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Over-expression of integrin β3 can partially overcome the defect of integrin β3 signaling in transglutaminase 2 null macrophages

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ABSTRACT

Transglutaminase 2 (TG2) is a protein crosslinking enzyme with many additional biological functions. We have previously shown that in TG2$^{-/-}$ mice the *in vivo* clearance of apoptotic cells is defective leading to autoimmunity. TG2 contributes to the formation of phagocytic portals by binding to both integrin β_3, a known phagocytic receptor, and its bridging molecule, MFG-E8. In TG2 null macrophages integrin β_3 cannot accumulate around the apoptotic cells and its signaling is impaired. In the present study we describe a subline of TG2 null mice, in which a compensatory increase in integrin β_3 expression, which resulted alone in a high receptor concentration around the apoptotic cells without the requirement for accumulation, partially corrected the defect in integrin β_3 signaling. Our data provide a proof for the concept that the function of TG2 is to stabilize accumulated integrin β_3 concentration in the phagocytic cup.

1. Introduction

Apoptotic cells are generated by diverse physiological processes, ranging from the elimination of damaged (or precancerous) cells to deletion of cells during developmental morphogenesis [1]. The culmination of the apoptotic program *in vivo* is the phagocytosis of the apoptotic cell. In mammals prompt removal of apoptotic cells is required to prevent the release of potential self-antigens and the onset of autoimmune-like syndromes [2].

A number of receptors have been identified on macrophages that either directly or indirectly facilitate apoptotic cell recognition and uptake [3]. These receptors converge on two evolutionally conserved pathways upstream to the activation of the low molecular weight GTPase Rac1, which is obligatorily required for the uptake [4]. One of these receptors is the integrin $\alpha_v\beta_3$ (vitronectin receptor), which is bridged via the milk fat globulin EGF factor 8 (MFG-E8) molecule to the phosphatidylserine appearing on apoptotic cells [5]. The integrin β_3 pathway regulates Rac1 activity via the 180 kDa protein downstream of chicken tumor virus no. 10 (CT10) regulator kinase II (Dock180) and the engulfment and migration protein (ELMO), which form together an unconventional two-part guanine nucleotide exchange factor for Rac1 [6]. The formation of the DOCK180/ELMO complex is induced in the integrin β_3 pathway by the Trio-controlled RhoG-GTP [7]. Rac activation then leads to the formation of an actin-rich phagocytic cup, followed by the internalization of the target [8].

In addition to its role in the phagocytosis of apoptotic cells, the RhoG-mediated pathway also participates in cell migration, and is transiently activated wherever lamellopodias are formed [9] resulting in the formation of a basal Rac-GTP level in the continually migrating macrophages.

Transglutaminases are a family of thiol- and Ca^{2+}-dependent acyl transferases that catalyze the formation of a covalent bond between the γ-carboxamide groups of peptide-bound glutamine residues and various primary amines, including the ε-amino group of lysine in target proteins [10]. Eight distinct enzymatically active transglutaminases have so far been described [11]. TG2 is very unique among the TG family members, because besides catalyzing the formation of protein crosslinks, it is also a G protein, and possesses protein disulfide isomerase and protein kinase activities [12]. In addition, TG2 also interacts with integrins of the β_1 and β_3 subfamilies, and integrin/TG2 complexes are detected inside the cell during biosynthesis and accumulate as coreceptors on the cell surface [13,14]. TG2 can also bind to the major extracellular protein

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fibronectin [15], and cooperate with integrins in cell adhesion and motility through either a direct noncovalent interaction with the β_1 and β_3 integrin subunits or formation of stable ternary complexes with integrins and fibronectin [13,14]. These interactions induce integrin clustering and modify integrin signaling [16].

We have previously reported that TG2$^{-/-}$ mice develop an age-dependent autoimmunity due to defective *in vivo* clearance of apoptotic cells [17]. Recently we found that TG2 expressed on the cell surface of macrophages promotes the engulfment of apoptotic cells by forming a complex with both integrin β_3 and MFG-E8 [18]. In the absence of TG2 the apoptotic cell-induced integrin β_3 signaling leading to RhoG and Rac1 activation is impaired. Additionally, we also described that the uptake of apoptotic cells by wild-type macrophages occurs via one or two phagocyte portals that are characterized by the accumulation of phagocytic receptors in the phagocytic cup and concentration of Rac molecules to this pole of the macrophages. In the absence of TG2 both the formation and the uptake rate of these portals are less efficient than that of wild-type macrophages. TG2 null macrophages try to compensate the loss of TG2 by increasing the expression of both integrin β_3 and RhoG [18]. Here we describe a subline of TG2 null mice, in which a strong compensatory increase in integrin β_3 expression was able to correct partially the defect in integrin β_3 signaling.

