TRANSGLUTAMINASE 2

AS AN ESSENTIAL REGULATORY FACTOR

OF NEUTROPHIL GRANULOCYTE DIFFERENTIATION

POTENTIAL CONTRIBUTION

IN RETINOIC ACID SYNDROME

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Transglutaminase 2 as an essential regulatory factor of neutrophil granulocyte differentiation

Potential contribution in retinoic acid syndrome

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LIST OF ABBREVIATIONS

AD	Alzheimer disease
APL	acute promyelocytic leukemia
ATO	arsenic-trioxide
ATRA	all-trans retinoic acid
BH3	Bcl-2 homology domain 3
$BNPH_2$	biotinamido-pentylamine
CT	cycle threshold
DC	dendritic cells
DIC	disseminated intravascular coagulopathy
DS	differentiation syndrome
ECM	extracellular matrix
FBS	fetal bovine serum
GM-CSF	granuloctye-macrophage colony-stimulating factor
HD	Huntington's disease
HDACs	histone deacetylases
HLH	helix-loop-helix
HPR	N-(4-hydroxyphenyl)retinamide
ICAM-1	intercellular adhesion molecule-1
iDC	monocyte-derived dendritic cells
IRFs	interferon regulatory factors
I-κBα	inhibitory subunit alpha of NF-κB
LIC	leukemia initiating cells
LPS	lipopolysaccharide
LTGF-beta	latent transforming growth factor-beta
MDC	monodansylcadaverine
NBT	nitroblue tertrazolium test
NCF2	neutrophil cytosolic factor 2
NF-κB	transcriptional activator nuclear factor
NLS	nuclear localization signal
NPM	nucleophosmin
NUMA	nuclear mitotic apparatus protein

O^{2-}	superoxide anion
PGC-1α	peroxisome proliferator receptor-gamma coactivator 1 alpha
PGE2	synthase prostaglandin E2 synthase
PLC	phospholipase C
PML	promyelocytic leukemia
PLZF	promyelocytic leukemia zinc finger
PMA	phorbol myristate acetate
polyQ	polyglutamine
pRb	retinoblastoma protein
RA	retinoic acid
RARE	retinoic-acid responsible element
RARs	retinoic acid receptors
RARα	retinoic-acid receptor α
RAS	retinoic acid syndrome
ROS	reactive oxygen species
STAT	signal transducer and activator of transcription
TG2	transglutaminase 2
TG2-KD NB4	transglutaminase 2 knockdown NB4 cells
TGase	transglutaminase
VCAM-1	vascular cell adhesion molecule-1
WBCs	white blood cells

2. MAGYAR NYELVŰ ÖSSZEFOGLALÓ

A mieloid eredetű neutrofil granulociták differenciálódása a csontvelőben indul, majd az innen kijutó előalakok a keringésben válnak érett funkcióképes sejtekké. A transglutamináz 2 (TG2) egy tiol és Ca^{2+}-függő acil-transzferáz, amely peptidkötésben lévő glutamin γ-karboxamin csoportja és különböző elsődleges aminok, valamint fehérjék lizin oldalláncának ε-amino csoportja közötti kovalens izopeptid kötés kialakulását katalizálja. Az enzim az immunrendszer legtöbb sejtjében kifejeződik és bebizonyosodott róla, hogy számos biológiai folyamatban aktív szerepet játszik (pl. fagocitózis, a sejtek letapadása és vándorlása, látens TGF-β aktiváció stb). Annak ellenére, hogy a neutrofilokról ismert, hogy a differenciálódásuk során az enzim indukálódik és az érett sejtekben nagy mennyiségű aktív enzim mutatható ki, a TG2 ezekben a sejtekben, ill. a hozzájuk köthető immunológiai folyamatokban betöltött szerepe egyelőre tisztázatlan.

Korábbi vizsgálataink alapján bebizonyosodott, hogy a promielocita sejtek neutrofil granulocita irányú differenciációja során a TG2 alapvető szerepet játszik az érett sejtek immunológiai tulajdonágainak kifejlődésében. Eredményeink azt mutatják, hogy a TG2 transzlokálódik a sejtmagba és ott keresztkötő aktivitásától függő módon részt vesz a sejtek éréséhez vezető génregulációs folyamatok szervezésében. Jelen értekezés célja a TG2-től függő regulációs események felderítése, valamint az enzim ezen folyamatokban betöltött szerepének tisztázása.

A TG2 szerepének vizsgálatára rendelkezésünkre áll a TG2 knockout egértörzs, valamint az NB4 promielocita sejtvonal, melyből all-transz retinsav (ATRA) kezeléssel egy, a természetes neutrofil granulocita állapothoz közeli alak differenciáltatható. A TG2 közvetlen RARα célgénként a retinsavas kezelés hatására nagymértékben indukálódik a differenciáció folyamata során. Lentivirus alapú shRNS vektor felhasználásával az eredeti NB4 sejtvonalból létrehoztunk egy stabil TG2 knock-down sejtvonalat, mely az ATRA kezelés után is csak mintegy 15%-ban képes a TG2 enzimet expresszálni.

Megfigyeléseink arra utalnak, hogy amennyiben a sejtek differenciációja TG2 hiányában (KO egér vagy knock-down NB4 sejtvonal) mellett zajlik, a sejtproliferációval kapcsolatos számos gén, valamint a neutrofil immunfunkcióban szerepet játszó több gén expressziója eltérést mutat. Annak kiderítése érdekében, hogy a TG2 mely gének expresszióját befolyásolja a neutrofil granulociták differenciálódása során, teljes génexpressziós vizsgálatot végeztünk DNS microarray technika alkalmazásával.

Összehasonlítva a TG2-KD NB4 sejtek differenciálódására jellemző génexpressziós mintázatot a normál NB4 sejtekével több mint 300 olyan gént azonosítottunk, melyek kifejeződése függ a TG2-tól. Eredményeink azt mutatják, hogy a TG2 szinergista módon részt vesz az ATRA által indukált génexpressziós átrendeződésben, hiszen a hiányában elmarad számos sejtproliferációval kapcsolatos gén repressziója, valamint az immunfunkcióval kapcsolatos gének kellő mértékű indukciója. A TG2 hiányának következtében fellépő génexpressziós eltérések fenotípus szinten is megnyílvánulnak, mivel a TG2-KD sejtek magasabb proliferációs képességgel, ugyanakkor csökkent mértékű immunológiai tulajdonságokkal (pl. baktérium fagocitózis, migráció, szuperoxidgyök termelés stb) rendelkeznek.

A csökkent mértékű immunológiai sajátságok kifejlődésével függ össze, hogy a TG2 hiányos sejtek kisebb mértékben képesek a retinsav-szindróma létrejöttében kulcsszerepet játszó CC kemokinek termelésére. Mivel a retinsav-szindróma az akut promielocitás leukémia ATRA alapú terápiájának legmeghatározóbb, akár halálos kimenetelű mellékhatása, a TG2 szintjének, vagy aktivitásának befolyásolása a terápia során potenciális beavatkozási lehetőséget vet fel.

3. INTRODUCTION

3.1. Tissue transglutaminase (TG2)

3.1.1. The transglutaminase (TGase) family

First evidence for transamidating activity was provided by Clarke and Waelsch observed in guinea-pig liver (Clarke 1957). Subsequently it was revealed that transamidation is carried out by enzymes which cross-link proteins through an acyl-transfer reaction (Pisano 1968). Since these findings a growing number of studies demonstrated that beyond mammalians, transglutaminases are present in micro-organisms, plants, invertebrates, fish, amphibians and birds (Kanaji 1993, Del Duca 1995, Singh 1994, Yasueda 1994, Zhang 1997, Puszkin 1985).

Transglutaminases (TGs) are thiol- and Ca^{2+}-dependent acyl-transferase that catalyze the formation of a covalent cross-link between γ-glutamyl groups of peptide-bound glutamine residues and the ε-lysyl group of lysine residues in certain proteins (Figure 1). The reaction results in a covalent post-translational modification by establishing the γ-glutamyl-ε-lysyl cross-linkage between the two substrate proteins (Folk 1977). Transglutaminase 2 (TG2) is also able to incorporate mono- or polyamines into proteins by amidation of glutamine residues of proteins and also to eliminate the amino group of glutamine residues by deamidation. It has been likewise published that the enzyme is capable to cleave the isopeptide bond by its isopeptidase activity in a Ca^{2+}-dependent manner (Parameswaran 1997).

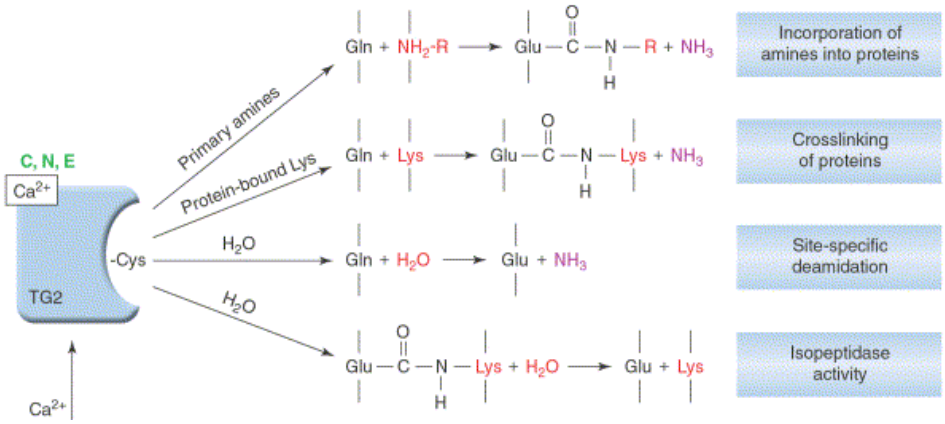


Figure 1. Biochemical activities of transglutaminase 2 (Fésüs 2002)

So far eight different enzymatically active transglutaminases have been described. They have evolved through duplication of a single gene and subsequent redistribution to distinct chromosomes (Grenard 2001).

Factor XIII is one of the best known Tgases, which circulates in a zymogen form in the blood and can be converted by thrombin-dependent proteolysis into the active transglutaminase (plasma TG) when it becomes able to covalently cross-link and stabilize the fibrin clot possessing an essential role in wound healing. Transglutaminase 1 (TG1) exists in membrane-bound and soluble forms and is called keratinocyte transglutaminase, while transglutaminase (TG3) is the epidermal or hair follicle transglutaminase. Both require proteolysis to become active and are involved in the terminal differentiation of the keratinocyte. The prostatic secretory TG4 is essential for fertility in rodents (Dubbink 1998) and TG5 once again has a role in epidermal differentiation (Candi 2001). Transglutaminase 6 (TG6) and transglutaminase 7 (TG7), the last two members of TG family, are less characterized (Grenard 2001). The protein, band 4.2 shows high similarity to the others, but lacks the catalytic site, therefore it is considered only structurally/evolutionarily related to the others (Korsgren 1990). Finally, transglutaminase 2 (TG2) is the most studied transglutaminase, because of its uniqueness among the others in that besides its cross-linking activity it also possesses GTPase (Nakaoka 1994), protein disulfide isomerase (Hasegawa 2003) and even protein kinase enzymatic activity (Mishra 2007). Furthermore it is characterized by broad tissue distribution and subcellular localization, and is also able to modify a number of cellular proteins post-translationally. Therefore, it has been demonstrated that TG2 contributes to various biological processes, much more than the other transglutaminases (Fesus L, Piacentini M. 2002)

3.1.2. Transglutaminase 2, the unique member of TGase family

Transglutaminase 2 (tissue transglutaminase, TG2) is usually characterized as a multifunctional protein, since in contrast with the other transglutaminases it can be involved in several wondrously diverse events in the living or dying cell due to its different functions.

3.1.2.1. Structure and functional elements of transglutaminase 2

The first reason for its multifunctionality can be found in its structure. Its basic structure is similar to the other transglutaminases, but it also bears several specific features,

which are not characteristic for the other types of transglutaminases. The human enzyme consists of 687 amino acid residues, which compose four distinct domains: an N-terminal β-sandwich, a catalytic core and two C-terminal β-barrel domains (Figure 2).

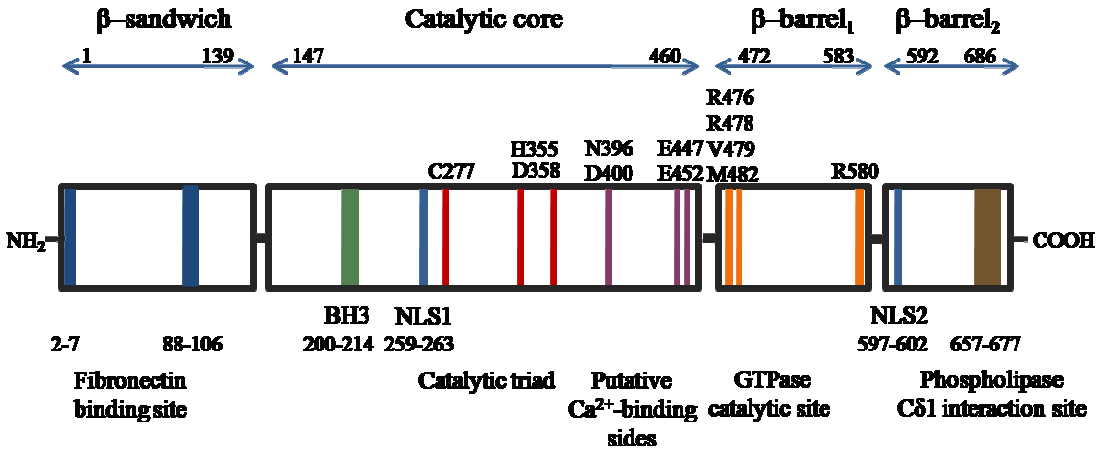


Figure 2. Functional elements of human transglutaminase 2 (Fésüs 2002)

For years the first 7 residues of the N-terminal β-sandwich domain were thought to be responsible for the fibronectin binding ability of TG2, which allows it to take part in specific biological processes such as cell adhesion, spreading and migration or wound healing (Jong-Moon 1995, Belkin 2001, Graudry 1999, Akimov 2001, Upchurch 1987, Martinez 1994). More recently the major functional sequence for the interaction with fibronectin was described within a region spanning amino acids 88-106 in the β5/β6 hairpin consisting of the antiparallel β strands 5 and 6 of the first domain (Hang 2005). The interaction surface with integrins has still not been located. The catalytic core domain carries the catalytic triad (Cys277-His335-Asp358) for the acyl-transfer reaction, and it is also responsible for the protein disulfide isomerase, ATPase and a protein kinase activity (Mishra 2004, Chandrashekar 1998). Recently, the structure of TG2 has been solved by X-ray crystallography from a new crystal, where human TG2 was trapped in complex with an inhibitor that mimics inflammatory gluten peptide substrates. The inhibitor stabilizes TG2 in an extended conformation, where the active site is exposed (Figure 3) (Pinkas 2007).

The core domain also contains the BH3 motif of the Bcl-2 protein family (Rodolfo 2004) and one of the predicted nuclear localization signals (NLS1) (Peng 1999). Based on these findings TG2 may interact with the pro-apoptotic protein Bax and sensitize cells toward apoptosis. TG2 can be transported into the nucleus by the importin-α3 system and once there

it is able to modify certain proteins including such, which regulate gene expression (e.g. histones or retinoblastoma) (Singh 1995, Lesort 1998, Shimizu 1997, Oliverio 1997).

The first β-barrel domain is responsible for one of the most important distinctivenesses of TG2, since the GDP-binding and GTPase catalytic site residues are mainly localized here. The GDP-binding capacity of TG2 has been known since the 1980s (Achyuthan 1987). The guanine binding site was identified in a cleft between the core and the first β-barrel domains (Liu 2002). The nucleotide binds to residues from the first and last strands (amino acids 476-482 and 580-583) of β-barrel 1 and to two core domain residues (Lys-173 and Phe-174). This site does not resemble that of the small Ras-related or heterotrimeric G-proteins, although like the latter it can also bind GTP/GDP in the abscence of Mg2+. In TG2 several positively charged residues surround the phosphates of the bound nucleotide. R580 forms two ion pairs with the α- and β-phosphates of GDP with the β-phosphate also being bound by R476 and positioned near R478 and the α-phosphate near K173.

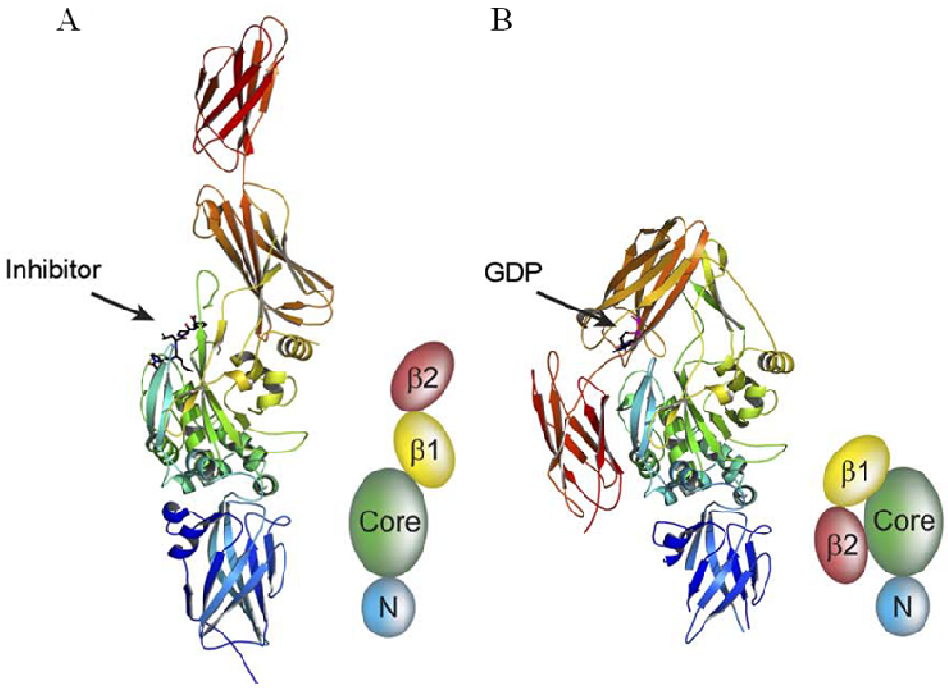


Figure 3. Overall structures of inhibitor-bound (A) and GDP-bound TG2 (B) (Pinkas 2007)

This exon of the TG2 sequence shows very poor sequence homology with the same parts of other TGases (Liu 2002). Nakaoka et al. demonstrated in 1994 that TG2 has GTPase activity and is able to function as a G-protein. In the liver TG2 couples to α1-adrenergic receptor,

activates phospholipase C (PLCδ1) and therefore it takes part in signal transduction (Nakaoka 1994). It is important to note, that the GDP-bound enzyme forms a closed conformer (Figure 3) contrary to the inhibitor-bound form, suggesting that the transition from the acyl-transferase activity to the GTPase one is accompanied by a large radical conformational change involving the whole structure of the protein (Pinkas 2007).

The second β-barrel domain carries the second predicted nuclear localization signal (NLS2) and the interaction site for phospholipase C is also localized at the C-terminal end of this domain (Peng 1999, Hwang 1995).

3.1.2.2. Tissue distribution and subcellular localization of transglutaminase 2

The second reason for that TG2 is involved in so diverse biological processes comes from its wide tissue distribution. Contrary to the other TGases its expression is not confined to a certain cell type. There are different cells or tissues that constitutively express TG2 (such as mesangial or interstitial cells, thymic subcapsular epithelium, colonic pericryptal fibroblasts), while in others it seems to be induced either by external stimuli (epithelium of the female breast, e.g.) or as part of their differentiation/maturation program (developing nephrons, enterocytes of the small intestine) (Thomázy 1989).

Furthermore, it has to be noted, that the subcellular localization of the enzyme is also diverse, since inside the cell its presence was detected not only in cytosol, but also in the mitochondrion (Piacentini 2002, Rodolfo 2004) and even in the nucleus. It can be transported into the nucleus with the help of the nuclear transport protein importin-α3 (Peng 1999). In the nucleus, TG2 can function as a G protein or as a transamidase in response to nuclear Ca^{2+} resulting in the cross-linking of proteins such as histones, the retinoblastoma protein (pRb) and the SP1 transcription factor (Oliverio 1997, Keresztessy 2006). TG2–catalyzed cross-linking of these proteins can induce significant structural and functional changes in them.

Although predominantly a cytosolic, TG2 can translocate to the plasma membrane in a 1:1 complex with β integrin (Mangala 2007, Akimov 2001). In the plasma membrane, TG2's association with integrins on the cell surface and its high affinity for fibronectin promote cell attachment to the proteins of the extracellular matrix (ECM) and the transduction of signaling pathways (Akimov 2000). Although its detailed externalization mechanism is unclarified so far, the enzyme resides in the extracellular matrix either freely, or coupled to ECM macromolecules and plays role in the remodeling of the ECM (Aeschlimann 1995).

3.1.2.3. Substrate specificity and cellular substrate proteins of transglutaminase 2

Next, one of the most important characteristic features of the type-2 transglutaminase among the other members of the family is its broad substrate specificity for proteins. Extensive research efforts have been invested to identify natural protein substrates for TG2 applying several types of approaches (e.g. identification either of the components of highly cross-linked insoluble polymers upon TG2 activation or the proteins labeled by monoamines by TG2). As a result of these the number of potential substrate proteins for TG2 is close to 150 (Csosz 2009). However, the physiological relevance of the accumulated data has remained an opened question, since many of the potential substrates were identified by *in vitro* cross-linking experiments in cell extract or by inducing a non-physiological Ca^{2+} overload in whole cells in the presence of high concentrations of labeled primary amines. Furthermore, experimental conditions, tissue specificity, post-translational modifications or loss of the native compact structure of a certain protein may also affect their behavior to act as a substrate for TG2. Therefore, it is further needs to be addressed how many of the indicated substrates are really relevant in cell functions. Nevertheless, it is evident that a huge number of TG2 substrate proteins are involved in cell motility (actin, myosin, troponin, b-tubulin, tau, Rho A), in the interaction of cells with extracellular matrix structures (fibronectin, fibrinogen, vitronectin, osteopontin, nidogen, laminin), and in key steps of energetic intermediate metabolism (aldolase A, glyceraldehyde-3-phosphate dehydrogenase, phosphorylase kinase) (Lee 1992, Orru 2002, Nadeau 1998, Eligula 1998, Bergamini 1995, Singh 2001, Kleman 1995, Mosher 1984, Martinez 1989, Sane 1988).

