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Role of tissue transglutaminase in phagocytosis of apoptotic cells by macrophages

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

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# TABLE OF CONTENT

<table>
<thead>
<tr>
<th>LIST OF ABBREVIATIONS</th>
<th>.................................................................</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>.................................................................................................................................</td>
<td>6</td>
</tr>
<tr>
<td>PHAGOCYTOSIS</td>
<td>.................................................................................................................................</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Recognition signals ...............................................................................................</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Phagocytic receptors ............................................................................................</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Engulfment pathways ...............................................................................................</td>
<td>11</td>
</tr>
<tr>
<td>TISSUE TRANSGLUTAMINASE (TG2)</td>
<td>.........................................................................................................................</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Transglutaminases .................................................................................................</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>TG2 is a multifunctional protein .........................................................................</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Structure of TG2 .................................................................................................</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Localisation ..........................................................................................................</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Role of TG2 in apoptosis and phagocytosis ................................................................</td>
<td>25</td>
</tr>
<tr>
<td>AIMS</td>
<td>.................................................................................................................................</td>
<td>30</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>.................................................................................................................................</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Antibodies and reagents .......................................................................................</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Animals ..................................................................................................................</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Cells .......................................................................................................................</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Phagocytosis assay ...............................................................................................</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Site-directed mutagenesis ....................................................................................</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Sequencing .............................................................................................................</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Cloning of TG2 gene from pSP-TG2 into the CMV-pShuttle vector ..........................</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Adenoviral gene delivery system ..........................................................................</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Western blot analysis of α-actin and TG2 expression ........................................</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Biotinylation and isolation of cell surface proteins ...........................................</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Q-PCR analysis for detecting of the expression of various phagocytosis receptors</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Detection of active Rac1 and RhoG .....................................................................</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Immunofluorescence staining and confocal microscopy .........................................</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Binding of TG2 to lipids .......................................................................................</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Biacore assays .......................................................................................................</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Time- lapse video ..................................................................................................</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Statistical analysis ..............................................................................................</td>
<td>40</td>
</tr>
<tr>
<td>RESULTS</td>
<td>.................................................................................................................................</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>1. Role of TG2 in phagocytosis of apoptotic cells ..............................................</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>1.1. The efficiency of apoptotic cell and carboxylate-modified latex bead uptake by TG2-/- macrophages is lower compared to their wild type counterparts ......................</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>1.2. Crosslinking activity of TG2 is not required for the phagocytosis of apoptotic cells</td>
<td>44</td>
</tr>
</tbody>
</table>
1.3. Adenovirus gene delivery system carrying TG2 can be used to rescue the phagocytosis deficiency of TG2^-/- macrophages ......................................................................................... 45
1.4. Phagocytosis of apoptotic cells by macrophages infected with adenoviruses carrying various TG2 mutants .......................................................... 47
1.5. Cell surface TG2 is involved in engulfment of apoptotic cells ........................................ 48
1.6. In the absence of TG2 the mRNA expression of β3 integrin receptor is higher, while the expression of other phagocytosis receptors is only slightly altered ........................................... 50
1.7. TG2 is required for proper integrin β3 signaling .......................................................... 51
1.8. The uptake of apoptotic cells by wild-type macrophages is mediated by an efficient engulfing centre ..................................................................................................................... 53
1.9. In TG2^-/- macrophages Rac1 cannot be concentrated around the apoptotic cells and the efficient engulfing centre is not formed ......................................................... 56
1.10. In lack of TG2 the expression of the integrin β3 protein was elevated but the receptors were not concentrated at the sites of apoptotic cells ................................................................... 59
1.11. TG2 interacts with milk fat globulin-8 ........................................................................ 60

2. A subline of TG2^-/- mice compensate the loss of TG2 by a much higher expression of integrin β3 ............................................................................................................................... 63

2.1. Macrophages from a subline of TG2^-/- mice have very low phagocytic activity .......... 63
2.2. Macrophages from a subline of TG2^-/- mice have altered morphology ....................... 64
2.3. Altered macrophage morphology in the TG2^-/- subline is related to an enhanced integrin β3 signaling .......................................................................................................................... 65
2.4. Apoptotic cells can induce the activation of RhoG in macrophages from the TG2 subline .. 67
2.5. Over-expression of wild-type Rac1 can compensate the defect of phagocytosis in macrophages of the TG2^-/- subline ......................................................................................... 68
2.5. Apoptotic cell-induced formation of 3-phosphoinositides is impaired in TG2^-/- macrophages ............................................................................................................................. 70

LEGEND TO SUPPLEMENTARY VIDEO ........................................................................... 71
DISCUSSION .................................................................................................................... 74
SUMMARY ...................................................................................................................... 79
ACKNOWLEDGEMENTS .............................................................................................. 81
REFERENCE .................................................................................................................. 82
PUBLICATION .............................................................................................................. 104
LIST OF ABBREVIATIONS

ACAMP                    apoptotic cell-associated molecular patterns
ABC                         ATP-binding cassette transporter
BAI1                        brain-specific angiogenesis inhibitor 1
CED                        cell corpse-engulfment defective
CEMMs                    cholesterol-enriched membrane microdomains
CRK II                     chicken tumor virus no. 10 (CT10) regulator kinase II
CRP                         C-reactive protein
CRT                        calreticulin
DOCK180               dedicator of cytokinesis (180 kDa)
ECM                        extracellular matrix
ELMO                     engulfment and migration protein
ERK                        extracellular signal–regulated kinase
FAK                        focal adhesion kinase
Fn                          fibronectin
Gas6                        growth arrest-specific 6
GFP                         green fluorescent protein
GULP                      engulfment adaptor protein
KO                           knock out
IL                           interleukin
Mer RTK                 c-mer proto-oncogene receptor tyrosine kinase
MFG-E8                   milk fat globule EGF-factor 8
LDL                        low-density lipoprotein
LRP, CD91              low-density lipoprotein receptor-related protein
MDC                       monodansylcadaverine
PAK                        p21-activated kinase
PRR                      pattern recognition receptor
PFU                        plaque forming units
PH                          pleckstrin homology domain
PI                               phosphoinositide
PI3K                     phosphatidylinositol-3-OH kinase
PLCδ                   phosphoinositide phospholipase C
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
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<td>PSR</td>
<td>phosphatidylserine receptor</td>
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<tr>
<td>PtdEtn</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>Rb</td>
<td>retinoblastoma</td>
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<tr>
<td>SIRPα</td>
<td>signal regulatory protein α</td>
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<td>SR</td>
<td>scavenger receptor</td>
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<tr>
<td>TG</td>
<td>transglutaminase</td>
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<td>TGF-β</td>
<td>transforming growth factor β</td>
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<td>TGF-R</td>
<td>TGF-β receptors</td>
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<td>Tim4</td>
<td>T-cell immunoglobulin- and mucin-domain-containing molecule</td>
</tr>
<tr>
<td>TNF-α</td>
<td>cytokines tumor necrosis factor α</td>
</tr>
<tr>
<td>TRIO</td>
<td>triple functional domain (PTPRF interacting)</td>
</tr>
<tr>
<td>TSP</td>
<td>trombospondin</td>
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<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
INTRODUCTION

PHAGOCYTOSIS

Programmed cell death generates a large number of apoptotic bodies that must be cleared to avoid secondary necrosis, tissue damage and inflammatory response. Apoptotic cells are usually cleared by phagocytosis in vivo. Phagocytosis, defined by Metchnikoff in 1883, means the uptake of large particles or cells by other cells. This rapid and efficient process removes damaged cellular components and inhibits inflammation. The engulfment of apoptotic cells is distinct from other types of phagocytosis in that the particle being taken up does not elicit an inflammatory response, which occurs in other types of phagocytosis, such as pathogen clearance (Ren & Savill 1998). The clearance of apoptotic cells is mediated by either professional phagocytes, called macrophages or by amateur ones, which are usually the neighbours of dying cells.

Recognition signals

Phagocytes can recognise either ‘eat me’ or ‘don’t eat me’ signals on the surrounding cells. Normal, healthy cells present signals that prevent binding to phagocytes or inhibit the engulfment activity. One of these ‘don’t eat me’ signals is CD47, which is known to be an integrin-associated protein. It is widely distributed on cells and can act as a ligand for the heavily glycosylated, signal regulatory protein α (SIRPα) on the phagocyte.

In the initial part of apoptosis dying cells stop presenting these signals and start to express surface markers, which are positive signals for their engulfment. Phagocytic receptors recognize these specific ‘eat-me’ signals on the apoptotic cell, through either direct apoptotic cell–phagocyte interactions or serum opsonizing proteins that bridge apoptotic ligands and the phagocyte receptors (Fadok et al. 2003; Henson et al. 2001). Some of these signals are shared with molecular patterns on the
surface of microbial pathogens and have been termed apoptotic cell-associated molecular patterns (ACAMPs) (Savill et al. 2002). ACAMPs are composed of oxidized and covalently modified lipids and proteoglycans on the apoptotic cell that interact with pattern recognition receptors (PRRs) in a similar manner to the recognition of bacterial pathogens by the innate immune system (Stuart and Ezekowitz 2005). Since the uptake of apoptotic cells by phagocytes occurs in the absence of inflammatory responses, a distinction between apoptotic cells and microbial pathogens must be made. Phagocytic clearance of apoptotic cells is characterized by the active production of anti-inflammatory cytokines, such as transforming growth factor β (TGF-β) and interleukin 10 (IL-10), and the down modulation of the pro-inflammatory cytokines tumor necrosis factor α (TNF-α) and IL-12 that promote an immunosuppressive environment in tissues (Fadok et al. 2001).

The redistribution of phosphatidylserine (PS) on the surface of the apoptotic cell is the best characterized signal that distinguishes cellular life from death. Cellular plasma membranes maintain an asymmetric distribution of lipid molecules in the bilayer: almost all of the phospholipids that contain terminal primary amino groups, phosphatidylethanolamine (PtdEtn) and PS are localized on the cytoplasmic surface. This intracellular localization of PS is maintained by an ATP-dependent aminophospholipid translocase that shuttles PS from the outer to the inner leaflet in living cells. During apoptosis and cellular events that are associated with cell death (i.e. activation of caspases, oxidative stress and alterations in cytoplasmic calcium concentrations), cellular mechanisms that maintain PS asymmetry become inactivated and PS concentrates in patches on the outer leaflet (Henson et al. 2001). Fattyacid oxidation of PS is required for PS externalization and recognition as an apoptotic signal. Milk fat globule EGF-factor 8 (MFG-E8), for example, has more preference for oxidized PS than non-oxidized PS (Borisenko et al. 2004), and C-reactive protein (CRP) binds to oxidized PS and can promote the clearance of CRP-opsonized apoptotic cells (Mevorach 2000).

Other molecules in addition to PS that are normally confined to the cytosolic side of the cell membrane appear on the cell surface. These include annexin I that colocalises with PS (Arur et al. 2003), the endoplasmic reticulum protein calreticulin (CRT) whose levels increase on the cell surface during programmed cell death by unknown mechanisms.
**Phagocytic receptors**

Several phagocytic receptors are attracted to PS through indirect interactions mediated by bridging molecules, such as MFG-E8, Gas6 and protein S. MFG-E8 is a glycoprotein, which is secreted by subset activated macrophages. Phagocytic receptors, $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins bind an RGD motif in the EGF-like domain of the soluble molecule MFG-E8. Gas6 (Chen et al. 1997) and protein S (Hall et al. 2005) are the ligands for Mer. Gas6 and protein S are secreted proteins characterized by post-translational $\gamma$-carboxylation modification of glutamic acid residues that bind PS and activate Mer family tyrosine kinases by inducing receptor dimerization and trans-phosphorylation (Ling et al. 1996).

Characteristically, deficiencies in PS opsonins or their receptors in knockout models leads to a failure to maintain self-tolerance and mice often show a prototypic systemic autoimmune disease. Targeted disruption of the MFG-E8–$\alpha_v\beta_5$ (or $\alpha_v\beta_3$) integrin or the growth arrest specific gene 6 (Gas6)–Mer RTK pathways lead to defects in physiological clearance of apoptotic cells and produce auto-antibodies to self-antigens, resulting in splenomegaly and glomerulonephritis (Cohen et al. 2002).

Simultaneous efforts by three separate laboratories, using very different strategies have recently identified three directly binding PS receptors (PSR) suggesting that PS-binding bridge molecules are not the only way of PS recognition. T-cell immunoglobulin- and mucin-domain-containing molecule (Tim4) was identified in a screen for engulfment-blocking monoclonal antibodies raised against naive peritoneal macrophages (Miyanishi et al. 2007). Meanwhile, brain-specific angiogenesis inhibitor 1 (BAI1) was identified as an interacting partner of engulfment-promoting protein ELMO, which associates and acts with Dock180 as a bipartite guanine nucleotide exchange factor for the small GTPase Rac (Park et al. 2007). The third PSR is stabilin-2, a multifunctional receptor binding a large array of ligands, and perhaps best known for its scavenger receptor and endocytic functions (Park et al. 2007). Park hypothesized that stabilin-2 might participate in the removal of aged, PS-exposing red blood cells (RBCs) and apoptotic cells. Engagement of stabilin-2-expressing cells with either aged RBCs or the monoclonal antibody resulted in production of TGF$\beta$, a hallmark of the anti-inflammatory program associated with
recognition of apoptotic cells. Intriguingly, each of these receptors seems to use different sequence structures to recognize PS and these may be different again from such recognition domains in the PS-binding bridge molecules, or the scavenger receptors.

Since scavenger receptors (SRs) can recognize diverse classes of molecules, including lipids and modified proteins, they also can detect apoptosis-related changes on cells. CD36, one of the first macrophage receptors to be involved in recognition of apoptotic cells (Savill et al. 1992; Ren et al. 1995), is a class-B scavenger receptor that also has an important role in endocytic clearance of oxidized low-density lipoprotein (oxLDL). There is also evidence that oxidation of the apoptotic-cell surface is an important component of recognition by phagocytes (Sambrano and Steinberg 1995; Chang et al. 1999; Kagan et al. 2002). CD36 has two transmembrane domains, and they are concentrated in a specific plasma membrane microdomain, in the caveolae. Trombospondin (TSP) is a multifunctional, 450 kDa adhesive glycoprotein, which facilitates the adhesion with interacting fibronectin or fibrinogen, and during phagocytosis it acts as a „molecular bridge” between the apoptotic cells and macrophages. A model was proposed in which the alpha\(_{v}\)beta\(_3\) integrin and CD36 on phagocytes cooperate to form a high affinity binding site for TSP, which binds to the apoptotic cells (Savill et al. 1992).

The low-density lipoprotein receptor–related protein 1 (LRP1) or CD91 is a multifunctional scavenger and signaling receptor (Herz and Strickland 2001). LRP1 can interact via a phosphotyrosine binding motif (NPXY motif) within their cytoplasmic tails with the adaptor protein, GULP (engulfment adaptor protein) (Su et al. 2002). CD91 interacts with calreticulin (CRT) which is highly expressed on the surface of apoptotic cells.

ATP-binding cassette (ABC) transporter (homologue of Caenorhabditis elegans CED7), is expressed in both dying and engulfing cells. ABC transporter is necessary for the efficient exposure of phosphatidylserine on apoptotic somatic cells. ABCA1, the prototype of the A subclass of mammalian ATP-binding cassette (ABC) transporter 1, has been implicated in the translocation of PS from inner to the outer leaflet. ABCA1 also facilitates the engulfment of apoptotic cells (Hamon et al. 2000). The inactivation of aminophospholipid translocase and the activation of phospholipid scramblases or ABC transporters together might lead to the exposure of PS on the
surface of apoptotic cells (Venegas and Zhou 2007). Mammalian ABCA7 enhances phagocytosis of apoptotic cells by macrophages, and it was proposed to be involved in the translocation of phospholipids across the lipid bilayer (Jehle et al. 2006). In response to apoptotic cells, ABCA7 moves to the macrophage cell surface and colocalizes with the LRP1 in phagocytic cups. ABCA7 appears to facilitate the cell surface localization of LRP1 and associated signaling via extracellular signal–regulated kinase (ERK) (Jehle et al. 2006).

