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

by Károly Jambrovics

Supervisor: Dr. Zoltán Balajthy

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

DEBRECEN, 2020

List of contents

List of Figures

List of tables

List of Abbreviations

ACTH	Adrenocorticotropic hormone
AL	Acute leukaemia
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
AMP	Adenosine-monophosphate
AP-1	Activator protein-1
APL	Acute promyelocytic leukaemia
ASC	Apoptosis-associated speck-like protein
ATO	Arsenic trioxide
ATP	Adenosine-triphosphate
ATRA	All-trans-retinoic acid
BIR	Baculovirus inhibitor of apoptosis repeat
BPCR	Breakpoint cluster regions
BSA	Bovine serum albumin
cAMP	Cyclic AMP
CARD	Caspase attracting domain
CD11a	Integrin receptor alpha
CD11b	Integrin receptor beta
CLR	C-type lectin receptors
CMW	Cytomegalovirus promoter
CNS	Central Nervous System
COX-1-2	Cyclooxygenase-1-2
CR	2-9-complement repeat
CRABP	Cellular Retinoic Acid Binding Protein
CRBP	Cytoplasmic Retinol Binding Protein
CRP	Reactive protein C
CYT-ARAB	Cytosine-arabinoside
DAMP	Damage associated molecular patterns
DBD	DNA binding domain
DC	Dendritic cells
DEX	Dexamethasone
DS	Differentiation syndrome
ECM	Extracellular matrix
ECL	Enhanced chemiluminescence
EDTA	Ethylene-diamine-tetraacetic acid
ELISA	Enzyme-linked immunoabsorbent assay
EGF	Epidermal growth factor
EGFP	Enhanced GFP
ER	Endoplasmic reticulum
F13A1	Factor XII Chain A
FAB	French-American-British classification
FACS	Fluorescence activated cell sorting
FAD	Flavin adenine dinucleotide
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine-diphosphate
GFP	Green fluorescent protein
GSH	Reduced-glutathione system
GTP	Guanosine-triphosphate
HAT	Histone acetyltransferase
HDAC	Histone deacetylase enzyme
HMGB1	High-mobility group protein B1
HOX	Homeobox genes
HRE	Hormone response element
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule-1
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
INF-α,-β	Interferon-alpha and beta
IRF3	Interferon regulatory factor 3
ITAM	Tyrosine-based activation motif
ITGAX	Integrin-alpha-X
LAD	Leukocyte adhesion deficiencies
LBD	Ligand binding domain
LFA-1	Lymphocyte function-associated antigen 1
LIC	Leukaemia initiating cells
LPS	Lipopolysaccharide
LRR	N-terminal leucine-rich region
MCP-1	Monocyte chemoattractive protein-1
MEA	Mercaptoethanol
mRFP	Monomeric red fluorescent protein
NADPH	Nicotinamide adenine dinucleotide-p
NB	Nuclear bodies
NBT	Nitroblue-tetrazolium salt
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor κappa B pathway
NFW	Nuclease-free water
NLR	NOD-like receptors
NLRP3	Nacht-, Leucine- Repeat-, PYD-Protein 3
NLS	Nuclear localisation signal
PAMP	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDI	Protein-disulphide isomerase activity
PE	Phycoerythrin
PHOX	phagocyte oxidase
PIP2	Phospho-inositol-di-phosphate
PIP3	Phospho-inositol-tri-phosphate
PLA_2	Phospholipase A_2
PLCγ	Phospholipase Cγ
PMA	Phorbol 12-myristate 13-acetate
PML	Promyelocytic leukaemia
PMSF	Phenylmethanesulfonyl fluoride
PPR	Pattern-recognition receptors
PVDF	Polyvinylidene difluoride membrane
Q-PCR	Real-Time Quantitative PCR
RALDHD	Retinaldehyde dehydrogenase enzymes
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RAS	Retinoic acid syndrome

RDH10	Retinol dehydrogenase enzyme
RFP	Red fluorescent protein
RLR	Receptors gene-I-like receptors
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase PCR
SDS	Sodium-dodecyl-sulphate
SOD	Superoxide dismutase
SSC	Side scatter
SUMO	Small-ubiquitin related modifier
TALEN	Transcription activator-like effector nucleases
TG1	Keratinocyte transglutaminase
TG2	Tissue transglutaminase
TG2-C	TG2-virus control NB4 cell line
TG2-KD	TG2-knocked down NB4 cell line
TG2-KO	TG2-knocked out NB4 cell line
TG3	Epidermal transglutaminase
TG4	Prostate transglutaminase
TGF-β	Transforming growth factor-β
TGM2	Tissue transglutaminase gene
TIR	Cytoplasmic Toll/ IL-1R homology domain
TLR	Toll-like receptors
TNF-α	Tumour necrosis factor-alpha
TrX/TrxR	Thioredoxin/thioredoxin reductase
TTBS	Tris-buffered saline and Tween 20
WHO	World Health Organization
US FDA	Food and Drug Administration

1. Introduction

Acute leukaemia (**AL**) is a bone marrow-derived neoplastic disease that is classified into two groups:

(1) Acute lymphoblastic leukaemia (**ALL**)
(2) Acute myeloid leukaemia (**AML**)

Both types are characterized by the accumulation of an increased number of immature white blood cells. In AML, it is the differentiation program of the myeloid lineage that is not capable of succeeding, and the immature cells from the bone marrow appear in the bloodstream. A differentiation block characterizes one of the subtypes in this group, acute promyelocytic leukaemia (APL) at the promyelocyte stage. At the molecular level, there is a reciprocal chromosomal translocation between chromosomes 15 and 17 in this subtype. The long arms of the chromosomes are exchanged, resulting in a chimeric-oncoprotein (PML-RARα) which will inhibit the differentiation and the apoptosis of these cells.

In the clinical practice, all-trans-retinoic acid (ATRA) is the primary treatment choice for the treatment of APL patients, with which remission can be achieved in 80-90%. ATRA induces the degradation of the PML-RAR oncoprotein and the terminal differentiation of the promyelocytes into neutrophil granulocytes after 3-5 days [Bennet et al., 1976; 1980; Fenaux et al., 2007; Wiernik et al., 2013]. Although, the ATRA treatment results in remission with the terminal differentiation of the APL cells and quick recovery in a significant percentage of the patients, in 5-10 % of the cases the so-called differentiation syndrome (DS) can develop. This phenomenon involves increased inflammatory cytokine/chemokine production, potentially followed by the death of the patients without aggressive treatment [Tallman et al., 2000; Luesink et al., 2009]. The ATRA treatment can induce the expression of thousands of genes, tissue transglutaminase or transglutaminase 2 (TG2) being one of the highest expressed ones [Balajthy et al., 2006; Csomós et al., 2010].

TG2 is a multifunctional enzyme; it is localized in various extra- and intracellular compartments. Intracellularly, it is detectable in the cytosol, nucleus, mitochondria and the endoplasmic reticulum (ER). It can act as a Ca^{2+}-dependent cross-linking enzyme, membrane-associated GTPase or even as a serine/threonine kinase. In the extracellular matrix, previously, it has been described as a co-receptor of the integrins [Kuo et al., 2011; Gundemir et al., 2011; Fesus et al., 2002].

It has been reported previously that arsenic trioxide (As_2O_3/ATO) can help the remission of the APL patient [Zhu et al., 2002; Ghavamzadeh et al., 2005]. The use of arsenic results in the induction of signal transduction pathways, and is also capable of activating transcription factors, which leads to enhanced reactive oxygen species (ROS) production via the NADPH-oxidase system, followed by mitochondrial depolarization and the activation of caspases [Miller et al., 2002; Kawate et al., 2007].

ATO as an alternative treatment in clinical trials could induce a partial differentiation of the APL cells, during which ROS production was increased significantly, and in parallel apoptosis was initiated [Chen et al., 1997]. Unfortunately, the increased amount of ROS can lead to the degradation of the surrounding tissue; moreover, the inappropriate dosage of the arsenic can lead to a high cytotoxic effect and the early death of the patient [Boukhenouna et al., 2018; Chen et al., 2011].

Combination therapies with chemotherapy are used to reach better outcomes. In the last few years, even more often combined treatments are used in which ATRA is administered together with ATO [Rego et al., 2000; Jeanne et al.; 2010; Chen et al., 2011; Powell et al., 2013]. The treatment generates a synergistic effect, in which the terminal differentiation of the cells is induced by the ATRA, whereas the presence of ATO can trigger the early apoptosis of the cells.

In the NB4 APL cell line, treatment with ATRA for three days results in the induced expression of several genes, from which TG2 is upregulated to a significantly higher level compared with the undifferentiated cells. In connection with the increase of TG2 mRNA and protein expression, these cells are capable of producing high amounts of ROS and inflammatory cytokines (MCP-1, TNF-α, IL-1β) [Csomós et al., 2010]. However, in the TG2 silenced (NB4 TG2-KO) or knockdown cells (NB4 TG2-KD) the phagocytotic capacity, adhesion, migration and ROS production are attenuated significantly [Balajthy el al.,2006; Csomós et al., 2010].

Based on these previous results, we further investigated whether the knocking down/silencing/ or inhibition of TG2 in the NB4 cell line has any beneficial effect alongside a combined treatment of ATRA + ATO, assessed by cell viability, gene expression pattern, endogenous/induced ROS production, inflammatory cytokine/chemokine production and the cell survival processes.

1.1. 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].

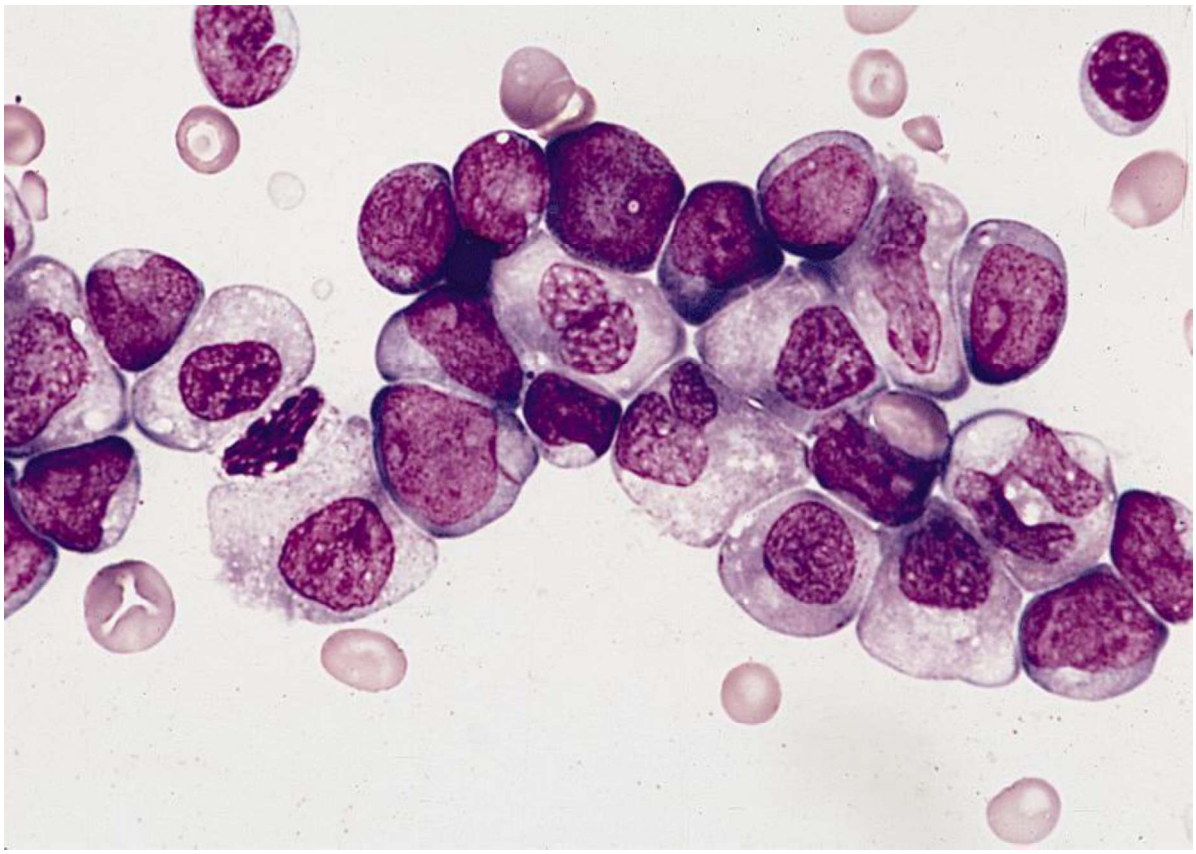


Figure 1. Morphology of the APL cells *[obtained from http://www.pathologyoutlines.com]. The APL cells have a large non-segmented cell nucleus with a slight cytoplasmic region. The morphological changes are monitored by Giemsa-May-Grünwald staining, by which the cell nucleus and cytoplasmic regions can be visualised.*

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].

Type	Full name and description	Genetic alterations
AML-M0	MINIMAL DIFFERENTIATED ACUTE MIELOBLASTIC LEUKAEMIA	
AML-M1	ACUTE MYELOBLASTIC LEUKAEMIA WITHOUT DIFFERENTIATION	
AML-M2	ACUTE MIELOBLASTIC LEUKAEMIA WITH DIFFERENTIATION	t(8;21)(q22;q22)(t(6;9)
AML-M3	ACUTE PROMYELOCYTIC LEUKAEMIA	t(15;17)(q22;q12)
AML-M4	ACUTE MYELOMONOCYTE LEUKAEMIA	inv(16)(p13;q22), del(16q)
AML-M4eo	ACUTE MYELOMONOCYTE LEUKAEMIA WITH BONE MARROW EOSINOPHILIA	inv(16), t(16;16)
AML-M5a	ACUTE MONOBLASTIC LEUKAEMIA	del(11q), t(9;11)
AML-M5b	ACUTE MONOCYTIC LEUKAEMIA	del(11q), t(11;19)
AML-M6	ACUTE ERYTHROID LEUKAEMIA	
AML-M6a	ERYTHROLEUKAEMIA	
AML-M6b	PURELY ERYTHROID SOURCED LEUKAEMIA	
AML-M7	ACUTE MEGAKARYOBLASTIC LEUKAEMIA	t(1;22)

Table 1. French-American-British (FAB) classification of leukaemias [Bennet et al., 1976]

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].

1.1.1. RARα

RARα belongs to the steroid/thyroid receptor family, and functions as a ligand-dependent transcription factor [Kurowaka et al., 1994; Chambon et al., 1996]. RARα is a nuclear receptor. It has a DNA binding domain (DBD) and a ligand-binding domain (LBD). The former has two Zn-finger motifs which are necessary for accurate DNA binding. RARα is capable of binding to the retinoic acid response element (RARE), which results in the regulation of cell differentiation and the basic homeostatic processes of the cell [Wiernik et al., 2013].

In physiological circumstances, RARα forms a heterodimer with the RXR receptors (RAR-RXR). If the ligand is not present, corepressor molecules attach to it (SMRT/N-Cor), by which the transcription is inhibited. The inhibition is mediated by histone deacetylase enzymes (HDACs). The HDACs condense the chromatin through the removal of the acetyl groups from the histones, hence the RNA polymerase enzyme is unable to bind to the sequence and transcription is inhibited (**Figure 2**).

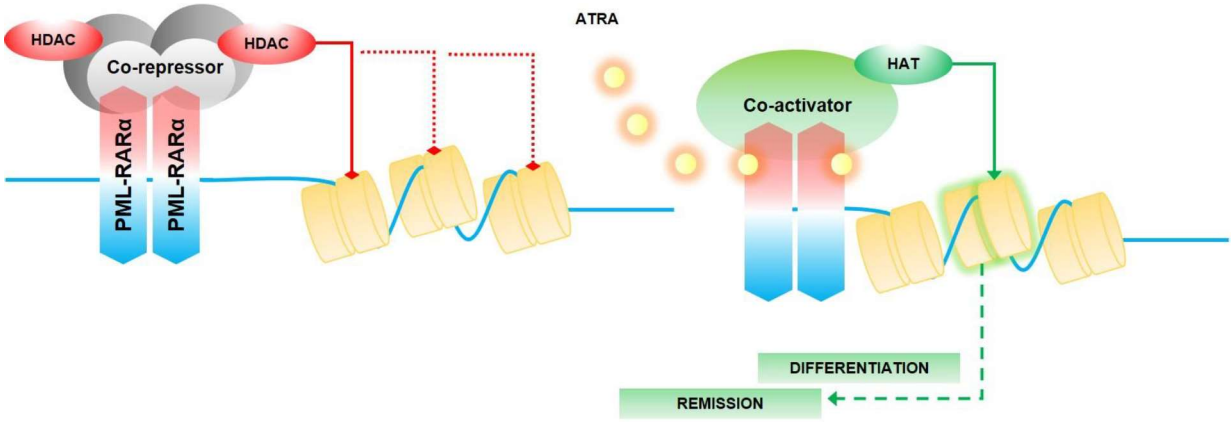


Figure 2. PML-RAR transcription regulation by histone acetylation and deacetylation

In the presence of the ligand, the corepressor molecules are released from the receptor, followed by the attachment of coactivator molecules with histone acetyltransferase (HAT) activity (p106/coactivator complex). By the acetylation, the chromatin is decondensed; thus, the DNA becomes active, and transcription can proceed (**Figure 2**).

1.1.2. Promyelocytic leukaemia protein (PML)

The promyelocytic leukaemia protein-coding region contains a TRIpartite motif that consists of a RING domain, two alternative cysteine and histidine-rich boxes (B-Box), and an alfa-helical coiled-coiled domain (CC) (**Figure 3**). These regions altogether form the so-called RBCC dimerization domain, which makes PML-PML homodimerization possible [Jensen et al., 2001].

Figure 3. Promyelocytic leukaemia gene structural features with the RBCC domain

PML is responsible for the proper regulation of cell proliferation pathways; it can also initiate apoptosis signalling. Borden and colleagues found that nuclear bodies (NB), 0.2-1 μm structures present in the nucleus in various numbers (1-30) have high PML content [Bordern et al., 2008].

It has been published that PML is a tumour suppressor since it can inhibit cell proliferation and stimulate apoptosis [Wang et al., 1988; Bernardi et al., 2008]. PML also plays a role in the initiation of the degradation of the fusion protein (PML-RAR) by a small-ubiquitin related modifier (SUMO)-dependent process. The latter is responsible for the posttranslational modification of the PML part [Zhong et al., 2000]. The degradation of PML can happen through proteasomal degradation, which can be also be initiated by phosphorylation or acetylation.

The fusion transcript of PML-RAR on chromosome 15 can be generated by many different optional breaks in the PML gene. Altogether three regions have been identified in PML, which are called breakpoint cluster regions (BPCR). If the 6th intron is involved in the breakage of the DNA, the long (L)-PML-RARα is formed. This is BPCR type 1. In another BCPR type, a triple intron break is involved, resulting in the short (S)-form. Alternatively, other variants can occur (V-form), in which the BCPR2-6 exons are randomly broken, followed by a deletion. These alterations are detectable in 75 % of the APL patients [Wiernik et al., 2013].

1.1.3. RARα-PML

Out of the morphological and cytogenetic features of APL, the presence of PML-RARα is the key to the pathomechanism, and it bears great importance to the therapy. Through the translocation, dominant-negative fusions can be generated, such as PML-RARα and RARα-PML (**Figure 4**).

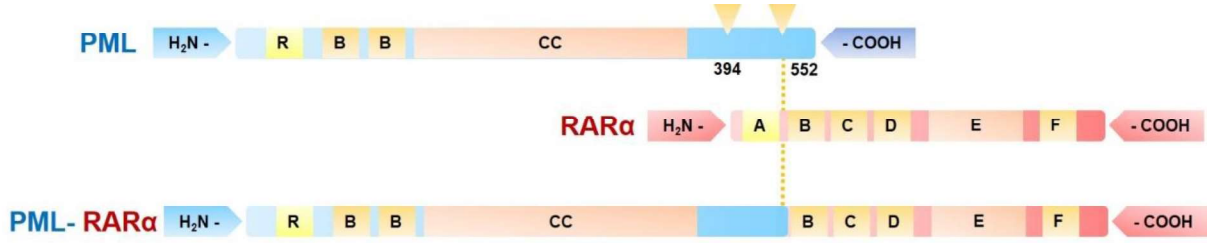


Figure 4. Generation of PML-RAR fusion type I [based on Rowley et al., 1977, Julien et al., 2001]

Several other gene rearrangements have been published in APL/AML patients: *e.g.* the PLZF-RARα, the NuMA-RARα, or the NPM-RARα translocational products [Chen et al., 2011]. The latter three new formations are generated through the involvement of chromosome 5 or 11 and chromosome 17 in the translocations.

The presence of the fusion protein was first published in 1991 [Lanotte et al., 1991]. In order to investigate the role of the fusion protein and the hypothesis that the fusion transcript brings about the differentiation inhibition, transgenic mouse experiments were carried out, focused on the presence of the leukaemia initiating cells (LIC) and the strategy of the treatment of APL [Gallagher et al., 1979]. During the treatments, they induced proteolytic degradation with different agents [Chen et al. 1997; Guidez et al., 1998; Breitman et al., 1981], which lead to granulocyte-directed differentiation (ATRA) or to the induction of apoptosis (ATO) [Raelson et al., 1996; Yoshida et al., 1996].

