SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
(PhD)

Role of tissue transglutaminase (TG2) in the differentiation, death and cytokine production of all-trans retinoic acid (ATRA) and arsenic-trioxide (ATO) treated NB4 leukaemic cells

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Introduction

Acute promyelocytic leukaemia (APL)

The acute promyelocytic leukaemia is one of the subtypes of the acute myeloid leukaemias (AML) (**Figure 1**). In 1957, Hillestad and colleagues published first that APL is a progressive, fast and lethal haematological disease. The neoplastic immune cells of an APL patient stay in the stage of promyelocyte while the differentiation is blocked [Bennet et al., 1980]. This subtype can be distinguished from the others, based on the cells' morphological and cytogenetic features [Bennet et al., 1985].

AML is a large group of bone-marrow-derived malignancies, the classification of its subtypes has been undertaken by two systems. In the first classification, called the French-American-British (FAB) classification system [Bennet et al.,1976] APL corresponds to the AML-M3 subtype (Table 1). Later on, the World Health Organization (WHO) published another system in which APL was identified based on the patients' symptoms and was assorted into the "AML - with genetic abnormalities" category [Daniel et al., 2001; Vardiman et al., 2002].

Based on the cytogenetic features of the cells we can distinguish APL from other myeloid leukaemias by a translocation involving the long arms of chromosome 15 and 17 [Goldman et al., 1974; Wang et al., 2008; Wiernik et al., 2013]. The result of the translocation is that the PML gene on chromosome 15 and the RARα gene from chromosome 17 are fused, giving rise to a chimeric transcript, PML/RARα. The fusion protein of these genes blocks the differentiation and the apoptosis of the blast cells [Kakizuka et al., 1991; Julien et al., 2011]. In most current therapies, the degradation of the fusion transcript and the elimination of its repressor function is the highest priority.

Patients with APL can experience severe bleeding in any part of the body due to thrombocytopenia, secondary fibrinolysis, and intravascular coagulation [Goldman et al., 1974]. A high number of azurophil-staining granules appear in the atypical promyelocytes, whereas the structure of the cell nucleus changes in parallel with the low fibrinogen level [Bennet et al.,1989; Daniel et al., 2001; Kühnl et al., 2012]. Since the changing structure of the nucleus is accompanied by great granularity, the disease used to be also called "hyper-granular promyelocyte leukaemia" [Goldman et al.,1974]. In the pathological classification, the presence of the Auer-rods are typical, which can be detected more often in AML patients [Rowley et al., 1977; Karen et al., 2008].

Years ago, APL was lethal and could not be treated with a good outcome, but in 1973 Bernard and colleagues found that APL cells were sensitive to daunorubicin chemotherapy, which lead to the full remission of the patient with APL [Bernard et al., 1973]. In the following years, the treatment of APL became more diverse by incorporating different chemical agents, for example, anthracycline, idarubicin, arsenic-trioxide or cytosine-arabinoside (CYT-ARAB). The latter gives a 75-80% remission in the newly diagnosed patient; however, at the beginning of the treatment results in a high death ratio due to bleeding complications. In 1985, a patient with APL received the first ATRA + ATO treatment, which years later was developed into a regimen consisting of four segments:

1) pre-**ATRA**
2) **ATRA**
3) **ATO** [Niu et al., 1999]
4) **ATRA** + **ATO** synergistic therapy [Abaza et al., 2017]

In the first induction therapy, 45 mg/kg ATRA was given *per os* twice a day, followed by 60 mg/kg/day Daunorubicin treatment for three days. This was followed by 100 mg/kg CYT-ARAB intravenous infusion for seven days, which in turn was followed by two rounds of arsenic treatment [Sanz et al., 2009; Powel et al., 2013].

All trans-retinoic acid (ATRA)

All-trans-retinoic acid is a small lipophile molecule. It stems from vitamin A in a two-step oxidation-based synthesis [Duester et al., 2008]. From vitamin A firstly retinol is produced, then, by the retinol dehydrogenase enzyme, RDH10 retinaldehyde is generated. In the next steps, the retinaldehyde dehydrogenases, RALDHD1-3 convert it into its final form of retinoic acid. Retinoic acid is required for healthy development. It can bind to the RARα receptor as a ligand, induces a conformational change, through which gene expression can be regulated. The transcription of the target genes can be either enhanced or inhibited. Examples of ATRA-regulated genes involved in ontogenesis are homeobox genes (HOX) and transglutaminase 2 (TGM2) [Duester et al., 2008].

In the literature, there are two main hypotheses about the molecular mechanism of the ATRA action. The first one involves ATRA's binding to a cell surface receptor, which results in intracellular signalling, whereas according to the second hypothesis ATRA first goes into the nucleus and induces gene expression directly [Duester et al., 2008; Csomós et al., 2010].

The ATRA, in physiological circumstances, can bind to cytosolic proteins such as Cellular Retinoic Acid Binding Protein (CRABP) and Cytoplasmic Retinol Binding Protein (CRBP). The intranuclear level of retinoic acid can be regulated by CRAPB levels [Corninc et al., 1992].

In 1996, Yoshida and colleagues observed that ATRA helped the remission of a patient with APL. They described an early change in PML-RARα expression 6 hours after the treatment that resulted from the degradation of the fusion protein [Yoshida et al., 1996].

Arsenic trioxide (As_2O_3/ATO)

Arsenic has several oxide forms, for example, arsenic tri- or dioxide. Arsenic is a white powder, solid material at room temperature. It is hardly water-soluble, upon heating it sublimates.

For therapy, it has been used before both parenterally and *per os*. Even in small doses, it can trigger severe reactions such as vomiting, tremours, digestions problems. A higher concentration of arsenic can induce severe cell death and could be lethal within a short time. In clinical trials, not only the oxide forms were used, but the sulphide forms also were applied (As_4S_4).

Arsenic was first used centuries ago in China to treat leukaemia. The main effect of arsenic is cell death initiation through the mitochondrial apoptotic pathway as a result of oxidative

damage and increased superoxide production. In the literature, the concentration of ATO was variable; however, in a small concentration of 0.5 μM ATO already induces apoptosis. Data from the literature also proves that this concentration can induce a partial differentiation of the leukemic cells [Chen et al.,1997].

The main target of the arsenic treatment is the PML component in the PML-RARα fusion protein. As it has been described previously, the PML domain contains the RBCC motif. The arsenic can bind to the cysteine residues in the RBCC's two zinc-ion binding motifs [Zhang et al.,2010]. Upon the binding of the arsenic a conformational change is initiated, the corepressor molecules are released (SMRT) followed by a MAP kinase-mediated signal transduction, where the end progress is the induction of apoptosis [Zhang et al., 2010; Sumi et al., 2010; Emi N., 2017].

At concentrations of 1 μM or above apoptosis can also be triggered by arsenic independently of the presence of the PML-RARα fusion protein [Zhu et al., 1999]. The molecular signal through which arsenic can induce apoptosis is an oxidative stress process via the reduced-glutathione (GSH) system. In the presence of arsenic, the activity of the NADPH-oxidase system increases and a higher amount of endogenous reactive superoxide (ROS) is produced. The high amount of ROS can destroy intracellular components, which, in turn, leads to apoptotic cell death [Zhang et al., 2010].
After the ATO induced conformational change of the PML-RARα, the PML protein becomes sumoylated, and this is followed by the proteasomal degradation of the fusion protein. The sumoylation is facilitated by increased kinase activity [Kogan et al.,2000; Zhang et al., 2010; Sumi et al., 2010]

ATRA + ATO

The reason why arsenic is widely used today in the clinic is its ability to promote the elimination of the LIC cells. The leukaemia initiating cells originate in the bone marrow, and although remission can be achieved by ATRA treatment alone, unfortunately, the LIC cells are not removed from the bloodstream which can lead to a second or third recurrence of the disease after a remission. By the inclusion of ATO in the treatment regimen this phenomenon can be avoided, thanks to targeting of the LIC by the arsenic [dos Santos et al., 2013]

Tissue transglutaminase (TG2)

Tissue transglutaminase, TG2 consists of 687 amino acids and has a molecular weight of 78 kDa. The enzyme is composed of four different domains:

1) N-TERMINAL - BETA SANDWICH
2) CATALYTIC CORE
3) C-TERMINAL - BETA BARREL 1
4) C-TERMINAL - BETA BARREL 2

It is localized in extra- and intracellular compartments, such as the cell membrane, cytosol, mitochondria and the nucleus. It has a role in signalling processes, cell proliferation, apoptosis, phagocytosis, wound healing progression and is a key player in the pathophysiology of different diseases (coeliac disease, cancer, and neurodegenerative diseases). TG2 can be detected in apoptotic cells and is involved in the crosslinking of Lys and Gln sidechains in proteins in apoptotic bodies [Fésüs et al., 2002; Pinkas et al., 2007].