CD14 is found as a plasma-membrane-anchored molecule (mCD14) on the surface of monocytes, macrophages and granulocytes as well as in soluble form (sCD14) in plasma. This molecule previously was much better known as a receptor for bacterial lipopolysaccharide (LPS). Apoptotic cells are docking through CD14 receptor and the macrophage CD14 mediates clearance of apoptotic cells without indicating inflammation. The CD14 clearance pathway more probably can interact with common or multiple apoptosis-associated plasma-membrane structures, most probably ACAMPs. The apoptotic signals and their recognition receptors are summarized in Figure 1.
Figure 1. The phagocyte recognition pattern and “eat –me-signals” on apoptotic cells in the mammalian clearance of apoptotic cells (Savill et al. 2002).

Engulfment pathways

Independent of the ingested cell (apoptotic or necrotic) and the receptors involved a number of common principles have been established: following receptor-mediated particle recognition actin polymerisation occurs at the site of ingestion and the particle is internalized via an actin-dependent mechanism. After internalization, actin is shed from the phagosome and the phagosome matures by a series of fusion and fission events with components of the endocytic pathway culminating in the mature phagolysosome. The signal pathways involved in uptake of apoptotic cells by either macrophages or non-professional phagocytes appear to be unique and very highly conserved evolutionarily.

Several studies in the nematode Caenorhabditis elegans and mammalian cells revealed conservation of the engulfment machinery (Reddien and Horvitz 2000;
(Gumienny and Hengartner 2001) involving genes working within two parallel signalling pathways promoting cytoskeletal reorganization and influencing the balance of membrane Rho family GTPases activity (Gardai et al. 2006). In Caenorhabditis elegans (C. elegans), there are no professional phagocytic cells, cell corpses are swiftly engulfed and digested by their neighboring cells (Zhou et al. 2004). CED (cell corpse-engulfment defective) 1, -6, -7, and dyn-1 in one pathway, and CED-2, -5, -10, and -12 in the other pathway (Zhou et al. 2004; Mangahas and Zhou 2005; Yu et al. 2006). The first pathway includes the CED-7 ABC transporter, which likely presents a death ligand on the surface of the dying cell; the CED-1 transmembrane receptor, which recognizes this signal; dynamin and the CED-6 adaptor protein (Wu and Horvitz, 1998), which may transduce a signal from CED-1. The second C. elegans pathway acts in parallel and involves a novel Rac GTPase signaling pathway, with the components CED-2, CED-5, CED-12, and CED-10 (Liu and Hengartner 1998). These pathways are summarized in Figure 2.
Figure 2. Two parallel signaling pathways control the engulfment of apoptotic cells in C. elegans. (a) Seven proteins are essential for the efficient engulfment of apoptotic cells and they are separated in the two pathways. The mammalian homologs of the corresponding C. elegans proteins are indicated inside the parentheses. (b) Diagram of the molecular functions and interactions of these seven proteins with addition other newly identified proteins involved in engulfment. Double thin lines indicate plasma membrane. Question marks indicate missing components and/or unknown events. (Mangahas et al. 2005).

Assembly of a trimolecular complex of ELMO, CrkII and DOCK180 in mammals (known respectively as CED-12, CED-2 and CED-5 in the worm) (Henson et al. 2005.) activates the guanine nucleotide exchange activity of DOCK180 for Rac (CED-10), which in turn promotes cytoskeletal rearrangements crucial for the uptake of target particles (Krysko et al. 2006). CED-2 and CrkII each have one N-terminal SH2 (Src-homology 2) domain followed by two SH3 (Src-homology 3) domains (Reddien and Horvitz 2000). SH2 domains bind phosphotyrosines, and SH3 domains bind proline-rich sequences (PXXP; where X is any amino acid) (Koch et al. 1991).
DOCK180 was identified on the basis of its physical interaction with Crk (Hasegawa et al. 1996). DOCK180 can localize with CrkII to focal adhesions and affect cell spreading (Kiyokawa et al. 1998). CED-5 likely acts within engulfing cells (Wu and Horvitz 1998) and can bind CED-2 (Reddien and Horvitz 2000), suggesting Crk-like and DOCK180-like proteins interact in engulfing cells to mediate engulfment in vivo. Both CED-5 and DOCK180 contain an N-terminal SH3 domain, a large central region with a DOCKER domain (Brugnera et al. 2002) and a C-terminal proline-rich region that likely binds the first SH3 domain of CED-2 and CrkII, respectively. The two mammalian CED-12-like proteins having PH domain (Pleckstrin Homology) were named ELMO1 and ELMO2 (Gumienny et al. 2001). PH domains can target interacting proteins to cell membranes (Shaw 1996), and ELMO1 can indeed facilitate the recruitment of DOCK180 to cell membranes (Gumienny et al. 2001). CED-12 likely acts within engulfing cells and can physically interact with CED-5 (Gumienny et al. 2001; Wu et al. 2001; Zhou et al. 2001), possibly as part of a CED-2/CrkII-CED-5/DOCK180-CED-12/ELMO ternary complex (Gumienny et al. 2001, Wu et al. 2001). CED-12 has a proline-rich candidate SH3-binding domain that may mediate interaction with the N-terminal SH3 domain of CED-5/DOCK180. Both the proline-rich and PH domains are needed for CED-12 function in vivo (Zhou et al. 2001). Although neither DOCK180 nor ELMO1 has clear homology to established GEFs, a domain of DOCK180, termed DOCKER, can act as a novel type of Rac GEF in vitro (Brugnera et al. 2002), and DOCK180 and ELMO1 can synergize to promote GTP loading of Rac in mammalian cell culture (Gumienny et al. 2001; Brugnera et al. 2002). DOCK180 and ELMO proteins appear to act together as a newly identified type of GEF.

Rac GTPases are members of a Ras superfamily subgroup that includes Rho, Rac, and Cdc42 and that controls cell morphology via regulation of the cytoskeleton (Van Aelst and D'Souza-Schorey 1997). RhoA and Rac-1 regulate phagocytosis of apoptotic cells in opposite manner—RhoA inhibits this process while Rac-1 is obligatorily required for engulfment (Morimoto et al. 2006). GTPases act as molecular switches that regulate the activities of a variety of signal transduction pathways: When bound to GTP, the protein is active for signal transduction and when bound to GDP is typically inactive (Bourne et al. 1991).

The upstream components required for Rac activation are different. One scheme of the upstream regulation of the CrkII/DOCK180/ELMO/Rac signaling is the
following: engagement of a ligand with the receptor leads to the tyrosine phosphorylation of that receptor or of a receptor-associated protein that interacts with the SH2 domain of CrkII (Reddien and Horvitz 2000). An SH3 domain in CrkII binds the C-terminal proline-rich region of DOCK180 and recruits it to cell membranes (Reddien and Horvitz 2000). Rac is localized to cell membranes by geranylation modification (Reddien and Horvitz 2000). The proline-rich region of ELMO binds the N-terminal SH3 domain of DOCK180 (Gumienny et al. 2001; Wu et al. 2001; Zhou et al. 2001), which then promotes the GTP loading of Rac (Brugnera et al. 2002). GTP-bound Rac drives reorganization of the actin cytoskeleton and extension of lamellipodia around cell corpses via an undiscovered effector.

Integrin receptors are candidate regulators of this pathway (Albert et al. 2000). αvβ5 integrin-dependent phagocytosis activates a signal cascade involving CrkII/DOCK 180/ Rac1 to promote early phagosome formation (Akakura et al. 2004). It was also shown that during cell adhesion integrins can also control the translocation of the GTP-bound Rac at specific plasma membrane microdomains. These integrin-regulated Rac binding sites are within CEMMs (cholesterol-enriched membrane microdomains). Integrins control Rac signalling by preventing the internalization of its binding sites in CEMMs (Grande-García et al. 2005).

Another upstream pathway of Rac identified in both mammalian cells and worms involves RhoG (homologue with MIG-2 in C. elegans) and the guanine nucleotide exchange factor TRIO (UNC-73 is the nematode equivalent) (deBakker et al. 2004). αvβ3 integrin induced apoptotic cell engulfment, in the presence of MFG-E8, could be inhibited by dominant-negative forms of RhoG or Rac1, which suggested that RhoG and Rac1 are also involved in the integrin-mediated engulfment (Nakaya et al. 2006).

The recently identified phosphatidylserine receptor, BAI, can also form trimeric complex with ELMO and Dock180 and can activate Rac (Park et al. 2007)(Fig.3).
Figure 3. PS receptors and possible signaling pathways.

The three new PS receptors are represented in context with some of the other PS recognizing systems thought to be involved in apoptotic cell removal. The bridge molecules here are shown with their proper signaling receptors and may act in part through the ELMO, DOCK180 and CrkII signaling module that activates Rac. BAI1 was identified specifically by its binding to ELMO. Tim4 has a very short cytoplasmic domain and most likely needs a partner (at this point unidentified) to signal for uptake. Stabilin-2 acts like a scavenger receptor, several of them are already known to bind native and/or oxidized PS, though generally with additional recognition of other anionic phospholipids (Bratton et al. 2008).

PS dependent signals, via MFG-E8 and Gas6 and their receptors, converge on a common downstream pathway and ‘synapse’ to amplify internalization signals for the phagocytosis of apoptotic cells. Mer activation by Gas-6 also induced a post-receptor signaling cascade involving Src-mediated tyrosin phosphorylation of FAK on Tyr^{861}, the recruitment of FAK^{Tyr861} to the αvβ5 integrin, and increased formation of the p130^{CAS}/CrkII/DOCK180 complex to activate Rac1. Gas6 failed to stimulate phagocytosis in β5-deficient phagocytes which indicates that Mer is directly and
functionally linked to the integrin pathway (Wu et al. 2005). These pathways are summarized in Figure 4.

**Figure 4.** Schematic model for convergence of PS-dependent receptors αvβ5 integrin and Mer signaling.

The second pathway involves the cell surface receptor proteins ABC1/CED-7, stabilin-2 and LRP1/CED1 and the adaptor protein GULP/CED-6. ABC1/CED-7 downstream ligands have not been identified yet (Zhou et al. 2001). It was also shown that CED-1, CED-6 and CED-7 are required for actin reorganization around the apoptotic cell corpse, and that the CED-10/Rac GTPase acts genetically downstream of these proteins to mediate corpse removal, functionally linking the two engulfment pathways as upstream regulators of Rac activation (Kinchen et al. 2005).
TISSUE TRANSGLUTAMINASE (TG2)

Transglutaminases

Transglutaminases are a widely distributed group of enzymes that catalyse the post-translational modification of proteins by the formation of isopeptide bonds. They belong to the family of thiol- and Ca$^{2+}$-dependent acyl transferases that catalyze the formation of a covalent bond between gamma-carboxamide groups of peptide-bound glutamine residues and various primary amines, including the epsilon-amino group of lysine in certain proteins. The cross-linked protein products are highly resistant to mechanical force and proteolytic degradation, and their accumulation is found in a number of tissues, including skin, hair, blood clotting and wound healing (Lorand and Conrad 1984). Seven distinct transglutaminases have been described and their accumulation is found in a number of tissues and processes, including skin, hair, blood clotting and wound healing, stabilization of extracellular matrix (ECM) or apoptotic envelopes. Plasma factor XIIIA is involved in catalyzing formation of the fibrin clot at site of blood coagulation. (Tajahashi et al. 1986). Keratinocyte TGase (TG1) plays a major role in terminal differentiation of epithelia. Epidermal TGase (TG3) is involved in differentiating epidermal and hair follicule cells (Peterson et al. 1983). Prostate TGase (TG4) in rodents results in the formation of capulatory plug through cross-linking of proteins in the seminal vesicle secretions. (Ho et al. 1992). TGase X (TG5) is induced during the early stages of human keratinocyte differentiation and crosslinks loricin, involucrin, and small proline-rich proteins in vitro (Candi E et al. 2001). The function of TGase6 (TGaseY) and TGase7 (TGaseZ) have not yet been characterized.

TG2 is a multifunctional protein

Protein crosslinking by TG2, a ubiquitous enzyme, has profound effect on cells by regulating the biological activity of signalling molecules as TGF-β, interleukin-2 as well as by modulating cell-matrix interaction (Aeschlimann et al.
A large number of TG2 substrates are proteins involved in the organization of cytoskeleton. Upon activation by Ca\(^{2+}\), TG2 contributes to the organization of cytoskeleton by cross linking various cytoskeletal proteins, i.e. microtubule protein tau (Piredda et al. 1999; Tucholski et al. 1999), tubulin (Maccioni et al. 1986) actin (Nemes et al. 1997; Safer et al. 1997), myosin, spectrin (Orrú et al. 2003), troponin T (Bergamini et al. 1995; Gorza et al. 1996) and vimentin. Extensive polymerization, which occurs during the final steps of apoptosis, stabilizes the structure of the dying cells thereby preventing release of cell components that might give rise to inflammatory or autoimmune responses (Fésüs and Piacentini 2002). Also the retinoblastoma gene product is a TG2 substrate during apoptosis in vivo and its polymerization has been indicated as a key signal for the initiation of apoptosis (Oliverio et al. 1997). Moreover, nuclear proteins such as core histones are able to act as acyl-donor TG2 substrates during cell death. Besides its involvement in protein cross-linking, within the intracellular compartment, TG2 is more likely to catalyse the incorporation of polyamines into specific acyl-donor substrates especially when the concentration of polyamines in the cell/tissue is in the millimolar range. Numerous proteins are covalently modified by polyamination in intact cells, and polyamines can modulate the function and metabolism of the protein substrate. TG2 is also involved in the activation of members of the Rho-GTPase family (Schmidt et al. 1998; Singh et al. 2001; Umar et al. 1996; Hebert et al. 2000). In response to retinoic acid, TG2 causes transamidation of RhoA and formation of the RhoA-Rho-associated coiled-coil-containing protein kinase 2, a complex that promotes the formation of stress fibres and focal adhesion complexes. Thus TG2 is a signal transduction protein because it can alter the function of signalling growth/differentiation factors (Umar et al. 1996).

In signaling TG2 also can act as a G protein (Nakaoka et al. 1994). TG2 binds and hydrolyzes GTP with an affinity and catalytic rate similar to \(\alpha\) subunits of large heterotrimeric G proteins and small Ras-type G proteins. Gh protein is composed of two subunits: \(\alpha\) (74-85 kDa) and \(\beta\) (50 kDa). \(\text{Gh}\alpha\) subunit was initially identified as tissue transglutaminase 2 (Nakaoka et al. 1994), \(\text{Gh}\beta\) was identified as calreticulin, a \(\text{Ca}^{2+}\)-binding protein (Feng et al. 1999). Interaction of \(\text{Gh}\) with \(\alpha_1\)-adrenergic receptors (\(\alpha_1\)-AR), oxytocin receptors (Baek et al. 1996; Park et al., 1998) or thromboxane A2 receptors (Vezza et al. 1999) switches off transglutaminase activity.
and dissociates GTP bound Gα from Gβ. G protein function of TG2 has role in modulating dynamic adhesion formation in cell spreading and migration, taking part of intracellular signal transduction resulting activation of protein kinase Ca (Stephens et al. 2004).

The GDP/GTP-bound form cannot act as a transglutaminase. This inhibition is suspended by Ca$^{2+}$, which serves as a switch between the two distinct functions (Achyuthan and Greenberg 1987). In the presence of physiological intracellular concentrations of calcium and GTP, it has been suggested that TG2 exists largely as a latent transamidase (Bergamini 1988; Smethurst and Griffin 1996; Zhang et al. 1998) but as an active G-protein involved in receptor signaling. In pathological states, when cells become leaky to calcium and GTP generation is impaired, the TG activity of TG2 contributes to programmed cell death events, such as apoptotic body stabilization (Smethurst and Griffin 1996; Nanda et al. 2001).