2. Materials and methods

2.1. Antibodies and reagents

Purified mouse anti-Rac1 monoclonal (clone 102), and phycoerythrine (PE)-conjugated anti-mouse β_3 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 goat anti-mouse IgG, and Prolong antifade reagent were purchased from Invitrogen (Carlsbad, CA). Horseradish peroxidase-conjugated anti-rabbit, anti-goat and anti-mouse IgGs, anti-mouse IgG-FITC, and vitronectin were purchased from Sigma–Aldrich (Budapest, Hungary). Anti-RhoG (clone C-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The enhanced chemiluminescence for immunoblots analysis was purchased from Millipore (Millipore, Billerica, MA).

2.2. Cell culture

TG2$^{+/+}$ and TG2$^{-/-}$ [19] mice were injected with 2 ml 4% thioglycollate and 4 days later macrophages were obtained by peritoneal lavage. Macrophages were allowed to adhere in 24-well plates and non-adherent cells were washed away. For phagocytosis assays, macrophages were stained overnight with CMTMR (10 μM). Thymocytes from 4 weeks old WT mice were used as apoptotic target cells. Thymi were mechanically disrupted, and isolated thymocytes were labeled overnight with CFDA (6 μM). To induce apoptosis cells were treated with 4 μM ionomycin (Sigma–Aldrich) for 6 h, at which time, 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). Thymocytes and macrophages were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, penicillin and streptomycin (Gibco, Grand Island, NY). The study protocol was approved by Animal Care Committee of University of Debrecen.

2.3. Phagocytosis assay

CMTMR-stained macrophages were incubated with apoptotic thymocytes labeled with CFDA in 40:1 target/macrophage ratio for 1 h. Cells incubated with apoptotic thymocytes incubated at 4 °C were used as controls. 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) to determine the percentage of double labeled (engulfing) macrophages.

2.4. Immunofluorescence staining and confocal microscopy

Peritoneal macrophages isolated from WT and TG2$^{-/-}$ mice were plated in two-well chamber-slides (5×10^5/well) and cultured for 48 h before use. After co-culturing macrophages with apoptotic cells for 30 min, cells were washed, fixed in ethanol/ acetone 1:1 for 10 min at −20 °C. For integrin β_3 staining macrophages were blocked with 50% FBS for 30 min at 37 °C, then washed with ice cold HEPES buffer and stained with PE-conjugated anti-mouse β_3 integrin antibody for 15 min on ice. For intracellular staining, cells were permeabilized with 0.1% Triton X after fixation and blocked with 1% BSA. For detecting Rac1 cells were labeled 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 a secondary antibody. Images were taken with a Zeiss LSM 510 or Olympus FV1000 confocal laser scanning microscope. For visualizing the distribution of integrin β_3 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.

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

Total RNA was isolated by TRI reagent (Sigma–Aldrich). Total RNA concentrations were determined by spectrometry after DNAse treatment (Sigma–Aldrich). TaqMan Reverse Transcription Reagent Kit (Applied Biosystems, Foster City, CA) was used for generating cDNA according to manufacturer's instructions. 200 ng total RNA was used in a reaction volume of 50 μl. ABI PRISM 7700 Sequence Detection System (Applied Biosystems) was used to determine the relative gene expression. Gene primers and probes were designed and supplied by Applied Biosystems. 18S ribosomal RNA was used as an endogenous control to normalize the amount of the sample cDNA added to the reaction. The 18S primers were labeled 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.

2.6. Detection of active Rac1 and RhoG

Macrophages plated overnight were exposed to 2 μm carboxylated latex beads for 40 min, or left untreated as controls, to detect GTP-bound forms of Rac1 and RhoG. Pull-down assay was performed with the EZ-Detect Rac1 Activation Kit (Pierce, Rockford, IL) according to manufacturer's instruction. For the RhoG pull-down assay PAK-GST was replaced with an ELMO-GST protein. Active RhoG was detected by Western blot analysis using anti-RhoG antibody. For these assays 1–1.5 mg of total cell proteins (determined by the Bradford method) was used in the pull-downs.