TG2 has pleiotropic effects in signalling. For its activity calcium is required. Guanine-nucleotides and redox processes also regulate the activity of the enzyme. The binding of the calcium or GDP/GTP results in a conformational change, which can regulate the activity. When TG2 is GTP bound, the conformational change facilitates the signalling roles.

The binding of calcium results in an open conformation, in which GDP/GTP is not capable of binding to the enzyme facilitating the crosslinking function. For the catalytic activity of TG2, a high calcium level is required, however, under normal circumstances, the intracellular level of the calcium is low, while the GDP/GTP level is high; consequently, the crosslinking activity is inhibited.

In tumour cells/stem cells, TG2 is preferably in the closed conformation, which is suspected of contributing to cell survival, whereas the open conformation is responsible for tumour inhibition [Fesüs et al., 2002; Eckert et al., 2006; Karen et al., 2011].

TG2 has protein-disulphide isomerase activity (PDI), through which the sidechains of cysteine amino acids can be connected through di-sulphide bonds. This feature of the enzyme can be linked to the regulation of apoptosis. Additionally, several studies in the literature have shown that TG2 has a serine/threonine kinase activity. Moreover, TG2 has a role in cell adhesion and

is capable of forming a complex with fibronectin in a trans-amidation activity-independent way. [Fesüs et al., 2002; Pinkas et al., 2007]

Production of ROS

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, nitrogen and hydrogen. The radicals are divided into two major groups: nitrogen and oxygen-containing ones. The peroxides, superoxide, the hydroxyl radical, singlet oxygen and alpha-oxygen are the most typical forms of the reactive species.

The produced ROS are capable of damaging the cell membrane, the DNA and proteins also, they have a cytotoxic effect and can be produced by several extra- and intracellular processes. The requirement of their production is a higher activity of the mitochondria. ROS can be produced by several immune cells via the nicotinamide adenine dinucleotide phosphate oxidase system (NADPH-oxidase system).

Experiments with NB4 cells have shown that the ROS triggered damage can be avoided by intracellular reductases [Jing et al., 1999]. The elimination of the reactive species is carried out by the reduced glutathione (GSH) and the thioredoxin/thioredoxin reductase (TrX/TrxR) system [Lu et al., 2007]. In the presence of ROS inducers like ATO, the neutralization systems are inhibited, while the production of the ROS increases [Schröder K. 2019].

The NADPH oxidase family is related to the NOX enzyme family. The name stems from the neutrophil NADPH oxidase system. The NOX enzymes' N- and C terminal regions are homologous with the prokaryote ferredoxin reductase. In the N-terminal region of the enzymes, there are four calcium-binding EF-hands. The gp91 phagocyte oxidase (phox) and the p22phox subunits also contain transmembrane domains that are composed of six and two alpha-helices, respectively. p22phox can bind to the p47phox, p67phox and p40phox molecules which have SH3 domains. The catalytic core of the enzyme is the gp91phox membrane integrated glycoprotein. It contains two heme molecules, localized in the enzyme's N-terminal transmembrane regions. The cytoplasmic C-terminal region contains the NADPH and flavin adenine dinucleotide binding domains (FAD-binding domains).

To activate the complex, the RAC proteins need to bind to the p67phox component, followed by a GTPase reaction. Phosphorylation of all the components is crucial in their regulation by signalling pathways [Sumimoto et al., 2008]. The level of the p22phox can be increased by inorganic arsenic III, and the subsequent NADPH-oxidase activation can trigger DNA-breaks

[Lynn et al., 2000]. A concentration as low as 0.75 μM of ATO can increase the production of the ROS by increasing the expression of p67 and p47phox in NB4 cells [Wang et al., 2008].

Free radicals trigger phospholipase A_2 (PLA_2) activity in the membrane, resulting in the formation of leukotrienes. Neutrophils are protected from the damaging effects of free radicals by the activities of superoxide dismutase (SOD), catalase and glutathione peroxidase (GP) enzymes and high-intensity NADPH-synthesis via the pentose phosphate pathway [Schröder K., 2019].

Inflammation and production of inflammatory cytokines/chemokines

Inflammation is the cellular response of the immune system to the damaging effects of pathogens, toxic substances or radiation, and it is intended to eliminate the damaging effect and initiate the tissue regeneration process. The immune interactions sufficiently reduce the injury or threat of infection. This reducing effect allows the restoration of tissue homeostasis and the attenuation of acute inflammation. This biological response is already functioning properly at the time of birth. At the level of the tissues, inflammation is characterized by *erythema*, swelling, heat, pain, and deterioration of tissue function as a result of local immune, vascular, and inflammatory cellular responses to infection and injury [Ferrero-Miliani et al., 2007; Granger et al., 2010].

Proinflammatory cytokines and chemokines produced by tissue macrophages initiate the migration of various cells from the blood. The first cells arriving are neutrophils, followed by monocytes, lymphocytes, and mast cells. Monocytes can differentiate into macrophages and dendritic cells and enter damaged tissues by chemotaxis. For transmigration to begin, cells in the blood must sense where they are needed. Sensing is mediated by P- and E selectins on the surface of capillary endothelial cells, which are mediated by IL-1-or TNF pro-inflammatory cytokines.

1. Aim of the study

Differentiation syndrome (DS) is a life-threatening, severe complication developed during retinoic acid (ATRA) treatment of acute promyelocytic leukaemia (APL). Administration of ATRA leads to impressive changes in gene expression, among the most induced is tissue transglutaminase (TG2), which is normally not expressed in neutrophil granulocytes in the bloodstream.

To evaluate the pathophysiological function of TG2 in the context of immunological and biochemical functions, such as great superoxide production, cytokine/chemokine production in differentiated NB4 cells, we used an NB4 TG2-KO cell line and an irreversible transglutaminase inhibitor, NC9, to clarify the contribution of TG2 to the development of potentially lethal DS upon all-trans retinoic acid treatment of APL patient.

DS, which develops in approximately 5–25% of patients and also triggered by the release of several inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β) and chemokines like monocyte chemoattractant protein-1 (MCP-1/CCL2). These cytokines/chemokines are released from promyelocytes as they differentiated towards to neutrophil granulocytes in response to both ATRA and/or ATO therapy. Endothelium damage with a severe capillary leak, oedema, fever, hypotension, dyspnea, pulmonary or pericardial effusions and tissue infiltration of differentiated immune cells may ensue during this process.

This study demonstrates that TG2 expression induced by ATRA treatment reprograms the inflammatory signalling pathways, facilitated by nuclear factor κ-light-chain-enhancer of activated B-cell activation, resulting in the overexpression of TNF-α and IL-1β in differentiating APL cells. At the same time, the ROS production is enhanced further, suggesting that atypically expressed TG2 is a promising target for leukaemia treatment.

Material and methods

Cell culturing

APL cell line, NB4 (DSMZ GmbH) was cultured in RPMI 1640 HEPES-containing medium (Thermo Fisher; Life Technologies), supplemented with 10% (v/v) foetal bovine serum (FBS) (Gibco, Paisley, Scotland), 2 mM L-glutamine and 1% (v/v) 100 U/mL penicillin-streptomycin and 1% (v/v) 100 mM Sodium-pyruvate solution (Sigma-Aldrich, Inc.). Cultures were maintained at 37°C with 5% CO_2 under normal cell culturing circumstances.