1.2. Treatment of APL

1.2.1. 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 (**Figure 5**)

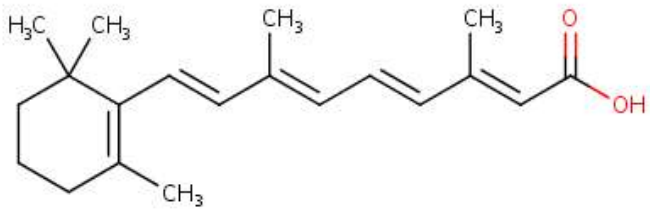


Figure 5. Structure of the ATRA [obtained from http://medchem.com]

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].

1.2.1.1. Differentiation of acute promyelocytic cells

The all-trans retinoic acid acts as a ligand for the retinoic acid receptors, which bind to the RARE elements in the regulatory region of the target genes. In the absence of ATRA, the heterodimer of RAR/RXR recruits a repression complex containing the SMRT/N-Cor and the mSin3 corepressors, and a histone deacetylase [Chen et al., 1995; Evan et al., 1997; Nagy et al., 1997]. The deacetylation of the histones results in chromatin condensation and transcriptional repression. ATRA can induce the dissociation of the complex and facilitate the association of a coactivator complex containing nuclear receptor coactivator p160 family members and histone acetyltransferases CBP/p300 [Chen et al., 1997]. The acetylation of the core histones leads to the loosening up of the chromatin, the promoter region becomes accessible for transcription initiation factors, and gene expression can commence.

Retinoic acid in a high concentration can induce the remodelling of the transcriptional complex in the APL cells and can lead to activation of hundreds of genes, which are regulators of different processes, for example, apoptosis, cell proliferation, metabolism and protein synthesis [Koyama et al., 1994; Csomós et al., 2010].

In the case, APL due to the characteristic chromosomal translocation, the retinoic acid in physiological concentrations (10^{-9}-10^{-8}) is unable to bind to the PML-RARα fusion receptors. However, in a pharmacological concentration – which means ~1 μM ATRA – it does induce the conformational change of the multifunctional complex around the chimaera. The corepressors are released, and the normal response of the RARα target genes is restored; thus the terminal differentiation of the APL cells is triggered [Girgnani et al., 1998, Liu et al., 2003] (**Figure 6**).

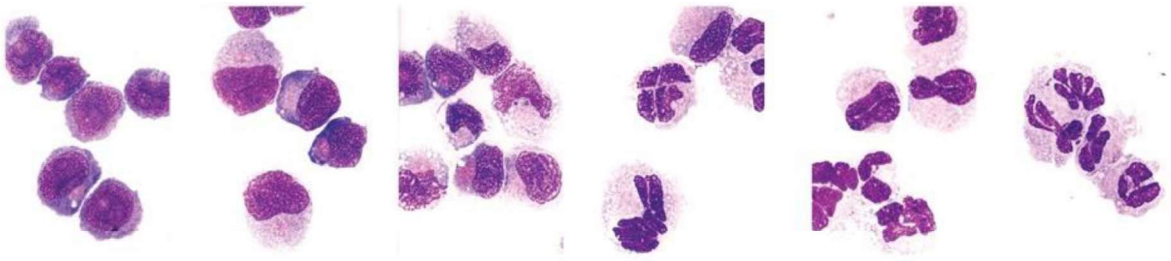
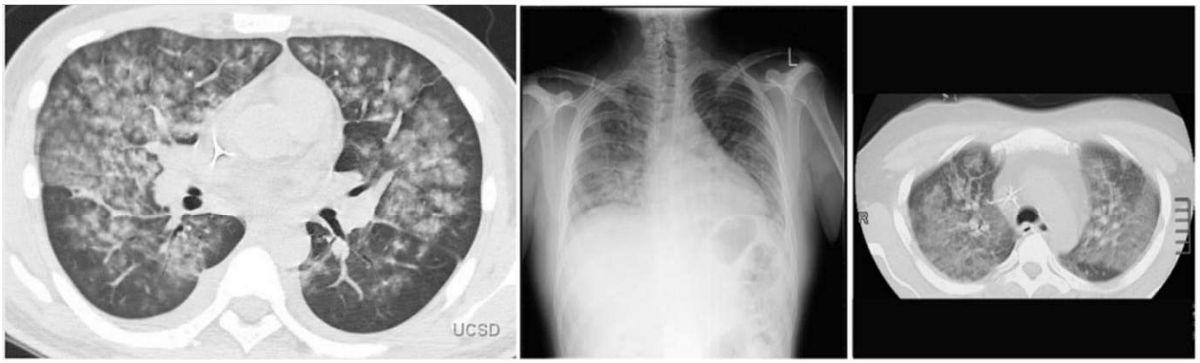


Figure 6. ATRA induced differentiation for five days [Lie et al., 2003] *ATRA-induced granulocytic differentiation in NB4 cells. Differentiation of the NB4 cells was evaluated by (a) morphological changes (Wright–Giemsa staining, magnification × 1000)*

1.2.1.2. Differentiation syndrome (DS)

The differentiation syndrome or the so-called retinoic acid syndrome is a rapidly developing and lethal consequence of the ATRA treatment [Frankel et al., 1992, Nim et al., 2007] (**Figure 7**).



1. Figure Differentiation syndrome in APL patients [Nim et al., 2007, https://radiopaedia.org/cases/all-trans-retinoic-acid-atra-syndrome] Left part: *second chest X-ray obtained six days later. The patient was found to have acute promyelocytic leukaemia (APL) and started treated with ATRA two days before this X-ray. Despite treatment for patients with Congestive Heart Failure (CHF), symptoms progressed.* ***Middle and right part:*** *Chest X-ray and CT performed on the 12th day of arsenic therapy. Interstitial infiltrates (septal lines and ground-glass opacity) are shown*

The syndrome itself is characterized by an unexpected and sudden fever, weight gain, pleural and pericardial effusions with interstitial pulmonary infiltration of immune cells. The number of the leukocytes are abnormally high in the bloodstream, and the overactivated/differentiating neutrophils will infiltrate into soft tissues like the heart, lungs, kidneys and liver. Retinoic acid, by itself, can induce a high production of inflammatory cytokines. This cytokine storm makes the case more severe and leads to that even more cells target the tissues, causing the death of patients in days. Besides the inflammatory cytokine and chemokine production, the levels of the CD45/CD11b and CD18 are also elevated [Luesink et al., 2010]. DS is often associated with hyperleukocytosis, edemas and bone pain. 2.5-31 % of APL patients, who have received induction therapy with all-trans retinoic acid and/or arsenic trioxide are reported to develop DS [de Botton et al., 1998, Tallman et al., 2000]

The pathogenesis of the disease/syndrome is not fully understood yet; however, several key molecules were identified over time. These important proteins are the integrin alpha chains, CD11a and CD11b, interleukin 1-beta (IL-1β), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), CD15 and cathepsin G [Seale et al., 1996; Dubois et al., 1994]. These proteins affect the consequences of the treatment and determine their severity (**Table 2**).

	Type of protein	ACT via	Reference
CD11a	INTEGRINS RECEPTORS	interacts with the endothelial cell receptor called intercellular adhesion molecule 1 (ICAM-1)	*Di Noto et al., 1994*
CD11b			*Dubois et al., 1994*
IL-1β	CYTOKINE	human leukocytic pyrogen generates fever	*Di Noto et al., 1996*
IL-6		acts as a pro-inflammatory cytokine, pyrogen	*Di Noto et al., 1996*
TNFα		regulation of the immune cells	*Marchetti et al., 1996*
Cathepsin G	SERINE TYPE OF PROTEASE	enhances the capillary permeability	*Seale et al., 1996*
CD15	TETRASACCHARIDE CARBOHYDRATE	mediates inflammatory extravasation of immune cells	*Liang et al., 2016*

Table 2. List of potential players in generating the differentiation syndrome in APL patient upon ATRA or ATO treatment

In the differentiation syndrome triggered by ATRA and/or ATO, a severe systemic inflammatory response can be observed, which is followed by extensive infiltration of differentiated APL cells into the patients' tissues. Among the organs, the lung is the most threatened, which was established by histological and respiratory changes in these patients [de Botton et al., 1998; Tallman et al., 2000; Tallman et al., 2002; Camacho et al., 2000; Ninomiya et al., 2004].

In the DS early recognition and prompt treatment can be the solution to avoid the severe consequences. When the DS is diagnosed, a high dose of dexamethasone (DEX) is administered, with which the hyperinflammatory cascade is inhibited. The progression of the DS is not fully understood; however, there are putative target pathways through which the differentiated APL cells are triggered (Nuclear factor kappa-B pathway).

The initial migration of the APL cells to the target tissues such as the lungs is facilitated and triggered by the secretion of specific chemokines and cytokines from the lung tissue. These alveolar proteins are the results of the ATRA treatment; the production of CCL2 and CXCL8 are significantly higher in alveolar epithelial cells. Tsai and colleagues have reported that the increased level of the alveolar chemokines resulted in increased migration of differentiated APL cells towards the alveolar epithelial cells. To suppress the production of the alveolar chemokines and cytokines, dexamethasone was used, by which the hyperinflammatory response could be attenuated [Tsai et al., 2007; 2008].

1.2.2. 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] (**Table 3**).

	CONCENTRATION IN μM										mM
I.	0.1	0.15	-	0.25	0.5	1	1.25	1.5	2.0	-	
II.	0.1	-	0.2	-	0.5	1	-	-	2.0	-	
III.	-	-	-	-	0.5	-	-	-	-	2.5	
IV.	-	-	-	-	-	-	-	-	-	-	5.0
FAILS TO INDUCE APOPTOSIS, HAS NO EFFECTS ON CELL CYCLE DISTRIBUTION											
PROAPOPTOTIC/APOPTOTIC FEATURES											
APOPTOSIS INDUCTION AND PML-RARα DEGRADATION											
ENTIRE PML-RARα DEGRADATION AND SEVERE APOPTOSIS											

Table 3. Various concentration of arsenic trioxide to treat APL from different trials in therapy

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]

1.2.3. 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 (**Figure 8**) [dos Santos et al., 2013]

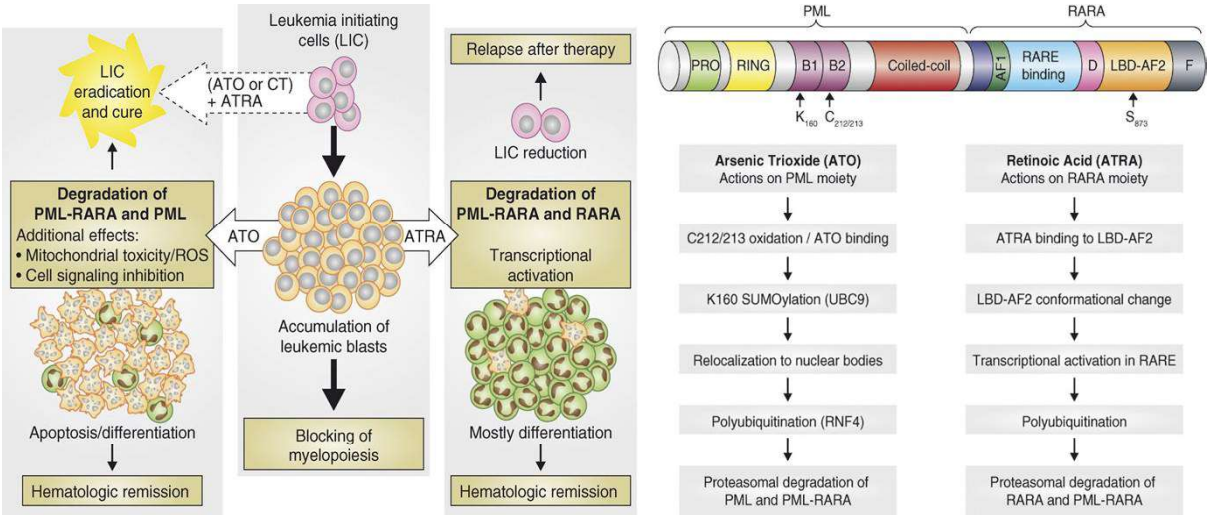


Figure 8. Elimination of leukaemic blasts and LICs is necessary for a definitive cure of APL. Left: *LICs posses high self-renewal capability and give rise to leukemic blasts that form the bulk of the disease* ***Right****: Mechanisms of proteolysis of PML-RARα by ATRA and ATO. ATRA induces degradation of RARα and PML-RARα via the RARα moiety. [Dos Santos et al., 2013]*

1.3. The transglutaminase family

The transglutaminase enzymes play essential roles in the homeostasis of the cell. Transglutaminases are thiol- and calcium-dependent acyl-transferase enzymes, catalysing a covalent crosslink between the glutamine- and lysyl residues in different proteins. Over time, new transglutaminases were discovered in different tissues, so as currently there to exist nine members with various tissue distribution and discrete functions (**Table 4**). Here we do not mention the enzymatically inactive Protein 4.2/EPB42 which can be found in erythrocytes, bone marrow and spleen.

GENE	~ Transglutaminase	ROLE IN	CHROMOSOME
TGM1	Keratinocyte	Skin	14q11.2
TGM2	Tissue	Apoptosis, Immune System	20q11.2-q12
TGM3	Epidermal	Skin	20q12
TGM4	Prostate	Prostate	3p22-p21.33
TGM5	TGM X	Skin	15q15.2
TGM6	TGM Y	Central Nervous System (CNS)	20q11-15
TGM7	TGM Z	Testis, Lungs	15q15.2
F13A1	Factor XIII Chain A	Blood Coagulation	6p25-p24

Table 4. The transglutaminase family members based on the tissue distribution

As previously mentioned transglutaminases have a characteristic structural organization in which we distinguish four domains. Immunoglobulin-like domains are surrounding the central catalytic domain of the enzyme. The core domain belongs to the papain superfamily and contains Cys-His-Asp catalytic triad in its active site. Crosslink formation is essential in blood coagulation/clot formation, apoptosis, adhesion, migration processes and in particular immune responses, as well, aimed at the elimination of pathogens (neutrophil extracellular trap (NET) formation) [Fesus et al., 2002; Csomós et al., 2016]. The enzyme catalysed reactions are categorized based on the biochemical targets:

1) incorporation of amines into proteins
2) crosslinking of proteins
3) site-specific deamidation
4) iso-peptidase activity
5) promotion of the extracellular matrix (ECM) connection
6) transmembrane signalling through the phospholipase activation

The cellular distribution of the transglutaminases is ubiquitous, and they can be found in the cytosolic and nuclear fractions, in the extracellular matrix or even in a membrane-bound form [Fesus et al., 2002, Grenard et al., 2001] (**Figure 9**).

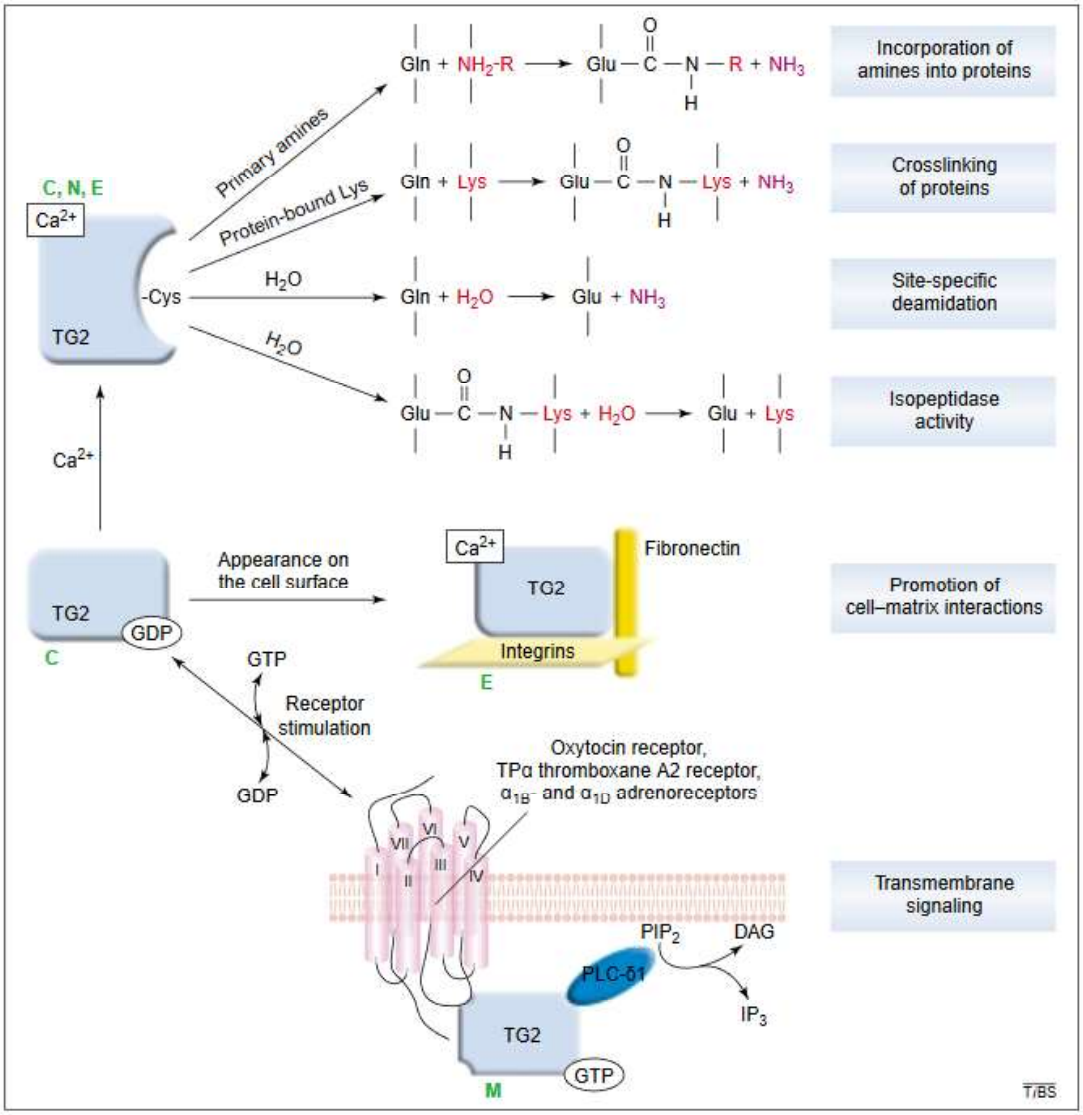


Figure 9. Biochemical activities of transglutaminase 2 (TG2), a member of the transglutaminase family [Fesus et al., 2002] *The localization of the transglutaminase especially the TG2 is abbreviated as the followings: C stands for the cytosolic, N for the nucleus and E for extacellular matrix.*

Transglutaminase 1 (TG1) is the so-called keratinocyte TG which can appear in a membrane-bound form or a soluble one, however, for its activation there a proteolytic event is required [Eckert et al., 2005].

TG3 has been described previously in the hair and follicles, together with TG1 after activation they play a role in the differentiation of the keratinocytes.

In the prostate, TG4 is dominantly expressed, playing a role in the maintenance of fertility[Dubbink et al., 1998].

TGM5 encodes TG5 which plays a role in skin development and differentiation. It is also involved in protein cross-linking by catalysing the formation of gamma-glutamyl-lysine iso-dipeptide bonds between adjacent polypeptides [Candi et al. 2005; Eckert et al. 2005]. TG5 can heavily cross-link keratin molecules and a range of differentiation-specific structural proteins, it facilitates the formation of the cornified cell envelope in the biogenesis of the stratum corneum [Kalinin et al. 2002].

The family has other members such as TG6 and TG7, the roles of which have not been fully understood or even studied yet. While TG6 plays a role in neuronal regulation and differentiation, TG7 is the least studied within the family.

Among the transglutaminases, the most evaluated and studied members are factor XIII and TG2. The former is probably the best-known enzyme of the family. In normal circumstances, it is in a zymogen form in the bloodstream and can be converted into the active form via thrombin-dependent proteolysis. The active FXIII, also called plasma transglutaminase requires calcium for covalent crosslink formation between fibrin fibres or even with the ECM. It has a crucial role in the formation of the haemostatic plug and wound healing [Muszbek et al., 2011].

Tissue transglutaminase (TG2) is a widely studied and evaluated member of the transglutaminase family since it has not only a transamidation activity but also iso-peptidase, disulphide-isomerase, GTPase and protein kinase activities [Fésüs et al. 2002; Mishra et al., 2007]. While the other members of the family are responsible for one particular function in the cell, TG2 carries a lot more potential for regulating the homeostasis of the cell.