2.7. Adenoviral gene delivery system

Recombinant, replication-deficient adenoviral vectors encoding either LacZ and the murine wild-type or constitutively active Rac1 [20] gene were produced using the AdEasy XL system (Stratagene) according to the manufacturer's instruction. Virus titers were determined by plaque assay in 293 cells after exposing them to

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virus for 48 h in DMEM medium supplemented with 2% serum and antibiotics. For gene delivery, 10^6 macrophages were exposed to the indicated amount of virus particles for 48 h in the same medium. LacZ expression was determined with X-gal staining, while Rac1 expression by Western blot analysis using anti-Rac1 specific antibodies.

2.8. Time-lapse video

For time-lapse video macrophages were or were not 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 [21]. 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 40×/1.2NA water immersion objective. 1024 × 1024 pixel images were taken every 10 s 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.

2.9. 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.

3. Results

3.1. Macrophages from a subline of TG2 null mice have very low phagocytic activity

To generate enough TG2 null macrophages for the phagocytosis experiments, we crossed TG2 null mice with each other through several generations and noticed that after a one year period of crossing macrophages from these mice had a lower phagocytic activity than macrophages from those TG2 null mice which were originated from crossing heterozygous mice. While following exposure to apoptotic cells in 40:1 target cells:macrophage ratio for 1 h 45 ± 12% of TG2 null macrophages were able to engulf at least one apoptotic cell [18], 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. 1a) and TG2 null macrophages engulfed various numbers of apoptotic cells [18], macrophages from this subline engulfed predominantly one single apoptotic cell (Fig. 1b). In most of the cases the uptake even of this single apoptotic cell has not been finished following a 1 h phagocytosis, as it is illustrated in a 3D reconstruction (Fig. 1c).

3.2. Macrophages from a subline of TG2 null mice have altered morphology

Not only the phagocytic capacity, but the appearance of the macrophages from this subline has also dramatically changed.