TG2 can act as an adaptor protein that facilitates extracellular interaction between fibronectin and integrins (Akimov and Belkin 2001). Fibronectin binding is believed to play a role in localizing TG2 in regions of tissue damage (Upchurch 1987), where it acts as a matrix-associated membrane-bound exoenzyme (Barnes et al. 1985), and it able to polymerize fibrinogen and fibronectin on the cell surface (Martinez et al. 1994), and is cleavable by membrane-bound matrix metalloproteinases (Belkin et al. 2001). TG2 has role in the stabilization of the extracellular matrix, since the loss of TG2 by proteolytic cleavage would facilitate increased cell migration and invasion in the metastatic process (Zirvi et al. 1993; Knight et al. 1991).

Some other function of TG2 has been also revealed like protein disulfide isomerase function (Hasegawa et al. 2003), and it also can act as a protein kinase for insulin growth factor-binding protein-3 (Mishra and Murphy 2004), p53 (Mishra and Murphy 2006), and histones (Mishra et al. 2006).

**Structure of TG2**

TG2 has four distinct domains: an N-terminal β-sandwich (with fibronectin and integrin binding site), catalytic core (containing the catalytic triad for the acyl-
transfer reaction: Cys\textsuperscript{277}, His\textsuperscript{335}, and Asp\textsuperscript{358}) and transition state stabilizing residue (Trp\textsuperscript{241}) (Murthy et al. 2002) and two C-terminal β-barrel domains (Fig.5).

![Molecular model of TG2](image)

Figure 5. Molecular model of TG2. Different colors indicate the four different domains (N-terminal β-sandwich, catalytic core domain and 2 β-barrels. Yellow arrows show the specific binding sites for fibronectin, GTP/GDP binding pocket and active site.

Although TG2 is localized predominantly in the cytoplasm, substantial amounts of the protein is present also on the surface in various cell types and in the extracellular matrix (Upchurch et al. 1991), despite of the fact that TG2 has no leader sequence, hydrophobic domains or posttranslational modifications for targeting the endoplasmic reticulum or Golgi apparatus. It was shown that Tyr\textsuperscript{274} is important for the externalization mechanism of the enzyme. Mutation of Tyr\textsuperscript{274}, thought to provide cis rather than the preferred trans peptide bond conformation, leads to lack of enzyme secretion. Secretion of the enzyme may be connected to the cis-to-trans isomerization of the non-proline cis peptide bonds. It’s hypothesized that the active trans conformation may occur in the enzyme upon the binding of Ca\textsuperscript{2+} and substrate. This
process can occur once the enzyme reaches the cell surface, where both Ca\(^{2+}\) and substrates such as fibronectin are available to the enzyme (Balklava et al. 2002).

The GDP-binding site of TG2 is located in a hydrophobic pocket between the core and \(\beta\)-barrel 1, on the opposite face to the proposed glutamyl substrate-binding site (Liu et al. 2002). It has a unique architecture that, although conserved in TG3 (Candi et al. 2004), is entirely distinct from that of other GTP-binding proteins and involves mainly residues from \(\beta\)-barrel 1 (amino acids 476–482 and 580–583) and Phe174 from the core. Additionally impaired GDP/GTP binding and hydrolysis are observed by site-directed mutation of Lys\(^{173}\) (Iismaa et al. 2000). Phe\(^{174}\) in the core domain forms part of a hydrophobic pocket around the guanine base, where it is thought to stabilize one side of the guanine ring through an aromatic stacking interaction. In \(\beta\)-barrel 1, Arg\(^{580}\), one of four positively charged residues surrounding the phosphate groups of GDP in the crystal structure, forms two ion pairs with the \(\alpha\)-and \(\beta\)-phosphates of GDP, as well as interacting with the guanine base. This confirms the importance of the arginine residue for nucleotide binding. In contrast, substitution of Arg\(^{478}\), which interacts with the \(\beta\)-phosphate, reduced but did not abolish GTP binding. Similarly, alanine substitution of Arg\(^{476}\) or leucine substitution of Lys\(^{173}\) (Iismaa et al. 2000), residues postulated to stabilize the \(\gamma\)-phosphate of GTP during binding and hydrolysis (Liu et al. 2002), caused only a minor reduction in GTP photolabeling. These results indicate that residues interacting only with phosphate groups contribute less to guanine-nucleotide binding than residues interacting with the guanine base.

TG2 can interact with fibronectin with its N-terminal region (Gaudry et al. 1999). The \(\beta5/\beta6\) hairpin of the first domain represents the major recognition site on the TG2 molecule for the interaction with fibronectin. In this regard, it is worth mentioning that this hairpin forms a prominent “finger” which is extended well beyond the globular first domain with its tip located aside from any other parts of the TG2 molecule (Liu et al. 2002). Probably, this lack of structural constraints makes the \(\beta5/\beta6\) hairpin well positioned for the interaction with the extremely large fibronectin molecule. Importantly, single point mutations of either Asp\(^{94}\) or Asp\(^{97}\) decreased markedly the affinity for fibronectin. Moreover, a substitution of both Asp\(^{94}\) and Asp\(^{97}\) with Ala in the double mutant caused a further steep decrease in the affinity with the 42-kDa fibronectin fragment, demonstrating a synergistic effect of these mutations on fibronectin binding.
TG2 can also bind to integrin receptor on the cell surface, but the exact location of this binding site has not identified yet. Depending on the cell type, 10–20% of β1-integrins on the cell surface can exist as a complex with TG2 in a 1:1 ratio (Akimov et al. 2000; Akimov et al. 2001). Other members of β-integrins (β3-, β4- and β5-) also were identified that could form stable complex with TG2 in cancer cell membranes (Herman et al. 2006; Mangala et al. 2007). The association of TG2 with integrins is known to occur primarily at extracellular domains of integrins and promotes their interaction with the ECM ligands such as, fibronectin, collagen, and vitronectin (Akimov et al. 2001). Importantly, the interaction between TG2 and integrin is independent of the cross-linking activity of TG2.

The second β−barrel domain contains the phospholipase C binding sequence (Hwang et al. 1995). The analysis of the TG2 primary sequence showed the presence of an eight amino acid domain, sharing 70% identity with the Bcl-2 family BH3 domain. BH3 domain (amino acids 204-212) is located in the catalytic core of TG2 and structured in two inversely oriented α-helices that are exposed to the interaction with solvent (Rodolfo et al. 2004).

**Localisation**

TG2 is localised inside the cell in free cytosolic, mitochondrial, and nuclear forms, in the extracellular environment and in association with the cell surface (Griffin et al. 2002). The presence of TG2 within the different cellular compartments is thought to serve distinct physiological functions.

TG2 expression is constitutive in many different cell types, and depending on the cell type can be regulated by several transcriptional activators, such as cytokines, retinoids, vitamin D and steroid hormones.

In the cytosol TG2 participates in several signaling events. TG2 expression in cancer cells leads to constitutive activation of the focal adhesion kinase (FAK) and its downstream PI3K/Akt survival pathway (Verma et al. 2006). It was demonstrated that TG2 expression augmented the autophosphorylation of FAK (Y397), the first step in the FAK activation pathway. It is well established that the autophosphorylation of FAK is followed by the recruitment of Src kinase that phosphorylates other tyrosine

23
sites on FAK (Y407, Y576, Y577, Y861, and Y925) leading to the activation of several downstream signaling pathways such as, RAS/ERK, PI3K/Akt, and CrkII/Dock180/Rac (McLean et al. 2005.). Among various pathways regulated by FAK, TG2-induced activation of FAK leads to the activation of the PI3K/Akt pathway.

TG2 can be transported to the nucleus, probably through interaction with the nuclear transport protein importin-a3 (Peng et al. 1999). In the nuclear envelope, where it exhibits GTP-binding activity, TG2 forms a complex with the nuclear pore p62 protein (Singh et al. 1995). Moreover it can form a ternary complex with IkappaB/p65:p50 and results in constitutive activation of the nuclear transcription factor-kappaB (NF-kappaB) (Mann et al. 2006).

The enzyme is also localized in the mitochondria (Piacentini et al. 2002; Krasnikov et al. 2005), where it regulates the energy balance of the cell (Piacentini et al. 2002; Rodolfo et al. 2004). TG2 critically affects the balance between complex I and complex II of the mitochondrial respiratory chain and the enzyme is involved in the regulation of the respiratory chain both in physiology and pathiology. Ablation of TG2 in knockout mice causes a reduced activity of mitochondrial complex I associated with an increased activity of complex II. Proteins involved in the mitochondrial respiratory chain, such as prohibitin and the β-chain of ATP synthase, are substrates for TG2 (Piacentini et al. 2002; Rodolfo et al. 2004). In addition it was also shown that TG2 contains a BH3 domain, co-localizes with mitochondria during apoptosis, and might promote apoptosis by interacting with members of the Bcl-2 family (Rodolfo et al. 2004).

Despite the lack of a leader sequence, TG2 is externalized from cells into the extracellular space where it has been implicated in the stabilization of the extracellular matrix (ECM) and in cell–ECM interactions (Aeschlimann and Thomazy 2000). The majority of extracellular TG2 is inactive under normal physiological conditions in cell culture and in vivo. However, abundant TG2 activity was detected around the wound in a standard cultured fibroblast scratch assay. Under ‘normal conditions’ TG2 externalized from cells becomes tightly bound to fibronectin and forms ternary complexes with collagens that function as a cementing substance in the ECM. The effect of TG2 on cell adhesion is mediated by non-catalytic mechanism involving the formation of a trimeric complex through simultaneous interaction of enzyme with the
gelatin-binding, 42kDa fragment of fibronectin and $\beta_1$- and $\beta_3$-integrins (Akimov et al. 2000). Beside cell adhesion TG2 associated with $\beta$-integrins (Ekmekcioglu et al. 2006.) may have important implications in terms of deregulated proliferation, survival and migratory functions of cancer cells.

TG2 also contributes to the organization of the ECM by stabilizing the latent transforming growth factor binding protein. Latent transforming growth factor binding protein-1 is particularly interesting because only after its TG2-catalysed linkage to the matrix does it release the active TGF$\beta$. Consequently, TG2 is presumed to be involved in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis and osteoarthritis via regulation of the availability of this cytokine in the matrix (Nunes et al. 1997).

The downregulation of surface transglutaminase may occur through internalization and subsequent lysosomal degradation. Constitutive endocytosis of cell-surface transglutaminase depends on plasma membrane cholesterol and the activity of dynamin-2, and involves both clathrin-coated pits and lipid rafts or caveolae. Cell-surface TG2 interacts directly also with LRP and promotes the association of LRP with $\beta_1$ integrins and the ECM. For the efficient internalization of TG2 from the cell surface through a dynamin-dependent manner which involves clathrin- and caveolin-dependent endocytic pathways LRP1 is required. This process might be crucial for regulation of its adhesive and signaling functions on the cell surface and reveal a novel functional link between cell-matrix adhesion and endocytosis (Zemskov et al. 2007).

Role of TG2 in apoptosis and phagocytosis

TG2 has a Janus face activity in the aspect of apoptosis. TG2 has both pro-and antiapoptotic effects depending upon the type of cell, kind of death stimuli, the cellular localization of the enzyme and the type of activities switched on.

Previous work done in our department has described that TG2 is induced and activated in cells undergoing apoptosis in the liver and thymus forming highly cross-linked protein polymers and proteinaceous shells which were resistant to detergents (Fésüs et al. 1987; 1989). The induction of the enzyme in apoptotic cells may be
mediated through various nuclear receptors and response elements including retinoids and tumor growth factor β (TGFβ) (Fésüs et al. 1996; Szondy et al. 1997; Szegezdi et al. 2000). TG2 with its BH3 domain can contribute to the conformational change and translocation of Bax to the mitochondria, release of cytochrome c and death of neuroblastoma cells (Rodolfo et al. 2004). In addition, Bax acts as a substrate of TG2 at the mitochondrial level. TG2 can affect the activity of the cysteine proteinase calpain as well (Sarang et al. 2007), which plays a key role in the activation of the proteolysis during the caspase-independent death program of red blood cells (Bratosin et al. 2001). Following the initiation of apoptosis the enzyme also can transamidate the actin and retinoblastoma (Rb) proteins (Nemes et al. 1997; Oliverio et al. 1997) but in the other hand transamidation of Rb TG2 can protect Rb from caspase-induced degradation (Boehm et al. 2002).

Adhesion-dependent survival signaling of cells is mainly mediated by the adhesive glycoprotein fibronectin binding to cell-surface matrix receptors (primarily the α5β1 integrins) through their Arg-Gly-Asp (RGD) sites. Synthetic RGD peptides inhibit this binding leading to detachment induced apoptosis called anoikis. Griffin and his coworkers have described a novel RGD-independent cell adhesion mechanism of osteoblasts and fibroblasts (Verderio et al. 2003) that rescues cells from anoikis induced by blocking the RGD-dependent survival signaling. This newly proposed pathway is mediated by externalized TG2 bound to fibronectin and cell surface heparan sulfate chains, is integrin-independent, requires the function of protein kinase Cα and leads to activation of Rho and the focal adhesion kinase. Cell adhesion to TG2-fibronectin does not require the transamidating activity of the enzyme, though at the high calcium concentration of the extracellular space TG2 is very likely in the active conformation (Aeschlimann and Thomazy 2000)

According to Aeschlimann (Stephens et al. 2004) TG2 can also promote cell adhesion by regulating and being regulated by phospholipase C through binding to it with the non-transamidating GTP form of the enzyme (Baek et al. 2001). This pathway also involves protein kinase Cα, Rho and focal adhesion kinase, but the mechanism is independent of the externalization and binding of TG2 to fibronectin. Therefore, it is still an open question how TG2 is involved in the extracellular matrix-dependent cells survival mechanism.
Cerione and his coworkers have recently shown that in some cell lines treated with retinoic acid the retinoic acid-induced TG2 can protect also against apoptosis induced by the synthetic retinoid HPR (Antonyak et al. 2001). Phosphoinositide 3-kinase activity was required for both the retinoic acid-stimulated expression and GTP-binding activity of TG2 (Antonyak et al. 2002), while its induction was antagonized by the Ras-ERK pathway (Antonyak et al. 2003). GTPase activity of TG2 was found sufficient to protect cells from HPR-induced death suggesting that the survival signaling does not require transamidating activity of TG2 (Antonyak et al. 2001). On the other hand, in their next set of experiments it was shown that TG2 protects Rb from caspase-induced degradation in a transamidation dependent manner (Boehm et al. 2002), and suggested that transamidation of Rb by TG2 is necessary for its ability to inhibit apoptosis. This apparent controversy may arise from the use of monodansylcadaverine which is a competitive but non-specific inhibitor of transglutaminases and is known to accumulate in membrane structures. Therefore, without direct demonstration of in situ changes in protein transamidation the use of monodansylcadaverine by itself is not sufficient to prove that the observed effect is linked to transamidation function of TG2.

In breast cancer cell lines EGF could inhibit doxorubicin-induced apoptosis while upregulated TG2 (Antonyak et al. 2004). Expression of exogenous TG2 could mimic the survival advantage of EGF. In addition, the observation that transfection of cells with transamidation-defective TG2 before EGF treatment could block the death-preventing effect of EGF argued for the role of transaminidation in the protection against death.

All these results discussed previously have been obtained from cell culture experiments, to check how relevant these data are in vivo system TG2 knock out mice have been developed (DeLaurenzi et al. 2001; Nanda et al. 2001). These animals were found viable, to grow up to normal size and weight with no apparent abnormalities in organ functions including the extracellular matrix or the heart (where the need for its G protein function has been most expected). Cells taken from these animals did not show any defect in apoptosis in either way, that is they were not less or not more resistant to death stimuli than their normal counterpart. Certainly, these observations may question both the pro-apoptotic and pro-survival function of TG2.