1.3.1. 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 (**Figure 10**)

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

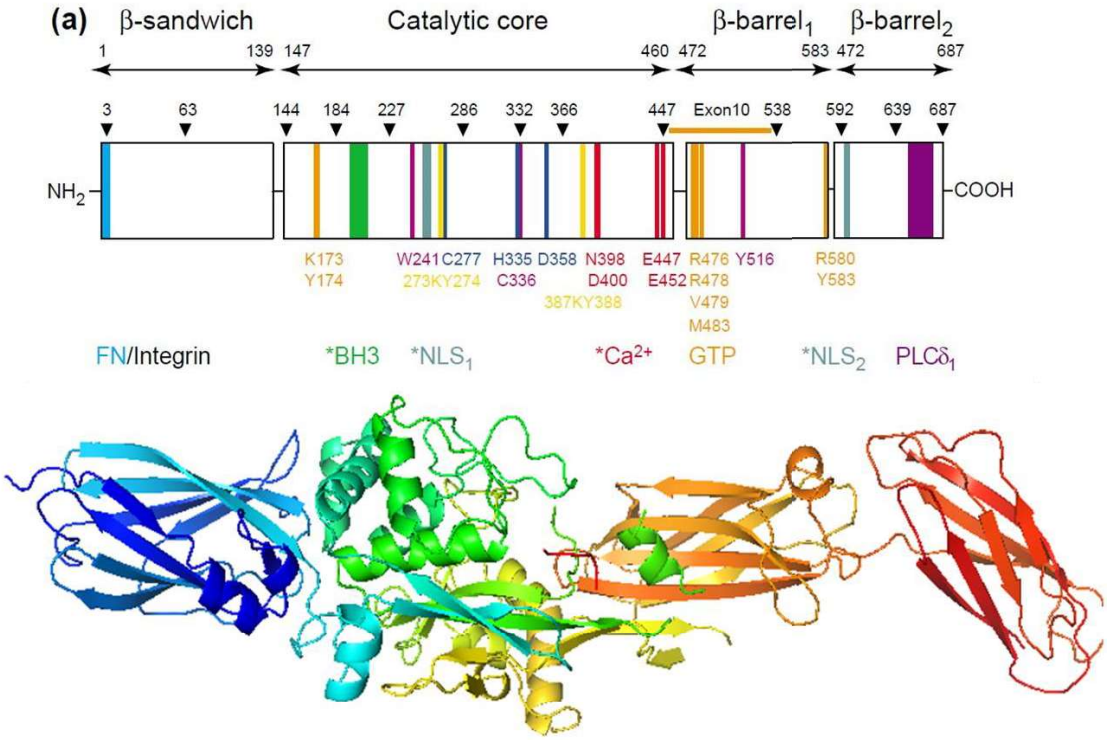


Figure 10. Domain composition of the tissue transglutaminase [Fesüs et al., 2002; PDB: 2Q3Z]

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 (**Figure 11**).

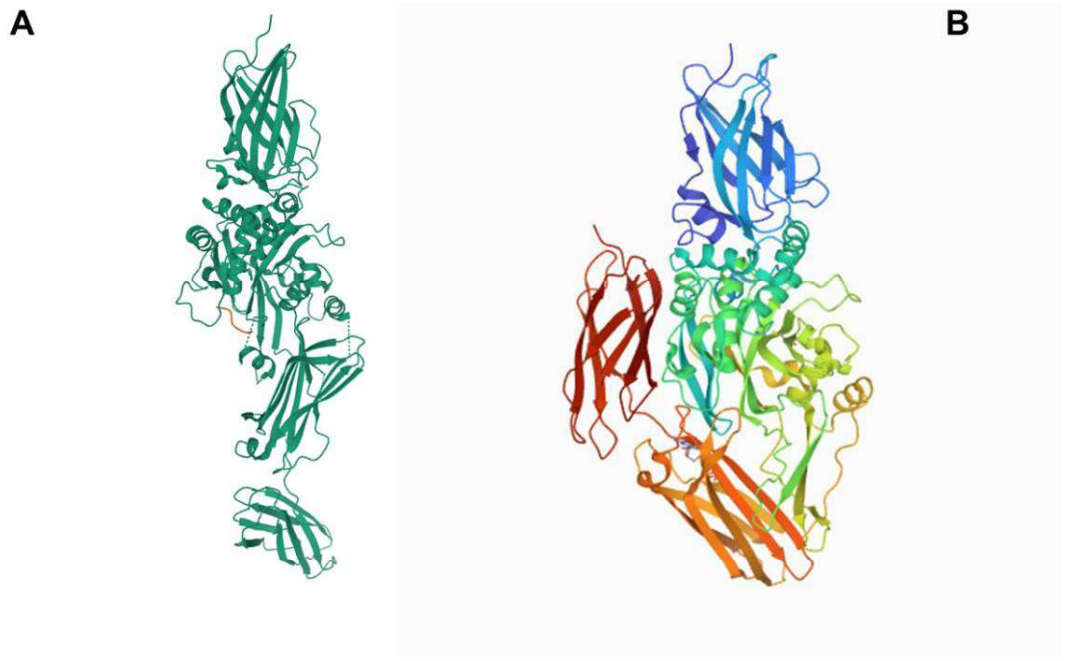


Figure 11. Structural features of human tissue transglutaminase. *(**A**) The open conformation of TG2 no GTP is bound to the enzyme [2Q3Z] (**B**) Closed conformation with the GTP [PDB:4PY6]*

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]

1.3.2. Structure elements of the TG2

In the very first studies, it was shown that the *N-terminal beta-sandwich* part is responsible for fibronectin-binding, which explains its involvement in the cell adhesion, migration and wound healing roles of the enzyme [Beklin et al., 2001]; however, a later functional analysis of the protein found that the interaction occurs through the first domain's hairpin structures (β5/β6 hairpins) [Hang et al., 2005].

In the *catalytic core domain*, there is a triad composed of Cys277-His335-Asp358. This triad is responsible for the acyltransferase activity, whereas the protein kinase, protein disulphide isomerase and ATPase activities have been connected to other sites [Mishra et al., 2004; Fésüs et al., 2002]. TG2 can regulate apoptosis, which was suggested to involve a putative BH3 motif in the protein. This part is located in the catalytic core domain, as well, and can interact with pro-apoptotic BCL-2 protein family members [Rodolfo et al., 2004]. If the enzyme interacts with BAX, apoptosis is facilitated. The core domain carries the nuclear localisation signals (NLS), as well, which makes nuclear translocation possible. When the enzyme has bound an inhibitor, its structure changes into an extended open conformation, in which the active site is exposed [Pinkas et al.; 2007].

The *first β-barrel* is involved in the acquisition of the closed conformation due to the presence of some of the GTP-binding residues there. Liu et al. have shown that the GDP/GTP binding capacity of the enzyme depends on the sequence between the core domain and the first beta-barrel [Liu et al.; 2002]. The GDP or GTP can bind to the last amino acid strands of this part of the enzyme (476-482; 580-583) and the core domain amino acid residues (173-174). Based on the existence of the GDP/GTP bound form, several studies established that TG2 has G-protein function as well, which could partially explain the cell signalling roles of the enzyme [Kumar et al., 2012, 2013; Eckert et al., 2015; Akbar et al., 2017].

The *second barrel* contains the second NLS and an interaction site for phospholipase C [Fésüs et al., 2002]. This part of the enzyme is required for the membrane bond form to support the conversion of phosphatidylinositol-bisphosphate to phosphatidylinositol-tris-phosphate (PIP_2-$PIP_{3)}$, which promotes cell survival [Caron et al., 2012; Eckert et al., 2014].

1.3.3. Tissue distribution and subcellular localization of TG2

Tissue transglutaminase has a broad tissue and cell distribution, and we can distinguish tissues and cells that express the enzyme constitutively, and those who show expression only upon differentiation (**Table 5**).

TISSUES CONSTITUTIVELY EXPRESSING TG2	INDUCIBLE EXPRESSED TG2 BY DIFFERENTIATION/MATURATION
mesangial cells	epithelium
interstitial cells	breast cells
thymic subcapsular epithelium	nephrons
colonic precryptial fibroblast cells	enterocytes

Table 5. Tissues and cells which are expressing TG2 constitutively or inducible

The subcellular localization of TG2 is also really diverse, based on the literature it appears in a wide range of cell compartments (**Figure 12**).

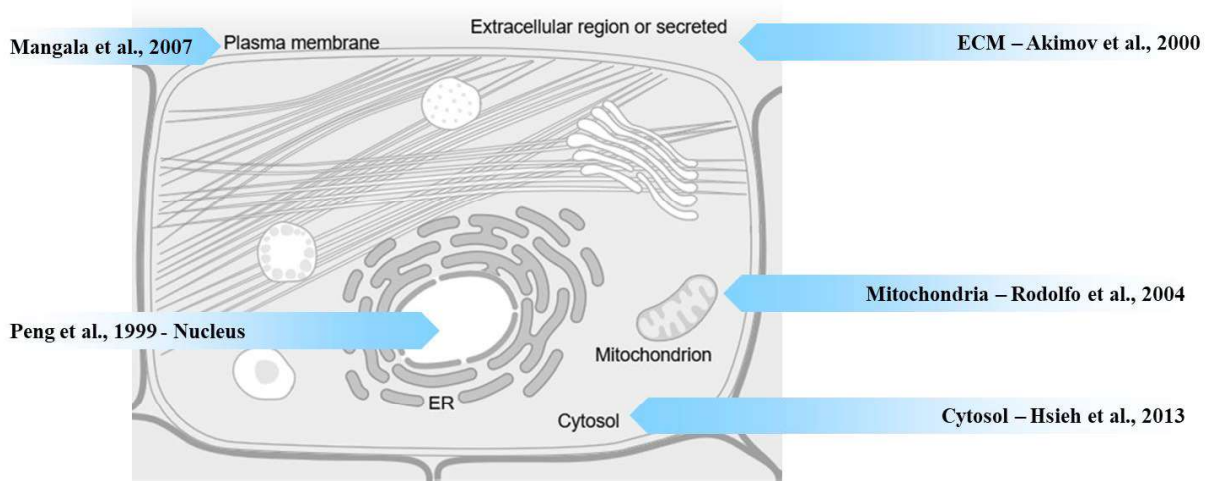


Figure 12. Distribution of the TG2 inside the cell compartments *[The cell's schematic figure was obtained from the http://www.uniprot.org website] The blue arrows are showing the first publication by which the TG2 has been localised in the marked tissue/cell compartment.*

Piacentini and also Rodolfo et al. reported that TG2 was detectable not only in the cytosol but also in the mitochondria [Piacentini et al., 2002; Rodolfo et al., 2004]. TG2 can be transported into the nucleus with the help of the NLS1-2, in a process carried out by the importin-α3 [Peng et al.; 1999].

Similarly to the cytosolic TG2, the nuclear enzyme also acts as a multifunctional one, performing enzymatic and non-enzymatic functions. It has a role in the pathogenesis of neurodegenerative diseases, as well as, in the development of tumours of the liver and other organs [Kuo et a.,2010]. Shimizu and colleagues published first that in starfish sperm, there is a crosslink catalysed by TG2 between H2B and H4 in the nucleus. It was described later that

the mammalian core histone proteins (H2A, H2B, H3, H4) are also glutamyl substrates of TG2 and the crosslinks *in vivo* lead to the chromatin condensation, showing the signs of apoptosis.

In human acute promyelocyte leukaemia cells upon all-trans-retinoic acid (ATRA), a fraction of TG2 can be translocated into the nucleus and be associated with the chromatin. Additionally, it has a role in the regulation of the inflammatory cytokine and chemokine production, the cells' phagocytic capacity and the cells' reactive superoxide production capacity. [Kuo et al., 2010; Balajthy et al., 2006, Csomós et al.; 2010]

Not only cell signalling processes be affected by TG2, but the cell's survival processes also. Under hypoxic circumstances, the neural system of the body is highly threatened. Under these conditions, TG2 localizes in the nucleus. This has been studied in SH-SY5Y human neuroblastoma cells *in vitro*. The increased amount of TG2 is capable of binding to the HIF1 transcription factor by which the upregulation of hypoxia response element-containing genes is decreased. This binding is trans-amidation independent and does not affect the HIF1 proteins. By this mechanism, TG2 can prevent further damages to the affected region, which can be important in ischemic circumstances. [Kuo et al., 2011; Mangala et al., 2005]

TG2 can support the activation of the nuclear factor kappa-B pathway (NF-κB), can regulate the inflammatory gene expression and via forming a complex with the p65/relA subunit can be directly translocated into the nucleus and regulate the transcription [Kuo et al., 2011].

Mangala et al. have reported that TG2 can form a complex with the cell membrane component beta-integrin molecules, which promotes cell-cell interaction and also involves fibronectin attachment to other proteins or ECM components [Mangala et al., 2007]. TG2 can play a role in the reorganization of the ECM, most frequently in the case of a pathogen-induced infection, neutrophils can create a NET, which captures the pathogens for elimination [Csomós et al., 2016].

1.3.4. Role of the TG2 in pathological processes

Tissue transglutaminase is widely expressed protein, playing a wide range of roles in the cell homeostasis. This multifunctionality may answer for the patho-biochemical roles of the enzyme as well. There are several diseases to which TG2 is connected. Diseases in which TG2 plays crucial roles can be categorised into three big groups:

1) inflammatory pathologies (production of pro-inflammatory cytokines and chemokines)
2) autoimmune diseases (Alzheimer disease, Coeliac disease)
3) malignant diseases (tumour formation and cancer progression)

The transglutaminases are responsible among the others for tissue repair, wound healing, and cell adhesion, and can also contribute to the development of fibrosis [Grenard et al., 2001]. TG2-dependent fibrosis can develop in the liver tissue, associated with a very severe inflammatory state of the patient. Johnson and colleagues reported that in renal and respiratory fibrosis, an excessive amount of scar tissue develops which is triggered by transglutaminases [Johnson et al., 1997].

In 2000, another study revealed that the high expression of TG2 in ageing articular chondrocytes leads to a chronic inflammation state, where the production of the inflammatory cytokines and chemokines was extremely high, and more specifically tumour necrosis factor-alpha and interleukin-6 had causative roles in the development of age-related arthritis [Rosenthal et al., 2000].

A lot of new studies have reported that the presence of the anti-TG2 antibodies or the crosslinking activity of the enzyme was highly detectable in coeliac and Huntington diseases. In the case of coeliac disease, the intestinal mucosa of the patients was TG2 positive, whereas the deamination of the glutamine residues of gliadin results in a strong immune response, in which highly activated T-lymphocytes damage the intestinal villi (**Figure 13**) [Marzari et al., 2001; Martuciello et al., 2018].

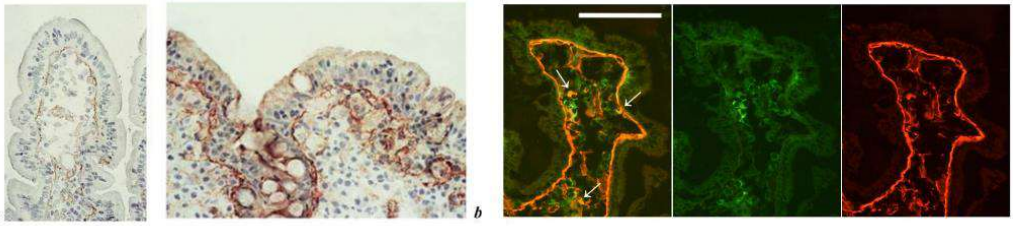


Figure 13. Immunolabelled section of healthy and coeliac disease patients [Esposito et al., 2005; Hietikko et al., 2018] *Immunostaining of the localization of TG2 protein in duodenal sections from a control patient* ***(a)*** *and a CD patient* ***(b). Right part:*** *Immunofluorescence staining for TG2 antibodies and plasma cells in small-bowel mucosal sections.*

In neurogenerative pathologies like in Huntington disease or Alzheimer disease, the activity of the enzyme is high, which is accompanied by the formation of deposits of crosslinked non-functional proteins. Thus, the tissue is disrupted, which can cause later on dementia and other dysfunctions in the CNS. [Karpuj et al., 2002; Szondy et al., 2017; Tangaraju et al., 2016]

The expression of tissue transglutaminase and its transamidation activity is often associated with inflammatory responses, due largely to the fact that cytokines/chemokines and growth factors are capable of regulating TG2 expression and consequently, its activity. The expression of the *TGM2* gene is regulated by the so-called "canonical and non-canonical" NF-κB pathways. These pathways are important in the production of pro-inflammatory molecules among which TNF-α, IL-1β, MCP-1 and IL-6 are crucial target genes of NF-κB signalling, besides these proteins are the potent inducers of TG2 expression (**Table 6**) [Mangala et al., 2005; Kumar et al., 2013].

AGENT INDUCES TG2	TARGET CELLS
CYCLIC AMP (cAMP)	Cerebellar granules
DEXAMETHASONE	Hamster fibroblasts and fibrosarcoma
DIMETHYL SULFOXIDE	Friend's erythroblastoma
INTERLEUKIN-6	Human hepatoblastoma
	Human myeloid cells
RETINOIC ACID	Human neuroblastoma cells
	Squamous carcinoma cells
SODIUM BUTYRATE	Human lung fibroblasts
TUMOUR NECROSIS FACTOR α	Human hepatoblastoma
PHORBOL ESTERS	Human myeloid
	Human colon carcinoma
TRANSFORMING GROWTH FACTOR β (TGF-β)	Human retinal pigment epithelial cells
EPIDERMAL GROWTH FACTOR (EGF)	Primary cultured hepatocytes
	Breast cancer cell lines
INTERFERON-ALPHA AND BETA (INFα-;β)	Human non-small cell lung cancer
HYDROGEN PEROXIDE	3T3 fibroblasts
GLUCOSE	Kidney epithelial cells
STATINS	Endothelial cells
UV-RADIATION	Lens epithelial (HLE-B-3)

Table 6. Potential inducers of the TG2 in different tissues [based on Mangala et al., 2005]

1.3.5. Role of the TG2 in the immune system

Among the many types of tissues and cells in which TG2 is expressed, its presence is noteworthy in the cells of the immune system, mostly in macrophages. TG2 is equally present in peritoneal and the bone marrow-derived macrophages.

In one of the very first experiments that demonstrated this, TG2 was induced in response to pathogen-derived lipopolysaccharide (LPS) and the concomitant enhanced superoxide production in activated macrophages which had been previously exposed to LPS *in vitro*. [Akimov et al., 2001].

The phagocytosis capacity of the macrophages in the presence of the TG2 was high, and Szondy and colleagues found that the macrophage-mediated complete elimination of apoptotic cells was carried out by a TG2-dependent process [Szondy et al., 2003]. *In vivo* experiments which showed that TG2 is connected to the immune system responses were first performed in 2005, after $TG2^{-/-}$ knock out mice had been generated. Without TG2, the immune response was less effective, and it was accompanied by altered TGF-beta and interleukin production [Falasca et al., 2005].

The importance of TG2 for inflammation and the clearance of apoptotic cells was proven by other studies in which it affected the LPS-induced production of pro-inflammatory factors via NF-κB pathway [Kim et al., 2002]. In 2010, Hodrea and colleagues published that in the presence of tissue transglutaminase there was CD11c positivity on the surface of dendritic cells (DC); however, antigen-presenting DCs did not show catalytically active TG2 on their surface [Hodrea et al., 2010].

In the same year Csomós et al. reported that knocking down TG2 in NB4 cells, resulted in a less active immune response with lower expression of pro-inflammatory cytokines, and a significantly diminished migration/adhesion and phagocytotic capacity of the NB4 cells due to the lack of TG2 [Balajthy el al., 2006; Csomós et al., 2010].

1.4. 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) (**Figure 14**).

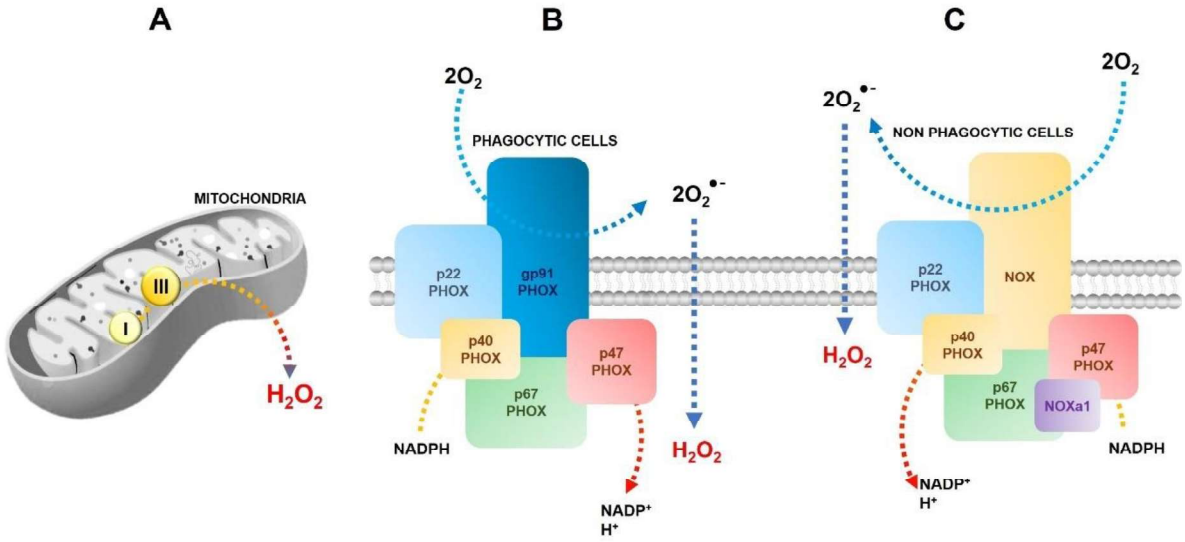


Figure 14. Potential ways to produce ROS (A) *ROS produced by the mitochondria, via the electron transport chain (ETC) first and third complexes and by the help of the mitochondrial Superoxide dismutase (SOD) enzyme.* ***(B, C)*** *Production of ROS through the NADPH-oxidase system composed of the different-proteins in phagocytic and non-phagocytic cells.*

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].