Generation of NB4 cells lines for the study

APL cell lines were used in the study:

1) **NB4 WT** (the wild type of the APL cell line, purchased from DSMZ GmbH, Germany)
2) **NB4 TG2-C** (non-target shRNA control) [Csomós et al., 2010]
3) **NB4 TG2-KD** (target anti-TG2 shRNA) [Csomós et al., 2010]
4) NB4 TG2-ha (TALEN-based silencing of wild type NB4, heterozygous TG2-KO)
5) **NB4 TG2-KO** (TALEN-based silencing of wild type NB4)

3.1. Generation of NB4 TG2-KO cell line

As a basic cell line for the TG2-knock-out experiments: APL wild-type NB4 (NB4 WT) were cultured in RPMI 1640 HEPES-containing medium, supplemented and cultured as it was described in the *3.1 Cell culturing* section.

Transcription activator-like effector nucleases (TALENs) plasmids

Talen Library Resource (National University of Seoul) designed the plasmids for the human tissue transglutaminase gene (*TGM2*). To validate the effectiveness of transfection a green fluorescent protein (GFP)-reporter-containing plasmid and a red fluorescent protein (RFP) containing surrogate reporter plasmid (pRG2S fluorescence surrogate reporter PRG2S-CS) were used from the company, LAMOBICS (GeneCopoeia, Labomics, Nivelles, Belgium).

Flow cytometry and GFP positive cell sorting

Cells were analysed and sorted out with the help of BD FACSAria™ III flow cytometer (BD Biosciences, San Jose, CA). The apoptotic cells were excluded from the validation by forward scatter (FSC), and side scatters (SSC) gating. For the proper sorting, the 488nm and the 561nm lasers were applied for excitation of enhanced-green fluorescent-protein (EGFP) and monomeric-red-fluorescent protein (mRFP). The EGFP/mRFP signals were detected using 530/30nm and 582/15nm band-pass filters. The successfully transfected NB4 WT cells were sorted out based on the EGFP and mRFP positivity, whereas approximately 10-12.000 EGFP/ mRFP double-positive cells were sorted out into a new plate for further cell culturing.

Treatment of the NB4 cell lines

Using the successfully silenced NB4 TG2-KO cell line, among the previously generated ones (NB4 WT, NB4 TG2-C, NB4 TG2-KD) differentiation upon all-trans retinoic acid were performed for 11 days.

As a combination therapy, ATRA together with ATO was used in two concentrations, 0.5 and 2.0 µl for 3 and 5 days. To evaluate the role of the TG2 *in vitro* like, we have applied an irreversible TG2 inhibitor, NC9 in 30 µM concentration together with ATRA, or with ATO for 3 and 5 days. The NC9 compound was a gift from our friend Jeffrey Keillor W. from the University of Ottawa.

Preparation of cell lysate for Western blot

A total of 1–2x 10^6 cells were harvested and centrifuged at 12.000 rpm at 4ºC for 15 minutes, followed by the washing of pellet with pre-cooled 1x phosphate-buffered saline (PBS). The pellets were lysed with lysis buffer (50 mM Tris, 1mM EDTA, MEA, 0,5% Triton X 100, 1 mM PMSF) containing protease inhibitor cocktail (Sigma-Aldrich) with 1:100 dilution ratio. Lysates were homogenized with 5-7 strokes by sonicator at the intensity of a 40% cycle (Branson Sonifer® 450). After sonication, samples were centrifuged at 12.000 rpm at 4ºC for 15 min. The resulted supernatant was collected for protein concentration measurement with a Bradford assay (BIO-RAD).

Preparation of SDS-PAGE samples

The collected clear supernatant protein concentration was measured using 96-well plates (Wallac 1420 VICTOR2™ Multilabel counter) with a Bradford assay (BIO-RAD) at a wavelength of 595 nm. Every sample was measured three times in parallel, using BSA standard

calibration (stock concentration of 0.5 mg/ml). The samples were diluted up to a 2 mg/ml concentration, mixed with equal volumes of SDS denaturation buffer (0.125 M Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol, 10% MEA, and 0.02% bromophenol blue) and incubated at 100°C for 10 min.

Preparation of cytosolic and nuclear fractions

After the treatments cell were harvested and lysed as previously described in section "*Preparation of cell lysate for Western blot"*. The collected cell pellets were re-suspended in pre-cooled Nuclei isolation buffer (Nuclei Isolation Kit - Sigma-Aldrich NUC101-1KT) supplemented with protease inhibitor cocktail (Sigma-Aldrich), PMSF (Sigma-Aldrich) with:100 dilution ratio and with phosphatase inhibitor cocktail (Sigma-Aldrich) with 1:50 dilution ratio. After the resuspension, the lysed samples were centrifuged at 500 rcf at 4ºC for 5 minutes, followed by the saving the supernatant, as it was considered as the cytosolic fraction. The remaining pellets were washed another two times with pre-cooled Nuclei isolation buffer supplemented with inhibitors.

After the supernatant was removed, the pellet was lysed in lysis buffer (50 mM Tris, 1mM EDTA, MEA, 0,5% Triton X 100, 1 mM PMSF), homogenized with 7-10 strokes in a sonicator at the intensity of a 40% cycle. The nuclear and cytosolic fractions were centrifuged at 12.000 rpm at 4ºC for 20-25 minutes. The resulting clear supernatants were collected for protein concentration measurement considered as the nuclear fractions of the samples.

Western blot analysis

A total of 20-25 µg total protein/nuclear/cytosolic fraction were electrophoresed on 10-15% SDS-polyacrylamide gels followed by blotting onto a polyvinylidene difluoride (PVDF) membrane (Millipore) using a semi-dry/wet blotting method. After blotting with 5% milk powder/1x Tris-buffered saline and Tween 20 (TTBS) blocking procedure were carried out for 1 hour at room temperature (or overnight at 4 °C).

The membranes were washed three times with 1x TTBS each time for 15 minutes at room temperature and then incubated with horseradish peroxidase-labelled (HRP), affinity-purified goat anti-mouse IgG secondary antibody (Advansta) with 1:10.000 dilution ratio for 1 hour at room temperature. TG2 bands were visualized using the ECL Kit chemiluminescence-based method (Advansta). Blot images were taken using a ProteinSimple AlphaImager® HP instrument.

Cytospin slides preparation

All samples for the cytospin were taken from a homogeneous suspension of NB4 cell cultures. After pre-cleaning the slides (70% alcohol or methanol), ten μL of a homogeneous sample together with 90 μL of 1x phosphate-buffered saline (PBS) was added to the Cytospin™ tube (Shandon-CYTOSPIN II, 6511 Bunker Lake Blvd. Ramsey, USA), followed by centrifugation at 800 rpm for 3 minutes. Slides were fixed at room temperature with methanol and were prepared for further staining with Giemsa-May-Grünwald dyes.

May-Grünwald Giemsa Staining

May–Grünwald and Giemsa (Molar Chemicals) dyes were diluted with distilled water at a 1:10 ratio. Previously methanol fixed samples were stained with a May–Grünwald solution for 10 minutes and were rinsed with diluted Giemsa solution for 5–30 minutes. Cytospin slides were washed with distilled water and dried after at room temperature for 10 minutes. Light microscope images and documentation were captured by using a FLoid® Cell Imaging Station instrument (Life Technologies) at a scale of 200 μm. The ratio of different phenotyped cells in NB4 cell lines was determined by morphological features during the ATRA, ATO alone and the ATRA + ATO treatments. Based on the morphological changes, we classified the morphological types of ATRA-, ATO- and ATRA + ATO-differentiated NB4 cells into six groups:

1) undifferentiated (unsegmented nucleus and thin cytoplasmic region)
2) differentiated (segmented nucleus with the white-grey higher proportion of the cytoplasmic region)
3) apoptotic (well-defined membrane changes, with shrinkage)
4) necrotic (severe destruction of the membrane structure)
5) apoptotic-necrotic (dark blue stained nuclear remnants, disarrayed membrane structure)
6) mitotic (chromatin changes, round shape)

Nitroblue-tetrazolium (NBT) test

NB4 cell lines' differentiation was evaluated by using nitroblue-tetrazolium (NBT) reduction assay (Sigma Aldrich) followed by the manufacturer's instructions. All the documentation and pictures were obtained using the FLoid® Cell Imaging Station instrument (Life Technologies).