Closer examination of the in vivo apoptosis program of the thymus has revealed that thymus disappears slower in the TG2−/− animals than in their wild-type
counterparts following injection of various apoptotic stimuli (Szondy et al. 2003). This was partially the result of an impaired phagocytosis of apoptotic cells. Although TG2 could promote phagocytosis from the side of apoptotic cells by facilitating the phosphatidylyserine exposure that is required for the recognition of apoptotic cells (Fadok et al. 1992), or by crosslinking the S19 ribonuclear protein that acts as chemotactic factor for macrophages (Nishiura et al. 1998) but the main defect was found in macrophages. This was partially related to a defect in TGFβ activation (Kojima et al. 1993), as TGFβ was shown to promote phagocytosis of apoptotic cells (Szondy et al. 2003; Rose et al. 1995). TGFβ is also needed for in vivo induction of TG2 in both apoptotic cells and macrophages. So in one hand TG2 crosslinks proteins in apoptotic cells and forms protective proteanaceous shell, which prevents the leakage of harmful cell content and in the other hand it also promotes the speed of phagocytosis and further formation of TGFβ in macrophages, which is also required to downregulate proinflammatory responses (Fadok et al. 1998). It seems that the main role of TG2 in vivo to ensure that whenever apoptosis is initiated, it is finished without causing inflammation and apparent tissue injury (Fig.6). If, however, necrosis still occurs TG2 promotes both tissue stability and repair (Nardacci et al. 2003; Nicholas et al. 2003). In TG2−/− animals all these anti-inflammatory actions are compromised resulting in the appearance of inflammatory cells at the apoptotic sites in short term and leading on long term to autoimmunity.
Figure 6. Relationship between tissue transglutaminase and TGF-β in the regulation of apoptosis and clearance of apoptotic cells in the thymus. Recognition of apoptotic cells via phosphatidylserine receptors (PSR) triggers latent TGF-β release and activation by the simultaneously released TG2. There are TGF-β receptors (TGF-R) on both macrophages and apoptotic cells. In one hand TGF-β promotes apoptosis in apoptotic thymocytes and induces TG2, and in the other hand in macrophages TGF-β promotes phagocytosis and downregulation of the production of proinflammatory cytokines. An autoregulatory loop is generated with induction of TG2 by TGF-β in macrophages resulting further TGF-β formation and release (Fésüs and Szondy 2005).
**AIMS**

Previous studies in our laboratory have revealed the loss of TG2 in mice leads to an *in vivo* defect of phagocytosis of apoptotic cells in mice, and the defect affects the macrophage site. The main aim of the present study was to investigate the phagocytosis defect also *in vitro* and to characterize the deficiency that develops in the engulfment process in the absence of TG2.

For this purpose we decided

1. To set up a phagocytosis model and techniques with the help of which the phagocytosis of apoptotic cells by macrophages can be studied *in vitro*.
2. To characterize the phagocytosis of apoptotic cells by wild-type macrophages using time-lapse video.
3. To characterize the phagocytosis of apoptotic cells by TG2^{-/-} null macrophages using time-lapse video.
4. To develop an adenoviral gene delivery system to determine which biological function of the TG2 is required for the phagocytosis process.
5. To characterize changes in the phagocytic receptors expression which try to compensate the loss of TG2.
6. To characterize the changes in the phagocytic signaling pathways that develop in the absence of TG2.
MATERIALS AND METHODS

Antibodies and reagents

Purified mouse anti-Rac1 monoclonal- (clone 102) and phycoerythrine (PE)-conjugated anti-mouse β3 integrin (clone 2C9.G2) antibodies were purchased from BD Pharmingen (San Diego, CA). 6-carboxy-3’,6’-diacetylfluorescein (CFDA), 5-(and 6-)-(4-chloromethyl)benzoyl)amino) tetramethylrhodamine (CMTMR), Alexa 647-conjugated anti-mouse and anti-goat IgGs, and Prolong antifade reagent were purchased from Invitrogen (Carlsbad, CA). Horse radish peroxidase-conjugated anti-rabbit, anti-goat and anti-mouse IgGs, phycoerythrine-labeled hamster anti-β3 integrin antibody, anti- mouse β-actin, 2 µm carboxylate-modified red fluorescent beads, NeutrAvidin agarose and vitronectin were purchased from Sigma –Aldrich (Budapest, Hungary). Anti-hexon AK was purchased from Chemicon (Billeric a, MA), anti-RhoG (clone C-20), anti-LRP (clone N-20) and polyclonal anti-TG2 (H-237) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 6B9mAb used for cell surface TG2 labeling of human macrophages was a kind gift from Thomas B. Issekutz (Dalhousie University). FITC-labelled rabbit anti-mouse IgF(ab)2 was purchased from DAKO (Glostrup, Denmark). Immunoblots were analysed by enhanced chemiluminescence (Millipore, Billerica, MA).

Animals

TG2 null mice lacking of part of exon 5, intron 5, exon 6, and a piece of intron of the transglutaminase gene (De Laurenze V, Melino G, 2001) and their wild-type counterpart were obtained from Gery Melino’s laboratory. Study protocol was approved by Animal Care Committee of University of Debrecen.
Cells
Peritoneal macrophages derived from TG2 wild type and knock out mice were used for phagocytosis experiments. Mice were injected with 2ml 4% thioglycollate and four days after peritoneal lavages were performed to receive differentiated macrophages. After washing, 5x 10⁵ peritoneal macrophages were allowed to adhere in 24-well plates for 24 hours in the presence of 10µM red fluorescent CMTMR at 37°C/ 5%CO₂. Thymocytes from 4 weeks-old WT mice were used as apoptotic target cells. Thymuses were mechanically disrupted, isolated thymocytes were labeled overnight with 6 µM green fluorescent CFDA, and subsequently treated with 4 µM ionomycin (Sigma-Aldrich, Budapest) for 6 hrs. 40–50% of thymocytes were Annexin V positive (i.e. apoptotic), and less than 5% of Annexin V positive cells were propidium iodide positive (i.e. necrotic). Necrosis was induced by heat shock (50 °C, 25min). Thymocytes and macrophages were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, penicillin and streptomycin (Sigma).

For adenovirus generation AD-293 cells were used. AD-293 cells are derived from the HEK293 cell line, but have improved cell adherence and plaque formation properties. HEK293 cells are human embryonic kidney cells transformed by sheared adenovirus type 5 DNA. It has adenovirus E1 gene in trans, allowing the production of infectious virus particles after transfection pAdEasy-1 vector. The packaging cell line was culture in DMEM (Sigma) (containing 4.5g/L glucose and 110mg/L sodium pyruvate and 4mM L-glutamine) supplemented with 10% FCS.

Phagocytosis assay
CMTMR labeled macrophages were incubated with 2 µm carboxylate-modified red fluorescent beads or with apoptotic thymocytes stained with CFDA in 40:1 target/macrophen ratio. Routinely, the assays were carried out in duplicates or triplicates for each condition with a 30 or 60 min incubation for thymocytes. Cells incubated with apoptotic thymocytes incubated at 4 °C were used as a control for efficient washing. For engulfment assays with 2 µm carboxylate-modified beads, the cells were incubated with beads in serum free medium for 2 h. After washing, the cells
on the plate were trypsinized, resuspended in cold medium with 0.5% sodium azide, and 10,000–20,000 cells were analyzed for each point by two-color flow cytometry (Epix Coulter). For visualizing apoptotic cells in macrophages, thymocytes that had not been taken up were washed away, after which the adherent cells were prefixed with 0.5% paraformaldehyde in 0.1 M Sörensen phosphate (SP) buffer (pH 7.4) for 30 min, fixed with 2.5% glutaraldehyde in SP buffer for 3 hrs at room temperature and than stained with 2% osmium tetroxide for contrast enhancement and studied by phase contrast microscopy.

**Site-directed mutagenesis**

Site-directed mutagenesis was performed on plasmid pSP73 carrying the murine TG2 gene. The Quik-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used for introduction of the desired mutations (See text). The following primers were used for cross-linking deficient mutant: TG2-X up: 5’-GTGAAGTACGGGCAGAGTTGGGTGTTTG-3’ and TG2-X down: 5’-CAAAACACCCAACTCTGCCCCGTACTTCAC-3’; for guanine nucleotide-binding site mutants: TG2-G1 up: 5’-CTACCAAGGCTCTGTCAACGACATCAAGAGTG TGCC-3’ and TG2-G1 down: 5’-GGCACACTCTTTAGTAGTGCCACAGAGCCCTT GGTAG-3’; TG2-G2 up: 5’-CAGCTACCTGCTGGCTCAAGAAGATCTCTACCTG GAG-3’ and TG2-G2 down: 5’-CTCCAGGTAGAGATCTTCTTGAGCCACAGGT AGCTG-3’; for fibronectin binding deficient mutant: TG2-FN up: 5’-CAGTGCTGCGCAGAGGcCAATGTCCTCTC-3’ and TG-FN down: 5’-GAGAGGACATTGGCCTTTGAGGCCAGCAGCT-3’; for secretion deficient mutant: TG2-S up: 5’-CACCCAGCACTGCCGGACTCTCAGCTG-3’ and TG2- S down: 5’-CAGCAAGTGATCCCGGCCAGCTGCTGGT-3’.

**Sequencing**

For sequencing BigDye Terminator v3.1 kit was used (Applied Biosystems). The reaction is based on principle of chain-terminating. The reaction
mixture contains enzyme, dNTP and the four differently labeled dideoxi-NTPs. During sequencing due to the chain termination fragments with different lengths are formed with having ddNTP labeling on their ends. The fragments are separated by capillary electrophoresis. After laser excitation the fragments are emitting at different wavelength according to their labeling. The signals are recorded by a CCD camera.

Sequencing primers:
1. 5’-GACTGCATCTTCACTGTGGAG-3’
2. 5’-CCGTGCCTTGACCAAATCGC-3’
3. 5’-ATGTGGGCAAACACGTCGAAG-3’
4. 5’-GAAGTTCCAGATCATCTGCTC-3’
5. 5’-CAAGAGCATACTAGGCAGGTATC-3’

**Cloning of TG2 gene from pSP-TG2 into the CMV-pShuttle vector**

The site directed mutagenesis was performed in pSP73 cloning vector. To insert TG2 gene into the adenovirus genom first we put our gene into a shuttle vector, which transfers TG2 and its mutant variants into the adenogenom by homologous recombination. KpnI and HindII restriction site were designed at the ends of TG2 cDNA by PCR (95°C-3min, 30x(95°C-30sec, 54°C-1min, 68°C-9min) 68°C-5min). The PCR products were inserted into a PCR-Blunt vector (Invitrogen). PCR-Blunt-TG2 plasmid and CMV-pShuttle vector were digested with Hind II and KpnI (Promega). Digested DNA was run on 1% agarose TAE gel, the TG2 cDNA (2,1kb) and the linearized CMV-pShuttle vector (7,5kb) were cut and purified by gel extraction kit (Quiagene, Hilden, Germany). Ligation was performed at 14 °C overnight.

Primers to create KpnI and Hind II restriction site:
TG2-KpnI: 5’-CGGGGTAACATGGCAGAGGCTGCTGAGG-3’
TG2-HindII: 5’-CCCAAGCTTTTAGGCGGGGCGGATGATAACAT-3’
Adenoviral gene delivery system

cDNA encoding murine TG2 and its mutant variants were introduced into adenovirus. To generate recombinant adenovirus a pCMV-shuttle vector was used. The wild type TG2 and its mutant variants were cloned into the shuttle vector, which transfers the inserts into the adenovirus genom by homologous recombination. The pShuttle-CMV contains a multiple cloning site between the CMV promoter and the SV40 polyadenylation signal and is suitable for insertion of the 2.2 kb TG2 cDNA, including specialized promoter and termination signal. The arm regions have sequence homology with pAdEasy-1. The control for production of recombinant adenovirus was a pShuttle-CMV-LacZ vector, where LacZ gene was inserted in the pShuttle-CMV. The recombination process occurs in a specific E. coli, called BJ183-AD-1, which was previously pretransformed with adenoviral backbone plasmid, pAdEasy-1. The BJ183-AD-1 strain is a recA proficient and supplied the machinery necessary to execute the recombination event between the shuttle vector and pAdEasy vector. Before transformation the shuttle vector was linearized with PmeI. The efficient transformation required 0.03-0.1 \( \mu \)g linearized shuttle vector DNA, and the transformation step was performed by an electroporator using 200Ω, 2.5kV, 25\( \mu \)F. The transformants were selected for kanamycin resistance, and the recombinants were identified by restriction digestion. After identification the right recombinants the adenogenom was produced in bulk in a recombinant-deficient XL10-Gold strain. The assembly and the virus generation occur in HEK 293-derived cells. The recombinant Ad plasmid had to be digested with Pac I before transfection of AD-293 cells. The transfection was performed with lipofectamine-2000 (Invitrogen) in transfection medium using 70% cell confluence. To prepare primary viral stock infected AD-293 cells were disrupted by freezing-thawing technique.

Adenoviruses carrying wild type and constitutive active Rac genes were a kindly gift from Sakae Tanaka (Fukada et al. 2005). Virus titers were determined by plaque assay with anti hexon-AK (Chemicon) antibody in 293 cells after exposing them to virus for 48 hours in DMEM medium supplemented with 2% serum and antibiotics. For gene delivery \( 10^6 \) macrophages were exposed to virus for 48 hrs in the same medium. LacZ expression was determined with X-gal staining, while TG2 expression was detected Western blot analysis.
Western blot analysis of α-actin and TG2 expression

Peritoneal macrophage’s lysate containing 2 mg/ml protein was mixed with an equal volume of sample buffer (0.125M Tris-HCl, pH6.8 containing 4% SDS, 20% glycerin, 10% 2-mercaptoethanol and 0.02% bromphenol blue). Electrophoresis was performed in SDS polyacrylamide gel. Separated proteins were electroblotted, blocked with 5% BSA and probed with anti- mouse β-actin and polyclonal mouse anti-TG2 (Santacruz) antibody. Bound antibodies were visualized using ECL detection system.

Biotinylation and isolation of cell surface proteins

Cell monolayers, corresponding to 4x10^6 cells, were rinsed with ice-cold PBS, preincubated in PBS on ice for 45 min, then were further incubated in Biotin-XX SSE (Sigma-Aldrich, Budapest) dissolved in PBS (0.5 mg/ml) for 20 min. Cells were scraped in lysis buffer (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM EGTA) and sonicated. Lysates were boiled for 5 min and cleared by centrifugation (13,000 rpm, 20 min) at room temperature. To isolate biotinylated (cell-surface) proteins, the lysates were incubated with NeutrAvidin-agarose. Proteins bound to NeutrAvidin-agarose were separated by 10% gel electrophoresis and analyzed by Western blot analysis by using anti-TG2 antibodies.

Q-PCR analysis for detecting of the expression of various phagocytosis receptors

Total RNA was isolated by TRI reagent (Sigma-Aldrich, Budapest). Total RNA concentrations were determined by spectrometry after DNAse treatment (Sigma-Aldrich, Budapest). TaqMan Reverse Transcription Reagent kit (Applied Biosystems, Foster City, CA) was used for generating cDNA according to manufacturers instructions. 200ng total RNA was used in a reaction volume of 50 µl. ABI PRISM 7700 Sequence Detection System (Applied Biosystems) was used to determine relative gene expression. Gene primers and probes were designed and
supplied by Applied Biosystems. 18S ribosomal RNA was used as endogenous control to normalize the amount of the sample cDNA added to the reaction. The 18S primers were labelled with VIC and sample primer with FAM. All samples were run in triplicate. Relative mRNA expression was quantified by comparing the cycle threshold (CT) between control and knockout cell samples.

Detection of active Rac1 and RhoG

Macrophages plated overnight alone or exposed to 2 µm carboxylated latex beads for 40 min were used. Pull down assay was performed with the EZ-Detect Rac1 Activation Kit (Pierce, Rockford, IL). The kit uses a GST-fusion protein containing PBD of human Pak1. GST human Pak1-PBD is incubated with 1mg total cell lysate (determined by the Bradford method). The pulled-down active Rac1 was detected by Western blot analysis using Anti-Rac1 antibody.