3.1.3. Role of transglutaminase 2 in pathological states

In the light of the multifunctionality and wide tissue distribution of this enzyme it is not surprising that its involvement was demonstrated in numerous pathological conditions. Chronic diseases, which were correlated to TG2, belong to the following three main categories: inflammatory diseases, including wound healing, tissue repair and fibrosis, and autoimmune conditions; chronic degenerative diseases (e.g. arthritis, atherosclerosis and neurodegenerative pathologies); and malignant diseases.

TG2 is probably involved in the angiogenic phase of wound repair as well as in its interaction with and stabilization of the extracellular matrix, possibly through its role as an independent cell-adhesion protein or as an integrin co-receptor (Jones 1997, Griffin 1999,

Akimov 2000). Among its physiological roles it also could contribute to the development of tissue fibrosis and scarring. Examples include the severe chronic inflammatory states found in liver diseases (cirrhosis and fibrosis, alcoholic hepatopathy and type C hepatitis) (Mirza 1997, Grenard 2001) and in renal and lung fibrosis via deposition of excessive scar tissue (Johnson 1997, Johnson 1999). In addition, involvement of TG2 in the pathogenesis of chronic inflammatory diseases of the joints, such as rheumatoid arthritis and osteoarthritis by activating of pro-inflammatory cytokines (TGFβ1, IL-6, TNFα) has been reported (Verderio 1999, Rosenthal 2000, Dzhambazov 2009).

Anti-transglutaminase antibodies have been found in several autoimmune diseases. Data in this perspective have been collected mostly for celiac disease (Arentz-Hansen 2000), type 1 diabetes (Seissler 1999), and, more recently, systemic lupus erythematosus and Sjögren syndrome (Villalta 2002). In case of celiac disease in the intestinal mucosa of gliadin-sensitive individuals, TG2 is apparently involved in deamidation of glutamine residues in gliadin and in formation of aggregates of itself and of gliadin, which are highly immunogenic through local activation of T-lymphocytes (Marzari 2001).

TG2 is related to the pathogenesis of several chronic neurodegenerative diseases, which are characterized by the accumulation of highly cross-linked insoluble protein materials. These include senile dementia of the Alzheimer type (Alzheimer disease, AD) and the polyglutamine (polyQ) tail diseases, such as Huntington's disease, rubropallidal atrophy and spinocerebellar palsy.

In AD, TG2 is expressed at a high level and also a truncated form (lacking portions of domain 4) appears. In patients TG2 activity is manifested by polymerization of a number of proteins, including Aβ peptide, β-amyloid precursor protein and the microtubule-associated tau protein, with formation of neurofibrillary tangles, as well as deposition of amyloid-like materials in the extracellular compartments. These abnormal protein polymers might be relevant to the pathogenesis of AD brains, and their formation has been ascribed to increased TG2 activity and to an altered distribution of the truncated protein (Rasmussen 1994, Citron 2001).

In contrast, the polyQ diseases (e.g. Huntington's disease) are primarily characterized by transcriptional defects in the substrates due to abnormal tail extensions that represent sites of TG2-mediated protein cross-linking. The presence of multiple glutamine repeats directly promotes stickiness in the altered proteins, which tend to rapidly polymerize. This phenomenon would be further promoted by covalent cross-linking by TG2 (Gentile 1998). Another study has provided further evidence for the involvement of TG2 in Huntington’s

disease, since it was demonstrated that administration of the transglutaminase inhibitor cystamine to transgenic mice (expressing exon 1 of huntingtin containing an expanded polyglutamine repeat) altered the course of the disease in a favorable way (Karpuj 2002).

The third field of active research on the importance of TG2 in human pathology is that of neoplastic diseases. The general conception is that tumor cells have a lower TG2 content than their normal counterparts and may differ in its subcellular localization. A decline in TG2 activity in tumors is possibly related to tumor metastatic potential, since reduced enzyme expression and activity would indeed lead to reduced cell adhesion, increased migration and a less stable extracellular matrix, thus facilitating the initial invasive stage of the tumor (Birckbichler 2000, Hand 1990). However, reports of increased TG2 expression in highly invasive tumors have also been reported, e.g. in the breast (van Groningen 1995, Hettasch 1996, Joshi 2006) and increased TG2 expression has been found in secondary metastatic tumors (Knight 1990). Other intriguing issues arise from the fact that powerful inducers of cell differentiation and apoptosis (e.g. 9-cis-retinoic or all-trans- retinoic acid) are also strong inducers of TG2 as well. These data suggest that the induced cell cycle arrest and the consequent cell differentiation are coupled to the high intracellular level of TG2 as observed with squamous-cell carcinoma in vitro and in promyelocytic leukemia in vivo (Jetten 1990, Davies 1985, Benedetti 1996). Nevertheless, the precise role of TG2 in cancer development seems to depend on the cell or tissue origin of the cancer and also on the stage of tumor progression. Therefore detailed analysis of the effect of TG2 in each specific neoplastic disease is further required to make us able to intervene in tumor formation or metastasis by regulating TG2 function.

3.1.4. Mechanism of the induction of TG2

The multiple activities and the very selective pattern of tissue-specific expression tissue of tissue transglutaminase suggest that its expression and activation must be tightly regulated. First studies took aim at clarifying the mechanism of the induction of the TG2 gene and presented evidence that it can be stimulated in resident peritoneal macrophages by exposure to factors present in serum (Murtaugh 1983) and also human peripheral blood monocytes differentiated into macrophages in serum-containing media *in vitro* were able to induce TG2 (Murtaugh 1984). Shortly afterwards *all-trans* retinoic acid was identified as a serum-derived factor which is responsible for the massive induction of TG2 in murine and human monocytes or macrophages. Studies on vitamin A-deficient rats have revealed that retinoids appear to be

generalized regulators of tissue transglutaminase expression, since a marked depression in the level of TG2 activity was observed in many tissues of those rats, and this decrease in the activity and protein level was found to be restorable by administration of *all*-trans retinoic acid (ATRA) (Verma 1992). Administration of retinoids to rats with normal retinoid nutritional status or exposure of human macrophages to ATRA *in vitro,* results in a dramatic increase in TG2 activity (Piacentini 1992, Moore 1984). Retinoids also induce the enzyme in human promyelocytic leukemia cell lines such as in HL-60 cells differentiating to myeloid cells (Maddox 1985, Davies 1985) and in NB4 differentiating to granulocytes (Benedetti 1996). These results suggested a key regulatory role for ATRA and, therefore, for the retinoic-acid receptor α (RARα) in the induction of TG2 gene. Indeed, a 3.8-kb fragment from the DNA flanking the 5'-end of the TG2 gene was found to be responsible for the retinoic acid-dependent induction of TG2 supposing that the retinoic acid responsible element (RARE) is embedded in the TG2 promoter (Chiocca 1988). Subsequent detailed analysis of the TG2 promoter revealed that two regions of the promoter are critical for the activation. One of these, the most upstream, contains a triplicate retinoid receptor binding motif that functions as a ligand dependent enhancer element. Although this retinoid-response element (mTGRRE1) can activate a heterologous promoter such as the thymidine kinase promoter, it cannot by itself confer ligand-dependent transcriptional activity on the TG2 promoter. For this activity it must be coupled with a second short DNA segment (HR-1) that is located 1 kb upstream from the transcription start site. This segment of DNA is unusual because it is one of the very few segments of DNA that is highly conserved between the human and the mouse TG2 promoters (Lu 1995). This conservation of sequence appears to reflect important functional properties of the promoter, since combination of this element with the upstream retinoid-responsive motif is permissive for retinoid-dependent transcriptional activation (Nagy 1996). Elaborated regulatory elements in TG2 promoter provide the basis for the complex regulatory mechanism characteristic for the TG2 gene regulation which is mainfested in the diverse induction of TG2 in different cell or tissue types, in various stage of cell differentiation or even in different developmental stages as it was demonstrated by riporter construct in transgenic animals (Nagy 1997). It appears that the regulation of TG2 gene is much more chiseled, since upon retinoid-specific cis-acting elements, other factors could be involved in the regulation of TG2 gene. It was demonstrated that TGF-β induces TG2 activity in tracheal epithelial cells (George 1990), IL-6 and TNF-α induce the enzyme in hepatocytes (Suto 1993, Kuncio 1998), and LPS also could contribute to its induction (Bowness 1997). Furthermore, interferon-γ and

cAMP were also described to be able to induce or at least be involved in the expression of TG2 (Metha 1985, Maddox 1988).

3.1.5. TG2 in the function of the immune system

There are a number of examples that TG2 is expressed in most cell types of the immune system. In most cases its presence was shown in macrophages. As early as 1981, TG2 was considered as a marker for murine macrophages since its presence and activity were detected in peritoneal and bone marrow-derived macrophages and also in several types of macrophage-like cell lines such as IC21, J774.2 and P388-D1 (Schroff 1981). Since then, extensive investigations were carried out to find out the exact role of this protein in macrophage function.

Evidences were arisen that the induction of TG2 is strongly associated to the differentiation of macrophages as it was demonstrated first during the course of the differentiation of murine leukemia cells into mature macrophage-like cells (Kannagi 1982). An increase in transglutaminase induction and activity seems to be a general phenomenon accompanying the differentiation and functional maturation of macrophages. It has been also suggested that transglutaminase is involved in the process of receptor-mediated endocytosis (Davies 1980, Levitzki 1980, Khera 1989, Abe 2000). In agreement with this proposal, a high enzymatic activity and presence of high-molecular-weight complex were specifically observed in differentiated type cells, which have a high phagocytic activity. Another physiological function of the enzyme has been suggested to be the modification of cell-surface protein, especially cross-linking of membrane proteins (Folk 1980). Next, a relationship was demonstrated between the induction of TG2 and the phorbol myristate acetate-stimulated release of superoxide anion (O^{2-}). Macrophages obtained from mice injected with either viable bacillus Calmette-Guérin, muramyl dipeptide, or killed Corynebacterium parvum released three- to six-times more O^{2-} than did resident macrophages and the TG activity in these macrophages was also two- to six- times higher than in resident cells (Harris 1984). However, they conclude that enhanced TG activity is not a prerequisite for the enhanced O^{2-} production observed in activated macrophages, since macrophages that were primed by exposure to LPS in vitro exhibited increased production of O^{2-} but no increase in TG activity.

It was indicated that the induction of TG2 by 1 alpha,25(OH)2D3 by a spermidine-dependent mechanism is involved in the induction mouse alveolar macrophage fusion

(Tanaka 1991). After identifying TG2 as a key element in the conversion of latent transforming growth factor-beta (LTGF-beta) to active TGF-β by bovine aortic endothelial cells (Kojima 1993), this phenomenon was described in macrophages as well (Nunes 1995). Another new significant function of macrophage TG2 was published by Akimov et al. in 2001. Their data demonstrate that cell surface TG2 serves as an integrin-associated adhesion receptor that might be involved in extravasation and migration of monocytic/macrophage cells into tissues. According to their results TG2 acts as an adhesion and migration receptor for fibronectin and it was found to be able to associate with multiple integrins of the beta1 and beta3 subfamilies (Akimov 2001).

In the aspect of phagocytosis it was demonstrated first in 2003 that TG2 is also required for the macrophages-mediated complete elimination of the large number of apoptotic cells arising every day (Szondy 2003). Subsequent analysis revealed that TG2 plays essential role also in the anti-inflammatory response induced by macrophages after the engulfment of apoptotic cells because $TG2^{-/-}$ mice developed autoimmune phenotype as a consequence of altered TGF-β and IL-12 production (Falasca 2005). Role of TG2 in the respect of anti-inflammatory response was also confirmed in murine peritoneal acute gout-like inflammation (Rose 2006). The liver X receptor/retinoic acid receptor α pathway was described by Rébé as the key mechanism of the induction of TG2 in macrophages in the aspect of apoptotic cell clearance and also suggesting promising strategy to treat the atherosclerosis (Rébé 2009). Successful investigations were performed to understand the mechanism by which TG2 contributes to the formation of phagocytic portal of macrophages in the process of engulfment of apoptotic cells (Tóth 2009). The importance of TG2 with respect to inflammation is manifested not only from the aspect of apoptotic cells but also in LPS-induced inflammation. LPS was found to be a potent inducer of TG2 (Bowness 1997) and in LPS-induced septic shock TG2 was found to be an pro-inflammatory factor, since it is able to cross-link the inhibitory subunit of NF-κB, hereby activate the NF-κB signaling, which leads to the production of pro-inflammatory cytokines (Piacentini 2008).

TG2 was also expressed by duodenal CD11c(+) dendritic cells (DC) and externalized to their surface with a possible role in T cell activation in the celiac lesion (Ráki 2007). Peripheral blood monocyte-derived dendritic cells (iDC) contain large amounts of TG2 and it is also detectable on the surface of these cells. It was described that TG2 on the surface of the antigen presenting DC can be catalytically active; hence it might directly be involved in the deamidation of gliadin peptides promoting the development of celiac disease (Hodrea 2010). Moreover, TG has an important role in DC not only in the relationship of antigen presentation

but in the final, functional maturation of DCs consequent to LPS treatment. The inhibition of the enzyme cross-linking activity led to impairment of DC function highlighted by wide phenotypic changes associated with a reduced production of cytokines (IL-10, IL-12) after LPS treatment and a lower ability to induce IFN-gamma production by naïve T cells. *In vivo* analysis of DCs obtained from TG2(-/-) mice confirmed that the enzyme ablation leads to an impairment of DC maturation and their reduced responsiveness to LPS treatment. Taken together, these data suggest that TG2 is an important element of DC differentiation in response to LPS and could be a candidate target for treating endotoxin-induced sepsis (Matic).

The role of transglutaminase 2 in polymorphonuclear granulocytes was examined much less. However, its presence was described and its cross-linking-dependent role in the zymosan-induced superoxide production was also shown (Giinzler 1982). TG2 was found to be involved in the induction of Il-1β in human peripheral blood-derived neutrophils in response to *Anaplasma phagocytophila* (Kim 2002). Furthermore, it was demonstrated that TG2 can be induced in neutrophil granulocytes by granulocyte-macrophage colony-stimulating factor (GM-CSF) treatment (Katano 2009). It was also revealed in study on rat basophilic leukemia cells that antigen stimulation induced expression and activity of TG2 by activation of NF-κB leads to increased production of reactive oxygen species, expression of prostaglandin E2 synthase (PGE2 synthase) and was responsible for increased secretion of prostaglandin E2 (Kim 2010). These findings suggest that increased expression of TG2 is closely associated to the inflammatory phenotype of polymorphonuclear granulocytes, since there are independent experiments showing that superoxide or inflammatory cytokine production of these cells – which are hallmarks of the maturity of polymorphonuclear cells – are strongly dependent on the presence and cross-linking activity of TG2.

Microglia also expresses TG2 and it is further inducible by LPS treatment and may contribute to brain inflammation mediated by nitric oxide (Park 2004). In lymphocytes the TG2 expression and activity was demonstrated with a possible role in their proliferation (Giinzler 1982). Taking together these findings suggest that TG2 is widely present in immune cells and acts either as an important mediator of the differentiation of these cells or as a direct participant of their immune function. Multiple effect of TG2 in immune cells is derived from its widespread distribution and the aforementioned functional diversity and substrate specificity. Due to the comprehensive investigations on this field of the transglutaminase research a number of TG2 functions were clarified. However, many of the precise functions of TG2 in the complex operation of the immune system are still unknown; hence further

investigations should be done to address the questions arising in the connection of immune system and type 2 transglutaminase.

3.1.6. TG2 in the regulation of the gene expression

An extremely new and interesting but poorly investigated aspect of transglutaminase research is the clarification its role in the regulation of gene expression. There are low but increasing number of publications concerning this regard of TG2 function. There is ample evidence that TG2 may influence effectors of gene expression (histones, transcription factors etc.) or components of signaling pathways, which in turn impact on transcription.

First indirect example for the involvement of TG2 in the regulation of gene expression was obtained in U937 cells undergoing apoptosis. In the early phases of apoptosis, the retinoblastoma gene product (pRb) – as a potential TG2 substrate – was posttranslationally modified by a TG2-catalyzed reaction. The reaction was prevented by dansylcadaverine, a potent enzyme inhibitor. In fact, TG2 was able to cross-link and achieve the polymerization of pRb, which was paralleled by the rapid disappearance of E2F-1. Therefore, according to this finding TG2 may contribute to the cell cycle regulation at gene expression level (Oliverio 1997.) In another study Boehm et al. suggest a different role of TG2 in the aspect of regulation of retinoblastoma protein function. They observed that the expression and activation of TG2 up-regulated in response to retinoic acid (RA) leads to the protection of several cell lines against N-(4-hydroxyphenyl)retinamide (HPR)-induced apoptosis. The protection from apoptosis is achieved by the rescue of pRb from caspase-induced degradation in a transamidation-dependent manner (Boehm 2002).

Next indirect example was described in human peripheral blood-derived neutrophils. Using nonspecific cross-linking inhibitor of TG2, monodansylcadaverine (MDC) they shown that the induction of Il-1β lags behind in response to *A. phagocytophila*, suggesting that clustering and/or internalization through binding to neutrophil receptors of *A. phagocytophila* is required for IL-1β mRNA induction (Kim 2002). Next result was observed in BV-2 microglia cell line. They found that TG2 activates the transcriptional activator nuclear factor (NF-κB) and thereby enhances LPS-induced expression of inducible nitric-oxide synthase. Activation of NF-κB by TG2 occurs via a novel pathway. Rather than stimulating phosphorylation and degradation of the inhibitory subunit alpha of NF-κB (I-κBα), TGase2 induces its polymerization. This polymerization results in dissociation of NF-κB and its translocation to the nucleus, where it is capable of up-regulating a host of inflammatory

genes, including inducible nitric-oxide synthase and TNF-α (Lee 2004). Similar activation of NF-κB was observed in doxorubicin-resistant breast cancer cell line, where again the TG2 was found to be responsible for the constitutive activation of the transcription factor, hereby for the maintenance of tumor progression (Park 2009).

Direct effect of TG2 on transcription factor was observed in case Sp1 inactivation. Ethanol induced apoptosis in hepatic cells enhances activity and nuclear accumulation of TG2. Nuclear TG2 is able to directly cross-link and inactivate the Sp1 transcription factor, as high level of cross-linked Sp1 was detectable both in alcoholic injured hepatocyte nuclei derived from rat models and in of patients with alcoholic steatohepatitis. As a consequence, reduced expression of the Sp1-responsive gene, c-Met and a subsequent apoptosis was observed in the liver (Tatsukawa 2009).

The most investigated and understood part of the relationship of TG2 and gene regulation is related to the post-translational modifications of histones by TG2. Modulation of chromatin through covalent histone modification is one fundamental way of regulating DNA accessibility during processes of gene transcription. According to the histone code hypothesis, the biological outcome of histone modifications is manifested by direct physical modulation of nucleosomal structure or by providing a signaling platform to recruit downstream regulatory or effector proteins (Strahl 2000, Jenuwein 2001). It was recognized a great while ago that nuclear histone proteins are proven excellent substrate for the cross-linking activity of TG2. First evidence arose from experiments on chicken erythrocyte nucleosomes *in vitro*, where particularly monodansylcadaverine (MDC), but also methylamine and putrescine as donor amine were incorporated into different core histones by TG2-catalysed reaction, suggesting histone proteins as glutamyl donor for TG2 (Ballestar 1996). In the same year the occurrence of epsilon-(gamma-glutamyl)lysine cross-link between a glutamine residue of histone H2B and a lysine residue of histone H4 was also reported in the testis of the starfish, *Asterina pectinifera* (Shimizu 1996). In 2000, histone H1 was identified as excellent lysyl donor substrate of pig liver tissue transglutaminase (Cooper 2000). Several another investigations conducted on histones solve examples and evidence for that TG2 is able to either covalently modify core histone proteins by the incorporation of monoamines or cross-link them to each other (Kim 2001, Ballestar 2001, Kim 2002, Sato 2003). Moreover, it was recently published that TG2 is also able to phosphorylate histones by its kinase activity *in vitro* suggesting a novel role for TG2 in the regulation of chromatin structure and function (Mishra 2006). These lines of evidence provide convincing arguments for the fact that TG2 supplies a possible tool for histone modifications. However, in most cases the modification of

histones by TG2 was carried out *in vitro* or under artificial conditions (e.g. at high Ca^{2+} concentration). So far, the physiological relevance of TG2-mediated histone modifications was not demonstrated, therefore further investigations are required to be performed in the aim of clarification of the biological significance of this phenomenon.

Recently, an extremely intriguing discovery has been published in regard to direct involvement of TG2 in the regulation of two nuclear-encoded mitochondrial proteins (McConoughey 2010). According to their results in Huntington's disease – in which essential role is attributed to the TG2 as mentioned above – the normal expression of cytochrome c and of its coactivator, peroxisome proliferator activated receptor-gamma coactivator 1 alpha (PGC-1α) is suppressed by TG2 due to a direct bound to proximal promoter of the cytochrome c gene and to the coding region of PGC-1α gene. This function of TG2 was Ca^{2+}-dependent and inhibited by ZDON (a product of the biotechnology company, Zedira), a novel peptide-based specific irreversible cross-linking inhibitor of TG2. These results serve evidence that TG2 is able to function as a selective corepressor of transcription of genes involved in mitochondrial function and, therefore, contribute to the transcriptional dysregulation appears in HD.