Surface marker detection with flow cytometry

Validation of the levels in differentiation, cell surface markers of CD11c/CD11b and Selectin-L were investigated. Total $4x10^5$ cells were plated into six-well plates, treated with ATRA;

ATRA+ATO, ATRA + NC9 and with ATRA + ATO + NC9. The treatment of the cells lasted for 11 days. By the end of the treatment, cells were collected and washed with pre-cooled 1x PBS, followed by centrifugation at 3000 rpm for 3 minutes. All the following steps were performed at 4°C. Pellets were washed and incubated with 2% bovine serum albumin (BSA) containing 1x PBS for 15 minutes and then centrifuged and washed and incubated at dark for 2 hours with the phycoerythrin (PE) labelled CD11c/CD11b, and with fluorescein isothiocyanate (FITC) labelled Selectin-L human antibody in 1:25 dilution ratio (R&D Systems). For each treatment and sample type, the appropriate isotype control (IgM/IgG) was prepared. The antibody labelling incubation followed by repeated washing steps; finally, the samples were measured by FACS (BD FACScalibur instrument). The geometric mean fluorescence of the cells was used to calculate the CD11c/CD11b/Selectin-L surface expression regarding each treatment and samples isotype control values. Data validated by WINMDI 2.9/Flowing software, normalized and corrected to the isotype controls.

Determination of superoxide anion production by luminol-amplified based chemiluminescence

Differentiated NB4 cell lines induced reactive O_2^- production was measured with the help of chemiluminescence assay using L-012 dye (Sigma Aldrich). The reaction volume of 100 µl containing 1×10^5 cells and 5.0 µl L-012 (100 µM). 2 µl PMA (100 nM) was added after and the mixture was incubated for another 5 minutes, followed by the chemiluminescence measurement with Synergy Multi-Mode Microplate Reader (BioTek Instruments, Inc) at intervals of 10 seconds. Production of generated ROS (light) by the reaction was recorded in relative luminometer units (RLUs) and corrected with the protein concentration level of each sample.

NF-κB induced luciferase activity measurement

The luciferase activity measurement was performed using Bright-Glo™ Luciferase Assay system (Promega). Before the luciferase measurement, NB4 cell lines were cultured in RPMI-1640 medium and treated as described in the previous sections. The measurement was performed according to the manufacturer's protocol. Results were validated by Graphpad Prism 8.00 software, using normalizing methods based on cell numbers and protein concentration, respectively.

Gene Expression Analysis with Real-Time Quantitative PCR (Q-PCR)

For real-time quantitative PCR measurements, total RNAs were isolated by TRIzol® (Invitrogen), following the company's instructions. Total RNA was determined by a NanoDrop

2000 Spectrophotometer (Thermo Fisher, Waltham, MA USA). Each sample was diluted up to 200 ng/μL for reverse transcriptase PCR (RT-PCR), The RT-PCR was performed using "High Capacity cDNA Reverse Transcription Kit" (Thermo Fisher) in a reaction of 10 μL sample + 10 μL reverse transcriptase-master mix. The assay and the PCR reaction were performed according to the manufacturer's manual.

For the real-time quantitative PCR reaction, the following TaqMan probes (ABI, Applied Biosystems, Waltham, MA USA 02451) were used: *TGM2*, *NCF2/p67PHOX*, *GPPhox91*, *IL-1β, TNF-α, MCP-1*, and *GAPDH*. The mRNA expression analysis was performed by the ABI Prism 7900 (ABI, Applied Biosystems). Relative mRNA expression levels of the genes were normalized to the level of *GAPDH* using the ΔΔ-Ct method.

Enzyme-linked Immunoabsorbent assay (ELISA)

The determination of the secreted proinflammatory cytokines and chemokines were measured by ELISA (deluxe ELISA, Biolegend). After each treatment, the supernatant of the cells was collected, diluted up to 10 times and analysed by separate plates focusing on MCP-1, IL-1β and TNF-α. ATRA and ATRA + NC9 treated and NB4 cells (for five days) supernatant were pooled together and analysed by the company Raybiotech Inc. by a Human cytokine array. The data received from the company after the analysis was normalized following the companies protocol.

Statistical analysis

The GraphPad Prism software was used to perform statistical analysis, version 8.02, by student T-test and Two-Way ANOVA (Bonferroni posthoc test; *$p<0.05$, **$p<0.01$ and ***$p<0.001$ ****$p<0.0001$).

Results

TG2 increases phagocytotic and antimicrobial power of maturing NB4 cell lines

With TALEN technology, we have generated a cell line where the *TGM2* gene was modified, resulting in a lack of TG2 protein. Without the TG2, cells were dividing properly, and the differentiation of the cells was not distracted. In the presence of 1 μM ATRA, NB4 cells in three days are differentiating into neutrophil granulocytes. In some of the literature data, it has been stated that DMSO can induce a partial differentiation of the cells, which has been evaluated with negative results in our experiments. Alone DMSO or DMSO + ETHANOL was

unable to induce any differentiation which proved by the lack of characteristics of differentiated cells.

To measure the differentiation efficacy, the nitro-tetrazolium-blue dye is used in diagnostics. This test is a very simple method for examining the phagocytic and oxygen-dependent antimicrobial ability of differentiated neutrophil granulocytes due to the reductive circumstances which stem from the NADPH-oxidase system. NB4 cell lines were differentiated in the presence of ATRA or within a combination of TG2 inhibitor. The results demonstrate that the differentiation process has taken place, which was not altered, but delayed in the lack of TG2 or the presence of an irreversible TG2 inhibitor either.

Csomós and colleagues previously have reported that NB4 TG2-KD cells showing NBT positivity, however, compared to the NB4 WT cells it is significantly less after three days of 1 μM ATRA treatment [Csomós et al., 2010]. To validate the correlation of TG2 expression levels to stages of differentiated neutrophil granulocyte, we determined the NBT positivity in percentage in the different cell lines parallel the expression levels of the human TG2, based on the NBT test and based on the mRNA expression patterns.

TG2 mRNA expression and protein expression were determined (not shown) in NB4 cell lines; whereas we have found that while the NB4 TG2-KO cell line did not express TG2, the wild type and NB4 TG2-C (virus control) cell lines were expressing TG2 in a great extent. When TG2 was knocked down, lower TG2 mRNA expression was observed. If we inhibited the TG2 by NC9, in 30 μM concentration, the TG2 mRNA expression did not show significant differences comparing to the ATRA treatments.

These results demonstrate that the lack of TG2 (NB4 TG2-KO cells) led to a shift in the NBT-positivity response curve compared to NB4 WT cell line. This phenomenon explains that the moderate or total expression of TG2 induced by ATRA has accelerated the differentiation process, resulting in a faster increase of NBT-positivity with a saturation curve over the 11-day time scale.

ATRA induces expression of L-selectin and leukocyte β2 integrin receptors (CD11b, CD11c) with their high-affinity state on the cell surface of NB4 cell lines

To investigate the differentiation ratio in parallel with the NBT test, flow cytometric analyses were performed on cell surface markers of L-selectin, CD11b, and CD11c. These proteins are accurate indicators of the degree of differentiation of NB4 cells. Our result showed that while

the expression of L-selectin, CD11b, and CD11c increased significantly from day 0 until day three and then remained almost unaltered by day 11 regarding cell surface positivity (data not shown here), the mean fluorescence intensity of the cells significantly increased during the time of treatments. Neither knockdown of TG2 nor the lack of it changed the cell surface expression of L-selectin, CD11b, and CD11c in the ATRA-induced differentiation. To investigate the fully active induced expression pattern, PMA was used for 20 minutes to see whether the surface expression can be further enhanced. The measurements showed that the PMA did not stimulate cell surface expression of CD11b and CD11c over the basic expression.

These results showed that retinoic acid treatment not only enhanced the expression of CD11b and CD11c on the cell surface but also triggered their high-affinity state detected by CBRM1/5 and CD11c (Clone type 3.9) mAb-s, specific for high-affinity CD11b and CD11c.