1.5. 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].

Microcirculatory phenomena associated with inflammation include alterations in vascular permeability, aggregation of white blood cells, and release of inflammatory mediators, including the following reaction:

1) receptors recognizing motifs in tissue macrophages recognize the dangerous stimuli
2) inflammatory signalling pathways are activated
3) inflammatory mediators are released
4) inflammatory activated cells migrate to infected or damaged tissue

The receptors which are recognized by the macrophages characterized by the inducing factor, it can be a pathogen or a damage-induced pathway triggered afterwards.

Pathogen associated molecular patterns (PAMPs), such as bacterial lipopolysaccharide or double-stranded RNA, trigger the activation of pattern-recognition receptors (PRRs) in both immune and non-immune cells. Some PRRs are also capable of recognizing damage-associated molecular patterns (DAMPs) that are formed or released in connection with a tissue or cell damage [Kumar et al., 2004].

DAMPs are biomolecules of the host (human body) that are capable of initiating or enhancing a sterile (non-infection-related) inflammatory response. A typical example of a sterile inflammation initiation is the response to uric acid crystals in gout. Damaged cells are also capable of attracting inflammatory cells by releasing hazardous molecules such as ATP, high-mobility group protein B1 (HMGB1), or bacterial mitochondrial proteins and DNA. The families of PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic

acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) [Ferrero-Miliani et al., 2007; Granger et al., 2010].

TLRs are highly conserved members of the PRR family that can sense pathogens extracellularly and intracellularly in the endosome or the lysosome. TLRs are characterized by an N-terminal leucine-rich region (LRRs) single-membrane region and a cytoplasmic Toll/ IL-1R homology (TIR) domain. Several toll-like receptors are expressed in humans. Tissue macrophages express these receptors, and their activation induces the transcription of inflammatory cytokines (IL-1β, TNF-α, IL-6) and chemokines/chemoattractants. TLRs activate signalling pathways involving Ser/Thr kinase complexes that eventually activate various transcription factors such as activator protein-1 (AP-1), NF-κB dimer, or interferon regulatory factor 3 (IRF3) [Larson et al., 1983; Kumar et al., 2004; Granger et al., 2010].

The NOD-like receptor (NLR) family consists of cytoplasmic pathogen sensors consisting of a central nucleotide-binding domain and a C-terminal leucine-rich repeat region. At the N-terminus, protein binding domains are found, such as the caspase attracting domain (CARD), the pyrin domain, or the baculovirus inhibitor of apoptosis protein repeat (BIR) domain. They do not regulate transcription, but form parts of inflammasomes and regulate caspase-1 activation [Granger et al., 2010].

Nacht Domain-, Leucine-Rich Repeat-, and PYD-Containing Protein 3 (NALP3) is the most studied NOD-like receptor that forms an inflammasome in tissue macrophages. Originally repressed NALP3 is activated by viral (RNA), bacterial (proteoglycans, toxins) or endogenous danger signals (uric acid, ATP). NALP3 oligomerizes in an ATP-dependent manner, attaching to the apoptosis-associated speck-like protein (ASC) and pro-caspase 1 protein, leading to its activation in the cytosol. Caspase-1 then cleaves and activates the precursor forms of IL-1β and IL-18. The effect of IL-1β is the appearance of selectin molecules on the surface of capillary endothelial cells [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.

Selectins are cell surface molecules composed of lectin, epidermal growth factor, and 2-9-complement repeat (CR) domains. Neutrophils and monocytes continuously express their ligands for binding to selectins. Selectin ligands are transmembrane glycoproteins that exhibit an oligosaccharide structure to the selectins. All three selectins recognize glycoproteins and glycolipids that contain a tetra-saccharide called sialyl-LewisX. It consists of sialic acid, galactose, fucose and N-acetyl galactosamine [Liang et al., 2016].

The formation of transient bonds between the receptor and the ligand initiates early steps in the adhesion cascade, resulting in the rolling of neutrophils arriving rapidly in the bloodstream and thus slowing down the surface of capillary endothelial cells. At the same time, the selectin/selectin ligand interaction initiates a signalling pathway in neutrophils that actively converts their integrin receptors (CD11/CD18, which can serve as the Lymphocyte function-associated antigen 1 (LFA-1)) and thus binds to their ligands on the surface of capillary endothelial cells (ICAM-1 and 2). The ligated integrin receptors then direct the adhesion of the slowing neutrophils to the surface of the capillary endothelium, followed by the process of transmigration [Loike et al., 1991].

The transmigration is controlled by chemokines released by tissue macrophages that accumulate at the site of infection or injury. Neutrophil's integrins bind to extracellular matrix proteins (collagen, fibronectin) during migration. Without this connection, diseases can develop like Leukocyte adhesion deficiencies (LAD). The lack of CD11 or CD18 integrins is a known human disease characterized by recurrent bacterial and fungal infections after birth. Lack of selectins, or the small G-protein RAC2 (which regulates rearrangement of the cytoskeletal skeleton), is associated with similar symptoms. The immune cells are not capable of slowing down, to reach the infection place and resulting in a non-healing wound at the current tissue [Bainton et al., 1987; Borregard et al., 1997; Nupponen et al., 2001; Sadhu et al., 2007].

Neutrophils arriving at the site of infection firstly, phagocytose infectious microorganisms and eliminate them. In the case of complement or repeated infection, neutrophils are able to recognize and phagocytose bacteria and fungi covered with pre-existing antibodies. Antibody-coated microorganisms are taken up by Fc gamma receptor-mediated phagocytosis. Signalling

is initiated by SRC kinase phosphorylation of the tyrosine-based activation motif (ITAM) motifs of the immunoreceptor in the cytoplasmic domain of the receptor. This attracts tyrosine kinases belonging to the SYK family to the receptor, leading to the activation of additional downstream targets such as phospholipase Cγ (PLCγ). As a result, both calcium and PKC signalling are activated. Fc gamma receptors also regulate tyrosine phosphorylation-based activation of several other proteins, leading to rearrangement of the cytoskeletal skeleton and resulting in phagosome formation. Nascent phagosomes undergo maturation and fuse with lysosomes. The formation of oxygen free radicals and the entry of the contents of the lysosome and neutrophil granules into the phagosome results in the killing and degradation of the pathogen.

Neutrophils increase vascular permeability by producing histamine upon TLR stimulation. The main function of the vascular pathway is to serve as a conductor for blood circulation to supply oxygen and nutrients to tissues. In most organs, endothelial cells form the boundary between blood and tissue. Under resting conditions, fluid and smaller molecules, but larger molecules, do not continuously escape from the vascular pathway. Molecules smaller than 40 kDa may spontaneously leave the vascular pathway, and larger molecules require the removal of the vascular barrier continuity to be applied. This process is called increased vascular permeability and can be induced by histamine. As a result, larger molecules may be released from the plasma at the site of inflammation to help identify, neutralize, kill pathogens (complement proteins, C-reactive protein, antibodies), or to protect tissues from molecules released from activated neutrophils (such as antiproteases). The process is aided by PGE_2 which induces vasodilation, thereby increasing the number of molecules exiting. The process causes the disintegration and red colour of the inflammatory environment.

Activation of phospholipase A_2 in neutrophils increases the free radicals and membrane-bound PLA_2 and IL-1/TNF-β, a high calcium concentration in the cytosol leads to the release of arachidonic acid by activating cytosolic PLA_2. Neutrophils continuously express lipoxygenases, which convert arachidonic acid to leukotrienes. The leukotrienes are chemo-attractants for later-arriving neutrophils and also help their activation. Besides, they interact with TNF-β / IL-1 to induce the expression of cyclooxygenase-2 (COX-2), which in association with a prostaglandin synthetase leads to PGE_2 formation and release [Larsen et al., 1983].

PGE_2 acts as a vasodilator to help as many neutrophils, monocytes, and mast cells emigrate. Also, PGE_2 stimulates sensory nerves to enhance the pain response. In the late stages of the

inflammatory response, PGE_2 regulates several processes that result in the silencing of inflammation and initiate subsequent tissue repair. Inhibition of COX-2 in the late stages of the inflammatory response has been shown to inhibit complete tissue healing in the liver, lungs, and colon [Downey et al., 1988]. By acting on the heat centre of the hypothalamus, fever-induced through the initiation of local PGE_2 synthesis. In the hypothalamus, arachidonic acid is not released from phospholipids, but from 2-arachidonoyl-glycerol by lipo-acylglycerol lipase. Higher body temperature inhibits the growth of microorganisms, so it is one of the body's protective mechanisms [Larsen et al., 1983].

IL-6 released from the site of inflammation initiates the acute phase response of the liver by acting on hepatic IL-6 cytokine receptors. Hepatocyte IL-6 receptors regulate the expression of approximately 400 genes in the liver. Their protein products, when they enter the bloodstream, play a variety of roles in protecting against pathogens. One of these is reactive protein C (CRP), a single pentamer that binds to lysophosphatidylcholine on the surface of dead cells and in certain bacteria, activating the complement system to bind to them and helping their phagocytosis. Because the levels of this protein rise very early and very sharply in connection with inflammation in the blood, clinicians look at the levels of this protein to detect possible inflammation in the body [Harada et al., 1994].

After the microorganisms or damaged cells are cleared out at the site of inflammation, the migration of neutrophils attenuates. Half of the dying cells reprogram inflammatory macrophages into healing macrophages, which stop the production of inflammatory cytokines and chemokines, and instead produce anti-inflammatory resolvins, adenosine, TGF-β and PGE_2 to stop inflammation and tissue damage. Then they return to the bloodstream [Larsen et al., 1983; Kojima et al., 1993].

Inhibition of inflammation also occurs in other ways. TNF receptors are released, which inhibit TNF-alpha, adrenocorticotropic hormone (ACTH) is released from the pituitary gland, which initiates glucocorticoid hormone synthesis in the adrenal cortex. By acting on the nuclear receptor of the hormone glucocorticoid, the inflammatory response in many cells stops [Stuart et al., 1996; Takami et al., 2002].

2. Aim of the study

NB4 cells differentiate into neutrophil granulocytes upon all trans retinoic acid (ATRA) treatment, in which one of the most highly expressed genes is tissue transglutaminase (TG2). Previously, our group showed that reduced both activity and epression of the enzyme, NB4 cells show reduced phagocytosis, adhesion, migration, and ROS-producing ability.

My primary goal was to functionally examine TG2. For the analysis, a transglutaminase-knock out, TG2-KO cell line was generated from wild-type cells using TALEN technology. Involving both wild-type and TG2-KD and TG2-KO cell lines in my work, we investigated the form in which the ATRA-induced TG2 enzyme contributes to the development of differentiation syndrome (DS).

For the interpretation of biochemical functions, my primary goal was to determine the inflammatory cytokine / chemokine and ROS-forming activity, which has been previously studied in our laboratory in order to determine whether the amount or presence of TG2 influences the severity of DS side effects. My aim was to investigate the cause of inflammation at the level of gene expression and secreted proteins.

In the second part of my work, I aimed to investigate the effects of a combination treatment with an arsenic trioxide and with a TG2 inhibitor, based on the ATRA-induced differentiation. Regarding to this, I examined the inflammatory cytokine/chemokine and ROS-producing ability of NB4-WT, TG2-C, TG2-KD and TG2-KO cells, respectively.

Material and methods

3.1. 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.

3.2. 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)

3.3. 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.

3.4. 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).

3.5. The sequences of the Human TALEN constructs on the *TGM2* gene (Human-H27143)

The sequences of the TALEN constructs containing the second exon of *TGM2* are highlighted in different colours (**Figure 15**).

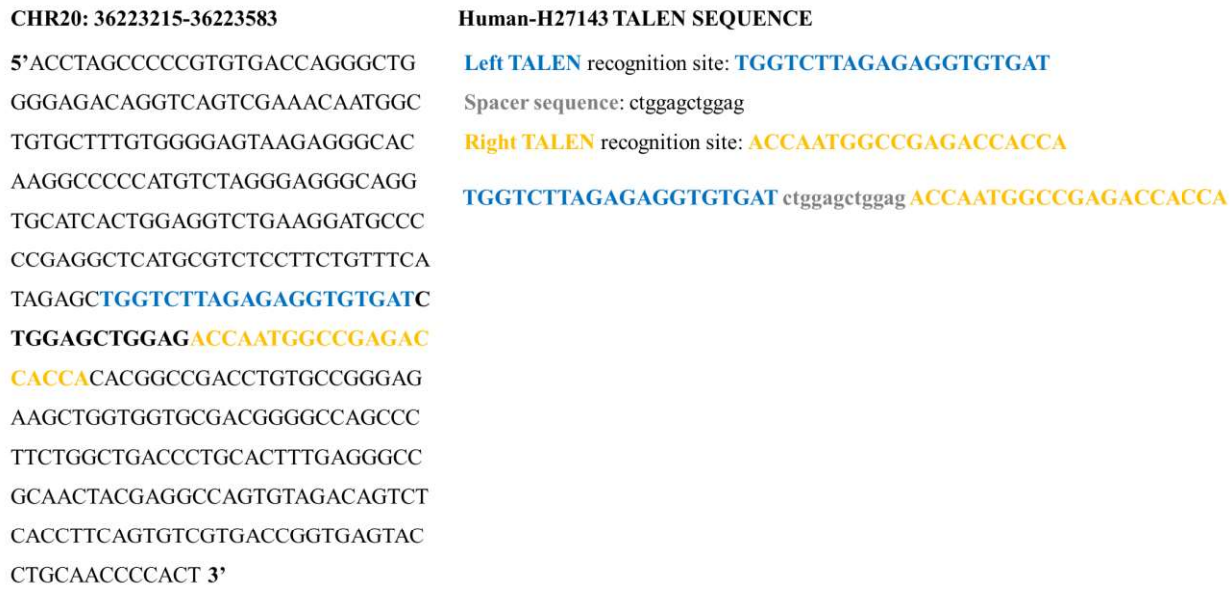


Figure 15. Sequences of the human TGM2-TALEN H27143 sites and the second exon of the TGM2. (Left panel) *Part of the chromosome 20 sequence where the second exon used for the TGM2 knock out generation. The highlighted sequences represent TALEN target sites with the spacer sequence between them.* ***(Right panel)*** *TALEN recognition sites highlighted with blue and orange colours covering the spacer sequence with grey.*

3.6. Oligonucleotide primer for PCR and sequencing

The TALEN, Human H27143 plasmids are 6.763 bp long with a cytomegalovirus (CMV) promoter. We have designed a primer pair from Sigma-Aldrich Inc. for sequence analysis (**Figure 16**).

Figure 16. Primer pair designed for sequence analysis of the Human H27143 TALEN sequence

The length of the sequence in the genomic DNA spanned by the primers was 369 bp, which can be detected later with polymerase chain reaction (PCR) and agarose gel-electrophoresis methods.

3.7. Transformation of competent *E. coli* cells

Competent DH5α and NEB® 5-alpha *E.coli* cells were separately transformed by the TALEN and surrogate reporter plasmids using the competent cell protocol (**Figure 17**).

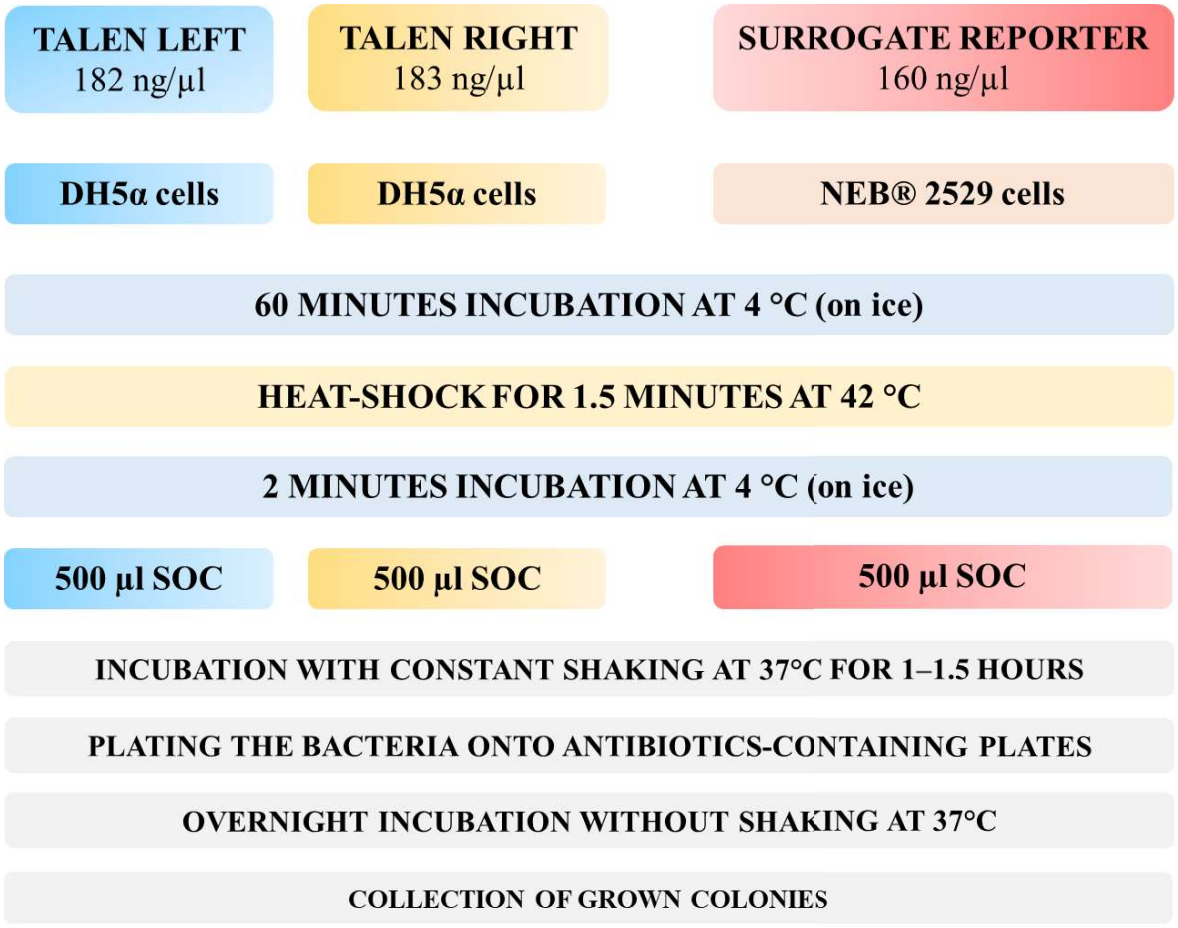


Figure 17. Transformation of competent E. coli cells with TALEN plasmids and with Surrogate reporter plasmids

After the successfully transformed bacterial cultures were harvested, and the plasmids were isolated for further experiments.

3.8. Plasmid DNA preparation

TALEN and Surrogate plasmids DNA-isolation/preparation was done by using a NucleoSpin® Plasmid isolation kit (Macherey-Nagel, Düren, Germany) from *E.coli* cells, following by the manufacturer's instructions.

3.9. Restriction digestion

We carried out restriction analysis with all the three plasmids (Talen left/right and Surrogate reporter). Before the digestion steps using Serial Cloner software, involving XbaI and BamHI restriction enzymes an in silico was performed (**Table 7**).

Restriction reaction mixture:	**Volume in µl**
10x Tango buffer	2.0
BamHI	1.0
XbaI	1.0
DNA plasmid	500.0 ng
Nuclease free water (NFW)	15
The total volume of	20

Table 7. Reaction mixture for restriction digestion analysis with BamHI and XbaI enzymes

3.10. Transfection of NB4 cells

NB4 WT cells were transfected by Amaxa® Cell Line Nucleofector® V Kit following the manufacturer's instructions. During transfection process, the plasmid DNA ratios were 1:1:1. The maximum plasmid DNA amount was not exceeding the total of 2µg DNA in the tube. The transfection efficiency was evaluated by the degree of GFP/RFP positivity in the transfected NB4 cells after each day.

3.11. 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.

3.12. Limiting dilution and cloning

A total of 1–5x 10^5 double-positive and sorted NB4 cells were diluted and plated in a round bottom (U), 96-well plates (TPP, Biotech Inc.) with a density of 1–2 cells/well. Clones underwent a clonal expansion under normal cell culturing circumstances.

3.13. Genomic DNA isolation

The isolation of the genomic DNA was performed using TRIzol® (Invitrogen) and column-based methods (Machinery & Nagel; UD GenoMed & Covalab tissue and blood DNA isolation kit). Both methods were performed following the manufacturer's protocol.

3.14. Determination of gene mutation in the TALENs KO cells

The previously isolated gDNA from the successfully transfected NB4 TG2-KO cells were further analysed by sequencing methods, performed in the Genomic Medicine and Bioinformatics Core Facility of the University of Debrecen, Hungary. The sequencing/analysis was done according to the Core Facility's protocols. The results were analysed using Chromas LITE/Finch TV software; the NB4 TG2-KO sequences were aligned to reference sequences. With the help of the National Centre for Biotechnology Information, basic local alignment search tool method was used to evaluate the result of the sequencing.

3.15. PCR with gDNA

The first PCR reactions with the genomic DNA from NB4 transfected cells were performed in 8-well strips in a total volume of 50 µl (**Figure 18**).