For the RhoG pull-down assay GST human Pak1-PBD was replaced with an ELMO-GST protein. Active RhoG was detected by Western blot analysis using anti-RhoG antibody. For RhoG assays 1.5mg of cell lysates were used.

Immunofluorescence staining and confocal microscopy

Peritoneal macrophages isolated from WT and TG2<sup>-/-</sup> mice were plated in two-well chamber-slides (5x10<sup>5</sup>/well) and cultured for 48h before used. After exposing or not macrophages to apoptotic cells for 30 min, cells were washed than fixed in ethanol/acetone 1:1 for 10min at -20 °C. F-actin was stained with phalloctoxin-Alexa488 for 20 min at room temperature. After three to five rinses in PBS, the slides were mounted with Prolong antifade reagent. Samples were examined with a Leica TCS SP confocal laser scanning microscope. For integrin β3 and LRP staining macrophages were blocked with 50% FBS for 30 min at 37°C, then washed with ice cold HEPES buffer and stained with phycoerytrin labeled hamster anti-β3 integrin or anti-LRP antibody for 15 min on ice. For LRP staining, Alexa 647 anti-goat IgG was used as a secondary antibody. After immunolabeling cells were washed with HEPES
and fixed with 4% paraformaldehyde for 20 min. For intracellular staining cell were permeabilized with 0.1% Triton X after fixation with 1% PFA and blocked with 1% BSA. For detecting Rac1 cells were labelled with purified mouse anti-Rac1 monoclonal antibody for 30 min at room temperature. After washing Alexa 647-conjugated goat, anti-mouse IgG was used as secondary antibody. Images were taken with a Zeiss LSM 410 or Olympus FV1000 confocal laser scanning microscope. For visualizing the distribution of integrin β3 and Rac1, overview images and 3D stacks were acquired at 1 μm optical thickness. 3D reconstructions and XYZ projections were created with the LSM 4.0 software.

**Binding of TG2 to lipids**

The membrane lipid strips (Echelon Biosciences, Inc. p6002) were blocked in 3% fatty-acid-free BSA in TBS-T containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.1% Tween-20 for 1 h at room temperature in the dark. The membrane was then incubated at 4 °C with gentle agitation in the same solution containing 1μg/ml GST (negative control), GST-PX (phosphoinositide-binding structural domain-positive control) or GST-TG2. The membranes were washed four times in TBS-T and then incubated for 1 h with anti-GST antibody (clone Z5; Santa Cruz) followed by routine immunoblotting steps.

**Biacore assays**

Surface plasmon resonance experiments were performed on a Biacore 3000 instrument equipped with research grade CM5 sensor chips (BIAcore AB, Uppsala, Sweden). Anti-GST surfaces were prepared at 25 °C by an amine-coupling method using reagents available from BIAcore AB (N-ethyl-N’-dimethylaminopropyl-carbodiimide [EDC], N-hydroxysuccinimide [NHS], and 1 M ethanolamine-HCl at pH 8.5). 30 μg/ml anti-GST antibodies in 10 mM sodium acetate coupling solution (pH 5.0) were injected at a flow rate of 5 μl/minute. The remaining reactive groups were blocked with a 7 min injection of 1 M ethanolamine-HCl at pH 8.5. Typical
immobilization level of the anti-GST antibody was between 12000 and 15000 RU. During the immobilization 2.5 µM GST-TG2, (~1300-1500 RU) and molar proportion of GST (~ 400-500 RU) were captured on the antibody surface. The GST and GST-TG2 were diluted in the running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM CaCl₂, 0.005% v/v Surfactant P20) and the flow rate was 10 µl/min. Recombinant human MFG-E8 (R&D Systems, Minneapolis, MN) or fibronectin (Sigma-Aldrich) were injected at five concentrations between 0.01 and 2 µM on either the GST or GST-TG2 surface at a flow rate of 10 µl/minute 7 minutes and the changes in the response units (RU) were recorded. After every injection the dissociation phase was 6 minutes and the regeneration was performed three times at flow rate of 20 µl/minute with 10mM glycine, pH 2.5. Surface covered with GST was used as reference surface. The response from the reference surface was subtracted from the target surface. All data were interpreted using the BiaEvaluation 4.1 software (Biacore). Kinetic data were interpreted by global fit of the data using a simple Langmuir interaction model. Kinetic (kₐ and k₅) and equilibrium parameters (Kₐ) were derived from the sensograms using BIAevaluation 3.1 software with fitting the data to a simple 1:1 Langmuir interaction model. Simulations were performed using the BiaSimulation software (Biacore).

**Time-lapse video**

For time lapse video macrophages were labeled with CMTMR as above and were exposed to unlabeled apoptotic thymocytes in a 6 target:1 macrophage ratio. For detecting the formation of 3-phosphoinositides in the cellular membranes, cells were transfected by a fusion protein consisting of GFP and the PH domain of phospholipase Cδ by adenoviral gene delivery (Varnai et al. 2002). Time-lapse movies were made using both fluorescence emission (in the standard rhodamine channel) and transmission of green (543 nm) light in a Zeiss LSM510 confocal laser scanning microscope equipped with a 40x/1.2NA water immersion objective. 1024x1024 pixel images were taken every 10 sec at 140 nm/pixel resolution. Regions of interest were extracted and exported to mpg format with 16 frames/s speed, yielding a compressed jpg video 160 times the actual speed of the process of phagocytosis.
Statistical analysis

All the data are representative of at least three independent experiments. Values are expressed as mean ± S.D. Statistical analysis was performed using the unpaired Student’s $t$-test.
RESULTS

1. Role of TG2 in phagocytosis of apoptotic cells

1.1. The efficiency of apoptotic cell and carboxylate-modified latex bead uptake by TG2-/- macrophages is lower compared to their wild type counterparts

Treatment of thymocytes with Ca-ionophore is known to induce apoptosis by increasing the intracellular Ca$^{2+}$ level. Adding these apoptotic cells to macrophages derived from TG2$^{-/-}$ and wild type mice different phagocytotic efficiency was observed. For precise determination of phagocytosis of apoptotic cells by TG2$^{+/+}$ and TG2$^{-/-}$ macrophages, both macrophages and apoptotic cells were fluorescently stained (Fig. 7A), and either the number of engulfed cells was counted under confocal microscope (Fig. 7B) or the percentage of macrophages engulfing apoptotic cells was determined by flow cytometry (Fig. 7C) at various time points. As shown in Figure 7, compared to their wild-type counterparts, a smaller percentage of TG2$^{-/-}$ macrophages engulfed apoptotic cells. During 1 hour period of phagocytosis approximately 85% of wild type (WT) macrophages engulfed at least one apoptotic cell. In contrast, 50% of TG2$^{-/-}$ cells were not able to engulf apoptotic cells at all, and the numbers of phagocytosed cells were much lower compared to WT counterparts. Since counting under confocal microscope is a time consuming technique, in all of the following experiments flow cytometry analysis was used to determine the percentages of macrophages with engulfed apoptotic cells. Though this method underestimated the real degree of the defect, not showing the difference in the number of engulfed cells, it reflected the changes in the phagocytotic capacity of TG2$^{+/+}$ macrophages treated in various ways.
Figure 7. Confocal images of wild type (TG2^{+/+}) and knock out (TG2^{-/-}) macrophages during phagocytosis (A). Under fluorescent microscope 500 TG2^{+/+} and TG2^{-/-} macrophages were counted and characterized according to the number of engulfed cells after 1 hour phagocytosis. Data represent one of three independent experiments. 'A' means the number of attached particles (B). Flow cytometric analysis of phagocytosis (C) performed by TG2^{+/+} and TG2^{-/-} macrophages. Phagocytosis assay was performed in 40:1 target/macrophage ratio for 1 hour. Apoptotic thymocytes were labeled with green fluorescent CFDA and the macrophages were stained with red CMTMR. The histogram shows the gated population for CMTMR and the markers show the percentages of the gated macrophages contain green particles. Increase in the percentage of macrophages engulfing apoptotic cells during a 2 hours period determined by flow cytometry (D). Data represent mean ± S.D. of three independent experiments.
2-µm carboxylate-modified latex beads were previously used to replace apoptotic cells in signaling studies (Park et al. 2007; Tosello-Trampont et al. 2003). The features of these latex beads are similar to apoptotic cells because of their size and the negative charges on their surface. To prove that these beads indeed mimic apoptotic cells in the cellular uptake, competition experiments were performed using J774 murine macrophage cell line, where the uptake of fluorescent latex beads was studied in the presence or absence of unlabeled necrotic and apoptotic cells. As shown in Figure 8, uptake of the latex beads competes with the uptake of the apoptotic cells during phagocytosis, but was not influenced by the presence of necrotic cells. This data demonstrate that these beads are indeed taken up by similar mechanism like apoptotic cells.

**Figure 8.** Flow cytometry analysis of carboxylate modified bead uptake in competition experiments. Unlabeled murine macrophages were incubated with fluorescent carboxylate modified beads alone or together with unlabeled apoptotic or necrotic thymocytes in 40:1 target/macrophage ratio for 1 hour. Data show three independent experiments under each experimental conditions.

The uptake of the 2 µm red-fluorescence carboxylate modified latex beads by wild-type and TG2⁻/⁻ macrophages was compared. As shown in Figure 9, their uptake,
similarly to apoptotic cells, was also significantly lower by TG2\(^{-/-}\) macrophages suggesting that whatever mechanism is used for their uptake, it must involve TG2.

Figure 9. TG2\(^{-/-}\) macrophages engulfed carboxylate modified latex beads by lower efficiency than their wild type counterparts detected by flow cytometry. The assays were carried out in 40:1 target/macroage ratio for 60 and 120 min. Data represent one of three independent experiments.

1.2. Crosslinking activity of TG2 is not required for the phagocytosis of apoptotic cells

TG2 is a multifunctional enzyme with Ca\(^{2+}\) dependent cross-linking activity and GTP-dependent G protein function. It is also present on the cell surface and can bind to fibronectin promoting the cell migration and focal adhesion. To test which biological functions of this enzyme participate in the engulfment process we treated TG2\(^{+/+}\) peritoneal macrophages with a competitive substrate, monodansylcadaverine (MDC). Macrophages were incubated for 18 hours with MDC before the phagocytosis assay and MDC was present during the phagocytosis as well. As shown in Figure 10, MDC was not able to significantly reduce the engulfment capacity of TG2\(^{+/+}\)
macrophages. These data suggest that the crosslinking activity of TG2 is not required for the phagocytosis of apoptotic cells by macrophages.

**Figure 10.** Crosslinking ability of TG2 is not involved in the phagocytosis of apoptotic cells by macrophages. Phagocytosis ability of wild type macrophages was examined in the presence and absence of a competitive substrate of TG2. After 18 hour preincubation with 15µM MDC, 5x10^5 macrophages were exposed to apoptotic thymocytes in 40:1 target/macrophage ratio for 60 min. Data represent one of three independent experiments.

1.3. Adenovirus gene delivery system carrying TG2 can be used to rescue the phagocytosis deficiency of TG2^-/- macrophages

To investigate which biological functions of TG2 were required to promote phagocytosis of apoptotic cells, an adenoviral gene delivery system was developed to transfect primary peritoneal macrophages with various mutants of TG2. The adenoviruses can infect a broad range of cell types and can be used to express human and non-human proteins. The infection is independent on active host cell division. High virus titer and high gene expression are responsible for the efficient protein production in mammalian cells. Because the recombinant adenovirus remains epichromosomal, they do not interrupt any of the host cell genes. TG2 cDNA was cloned into the adenovirus genom and this adenovirus infection was used to deliver
TG2 gene into the TG2−/− peritoneal macrophages. After 2 days of infection TG2 protein expression was observed in the TG2−/− macrophages. As shown in Figure 11A, depending on the amount of infecting viral particles increased amounts of WT TG2 could be delivered into WT and TG2−/− macrophages.

To prove that the lack of TG2 is responsible for phagocytosis deficiency, we infected TG2−/− macrophages with adenoviruses carrying the wild type TG2 or the LacZ gene (infection control), then phagocytosis assay was performed. As shown in Figure 11B, exposure to adenoviruses containing the wild type TG2 gene was able to restore the phagocytosis deficiency of macrophages derived from TG2−/− mice.

**Figure 11.** Wild-type TG2 can be successfully delivered into mouse peritoneal macrophages using increasing amounts of adenoviral particles. (A) Two days after infection 40 µg protein lysate from wild-type or TG2−/− macrophages infected with the indicated amounts of virus were subjected to immunoblot analysis. (B) Adenoviral delivery of wild-type TG2 fully reverts the phagocytosis phenotype of TG2−/− macrophages. Two days after adenoviral delivery of lacZ or wild-type TG2 (2x10⁹ plaque forming units (PFU)/ml), macrophages were tested for phagocytic activity for 30 min. Data represent one of three independent experiments.
1.4. Phagocytosis of apoptotic cells by macrophages infected with adenoviruses carrying various TG2 mutants

To determine which function of TG2 participates in phagocytosis, several TG2 mutants were generated. Adenovirus was used to deliver the WT, cross-linking (referred as TG-X by replacement of catalytic $\text{Cys}^{277}$ by Ser ([Lee et al. 1993]), fibronectin binding (TG-FN through substitution of Asp$^{94}$ and Asp$^{97}$ by Ala ([Hang et al. 2005])), GTP/GDP binding (TG-G1, TG-G2 by replacement of Lys$^{172}$ and Phe$^{173}$ by Asn and Asp, and of Glu$^{578}$, Arg$^{579}$ by Gln and Glu ([Iismaa et al. 2000])) and cell surface secretion (TG-S, replacement of Tyr$^{274}$ by Ala ([Balklava et al. 2002])) deficient TG2 mutants into TG2$^{-/}$ peritoneal macrophages. After 2 days of adenovirus infection the phagocytosis assay was performed. Adenovirus carrying lacZ gene was used as an infection control. Following adenoviral delivery all the mutants with the exception of the fibronectin binding mutant were stably expressed by the TG2$^{-/}$ cells (Fig.12A). The defect in the expression of the fibronectin mutant might be related to some sequence mistake in this adenovirus construct, or the immediate degradation of the expressed protein.

As shown in Figure 12B, from the stably expressed proteins the WT enzyme and the cross-linking activity mutant were able to revert the phagocytosis phenotype but the guanine nucleotide binding- or the secretion mutants could not
Figure 12. Cell surface TG2 promotes phagocytosis (A) All the TG2 mutants with the exception of the fibronectin binding mutants were stably expressed in TG2−/− macrophages. Nt means the non infected control sample. (B) Only the wild-type and the crosslinking mutant of TG2 are able to restore the phagocytosis defect of TG2−/− macrophages. Two days after adenoviral delivery of lacZ, wild-type or mutant TG2s (2x10⁹ PFU/ml) into TG2−/− macrophages, cells were tested for phagocytic activity for 60 min. Data represent one of three independent experiments.

1.5. Cell surface TG2 is involved in engulfment of apoptotic cells

Growing evidence indicates that TG2 takes part in the assembly and remodeling of extracellular matrix and promotes cell adhesion and spreading. Besides the adenoviral experiment, mentioned above exogenously added human recombinant TG2 (2 µg/ml) also stimulated phagocytosis of apoptotic cells in TG2+/+ and TG2−/− macrophages (Fig.13) proving further the involvement of cell surface TG2 in the phagocytosis.
**Figure 13.** Exogenous addition of human recombinant TG2 can rescue the phagocytosis deficiency in TG2−/− macrophages. Phagocytosis assay was performed after two hours of pretreatment with 2 µg/ml recombinant TG2 of TG2+/+ (A) and TG2−/− macrophages. The recombinant protein was present during the whole assay period as well. Apoptotic thymocytes were exposed to TG2+/+ and TG2−/− macrophages 40:1 target/macrophage ratio for 60 min. Data show one of three independent experiments.