3.2. Acute promyelocytic leukemia (APL)

Acute promyelocytic leukemia was first described as a distinct subtype of acute myeloid leukemia (AML) more than 50 years ago (Hillestad 1957). It accounts for approximately 10% of cases of AML. Diagnosis of this disorder is based on leukopenia coexisting with a marrow replaced with granulated dysplastic promyelocytes, disseminated intravascular coagulopathy (DIC), lack of Ia (HLA-DR) antigen expression, and translocation between the long arms of chromosomes 15 and 17 (t[15;17]) (Stone 1990). Chromosomal translocation leads to a blockade in the granulocyte differentiation of myeloid cells at the promyelocytic stage and causes the accumulation of promyelocytic cells in the bone marrow and circulation (Stone 1990, Raymond 1993).

3.2.1. Molecular genetics of acute promyelocytic leukemia

In the mid-1970s, acute promyelocytic leukemia was found to be consistently associated with a nonrandom chromosomal abnormality characterized by balanced and reciprocal translocations between the chromosomes 15 and 17 (Rowley 1977). In 1987, the gene encoding the retinoic acid receptor-alpha (RARα) was mapped to chromosome 17q21 (Mattei 1987). The location of the chromosomal breakpoint in acute promyelocytic leukemia prompted studies of a potential role for the RARα gene in this translocation (Chomienne 1990, Miller 1990, Longo 1990). Subsequently, several groups cloned the t(15;17) breakpoint and showed that the RARα gene was rearranged in all the patients tested. Moreover, the breakpoints on chromosome 15 were found to cluster tightly in a region containing a previously unknown gene, initially called myl44 and later renamed promyelocytic leukemia protein (PML) (de The 1990, Alcalay 1991, Goddard 1991, Kakizuka 1991, de The 1991).The 15;17 translocation fuses the genes for PML and RARα, resulting in the synthesis of two reciprocal fusion transcripts, PML/RAR-α and RAR-α/PML. Thus, in acute promyelocytic leukemia (along with certain other types of acute myelocytic leukemia, chronic myelocytic leukemia, acute pre-B-cell lymphoblastic leukemia, and Ewing's sarcoma), a translocation generates a disease-specific fusion protein that is involved in carcinogenesis (Solomon 1991). It is important to notice that the characteristic [t(15;17)(q22;q21)] translocation of 15 and 17 chromosomes appears in the 95-98% of patients. Nevertheless, there are rare variants of translocation resulting in other types of fusion proteins which also lead to arrest in myeloid differentiation and provoke APL. The first variant translocation in APL to be described, and the most intensively studied, is the

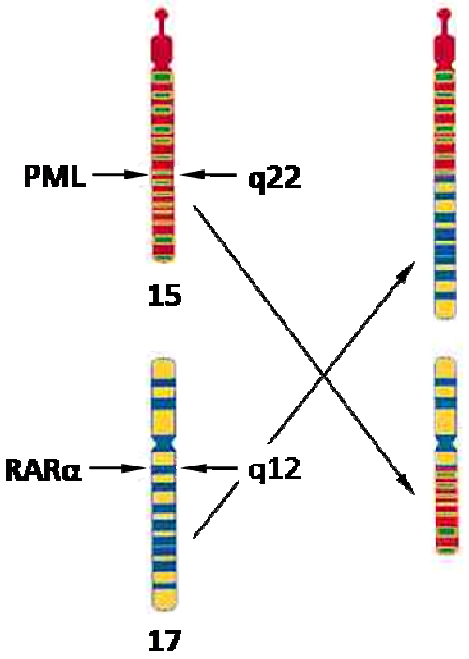


Figure 4. Chromosomal rearrangement in APL. *(Data on file, Pharmacia & Upjohn Company, Kalamazoo, Mich)*

t(11;17)(q23;q21) (Chen 1993). In this case the kruppel-like gene PLZF (promyelocytic leukemia zinc finger) is inserted within intron 2 of RARα, so that an inframe fusion protein is expressed as PLZF-RARα. A reciprocal RAR-PLZF fusion is also possible (Chen 1994). The second variant chromosomal translocation to be characterized is the t(5;17)(q35;q21) translocation (Corey 1994). A total of four such patients have been identified. This variant translocates the nucleophosmin gene on 5q35 into the RARα locus. Again, the breakpoint occurs within the second intron of RARα, so that a fusion protein is expressed that encodes the B-F domains of RARα in the same reading frame as the N-terminal domains of nucleophosmin (NPM). Nucleophosmin is a nucleolar phosphoprotein that plays a role in ribosomal RNA assembly and in regulation of centrosomal duplication; it also has chaperoning activities, as well as nuclease activity (Redner 1996, Okuda 2000). A reciprocal RAR-NPM fusion was described in three of the four cases. There is only one example where the fusion partner of RARα was NUMA, (nuclear mitotic apparatus protein) and the fusion product was an in-frame NUMA-RAR protein (Wells 1997). It was also described that the Stat5β, a member of the signal transducer and activator of transcription family, can be inserted into the second intron of RARα and result in a Stat5β-RARα fusion and leukemia (Arnould 1999).

3.2.2. Mechanism of transcriptional repression provoked by PML/RAR-α or RAR-α/PML in granulocytic differentiation

Retinoic acid (RA) acts as a ligand for the retinoic acid receptors (RARs), which are transcription factors that bind to particular sequences, the retinoic acid response element (RAREs), in the regulatory region of specific target genes. In the absence of RA, the RAR/RXR heterodimer recruits a repression complex containing nuclear receptor corepressors SMRT or N-CoR (Chen 1995, Evans 1995), corepressors mSin3, and histone deacetylases (HDACs) (Alland 1997, Heinzel 1997, Nagy 1997). Deacetylation of core histones results in chromatin condensation and transcriptional repression. RA induces the dissociation of this complex and promotes the association of a coactivator complex containing nuclear receptor coactivator p160 family members (Chen 1997, Torchia 1997) and histone acetyltransferases CBP/p300 (Chakravarti 1996, Kamei 1996). Acetylation of core histones leads to chromatin relaxation, promoter clearance, and activation of gene transcription. In the process of differentiation of myeloid precursors towards neutrophil granulocytes retinoic acid induces an extraordinary gene expression changes by the activation of RARα receptor. The

complex transcriptional remodeling involves the differential regulation of hundreds of different genes. These genes include transcription factors, chromatin-modifying factors, cell cycle regulators, as well as regulators of apoptosis, stress responses, metabolism and protein synthesis. Some of these genes harbor RAREs in their promoter regions and appear to be direct targets of the RARs, whereas others have no obvious RARE and may represent indirect downstream targets of the activated RARs. Moreover, some of these differentially expressed genes may be directly involved in mediating terminal granulocytic differentiation, whereas others may merely reflect the biochemical and functional characteristics of mature granulocytes (Zheng 2005, Park 2003).

Predominantly, cells from APL patients have the characteristic chromosomal translocation between chromosomes 15 and 17 [t(15;17)(q22; q21)] leading to a fusion of the RARα to the promyelocytic leukemia (PML). In case of this mutation retinoic acid is unable to bind to the RARα and induce the terminal differentiation process in physiological concentration (10^{-9} to 10^{-8} M). The fusion product acts as a dominant negative of RARα by forming homodimers, recruiting corepressors, and inhibiting expression of target genes necessary for granulocytic differentiation by binding to RAREs. However, because this repression of RARα target genes by PML-RARα is associated with the recruitment of DNA and histone-modifying enzymes such as histone deacetylases (HDACs), histone methyltransferases (Carbone 2006), and DNA methyltransferases (Di Croce 2002), the leukemogenic activity of this fusion product is mediated by mechanisms beyond the simple repression of RARα-regulated genes. It leads to a highly repressive chromatin environment, which affects multiple pathways. Furthermore, the PML-RARα product may also inhibit the normal function of the PML protein as a tumor suppressor and therefore acts as a dominant negative against both proteins (Scaglioni 2007).

3.2.3. Treatment of acute promyelocytic leukemia

Pharmacological concentration of ATRA leads to a conformation change of the multifunctional molecule complex around PML-RARα. Corepessors are released, normal regulation of RARα-responsive genes is restored, and hence, terminal differentiation of APL cells is induced (Grignani 1998).

The phenomenon is supported by several recent microarray and proteomic studies, which have identified hundreds of genes that are differentially regulated in the ATRA-induced differentiation of APL cell (Zheng 2005, Park 2003), including down-regulation of c-

myc (Bentley 1986) and up-regulation of C/EBP-ε (Park 1999), as well as PU.1 (Mueller 2006). Furthermore, genes governing increased protein synthesis such as PDCD4 or RTP801 are down-regulated in APL cells during ATRA exposure (Ozpolat 2007, Gery 2007) whereas genes associated with protein degradation are up-regulated by ATRA, leading to a degradation of the PML-RARα fusion product (Kitareewan 2002, Hattori 2007).

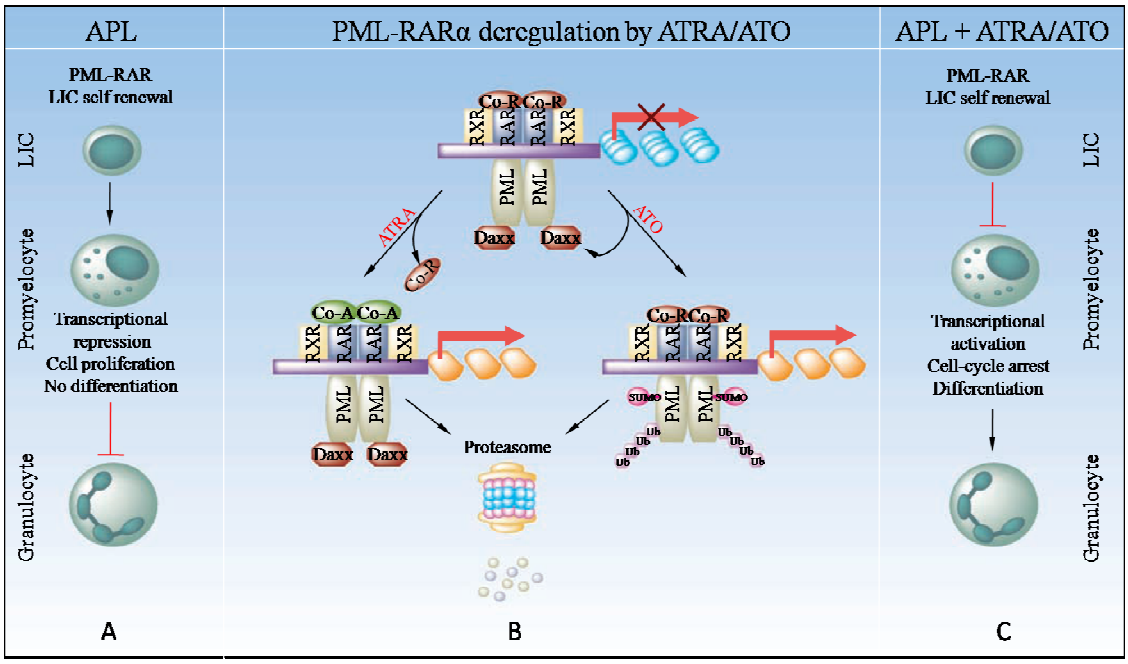


Figure 5. APL pathogenesis and mechanisms of action of the curable combination ATRA-ATO. *A, APL is characterized by the expression of PML/RARα that confers self-renewal properties of leukemia initiating cells (LIC) to committed cells and blocks promyelocyte differentiation. B, in APL cells, PML/RARα heterodimerizes with RXR and recruit corepressors (Co-R) onto master genes that control promyelocyte differentiation. ATRA releases corepressors and recruits the coactivators (Co-A), leading to transcriptional activation of targets and differentiation. ATRA also induces the recruitment of the proteasome that degrades PML/RARα. ATO enhances sumoylation of PML/RARα on Lys 160, which modulates Daxx binding (and thus repression), but also triggers SUMO-dependent polyubiquitination proteasome-dependent degradation of PML and PML/RARα. This will modulate Daxx binding to PML/RARα, leading to the release of transcriptional repression. C, PML/RARα degradation is responsible for the eradication of APL LIC. ATRA and ATO also induce PML/RARα transcriptional activation that leads to the differentiation of promyelocytes (Nasr 2009).*

Retinoic acid-based treatment of acute promyelocytic leukemia is often supplemented by the administration of arsenic-trioxide (ATO), which in itself leads to partial differentiation of leukemia initiating cells (LIC) and the consequent apoptosis of them (Niu 1999). Recent

studies brought to light that ATO binds directly to cysteine residues in zinc fingers located within the PML-RARα and PML. Arsenic binding induces PML-RARα oligomerization, which increases its interaction with the small ubiquitin-like protein modifier (SUMO)-conjugating enzyme UBC9, resulting in enhanced SUMOylation and degradation. Thus, ATO exerts its therapeutic effect by promoting degradation of the fusion protein PML-RARα (Zhang 2010) (Figure 5).

Therefore, degradation of PML-RARα may also represent an intersection, where the mechanisms of action of ATO converge on those of ATRA. A comparison of ATO- and ATRA-induced gene expression and proteome profiles showed that both compounds regulate similar cellular factors. However, ATO's emphasis was on altering the proteome and inducing apoptosis as opposed to predominant regulation of gene expression and differentiation by ATRA (Zheng 2005). Interestingly, differentiation of APL cells by either ATRA or ATO is highly dependent on stimulation by myeloid growth factors as evidenced by experiments using growth factor–neutralizing antibodies (Matsui 2005). In addition, both substances can induce a side effect known as APL differentiation syndrome, suggesting some overlapping mechanisms of action (Sanz 2008). However, ATO in itself causes less alteration of transcription than does ATRA, as mirrored in the lower number of genes changing upon ATO-treatment compared to the ATRA influenced ones and also in the partial morphologic and functional differentiation of promyelocytic cells (Zhang 2005). But, sharing similar pathways but exhibiting different foci of action, the two compounds complement each other achieving complete remission rates of up to 90% to 95% (Sanz 2008). Taken together, therapy of APL with ATRA and ATO is to date the most successful example of differentiation therapy.

3.2.4. The retinoic acid syndrome

Although ATRA is generally well tolerated, some patients develop a potentially lethal syndrome, the retinoic acid syndrome (RAS) or also known as differentiation syndrome (DS) as a side effect of ATRA-treatment (Frankel 1992). RAS is characterized by unexplained fever, weight gain, respiratory distress, interstitial pulmonary infiltrates, pleural and pericardial effusions, episodic hypotension, and acute renal failure (Frankel 1992). The diagnosis RAS is made on clinical grounds by the presence of at least 3 of the aforementioned signs and/or symptoms, in the absence of alternative explanations. In addition, RAS is often associated with the development of hyperleukocytosis, pulmonary edema, generalized edema,

headache, and bone pain. RAS is reported in 2.5% to 31% of the APL patients who receive induction therapy with ATRA and/or ATO (de Botton 1998, Tallman 2000). This syndrome is the most serious toxicity of ATRA and is often, but not always, associated with the development of hyperleukocytosis.

The pathogenesis of the syndrome is not completely understood. However, several possible mediators have been identified, including cathepsin G, a serine protease that enhances capillary permeability (Seale 1996); cell adhesion molecules on APL cells such as CD15 (Lex) and integrins CD11a and CD11b, which interact with the endothelial cell receptor ICAM (intercellular adhesion molecule)-1; and hematopoietic growth factors such as interleukin IL-1β , tumor necrosis factor TNF-α and IL-6, which promote leukocyte activation (Di Noto 1994, Dubois 1994, Di Noto 1996). Increased expression of IL-1β in leukemic promyelocytes may induce endothelial cell expression of ICAM-1 and vascular cell adhesion molecule (VCAM)-1, which may allow for leukemic cell binding to endothelium (Marchetti 1996). Exposure of the promyelocytic leukemia cell line NB4 to ATRA promotes formation of leukoaggregates via LFA-1/ICAM-2 interaction, providing another potential mechanism contributing to the syndrome (Larson 1997).

RAS is not observed during consolidation or maintenance therapy with ATRA and/or ATO in APL patients, nor is RAS observed during ATRA treatment in non-APL malignancies, implicating that the APL cells play a crucial role in the development of RAS (de Botton 1998, Tallman 2000, Tallman 2002). In RAS an excessive systemic inflammatory response is observed which can result in massive tissue infiltration of differentiating APL cells. The lung is one of the most relevant target organs of the inflammatory response in RAS, illustrated by marked histopathologic changes and respiratory problems, requiring mechanical ventilation in 15% to 55% of the APL patients with RAS (Camacho 2000, Ninomiya 2004). Furthermore, the excessive inflammatory response can induce a life-threatening capillary-leak syndrome and multiorgan failure (Puneet 2005).

Although early recognition of RAS and prompt treatment with high dose dexamethasone have reduced the RAS -related mortality significantly, still 15% of the induction deaths in APL are associated with RAS. The pathogenic mechanism of the hyperinflammatory cascade in RAS is not fully understood. Two different mechanisms play an important role in the development of RAS: the migration of APL cells to the lung and the differentiation of APL cells. The initial migration of APL cells to the lung seems to be triggered by alveolar chemokine secretion. ATRA is able to significantly increase the expression and production of specific chemokines (CCL2 and CXCL8) by alveolar epithelial cells *in vitro* and *in vivo*,

resulting in increased migration of APL cells toward alveolar epithelial cells (Tsai 2007, Tsai 2008). Suppression of alveolar chemokine secretion with dexamethasone or neutralization with specific chemokine antibodies resulted in reduction of the migration (Tsai 2007, Tsai 2008). Differentiation of APL cells may be an additional crucial factor in the development of RAS because it seems to be required for the pulmonary infiltration of APL cells, observed in RAS (Cunha De Santi 2007). In this respect, Tsai et al showed that ATRA stimulation of APL cells significantly increased the migration of APL cells toward alveolar epithelial cells *in vitro* (Tsai 2007, Tsai 2008). In addition, in a murine APL model, transplantation of ATRA-treated APL cells resulted in the development of RAS with pulmonary infiltration and lung edema, which was not observed when untreated APL cells or ATRA-resistant APL cells were used for transplantation (Ninomiya 2004).

Differentiation induction of APL cells is associated with increased expression of specific adhesion molecules and inflammatory cytokines, which may promote activation, migration, and adhesion of these cells. Furthermore, up-regulation of specific chemokines (CCL2 and CXCL8) has been reported during differentiation induction of APL cells (Tsai 2007, Tsai 2008, Cunha De Santis 2007, Marchetti 1996, Taraboletti 1997, Zang 2000, Hsu 1999, Behringer 2001, Di Noto 1996, Shibakura 2005, Dubois 1994, Burn 1994). Because chemokines coordinate the development, differentiation, and trafficking of leukocytes during inflammatory reactions and mediate the migration of leukemic cells, increased production of chemokines by differentiating APL cells may be important for the development of RAS (Cignetti 2003).

3.3. Aims of the study

We investigated the role of transglutaminase 2 in neutrophil granulocytes, with a particular focus on their differentiation.

For that purpose we used neutrophil granulocytes derived from mice and neutrophil granulocyte-like cells differentiated from the human promyelocytic leukemia cell line, NB4. We compared the phenotypes and immunological features of wild-type neutrophils and TG2 (-/-) cells or a TG2 cross-linking inhibitor-treated NB4-derived granulocytes.

We found several aspects of neutrophil granulocyte function to be dependent on TG2.

Having demonstrated that TG2 enters into the nucleus of neutrophils, and that there it is strongly associated to the chromatin, we were prompted to hypothesize that TG2 might be involved in direct genetic regulation in these cells. It is known that TG2 is able to translocate into the nucleus of several types of cells, but its nuclear function is poorly clarified and in neutrophils it is not investigated at all.

This made us to intend to uncover the gene regulatory changes that lead to the characteristic features of TG2-defficient cells. To that end we wanted to establish stable shRNA-mediated TG2 knock-down NB4 cells and then to identify those genes whose expression is dependent on TG2.

Based on the gene expression data we describe a novel function of TG2 on the level of gene expression regulation during the course of the differentiation of neutrophil granulocytes.

4. MATERIALS AND METHODS

4.1. Cell culture

NB4 (purchased from DSMZ GmbH) cell line was cultured in RPMI 1640 Medium (Sigma) supplemented with (10% v/v) fetal bovine serum (FBS) (Sigma), 2 mM glutamine (Sigma), 100 U/mL penicillin and 100 µg/mL streptomycin solution (Sigma). Differentiation of NB4 was induced at 1 x 10^5 cells/mL by administration of 1 µM ATRA (Sigma). The 293FT packaging cell line was purchased from Invitrogen and maintained in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% FBS, 4 mM glutamine, 100 units penicillin - 100 µg/mL streptomycin solution and 1 mM sodium pyruvate (Sigma).

4.2. Western blot analysis of transglutaminase 2 in lysates of cell fractions

The crude cytoplasmic and nuclear fractions were prepared from untreated and ATRA-treated NB4 cells. Cultured cells were lysed in buffer A (250 mM sucrose, 1 mM DTT, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 15 mM PIPES, pH 7.4, 1 mM PMSF at 4°C) containing 0.1% nonionic detergent nonidet P-40 and homogenized with 8 to 10 strokes in dounce homogenizer. The completeness of lysis was determined by microscopy using May-Grünwald-Giemsa staining. The nucleus and cytosol content of cells were separated by centrifugation at 1100g for 15 minutes at 4°C. Cytosol was centrifuged at 13 400g for 15 minutes at least 3 times to remove the remaining nuclei. The nuclear fractions were washed 3 times in buffer A containing 0.1% NP-40, then lysed in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 15 mM β-MEA, 0.1% Triton X-100, 0.5 mM PMSF. Lysates containing 2 mg/mL protein were mixed with equal volumes of lysis buffer (0.125 M Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol, 10% mercaptoethanol, and 0.02% bromophenol blue) and incubated at 100°C for 10 minutes. Protein (25 µg) was electrophoresed on 8% SDS–polyacrylamide gels and electroblotted onto a PVDF membrane. The blot was first saturated with 5% BSA in TTBS. Then, monoclonal antibody CUB7402 (Neomarkers, Fremont, CA) against TG2, diluted in 0.5% BSA in TTBS 1:2000 or 1:8000, was added and incubated at 4°C overnight, or at room temperature for 2 hours, followed by incubation with horseradish peroxidase (HRP)–labeled affinity-purified goat anti–mouse IgG (Sigma) overnight at 4°C, or at room temperature for 1 hour. Each step was followed by 3 15-minute washes in TTBS.