30 X CYCLES

95°C: 3 min	[95°C: 30 sec	60°C: 30 sec	72°C:1 min	72°C: 5 min	4°C

PCR reaction mixture:	Volume in µl
Taq polymerase buffer (10x)	5
dNTP mix (10 mM)	5
TALEN forward primer	1
TALEN reverse primer	1
$MgCl_2$ (25 mM)	8
gDNA (50 ng/µl)	1
Taq polymerase	1
Nuclease free water (NFW)	28
Total volume of	50

Figure 18. PCR reaction mixture and program with gDNA of NB4 TG2-KO candidates

For agarose analysis, 1/10 of the product was loaded onto a 2% agarose gel, ran for 90 minutes at 80 V constantly. The results were visualised using UV filter by Protein Simple AlphaImager® HP (ProteinSimple, 3001 Orchard Parkway, San Jose, California, 95134 USA)

3.16. 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.

3.17. 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).

3.18. 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.

3.19. 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.

3.20. 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).

Different primary antibodies were used (**Table 8**), diluted in 0.5% milk powder in 1x TTBS, with a dilution ratio of 1:5.000–1:10.000, and incubated overnight at 4 °C.

	Type	Cat. number	Company
anti-TG2	Monoclonal	CUB7402	Neomarkers, Fremont, CA
anti-p65	Monoclonal	#8242S	Cell signalling Technologies
anti-p65 (phospho - SER536)	Polyclonal	#3031S	Cell signalling Technologies
anti-p50	Monoclonal	#13586	Cell signalling Technologies
anti-GAPDH	Monoclonal	649202	Biolegend
anti-PCNA	Polyclonal	307902	Biolegend
anti-ACTIN	Polyclonal	A2066-100UL	Sigma Aldrich

Table 8. List of antibodies used in Western blot

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.

3.21. 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.

3.22. 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)

3.23. 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).

3.24. 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.

3.25. 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.

3.26. Transduction of NB4 cells with Luciferase lentiviral reporter

To evaluate the NF-κB-pathway in NB4 cell lines, ready-to-transduce transcription factor-responsive lentiviral reporter system (CLS-013-L8 -QIAGEN) were used in transient and in permanent transfections methods as well. All the steps were performed based on the protocol of the company.

3.27. Selection of the NF-κB reporter element containing cells

The permanent/transient transfected cells were selected using Puromycin solution (Sigma-Aldrich) dissolved in cell culture medium (RPMI 1640 HEPES using in 5-10 µg/ml final concentration.

3.28. 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.

3.29. Gene Expression Analysis with Real-Time Quantitative 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.

3.30. 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.

3.31. 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).

3.32. List of reagents and instruments were used during the study

	PRODUCT	COMPANY	CAT. NUMBER
Cell culturing	TALEN LEFT and RIGHT plasmids	Talen Library Resource	Human-H27143
	Surrogate reporter plasmid	LAMOBICS	PRG2S-CS
	Competent DH5α *E.coli*	Thermo Fisher Scientific	18265017
	NEB® 5-α *E.coli*	Thermo Fisher Scientific	New England Biolabs
	NucleoSpin® Plasmid isolation kit	Macherey & Nagel, Düren, Germany	740588.50
	XbaI restriction enzyme	Thermo Fisher Scientific	18754311
	BamHI restriction enzyme	Thermo Fisher Scientific	18957421
	Amaxa® Cell Line Nucleofector® V Kit	LONZA	VVCA-1003
	The NF-κB responsive lentiviral reporter system	QIAGEN	CLS-013-L8
	NB4 WT cell line	DSMZ GmbH Germany	ACC 207
	GIBCO Fetal Bovine Serum (FBS)	Life Technologies Kft.	10500064
	RPMI Medium 1640	Sigma-Aldrich, Inc.	R8758-500ML
	L-Glutamine Solution 200 mM		59202C
	Penicillin − Streptomycin − Neomycin Solution		P4083
	Sodium pyruvate solution		S8636
Treatment and lysis	Phorbol 12-myristate 13-acetate (PMA)		P1585-10MG
	Arsenic-trioxide (ATO)		202673
	All-trans retinoic acid (ATRA)		R2625-50MG
	Nitro-tetrazolium-blue (NBT)		N6876-50MG
	Bovine serum albumin (BSA)		A2153-50G
	Protease inhibitor cocktail		P8340
	Phosphatase inhibitor		P5725
	Sodium-dodecyl-sulphate (SDS)		L3771
	Polyvinylidene difluoride (PVDF) membrane		IPVH00010
	Nuclei Isolation Kit: Nuclei EZ Prep		NUC101-1KT
	Phenylmethylsulfonyl fluoride (PMSF)		10837091001
	WesternBright ECL HRP substrate	Advansta	K-12045-D50
Cytospin	May-Grünwald solution	Molar Chemicals Kft	1941-606-310
	Giemsa solution		05491-606-310
RT-PCR	TRIzol® reagent	Thermo Fisher Scientific	15596026
	High Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific	4388950
RT-qPCR	TaqMan assay *TGM2*	Thermo Fisher Scientific	hs01566408_m1
	TaqMan assay *MCP-1*		hs00234140_m1
	TaqMan assay *TNF-α*		hs00174128_m1
	TaqMan assay *IL-1β*		hs01555410_m1
	TaqMan assay *NCF2*		hs01084940_m1
	TaqMan assay *GPphox91*		hs00166163_m1
	TaqMan assay *GAPDH*		hs02786624_g1
ELISA	MCP-1 deluxe	BioLegend, Inc.	438804
	IL-1β deluxe		437004
	TNF-α deluxe		430204

4. Results

4.1. 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 (**Figure 19**).

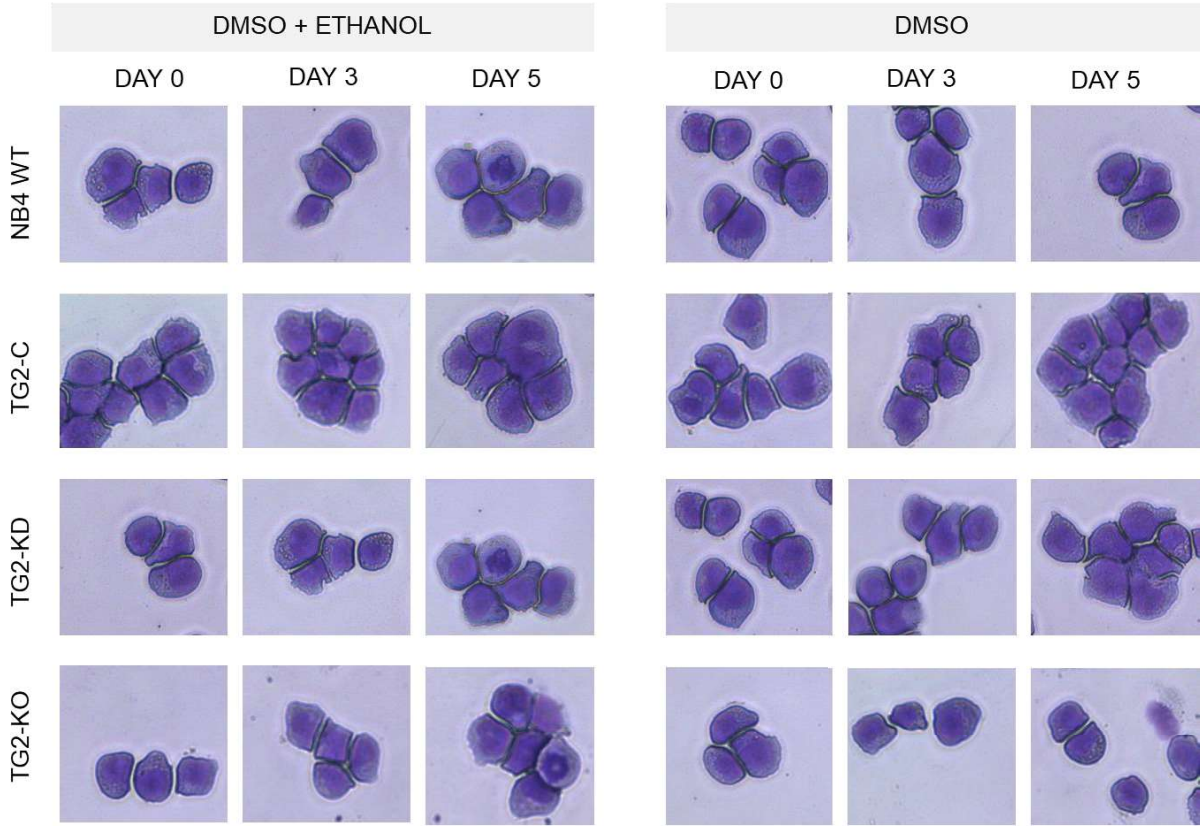


Figure 19. DMSO; DMSO + ETHANOL and the dissolvent solutions are not capable of inducing differentiation of NB4 cells *Representative images of May-Grünwald-Giemsa staining for the morphological examination of NB4 cell lines treated with DMSO +ETHANOL or with DMSO alone for three and five days (n = 3). Light microscope images and validation were obtained using the FLoid® Cell Imaging Station instrument (Life Technologies).*

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 (**Figure 20**).

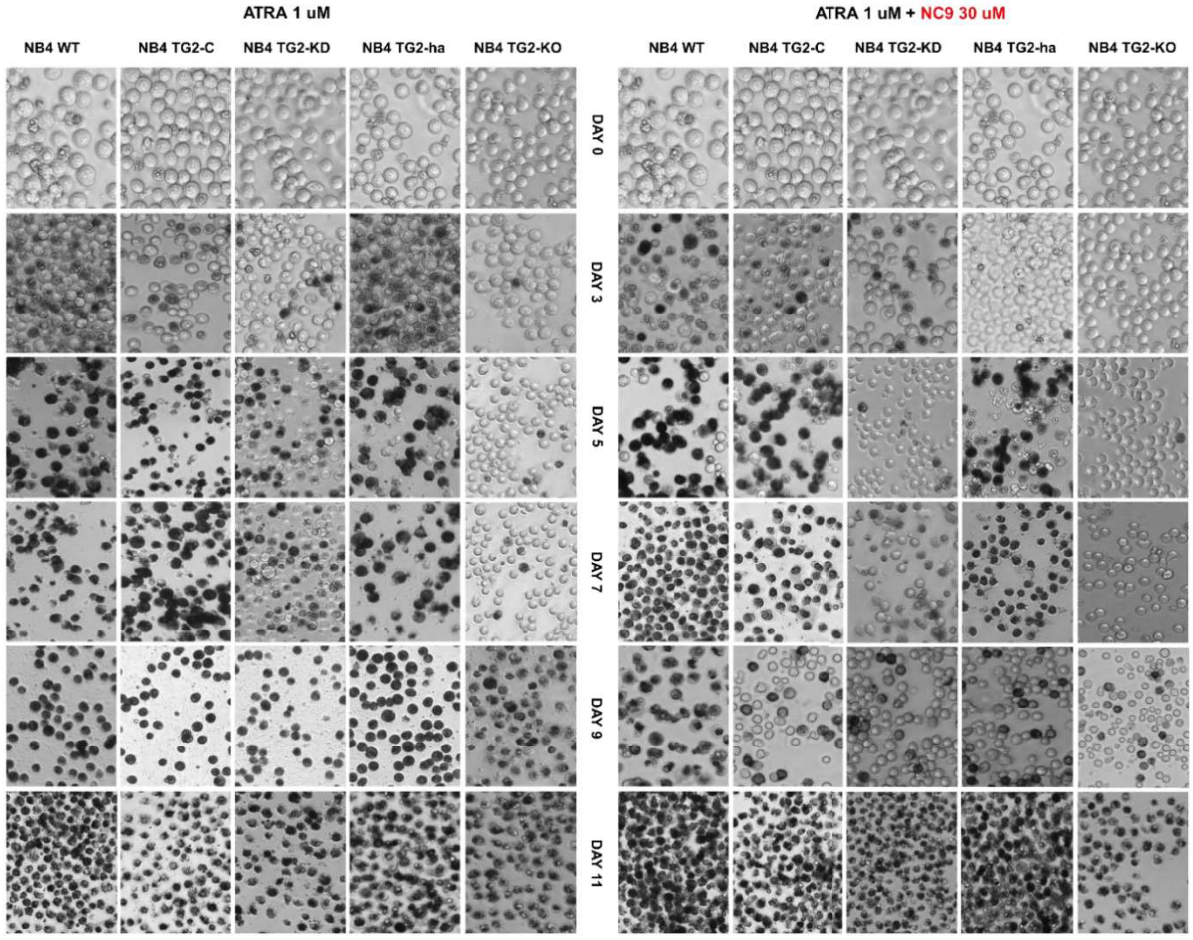


Figure 20. TG2 accelerates the phagocytotic and microbial killing functions of differentiating NB4 cell lines. *NB4 cell lines were cultured in the presence of 1 μM ATRA (left) and the presence of 1 μM ATRA together with 30 μM NC9 (right) for 11 days. NBT-reduction assay was performed at the indicated time points in triplicates. Images were obtained using the FLoid® Cell Imaging Station (Life Technologies) instrument with a magnification scale: 200 μm.*

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 (**Figure 21. A**).

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 (**Figure 21. A/red bars**).

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 (**Figure 21. B**).

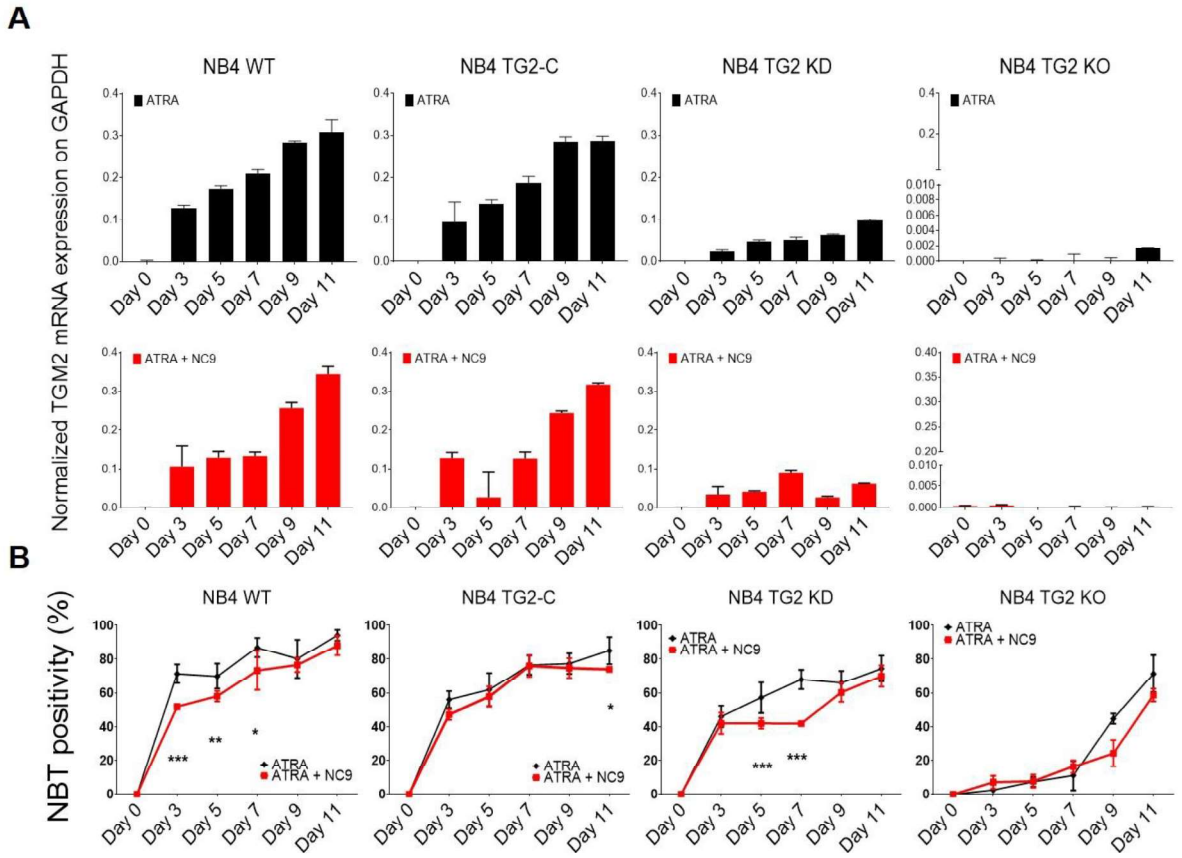


Figure 21. All trans-retinoic acid induce the differentiation of the NB4 cells, which characterised by increased expression of tissue transglutaminase

(A) *Relative mRNA expression of TGM2 in NB4 WT, NB4 TG2-C, NB4 TG2-KD and NB4 TG2-KO cells after one µM ATRA (black columns) treatment and 1 µM ATRA + 30 µM NC9 (red columns), measured at the indicated days (0-11) by real-time Q-PCR and normalised to GAPDH (n=3).* ***(B)*** *NB4 cell lines underwent the ATRA induced differentiation, characterised by the capability to reduce the nitro-tetrazolium-blue dye (NBT). The assay was performed at the indicated time points in triplicates. Percentage of the NBT-positivity represented as mean % ± SD from parallel experiments (n=3). Statistical analysis has been done with TWO-WAY analysis of variance (TWO-WAY ANOVA; Bonferroni post-hoc test; $P < 0.05$)*

4.2. 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 (**Figure 22**).

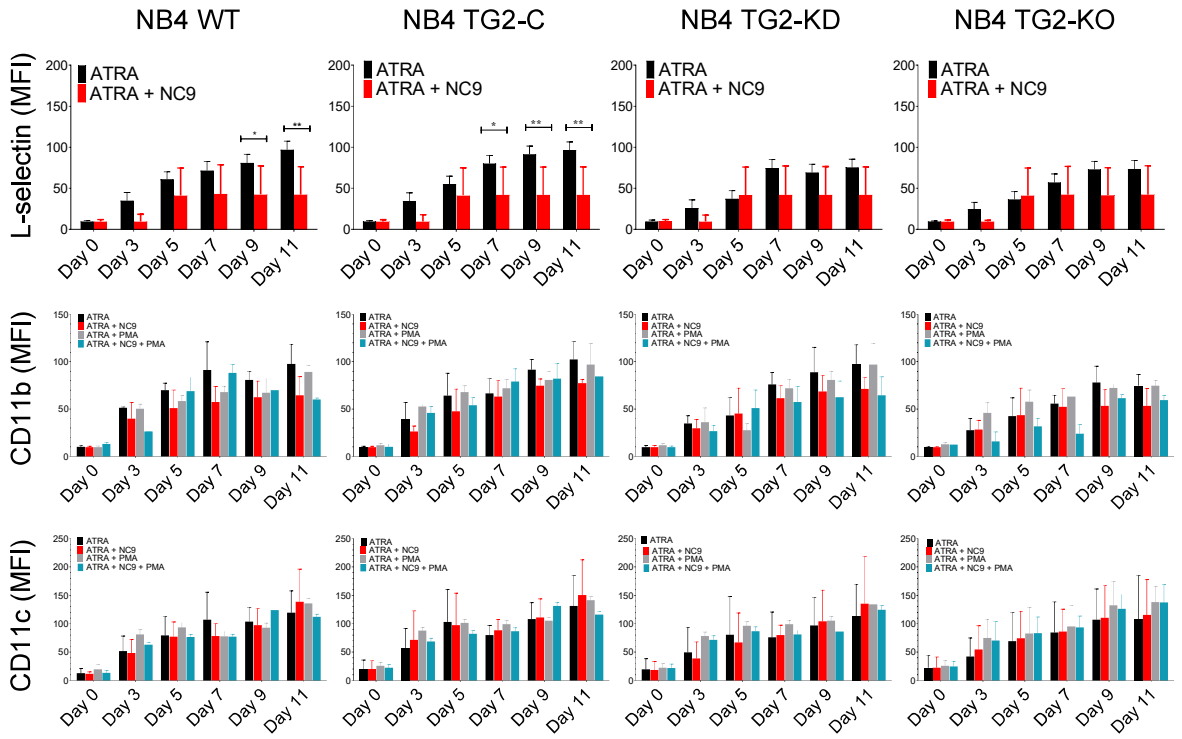


Figure 22. ATRA treatment induces protein expression of CD11b/CD11c integrin receptors and their high-affinity state on the cell surface. *NB4 cells were treated with 1 μM ATRA, 1 μM ATRA + 30 μM NC9, or additionally with 20 minutes stimulation of PMA (10 nM). Measurements were conducted in triplicates; results were validated by Flowing software 2.00. Graphs show the representation of the mean fluorescent intensity (MFI ± SD) values. MFI values were calculated based on each treatment's isotype control (n= 9). (**Upper part**) Flow cytometric analysis of cell surface expression of L-selectin, (**Middle part**) differentiation marker CD11b (**Lower Part**) and CD11c. Statistical analysis was performed using Two-way ANOVA (Bonferroni post-hoc test; $P < 0.05$).*

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 (**Figure 22**). 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 (**Figure 22. blue bars**).

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 (**Figure 22. Middle row**).

4.3. 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 (**Figure 23**). 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 (**Figure 23. A, B black bars**). 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 (**Figure 23. A, B red bars**). The PMA-induced total amount of ROS productions were measured in Relative light unit (RLU) normalised on 100 μg protein content of the cells. (**Figure 23. C**). 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 (**Figure 23. C, red bars**).