All trans-retinoic acid-induced TG2 facilitates the respiratory burst of NB4 cell lines

Immune cells and differentiated neutrophil granulocytes are capable of producing large amounts of reactive oxidants in response to a variety of inflammatory stimuli (pathogens, inflammatory cytokines, arsenic). Balajthy and colleagues have found that TG2-KO mice derived neutrophils had a lower expression of *GP91PHOX* mRNA and the production of ROS was less compared to the wild-type mice [Balajthy et al., 2006]. The two key protein of the neutrophil NADPH-oxidase system is the *GP91PHOX* and *NCF2/P67PHOX*. The TG2-dependency regarding the production of ROS was further evaluated by measuring the expression levels of mRNA expression of the *NCF2* and *GP91PHOX* component. We have found that retinoic acid-induced differentiation increased the mRNA expression of *NCF2/GP91PHOX* in NB4 cells in the presence of the TG2. However, in the NB4 TG2-KD and TG2-KO cells showed less expression of these genes. By the inhibition of the TG2, the mRNA expression remained at a lower level in a significant way, which showed the TG2 contribution of TG2 to the function of the NADPH-oxidase system. The PMA-induced total amount of ROS productions were measured in Relative light unit (RLU) normalised on 100 μg protein content of the cells. The NB4 WT and TG2-C cells produced significantly higher amount of ROS compared to the TG2-KD or TG2-KO cells, whereas the inhibition of the TG2 by NC9 resulted in a similar effect.

TG2 induces typical pro-inflammatory cytokines and chemokine expression through NF-κB and transcriptional activation

The pro-inflammatory cytokines tumour-necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and the chemokine, MCP-1/CCL2 have various functions in the progression of inflammation and the activation of other resting immune cells in the bloodstream. Regarding the mRNA and protein expression patterns of TNF-α, IL-1β, and MCP-1 in NB4 cells showed very similar results in the context of tissue transglutaminase expression. While high TG2 expression was accompanied by elevated mRNA and protein expression of cytokines/chemokine, at the low (NB4 TG2-KD) or the lacking expression of TG2 in NB4 TG2-KO cell line, both remained low in both ELISA and Q-PCR measurement.

Due to the previous results of the expression of the *GP91PHOX* gene, together with the expression data of TNF-α, IL-1 β and MCP-1, which all are NF-κB -dependent genes, we hypothesised that TG2 mediated the transcriptional activity of NF-κB proportional to TG2 quantity. This result was proven by applying NC9. In the presence of the inhibitor, the mRNA and protein expression level of the indicated cytokines/chemokine were significantly lower compared to the ATRA treated ones.

To confirm that the level of TG2 supports NF-κB-mediated transcriptional activity, a luciferase reporter assay was used, where an NF-κB promoter-driven luciferase construct was stably integrated into the genomic DNA of the NB4 cell lines.

We have found that the different expression levels of TG2 in the cell lines during the treatments were associated with proportionate NF-κB promoter-driven luciferase reporter activity and the well known exogenous inducer, TNF-α was not capable to further elevate it. Additionally, by inhibiting the TG2 with 30 μM NC9, the activity was demolished in those cell lines were the TG2 expressed.

TG2 expression level drives inflammatory cytokine expression quantitatively in resting ATRA-differentiated NB4 cells

To determine what kind of cytokines and chemokines are produced by the NF-κB pathway in a TG2 dependent way first, we have used the US Boston NF-κB target gene database. This database consists of 480 cytokines and chemokines, which are produced via the nuclear factor kappa B pathway. NB4 cells were treated with ATRA, and NB4 WT cells additionally were treated with ATRA + NC9. The supernatants of the cell cultures were collected, pooled, followed by the analysis using the ELISA-based RayBiotech 200 Human Biomarker Testing

Service. Inflammatory biomarkers were quantified from the supernatants of ATRA differentiated NB4 WT, TG2-KD, TG2-KO, and NB4 WT ATRA + NC9 treated cells. From the 200 proteins, 50 were detectable in the supernatant. From the 50 detectable proteins, 44 were expressed in a TG2-dependent manner, from which, 18 were identified as NF-κB transcription-factor target genes and can be found in US Boston NF-κB target gene database (Boston University Biology; 2018).

From the 18 proteins analysed and filtered out as TG2 dependent/$NF_{\kappa}B$ pathway, ten different cytokines were changing in parallel with changes in the expression level of TG2 protein: TNFα, I-309 (CCL-1), IP-10 (CXCL10), MIP-3alpha (CCL20), IL10, ICAM-1, MCSF, IL-1ra, and MDC (CCL22), and PAI-1 in NB4 WT, TG2-KD, and TG2-KO cells.

Eight NF-κB-pathway controlled cytokines and chemokines (MCP-1 (CCL2), MIP-1a (CCL3), MIP-1b (CCL4), IL-1b, IL-8, IL-9 CCL-28, and OPN (SPP1) were found to vary in parallel with TG2 expression to some extent.

Additionally, we found cytokines and chemokines which do not depend on NF-κB pathway, but were also expressed in a TG2 expression-dependent manner: MCP-3 (CCL7), MCSF R, TNF RII, GDF-15, angiogenin, VEGF R1, PECAM-1, lymphotactin (XCL1), and CXCL16.

Similarly, Cathepsin S, TNF RI, Resistin, and IL-2 Rb, but not Eotaxin-3 (CCL26) and Eotaxin-2 (CCL24), were expressed in a TG2-dependent manner.

TG2 contributes to expression and nuclear translocation of NF-κB, which is significantly reduced by the TG2 inhibitor NC9

NF-κB promoter-driven luciferase reporter activity and the extent of the expression of several NF-κB-controlled inflammatory biomarkers indicated that NF-κB transcriptional activity might depend on the magnitude of the expression of the TG2 protein.

The NB4 cells treated with 1 μM ATRA + 30 μM NC9 expressed fewer amounts of TG2, than those cells which were treated only with ATRA. However, the NC9 on the mRNA transcription did not change the TG2; the amount of TG2 protein was affected significantly.
These results suggested that the presence of NC9 by inhibiting the TG2 facilitates the proteolytic degradation of TG2. We have investigated how the low expression of the TG2 affects the expression of p65/RelA, p50, and phospho(Ser536)-p65/RelA components of NF-κB dimer. We found that the absence or reduced expression of TG2 by NC9 significantly

restricted the expression of p65/RelA, phospho-p65/RelA and p50 proteins in NB4 WT, TG2-C, TG2-KD, TG2-ha, and TG2-KO cells respectively at the level of the proteins.

Previously it has been published that in the ATRA-differentiated NB4 wild type cells, TG2 can translocate to the nucleus during differentiation [Balajthy et al., 2006]. To investigate the crucial role of nuclear TG2, after differentiation for 11 days, the cytosolic (C) and the nuclear fractions (N) of NB4 wild type cells were isolated and analysed for the expression of TG2, p65/RelA, phospho-p65/RelA, and p50.

In the ATRA differentiated cells, not only the total but also the other two fractions contain TG2 to a great extent. At the same time, the ATRA + NC9 treatment was associated with significantly increased cytosolic and decreased nuclear TG2 levels, compared to cells treated only with all-trans-retinoic acid. Additionally, the amount of total and nuclear p65/RelA was also reduced in the presence of the TG2 inhibitor.

Expression of phospho(Ser536)-p65/RelA, which is the "transcriptionally active" form of p65/RelA component of the dimer, was very similar to what we have observed in the case of TG2 protein in the total-, cytosolic and nuclear fractions. The total and nuclear lysates show reduced phospho-p65/RelA protein contents in the presence of NC9 inhibitor as well as total and nuclear lysates of p50 subunit of NF-κB showed.

These results support our hypothesis, in which the NC9 inhibits nuclear translocation of TG2 and considerably increases its cytosolic amount. These results are strengthened by the expression of phospho-p65/RelA and p50 proteins upon ATRA +NC9 treatment.

To further evaluate the effect of the irreversible TG2 inhibitor, we have differentiated NB4 wild cells for 11 days in the presence of ATRA or ATRA + NC9. These cells were treated for 3 hours with proteasome inhibitor, MG132 (Sigma Aldrich). Our western blot results of TG2 protein expression confirmed the enhanced proteolytic degradation of TG2 during the NC9 treatment.

In the presence of MG132 proteasome inhibitor, the TG2 protein expression level was not decreased but recovered to the original levels. These results also support the hypothesis of ours, that the NC9 treatment can accelerate the TG2 degradation.