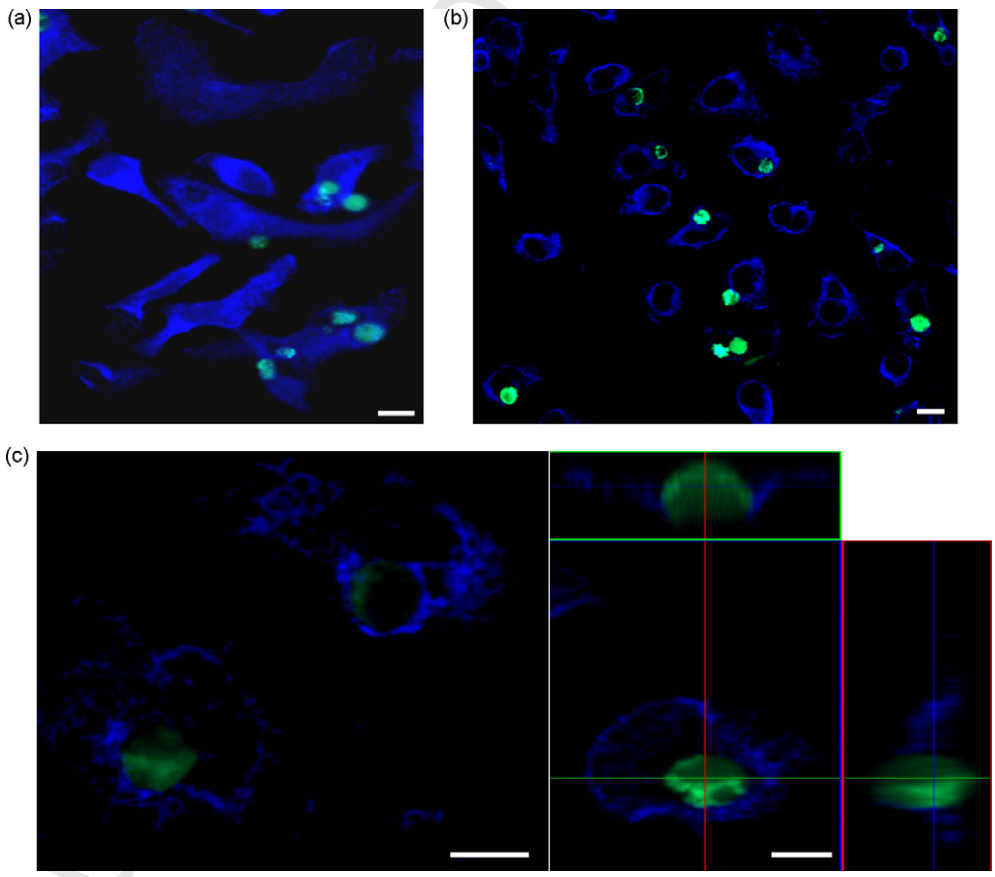

Fig. 1. Macrophages from a subline of TG2 null mice show altered Rac1 distribution and impaired phagocytosis. (a) Distribution of Rac1 protein in wild-type macrophages engulfing and non-engulfing macrophages. (b) Distribution of Rac1 protein in engulfing and non-engulfing macrophages from a subline of TG2 null mice. (c) Distribution of Rac1 protein in a TG2 null macrophage engulfing apoptotic cells detected by confocal microscopy (3D reconstruction) and XYZ projection from the 3D stack. Rac1 is blue and apoptotic cells are green. Bars represent 5 μm. Data show one representative experiments of three. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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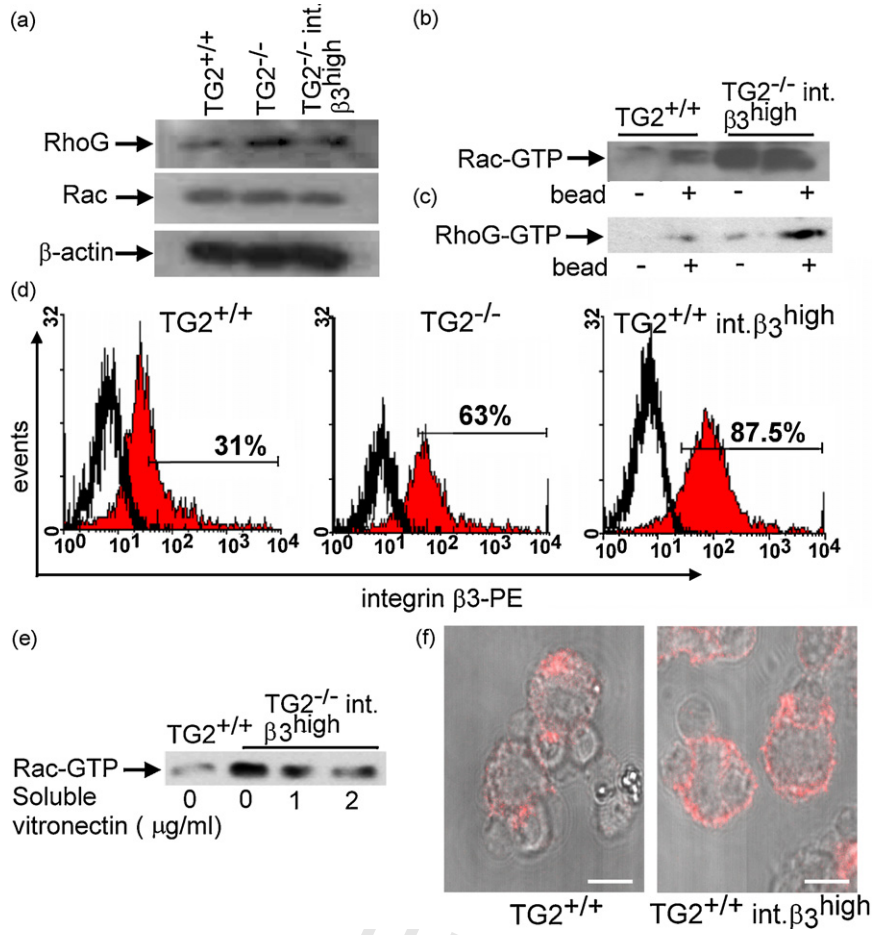