Transglutaminase bands were visualized by ECL Kit (Amersham, Little Chalfont, United Kingdom).

4.3. Cross-linking activity assay of TG2

Lysates of cytosolic and nuclear fractions of nontreated or ATRA-treated NB4 cells were prepared as for the Western blot analysis. TG2 activity was measured in a reaction mixture with a total volume of 100 μL consisting of 50 μL (2 mg/mL) crude cell fraction homogenate, 10 μL N, N-dimethylcasein (40 mg/mL), 20 μL [1,4(n)-3H] putrescine (30 Ci[37 MBq]/mmol), 10 μL 250 mM Tris-HCl, pH 7.5, containing 150 mM β-MEA, and 10 μL $CaCl_2$ (50 mM). The reaction was initiated by the addition of $CaCl_2$, incubated at 37°C for 5 minutes, and then 25 μL of sample was removed, dropped on filter paper, precipitated in cold trichloroacetic acid, and washed intensively with 10% and 5% TCA and ethanol, respectively. The radioactivity of the filter paper was measured in a liquid scintillation β-counter.

4.4. Immunolabeling of cells

Cells were fixed with (1) 4% paraformaldehyde in HEPES (4°C; 10 minutes), (2) 8% paraformaldehyde in HEPES (4°C; 50 minutes); (3) 4% paraformaldehyde in HEPES (4°C; 20 minutes), (4) 2% paraformaldehyde in PBS (15 minutes), and then (5) methanol (-20°C; 20 minutes). After fixation, cells were incubated in 25 mM glycine in PBS (20 minutes); permeabilized with 0.1% Triton X-100 in PBS (20 minutes); washed 5 times for 20 minutes in TTBS; blocked with TTBS, 5% BSA, or milk powder, pH 7.4 (20 minutes); incubated (2 hours) with mouse monoclonal antibody against TG2; washed 5 times for 30 minutes in TTBS; incubated with Alexa Fluor 633 goat anti–mouse IgG (Molecular Probes) (1 hour); washed 5 times for 30 minutes in TTBS; and then rinsed 3 times in PBS before coverslips were mounted in Mowiol (Vector Labs, Burlingame, CA) dissolved in glycerol.

Images were collected using a Zeiss LSM 510 confocal microscope (63 X Plan-Apochromat objectives, numeric aperture: 1.4; Carl Zeiss, Heidelberg, Germany). PI and Alexa Fluor 633 were excited by 543-nm and a 633-nm HeNe lasers, and fluorescence emission was detected through a 560- to 610-nm bandpass, and a 650 long-pass filter, respectively. Sequential excitation was used to avoid crosstalk between the detection channels. Images were recorded at identical instrument settings to allow direct comparison of

intensities. Micrographs were low-pass filtered to reduce noise using the data acquisition software of the LSM 510.

4.5. Measurement of protein-bound Nε-(γ-glutamyl)-lysine linkage

The amount of the Nε-(γ-glutamyl)-lysine cross-link content was measured in homogenized samples according to Tarcsa (Tarcsa 1990). Briefly, the protein content of cytosolic and nuclear cell fractions was precipitated and lyophilized. After rehydration, extensive enzymatic digestion, and derivatization with phenylisothiocyanate the obtained Nε-(γ-glutamyl)-lysine isodipeptide derivative was separated on a cation exchanger resin and then on silica high-pressure liquid chromatography column, and finally, quantified after reversed-phase high-pressure liquid chromatography.

4.6. In situ labeling of TG2 activity

Cells were labeled with 6 mM 5-(biotinamido)-pentylamine (BPNH2; Molecular Probes, Eugene, OR) for 12 hours, harvested, and fractionated into nuclear and cytosolic fractions. To visualize the proteins into which the 5-(biotinamido)-pentylamine had been incorporated, samples (25 μg of protein) were electrophoresed on 8% SDS–polyacrylamide gels, transferred to PVDF membrane, and probed with horseradish peroxidase–conjugated streptavidin (Amersham) at 1:1000 final dilution in TTBS with 0.5% BSA, at room temperature for 1 hour. The blots were washed and developed as described above.

4.7. Determination of superoxide anion production by isoluminol and luminol-amplified chemiluminescence

Cells were centrifuged at 200g for 5 minutes at 4°C. A measuring vial (1.0 mL) was prepared containing 0.1 mL (10^6) cells and 0.1 mL isoluminol or luminol (0.5 mM) in 0.8 mL modified Krebs-Ringer buffer (120 mM NaCl, 5 mM KCl, 1.7 mM KH2PO4, 8.3 mM Na2HPO4, 10 mM glucose, 1 mM CaCl2, and 1.5 mM MgCl2; pH 7.3). The samples were kept for 5 minutes at 37°C and activated by addition of 2 μL PMA (50 nM).

Mouse peritoneal polymorphonuclear neutrophils' O^{2-} production was measured by a chemiluminescence assay using L-012 dye. The reaction volume of 500 μL contained 10^5 cells and 5.0 μL L-012 (100 μM). After measuring the background signal, 2 μL PMA (50 nM)

was added and incubated for 5 minutes, and then the chemiluminescence was counted in a MOONLIGHT 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) at intervals of 10 seconds. Production of light was recorded in relative luminometer units (RLUs) (Dahlgren 1999, Nishinaka 1993).

4.8. Northern blotting

Total RNA was extracted with TRI Reagent (Sigma) according to the supplier's instruction; 30 μg was electrophoresed in 1.5% agarose gel containing 0.2 M formaldehyde, transferred to a supported nitrocellulose membrane (Hybond-c Extra; Amersham), and UV linked. The membrane was prehybridized with Perfect Hyb Plus (Sigma) for 30 minutes and hybridized with 32P-labeled probes (HexaLabel DNA Labeling Kit; MBI, Fermentas, Vilnius, Lithuania) overnight at 68°C. cDNA clone MGC: 45153 IMAGE: 5505170 corresponding to $GP91^{phox}$ was used as a probe.30 Membrane was washed twice at room temperature in 2 X SSC containing 0.1% SDS for 5 minutes, and twice for 20 minutes at 65°C in 0.1 X SSC containing 0.1% SDS. Wrapped blot was exposed to x-ray film (Kodak X-Omat AR; Rochester, NY) using an intensifying screen at -70°C for 5 days.

4.9. Recruitment of peritoneal neutrophils from mice

Wild-type and TG2-deficient mice were injected intraperitoneally with 1 mL 10% yeast extract (Sigma). At 4 hours, mice were injected intraperitoneally with 3 mL RPMI-1640 medium, their abdomens were massaged, and total lavage fluid was withdrawn. Peritoneal cells were washed in sterile saline, centrifuged (200 g, 10 minutes, 25°C), and resuspended in RPMI-1640 culture medium. To create a monolayer of PMN cells, the granulocytes were allowed to adhere for a half hour, followed by gentle washing of the monolayer with culture medium to remove nonadherent cells. Following cytospin and May-Grünwald-Giemsa staining, the percentage of neutrophil granulocytes and macrophages was determined.

4.10. Virus production and generation of stabile TG2 knockdown NB4 cell line

A set of 5 anti human TG2 short hairpin RNA expressing lentiviral plasmids (pLKO.1-puro) was purchased from Sigma. As a negative control a non-target shRNA vector was used (Sigma) which expresses a shRNA sequence containing 4 base pair mismatches to any known

human gene. Lentiviral vectors were produced and used according to the manufacturer's protocol. In brief, bacterial glycerol stocks were amplified in 250 mL LB Broth Base medium (Sigma) overnight in the presence of 100 µg/mL Ampicillin (Sigma). Lentiviral plasmids were purified using Wizard Plus Midipreps DNA Purification System (Promega). For virus production: 60-70% confluent 293FT cells plated in 75 cm^2 cell culture flask were used for transfection. A total of 12 µg of plasmid DNA was used for the transfection of one flask: 3 µg of the antiTG2 shRNA plasmid (or non-target shRNA control plasmid) and 9 µg of ViraPower Packaging Mix (Invitrogen) were delivered into the packaging cells by Lipofectamine 2000 reagent (Invitrogen) according to the manual. Transfection medium (10 mL) was replaced after 12 hrs, conditioned medium containing virus particles was collected on the second and the third day after transfection, cleared by low-speed centrifugation, filtered through 0.45-µm-pore-size cellulose acetate filters and stored at 4 °C until concentration. For concentration viral supernatants were ultracentrifuged at 100 000 g for 2 hours at 4 °C. Pellets were resuspend in 200 µl sterile PBS, aliquoted and stored at -70 °C.

Virus titers were determined by cell culture titration; 10-fold serial dilutions of the concentrated viruses were used to infect 10^5 HeLa cells in a six-well plate. After 24 h transduced cells were selected and expanded in the presence of puromycin (Sigma) at final concentration of 5 µg/mL. 8-10 days old colonies were counted and virus titers were calculated by multiplying the numbers of the colonies with the dilution.

For NB4 transduction 2 x 10^4 cells were transduced with TG2 shRNA 1-5 or non-target shRNA viral vectors at a final MOI of 1.0 in 100 µL medium in 48-well plate. 3-5 parallel infections were performed with each viral vector. After 24-36 hrs stable transduced cells were selected by the administration of puromycin at final concentration of 5 µg/mL. Efficiency of TG2 gene silencing was determined at different time points by real-time Q-PCR and by Western blot analysis after induction of TG2 by ATRA.

4.11. Microarray experiment: sample preparation, labeling, hybridization and data analysis

Differentiation of NB4 was induced at 1 x 10^5 cells/mL by the administration of 1 µM ATRA and cells were harvested at 0, 48 or 72 hrs thereafter. Total RNA from 10^7 cells was isolated using the RNeasy kit (Qiagen). Experiments were performed in biological triplicates representing samples from different differentiations. Further processing and labeling, hybridization to GeneChip Human Gene 1.0 ST Arrays (Affymetrix), and scanning were

conducted at the Microarray Core Facility of the European Molecular Biology Laboratory (Heidelberg, Germany). Image files were imported to GeneSpring 10.1 (Agilent). Raw signal intensities were normalized per chip (to the 50th percentile) and per gene (to the median). To identify significantly regulated genes between two compared samples, we identified probe sets that showed at least 2-fold up- or down-regulation by eliminating probe sets with a ratio of signal intensity between 0.5 and 2. Finally, we performed a t-test for each pair of probe sets and filtered for values of $p \leq 0.05$. The PANTHER classification system (www.pantherdb.org/tools/genexAnalysis.jsp) was used for functional classification of genes.

4.12. Real-time Q-PCR

Total RNA was isolated from cells using Trizol Reagent (Invitrogen) and reverse transcribed to cDNA by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturers' instructions. Transcript levels were determined by real-time Q-PCR using Taqman probes. Real-time monitoring was carried out using an ABI Prism 7900 (Applied Biosystems). Transcript levels were normalized to the level of cyclophilin D and gene expression was determined by the comparative cycle threshold (CT) method.

4.13. Flow cytometry

Flow cytometric analysis was performed on FACS Calibur (BD Biosciences) using Cell Quest Pro software. To detect the surface expression of CD11c 8×10^5 cells were incubated in dark with FITC-conjugated anti-human CD11c mAb or with anti-IgG1 mAb as isotype negative control for 2 hrs at 4°C in 1 % BSA-PBS. Cells were washed, fixed by 1% PFA and then analyzed by flow cytometry. The geometric mean fluorescence of the FITC-positive cells was used to calculate CD11c surface expression labeled by anti-IgG1 mAb.

For detection of phagocytic capacity, 10^5 three days differentiated NB4 cells were incubated in dark with FITC-labeled *Listeria monocytogenes* or *Staphylococcus aureus* with 1:50 ratio (cells/bacteria) for two hrs at 37°C or at 4°C (to assess the aspecific sticking of bacteria to the phagocytes). After 2 hrs cells were put on ice, washed 2-times with 5-fold volume of ice cold PBS, fixed by 1% PFA and then analyzed by flow cytometry. Percent of FITC-positive cells were used as a measure of phagocytosis, with uptake at 4°C as a control for bacterial adhesion.

Cell cycle analysis by flow cytometry was carried out by standard method (propidium iodide staining after alcoholic fixation).

4.14. Cell adhesion and migration

For determination of cell adhesion, cells were differentiated for 48 and 72 hrs, than plated into 1% BSA (Sigma) coated plastic tissue culture dishes for 1 hour. Nonadherent cells were removed by washing, then the remaining cells were fixed with ice cold methanol:acetic acid (4:1), stained with hematoxylin solution for 5 minutes and counted microscopically. Migration assays were performed in BD Matrigel Invasion Chamber (BD Biosciences) according to the manufacturer's protocol using 200 nM fMLP, 10ng/mL IL-8 and 10% FBS as chemoattractants. 2.5 x 10^4 differentiated cells were allowed to migrate for 14 hrs. Chemoattractive effect of ATRA-treated wild-type, virus control and TG2 KD NB4 cell supernatants on white blood cells (WBCs) was evaluated using transwell polycarbonate inserts (6.5 mm diameter) with 5 μm pores (Costar Corning) and carried out as described previously.

4.15. CC chemokine level measurements

Supernatant of undifferentiated, 2 and 3 days ATRA-treated NB4 cells and its sublines were used for quantification of secreted CC chemokines. Concentration of CCL2 and 24 were measured using the Quantikine human ELISA kit (R&D Systems), CCL3 and 22 were quantified by the RayBio human ELISA kit (RayBiotech). Assays were performed according to the manufacturer's protocols.

4. RESULTS

5.1. ATRA-induced differentiation of the acute promyelocytic cell line NB4 towards neutrophil granulocyte is accompanied by the induction of TG2 and its translocation into the nucleus

It was demonstrated previously, that TG2 is dramatically induced in NB4 cells upon ATRA-treatment (Benedetti L, Blood, 1996). To get a better insight into the role of TG2 in this differentiation process, first, the cytosolic and nuclear protein fractions of the differentiating cells were separated and the cellular distribution of the enzyme was analysed by Western blot. We demonstrated that undifferentiated NB4 cells do not express TG2, but after 24 hrs of the ATRA-treatment TG2 is starting to be accumulated in the cytosol and it reaches its maximum protein level on the third day of the differentiation (Figure 6A). From that day its level remained at the same high level until the seventh day which was the last time point for sampling. TG2 protein level was found to be detectable also in the nuclear fraction of the ATRA-treated NB4 cells, but its accumulation in the nucleus was observable only from the second day of the differentiation and peaked after 4-5 days.

Protein cross-linking activity of TG2 in the two fractions was also determined by detecting the incorporation of [3H]putrescine into casein. The cross-linking activity of TG2 in the cytosol lysate increased continuously in accordance with the protein level of the enzyme. Interestingly, in the nucleus the activity of TG2 was also detectable, however its level lagged behind compared to the cytosolic form and calculated to total protein level (Figure 6B).

Confocal microscopy and immunostaining of permeabilized untreated promyelocytes and differentiated NB4 cells confirmed that TG2 accumulates in the cytosol during differentiation. Furthermore, we also validated its presence in the nucleus by double staining for DNA and TG2 on the sixth day of differentiation (Figure 6C). To determine which part of the nucleus contained the TG2 protein, nuclei were fractionated into detergent-soluble and insoluble fractions. The latter was sonicated and further divided into salt-extractable and salt-resistant fractions with 5 M NaCl. As in Wan et al. 1999, these fractions were identified as free/loosely bound, chromatin-associated and nuclear matrix factors. All three fractions were analyzed by Western blotting (not shown). These experiments revealed that a fraction of nuclear TG2 is loosely bound, while the rest is likely associated with the chromatin, and the nuclear matrix.

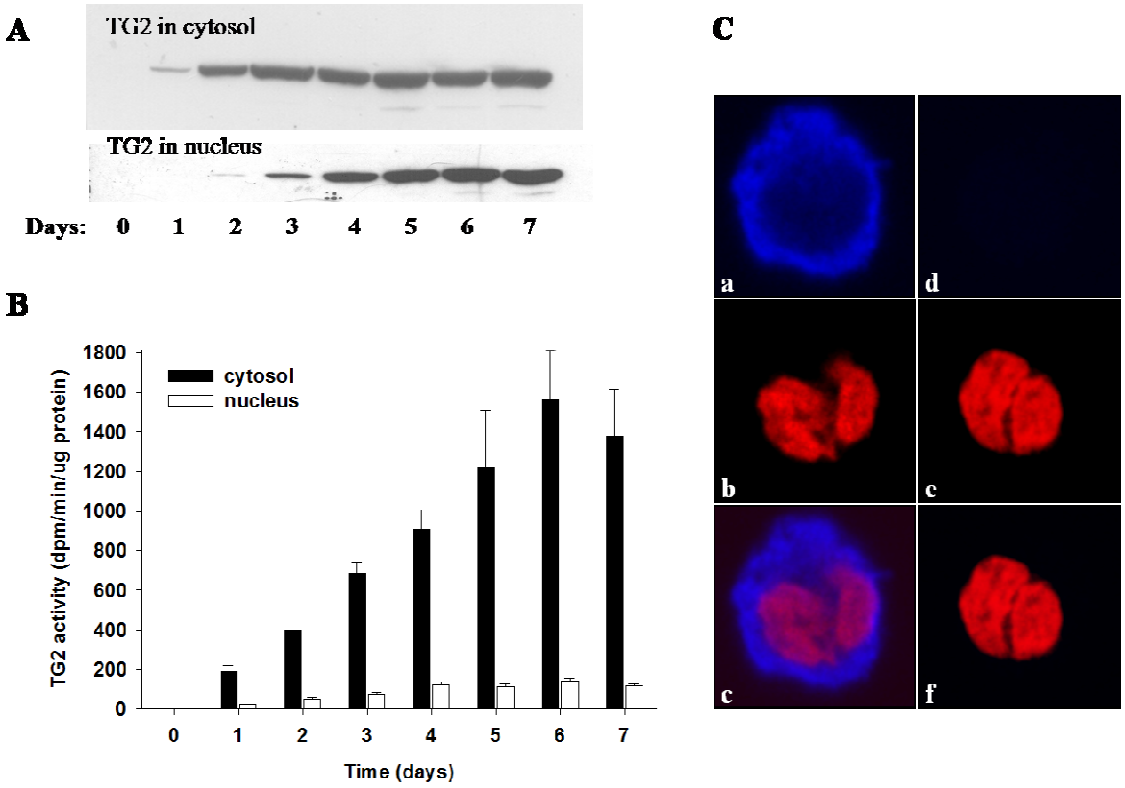


Figure 6. ATRA-induced neutrophil granulocyte differentiation induces the expression and activity of TG2 both in the cytosol and the nucleus of NB4 cells. *(A) Western blot analysis of TG2 in differentiating NB4 cells. 25 μg total protein of cytosolic and nuclear fractions were separated by SDS-PAGE and then blots were developed with the CUB7402 monoclonal antibody against TG2. (B) TG2 enzymatic activity in differentiating NB4 cells. Activity was measured by detecting incorporation of [3H]putrescine into casein in cytosolic and nuclear fractions. The amount of incorporated [3H]putrescine was determined in a beta-counter. Bars depict the means of 3 separate experiments each performed in duplicate. Error bars indicate standard deviation (SD). (C) Localization of TG2 in maturing NB4 cells analysed by confocal microscopy. NB4 promyelocytes were cultured in the presence of 1 μM ATRA for 6 days. Cells were fixed, permeabilized, and labeled with a monoclonal anti-TG2 antibodie followed by Alexa Fluor 633–tagged GAMIG (a, blue), and were also stained with PI (b, red). Panel c. is the overlay image. Note that TG2 is present in the nucleus. In the control, unspecific labeling by Alexa Fluor 633-GAMIG was checked by omitting the anti-TG2 antibody (d-f). The thickness of the optical sections shown is 800 nm.*

TG2, similarly to other mammalian transglutaminases, requires Ca^{2+} to become a catalytically active transamidating enzyme. To determine whether the induced TG2 in differentiating NB4 cells was indeed activated, the amount of the protein-bound Nε-(γ-glutamyl)-lysine cross-links were determined. It was found that ATRA treatment does not only increase the level of TG2 protein in NB4 cells, but it also leads to increased amount of cross-links in both the cytosolic and the nuclear fractions (Figure 7A). To evaluate whether

ATRA-induced TG2 in NB4 is active on primary amines such as polyamines as well, in situ biotinamido-pentylamine ($BNPH_2$) incorporation assay was applied. On the fourth day of the differentiation high numbers of proteins were modified by $BNPH_2$ in the nucleus and the cytosol of intact cells (Figure 7B). Both experiments indicate that TG2 is present and active in the cytosol and as well as in the nucleus of the differentiating NB4 cells.

