we sought to investigate whether CD IgA-increased extracellular TG2 transamidating activity was associated with augmented cell surface TG2 presence. We have previously shown that celiac disease autoantibodies do not up-regulate the total TG2 protein expression level in endothelial cell lysates [15]. In contrast, in endothelial cells treated with CD IgA autoantibodies there was a small but statistically significant increase in the relative TG2 presence on the cell surface (Figure 3A and 3B) which nonetheless did not account for the increased extracellular TG2 activity. This supports the hypothesis that extracellular TG2 activation by CD IgA in endothelial cell is a more complex and elaborate process involving other important biological players. If the increased extracellular TG2 expression in the presence of CD IgA has a physiological significance or not, needs to be further investigated.

Bearing in mind that TG2 is subject to oxidation and as a result gradually loses activity upon handling and storage [20], we examined how the reducing or oxidizing environmental conditions might affect CD IgA-mediated modulation of TG2. Interestingly, in an *in vitro* system, CD IgA was able of inducing TG2 activation only in the presence of a reducing agent without the addition of exogenous Ca^{2+} (Figure 4A), supporting the conception that most probably their biological effects in endothelial cells are exerted under a reducing extracellular environment. Moreover, here we have shown that oxidation alters the protein's conformation in such ways that the antigenic epitopes are less accessible and thus the reducing environment helps maintain longer and more effective binding of the antibodies to the TG2 antigen (Figure 4B). Recently, Jin and co-workers [14] elegantly demonstrated in cell culture that extracellular TG2 can be activated by the cellular secreted redox protein TRX, which helps to keep the protein in an open and active conformation. Interestingly, we found here that CD IgA-treated HUVECs secreted TRX into the extracellular space and that this was coupled with reduced surface expression of the protein (Figure 5A and 5B), this suggesting that TRX might have a role in CD IgA-mediated TG2 activation. Such assumption was supported by our results in that pretreatment of endothelial cultures with PX12 prevented the extracellular TG2 activation mediated by CD IgA (Figure 6A). It is of note that, PX 12, similarly to other alkylating agents [28], was able to prevent TG2 enzymatic activity in the presence of Ca^{2+} and DTT. Further to the above, we observed that the inhibition of extracellular TRX by PX12 was able to prevent secretion of the protein and to normalize TG2 surface expression in endothelial cells treated with CD IgA but did not prevent its decrease from the surface (data not shown). Previous works have shown that the pro-inflammatory cytokine INF-γ can induce secretion of TRX at levels capable of activating TG2 [29]. In contrast, our findings exclude a role for INF-γ in the constitutive activation of extracellular TG2 mediated by CD IgA, since this was not detectable in our experimental settings (data not shown). In summary, our data suggest that CD IgA mediates TG2 activation in the extracellular environment where the protein is mainly inactive under physiological conditions [30], by a mechanism involving the redox sensor protein TRX. In our system, disease-specific autoantibody-induced INF-γ-independent secretion of TRX probably helps to keep TG2 in a conformation suitable for the constitutive antibody binding and activation described in the present work. Intriguingly, the decreased surface expression of TRX and the augmented TG2 expression on HUVECs may lead us to speculate that at the cell surface TG2 might be present in a conformation which is not suitable for the enzymatic activation mediated by CD IgA.