Since there are no antibodies available, which could specifically label mouse TG2 on the cell surface, to prove that TG2 is indeed expressed on the surface of mouse macrophages, cell surface proteins were labeled with Biotin-XX SSE and isolated with NeutrAvidin-agarose and probed for TG2 on Western blot. As shown in Figure 14.A, around 10% of the total TG2 expressed by mouse macrophages appears on the cell surface.

To investigate whether the guanine nucleotide binding mutants are ineffective, because they cannot be transported to the cell surface, their cell surface appearance was tested with the biotin-labelling technique. As shown in Figure 14B, the crosslinking mutant and both guanine nucleotide binding variants, but not the secretion mutant appeared on the cell surface suggesting that guanine nucleotide binding is required not for the cell surface transfer. Together, these data indicate that cell surface TG2 in guanine nucleotide bound conformation promotes phagocytosis of apoptotic cells.
Figure 14. TG2 is expressed on the cell surface of mouse macrophages and guanine nucleotide binding is not required for the cell surface delivery. (A) 10% of total transglutaminase is expressed on the surface of mouse macrophages. Biotin-XX SSE-labelled cell surface TG2 of mouse macrophages was isolated with NutrAvidin agarose, and its amount was compared to that of total amount of TG2 by immunoblot analysis. (B) Crosslinking and guanine nucleotide mutants of TG2 can, but the secretion mutant cannot be delivered to the cell surface of mouse macrophages. Two days after adenoviral delivery of various mutants of TG2 (2x10^9 PFU/ml) into TG2^-/- macrophages the appearance of TG2 on the cell surface was detected as in (A). T, total; S, surface.

1.6. In the absence of TG2 the mRNA expression of β3 integrin receptor is higher, while the expression of other phagocytosis receptors is only slightly altered

To test whether the loss of TG2 could affect the expression of any of the various phagocytosis-related receptors, we determined their mRNA expression by TaqMan assay. To our surprise mRNA of integrin β3 was expressed at a higher level in TG2^-/- cells (Fig.15).
Figure 15. mRNA expression of various receptors participating in the phagocytosis of apoptotic cells in TG2^−/− versus wild-type macrophages. The expression of various receptors was determined by Q-PCR technique in the two types of macrophages. Data represent relative expression (TG2 null/wild-type) values. c1qr, complement 1q receptor; av, integrin αv; b3, integrin β3; b5, integrin β5; TSP, thrombospondin-1; LPR-1, low-density lipoprotein receptor related protein-1; SRA, scavenger receptor A; ABC1, ATP-binding cassette transporter

1.7. TG2 is required for proper integrin β3 signaling

The engulfment process is always an actin-dependent mechanism. The actin reorganization during the phagocytosis is regulated by GTP bound Rac. The upstream components required for Rac activation are different. One pathway identified in both mammalian cells and worms involves RhoG/MIG-2 and the guanine nucleotide exchange factor TRIO/UNC-73 (deBakker et al. 2004) where the cell surface receptor is the integrin (Albert et al. 2000). Since cell surface TG2 was reported to be a coreceptor for integrin β3 (Akimov et al. 2000; Janiak et al., 2006), and integrin β3 plays a central role in the uptake of apoptotic cells (Savill et al. 2002), we speculated that TG2^−/− cells upregulated integrin β3 to compensate the loss of TG2. That is why we investigated the functionality of the integrin β3 signaling pathway in TG2^−/− cells by determining the activation of RhoG and Rac1 (Kinchen et al. 2005; Brugnera et al. 2002; deBakker et al. 2004; Akakura et al. 2004). Since we found GTP-bound Rac1
in apoptotic cells which could have interfered with the pull-down assay, we used 2-
µm carboxylate-modified latex beads in these studies.

Loss of TG2 did not affect the total amount of Rac1, while RhoG levels were
slightly elevated (Fig.16A). Addition of beads to WT macrophages triggered
activation of both RhoG (Fig.16B) and Rac1 (Fig.16C) detected by ELMO and PAK
pull down assays, respectively. In the absence of TG2 RhoG and Rac1 could not be
activated in detectable amounts following exposure to carboxylate-modified latex
beads (Fig.16B and C). These data indicate that the integrin β3 pathway is impaired in
TG2−/− cells.
Figure 16. Altered phagocytosis signaling is detected in TG2\textsuperscript{+/-} macrophages. (A) Total amounts of Rac have not changed, while RhoG levels are slightly elevated in TG2\textsuperscript{+/-} macrophages determined by immunoblot analysis. (B) Rho G is activated in wild type but not in TG2\textsuperscript{+/-} macrophages exposed to carboxylate-modified latex beads. Wild type and TG2\textsuperscript{+/-} macrophages were exposed or not to carboxylate-modified latex beads. Following 40 min of phagocytosis GTP-bound Rho G was collected by pull down with ELMO-GST protein from equal amounts of protein lysates and subjected to immunoblot analysis. (C) Rac1 is activated in wild-type but not in TG2 null macrophages exposed to carboxylate-modified latex beads. Wild type macrophages were exposed or not to carboxylate-modified latex beads. Following 40 min of phagocytosis GTP-bound Rac1 was collected by pull down with PAK-GST protein from equal amounts of protein lysates and subjected to immunoblot analysis.

1.8. The uptake of apoptotic cells by wild-type macrophages is mediated by an efficient engulfing centre

Rac1 cycles between the membrane and cytosol and at the plasma membrane activated GTPases interact with downstream effectors. Translocation of Rac to
membrane seems to be critical for the activation of its effector (del Pozo et al. 2004; Moissoglu et al., 2006). In its inactive status, Rac remains in the cytoplasm and becomes activated upon cell stimulation, when it is translocated to the membrane (Dinauer 2003). GTP loading and membrane targeting of Rac are separable events and Rac activity is regulated by both the GDP/GTP exchange cycle and the membrane association/dissociation cycle.

In non-engulfing WT cells Rac1 was found in the perinuclear region (Fig.17A), while in engulfing cells it concentrated at one pole of the macrophage (Fig.17B). This pole was surrounded by well organized F-actin filaments, and F-actin was also concentrated in the phagocytic cup (Fig.17C). Seeing many sections we noticed that the uptake of the apoptotic cells occurred through one or two Rac1 rich poles, which acted as an effective engulfing gate in the macrophage. Figure 17B illustrates how such an engulfing pole works: one apoptotic cell is already engulfed, the second one is still being taken up, while the macrophage grabs already the third apoptotic cell. The apoptotic cells taken up by one gate seem to be compartmentalized by Rac-rich structures from those apoptotic cells, which were taken up by the other gate (Fig.17D). As a result, in each macrophage, which engulfed more than 3 apoptotic cells one or two groups of apoptotic cells could be detected when analyzed with 2% osmium tetroxide for contrast enhancement before phase contrast microscopy (Fig. 17E).

To support our statements, we also analyzed time-lapsed microscopic images of the engulfing fields. For example, in video 1 it is demonstrated how a wild-type macrophage takes up three apoptotic cells through two portals when the target cell number is lower (4 target:1 macrophage). In video 2 we demonstrate the uptake of 6 apoptotic cells via two portals at higher target cell number (6 target:1 macrophage). For better vision the macrophages are labeled with CMTMR and the apoptotic cells engulfed are seen as black wholes. A shot from the movie is marked for the two centers in Figure 17.F.
Figure 17. Wild-type macrophages form two phagocyte poles characterized by Rac1 accumulation for the uptake of apoptotic cells. (A) Distribution of Rac1 protein in wild-type engulfing and non-engulfing macrophages detected by confocal microscopy. Please note that in non-engulfing macrophages the localisation of Rac1 (blue colour) is perinuclear. In engulfing macrophages Rac1 is concentrated at one pole of the cell around the apoptotic cells (green). (B) Illustration in a 3D reconstruction of the efficient functioning of the engulfment centre formed in wild-type macrophages by accumulation of Rac1 molecules at one pole of the cell: one apoptotic cell is already engulfed, the second one is still being taken up and is surrounded by Rac1 rich lamellipodia (seen on the right panel with XYZ projections), while the macrophage grabs already the third apoptotic cell. (C) Distribution of F-actin molecules in wild-type macrophages digesting apoptotic cells detected by confocal microscopy. F-actin molecules are concentrated in the phagocytic cup and the well organized F-actin filaments occur surround the engulfing centre.(D) Apoptotic cells taken up by distinct engulfing centers are separated within the macrophages (XYZ projection from a 3D stack). Bars represent 10 µm. (E) Following staining with 2% osmium tetroxide for
contrast enhancement before phase contrast microscopy apoptotic cells can be detected in macrophages forming one or two groups. Arrows point at the two distinct groups of apoptotic cells within the macrophages. (F) A shot from video2.mov to mark the 2 separate engulfing centers in a CMTMR labeled macrophage taking up 4 and 2 apoptotic cells (appearing as black holes), respectively.

1.9. In TG2<sup>-/-</sup> macrophages Rac1 cannot be concentrated around the apoptotic cells and the efficient engulfing centre is not formed

In contrast, in TG2<sup>-/-</sup> cells most of the Rac1 was found in the perinuclear region in both non-engulfing (Fig. 18A) and engulfing macrophages (Fig.18B) implying that in the absence of TG2 Rac1 cannot be fully activated and concentrated around the apoptotic cells and the Rac rich pole could not be formed. In addition, due to the improper signaling an abnormal F-actin polymerisation was detected at the apoptotic cell binding sites (Fig.18C). Because in the absence of TG2 an efficient engulfing pole could not be built up, TG2<sup>-/-</sup> macrophages tend to take up the apoptotic cell at different sites (Fig.18B and D).

To provide a further proof of concept for the statements, time-lapse videos were prepared following labeling TG2<sup>-/-</sup> macrophages with CMTMR at 1macrophage: 6 target cell ratio. Video3 shows a macrophage, which all the time interacts with apoptotic cells, but is unable to form the phagocyte portal in the video timeframe (consistent with Fig.7B, more than 50% behave like this). Next we analyzed those macrophages, which engulfed apoptotic cells. Video 4 shows a macrophage, which takes up 2 apoptotic cells at two different sites synchronously. However, the uptake of both cells is much slower than seen in video 1 or 2 for the wild-type macrophages and not finalized in the video timeframe. Video 5 shows a macrophage which engulfs also two apoptotic cells and has difficulties in taking up the second one. A third apoptotic cell may sense this portal, is moving around it, but its engulfment was not initiated during the time period the video was taken. This video also indicates that the apoptotic cell is very likely not a passive target of the uptake process, but actively interacts with the macrophage to initiate the engulfment. Finally, we found very rarely
(1-2% at this cell density) TG2<sup>−/−</sup> macrophage that bypassed the loss of TG2 and took up more than one apoptotic cell via the same portal during the video timeframe. To demonstrate this, video 6 shows a TG2<sup>−/−</sup> macrophage, which took up three apoptotic cells via the same portal. This macrophage engulfed an additional apoptotic cell via a second portal. The presence of such TG2<sup>−/−</sup> macrophages might indicate that the macrophage population we investigate is heterogeneous in their demand for the presence of TG2 to mediate phagocytosis. This would be consistent with the observation of Miyanishi (Miyanishi et al. 2007), who found that phagocytosis of non-treated peritoneal macrophages is dependent on the phosphatidylserine receptor Tim4, whereas thioglycolate-elicited macrophages mediate phagocytosis via MFG-E8. Tim4 and MFG-E8, however, are expressed by both macrophage populations, though at markedly different levels.
**Figure 18.** In the absence of transglutaminase 2 Rac1 is not concentrated at the sites of apoptotic cells and the efficient engulfing centre is not formed. (A) Distribution of Rac1 protein in TG2<sup>−/−</sup> non-engulfing macrophages detected by confocal microscopy. Localisation of Rac1 (blue colour) is perinuclear in non-engulfing macrophages. (B) Distribution of Rac1 protein in TG2<sup>−/−</sup> engulfing macrophages detected by confocal microscopy. Please note that Rac1 is not concentrated around the apoptotic cell (green) in large amounts to form an engulfing centre in TG2<sup>−/−</sup> macrophages (3D reconstruction). (C) Distribution of F-actin molecules in TG2<sup>−/−</sup> macrophages digesting apoptotic cells detected by confocal microscopy. Abnormal F-actin organisation at the sites of the apoptotic cells is visible and the fine actin filament network, which normally surrounds the engulfing centre, is missing. (D) TG2<sup>−/−</sup> macrophages take up the second apoptotic cell at different site of the cell, rather than through the same gate. Arrows point at macrophages that took up apoptotic cells at different sites.
1.10. In lack of TG2 the expression of the integrin β₃ protein was elevated but the receptors were not concentrated at the sites of apoptotic cells

In line with the mRNA data, the expression of integrin β₃ was elevated in TG2⁻/⁻ cells (Fig.19A) as compared to their WT counterparts (Fig.19B). Despite of the increased protein levels, we did not detect any significant change in the cell surface distribution of integrin β₃ receptors in the absence of TG2 on non-engulfing macrophages (Fig.19D) as compared to WT cells (Fig.19C). During phagocytosis, however, WT cells concentrated integrin β₃ at one pole of the cell, where the uptake of the apoptotic cells occurred, while TG2⁻/⁻ macrophages were unable to do so.

**Figure 19.** In the absence of transglutaminase 2 integrin β₃ is not concentrated at the sites of apoptotic cells and the efficient engulfing centre is not formed. Cell surface expression of integrin β₃ on wild type (A) and TG2⁺/⁺ (B) macrophages determined by flow cytometric analysis. M, mean. Cell surface distribution of integrin β₃ on wild type (C) and TG2⁻/⁻ (D) engulfing and non-engulfing macrophages determined by confocal microscopy analysis. Note accumulation of the protein in the phagocytic cup in case of wild type macrophages while this accumulation of the protein alongside the apoptotic cell is missing on the surface of TG2 null macrophages. Bars represent 10 µm.
The lack of integrin β3 accumulation around the apoptotic cells in TG2−/− macrophages seems to be specific for integrin β3, since accumulation of another phagocytic receptor, LRP-1, which was also shown to interact with TG2 (Zemskov et al. 2007) was not affected (Fig.20). These data indicate that TG2 is required for the recognition of apoptotic cells by the MFG-E8/integrin β3 ligand/receptor complex and for its consequent accumulation around the apoptotic cell. In the absence of integrin β3 signaling the initiation of the engulfing centre, which efficiently take up the additional apoptotic cells, is also impaired.

![Image](image.png)

**Figure 20.** Cell surface distribution of LRP-1 on engulfing macrophages determined by confocal microscopy analysis. Note accumulation of the protein in the phagocytic cup in both wild type and TG2−/− macrophages. Bars represent 10 µm.

1.11. TG2 interacts with milk fat globulin-8

To investigate the mechanism by which TG2 might promote recognition of apoptotic cells by MFG-E8/integrin β3, we tested possible molecular interactions of TG2 besides its known binding to integrin β3 (Akimov et al., 2000). Since recognition of phosphatidylserine on apoptotic cells plays a key role in the removal of dying cells, and MFG-8 was shown previously to bridge integrin β3 to phosphatidylserine on apoptotic cells (Hanayama et al., 2002), we tested whether TG2 can also recognize phosphatidylserine using membrane strips spotted with various phospholipids. As
shown in Figure 21A, TG2 did not bind to phosphatidylserine on the membrane strips indicating that while MFG-E8 acts as a bridging molecule between phosphatidylserine and integrin β3, TG2 does not.