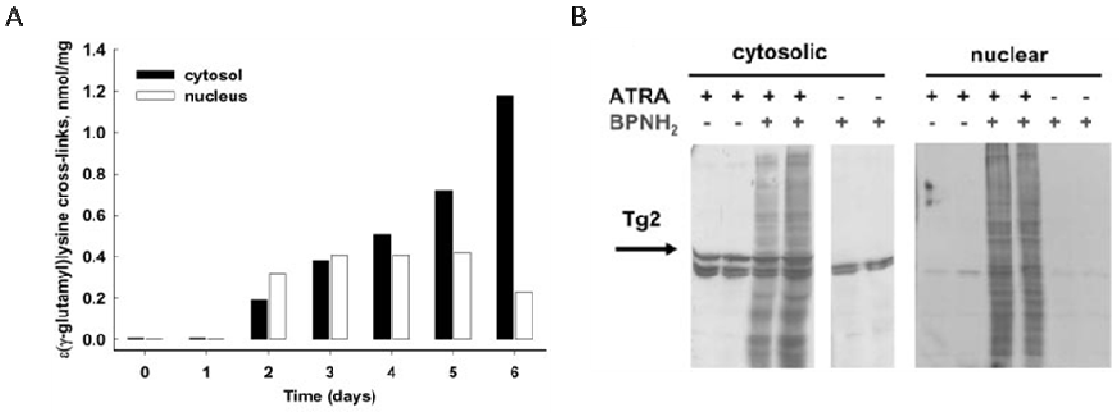


Figure 7. In ATRA-treated NB4 cells, both cytosolic and nuclear TG2 are active. *(A) Protein-bound Nε-(γ-glutamyl)-lysine cross-links in differentiating NB4 cells. NB4 cells were treated with 1 μM ATRA for 6 days, then cytosolic and nuclear fractions were separated. The Nε-(γ-glutamyl)-lysine cross-link content from each fraction containing 1 to 2 mg protein was determined as described in "Materials and methods." (B) In situ labeling of proteins of NB4 cells by TG2. Following 1 μM ATRA treatment for 4 days, cells were incubated in the presence of 6 μM 5-(biotinamido)-pentylamine ($BPNH_2$) for an additional 12 hours and then separated into cytosolic and nuclear fractions. $BPNH_2$-labeled proteins were analyzed by SDS-PAGE following immunoblotting with horseradish peroxidase (HRP)–conjugated streptavidin. To detect TG2 in the cytosol, the same blot (left panel, lanes 1 through 4) was probed with monoclonal anti-TG2 antibody. The arrow points to the TG2 bands. Untreated NB4 control cells revealed the endogenous biotinylated proteins. The parallel lanes represent 2 independent experiments.*

5.2. Effect of inhibition of TG2 activity on ATRA-induced differentiation of NB4

Since we observed the activation of the cross-linking activity of TG2 during the differentiation of NB4 promyelocytic cells towards neutrophil granulocytes we wanted to know whether the inhibition of its activity would influence the differentiation process. Therefore, we applied monodansylcadaverine (MDC), a nonspecific competitive inhibitor of transglutaminase during the whole time of the differentiation. On days 4 and 5 after MDC treatment, a 3- to 4-fold decrease of transglutaminase activity measured in whole cell lysates

was observed, while the level of its expression did not change (Figure 8A and B). Then, to confirm the inhibitory effect of MDC on crosslinking activity of TG2 we determined the amount of the protein-bound Nε-(γ-glutamyl)-lysine cross-links in four day differentiated NB4 cells. Administration of MDC resulted in a significant decrease in the cross-link content of cytosol and nucleus (Figure 8C).

Superoxide (O_2^-) production is characteristic for neutrophil granulocytes and it is considered as reliable marker of differentiation of myeloid cells determined by the NBT reducing ability (Dahlgren 1999). After the stimulation of cells by phorbol myristate acetate (PMA) it can be evaluated by the detection of the insoluble formazan deposits in the cells as blue precipitates. Untreated, proliferating NB4 cells are not able to reduce NBT, but after 24 hrs of ATRA-treatment the proliferation rate of the cells starts to slow down and in line with this they develop the ability to reduce NBT and formazan deposits are observable intracellularly. In normal case, after 72-96 hrs of ATRA administration the NBT positivity reaches the 100% percent indicating that all of the blast cells are differentiated (Figure 8D). When the differentiation of NB4 cells were induced in the presence of MDC, significant reduction was observed in the NBT reducing capacity of NB4 cells, especially at day 4 and 5. MDC alone did not influence the NBT positivity. These results suggest that crosslinking activity of TG2 is a constituent part of the ATRA-induced differentiation of these leukemic cells.

Another way to measure the production of O_2^- is based on isoluminol- or luminol amplified chemiluminescence assay. Isoluminol can be used to detect superoxide production by the extracellular NADPH-oxidase system, while luminol allows us to follow the production of O_2^- by the intracellular system (Dahlgren 1999). Extracellular and intracellular NADPH-oxidase activity was induced either by 50 nM PMA or by opsonized *S. aureus* and *C. albicans*, and, than measured in the presence of isoluminol or luminol, respectively. O_2^- production measured by chemiluminescence assay correlated well with the results obtained by NBT-assay, since MDC-treated differentiated NB4 cells produced significantly lower amount of superoxide both extra- and intracellularly (Figure 8E and F).

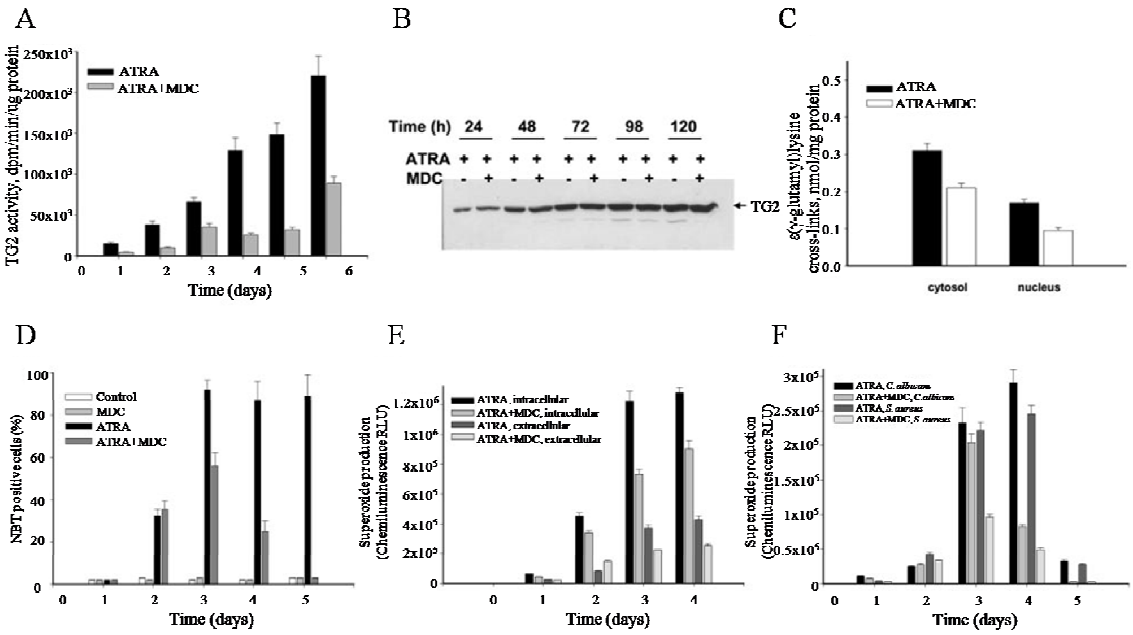


Figure 8. Inhibition of TG2 transamidation activity decreases both amine incorporation into proteins and the protein cross-link content in the cytosol and the nucleus of maturing NB4 cells. *NB4 cells were cultured for 96 hours in the presence of 1 μM ATRA or 1 μM ATRA plus 15 μM MDC. (A) Transglutaminase activity in MDC-treated cells. Total cell lysate (50 μg) was used for assaying TG2 activity. Activity was measured by incorporation of [3H]putrescine into casein. The means of 3 separate experiments performed in duplicate are shown. (B) Western blot analysis of TG2 in NB4 cells. The amount of TG2 in ATRA- and ATRA plus MDC-treated samples was determined by SDS-PAGE following immunoblotting with monoclonal anti-TG2 antibody. The arrow points to the TG2 bands. (C) Cytosolic and nuclear Nε-(γ-glutamyl)-lysine cross-link content in the absence or presence of TG2 inhibitor on day 4 of differentiation. Results are expressed as the mean SD of 3 independent experiments.*

Inhibition of TG2 activity decreases superoxide production in differentiating NB4 cells. *(D) Ratio of NBT-positive cells following treatment with MDC. At the indicated days, cell smears were fixed and analyzed for nitroblue tetrazolium reduction (at least 300 cells were scored for each experimental condition). (E-F) Chemiluminescence reaction of stimulated cells. (E) In NB4 cells extracellular and intracellular NADPH-oxidase activity was induced by 50 nM PMA and measured in the presence of isoluminol (0.5 mM) or luminol (0.5 mM), respectively. (F) NB4 cells were stimulated by opsonized S aureus and C albicans. Extracellular and intracellular NADPH-oxidase response was determined in the presence of isoluminol (0.5 mM) or luminol (0.5 mM), respectively. Results are the mean SD of 3 experiments.*

These results indicate that during the differentiation process TG2 with its crosslinking activity is necessary for the development and assembly of the NADPH-oxidase system which is one of the most important immunological features of neutrophil granulocytes, since it plays essential role in the killing of phagocytosed pathogens. A so called $gp91^{phox}$ protein, a subunit of the NADPH-oxidase system was described to be responsible for the lower production of

superoxide in certain cases (Dahlgren 1999). Therefore, and to further examine our hypothesis we determined the expression level of gp91^{phox} by Northern blot and real-time QPCR. With both methods we detected a reduction in the expression of gp91^{phox} mRNA level in case of MDC treated differentiated NB4 cells on the third and fourth days of the differentiation (Figure 9A and C). According to these results the lower level of superoxide production in MDC-treated differentiating NB4 is caused by the lower expression of gp91^{phox}. These finding suggest that during the differentiation process active TG2 is required for the optimal generation of the NADPH-oxidase system and TG2 contributes to this process at gene expression level.

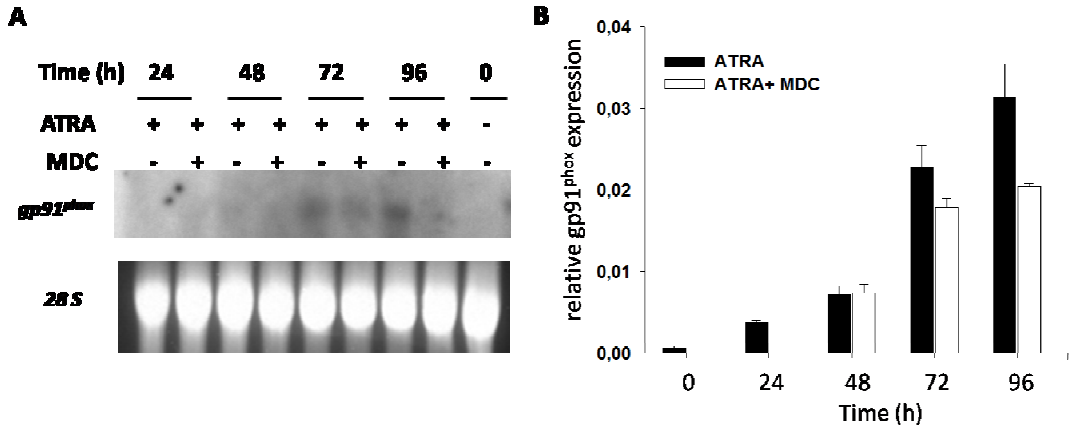


Figure 9. Neutrophils with decreased TG2 activity express lower levels of GP91^{phox} mRNA and generate less superoxide. *(A) NB4 cells were treated with 1 μM ATRA or 1 μM ATRA plus 15 μM MDC for 3 days. RNA was prepared and subjected to Northern blot analysis as described in "Materials and methods." (B) NB4 cells were incubated with MDC from day 1 in the presence of ATRA and harvested from following days. Relative expression of GP91^{phox} was normalized to the expression of human cyclophilin.*

5.3. Role of TG2 in mouse neutrophils

In the aim of evaluation of the role of TG2 in neutrophil function in vivo, we compared NBT positivity and superoxide anion generation in neutrophils derived from TG2+/+ and TG2-/- mice. Neutrophils were isolated from peritoneal exudates of mice previously injected with yeast extract. In accordance with the NB4 cell culture model, neutrophils of TG2-/- mice showed a 10-fold decrease in NBT positivity compared to the cells derived from WT mice (Figure 10A). To quantify the production of O_2^- specifically, a luminol analog L-012, by which superoxide can be detected as a chemiluminescence signal with a high sensitivity at

lower cell numbers, was used. The neutrophils of TG2-/- mice generated one order of magnitude less superoxide anion than the TG2+/+ neutrophils when they had been stimulated with PMA (Figure 10B).

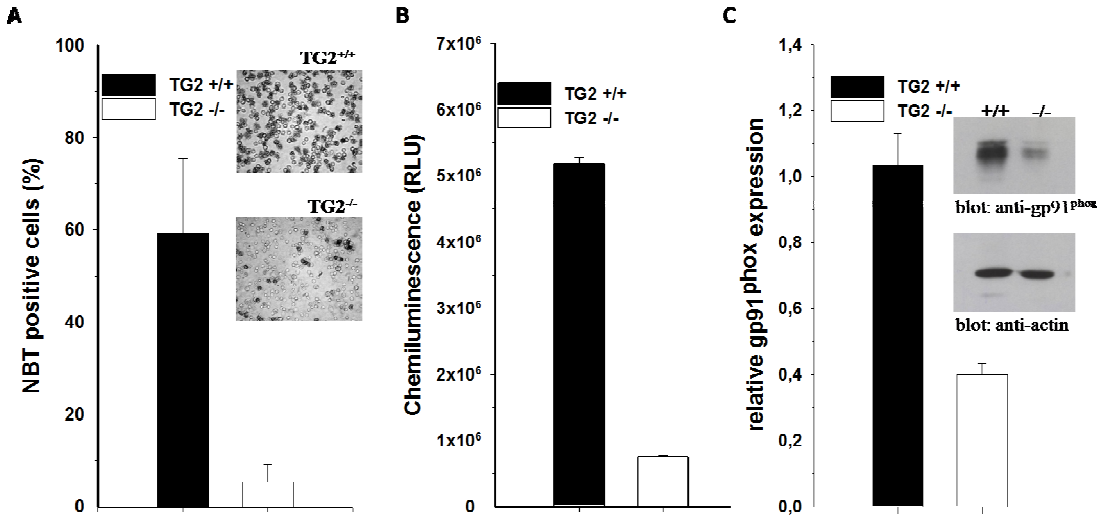


Figure 10. NBT positivity and the expression of gp91^{phox} in TG2+/+ and TG2-/- mouse neutrophils. *Wild-type and TG2 knockout mice were injected intraperitoneally with 1 mL 10% yeast extract. Four hours after the injection, the peritoneal lavage fluid was withdrawn and washed. (A) Granulocytes were allowed to adhere for 0.5 hours, and were then analyzed for NBT reduction. Inset images are NBT-stained neutrophils in a 96-well microplate. Visualization was done using an inverted microscope (Axiovert 135; Zeiss, Oberkochen, Germany), an Achrostigmat 20/0.45 Ph2 objective, adapter ring VAD-S70, and a DSC-S70 digital still camera (Sony, Tokyo, Japan). Film was imaged in AlphaImager 2200 (Alpha Innotech, San Leandro, CA). (B) Superoxide generation of TG2+/+ and TG2-/- mouse neutrophils. Wild-type and TG2-/- mouse neutrophils were collected and separated as previously described. ROS was generated by adding 50 nM PMA. Chemiluminescence was detected as described in "Materials and methods." (C) RT-Q-PCR analysis of gp91^{phox} mRNA expression of TG2+/+ and TG2-/- mouse neutrophils; gp91^{phox} mRNA levels were normalized to mouse cyclophilin. Data are representative of 2 independent experiments; error bars represent SD. Inset gp91^{phox} protein level of TG2+/+ and TG2-/- mouse neutrophils. Proteins of wild-type and TG2-/- mouse neutrophils were analyzed by Western blotting using an antibody against the gp91^{phox} and beta-actin.*

To ascertain whether the lower expression of gp91^{phox} is responsible for the reduced superoxide production in murine neutrophils similarly to the human cells we determined the relative mRNA expression of this subunit of the NADPH-oxidase system by real-time QPCR. In case of neutrophils isolated from TG2-/- mice, we experienced significant decrease in the expression of gp91^{phox} (Figure 10C). By Western blot analysis we managed to confirm that the reduction in gp91^{phox} mRNA level in TG2-/- neutrophils leads to, indeed, a diminish in the protein amount of this protein (Figure 10C).

5.4. Generation of stable TG2 knockdown NB4 cell line

To evaluate the role of TG2 in neutrophil granulocyte differentiation, the TG2 gene was silenced in NB4 acute promyelocyte cell line through RNA interference. NB4 cells were transduced by anti-TG2 shRNA expressing lentiviral vector (TG2-KD NB4). Non-targeting shRNA control vector was also used to create virus control NB4. The expression of TG2 mRNA was dramatically induced by ATRA treatment and continually increased during the 4 days of differentiation (Figure 11A). In virus control NB4 TG2 mRNA levels were not affected, while expression of specific shRNA sequence against TG2 (TG2-KD NB4) significantly kept TG2 mRNA and protein levels down even at the fourth day of differentiation (Figure 11A and B).

We have previously demonstrated that during ATRA-induced differentiation of NB4 cells, TG2 translocates into the nucleus. To examine the effects of shRNA on both endogenous expression and translocation of TG2 into the nucleus, we analyzed the cytosolic and the nuclear fraction of control, virus control and TG2 knockdown NB4 cells by Western blot (Figure 11B). Expression of TG2 was detectable at the first day and increased up to the fourth day in cytosolic fraction during the differentiation process. Virus control NB4 cells showed similar expression pattern. In TG2-KD NB4 cells treated in the same way, there were no detectable signs of TG2 in the cytosol at the first and the second day and it remained at a low level at the third and fourth day as compared to the control or the virus control samples. Determination of TG2 crosslinking activity in cytosolic fractions also confirmed that TG2 activity was not affected in virus control cells, but markedly decreased in TG2-KD NB4 cells (Figure 11B). In nuclear fractions of control and virus control cells, TG2 appeared at the second day of the differentiation process. However, in TG2-KD NB4 cells TG2 was hardly detectable, even on the third day. Nuclear crosslinking activity and protein level of TG2 changed in a parallel manner.

Taking together, these results indicated that ATRA-treated TG2-KD NB4 cells prove an appropriate model for studying the role of TG2 in neutrophil granulocyte differentiation, furthermore, the virus control NB4 cells served as the proper control to interpret results of the silencing experiments.

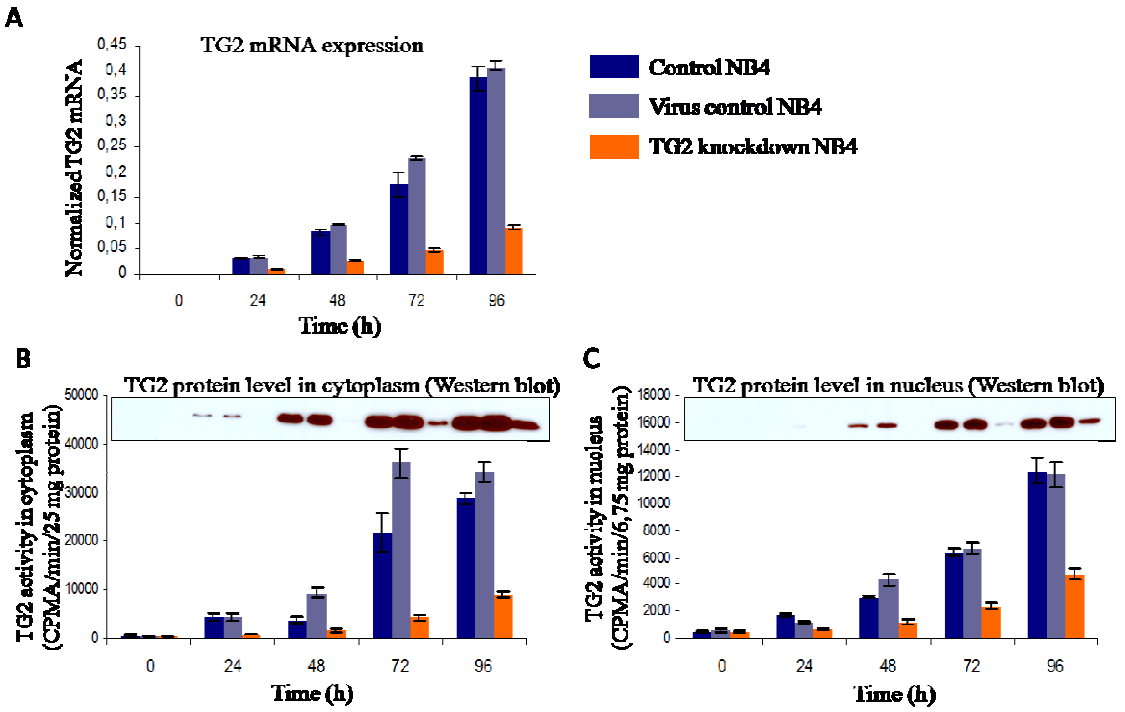


Figure 11. Generation of stabile TG2 knockdown NB4 cell line. *(A) NB4 cells (control NB4), NB4 cells transduced by random, non-target short-hairpin RNA expressing lentivirus (virus control NB4) and NB4 cells transduced by specific short-hairpin RNA against TG2 expressing lentivirus (TG2 knockdown NB4) were differentiated in the presence of 1 μM ATRA for 0, 24, 48, 72 and 96 hrs. mRNA expression of TG2 was determined at the indicated time points by real-time Q-PCR, measurements were conducted in triplicates, values are expressed as mean% ± SD of the mean. (B-C) Control, virus control and TG2 knockdown NB4 cells were differentiated in the presence of 1 μM ATRA for 96 hrs. B shows cytosolic activity of TG2 measured at the indicated time point by detecting incorporation of [3H]putrescine into casein. The amount of incorporated [3H]putrescine was determined in a beta-counter. Bars depict the means of 3 separate experiments each performed in duplicates. Inserted panel shows the cytosolic protein levels of TG2 at the indicated time points detected by Western blot. 25 μg total protein was loaded in each line, separated by SDS-PAGE, blotted and developed with CUB7402 monoclonal antibody against TG2. C shows nuclear activity of TG2 measured by detecting incorporation of [3H]putrescine into casein. Inserted panel shows the Western blot analysis of nuclear TG2. TG2 enzyme activity assays and Western blot analyses were performed as described above.*

5.5. shRNA-induced knock down of TG2 delays the differentiation process of NB4 cells

NBT reducing ability and the expression of CD11c are considered as reliable markers of differentiation of myeloid leukemia cells. Untreated cells were not able to reduce NBT, while in the case of control and virus control NB4 cells the reducing activity of NBT were observed

24 hrs after ATRA treatment and reached maximum levels at 72-96 hrs. Differentiated TG2-KD NB4 cells were also able to reduce NBT at 24 hrs but the number of NBT positive cells was 3-times less than those in control and the virus control NB4 cells and did not reach the maximum level even into 96 hrs of differentiation (Figure 12A).