ATO induces morphological, and cell number changes upon ATRA + ATO treatment in differentiated NB4 cells in a dose-dependent manner

The arsenic-trioxide was approved by the Food and Drug Administration (US FDA) to treat acute promyelocytic leukaemia. ATO in combination with other agents or either as a single therapy can significantly improve the remission potential of the newly diagnosed and relapsed APL patient compared to retinoic acid treatment alone [Niu et al., 1999; Wang et al., 2008; Sumi et al., 2010; Huyn et al., 2016].

ATO can trigger a cytotoxic effect in a concentration-dependent manner (2.0 μM < x). In contrast, at lower concentrations (x < 0.5 μM), it can induce partial differentiation and at higher dosages initiates apoptosis of the acute promyelocytic cells in the early period of the treatment. To investigate how the ATO affects the cell division and proliferation we have differentiated NB4 WT, TG2-C, TG2-KD and TG2-KO cells for 5 days in the presence of ATRA or combination with ATO in two concentrations: ATRA + ATO 0.5 μM and ATRA + ATO 2.0 μM.

Upon ATRA and ATO treatment the cell morphology changes. By the presence of the arsenic apoptotic and necrotic cells appeared, exhibiting a variable size and quality of cytoplasm [Zang et al., 2010]. To investigate how the NB4 cells respond to the single ATRA, ATO or various combined treatment of ATRA +ATO, cells were examined by morphological changes on cytospin slides. Each slide was examined three times, from which altogether 600 cells were counted. Based on the changed morphology, five big clusters were generated. These morphological changes are represented a mean value of the living, differentiated, mitotic, apoptotic and necrotic cells.

In the case of retinoic acid treatment, the cells mainly represent mitotic and differentiated cells. Our results show that after five days of differentiation, ATO induces a time- and dose-dependent cytotoxic effect, resulting in damaged and late apoptotic-necrotic NB4 cells. Higher concentrations of ATO were strongly associated with an increased number of apoptotic and necrotic cells. A higher apoptotic ratio was detectable in the ATRA + ATO 2.0 μM combined treatment compared to the ATRA + ATO 0.5 μM, whereas the number of differentiated cells was lower in NB4 WT and NB4 TG2-C cell lines. Overall based on the Giemsa-May-Grünwald staining, we observed a higher degree of apoptosis or necrosis in TG2-KD or TG2-KO cells. ATO treatment alone results in partial differentiation and dose-dependent apoptosis.

ATRA and ATO alone induce the production of ROS and expression of *NCF2* and *GPphox91* in differentiated NB4 cell lines, which altered by ATO in a dose-dependent manner.

In previous chapters, we showed that the atypical expression of TG2 could enhance neutrophil granulocytes' reactive superoxide production by enhancing the mRNA expression of two key components of the NADPH-oxidase system, *NCF-2/P67PHOX* and *GP91PHOX*. ATRA induced differentiation resulted in a high expression of the current genes, additionally a ROS production to a great extent, which could be reduced by applying an irreversible TG2 inhibitor, NC9. Since the NADPH-oxidase system is responsible for ROS production, we examined the extent of ROS production after ATRA +ATO treatments respectively.

Both genes, the *NCF-2/P67PHOX* and *GP91PHOX* mRNA expression levels were measured after 1 μM ATRA, 0.5 μM- and 2.0 μM arsenic, and in a combination, ATRA + ATO treatments for five days. We observed a TG2-dependent mRNA expression after ATRA treatment, whereas the ATO treatments resulted in almost a similar compared to the retinoic acid ones in NB4 wild type cells.

Based on the literature data, as we expected ATRA or ATO alone could enhance the production of ROS, via enhancing the NADPH-oxidase system, we confirmed this phenomenon, but in a combination (ATRA + ATO, 0.5 and 2.0 μM) the gene expression values remained low compared to treatments alone. The mRNA expression values of NCF2 and GP91PHOX were synchronised with the production of ROS, especially after the ATRA + ATO 2.0 μM treatment.

The expression of NCF2 and GPphox91 in differentiated NB4 cell lines attenuated by inhibition of TG2 with NC9

In the NB4 cell lines we found that where TG2 was induced by ATRA treatment, while lower expression levels of both *NCF-2/P67PHOX* and *GP91PHOX* mRNA were measured in the presence of NC9 beside the ATRA, compared to the ATRA.

The mRNA expression values were less in the presence of TG2 inhibitor. In ATRA + ATO + NC9-treated cells, the NC9 significantly reduced production of ROS after five days in an ATO concentration-dependent manner. The reactive superoxide production of differentiated NB4 WT cells was decreased by 2.4- with 0.5 μM ATO and 3.5-fold with 2.0 μM ATO. The TG2 inhibitor further reduced these values.

Presence of TG2 regulates the gene expression and the protein secretion of TNF-α, IL-1-β, and MCP-1 in differentiated NB4 cells.

From the literature, we know that the differentiation syndrome can be lethal in 2.5–30% of total cases. Severe complications are characterised by the presence of a large number of differentiated leukaemic cells in the bloodstream, additionally a great extent of the inflammatory cytokines produced via the NF-κB pathway, triggering the "cytokine storm."

In the previous sections, we have shown that MCP-1, IL-1-β and TNF-α proteins were secreted in a TG2-dependent manner in ATRA differentiated NB4 cell lines. NB4 cells were treated for five days and the secreted MCP-1, IL-1-β and TNF-α were measured at the mRNA and protein levels upon ATRA, ATRA + ATO 0.5 μM, and ATRA + ATO 2.0 μM.

ATO alone did not generate inflammatory cytokines and chemokines (data not shown here) however comparing to the ATRA treatment alone the combination with arsenic generated less protein of the current cytokines and chemokine.

The MCP-1, IL-1β and TNFα were approximately 50% lower for the combined ATRA + ATO 2.0 μM treatment than for retinoic acid alone, but these values were further reduced in a TG2-quantity-dependent manner.

After five days, we found that in the case of NB4 wild type cells in the ATRA + ATO 2.0 μM treatment MCP-1, IL-1β and TNFα production was reduced/inhibited accurately (with 5×, 10× and 20× lower values comparing to the controls).

Inhibition of TG2 decreases the gene expression and the protein secretion of TNF-α, IL-1-β, and MCP-1 in differentiated NB4 cells.

After we have examined how the TG2 protein expression altered the expression and secreted protein levels of MCP-1, IL-1β and TNFα inflammatory cytokines and chemokines in NB4 WT, TG2-C, TG2-KD and TG2-KO cells after ATRA + ATO treatments (0.5 and 2.0 μM), we have checked how the irreversible TG2 inhibitor, NC9 affects differentiating NB4 WT cells upon ATRA + ATO treatments.

Discussion

All-trans-retinoic acid (ATRA) therapy is one of the most frequently used agents to treat acute promyelocyte leukaemic (APL). Administration of ATRA induces the terminal differentiation of leukaemic cells towards neutrophil granulocytes, while arsenic trioxide (ATO) has recently been identified as another effective drug for treatment APL. ATO, in combination with ATRA, shows a synergistic effect, which further prolongs the survival of APL patients in a dose-dependent manner. ATO also affects different transcription factors resulting in activation of the cellular signalling pathways leading to reactive oxygen species (ROS) generation by the NADPH-oxidase system. These events explain the ability of ATO to induce partial differentiation and apoptosis, leading the remission in relapsed APL patients with the initiation of the degradation of the PML-RARα [de Thé et al., 2017].

ATRA-induced differentiation can be modelled using NB4/APL cells. The differentiation process involves modulation of several genes to produce functional neutrophil granulocytes at the end of the progress. The most upregulated gene in ATRA-induced maturation of NB4 cells is the tissue transglutaminase. Knocking down the expression of TG2 in NB4 cells (NB4 TG2-KD) has revealed modulation of gene expression; reactive oxygen species (ROS) generation; cytokine expression, secretion; adhesion, migration and phagocytic capacity of mature neutrophil granulocytes [Balajthy et al., 2006; Csomós et al., 2010].