Fig. 2. Macrophages from a subline of TG2 null mice express high levels of integrin β_3 and show altered integrin β_3 signaling. (a) Basal levels of RhoG and Rac1 in the wild-type, in the average and in the integrin β_3 high TG2 null macrophages determined by immunoblot analysis. (b) Rac1 is activated in wild-type but not in TG2 null/integrin β_3 high macrophages exposed to carboxylate-modified latex beads. 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. Please note that the basal Rac1-GTP levels are strongly elevated as compared to the wild-type macrophages. (c) RhoG is activated in both wild-type and TG2 null/integrin β_3 high macrophages exposed to carboxylate-modified latex beads. Wild-type and TG2 null macrophages were exposed or not to carboxylate-modified latex beads. Following 40 min of phagocytosis GTP-bound Rho Gwas collected by pull-down with ELMO-GST protein from equal amounts of protein lysates and subjected to immunoblot analysis. Please note that the basal RhoG-GTP levels are elevated as compared to the wild-type macrophages. (d) Cell surface expression of integrin β_3 determined by flow cytometric analysis on wild-type, on the TG2 null and on macrophages from the TG2 null subline. M, mean. (e) 1 h preincubation with increasing concentrations of vitronectin decreases the basal levels of active Rac1 in the TG2 null/integrin β_3 high macrophages. Basal levels of Rac1-GTP were determined as described in (c). (f) Cell surface distribution of integrin β_3 determined by confocal microscopy analysis on wild-type and TG2 null engulfing macrophages. Please note accumulation of integrin β_3 around the apoptotic cells in wild-type macrophages (arrows), while the even distribution of it in TG2 null/integrin β_3 macrophages.

While non-engulfing WT (Fig. 1a) and TG2 null cells had fibroblastoid forms [18], and Rac1 was located in the cytoplasm in the perinuclear region [18], in non-engulfing macrophages of the TG2 subline a clear recruitment of Rac1 to the periphery could be detected (Fig. 1b). This was accompanied by an enhanced lamellopodia formation and motility, which as compared to wild-types (Video 1) seemed to inhibit rather than to promote phagocytosis of apoptotic cells (Videos 2 and 3).

3.3. Altered macrophage morphology in the TG2 null subline is related to an enhanced integrin β3 signaling

Since enhanced motility and membrane localization of Rac1 is related generally to Rac1 activation [9], we decided to determine the levels of active Rac1 in these macrophages with PAK pull-down assay. As shown in Fig. 2a, the absolute levels of Rac1 have not changed in these TG2 null macrophages as compared to wild-type or to the average TG2 null macrophages. However, the amount of active Rac1 was significantly elevated (Fig. 2b).

Since integrin β_3 signaling can be coupled to Rac1 activation leading to enhanced motility via activating RhoG [9], we decided to determine the basal and active levels of RhoG as well as the cell surface levels of integrin β_3. As shown in Fig. 2a, basal RhoG levels were elevated in all types of TG2 null macrophages, as compared to the wild-types. However, while RhoG-GTP levels were not elevated in the TG2 null macrophages [18], in the macrophages from the TG2 null subline an elevated RhoG-GTP level was detected (Fig. 2c).

Concomitantly, much higher levels of integrin β_3 were found on the cell surface of these macrophages (Fig. 2d). When these macrophages were kept in the presence of increasing concentrations of soluble vitronectin, which competes with the binding of the immobilized integrin β_3 ligands and thus interferes with the signaling [22,23], the levels of active Rac1 were decreased proving that the elevated amount of Rac1-GTP levels are indeed a consequence of the enhanced integrin β_3 levels and signaling in these cells (Fig. 2e).

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

Previous studies have shown that apoptotic cells cannot activate RhoG and Rac1 of the integrin β_3 signaling pathway in TG2 null