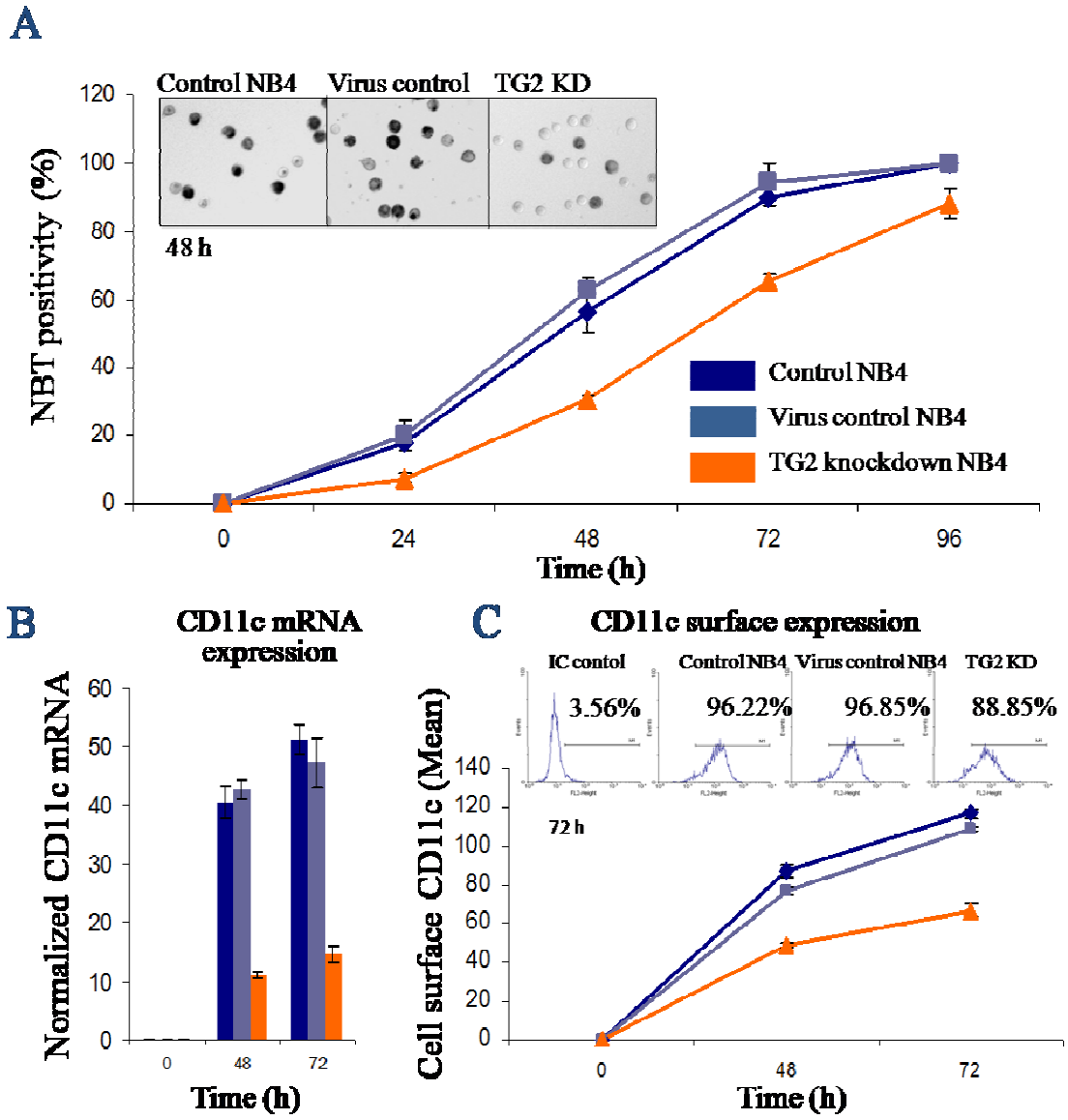


Figure 12. shRNA-induced knocking down of TG2 delays the differentiation process of NB4 cells. *NB4 cells were cultured in standard medium in presence of 1 μM ATRA for 0, 24, 48, 72, 96 hrs. (A) NBT reduction assays were performed (5 x 10^4 cells) at the indicated time points in triplicates in a 96-well plate by the addition of 1 mg/mL NBT and 2 μL (100 μg/mL)*

PMA for 30 min. At least 400 cells were counted in each sample and according to their intracellular blue formazan deposit contents percentage of NBT positivity was determined. Percentage of NBT positivity is expressed as mean% ± SD for n = 3 parallel experiments. Light-microscopic image inserts show typical NBT positivity of the ATRA-treated control, virus control and TG2 knockdown NB4 at the second day stage of the differentiation. (B) Induction of CD11c differentiation marker in ATRA-treated control, virus control and TG2 knockdown NB4 cells were determined at the given time points of differentiation by real-time Q-PCR. Measurements were conducted in triplicates; values are expressed as mean% ± SD of the mean. (C) Differences in surface expression of CD11c between control, virus control and TG2 knockdown NB4 cells were analyzed by flow cytometry at the indicated time points. 8 x 10^5 cells were labeled with mouse anti human CD11c IgG. Graph shows the average of the mean values of fluorescence measured in FL2 channel with ± SD. Typical flow cytometric profiles of CD11c surface expression in each cell types at the third day are shown by the inserted histograms.

The expression of the CD11c differentiation marker was measured at 48 and 72 hrs of differentiation and its expression in TG2-KD NB4 cells was 3-fold lower than in the control or in the virus control NB4 cells at each day of differentiation (Figure 12B). CD11c surface expression was also evaluated at the second and the third day of the differentiation by flow cytometric analysis, and it was further confirmed that while control virus infection did not influence CD11c surface expression, knockdown of TG2 lead to decreased expression of CD11c on the cell surface (Figure 12C).

5.6. Comparison of gene expression profiling of differentiating control and TG2 knockdown NB4 cells

To determine the influence of TG2 on NB4 differentiation, gene expression changes induced by ATRA in control NB4 cells were determined during the differentiation process (on days 0, 2 and 3) and compared to those in TG2-KD NB4 cells**.** During the differentiation process we detected 340 up-regulated genes on day 2 and 559 on day 3, and among these up-regulated genes 307 showed at least 2-fold increase on both days of the sampling in NB4 control cells (Figure 13A). Interestingly, we identified approximately 3-4-times more suppressed than induced genes by ATRA at both time points: 1281 genes showed decreased expression on day

two and 1586 on day three. 1059 genes were found to be repressed on both days of differentiation in control NB4 cells (Figure 13A).

Remarkably, in differentiating TG2-KD NB4 cells there were less up-regulated genes, 254 on day 2, 344 on day 3 and 208 overlapped, than in control ATRA-treated NB4 cells (Figure 13B). On the other hand, there were 1011 down-regulated genes on day 2, 1360 on day 3 and 808 overlapped in differentiating TG2-KD NB4 cells, as compared to control. These data suggest that the presence of TG2 has significant impact on ATRA-induced changes in gene expression in NB4 cells. Affected genes derived from the wild-type NB4 differentiation were found to be significantly enriched in the following categories: 'immune function', 'cell cycle regulation', 'nucleotide metabolism' and 'protein metabolism'. These results reflect well the biological events occurring upon ATRA-treatment, since during the differentiation cell cycle arrest is induced, which is accompanied by a significant decrease in nucleotide requirement and protein synthesis.

In TG2-KD NB4 cells, the gene ontological categories characteristic of differentiation of control NB4 cells contained fewer genes and several of those subcategories were not significantly overrepresented at reduced TG2 level. These data indicate that the induction of TG2 in NB4 cells contributes to the transcriptional remodeling, which launches the terminal differentiation program of these leukemic cells.

To validate the microarray results, we confirmed the expression patterns for ten selected genes by using real-time Q-PCR (Figure 13C).

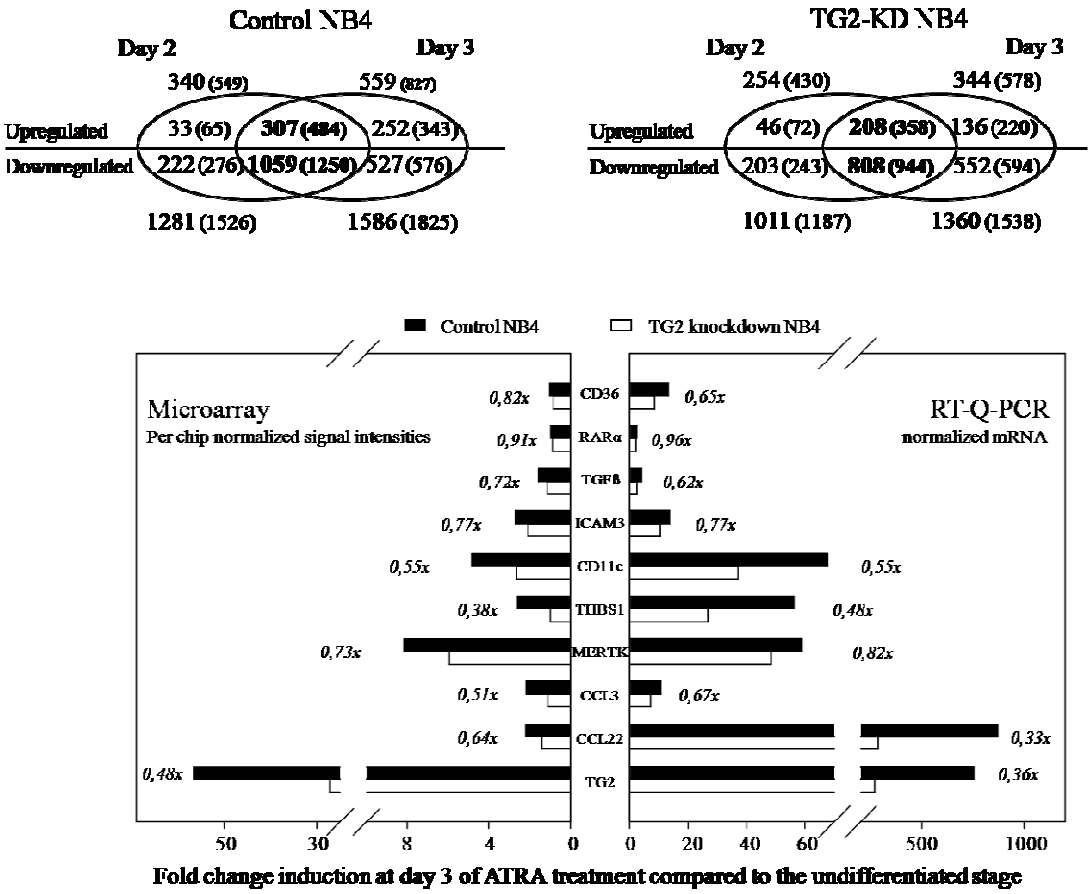


Figure 13. Number of genes (and entities) regulated by ATRA in differentiating control and TG2 knockdown NB4. *(A-B) Number of annotated genes (and the all entities in parenthesis) regulated by ATRA in control NB4 (A) and in TG2-KD NB4 cells (B) are visualized as a Venn diagram. 2x fold change difference was set and significantly changed genes were selected ($p \leq 0.05$ and Bonferoni Multiple Correction was used). The numbers of up- and down-regulated genes are separated. Genes significantly regulated at the both stages of the differentiation (day 2 and day 3) are presented in the overlapping region of Venn diagram (in bold). Note that among the genes up-regulated in both stages of the differentiation in TG2-KD NB4 cells almost 100 (307-209) genes were not up-regulated while about 250 (1059-808) genes were not down-regulated compared to the control cells. (C) Real-time Q-PCR verification of microarray data. Fold change induction of ten selected genes upon ATRA-treatment in control (black bars) and TG2-KD cells (white bars) are shown among the X-axis on the third day of differentiation. Left panel shows the results obtained from the microarray analysis and right panel demonstrates a representative expression pattern of the same genes determined by real-time Q-PCR. The genes analyzed were the cell surface antigen CD36, the retinoic acid receptor alpha (RARα), the transforming growth factor beta (TGFβ), the intercellular adhesion molecule 3 (ICAM3), the cell surface antigen CD11c/integrin alpha X (CD11c/ITGAX), the thrombospondin-1 (THBS1), the MER receptor tyrosine kinase (MERTK), the CC chemokines CCL3 and CCL22 and TG2. Ratios of fold change differences of analyzed genes in the TG2-KD NB4 cells and the control cells are presented.*

5.7. Functional classification of genes altered by ATRA in differentiating control and TG2-KD NB4 cells.

Since the gene expression changes are more pronounced on day 3, we focused on the third day differentiated stage for functional classification of the genes with altered expression. Gene sets representing the regulated genes in control and TG2-KD NB4 cells were classified into biological categories using the PANTHER Protein Classification System. As shown in Table 1, several functional categories were found to be significantly enriched (p value ≤ 0.05) among the 2144 genes regulated by ATRA in control NB4 cells at day 3. The identified categories are in accordance with the current knowledge about NB4 cells differentiation, ATRA-induced cell cycle arrest is accompanied by the repression of genes serving the metabolite requirements of cell division or necessary for the cell cycle and protein assembly. Differentiating NB4 cells develop the features of neutrophil granulocytes associated with induction of several immune function-related genes (Table 1).

		Control NB4			TG2-KD NB4		
Biological Process	# of genes in reference list	# of genes observed	# of genes expected	P-value	# of genes observed	# of genes expected	P-value
Immunity and defense	**1318**	**199**	**98,16**	**5,00E 19**	**140**	**78,15**	**1,55E 09**
Interferon-mediated immunity	63	20	4,69	1,77E-05	6	3,74	NS
MHCI-mediated immunity	22	12	1,64	3,35E-05	9	1,3	0,0018
Granulocyte-mediated immunity	64	16	4,77	0,0056	10	3,8	NS
T-cell mediated immunity	194	30	14,45	0,0311	22	11,5	NS
Cytokine/chemokine mediated immunity	125	22	9,31	0,0384	12	7,41	NS
Nucleoside, nucleotide and nucleic acid metabolism	**3343**	**360**	**248,97**	**1,59E 11**	**272**	**198,23**	**1,44E 06**
DNA metabolism	360	57	26,81	3,04E-05	44	21,35	0,0014
DNA repair	169	34	12,59	7,85E-05	27	10,02	0,0012
tRNA metabolism	42	13	3,13	0,0035	12	2,49	0,0017
Cell cycle	**1009**	**132**	**75,15**	**2,47E-08**	**102**	**59,83**	**7,42E-06**
Cell cycle control	418	67	31,13	1,72E-06	48	24,79	0,0028
Protein metabolism and modification	**3040**	**315**	**226,41**	**4,22E 08**	**249**	**180,27**	**3,61E 06**
Protein modification	1157	123	86,17	0,0108	97	68,61	NS
Protein complex assembly	68	16	5,06	0,0112	12	4,03	NS
Intracellular protein traffic	1008	103	75,07	0,0318	86	59,77	0,0202

Table 1. Classification of biological processes significantly enriched in the set of genes regulated by ATRA in control and TG2 knockdown NB4 cells at day 3.

In TG2-KD NB4 cells, the categories of biological process characteristic for differentiation of control NB4 cells, identified above, contained less number of genes and several subcategories were not significantly overrepresented at reduced TG2 level. These data clearly indicate that induction of TG2 in NB4 cells contributes to the transcriptional remodeling, which leads to the terminal differentiation program of these leukemic cells (Table 1).

5.8. TG2 facilitates the transcriptional alterations induced by ATRA

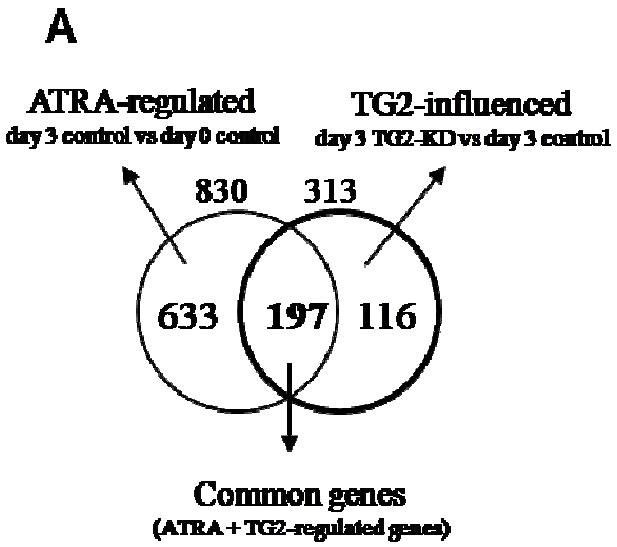
Since the effect of the knockdown of TG2 was found to be the most pronounced on the third day upon the phenotypic features of differentiating NB4, we identified the genes whose expression were affected by TG2 at that stage. We also considered that the regulatory effect of TG2 on gene expression might not be reflected in our results to its full extent due to incomplete (less than 100% efficient) silencing of the gene and the temporary nature of the knock-down effect. To avoid losing potentially TG2 regulated genes, threshold for inclusion in the study was set to 1.5 fold change, which is a relatively low value but a strict significance criterion (p value ≤ 0.05) was retained to select relevant genes. Figure 14A shows in a Venn diagram the overlapping genes regulated by ATRA and modulated by TG2 at the third day of differentiation. We identified 313 genes that are affected by TG2. An important observation is that 197 annotated genes from the 313 (63%) influenced by TG2, are also regulated by ATRA, showing that TG2 has a significant impact mainly on the expression of differentiation related genes. A list of all genes significantly up- or downregulated by ATRA has been appended to our paper (Csomós 2010) as supplementary information.

To visualize the relationship between the TG2 modulator effect and gene expression changes related to the differentiation process, the transcript level ratios of TG2-KD NB4 cells vs. control cells at day 3 (effect of TG2 knockdown), as well as differentiated control NB4 vs. undifferentiated NB4 (effect of differentiation), were determined and plotted against each other in case of the 313 TG2 dependent genes (Figure 14B). The modulator effect of TG2-KD is represented along the Y-axis, while effect of the ATRA-induced differentiation is visualized along the X-axis; genes regulated by ATRA are highlighted by black spots.

As shown by the scatter plot, a large proportion of the ATRA-inhibited genes fell into cluster 1, meaning that their expression remained high in TG2-KD NB4 cells. Knocking down of TG2 augments the ATRA-induced repression only in few cases (cluster 4). These results indicate that the inhibitory effect of ATRA, in case of these genes is mediated by TG2. In the

ATRA-induced genes there was only one gene, which remained up-regulated in TG2-KD cells (cluster 3), while 131 showed reduced expression in cells lacking TG2 (cluster 6). This means that TG2 has an important facilitating role in the induction of certain genes characteristic to the differentiation process. Taking together, these results suggest that TG2 and ATRA have mainly synergistic effects during differentiation.

Significantly overrepresented gene ontology categories derived from cluster 1 and 6 are presented in Table 2. Important genes from these two clusters, which are relevant to cell cycle regulation and immune function, are also listed.