TG2 is a Ca^{2+}-dependent cross-linking enzyme that is capable of deamidating γ-carboxamide groups of particular protein-bound glutamines [Fésüs et al. 2002]. TG2 has several enzymatic activities which are Ca^{2+} independent; it hydrolyzes guanosine-triphosphate (GTP) and adenosine triphosphate (ATP), can affect the G-protein-coupled receptor signalling and has protein kinase and protein disulfide-isomerase activities respectively. Recent studies have shown that TG2 in the GTP-bound/closed form drives cancer cell survival [Eckert et al., 2015; Kerr et al., 2017].

However, the treatment involving ATRA could result in a good outcome and a remission for APL patients, unfortunately in the form of hyperinflammatory reactions, ATRA treatment can cause severe side effects, including the infiltration of differentiated cells into soft tissues and organs, such as the lungs, liver and heart. These side effects of ATRA-treatment are among the signs of differentiation syndrome (DS), which is documented in 5-25% of APL patients who received ATRA induction therapy. First, the syndrome was called retinoic acid syndrome (RAS), years later it has been documented that not only ATRA, but also arsenic trioxide (ATO), or even with a combination can have such a side effect [Montesinos et al., 2011].

The differentiation syndrome is characterized by suddenly appearing fever; pleural and pericardial effusions; hypotension, vascular capillary leak, respiratory distress and due to the blood coagulation deficiency severe bleeding [Larson et al., 2003; Chen et al., 2011]. The differentiation syndrome is triggered by the release of several inflammatory cytokines/chemokine, such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and chemokines like monocyte chemoattractant protein-1 (MCP-1/CCL2) [Luesink et al., 2009]. These cytokines/chemokines are released from promyelocytes while they were differentiating in response to both ATRA and/or ATO therapies.

If the DS has occurred glucocorticoid treatment (mainly dexamethasone or prednisolone) is used to avoid the overproduction of the inflammatory cytokines/chemokines resulting in the recovery of the DS patients within 12 hours [Harada et al.,1994; Shibakura et al., 2005; Tsai et al., 2007], but the glucocorticoid treatments are not going to inhibit the induction of chemokines in differentiating APL cells [Montesinos et al., 2011].

However, the inflammatory signal-cascade is a well-known side effect of the ATRA treatment, or for ATO treatment, the exact molecular mechanism is not fully understood yet. From the literature of the last decade, we know that there are two optional molecular mechanisms which could be responsible for the severe side effects: the acute promyeloid cells' differentiation process and the infiltration of these differentiating cells into the soft tissues like lungs. These biochemical processes require cell adhesion molecules like selectins, integrins and soluble mediators (pro-inflammatory cytokines/chemokines), which trigger the activation of the receptors, and the adhesion/migration activation of the resting immune cells. ATRA-induced differentiation is associated with elevated expression of inflammatory cytokines/chemokines, and adhesion molecules called integrins [Balajthy et al., 2006; Luesink et al., 2009; Csomós et al., 2010].

From the ’90s to characterized the differentiation, CD11b/CD11c receptors have been used as surface marker proteins of differentiating APL cells. In normal circumstances, CD11b is mainly stored in the intracellular granules and in secretory vesicles of the cells [Bainton et al., 1987; Dertmers et al., 1990; Loike et al., 1991; Frankel et al., 1992; Takami et al., 2002]. Our results confirmed that during ATRA-induced differentiation of NB4 cells, CD11b and also the CD11c receptors were translocated to the cell surface in a great extent, whereas the amount of active CD11b on the surface can not be increased further by phorbol-esters (PMA).

CD11c is another potential diagnostic marker for sepsis or systemic inflammation [Shibakura et al., 2005]. CD11c is also known as Integrin-alpha X (ITGAX) receptor on the surface of dendritic cells, monocytes, macrophages, neutrophils and some B-cells, that helps to trigger the neutrophil respiratory burst with ROS; highly expressed in leukaemias, acute nonlymphocytic leukaemias, and some B-cell chronic lymphocytic leukaemias [Stewart et al., 1996]. In our results, we found that the CD11c has a gradually increased cell surface expression in the activated form, with a high affinity for ligands on ATRA treated NB4 cell lines.

The production of the pro- and inflammatory cytokines/chemokines play a role in rolling, in stable adhesion and the infiltration of differentiated APL cells, with a huge potential for APL cell-mediated organ damage, a process observed in differentiation syndrome [Harada et al., 1994; Luesink et al., 2009]. The transcription of the major cytokines and chemokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and chemokines like monocyte chemoattractant protein-1 (CCL2/MCP-1) occurs via the NF-κB pathway.

Our results indicate that ATRA induced differentiation is associated with a high expression of the inflammatory cytokines/chemokines at the level of the mRNA and the level of the secreted proteins in NB4 cells, which is not detectable with ATO treatment alone. The translocation of NF-κB dimer into the nucleus results in upregulation of various inflammatory genes followed by the secretion of their products, including TNF-α, IL-1β, IL-8, IL-9, and MCP-1.

Among the secreted inflammatory cytokines and chemokines, TNF-α and IL-1β are the most effective agents. TNFα was detected previously at the highest concentrations of 0.8–1.2 ng/L in APL/DS patients, which caused a capillary leak and attenuated lung, cardiac and renal function [Tang et al., 2017]. The differentiating APL/NB4 cells stimulate their extravasation and migration into various organs by producing inflammatory cytokines, chemokines and interleukins (TNF-α, MCP-1, IL-1β) in an autocrine way.

To investigate the whole range of potential pro-inflammatory or inflammatory cytokines/chemokines induced by ATRA, with the help of a Human 200 cytokines array (Raybiotech) after 11 days of retinoic acid-induced differentiation secreted molecules have been identified and divided into different groups:

1) TG2 dependent
2) TG2 & NF-κB dependent
3) TG2 independent but NF-κB dependent
4) TG2 dependent but NF-κB independent

Our results have been analysed regarding a big database from the University of Boston. From the well known NF-κB pathway involved, cytokines and chemokines were filtered and analysed by the Human array results. From the total 480 molecules, we have found 50 proteins which were detectable in the differentiated NB4 cells supernatant. From the 50 detectable proteins, 44 were expressed in a TG2-dependent manner, from which 18 were identified as NF-κB transcription-factor target genes.

After eleven days of differentiation with ATRA in wild type cells where the amount of TG2 protein is high, the NF-κB dimer protein expression together with the phosphorylated and transcriptional active p65 expression was high. The inhibition of TG2 parallels the considerably low levels of p50 and phosphor-p65/RelA (Ser536), which suggests that the expression of TG2 by retinoic acid induction, reprogramming the NB4 cells into inflammatory neutrophil granulocytes and induce the translocation of NF-κB dimer into the nucleus initiating the inflammatory cytokine production.

Destruction of inhibitory protein of the NF-κB dimer (IκB) is stimulated by several diverse signals such as lipopolysaccharide (LPS), reactive superoxide forms, TNFα, or Il-β. It could be one option that NF-κB activation is TG2-dependent, where TG2 interacts with IκB to initiate its non-proteasomal degradation causing both activation and nuclear translocation of NF-κB-s [Kumar et al., 2012]. An additional option could be that the TG2-mediated cross-linking of IκBα and activation of NF-κB has been described before, yet we were unable to detect any forms of IκBα-polymer in differentiated NB4 cells.

Besides the increased secretion of cytokines and chemokines, the differentiating APL cells are capable of producing reactive oxygen species (ROS) in a great extent which damages the surrounding tissue. ATRA-induced differentiation-associated ROS production with the elevated expression of two elements of the NADPH-oxidase system, *NCF2/P67PHOX* and

GP91PHOX, resulting in the possibility of increased ROS production and, consequently, more severe organ damage.

As we have seen, the ATRA induction the increased mRNA expression of the NADPH-oxidase elements lead to intensive production of ROS. In the case of arsenic therapy the so-called "oxidative stress" is one of the major consequences of the treatment caused by reactive oxygen species, a group of oxygen-based reactive molecules produced by ATO activated the NADPH-oxidase system. This will result in the disruption of mitochondrial membrane potential and subsequent apoptosis [Chen et al., 2011; Boukhenouna et al., 2018; Schröder K., 2019]. ATO alone can trigger the increase of the mRNA expression levels of *NCF2/P67PHOX* and *GP91PHOX* as well as the production of total ROS, similar to ATRA in differentiated NB4 cell lines. ATO alone can initiate the apoptosis of the cells, with a massive ROS production, while the inflammatory cytokine production is far less compared to the ATRA treatment.