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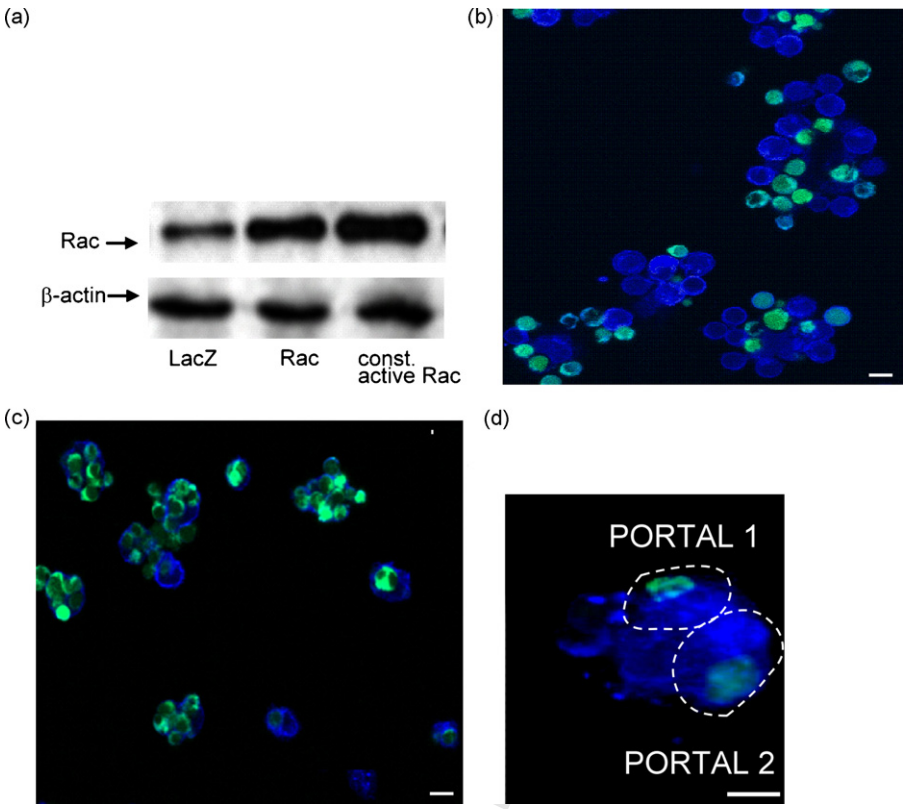

Fig. 3. Over-expression of wild-type Rac1 can compensate the defect of phagocytosis in macrophages of the TG2 null/integrin β_3 high macrophages. (a) Levels of basal Rac1 following adenoviral delivery of wild-type or constitutively active Rac1 into TG2 null/integrin β_3 high macrophages. (b) Phagocytosis of constitutively active Rac1-transfected TG2 null/integrin β_3 high macrophages. Please note the lack of uptake of apoptotic cells by these macrophages. Rac1 is blue and apoptotic cells are green. (c) Phagocytosis of wild-type Rac1-transfected TG2 null/integrin β_3 high macrophages. (d) Distribution of the Rac1 protein in a wild-type Rac1-transfected TG2 null/integrin β_3 high macrophage engulfing two apoptotic cells detected by confocal microscopy (3D reconstruction). Please note the concentration of Rac1 around the two apoptotic cells. Bars represent 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

macrophages [18]. But in the macrophages of this subline increased expression of integrin β_3 could maintain an elevated Rac1 level. That is why we decided to test the functionality of the integrin β_3 pathway during phagocytosis of apoptotic cells. In context with our previous observations [18] macrophages of this TG2 subline also did not concentrate integrin β_3 around the apoptotic cells (Fig. 2f). However, due to the increased receptor levels, intense stainings of integrin β_3 could be detected all around the macrophage including the phagocytic cup. Next the activation of RhoG and Rac1 was tested [4,6,7]. Since GTP-bound Rac1 in apoptotic cells could interfere with the pull-down assay, we used 2-μm carboxylate-modified latex beads in these studies, which have been previously used as surrogate apoptotic cells in signaling studies [24]. A shown in Fig. 2c, 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 β_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. 2b). 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. 1b and c).

3.5. Over-expression of wild-type Rac1 can compensate the defect of phagocytosis in macrophages of the TG2 null 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 β3, or as a result of the TG2-independent, but integrin β3-dependent enhanced motility the amount of free Rac1, which 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 β_3 signaling, macrophages from the TG2 null 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. 3a) completely inhibited the phagocytosis of apoptotic cells (Fig. 3b), indicating that switching on and off of Rac1 is required for proper phagocytosis of apoptotic cells. However, when these cells were transfected with wild-type Rac1 (Fig. 3a), Rac1 accumulation could be detected around the apoptotic cells to form the efficient phagocytic portal (Fig. 3d). Consequently the phagocytosis rate of the Rac1-transfected cells reached that of the wild-type cells ($75 \pm 12\%$ and $82 \pm 7\%$ for wild-type and TG2 null/integrin β_3 high macrophages, respectively). Not only the percentage of the engulfing macrophages has increased following Rac1 transfection, but Rac1-transfected TG2 null macrophages also efficiently took up apoptotic cells, though their enhanced motility has remained (Fig. 3c, Video 4). In contrast, transfection of wild-type Rac1 into average TG2 null macrophages resulted only in a mild increase of the phagocytosis rate (from $45 \pm 12\%$ to $56 \pm 13\%$).