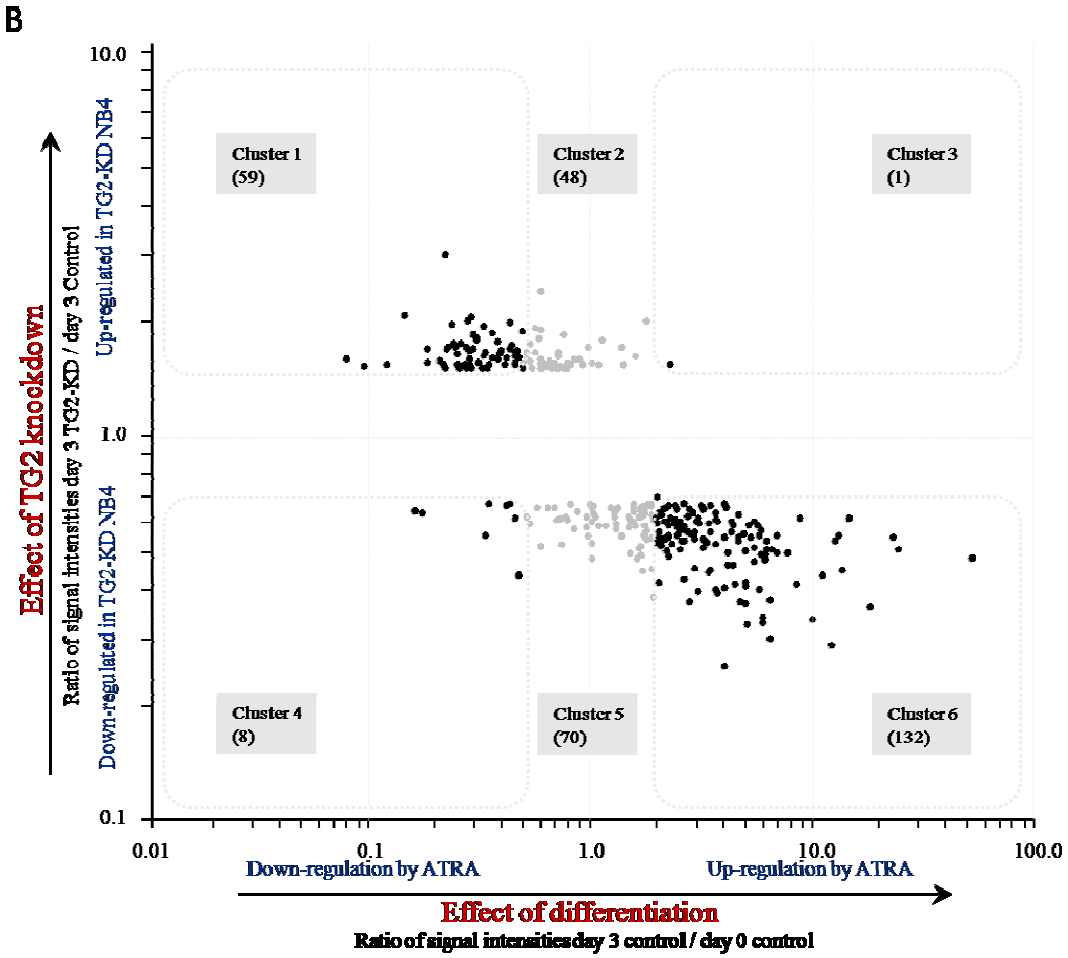


Figure 14. TG2 influences the expression of ATRA-regulated genes in differentiation of NB4 cell. *(A) 830 genes regulated by ATRA on day 3 belonging in the 4 most affected biological process categories (Table 1) were further investigated in the respect of TG2 effect. The Venn diagram illustrates the number of genes regulated by ATRA (830), the genes influenced by TG2 (313), and the number of the common part of these two gene sets (197). (B). The ratios of normalized transcript levels of 3-day ATRA-treated control NB4 cells versus untreated control NB4 cells were calculated in case of selected genes (effect of differentiation). Similarly, the ratios of normalized transcript levels of 3-day ATRA-treated TG2-KD NB4 cells versus 3-day ATRA-treated control NB4 cells were determined (effect of TG2 knockdown). The two ratios of transcript levels were plotted against each other resulting in a scatter plot that shows the relationship of transcriptional changes caused by the differentiation process and the TG2 knockdown effect. Down-regulation of 58 genes is behind (cluster 1) and the expression of 7 genes (cluster 4) is suppressed at higher degree than it occurs in ATRA-treated NB4 cells. While 1 gene is more up-regulated by ATRA in TG2-KD cells than normally expected (cluster 3), 131 genes are less induced in TG2-KD NB4 cells (cluster 6) as compared to the control. TG2 influenced genes regulated by ATRA are highlighted and genes that belong to a certain cluster are indicated.*

	Biological Process	# of genes in reference list	# of genes observed	# of genes expected	P-value
Cluster 1	**Cell cycle**	**1089**	**7**	**1,83**	**6,58E-02**
	CDK6 - cyclin-dependent kinase 6				
	E2F5 - E2F transcription factor 5, p130-binding				
	MPP6 - membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6)				
	ORC3L - origin recognition complex, subunit 3				
	OTUD6B - OTU domain containing 6B				
	TUBGCP5 - tubulin, gamma complex associated protein 5				
	Transport	**1306**	**8**	**2,36**	**6,78E-02**
Cluster 6	**Immunity and defense**	**1318**	**53**	**60,37**	**1,04E-32**
	CD74, 86, 93 - CD74, 86 93 molecule				
	CFP - complement factor properdin				
	CLC - Charcot-Leyden crystal protein				
	FCRR1G - Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide				
	HLA-B - major histocompatibility complex, class I, B				
	LGALS9 - lectin, galactoside-binding, soluble, 9 (galectin 9)				
	PLSCR1 - phospholipid scramblase 1				
	SAMHD1 - SAM domain and HD domain 1				
	TYROBP - TYRO protein tyrosine kinase binding protein				
	Interferon-mediated immunity	**63**	**12**	**0,3**	**8,06E-13**
	IFI35 - interferon-induced protein 35				
	IFIT1, 2, 3, 5 - interferon-induced protein with tetratricopeptide repeats 1, 2, 3, 5				
	IRF9 - interferon-stimulated transcription factor 3, gamma 48kDa				
	OAS1, 2, 3 - 2',5'-oligoadenylate synthetase 1, 2, 3				
	OASL - 2'-5'apos;-oligoadenylate synthetase-like				
	SLAMF7 - SLAM family member 7				
	Cytokine/chemokine mediated immunity	**125**	**12**	**0,6**	**2,05E-09**
	CCL2, 3, 22, 24 - chemokine (C-C motif) ligand 2, 3, 22, 24				
	IL1B - interleukin 1, beta				
	IL8 - interleukin 8				
	TNF - tumor necrosis factor				

	Biological Process	# of genes in reference list	# of genes observed	# of genes expected	P-value
Cluster 6	**Granulocyte-mediated immunity**	**64**	**5**	**0,31**	**2,05E-02**
	GCA - grancalcin, EF-hand calcium binding protein				
	NCF2 - neutrophil cytosolic factor 2 - 65kDa, chronic granulomatous disease, autosomal 2				
	T-cell mediated immunity	**194**	**7**	**0,94**	**7,02E-02**
	Signal transduction	**3406**	**39**	**160,47**	**4,01E-05**
	RARRES3 - retinoic acid receptor responder (tazarotene induced) 3				
	Cytokine and chemokine mediated signaling pathway	**252**	**14**	**10,22**	**6,01E-08**
	Ligand-mediated signaling	**421**	**12**	**20,04**	**2,02E-03**
	Cell surface receptor mediated signal transduction	**1638**	**22**	**70,92**	**1,08E-02**
	Cell communication	**1213**	**22**	**50,87**	**1,03E-04**
	Cell motility	**352**	**11**	**10,7**	**1,09E-03**
	ITGB3 - integrin, beta 3				
	LSP1 - lymphocyte-specific protein 1				
	S100A8 - S100 calcium binding protein A8				
	Cell adhesion	**622**	**13**	**30,01**	**3,05E-03**
	CEACAM1 - carcinoembryonic antigen-related cell adhesion molecule 1				
	ITGA6, AM, X - integrin, alpha 6, alpha M, alpha X				
	SELL, P - selectin L and P (lymphocyte adhesion molecule 1 and granule membrane protein 140kDa)				
	SIGLEC12 - sialic acid binding Ig-like lectin 12				
	SPP1 - secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)				
	TSPAN14 - tetraspanin 14				
	Apoptosis	**531**	**10**	**20,57**	**8,06E-02**
	BCL2A1 - BCL2-related protein A1				
	BIRC3 - baculoviral IAP repeat-containing 3				
	DAPK2 - death-associated protein kinase 2				
	NLRC4 - NLR family, CARD domain containing 4				

Table 2. Significantly overrepresented gene ontology categories and representative genes derived from cluster 1 and 6.

5.9. TG2 knockdown NB4 cells retain cell proliferation potential

ATRA induces cell cycle arrest and terminal differentiation of NB4 cells by down-regulating genes, which are responsible for the extensive proliferation of these leukemic cells. Based on the gene expression data, there are several cell proliferation-related genes (such as E2F5, CDK6) whose expression remained at a markedly higher level in TG2-KD NB4 cells. Studying the changes of cell proliferation and cell cycle distribution profiles a significant effect of antiTG2 shRNAs expression was observed on both. At 48 hrs there were approximately 27% more cells, and even at 72 hrs about 10% more proliferating cells, in culture of TG2-KD NB4 than in controls (Figure 15A). Cell cycle distribution in NB4 cell lines revealed that when TG2 is silenced significantly more cells were present in S phase on the third day of differentiation (Figure 15B).

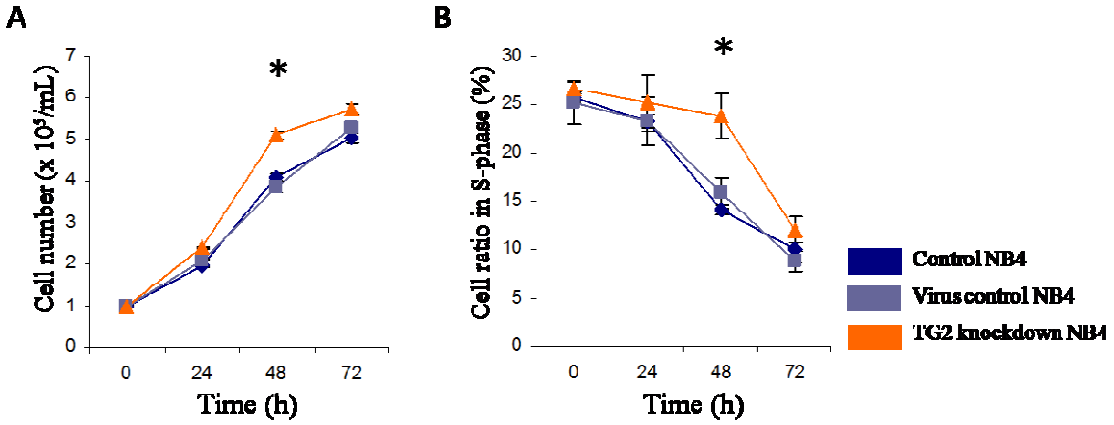


Figure 15. Knocking down of TG2 in NB4 cells enhances the cell proliferation potency during ATRA-induced differentiation. *10^5 cells/mL NB4 cells and its sublines (virus control and TG2-KD NB4 cells) were cultured in standard medium in presence of 1 μM ATRA for 48 and 72 hrs. (A) Cell numbers were determined by the trypan blue dye exclusion method at the 24, 48 and 72 hrs states of the differentiation. Figure shows growth curves of control, virus control and TG2-KD NB4 cells conducted in 3 parallel experiments. The increase in TG2-KD NB4 cell number was statistically significant ($p \leq 0.05$) at 48. (B) Cell-cycle of NB4 cells and its sublines (minimum 20.000 events collected from each) were analyzed by flow cytometry. Figure shows mean of S-phase ratios of cell cycles from three independent experiments at 0, 24, 48 and 72 hrs of differentiating cells. The error bars represent standard SD of means. Increased number of TG2-KD NB4 cells in S-phase at 48 hrs was calculated to be statistically significant ($p \leq 0.05$).*

5.10. Immune functions are compromised in differentiating TG-KD NB4

Based on the microarray data, several functional deficiencies in differentiating TG2-KD NB4 cells could be predicted. As many genes with reduced expression levels in TG2-KD NB4 cells belonged to the 'Cell motility' or 'Cell adhesion' categories (i.e. integrins and selectins), we tested whether these expression changes appear at the functional level. Adhesion of neutrophils is crucial for physiological processes of cell migration, chemotaxis and phagocytosis. Undifferentiated NB4 cells are floating in culture but after the administration of ATRA they start to adhere to plastic surfaces. Numbers of adherent cells were determined in the second and third day of the differentiation and a significant reduction in the adherence of TG2-KD NB4 cells was observed (Figure 16A). Microscopic analysis also revealed that differentiating NB4 cells without TG2 are less spread, remain spherical and are only weekly attached to surface. We also tested whether down-regulation of TG2 expression would affect the migration and chemotaxis of differentiating NB4 cells in response to the chemoattractant IL-8 and/or fMLP and we observed a reduction in migration of TG2-KD NB4 cells (Figure 16B).

Following chemotaxis, phagocytosis of microorganisms by neutrophils is an important prompt response to inflammation. As differentiated TG2-KD NB4 shows marked reduction in the expression of several phagocytosis-related genes, such as CD14, CD36, MERTK and others, we were interested to see whether phagocytic capacity was influenced by the reduced TG2 level. In phagocytosis of *Listeria monocytogenes* and *Staphylococcus aureus,* approximately 1.5-fold and 2-fold decreases were detected, respectively (Figure 16C).

Measurement of highly-reactive oxygen species (ROS) generation by neutrophils during activation of respiratory burst is of great importance to evaluate the bactericidal activity of neutrophils. Neutrophils increase their consumption of O_2 to generate ROS, as superoxide anion (O_2^-) and H_2O_2, by NADPH-oxidase. The expression of *gp91/phox*, a subunit of NADPH-oxidase, which we found to be down-regulated on the mRNA level in MDC-treated NB4 cells and both on the mRNA and protein levels in TG2(-/-) neutrophils (Balajthy 2006), also fell behind wild-type expression level in differentiating TG2-KD-NB4, however its change was less than 1.5-fold and p was >0.05. Nevertheless, NCF2 (neutrophil cytosolic factor 2; P67PHOX/NOXA2), another major component of the neutrophil NADPH-oxidase system, was also found to be less induced in TG2-KD NB4 cells. ROS production was determined during the differentiation process at the second and the third day. TG2-KD

NB4 cells generated approximately 2.5-fold less superoxide anion than controls in accordance with the lower expression of NCF2 (Figure 16D).

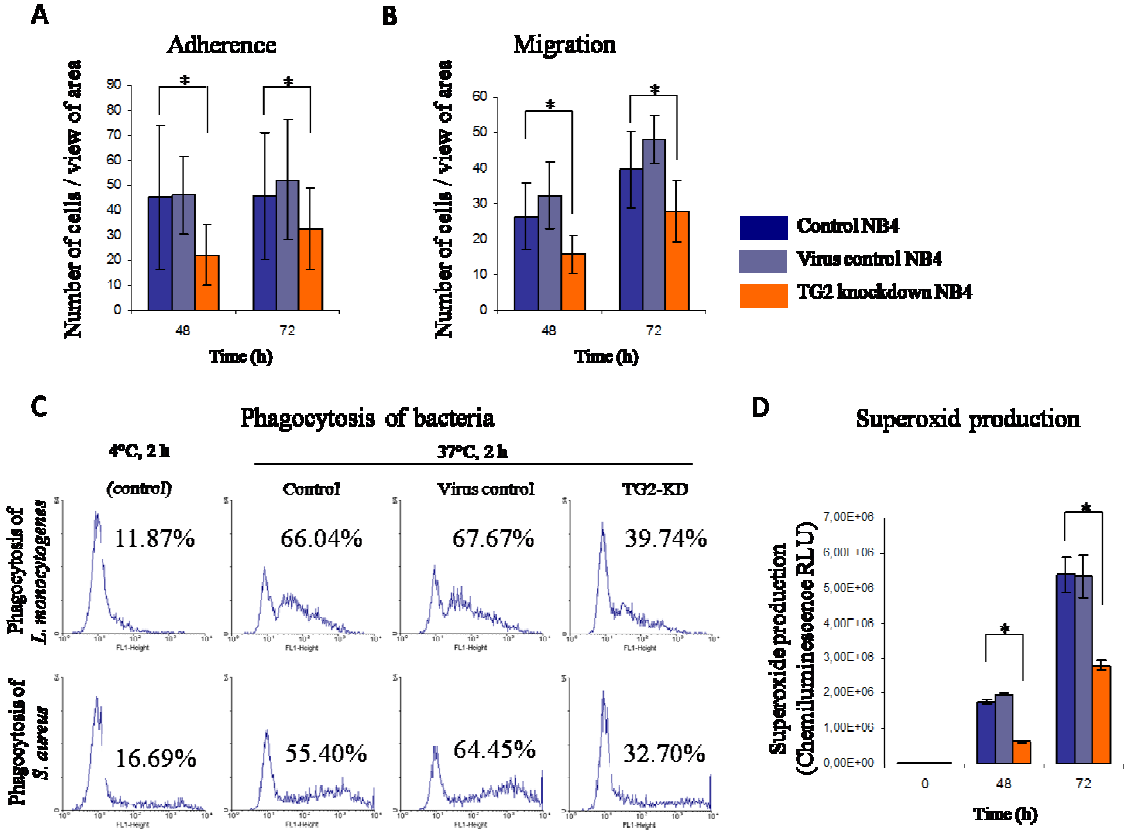


Figure 16. Knocking down of TG2 in NB4 cells reduces the immunological functions during ATRA-induced differentiation. *10^5 cells/mL NB4 cells and its sublines (virus control and TG2-KD NB4 cells) were cultured in standard medium in presence of 1 μM ATRA for 48 and 72 hrs. (A) After 48 and 72 hrs of initiation of differentiation 10^5 cells in 1mL were allowed to adhere to plastic surface of 24-well plate for 30 min. Following removal of non-adherent cells the number of adherent cells were determined by randomly selected 10 fields of view seen through the eyepieces of the microscope performed in 3 independent experiments. Decrease in adherence in case of TG2-KD NB4 cells was statistically significant ($p \leq 0.05$). (B) 2.5 x 10^5 cells from each cell lines were placed into the upper chamber of Matrigel Invasion Chamber after 48 hrs and 72 hrs of initiation of differentiation. Migration was elicited by 200 nM fMLP or 10 ng/mL IL-8 containing medium in lower chamber. Numbers of cells that migrated through the chambers were determined as described previously in panel A. The migration time was 14 hrs. The decrease in chemotactic activity was calculated to be significant at 48 and 72 hrs ($p \leq 0.05$). (C) ATRA differentiated control, virus control and TG2-KD NB4 cells were fed with two types of FITC-labeled bacteria (Listeria monocytogenes and Staphylococcus aureus) with the ratio of 1:50 cell/bacterium for 2 h in the presence of serum at 72 hrs stage of differentiation. Phagocytic capacity was evaluated by flow cytometric analysis. The bars represent mean and SEM of 3 independent experiments ($p \leq 0.05$). Typical flow cytometric profiles of L. monocytogenes (upper panel) and S. aureus (lower panel) phagocyting 72 hrs differentiated cells are shown. (D) Intracellular NADPH-oxidase activity of 48 and 72 hrs ATRA differentiated control, virus control and TG2-KD NB4*

cells (10^6 cells/mL) was induced by 50 nM PMA in 1 mL reaction volume containing 5 μL L-012 (100 μM). Results are the mean ± SD of three experiments. Chemiluminescence was detected by MOONLIGHT 2010 luminometer at intervals of 10s. The decrease in ROS production in TG2 knockdown NB4 was calculated to be statistically significant ($p \leq 0.05$).

5.11. Production of CC chemokines involved in the differentiation syndrome is restricted in TG2-KD NB4 cells upon ATRA-treatment

In the induction of the members of CC chemokine family microarray profiling showed that CCL2, 3, 22 and 24 were expressed significantly lower in TG2-KD cells at 48 and 72 hours after ATRA-stimulation (Figure 7A). We confirmed their reduced expressions both by real-time Q-PCR and measuring their concentration in the culture fluid (Figure 7B-C).

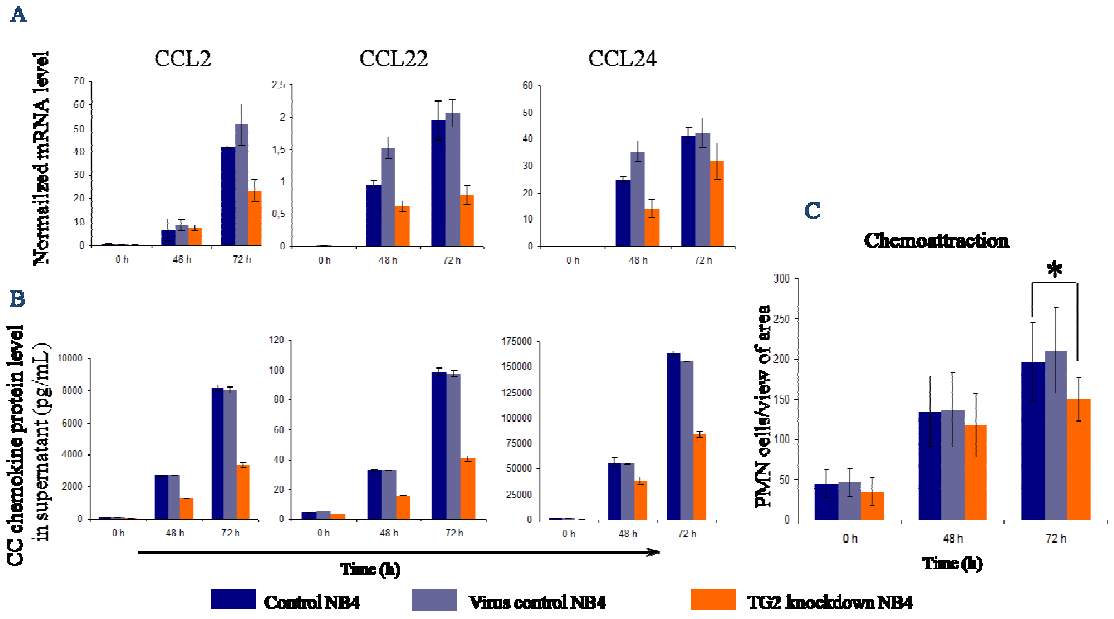


Figure 17. Production of CC chemokines induced by ATRA are restricted in TG2 knockdown NB4 cells. *10^5 cells/mL NB4 cells and its sublines (virus control and TG2-KD NB4 cells) were cultured in presence of 1 μM ATRA for 48 and 72 hrs. (A) Relative mRNA expressions of CC chemokines CCL2, CCL22 and CCL24 were determined at the indicated time points by real-time Q-PCR. Expression levels are normalized to the level of the cyclophilin. (B) Protein levels of CC chemokines were quantified in supernatants of NB4 cells at the indicated time points by ELISA. Figures show typical protein level patterns from 2 or 3 independent experiments measured in duplicates. (C) Chemoattractive effect of supernatant of differentiating control, virus control and TG2-KD NB4 cells on peripheral white blood cells was evaluated by migration assay. Migration experiments were performed two times with three parallels in each experiment. Statistical significance between wild-type and TG2 KD cells was determined by using the Students'-t-test $p < 0.05$.*

These results revealed that the suppressed TG2 expression can restrict the ATRA-induced CC chemokine productions of APL cells. We could also verify that the lower production of chemokines in TG2-KD NB4 cells leads to reduced chemotaxis of WBCs in a transwell system (Figure 7D).