Moreover, by the presence of ATO, TG2 was not induced. Beside the production of the ROS and the change in the mitochondrial membrane potential in NB4 cells, arsenic initiates morphological changes respectively [Zhang et al., 2010]. The number of apoptotic cells was concentration and time-dependent regarding the ATO treatment. Based on our results, the ATO concentration is cytotoxic even at the beginning of the treatment when it is above 0.5 μM. Since arsenic can eliminate the APL cells targeting the PML part more quickly, the combination therapies started to get more interest in the last decade. Combination therapies can be done by applying ATO together with other chemotherapeutic agents, glucocorticoids or with ATRA. Another beneficial effect of the ATO treatment in a combination that it can demolish the leukaemia initiating cells (LIC) avoiding the second/third generation of the disease [Dos Santos et al., 2013].

It has previously been published that the morphology of APL cells upon ATRA or ATO treatments have changed. ATO treatment was associated with apoptotic/necrotic cell death features, exhibiting various size and quality of cytoplasm [Zang et al., 2010]. In our study, we observed that the ratio of cell death was time and concentration-dependent in NB4 cells. Alone the ATO induces impressive cell death at the beginning of the treatment. However, this cell death can be moderated by the presence of ATRA induced TG2.

When we treated the cells with ATRA + ATO, the NB4 WT and TG2-C cells showed a higher survival ratio comparing to the just ATO treated ones alone. Moreover, we found that ATRA

induced TG2 showed up as a protective protein against the ATO induced cell death upon the differentiation. NB4 TG2-KD and TG2-KO cells were represented a higher cell death ratio with an increasing number of apoptotic and necrotic cells upon ATRA + ATO treatments. With flow cytometry, we have checked the Annexin-V and Propidium iodide percentage upon ATRA + ATO treatment, and we have found that without the TG2 the number of Annexin-V positive cells and by this the apoptosis was significantly higher compared to the wild type cells (not published data).

From these data, we concluded that reduced quantity of TG2 enhanced the sensitivity of NB4 cells to a combined ATRA + ATO treatment, with significantly higher apoptotic and necrotic ratios.

So far, no study has shown how ROS production changed upon combination therapies like ATRA +ATO. To investigate how a combination therapy influences the cell survival and differentiation processes, we first examined ROS production. Alone the ATRA and ATO can increase the production of superoxide in a significant way, a 2-fold decrease in ROS production was detectable in response to ATRA + ATO compared to single ATRA or ATO treatments.

While the atypically expressed TG2 in differentiated NB4 cells can enhance the function of the NADPH-oxidase system, resulting in a high ROS production, TG2 deficiency in TG2-KD or TG2-KO cells is associated with significantly low ROS production, which can be further reduced by the ATRA + ATO treatments.

Our results have revealed a novel, active role of TG2 in the expression and activation of the components of the NF-κB pathway respectively, thus the development of an unusual response to conventional ATRA treatment of APL. We examined how the combination with ATO can change the production of the inflammatory cytokines and chemokines.

Upon ATRA +ATO combination treatment, NB4 cell lines were differentiating properly, proved by the surface expression of CD11s markers (unpublished data) and cell death ratios were significant, higher compared to the ATRA treatment alone, but the inflammatory cytokines TNFα, IL-1β and chemokine, MCP-1 mRNA expression and the secreted protein levels were significantly lower compared to the ATRA treatment.

The pro-inflammatory and inflammatory protein production was significantly lower in the decreased (TG2-KD) or even less in the absence of TG2 (TG2-KO). These results also

strengthened the hypothesis that the ATRA induced TG2 expression to influences the NF-κB pathway activity positively. With the atypically high expression of the TG2, the NF-κB pathway showed significantly higher activity measured by luciferase reporter gene upon ATRA treatment, whereas the lack of TG2 or decreased expression led to a less luciferase reporter gene activities. It was demonstrated before that TG2 can form a complex in the cytosol as well as in the nucleus with p65 binding to the promoter of the HIF-1α, which may explain that TG2 could become a transcriptional co-regulator in the cell nucleus [Kumar et al., 2012].

In the presence of NC9, which is an irreversible transamidase site-specific inhibitor of tissue transglutaminase, the conformational equilibrium of TG2 will shifts from the closed GTP-binding form to the open conformation characterized by a disorganized GTP-binding site [Eckert et al., 2015]. According to our results, the NC9-induced conformational changes in the tissue transglutaminase protein attenuate the NF-κB signalling pathway in a significant way.

Upon ATRA+ NC9 treatment, the amount of total TG2 protein is decreased, and the nuclear translocation of the TG2 is diminished, increasing the cytosolic TG2. The reduced nuclear TG2 was correlated with decreased total levels of p50, p65/RelA, and phospho-p65/RelA proteins in the nucleus of NB4 WT cells, while the level of the transcriptionally active form of p65, phospho-p65 (Ser 536) was increased significantly in the cytosol.

These results suggest the possibility that TG2, in a GTP-bound, “closed” conformation can translocate into the nucleus and helps the translocation of p65 consequently. When NC9 modified the structure of TG2, the accumulation of both TG2 and p65/RelA in the cytosol generated the low transcription activity of the NF-κB pathway. As a result, the production of inflammatory cytokines/chemokines were inhibited.

As we have observed that the TG2 inhibitor, NC9 decreases NF-κB translocation to the nucleus and NF-κB activity, significantly reducing the production of inflammatory biomarkers, such as MCP-1, IL-1β and TNF-α these results also demonstrated that combined ATRA + ATO 2.0 μM with inhibition of atypically expressed TG2 by NC9, demolishes the expression and secretion of the three inflammatory markers and the production of ROS very effectively. ATRA + ATO 2.0 μM + NC9 treatment resulted in an 8-fold decrease in MCP-1, a 15-fold decrease in IL-1β levels and a 61-fold reduction of TNFα; whereas a 28-fold decrease in ROS production, compared to ATRA alone, after five days.

These experiments indicate that TG2 is a crucial component of the generation of inflammation and in tissue destruction indirectly, while the inhibition of TG2 by NC9 may prevent the development of this severe side effect of single retinoic acid therapy. Targeted suppression of the TG2-dependent processes may alleviate the common and potentially fatal toxicity of retinoid treatment in patients with APL/DS, representing a potential therapeutic strategy for the future.

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List of publications related to the dissertation

1. **Jambrovics, K.**, Uray, I. P., Keillor, J. W., Fésüs, L., Balajthy, Z.: Benefits of Combined All-Trans Retinoic Acid and Arsenic Trioxide Treatment of Acute Promyelocytic Leukemia Cells and Further Enhancement by Inhibition of Atypically Expressed Transglutaminase 2.
Cancers (Basel). 12 (3), 648-, 2020.
DOI: http://dx.doi.org/10.3390/cancers12030648
IF: 6.126 (2019)

2. **Jambrovics, K.**, Uray, I. P., Keresztessy, Z., Keillor, J. W., Fésüs, L., Balajthy, Z.: Transglutaminase 2 programs differentiating acute promyelocytic leukemia cells in all-trans retinoic acid treatment to inflammatory stage through NF-kB activation.
Haematol-Hematol. J. 104 (3), 505-515, 2019.
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IF: 7.116

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List of other publications

3. Volkó, J., Kenesei, Á., Zhang, M., Várnai, P., Mocsár, G., Petrus, M. N., **Jambrovics, K.**, Balajthy, Z., Müller, G., Dóczy-Bodnár, A., Tóth, K., Waldmann, T. A., Vámosi, G.: IL-2 receptors preassemble and signal in the ER/Golgi causing resistance to antiproliferative anti-IL-2R[alfa] therapies.
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4. Beyer, D., Tándor, I., Kónya, Z., Bátori, R. K., Roszik, J., Vereb, G., Erdődi, F., Vasas, G., Mikóné Hamvas, M., **Jambrovics, K.**, Máthé, C.: Microcystin-LR, a protein phosphatase inhibitor, induces alterations in mitotic chromatin and microtubule organization leading to the formation of micronuclei in Vicia faba.
   Ann. Bot. 110 (4), 797-808, 2012.
   DOI: http://dx.doi.org/10.1093/aob/mcs154
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