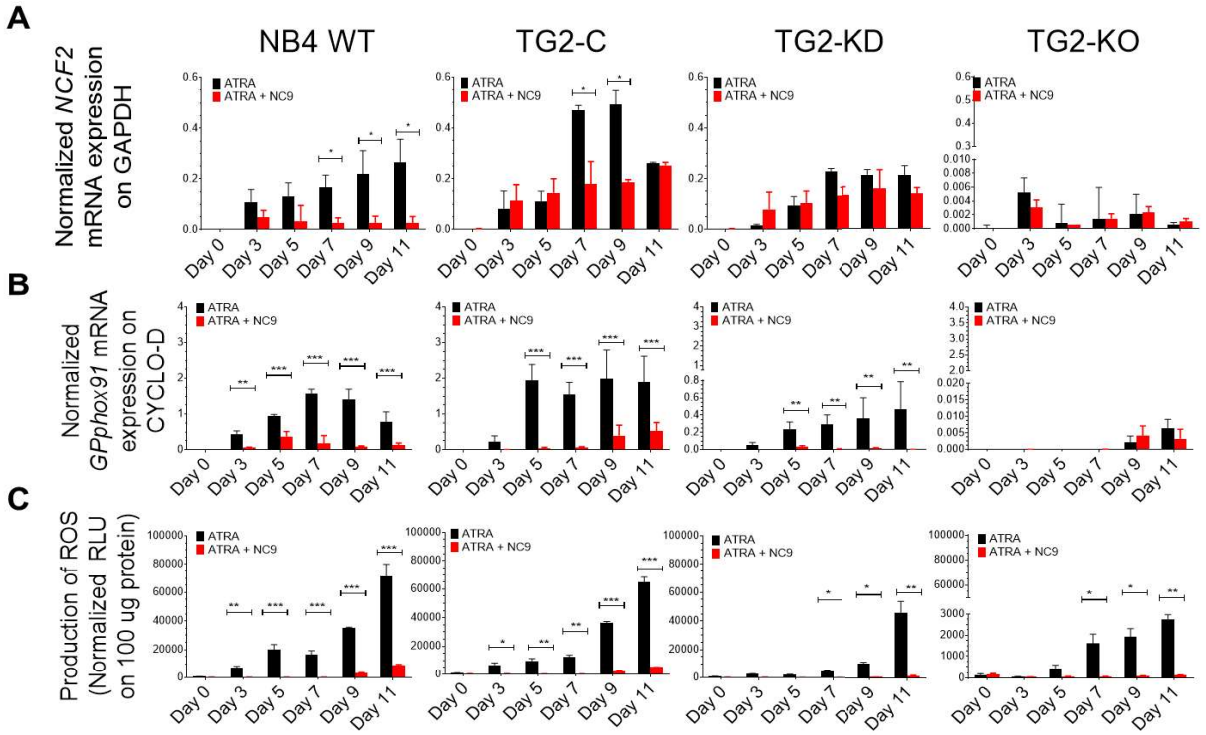


Figure 23. TG2 expression drives both expression of NCF2 and GP91PHOX respiratory burst responsible genes and the generation of reactive oxygen species (ROS). (A-B) *Relative mRNA expressions of NCF2 and GP91PHOX upon ATRA or ATRA + NC9 treatment for 11 days were determined by real-time Q-PCR and normalised to Cyclophilin-D mRNA levels in NB4 WT, TG2-C, TG2-KD and TG2-KO cells. Graphs represent the mean ± SD values (n=3).* ***(C)*** *Production of reactive oxygen species (ROS) was determined for each cell line by using PMA and L012-dye/luminescence-based method in triplicates (n=5) relative light units (RLU) were calculated and validated by normalisation to 100 µg protein of cell lysate content. Statistical significance was determined by using the One-way ANOVA (Bonferroni post-hoc test; P< 0.05).*

4.4. 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 (**Figure 24**). 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 (**Figure 24**).

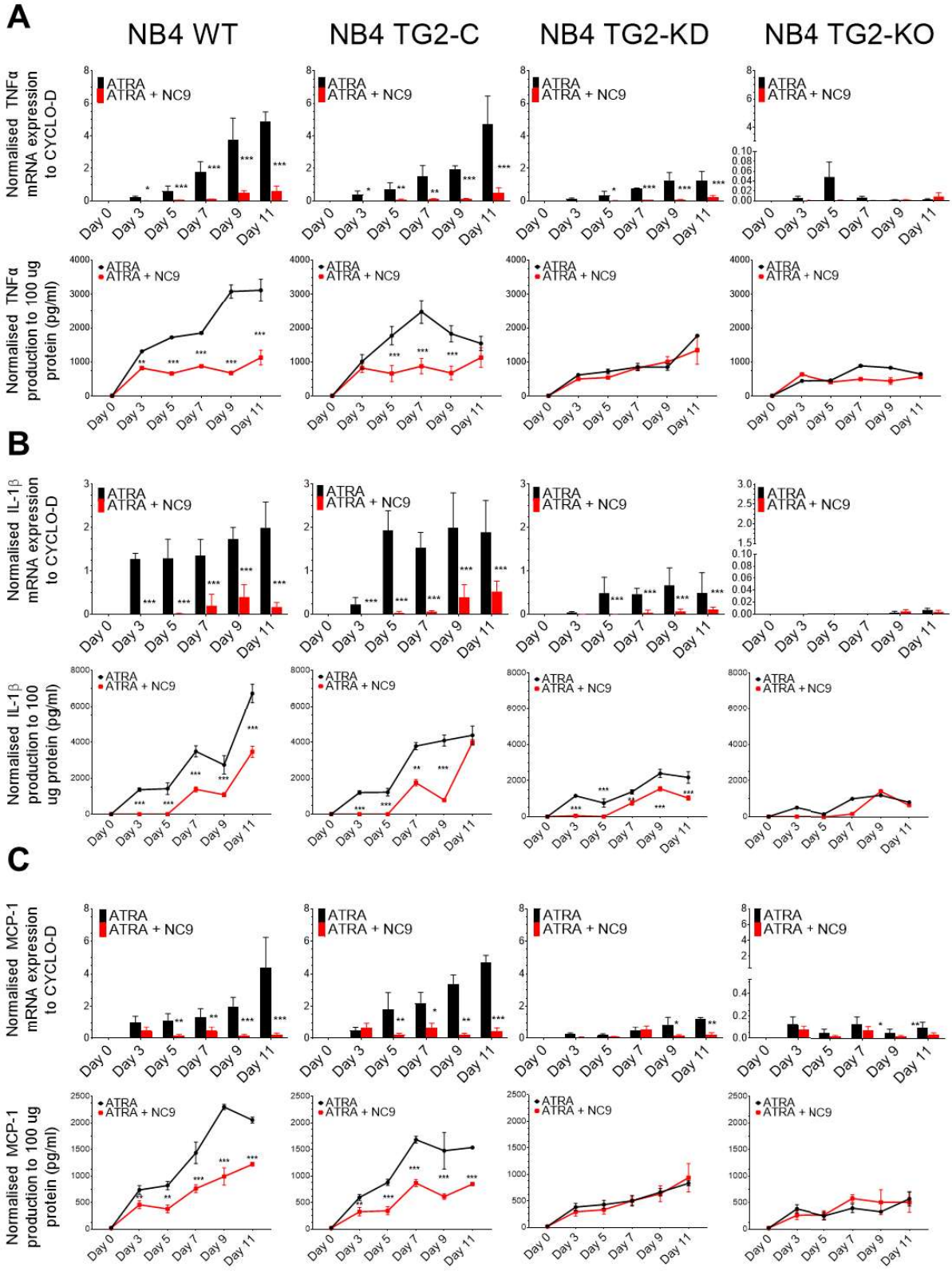


Figure 24. TG2 up-regulates both mRNA expressions of TNF-α, IL-1 β and MCP-1 through NF-κB transcriptional activation and their secretion *Relative mRNA expression of TNF-α (A), IL-1 β (B) and MCP-1 (C) in 1 μM ATRA or one μM ATRA + 30 μM NC9-treated NB4 cells for 11 days via Q-PCR and normalised to Cyclophilin-D. The inflammatory cytokines were quantified by ELISA (Biolegend Deluxe) and normalised to 100 μg protein content of each cell lysate. Graphs show secreted protein levels from three independent experiments measured in triplicates. Statistical significance was determined by using the Two-Way ANOVA (Bonferroni Post-Test; P < 0.05).*

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 (**Figure 22-24**). 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 (**Figure 24. red bars and lines**).

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 (**Figure 25**).

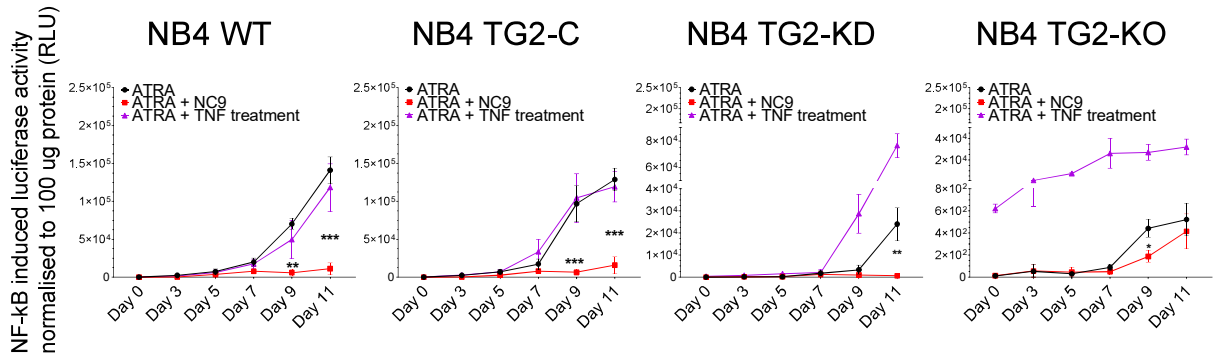


Figure 25. ATRA induced NF-κB transcriptional activity on NF-κB responsive luciferase reporter gene. *Treated and harvested cells were analysed for luciferase reporter activity as relative light units (RLU). Luciferase activity measurements were performed in triplicates (n=9). Statistical significance was determined by using the Two-Way ANOVA (Bonferroni Post-Test; P < 0.05).*

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 **(Figure 25. purple lines**). Additionally, by inhibiting the TG2 with 30 μM NC9, the activity was demolished in those cell lines were the TG2 expressed (**Figure 25. red lines**).

4.5. 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) (**Figure 26**).

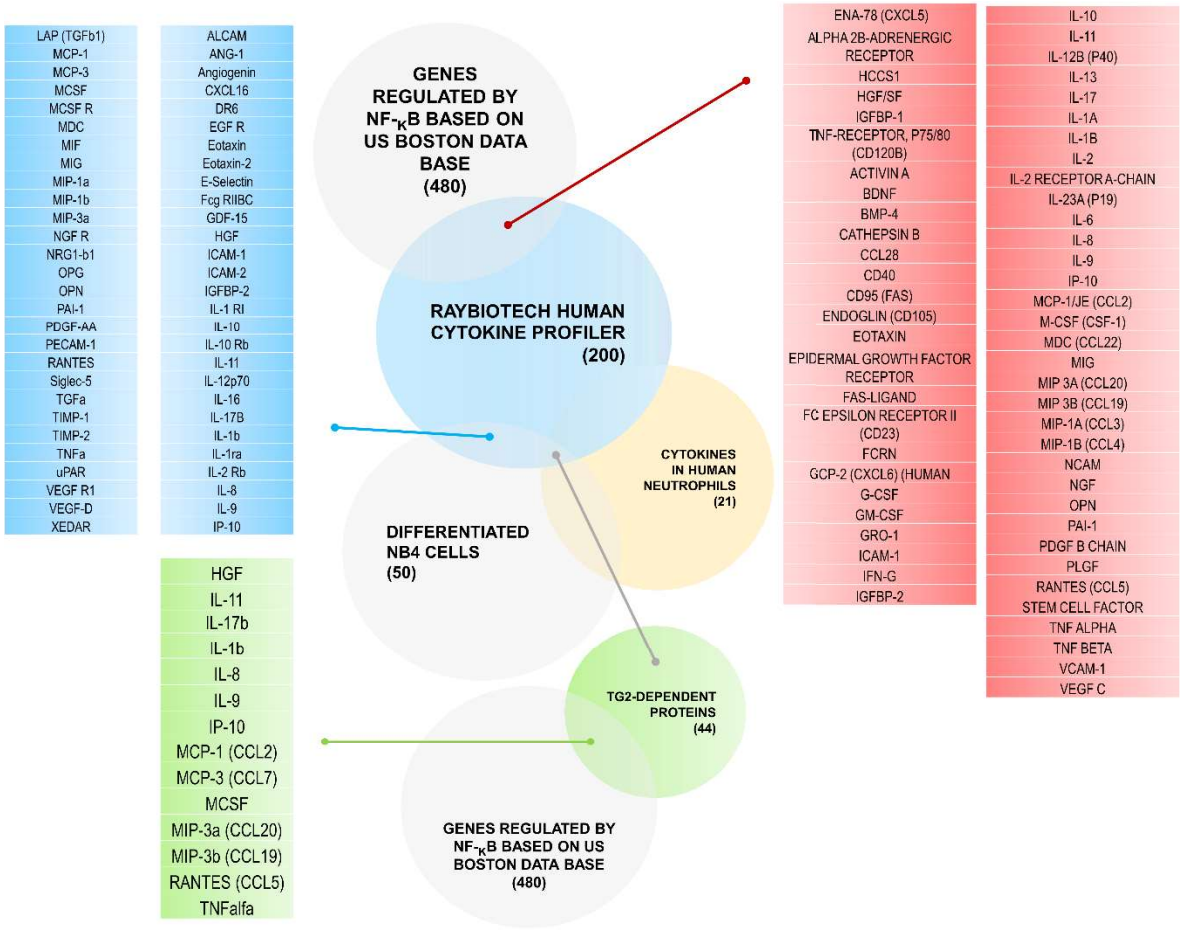


Figure 26. Filtering of TG2 dependent and NF-κB pathway produced cytokines and chemokines. *Supernatants of NB4 cells were collected and pooled together, after ATRA, ATRA + NC9 treatment. The supernatants were analysed and validated by the company, Raybiotech. From the Human cytokine 200 profiler array, 50 proteins were detected in the differentiated NB4 cells, from which 44 were TG2 dependent. Altogether 18 proteins were filtered out as a TG2 and NF-κB pathway-dependent.*

From the 18 proteins analysed and filtered out as TG2 dependent/NF_kB 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 (**Figure 27**).

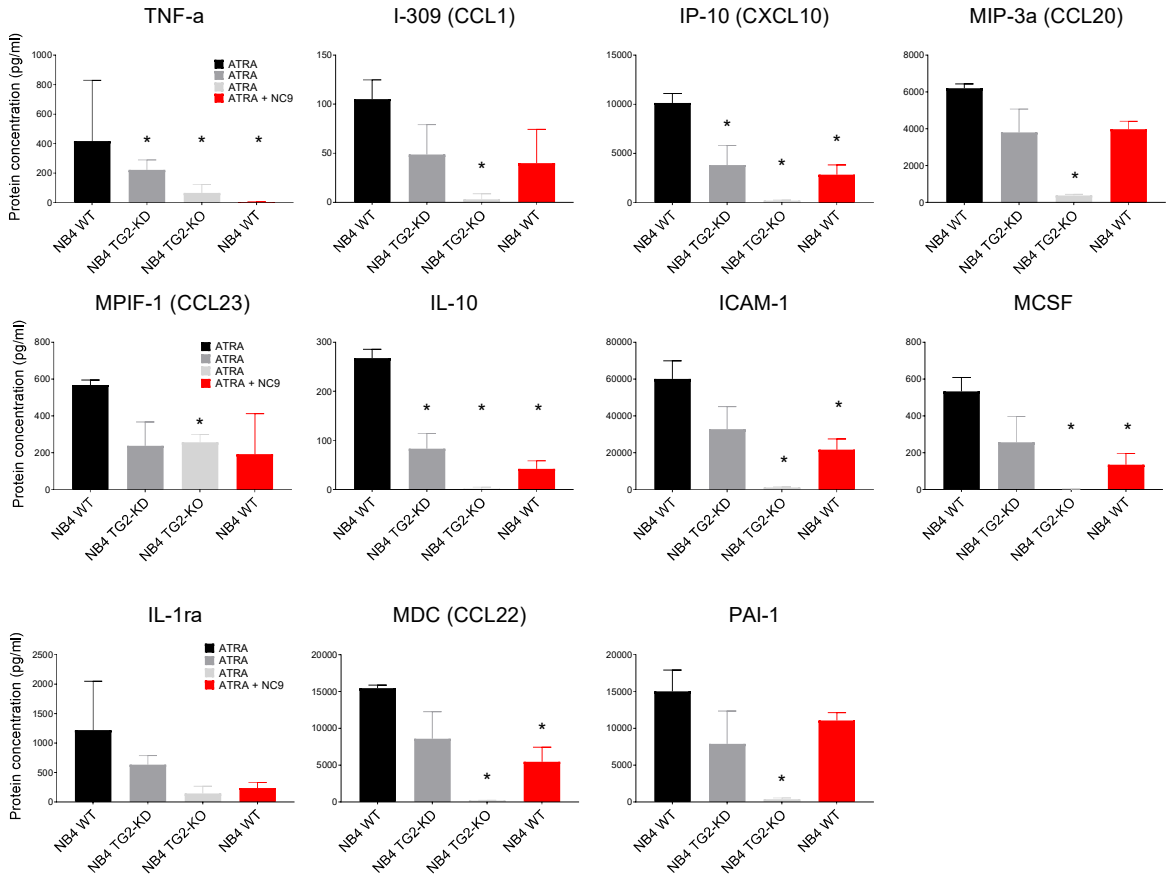


Figure 27. Different TG2 expression levels lead to altered inflammatory cytokine production. *In the cell culture, supernatant secreted chemokines and cytokines were quantified by ELISA-based fluorescent detection cytokine array by the company: RayBiotech. A Human Cytokine Array was used; the company has done the data validation. Graphs show the mean values ± SD of secreted protein levels from three independent experiments (n=3). (a) TG2 quantity-dependent regulated NF-κB transcription factor target genes, and TG2 inhibitor NC9-sensitive secretory proteins. Statistical significance was determined by using the Two-Way ANOVA (Bonferroni Post-Test; P < 0.05).*

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 (**Figure 28**).

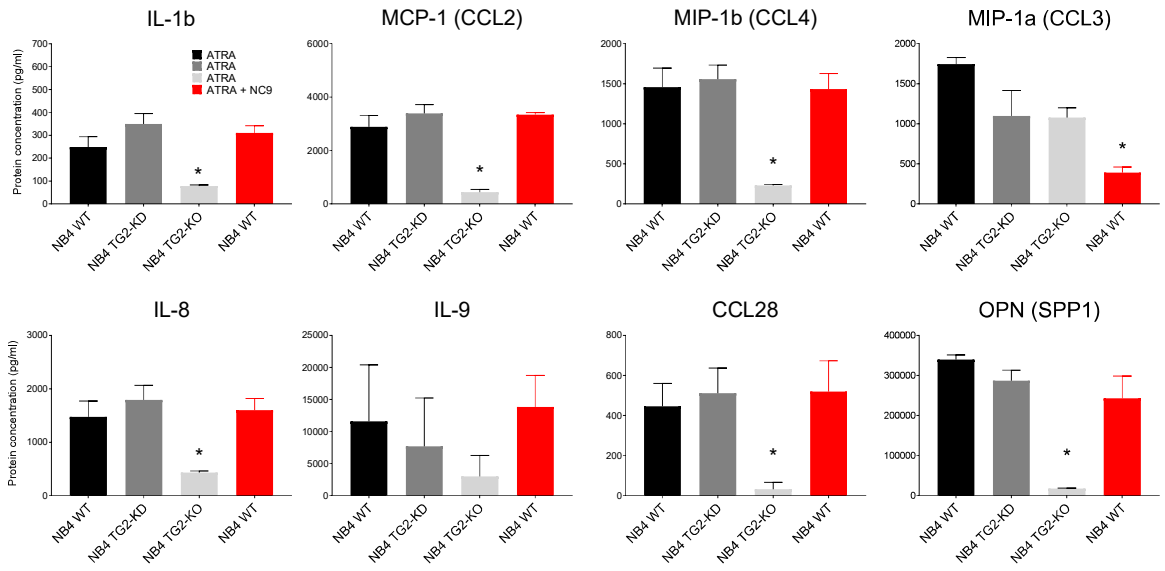


Figure 28. Different TG2 expression levels lead to altered inflammatory cytokine production. *In the cell culture, supernatant secreted chemokines and cytokines were quantified by ELISA-based fluorescent detection cytokine array by the company: RayBiotech. A Human Cytokine Array was used; the company has done the data validation. Graphs show the mean values ± SD of secreted protein levels from three independent experiments (n=3). TG2 quantity-dependent regulated NF-κB transcription factor target genes, and TG2 inhibitor NC9-sensitive secretory proteins. Statistical significance was determined by using the Two-Way ANOVA (Bonferroni Post-Test; P < 0.05).*

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, (**Figure 29**).

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 (**Figure 30**).