6. DISCUSSION

Our experimentsshed some light on the role of TG2 in the ATRA-induced differentiation of the NB4 promyelocytic leukemia cell line at the level of the regulation of gene expression. Undifferentiated NB4 cells do not express TG2, but after the administration of ATRA, TG2, a direct target gene of RARα, is strongly induced. Accumulating TG2 is present in the cytosol of differentiating NB4 cells (Benedetti 1996), and we have demonstrated that TG2 also enters into the nucleus (Figure 6). TG2 possesses cross-linking activity in both compartments, since increasing expression of TG2 is associated with an increase in the protein-bound Nε-(γ-glutamyl)-lysine content of the cells, and also administered 5-(biotinamido)-pentylamine is incorporated into cellular proteins (Figure 7). Despite extensive investigations on this field the intracellular activation of TG2 is still generally a dubious question due to the low level of intracellular Ca^{2+}. It is clear that Ca^{2+} is absolutely required for the activation of TG2 in millimolar concentrations according to *in vitro* assays (Folk 1967). Mammalian blood and interstitial fluid contain millimolar amounts of Ca^{2+} capable of activating extracellular TG2. Ongoing transglutaminase activity in organisms is supported by the presence of Nε-(γ-glutamyl)-lysine linkages in extracellular matrix and blood clots (Murthy 1991). In contrast to the high extracellular Ca^{2+} concentration cells typically maintain basal concentrations of cytosolic Ca^{2+} between 20 and 100 nM and allow increases of up to 500 nM following cellular activation. This range of intracellular Ca^{2+} concentration raises aquestion as to how intracellular TG2 is activated.

However, it has been described that one of the landmark events of transcriptional remodeling in differentiating PML cells is the modulation of genes involved in calcium signaling and effector cascades (Zheng 2005). Differentiation of NB4 cells is characterized by increased expression of inositol trisphosphate receptors (ITPR) and other factors which constitute major calcium-releasing channels on the endoplasmic reticulum (ER), and by decreased expression of key calcium-storage proteins inside the ER (Zheng 2005). Accordingly, a remodeling event of calcium-regulatory cascades may consequently result in a net increase of cytosolic influx from the internal stores during ATRA-induced differentiation. An increased level of cytosolic Ca^{2+} influx may stimulate various calcium-dependent activities that seem to be important for neutrophil function and also could lead to the activation of intracellular TG2. In this study, we have not examined the changes of cytosolic Ca^{2+} concentration during the course of the differentiation, but as the induction of TG2 upon ATRA-treatment is strongly accompanied by an increase in the Nε-(γ-glutamyl)-lysine cross-

linked content of the cells and the incorporation of 5-(biotinamido)-pentylamine – as monoamine substrate of transglutaminases – into cytosolic and nuclear proteins, we assumed that the enzyme was activated.

Treatment of differentiating NB4 cells with MDC, a competitive cross-linking inhibitor of TG2, provides further evidence for the presence of active TG2 in the differentiating cells, since the accumulation of cross-linked content can be diminished by using the inhibitor. It was an interesting finding that inhibition of TG2 activity is accompanied by reduced NBT positivity of the cells (Figure 8). Since NBT positivity is considered to be a reliable marker of differentiation, we suspected a role for TG2 in the differentiation. Reduced production of superoxide anion in the presence of MDC in differentiated cells suggested a failure in the function of the NADPH-oxidase system. Further investigation revealed that at least one component of the complex, the $gp91^{phox}$ subunit is expressed at a lower level in MDC-treated differentiated NB4 cells (Figure 9). These findings led us to ascribe a regulatory role in the induction of $gp91^{phox}$ to TG2. Experiments on TG2 KO mice confirmed our hypothesis; since TG2-/- mouse neutrophils also show reduced NBT-positivity and superoxide production and they also express $gp91^{phox}$ at a decreased level (Figure 10).

To clarify the general role of TG2 in the differentiation process of NB4 we generated stable TG2 knockdown NB4 cells using lentivirus-mediated gene silencing that allowed us to study the differentiation process at reduced TG2 expression, taking into account, that besides the numerous advantages of shRNA-mediated gene silencing, complete and sustained elimination of the expression of a particular gene cannot be achieved, which may attenuate the consequences of knocking down of TG2. Nevertheless, we managed to reduce the level of TG2 during differentiation as it was confirmed by the determination of protein amount of TG2 by Western blot and by the measurement of its activity (Figure 11).

ATRA-treated TG2-KD NB4 cells lag behind significantly in differentiation as indicated by the decreased level of CD11c mRNA, its surface expression and the diminished NBT reducing capacity of these cells (Figure 12). The delayed differentiation of the knockdown cells is also revealed in several functional consequences such as decreased chemotaxis, adherence, phagocytic capacity, superoxide production and their sustained proliferative ability (Figure 13-14). TG2 has been implicated in various physiological phenomena, which can individually bear importance to the function of neutrophils. For instance, TG2 was identified as a cell surface receptor for fibronectin and therefore implicated in the adhesion and migration of monocytes or fibroblasts (Akimov 2001, Balklava 2002). Ex-vivo matured macrophages showed significantly decreased phagocytosis when they were differentiated in

the presence of a TG2 inhibitor. TG2 has been shown to influence the phagocytosis of apoptotic corpses by macrophages, most probably through its interaction with integrins and MFG-E8 (Toth 2009).

Impairment of neutrophil function by the reduction of TG2 could, in principle, result from the compromise of one or more of the aforementioned processes. The unexpected comprehensiveness of phenotypic changes in TG2-KD NB4, however, suggests that these do not represent isolated instances of interference with the molecular machinery of specific granulocyte tasks (phagocytosis, adherence, migration), rather a break-up of the blueprint for the granulocyte specific molecular apparatus as a whole. This must entail a principally unique regulatory effect of TG2, executed higher upstream during the course of the signaling events which induce NB4 cell differentiation.

The immediate effect of ATRA on gene transcription results in comprehensive reprogramming of the transcriptome. In the literature there is ample evidence that TG2 may influence effectors of gene expression (e.g. histones, Sp1, cytochrome c, PGC-1α) or components of signaling pathways, which in turn impact on transcription (e.g. NF-κB) as it was described in detail in the introduction. To ascertain whether TG2 may be implicated in ATRA-induced gene expression regulation in NB4 cells total gene expression profiling was carried out.

So far a whole genome gene expression analysis of ATRA-treated NB4 cells has not been performed. Previous similar analyses involved approximately 13 thousand genes, while our study concerned more than 28 thousand genes. Besides identifying several new ATRA-regulated genes we managed to confirm the changes in the expression of several markers of differentiation such as the members of the CCAAT/enhancer-binding protein family (C/EBPβ and ε), the helix-loop-helix (HLH) family (BHLHB2, ID-2 etc.), the interferon regulatory factors (IRFs), signal transducer and activator of transcription (STATs) and SWI/SNF family of proteins (SMARCD).

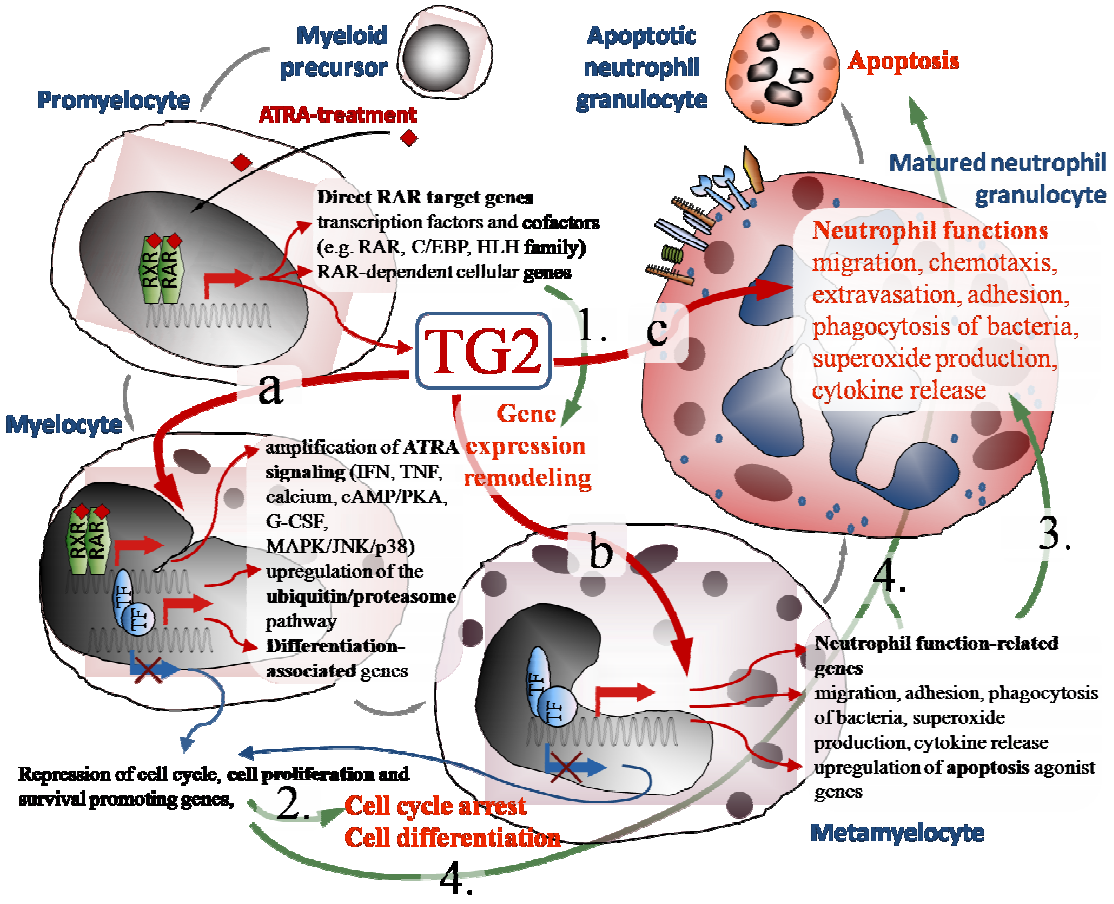


Figure 18. Gene expression alterations and consequent functional changes in promyelocytic leukemia cells during the course of their ATRA-induced differentiation into neutrophil granulocytes with the proposed diverse effects of TG2. *Stages of the differentiation process are shown (bone-marrow derived precursor, promyelocyte, myelocyte, metamyelocyte, neutrophil granulocyte, apoptotic neutrophil granulocyte). Direction of transitions is indicated by grey arrows. In APL ATRA-treatment is implemented on promyelocytic cells (black arrow, red rhomb). Angular arrows – red (activation), blue with X (repression) – represent the gene transcription; thin continual arrows indicate the gene sets induced (red arrow) or repressed (blue arrow) in a certain stage. Cell-biological processes evoked by gene expression changes are indicated with green arrows. 1. Gene expression remodeling: first, early up-regulation of direct RAR-target genes. There are several regulatory factors which are directly induced by ATRA and responsible for the amplification of ATRA signaling. For instance C/EBP and HLH transcription factor family and the RARα in itself serving a positive amplifier loop in the signaling, but also cellular genes such as CD11b, CD11c, and CD36 are also up-regulated. TG2 as a direct target gene of RARα is also strongly induced in the early phase. 2. In a later phase, a secondary wave of signal amplification appears which is responsible for the induction of gene sets involved in for example the Ca^{2+}-, IFN-, cAMP-, MAPK-signaling and for the repression of cell cycle, cell proliferation and survival promoting genes. Events occur in this phase lead to cell cycle arrest and differentiation. 3. Late gene expression changes provide the development of neutrophil functions and initiate the postmaturation apoptosis. Due to the up-regulation of numbers of immune function-related genes (e.g. cell surface receptors, genes involved in*

phagocytosis or ROS production) cells become functionally matured neutrophils. 4. As a late consequence of gene expression remodeling due to the repression of cell survival promoting genes and the up-regulation of apoptosis agonist genes matured cells die by apoptosis. ***Effects of TG2.*** *Based on our results we assume a possible synergistic effect of TG2 in the middle and late phase on the gene expression regulation induced by ATRA (a-b). In matured neutrophils TG2 may act on several cellular functions such as adhesion, migration and phagocytosis as it was described by others formerly (c).*

The complex modulatory role of TG2 in the differentiation process affects the remodeling of the gene expression profile in ATRA-induced differentiated NB4 cells. We identified at least 300 genes whose expression was dependent on the presence of TG2 to some degree and it turned out that they were mostly ATRA-dependent, as well. Genes affected by both differentiation (ATRA-dependent) and TG2 seem to be regulated in an opposite direction (Figure 4B). In details, 88.1% of the genes down-regulated by ATRA remained at a higher level in TG2-KD cells and most genes induced by ATRA in control NB4 cells showed lower expression level in the TG2-KD. These results raise the possibility of a synergic effect of TG2 with the regulatory role of ATRA.

Detailed analysis of the genes whose expressions were modified by TG2 revealed three important findings.

First, among the genes down-regulated by ATRA but held at higher level in TG2-KD cells the cell cycle and cell proliferation-related genes were significantly overrepresented (e.g. E2F5, CDK6). This result led us to assume that TG2-KD cells remain more proliferative for a longer time; therefore TG2 is necessary for the attenuation of cell proliferation. Indeed, TG2-KD cells have higher dividing capacity with more cells distributed in S-phase of the cell cycle in the early stage of the differentiation program. However, it has to be noted, that we did not observe a difference in the survival capability of differentiated TG2-KD cells as compared to the differentiated control NB4 cells during long-term culturing, or in the rate of apoptosis induced by arsenic-trioxide treatment.

Second, the genes, which are ATRA-induced but less expressed in TG2-KD cells during differentiation, mostly fall into the ‘Immunity and defense’ (Granulocyte-, Interferon- and Cytokine/Chemokine mediated immunity), the ‘Cell motility’ and the ‘Cell adhesion’ functional categories. Expression of key molecules involved in chemotaxis, phagocytic capacity and superoxide production and normally up-regulated in control ATRA-treated cells is diminished in TG2 KD cells.. One of these is paxillin that is necessary for adhesion and motility of leukocytes. PAK1/p21protein (Cdc42/Rac)-activated kinase 1 is known for taking part in the regulation of chemotaxis, chemokine-induced cytoskeletal actin polymerization,

and oxidative burst. We found that the expression of S100A8, a recently described pro-inflammatory protein expressed by phagocytes and implicated both in NADPH oxidase activation by interaction with NCF2/p67PHOX or transepithelial migration of neutrophils also remained at lower level in TG2-KD NB4 cells. These examples suggest that TG2 is required for the development of the full innate immune function of differentiating NB4 cells including their full inflammatory responsiveness.

Ther third conclusion is related to the role of TG2 in the process of inflammation. TG2 is implicated in the enhancement of inflammation since a TG2 inhibitory peptide significantly decreased the production of inflammatory cytokines and neutrophil infiltration into the lungsof LPS-treated mice. Furthermore, TG2 knockout mice are partially resistant to LPS-elicited experimental septic shock with increased survival, a diminished inflammatory response and attenuated organ damage. The expression level of the CCL2 inflammatory mediator was consistently up-regulated in ATRA treated APL cells. The simultaneous expression of cytokines such as TNF-α, IL-1β, IL8 and CCL2 by ATRA treated leukemia cells, such as NB4 cells, may result in both increased binding to epithelial cells and chemotactic transmigration and thereby further accelerate tissue infiltration. Despite corticosteroid treatment a massive induction of CC chemokines (e.g. CCL2) appears in ATRA treated APL patients that might lead to the differentiation syndrome with excessive inflammatory response. Since knocking down of TG2 in NB4 cells is accompanied by reduced chemokine production (CCL2, 3, 22 and 24) upon ATRA-treatment and therefore leads to decreased development of chemoattractive capacity, we propose that, the role of TG2 is essential in the regulation of inflammatory responsiveness and the development of differentiation syndrome.

Based on the findings presented here, it may be a viable option to interfere with such a pathological condition through modulating TG2 level in the differentiating leukemic cells. Specific down-regulation of TG2 expression by gene silencing or the use of chemical compounds to this effect may lead to suppression of cytokine secretion in retinoic acid syndrome patients. Such a therapeutic approach may also work in other TG2-relatedgain-of-function diseases like neurodegeneration, fibrosis, inflammation, cardiac failure, and celiac disease.

7. SUMMARY

Therapy of acute promyelocytic leukemia (APL) primarily consists of an *all-trans*-retinoic acid/ATRA-based treatment, which results in terminal differentiation of leukemic cells toward neutrophil granulocytes. However this differentiation-induced therapy is often accompanied by organ infiltration of differentiating leukemic cell leading severe hyper-inflammatory response in lung as an even lethal side effect of ATRA-treatment, called retinoic acid syndrome (RAS). Administration of ATRA leads to massive changes in gene expression in APL cells, including down-regulation of cell proliferation related genes and induction of genes involved in immune functions of neutrophil granulocytes. One of the most induced genes by ATRA in APL NB4 cells is transglutaminase 2 (TG2).

We have demonstrated that transglutaminase 2, after its induction, partially translocates into the nucleus, associates to the chromatin and is able to modify nuclear proteins by its acil-transferase activity during the differentiation process. The transglutaminase-catalyzed cross-link content of both the cytosolic and the nuclear protein fractions increased while NB4 cells underwent cellular maturation. Inhibition of cross-linking activity of TG2 by monodansylcadaverin in these cells led to diminished nitroblue tetrazolium (NBT) positivity, production of less superoxide anion, and decreased expression of $gp91^{phox}$, the membrane-associated subunit of NADPH oxidase. Neutrophils isolated from TG2-/- mice showed diminished NBT reduction capacity, reduced superoxide anion formation, and down-regulation of the $gp91^{phox}$ subunit of NADPH oxidase, compared with wild-type cells. These results clearly suggest that TG2 may modulate the expression of genes related to neutrophil functions and is involved in the process of differentiation of neutrophil.

To further investigate the role of TG2 in the differentiation process, RNA interference-mediated stable silencing of TG2 in NB4 cells (TG2-KD NB4) coupled with whole genome microarray analysis was performed. Our experiment revealed that TG2 contributes to the expression of a numerous ATRA-regulated genes. In the TG2-KD NB4 cells during ATRA-induced differentiation large number of genes related to neutrophil granulocyte function stayed partially suppressed. Down-regulation of these genes led to reduced adhesive, migratory and phagocytic capacity of neutrophils and less superoxide production. ATRA-controlled down-regulation of those genes, which are involved in cell cycle control and cell proliferation held at higher expression level and found to be manifested in a higher proliferative rate of TG2-KD NB4 cells. Since we observed that the induction of CC-

chemokines (CCL2, -3, -22, -24), which are responsible for the development of retinoic acid syndrome (RAS) in ATRA treated APL patients, were significantly lesser in TG2-silenced NB4 cells, significance of TG2 may have far-reaching consequences in clinical aspect of ATRA treatment APL patients.

Based on our results we suppose a complex regulatory effect of TG2 upon the retinoic acid-mediated differentiation of myeloid cells and propose that a reduced expression of TG2 in differentiating APL cells may suppress effector functions of neutrophil granulocytes and therefore moderate ATRA-induced hyper-inflammatory response in RAS.

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10. PUBLICATIONS AND POSTERS

This thesis is built on the following publications:

Tissue transglutaminase contributes to the all-trans retinoic acid induced differentiation syndrome phenotype in the NB4 model of acute promyelocytic leukemia.
Csomos K, Nemet I, Fesus L, Balajthy Z.
Blood. 2010 Aug 25.

Tissue-transglutaminase contributes to neutrophil granulocyte differentiation and functions.
Balajthy Z, **Csomos K**, Vamosi G, Szanto A, Lanotte M, Fesus L.
Blood. 2006 Sep 15.

Other publications:

Phage display selection of efficient glutamine-donor substrate peptides for transglutaminase 2.
Keresztessy Z, Csosz E, Harsfalvi J, **Csomos K**, Gray J, Lightowlers RN, Lakey JH, Balajthy Z, Fesus L.
Protein Sci. 2006 Nov 15.

PPARgamma-dependent regulation of human macrophages in phagocytosis of apoptotic cells.
Majai G, Sarang Z, **Csomos K**, Zahuczky G, Fesus L.
Eur J Immunol. 2007 May.

Poster presentations on international congresses:

Retinoic acid-induced tissue-transglutaminase contributes to neutrophil granulocyte differentiation by modulating the expression of $gp91^{phox}$
Balajthy Z, **Csomos K**, Fesus L.
8^{th} International Conference on Protein Crosslinking and Transglutaminases
Lübeck, Germany, September 2005.

PPAR gamma dependent programming of macrophage capacity for phagocytosis of apoptotic cells

Majai Gy, Sarang Zs, **Csomos K**, Fesus L.

13th ECDO Euroconference on Apoptosis

Budapest, Hungary, October 2005.

Studying the role of tissue-transglutaminase in neutrophil granulocyte differentiation

Csomos K, Balajthy Z, Zahuczky G, Fesus L

32nd FEBS Congress Molecular Machines

Wienna, Austria, July 2007.

Studying the role of tissue-transglutaminase in neutrophil granulocyte differentiation

Csomos K, Balajthy Z, Zahuczky G, Fesus L

9th International Conference on Protein Crosslinking and Transglutaminases

Marrakesh, Morroco, September 2007.

Tissue transglutaminase contributes to all-trans retinoic acid induced gene expression remodelling in acute promyelocytic leukemia

Csomos K, Nemet I, Fesus L, Balajthy Z.

Remodelling in Acute Promyelocytic Leukemia

Gordon Reseach Conferences: Transglutaminases In Human Disease Processes

Davidson College, NC, US. July 18-23, 2010.