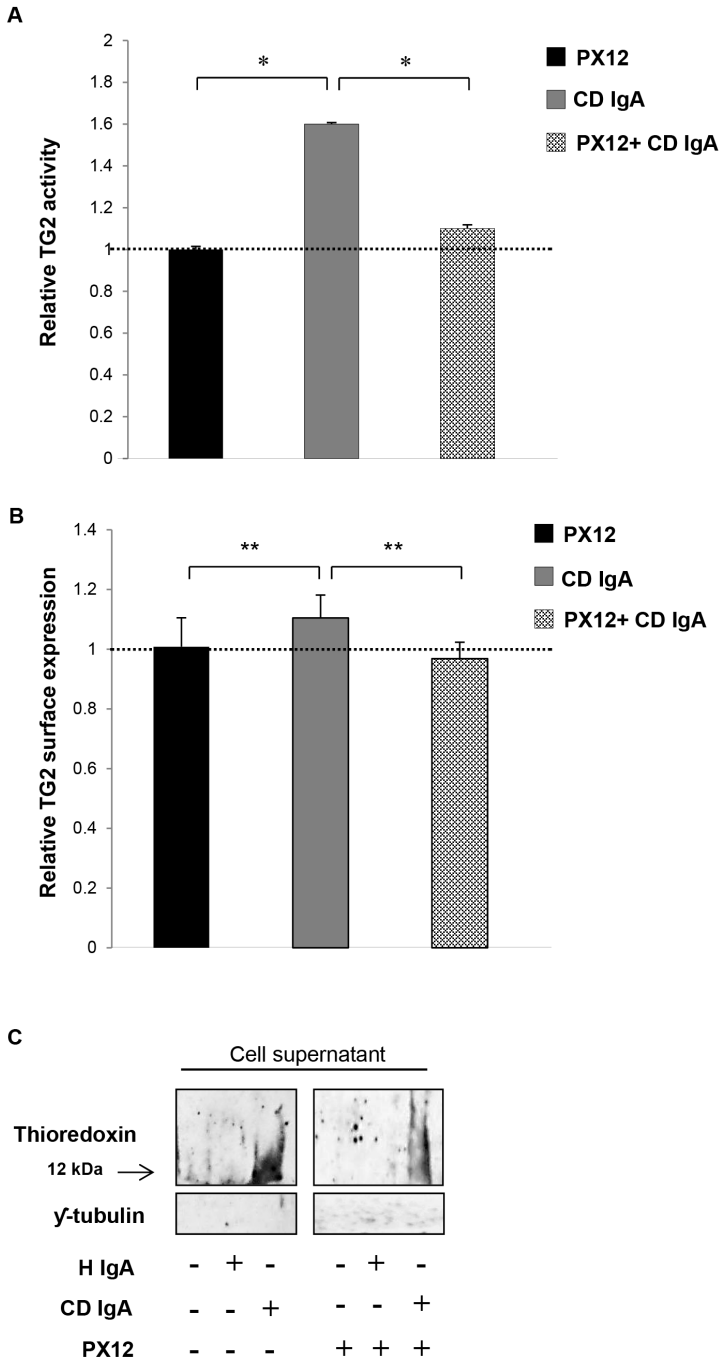

Figure 6. Celiac IgA CD IgA) mediated activation of extracellular transglutaminase 2 (TG2) by thioredoxin (TRX). (**A**) The relative surface expression of TG2 as well as (**B**) the relative TG2 activity on HUVECs treated with CD IgA autoantibodies was investigated by ELISA prior preincubation of endothelial cells with TRX inhibitor, PX12. The dashed line indicates TG2 expression and activity in untreated HUVECs. Bars represent mean values as fold of control and error bars indicate standard error of the mean. P-value < 0.05 was considered significant (*p<0.01 and **p<0.001). (**C**) The secretion of TRX in endothelial cell (HUVECs) culture media was analysed by Western blotting in the presence of a specific TRX inhibitor, PX12 (1-methylpropyl 2-imidazolyl disulfide). Representative Western blots of HUVECs culture supernatants show amounts of TRX and γ-tubulin used as loading control for quality sample. Data derived from at least three independent experiments, repeated in quadruplicate are shown. Endothelial cells were treated with CD IgA and non-celiac subject's immunoglobulin-A (H IgA).

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Author Contributions

Conceived and designed the experiments: CAN KL MM. Performed the experiments: CAN IRKS. Analyzed the data: CAN KL IRKS. Contributed reagents/materials/analysis tools: CAN MM KL KK IRKS MG ZW. Wrote the manuscript: CAN KL IRKS.

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