(The supernatant concentration of the remaining 16 biomarkers are not shown here).

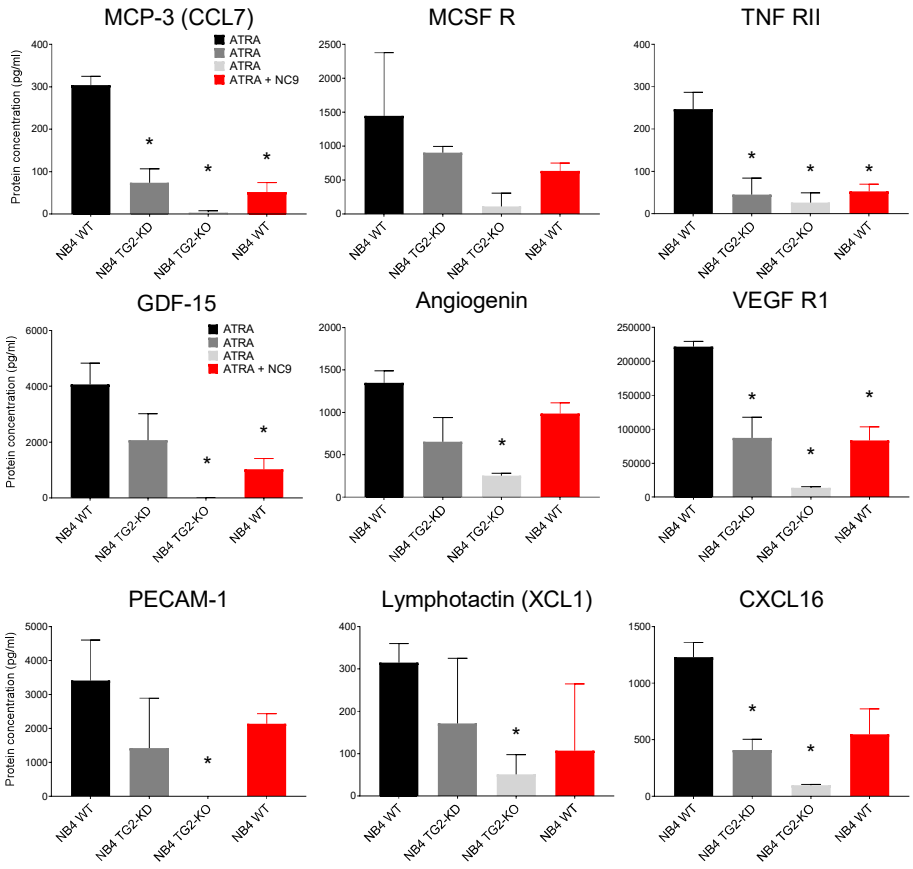


Figure 29. Different TG2 expression levels lead to altered inflammatory cytokine production. In the cell culture, supernatant secreted chemokines and cytokines were quantified by ELISA-based fluorescent detection cytokine array by the company: RayBiotech. *A Human Cytokine Array was used; the company has done data validation. Graphs show the mean values ± SD of secreted protein levels from three independent experiments (n=3). TG2 quantity-dependent regulated NF-κB transcription factor target genes, and TG2 inhibitor NC9-sensitive secretory proteins. Statistical significance was determined by using the Two-Way ANOVA (Bonferroni Post-Test; P < 0.05).*

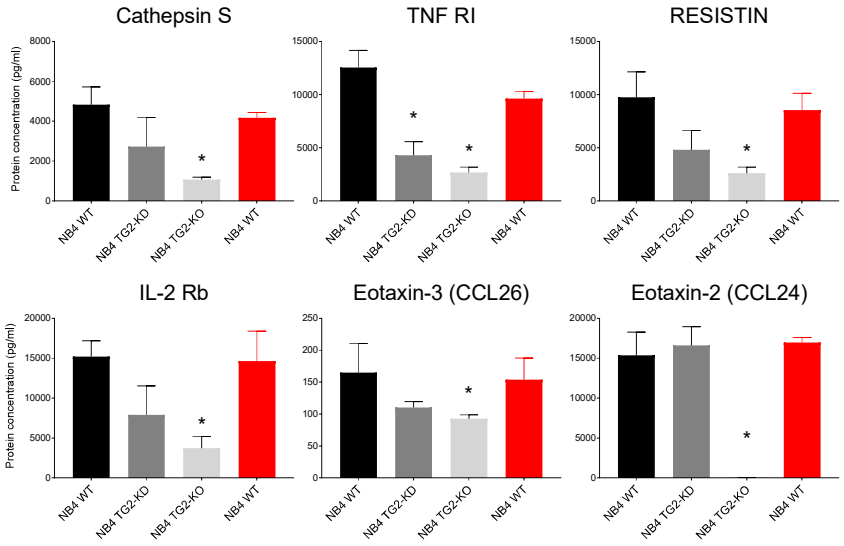


Figure 30. Different TG2 expression levels lead to altered inflammatory cytokine production. *In the cell culture, supernatant secreted chemokines and cytokines were quantified by ELISA-based fluorescent detection cytokine array by the company: RayBiotech. A Human Cytokine Array was used; the company has done data validation. Graphs show the mean values ± SD of secreted protein levels from three independent experiments (n=3). TG2 quantity-dependent regulated NF-κB transcription factor target genes, and TG2 inhibitor NC9-sensitive secretory proteins. Statistical significance was determined by using the Two-Way ANOVA (Bonferroni Post-Test; P < 0.05).*

4.6. 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 (**Figure 25**).

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 (**Figure 31. marked with red box/red bar**). However, the NC9 on the mRNA transcription did not change the TG2 (**Figure 21. A**); the amount of TG2 protein was affected significantly (**Figure 31. left panel, first line**).

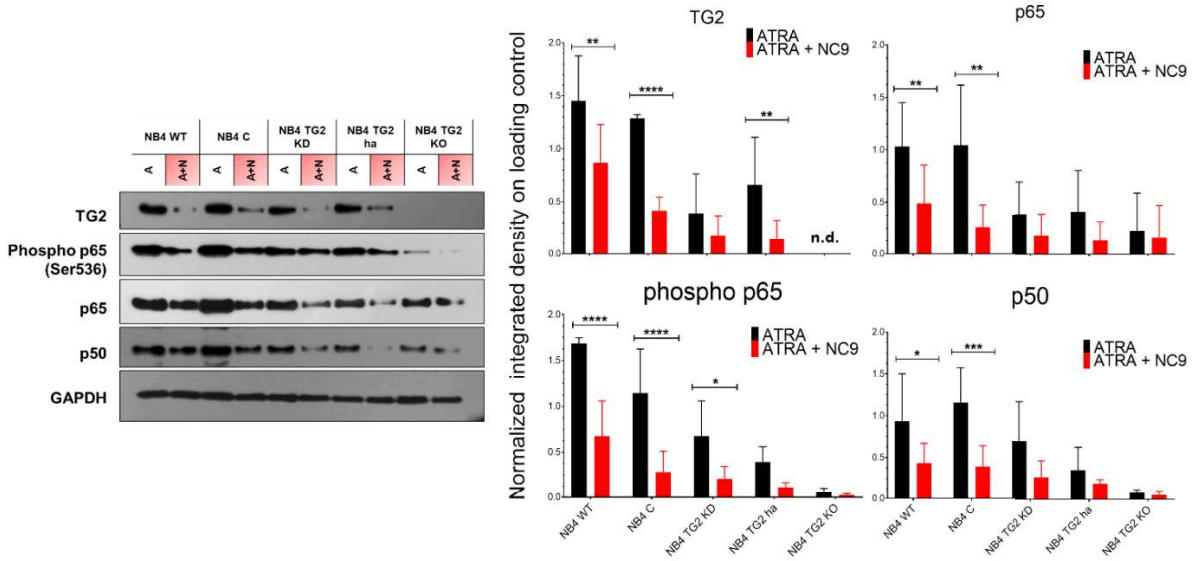


Figure 31. TG2 both induces and guides the nuclear translocation of NF-κB. (left) *Representative Western blot showing TG2 protein expression levels upon ATRA + NC9 treatment through 11 days (n=3). The blots are showing total cell lysate samples TG2, p65/RelA, phospho-p65/RelA and p50 protein expression levels in NB4 cell lines and its densitometric analysis (n=6)* ***(right).*** *Statistical significance was determined by using the Two-Way ANOVA (Bonferroni post-hoc test; P < 0.05).*

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 (**Figure 31, right panel black and red bars**).

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 (**Figure 32**).

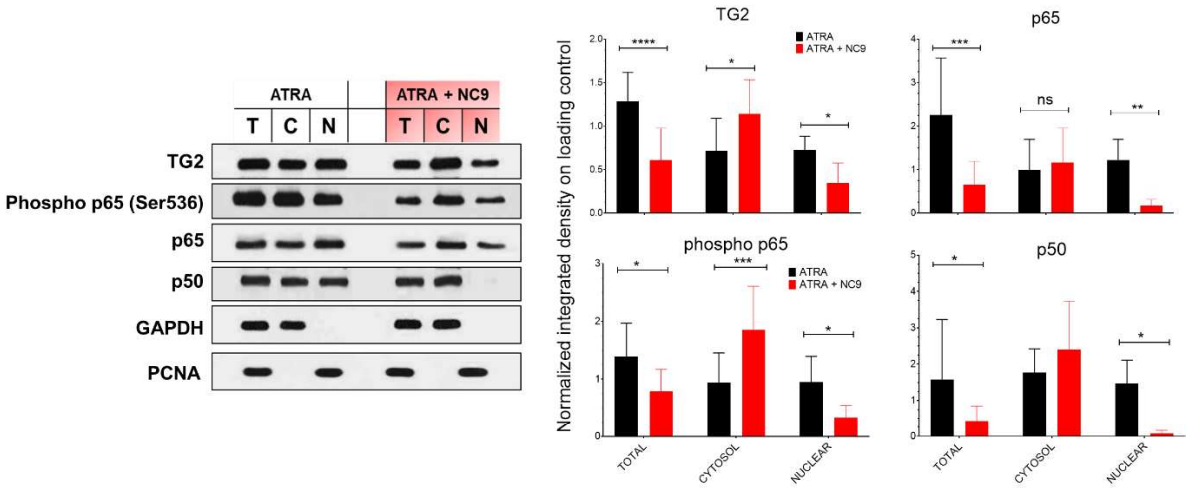


Figure 32. TG2 both induces and guides the nuclear translocation of NF-κB. (left) *Representative Western blot showing TG2 protein expression levels upon ATRA + NC9 treatment through 11 days (n=3). Western blots of total cell lysate (T), cytosolic (C) and nuclear fractions (N) of TG2 and p65/RelA, phospho-p65/RelA and p50 protein expression and its densitometry, respectively (n=10).* ***(right).*** *Statistical significance was determined by using the Two-Way ANOVA (Bonferroni post-hoc test; $P < 0.05$).*

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 (**Figure 32. left panel**). Additionally, the amount of total and nuclear p65/RelA was also reduced in the presence of the TG2 inhibitor (**Figure 32. right panel, right-top**).

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 (**Figure 32. right panel, left-bottom**). 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 (**Figure 32. right panel, right bottom-red bars**).

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 (**Figure 33**).

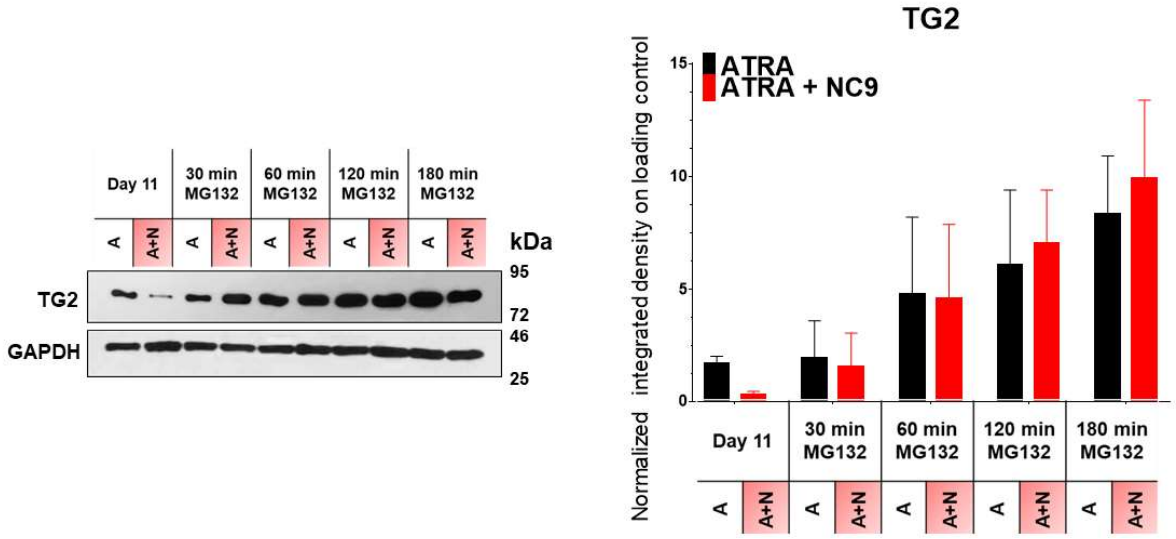


Figure 33. TG2 undergoes an enhanced proteolytic degradation during the NC9 treatment. *Western blot analysis of NB4 WT cells upon ATRA; ATRA + NC9 and five μM MG132 treatment (n=6). Statistical significance was determined by using the Two-Way ANOVA (Bonferroni post-hoc test; P < 0.05).*

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.

4.7. 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 (**Figure 34**).

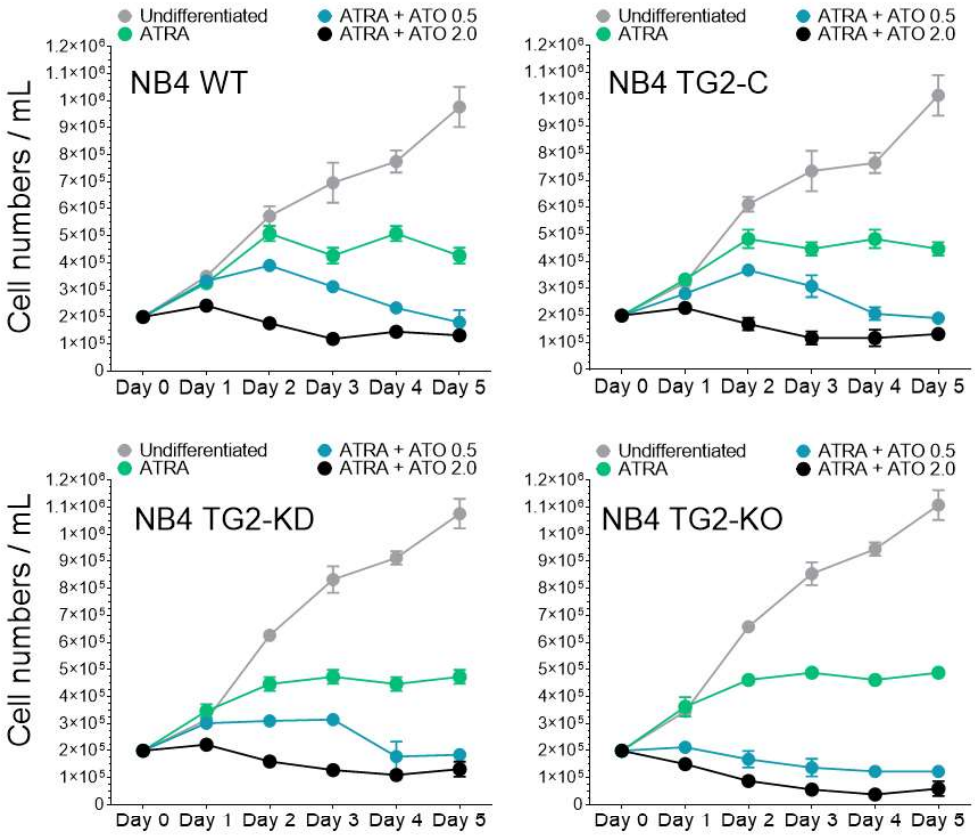


Figure 34. Cell division and proliferation of NB4 cells upon ATRA and ATRA + ATO treatment (not published yet). *NB4 cells were treated with 1 μM ATRA and with ATRA + ATO 0.5 μM, or with ATRA + ATO 2.0 μM for five days. KOVA Glasstic ® slide counted cEll numbers; each treatment/cell line was calculated from 5 parallel at the indicated time points (N=5). Graphs represent the mean cell number with SD.*

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 (**Figure 35**).

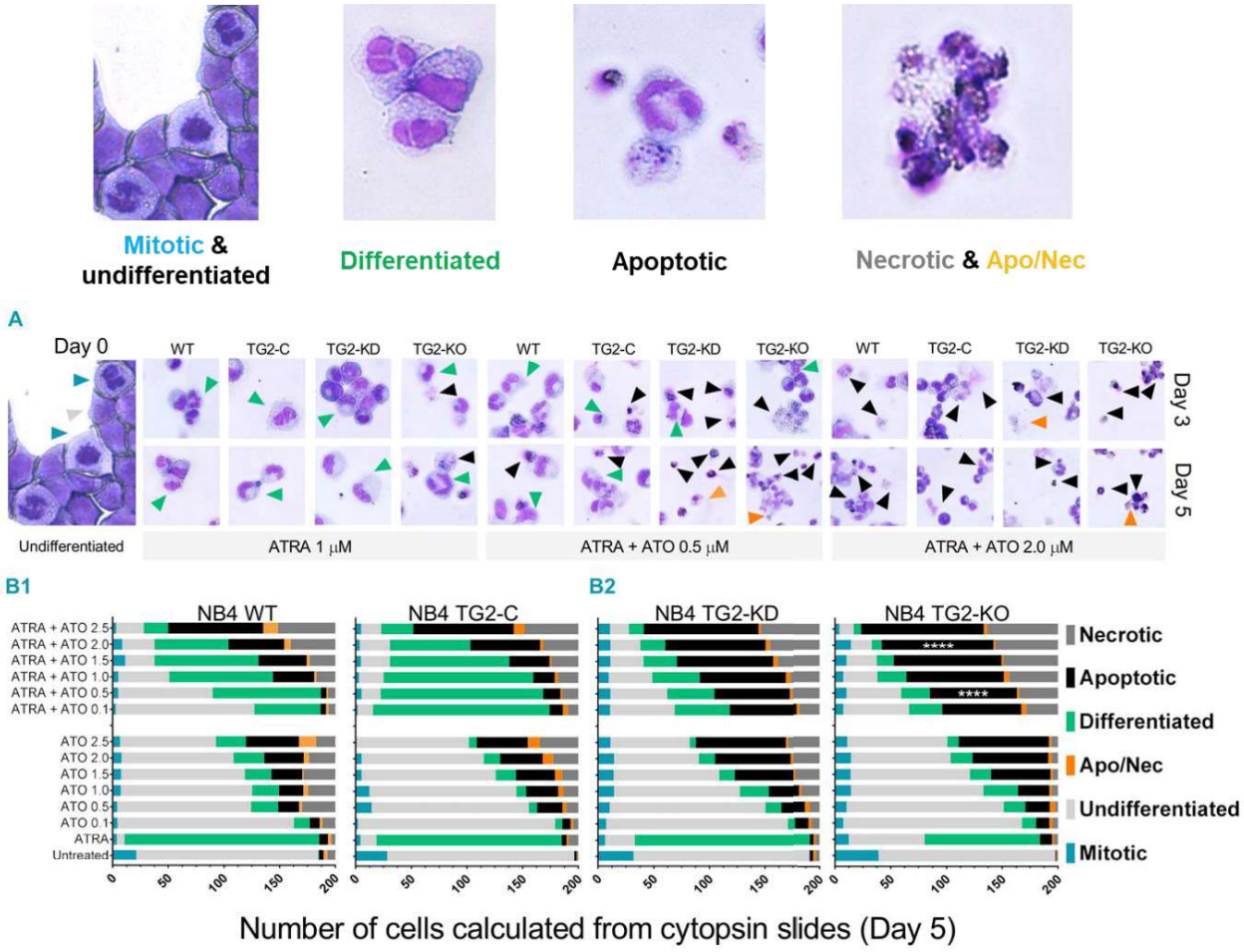


Figure 35. Tissue transglutaminase (TG2) expression alters arsenic-induced apoptosis. (A) *TG2 has a protective role against ATO-induced cell death, whereas its absence/lack results in a more sensitive response to the treatment. Representative images of May–Grünwald–Giemsa staining for the morphological evaluation of NB4 cell lines treated with 1 μM ATRA or with ATRA + ATO (0.5 or 2.0 μM) for three and five days (n = 5). The Cytospin pictures and documentation were obtained using the FLoid® Cell Imaging Station instrument (Life Technologies). Cell death features are marked with different triangles based on the colour code. (**B1, B2**) Quantification of May–Grünwald–Giemsa-stained Cytospin™ slides. From each Cytospin slide, 200 cells treated with ATRA or ATO for five days or in a combination thereof were counted from three different fields of view, were quantified based on cell death features listed and were marked with different colours at the right side of the panels. The graphs represent the mean values of the counted cells, where the black/grey/orange colours mark the cell death featuresStatistical significance was determined via two-way analysis of variance (TWO WAY ANOVA; Bonferroni post-hoc test; ATRA + ATO 2.0 WT: Apoptotic vs. ATRA + ATO 2.0 TG2-KO: Apoptotic **** p< 0,0001; ATRA + ATO 0.5 WT: Apoptotic vs. ATRA + ATO 0.5 KO: Apoptotic **** p< 0.0001).*

In the case of retinoic acid treatment, the cells mainly represent mitotic and differentiated cells (**Figure 35. blue and green triangles**). 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 (**Figure 35. B1 and B2**, lower panels, green and black bars). 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 (**Figure 35. B2, upper panel, black and dark grey columns**). ATO treatment alone results in partial differentiation and dose-dependent apoptosis (**Figure 35. B1, B2, lower panels)**.