Since in cell adhesion TG2 was shown to enhance integrin β3 signaling by forming ternary complexes with the integrin β3 ligand fibronectin and integrin β3 via binding to both (Akimov et al. 2000), we decided to check whether TG2 can similarly interact with MFG-E8, the phagocytic integrin β3 ligand, by using surface plasmon resonance technique. Sensograms obtained at 5 different MFG-E8 concentrations are shown in Figure 21B indicating interaction of MFG-E8 with TG2 in a concentration range of 5 to 200 nM. Fitting the sensograms according to a simple 1:1 binding model the association constant (K_a = 1.86 x 10^8) was determined for this interaction. As a comparison, the association constant (K_a = 1.00 x 10^7) for the binding of TG2 to fibronectin (a known interacting partner of TG2) was also determined. These data imply that the MFG-E8/TG2 complex is reasonably stable and suggest that this interaction may also occur under physiological conditions.
**Figure 21.** TG2 binds to MFG-E8 with higher affinity than to fibronectin, but does not interact with phosphatidylserine. (A) GST, GST-PX (phosphoinositide-binding structural domain) and GST-TG2 were incubated with PIP Strips membrane, and bound proteins were detected with HRP-conjugated anti-GST antibody. Phospholipids blotted on the PIP Strips membrane; LPA, lysophosphatidic acid; LPC, lysophosphocholine; PtIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-phosphate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; S1P, sphingosine-1-phosphate; PA, phosphatidic acid; PS, phosphatidylserine. (B) Sensograms for the interaction of MFG-E8 and fibronectin with TG2 detected at the indicated concentrations of MFG-E8 and fibronectin by surface plasmon resonance.
2. A subline of TG2⁻/⁻ mice compensate the loss of TG2 by a much higher expression of integrin β3

2.1. Macrophages from a subline of TG2⁻/⁻ mice have very low phagocytic activity

To generate enough TG2⁻/⁻ macrophages for the phagocytosis experiments, we crossed TG2⁻/⁻ mice with each other through several generations. After a one year period of crossing we noticed that macrophages from these mice had a lower phagocytic activity than macrophages from those TG2⁻/⁻ mice which were originated from crossing heterozygous mice. While following exposure to apoptotic cells for 1 hour 45±12% of TG2⁻/⁻ macrophages were able to engulf at least one apoptotic cell, only 25±8% (p < 0.05) of the macrophages from this subline had phagocytic activity. Then we looked by confocal microscopy at the number of apoptotic cells engulfed by individual macrophages. While we have found that phagocyting wild-type (Fig.22A) and TG2⁻/⁻ macrophages engulfed various numbers of apoptotic cells, macrophages from this subline engulfed predominantly one single apoptotic cell (Fig.22B). In most of the cases the uptake even of this single apoptotic cell has not been finished following a one hour phagocytosis, as it is illustrated in a 3D reconstruction (Fig.22C).
Figure 22. Macrophages from a subline of TG2<sup>-/-</sup> mice show altered Rac1 distribution and impaired phagocytosis. (A) Distribution of Rac1 protein in wild-type macrophages engulfing and non-engulfing macrophages. (B) Distribution of Rac1 protein in engulfing and non-engulfing macrophages from a subline of TG2<sup>-/-</sup> mice. (C) Distribution of Rac1 protein in a TG2<sup>-/-</sup> macrophage engulfing apoptotic cells detected by confocal microscopy (3D reconstruction) and XYZ projection from the 3D stack. Rac-1 is blue, apoptotic cells are green. Bars represent 5 µm. Data show one representative experiments of three.

2.2. Macrophages from a subline of TG2<sup>-/-</sup> mice have altered morphology

Not only the phagocytic capacity, but the appearance of the macrophages from this subline has also dramatically changed. While non-engulfing TG2<sup>+/+</sup> (Fig. 22A) and TG2<sup>-/-</sup> cells had fibroblastoid forms, and Rac1 was located in the cytoplasm in the perinuclear region, in non-engulfing macrophages of the TG2 subline a clear
recruitment of Rac1 to the plasma membrane could be detected (Fig. 22B). This was accompanied by an enhanced lamellipodia formation and motility, which as compared to wild-types (Video 7) seemed to inhibit rather then to promote phagocytosis of apoptotic cells (Video 8 and 9).

2.3. Altered macrophage morphology in the TG2\(^{-/-}\) subline is related to an enhanced integrin \(\beta_3\) signaling

Since enhanced motility and membrane localization of Rac1 is related generally to Rac1 activation (Katoh et al. 2006), we decided to determine the levels of active Rac1 in these macrophages with PAK pull down assay. The absolute levels of Rac1 have not changed in these TG2\(^{-/-}\) macrophages (Fig. 23A) as compared to wild-type or to the average TG2\(^{-/-}\) macrophages. However, the amount of active Rac1 was significantly elevated (Fig. 23B).

Since integrin \(\beta_3\) signaling can be coupled to Rac1 activation leading to enhanced motility via activating RhoG (Katoh et al. 2006), we decided to determine the basal and active levels of RhoG as well as the cell surface levels of integrin \(\beta_3\). As shown in Figure 23A, total RhoG levels were slightly elevated in all type of TG2\(^{-/-}\) macrophages, as compared to the wild-types. However, while RhoG-GTP levels were not elevated in the TG2\(^{-/-}\) macrophages (Fig. 16B), in the macrophages from the TG2\(^{-/-}\) subline an elevated Rho-GTP level was detected (Fig. 23C).
Figure 23. Macrophages from a subline of TG2<sup>−/−</sup> mice show altered integrin β₃ signaling. (A) Total levels of RhoG and Rac1 in the wild-type, in the average and in the integrin β₃ high TG2<sup>−/−</sup> macrophages determined by immunoblot analysis. (B) Rac1 is activated in wild-type but not in TG2<sup>−/−</sup>/integrin β₃ high macrophages exposed to carboxylate-modified latex beads in same experiment discussed in Figure 16. Please note, that the basal Rac1-GTP levels are strongly elevated as compared to the wild-type macrophages. (C) Rho G is activated in both wild type and in TG2<sup>−/−</sup>/integrin β₃ high macrophages exposed to carboxylate-modified latex beads. Please note that the basal RhoG-GTP levels are elevated as compared to the wild-type macrophages.

Concomitantly, much higher levels of integrin β₃ were found on the cell surface of these macrophages (Fig. 24A). When these macrophages were kept in the presence of increasing concentrations of soluble vitronectin, which competes with the binding of the immobilized integrin β₃ ligands and thus interferes with the signaling (Preissner et al.1991, Savill et al. 1990), the levels of active Rac1 were decreased proving that the elevated amount of Rac1-GTP levels are indeed a consequence of the enhanced integrin β₃ levels and signaling in these cells (Fig. 24B).
Figure 24. Macrophages from a subline of TG2⁻/⁻ mice express high levels of integrin β₃. (A) Cell surface expression of integrin β₃ determined by flow cytometric analysis on wild type, on the TG2⁻/⁻ and on macrophages from the TG2⁻/⁻ subline, respectively. M, mean. (B) 1 hour preincubation with increasing concentrations of vitronectin decreases the basal levels of active Rac1 in the TG2⁻/⁻/integrin β₃ high macrophages. Basal levels of Rac1-GTP were determined as described previously. (C) Cell surface distribution of integrin β₃ determined by confocal microscopy analysis on TG2⁺/⁺ and TG2⁻/⁻ engulfing macrophages, respectively. Please note accumulation of integrin β₃ around the apoptotic cells in TG2⁺/⁺ macrophages, while the even distribution of it in TG2⁻/⁻/integrin β₃ macrophages.

2.4. Apoptotic cells can induce the activation of RhoG in macrophages from the TG2 subline

Previous data discussed above have shown that apoptotic cells cannot activate RhoG and Rac1 of the integrin β₃ signaling pathway in TG2⁺/⁺ macrophages. But in the macrophages of this subline increased expression of integrin β₃ could maintain an elevated Rac1 level. That is why we decided to test the functionality of the integrin β₃
pathway during phagocytosis of apoptotic cells. In context with our previous observations macrophages of this TG2 subline also did not concentrate integrin \( \beta_3 \) around the apoptotic cells (Fig. 24C). However, due to the increased receptor levels, intense stainings of integrin \( \beta_3 \) could be detected all around the macrophage including the phagocytic cup. Next the activation of RhoG and Rac1 was tested. A shown Figure 23C, when macrophages from the TG2 subline were exposed to carboxylate-modified latex beads the amount of active RhoG increased indicating that increased levels of integrin \( \beta_3 \) compensated the loss of TG2 in the phagocytosis signaling at least at the level of RhoG activation. However, the elevated Rac1-GTP levels could not be further increased when macrophages were exposed to carboxylate-modified latex beads (Fig. 23B). In context with these observations, when these macrophages were exposed to apoptotic cells, Rac1 remained recruited all over the periphery and did not accumulate around the apoptotic cells (Fig. 22B and C).

2.5. Over-expression of wild-type Rac1 can compensate the defect of phagocytosis in macrophages of the TG2\(^{-/}\) subline

There are two possible explanations for the lack of Rac1 activation by apoptotic cells. Either TG2 is required for proper apoptotic cell-induced signaling to activate Rac1, which is not compensated by the enhanced levels of integrin \( \beta_3 \), or as a result of the TG2-independent, but integrin \( \beta_3 \)-dependent enhanced motility the amount of free Rac1, that could be activated and localized around the apoptotic cells, is exhausted. To test whether providing of additional Rac1 molecules could overcome the defect in integrin \( \beta_3 \) signaling, macrophages from the TG2\(^{-/}\) subline were transfected with wild type or constitutively active Rac1 molecules by adenoviral gene delivery, and their phagocytosis was detected by FACS analysis. Addition of constitutively active Rac1 (Fig. 25A) completely inhibited the phagocytosis of apoptotic cells (Fig. 25B), indicating that switching on and off of Rac1 is required for proper phagocytosis of apoptotic cells.
Figure 25. Over-expression of wild-type Rac1 can compensate the defect of phagocytosis in macrophages of the TG2+/integrin β3 high macrophages. (A) Levels of basal Rac1 following adenoviral delivery of wild-type or constitutively active Rac1 into TG2+/integrin β3 high macrophages. (B) Phagocytosis of constitutively active Rac-1-transfected TG2+/integrin β3 high macrophages. Please note the lack of uptake of apoptotic cells by these macrophages. Rac-1 is blue, apoptotic cells are green. (C) Phagocytosis of wild-type Rac-1-transfected TG2+/integrin β3 high macrophages. (D) Distribution of the Rac1 protein in a wild-type Rac1-transfected TG2+/integrin β3 high macrophage engulfing two apoptotic cells detected by confocal microscopy (3D reconstruction). Please, note concentration of Rac1 around the two apoptotic cells. Bars represent 5 µm.

However, when these cells were transfected with wild type Rac1 (Fig. 25A), Rac1 accumulation could be detected around the apoptotic cells to form the efficient phagocytic portal (Fig. 25D). Consequently the phagocytosis rate of the Rac1-transfected cells reached that of the wild type cells (75±12 % and 82±7% for wild-type and TG2+/integrin β3 high macrophages, respectively). Not only the percentage of the engulfing macrophages has increased following Rac1 transfection, but Rac1-transfected TG2+/ integrin β3 high macrophages also efficiently took up apoptotic cells, though their enhanced motility has remained (Fig. 25C, video 10).
Our previous studies have demonstrated that wild-type macrophages take up apoptotic cells via one or two portals. As demonstrated in Figure 25C and video 11, Rac1–transfected TG2/−/−/integrin β3 high macrophages engulf apoptotic cells also via two portals, indicating that not the number of integrin β3 molecules determine the number of phagocytic portals.

2.5. Apoptotic cell-induced formation of 3-phosphoinositides is impaired in TG2−/− macrophages

Activation of phosphatidylinositol-3-OH kinase (PI-3kinase) is required for proper phagocytosis (Leverrier et al. 2003), and both DOCK180 and ELMO contains recognition domains for 3-phosphoinositides for proper membrane localization and thus directing Rac1 activation (Ravichandran et al. 2007). To detect formation of 3-phosphoinositides during the phagocytosis of apoptotic cells both wild-type (Video 12) and TG2 null macrophages (Video 13) were transfected by PLCδ-PHD-GFP. Apoptotic cell recognition induced formation of 3-phosphoinositides around the apoptotic cells in wild-type macrophages, while this induction was impaired in TG2−/− macrophages indicating that not only the activation of RhoG, but proper activation of the phosphatidylinositol-3-OH kinase is also under the control of TG2.
LEGEND TO SUPPLEMENTARY VIDEO

**Video1.** A wild type macrophage engulfs three apoptotic thymocytes via two phagocytic portals. Wild-type macrophages were exposed to apoptotic thymocytes in 1 macrophage:4 target cell ratio. Time-lapse video was taken as indicated in the Materials and Methods. The process of phagocytosis is shown 160 times faster than the real speed.

**Video2.** A wild type macrophage engulfs six apoptotic thymocytes via two phagocytic portals. CMTMR labeled wild-type macrophages were exposed to apoptotic thymocytes in 1 macrophage:6 target cell ratio. Time-lapse video was taken as indicated in the Materials and Methods. The process of phagocytosis is shown 160 times faster than the real speed.

**Video3.** A TG2⁻/⁻ macrophage exposed to apoptotic cells incapable of forming phagocytic portals. CMTMR labeled TG2⁻/⁻ macrophages were exposed to apoptotic thymocytes in 1 macrophage:6 target cell ratio. Time-lapse video was taken as indicated in the Materials and Methods. The process of phagocytosis is shown 160 times faster than the real speed.

**Video4.** A TG2⁻/⁻ macrophage exposed to apoptotic cells engulfing two apoptotic cells synchronously via two phagocytic portals. CMTMR labeled TG2⁻/⁻ macrophages were exposed to apoptotic thymocytes in 1 macrophage:6 target cell ratio. Note that the uptake is not finished within the video time frame. Time-lapse video was taken as indicated in the Materials and Methods. The process of phagocytosis is shown 160 times faster than the real speed.

**Video5.** A TG2⁻/⁻ macrophage exposed to apoptotic cells engulfing two apoptotic cells synchronously via two phagocytic portals, but having difficulties in taking up the second one. Note that a third apoptotic cell seems to sense this portal, is moving around it, but is not taken up during the time period the video was taken. CMTMR labeled TG2⁻/⁻ macrophages were exposed to apoptotic thymocytes in 1 macrophage:6
target cell ratio. Time-lapse video was taken as indicated in the Materials and Methods. The process of phagocytosis is shown 160 times faster than the real speed.

**Video6.** A TG2\(^+\) macrophage exposed to apoptotic cells engulfing four apoptotic cells via two phagocytic portals. CMTMR labeled TG2\(^+\) macrophages were exposed to apoptotic thymocytes in 1 macrophage:6 target cell ratio. Time-lapse video was taken as indicated in the Materials and Methods. The process of phagocytosis is shown 160 times faster than the real speed.

**Video7.** Subsequent uptake of three apoptotic cells by wild-type macrophages using two phagocyte portals. Wild-type macrophages were exposed to apoptotic thymocytes in 1 macrophage:6 target cell ratio. Time-lapse video was taken as indicated in the Materials and Methods. The process of phagocytosis is shown 160 times faster than the real speed.

**Video8.** Enhanced lamellopodia formation and motility of high integrin \(\beta_3\) expressing TG2\(^-\) macrophages, which is not accompanied by an enhanced phagocytic activity. Macrophages isolated from the mice of the TG2\(^-\) subline were exposed to apoptotic thymocytes in 1 macrophage:6 target cell ratio. Time-lapse video was taken as indicated in the Materials and Methods. Please, note that the actively moving macrophage interacts all the time with apoptotic cells, still engulfment is not seen during the video time frame. The process of phagocytosis is shown 160 times faster than the real speed.