Our previous studies have demonstrated that wild-type macrophages take up apoptotic cells via one or two portals. As demonstrated in Fig. 3c and Video 5, Rac1-transfected TG2 null/integrin β_3 high macrophages engulf apoptotic cells also via two portals, indicating that not the number of integrin β3 molecules determine the number of phagocytic portals.

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3.6. Apoptotic cell-induced formation of 3-phosphoinositides is impaired in TG2 null macrophages

Activation of phosphatidylinositol-3-OH kinase (PI-3kinase) is required for proper phagocytosis [25], and both DOCK180 and ELMO contain recognition domains for 3-phosphoinositides for proper membrane localization and thus directing Rac1 activation [26]. To detect the formation of 3-phosphoinositides during the phagocytosis of apoptotic cells both wild-type (Video 6) and TG2 null macrophages (Video 7) 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 null 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.

4. Discussion

Previous studies done in our laboratory have shown that the *in vivo* loss of TG2 results in impaired phagocytosis of apoptotic cells leading to autoimmunity [17]. The loss of TG2 affected the macrophage side, where TG2 seems to act as an integrin β_3 coreceptor interacting with both integrin β_3 and its phagocytosis ligand MFG-E8 [18]. While in wild-type macrophages integrin β_3, similarly to other phagocytic receptors, is concentrated in the phagocytic cup, in the absence of TG2, integrin β_3 was evenly distributed on the macrophage cell surface and the apoptotic cell-induced signaling of integrin β_3 leading to RhoG and Rac1 activation was impaired. TG2 null cells try to compensate the loss of TG2 by upregulating the expression of both integrin β_3 and its signaling partner RhoG to enhance the efficiency of integrin β_3 signaling in the absence of TG2.

Wild-type macrophages use one or two phagocyte portals to engulf apoptotic cells. Loss of TG2 did not affect the number of phagocytic portals, but in the absence of TG2 both the formation and the engulfment rate of the phagocyte portals formed were less efficient. So we proposed that the role of TG2 is to stabilize the phagocyte portals [18].

In the present study we describe a subline of TG2 null mice, in which the macrophages compensated the loss of TG2 by a much higher expression of integrin β_3 than the average TG2 null macrophages. Integrin β_3 and RhoG signaling is involved not only in the phagocytosis of apoptotic cells, but also in cell motility [9]. As a result, increased expression of integrin β_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 null/integrin β_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 null macrophages [18]. When these macrophages were exposed to apoptotic cells, integrin β_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 null macrophages [18], triggered the activation of RhoG, indicating that the elevated integrin β_3 levels were sufficient to overcome the defect caused by the loss of TG2 in the initiation phase of integrin β_3 signaling. Still activation of Rac1, a downstream target of RhoG, could not be observed, and the activation of PI-3kinase 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 β_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 [18], integrin-dependent migration on laminin, for example, is inhibited [27] by TG2. Thus the loss of TG2 might not equally affect the phagocytic and motility signaling pathways, especially that of the activation of PI-3kinase, which seems to be independent of integrin signaling in the context of phagocytosis (Raymund Birge, personal communication). Moderate increase in integrin β_3 expression observed in TG2 null 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 β_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 β_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 β_3 high macrophages were lower than the ones induced by surrogate apoptotic cells in wild-type macrophages and remained inducible (Fig. 2c), basal levels of the GTP-bound Rac1 much exceeded the ones that were detectable in wild-type cells exposed to the surrogate apoptotic cells (Fig. 2b). So we decided to test by transfection of Rac1 molecules, whether the efficiency of the phagocytosis signaling could be altered by increasing the levels of the free Rac1, which forms the next step in the integrin β_3 signaling pathway. Transfection of constitutively active Rac1 completely inhibited the phagocytosis of apoptotic cells. This observation confirms that of Nagaya et al. [28] and suggests that dynamic switching on and off of Rac1 is required for proper phagocytosis. Addition of wild-type Rac1, however, efficiently increased the rate of phagocytosis. These macrophages were able to concentrate additional Rac1 around the apoptotic cells and engulfed apoptotic cells with a similar rate as wild-type macrophages.

Our data provide a further proof for the hypothesis that the role of TG2 in phagocytosis is to provide efficient integrin β_3 signaling around the apoptotic cells (either by promoting integrin β_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. 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 [29].

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.imlet.2009.07.009.

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