**Video9.** Enhanced lamellopodia formation and motility of high integrin \(\beta_3\) expressing TG2\(^-\) macrophages, which is not accompanied by an enhanced phagocytic activity. CMTMR-labeled macrophages isolated from the mice of the TG2\(^-\) subline were exposed to apoptotic thymocytes in 1 macrophage:6 target cell ratio. Time-lapse video was taken as indicated in the Materials and Methods. Please, note that the actively moving macrophage interacts all the time with apoptotic cells, still engulfment is not seen during the video time frame. The process of phagocytosis is shown 160 times faster than the real speed.
**Video10.** Transfection of wild-type Rac1 efficiently enhances the phagocytic activity of TG2−/− null/integrin β3 high macrophages. CMTMR labeled high integrin β3 expressing, wild-type Rac1 transfected TG2−/− macrophages were exposed to apoptotic thymocytes in 1 macrophage:6 target cell ratio. Time-lapse video was taken as indicated in the Materials and Methods. Please note, that the motility of the macrophage has not altered, but engulfment of several apoptotic cells is documented during the video time frame. The process of phagocytosis is shown 160 times faster than the real speed.

**Video11.** Increase in integrin β3 expression does not increase the number of phagocytic portals in TG2−/− macrophages. A macrophage is shown taking up 4 apoptotic cells via two portals. CMTMR labeled high integrin β3 expressing, wild-type Rac1 transfected TG2−/− macrophages were exposed to apoptotic thymocytes in 1 macrophage:6 target cell ratio. Time-lapse video was taken as indicated in the Materials and Methods. The process of phagocytosis is shown 160 times faster than the real speed.

**Video12.** 3-phosphoinositides are formed in the phagocytic cup of macrophages engulfing apoptotic cells. PLCδ-PHD-GFP transfected macrophages were exposed to apoptotic thymocytes in 1 macrophage:6 target cell ratio. Time-lapse video was taken as indicated in the Materials and Methods. The process of phagocytosis is shown 160 times faster than the real speed.

**Video13.** Formation of 3-phosphoinositides in TG2−/− macrophages exposed to apoptotic cells is impaired. PLCδ-PHD-GFP transfected TG2−/− macrophages were exposed to apoptotic thymocytes in 1 macrophage:6 target cell ratio. Time-lapse video was taken as indicated in the Materials and Methods. The process of phagocytosis is shown 160 times faster than the real speed.
DISCUSSION

Tissue transglutaminase is a multifunctional enzyme and its sequence motifs and domains can each be assigned distinct cellular functions, including the regulation of cytoskeleton, cell adhesion, intracellular signaling and cell death. To study the function of TG2 in vivo, TG2 knockout mouse strains have been generated in two laboratories (DeLaurenzi and Melino 2001; Nanda et al. 2001). These animals are viable, reach adulthood and show no apparent abnormalities. However, when our laboratory started to examine the in vivo apoptosis program of thymus, it was found that the thymus disappears slower in the TG2−/− animals than in their wild-type counterparts following injection of various apoptotic stimuli (Szondy et al. 2003). The delayed involution of the thymus to apoptotic stimuli was partially the result of an impaired phagocytosis of apoptotic cells. TG2 can promote in vivo phagocytosis from the side of apoptotic cells by facilitating the phosphatidylserine exposure (Sarang et al. 2007), or by crosslinking the S19 ribonuclear protein that acts as chemotactic factor for macrophages (Nishiura et al. 1998). But the main defect was found in macrophages. This was partially related to a defect in TGF-β activation (Szondy et al. 2003), as TGFβ released by macrophages digesting apoptotic cells (Fadok et al. 1998) can promote phagocytosis of apoptotic cells (Rose et al. 1995). However, addition of recombinant TGFβ could not fully activate phagocytosis of apoptotic cells by TG2 null macrophages indicating that TG2 has additional roles in the phagocytosis process.

In this study we investigated phagocytosis of apoptotic cells by peritoneal macrophages in vitro to understand how the TG2 contributes in macrophages to proper engulfment of apoptotic cells besides activating TGF-β. To clarify the role of TG2 we used peritoneal macrophages derived from mice lacking TG2 and wild type macrophages in an in vitro phagocytosis model.

The engulfment of apoptotic cells is regulated by a highly redundant system of receptors, bridging molecules and 'eat me' signals. The complexity of the system is reflected by the term: ‘engulfment synapse’, used to describe the interaction between a phagocytic cell and its target (Grimsley and Ravichandran 2003). Recruitment of integrins to the apoptotic cells to form phagocytic cup suggests that integrins are one of the components of the “engulfment synapse”. The role of integrin in the formation of “immunological synapse” between –cells and antigen-presenting cells has also
been reported. A major feature of the mature “immunological” synapse is the concentration of all the recognition receptor, similar to “engulfment synapse” (Bromley et al. 2001).

While in previous studies the development of the engulfment synapse has been described, hardly any kinetic analysis of the apoptotic engulfment process has been described so far. In our study we described for the first time that in contrast to the present view of stochastic uptake, phagocytosis of apoptotic cells occurs through one or two engulfing gates within the macrophages, which will take up the apoptotic cells. This observation is consistent with the recently published report by Nakaya et al. 2008, who demonstrated that the phagocytosis of apoptotic cells in NIH3T3 fibroblasts transfected with integrin $\alpha_v\beta_3$ occurs via a single portal. We propose that these portals are efficient engulfing centers characterized by phagocytic receptor and Rac1 accumulation following interaction with the apoptotic cells at a pole of the macrophage, but their place or number is very likely determined by yet unknown molecules. Accumulation and activation of integrin $\beta_3$ around the apoptotic cell is part of the initiation of such a centre, and occurs with the contribution of cell surface TG2, at least in thioglycollate elicited peritoneal macrophages. In the absence of TG2 the formation of phagocyte portal is less efficient, and even those portals, which are formed, take up the apoptotic cells at a much lower rate. As a result, the uptake of apoptotic cells becomes slow and random.

As an explanation for the slower phagocytosis, we found an altered integrin signaling in TG2 null macrophages. Neither activation of RhoG, nor that of Rac1 was found proper in TG2 null macrophages. As a result Rac1 could not accumulate properly at a pole of the macrophage and the F-actin structure was also disorganized. To compensate the loss of TG2 macrophages increased the expression of integrin $\beta_3$.

In a search to explain how TG2 promotes accumulation of integrin $\beta_3$ and consequently integrin signaling, in the phagocytic synapse, we found that it is able to form a complex with MFG-E8, a known bridging molecule for integrin $\beta_3$. According to our results the binding affinity of TG2 to MFG-E8 was higher than that to fibronectin (Fn), a bridging molecule of integrin $\beta_3$ to the extracellular matrix (ECM). Integrins are relatively low affinity receptors for ECM proteins, including Fn. In contrast, TG2 binds with high affinity and 2:1 stoichiometry to Fn and its 42-kD fragment (Turner and Lorand 1989; Radek et al. 1993), and forms stable complexes.
with integrins. As a result, TG2 promotes integrin $\beta_3$ signaling in cell adhesion via stabilizing the integrin-Fn interaction. In addition, TG2 also promotes integrin clustering in the membrane, and consequently, amplifies integrin outside-in signaling (Akimov et al. 2000). Our finding that TG2 is also a binding partner for MFG-E8 indicates that TG2 might similarly affect integrin signaling in the context of phagocytosis as it does on the context of cell adhesion or migration on Fn.

We found that to promote phagocytosis TG2 must have intact guanine nucleotide binding sites. According to our findings for the cell surface transfer of TG2 guanine nucleotide binding is not required, but Tyr-274 residue plays important role (Balklava et al. 2002), and possibly proceeds by a process similar to that proposed for translocation of TG2 into the nucleus (Fésüs and Piacentini 2002). Thus we propose that guanine nucleotide binding is required for TG2 to fulfill its phagocytic function on the cell surface. This is in line with a previous observation, which demonstrated that GTP binding affects the effect of TG2 on integrin signaling (Johnson et al. 2005).

Though TG2 can act as a G protein in many physiological settings, proper guanine nucleotide binding of TG2 is not only required for its GTPase/ATPase signaling activities. It is needed also for placement of TG2 in conformation states that can facilitate physical interactions with several $\alpha$ integrin cytosolic tails and drive integrin-mediated effects on p38 mitogen-activated protein kinase signaling and cell differentiation (Johnson et al. 2005). In addition guanine nucleotide binding conformation may allow TG2 to form a complex with one or more extracellular matrix proteins, bridging molecule or external domains of cell surface receptor to induce rapid signaling events leading to actin rearrangement and efficient engulfment. Thus we propose that guanine nucleotide binding of TG2 in the context of phagocytosis is also required for gaining the proper conformation for the interaction with MFG-E8 and integrin $\beta_3$. Since the engulfment process was not dependent on the transamidation activity of TG2, it is very likely that the interaction between TG2 and MFG-E8/integrin $\beta_3$ complex occurs at its inactive conformational state.

Interestingly on the cell surface TG2 interacts also with another phagocytic receptor, LRP1, which is involved in the removal of TG2 from the cell surface (Zemskov et al. 2007). However, neither the levels, nor the association of LRP-1 with the phagocytic synapse was affected by the loss of TG2. Still based on our present results, we cannot exclude, that LRP-1 signaling is also affected by the loss of TG2.
While we were studying TG2\textsuperscript{-/-} mice, we found a subline, in which the macrophages compensated the loss of TG2 by a much higher protein expression of integrin $\beta_3$ on the cell surface than the average TG2\textsuperscript{-/-} macrophages. Integrin $\beta_3$ and RhoG signaling is involved not only in the phagocytosis of apoptotic cells, but also in cell motility (Katoh et al. 2006). As a result, increased expression of integrin $\beta_3$ in these cells resulted in enhanced motility with high basal active RhoG and Rac1 levels. Concomitant with the elevated basal active Rac1 levels, in non-engulfing TG2\textsuperscript{-/-} /integrin $\beta_3$ high macrophages Rac1 was located mostly bound to the cellular membranes, while we found it in the cytosol in wild –type and in regular TG2\textsuperscript{-/-} macrophages. When these macrophages were exposed to apoptotic cells, integrin $\beta_3$ remained evenly distributed on the cell surface, but, due to the enhanced expression a high density was detected around the apoptotic cells as well. In these cells addition of carboxylate-modified latex beads, unlike in regular TG2\textsuperscript{-/-} macrophages, triggered the activation of RhoG, indicating that the elevated integrin $\beta_3$ levels were sufficient to overcome the defect caused by the loss of TG2 in the initiation phase of integrin $\beta_3$ signaling. Still activation of Rac1, a downstream target of RhoG, could not be observed, and the activation of PI-3 kinase was impaired. Concomitantly, we could not detect a significant accumulation of Rac1 around the apoptotic cells, and the phagocytosis of apoptotic cells was more severely affected.

Though integrin $\beta_3$ and RhoG are involved in both cell motility and phagocytosis, TG2 is not equally required for both. While phagocytosis of apoptotic cells is enhanced, integrin-dependent migration on laminin for example is inhibited (Lentini et al. 2008) by TG2. Thus the loss of TG2 might not equally affect the phagocytic and motility signaling pathways, especially that of the activation of PI-3 kinase, which seems to be independent of integrin signaling in the context of phagocytosis (Raymund Birge, personal communication). Moderate increase in integrin $\beta_3$ expression observed in TG2\textsuperscript{-/-} macrophages is expected to enhance the impaired phagocytosis in the absence of TG2 and to induce enhanced motility, as it enhances the number of integrin receptors and the efficiency of its signaling. However, if the levels of integrin $\beta_3$ increase further, the two pathways might become competitive, integrin-induced motility winning by being too efficient in Rac1 activation and using up free Rac1. This is because total RhoG levels adapted to the changes in integrin $\beta_3$ expression and enhanced integrin signaling, but there were no
alterations in the basal Rac1 levels. This was suggested by the findings that while basal GTP-bound RhoG levels of the TG2 null/integrin $\beta_3$ high macrophages were lower than the ones induced by surrogate apoptotic cells in wild-type macrophages and remained inducible, basal levels of the GTP-bound Rac1 much exceeded the ones that were detectable in wild-type cells exposed to the surrogate apoptotic cells. Increasing the free Rac level by transfection of Rac1 molecules, we could improve the phagocytosis, but transfection of constitutively active Rac1 completely inhibited the phagocytosis of apoptotic cells. This observation confirms that of Nagaya et al.2008 and suggests that dynamic switching on and off of Rac1 is required for proper phagocytosis. Macrophages transfected with wild type Rac were able to concentrate additional Rac1 around the apoptotic cells and engulfed apoptotic cells with a similar rate as wild-type macrophages.

These data provide a further proof for the hypothesis that the role of TG2 in phagocytosis is to provide efficient integrin $\beta_3$ signaling around the apoptotic cells (either by promoting integrin $\beta_3$ clustering in the phagocytic cup, or by enhancing the affinity of the receptor for its ligand MFG-E8/phosphatidylserine), which in the absence of TG2 can also be achieved by an enhanced receptor density. Our data, however, might also suggest that TG2 is required for additional signaling pathways involved in the activation of PI-3kinase. Activation of PI-3kinase is essential for proper phagocytosis (Leverrier et al. 2003), and both DOCK180 and ELMO contain recognition domains for 3-phosphoinositides for proper membrane localization and thus directing Rac1 activation (Ravichandran and Lorenz 2007). Based on our data, integrin signaling will not affect the number of phagocytic poles formed, but as it was suggested by others, it might be critical in the initiation of the formation of the cellular pole (Ho et al. 2009).
SUMMARY

The clearance of apoptotic cells by macrophages plays a crucial role in tissue repair, suppressing inflammation and regulating immune responses. Transglutaminase 2 (TG2) is a protein crosslinking enzyme with diverse biological functions. Among many others it acts as an integrin β3 co-receptor. We have previously shown that in TG2−/− mice the in vivo clearance of apoptotic cells is defective leading to development of SLE like autoimmunity. This was partially related to a defect in TGF-β activation, as TGFβ released by macrophages digesting apoptotic cells promotes phagocytosis of apoptotic cells and inhibits inflammatory responses.

In the present work the role of TG2 was studied in details in the engulfment of apoptotic cells by macrophages. Here we report that TG2 promotes phagocytosis of apoptotic cells by acting on the macrophage cell surface in guanine nucleotide bound form. Besides being a binding partner for integrin β3, a receptor known to mediate the uptake of apoptotic cells via activating Rac1, we also show that TG2 binds milk fat globule EGF-factor 8 (MFG-E8), a protein known to bridge integrin β3 to phosphatidylserine on apoptotic cells. We report that in wild-type macrophages one or two engulfing portals are formed during phagocytosis of apoptotic cells that are characterized by accumulation of integrin β3 and Rac1. In the absence of TG2, although the levels of integrin β3 are enhanced, integrin β3 and consequently Rac1 can not be concentrated and activated at one pole of the macrophage. The defect in the αvβ3 integrin signaling leads to an abnormal actin cytoskeletal organization and the efficient engulfing gate is not formed. Together, our data indicate that TG2 is a new protein member of the phagocytic cup, which together with MFG-8 is required for proper apoptotic cell recognition and integrin β3 signaling.

In the present study we also describe a subline of TG2−/− mice, in which integrin β3 expression compensatory increases resulted alone in a high receptor concentration around the apoptotic cells without the requirement for accumulation. The elevated integrin β3 levels were sufficient to overcome the defect caused by the loss of TG2 in the initiation phase of integrin β3 signaling, but a significant accumulation of Rac1 around the apoptotic cells did not occur, and the phagocytosis of apoptotic cells was more severely affected. The lack of Rac1 accumulation was partially related to a defect of PI-3-kinase activation. Our data provide a proof for the
concept that the function of TG2 is to stabilize accumulated integrin $\beta_3$ concentration in the phagocytic cup.
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PUBLICATION

Publications related to the thesis


Other publications


First authored posters on international meetings

Tóth, B., Kis-Tóth, K., Szondy, Z.: Role of Fas ligand and nur77 in T cell apoptosis induced by retinoids. From transcription to physiology: Regulation of gene expression and protein. FEBS Summer School. Spetses, Greece, 2003

Tóth, B., Kis-Tóth, K., Szondy, Z.: Role of Fas ligand and nur77 in T cell apoptosis induced by retinoids. 12th Euroconference on Apoptosis, Chania, Greece, 2004

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