4.8. 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 (**Figure 36**).

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 (**Figure 36. A1, A2, B1, B2, blue coloured bars**).

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 (**Figure 36. A3, A4, B3, B4**). The mRNA expression values of NCF2 and GP91PHOX were synchronised with the production of ROS, especially after the ATRA + ATO 2.0 μM treatment (**Figure 36. C1–C4**).

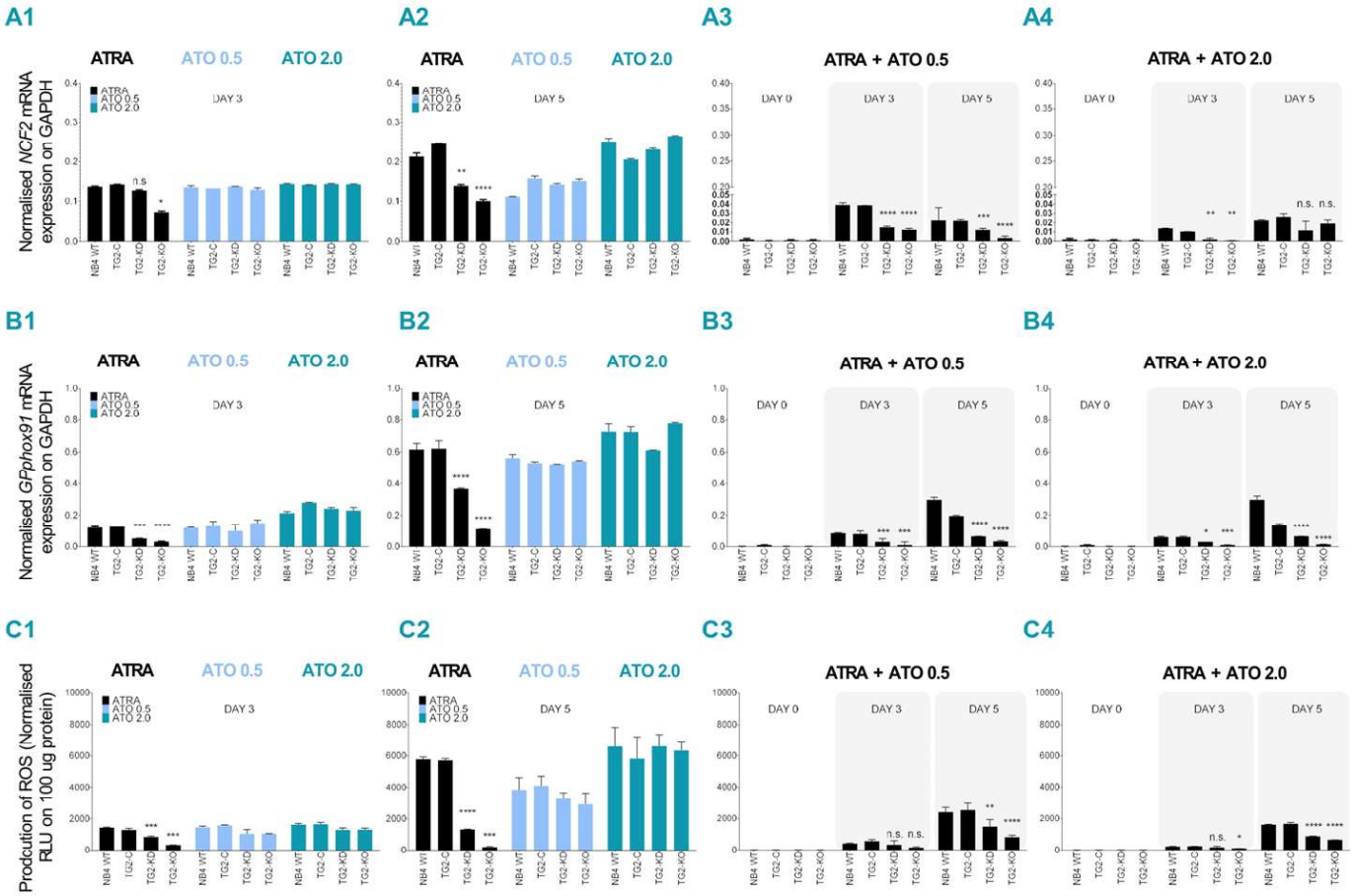


Figure 36. Combined ATRA + ATO treatment attenuates both expression of NCF2 and GP91PHOX respiratory burst oxidase genes and the production of reactive oxygen species. ***(A1–A4)*** *NB4 WT, Table. TG2-KD and TG2-KO cells were incubated with 1 μM ATRA, ATO (0.5 μM or 2.0 μM) and a combination of the two* ***(A3–A4)*** *for three* ***(A1)*** *and five days* ***(A2).*** *Relative mRNA expressions of NCF2/P67PHOX were measured on the indicated days by real-time Q-PCR and were normalised to GAPDH. The graph represents relative mRNA expression (means ± SD, n = 3).* ***(B1–B4)*** *NB4 cells were incubated with 1 μM ATRA, ATO (0.5 μM or 2.0 μM) and a combination of the two* ***(B3–B4)*** *for three* ***(B1)*** *and five days* ***(B2).*** *Relative mRNA expressions of GP91PHOX were measured on the indicated days by real-time Q-PCR and were normalised to GAPDH. The graph represents relative mRNA expression (means ± SD, n = 3).* ***(C1–C4)*** *NB4 cells were incubated with 1 μM ATRA, ATO (0.5 μM or 2.0 μM) and a combination of the two* ***(C3–C4)*** *for three* ***(C1)*** *and five days* ***(C2).*** *Production of ROS was determined for each cell line after the mentioned treatments, using a luminescence-based method in triplicate (n = 5), and was reported as RLUs. Graphs are the representation of mean ± SD values normalised to 100 μg protein of cell lysate content. Statistical significance was determined via two-way analysis of variance (ANOVA; Bonferroni post-hoc test; NB4 WT vs. TG2-KD, TG2-KO * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, **** $p < 0.0001$).*

4.9. 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 controls (**Figure 37. A1–A3, B1–B3, red bars**).

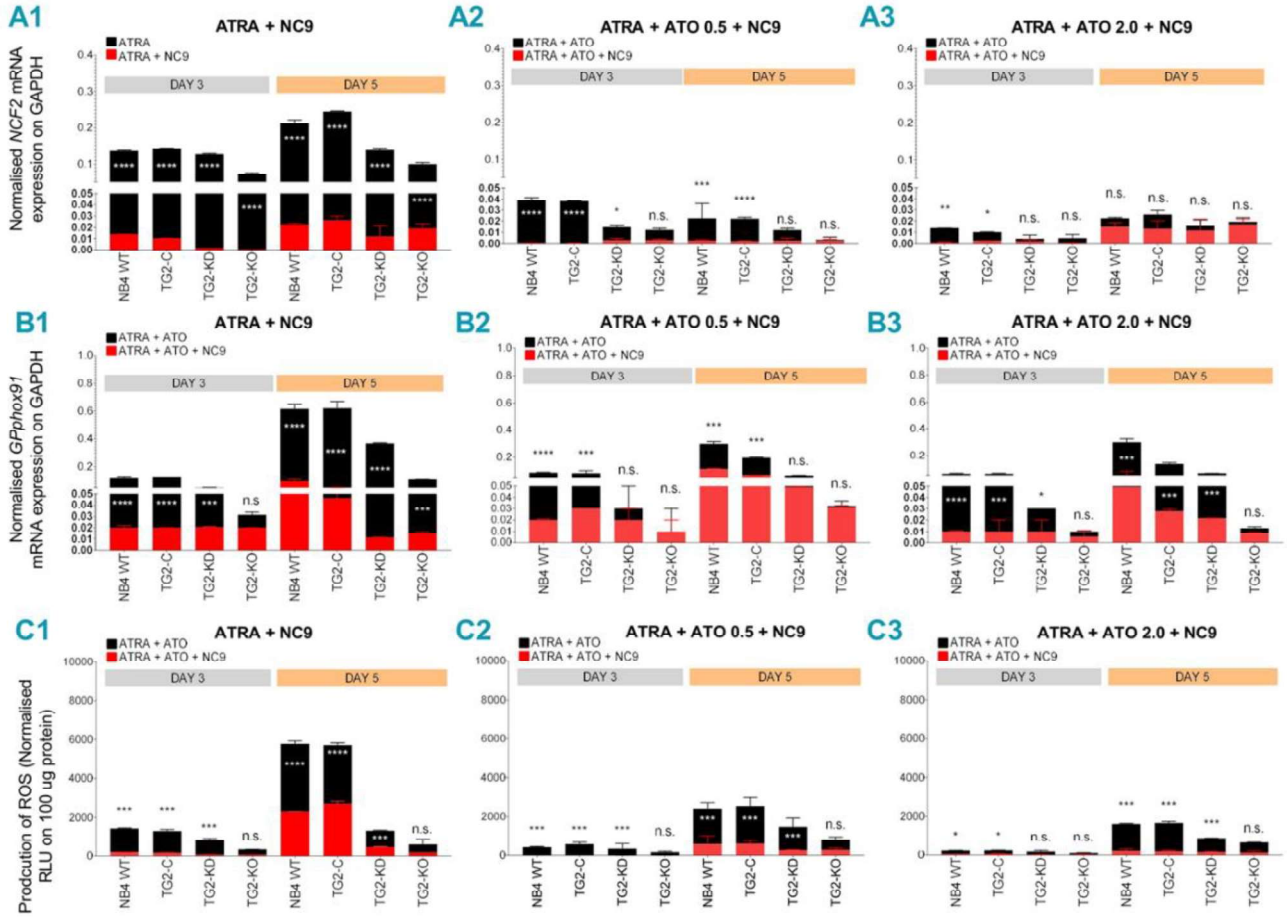


Figure 37. TG2 inhibitor NC9 diminishes the capability of ATRA + ATO-differentiated NB4 WT cell lines for ROS production. *(**A1–A3**) Relative mRNA expressions of NCF-2/P67PHOX and GP91PHOX (**B1–B3**) were measured in NB4 WT, TG2-C, TG2-KD and TG2-KO cells treated with ATRA + NC9, with ATRA + ATO 0.5 μM + NC9 and with ATRA + ATO 2.0 μM + NC9 at the indicated time points by Q-PCR, and they were normalised to GAPDH (black bars as control, without NC9). The mRNA expression levels from three independent experiments were measured in triplicate, and graphs show the representation of the mean ± SD values. (**C1–C3**) In the presence of 30 μM of the TG2 inhibitor NC9, production of ROS was determined for each cell line using a luminescence-based method in triplicate (n = 5) and reported in Relative Luminescence Units RLUs without NC9 (black bars as control). Graphs are the representation of mean ± SD values normalised to 100 μg protein of cell lysate content. Statistical significance was determined via two-way analysis of variance (ANOVA; Bonferroni and Tukey post-hoc test; ATRA vs. ATRA+NC9 * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, **** $p < 0.0001$).*

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 (**Figure 37. red bars**)

4.10. 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 (**Figure 38**).

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 (**Figure 38. middle part and right part of the panel**).

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 (**Figure 38. A1, B1, C1**).

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) (**Figure 38. right part of the panel**)

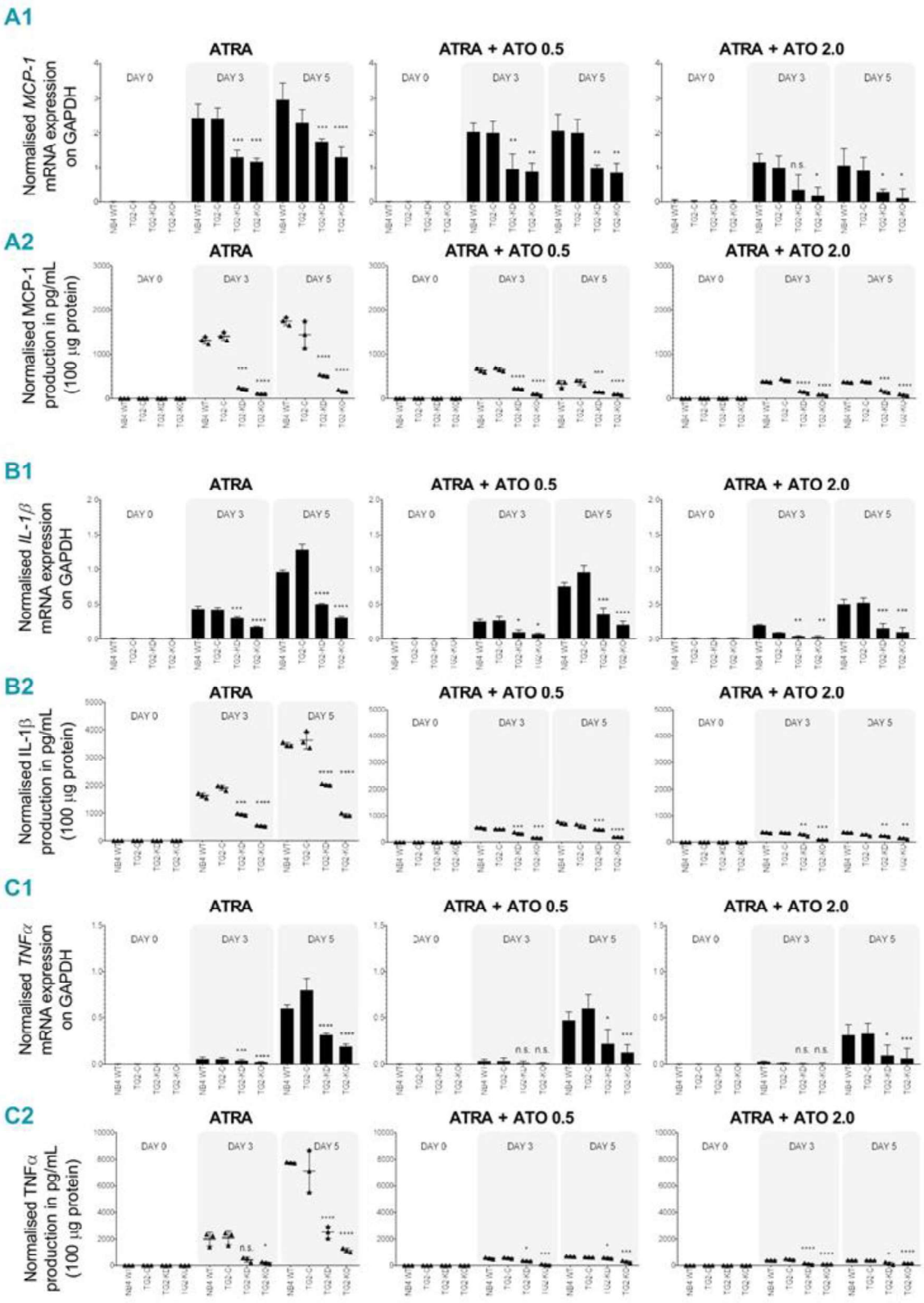


Figure 38. ATRA + ATO treatment can decrease the MCP-1, IL-1β and TNFα inflammatory biomarkers. *(**A1, B1, C1**) NB4 WT, TG2-C, TG2-KD and TG2-KO cells were incubated with 1 µM ATRA, ATO (0.5 µM or 2.0 µM) and a combination of the two for three and five days. Relative mRNA expressions of MCP-1 (**A1**), IL-1β (**B1**) and TNFα (**C1**) were measured on the indicated days by real-time Q-PCR and were normalised to GAPDH. The graph represents relative mRNA expression (mean values ± SD, n = 3). (**A2, B2, C2**) The protein content of the supernatants of NB4 cell lines was quantified by ELISA and was normalised to 100 µg protein content of NB4 cell lysates. The secreted protein levels of MCP-1 (**A2**), IL-1β (**B2**) and TNFα (**C2**) from three independent experiments were measured in triplicate and represented the mean ± SD values. Statistical significance was determined via two-way analysis of variance (ANOVA; Bonferroni post-hoc test; NB4 WT vs. TG2-KD, TG2-KO * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, **** $p < 0.0001$).*

4.11. 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) (**Figure 38**), we have checked how the irreversible TG2 inhibitor, NC9 affects differentiating NB4 WT cells upon ATRA + ATO treatments (**Figure 39**).

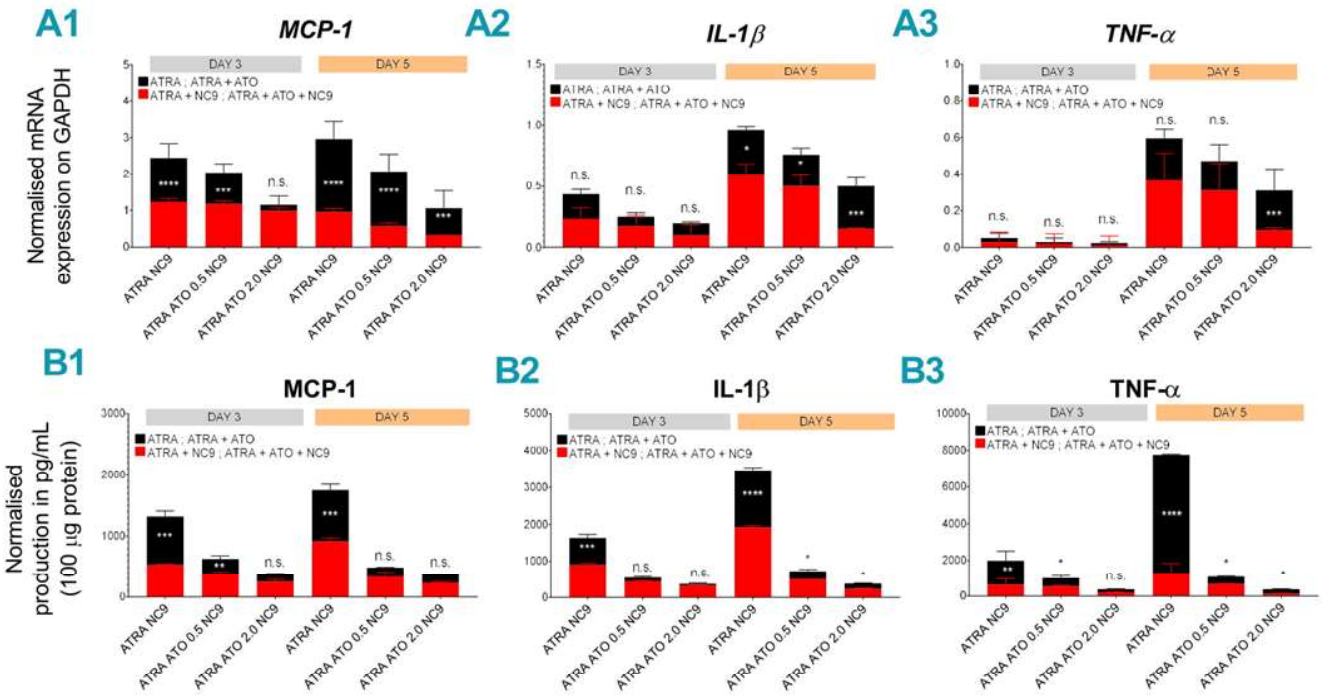


Figure 39. Inhibition of TG2 decreases the gene expression and the protein secretion of TNF-α, IL-1β and MCP-1 in differentiated NB4 cells. *Relative mRNA expressions of MCP-1 (**A1**), IL-1β (**A2**) and TNF-α (**A3**) were measured in ATRA and in ATRA + ATO (0.5 or 2.0 μM) in differentiated NB4 WT cell line with or without 30 μM NC9 (red bars) on the indicated days by Q-PCR and were normalised to GAPDH. The mRNA expression levels from three independent experiments were measured in triplicates, whereas the graphs show the representation of the mean ± SD values. The protein content of the supernatants of MCP-1 (**B1**), IL-1β (**B2**) and TNFα (**B3**) with or without 30 μM NC9 (red bars) was quantified by ELISA and was normalised to 100 μg protein content of NB4 WT cell lysates. The figures show secreted protein levels from three independent experiments measured in triplicate, and the graphs show the representation of the mean ± SD values. Statistical significance was determined via two-way analysis of variance (ANOVA; Bonferroni and Tukey post-hoc test; ATRA vs. ATRA+NC9 * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, **** $p < 0.0001$).*

After five days, we have found that the production of these inflammatory biomarkers significantly reduced the quantities of both mRNA and protein levels upon NC9 treatment, more effectively in the case of TNF-α, compared to results from a single ATRA treatment (**Figure 39. A1–A3, B1–B3**). These results also support our hypothesis, where the TG2 facilitate the NFkB pathway and the production of the inflammatory cytokines and chemokines, which have been evaluated previously in different chapters of this study.

5. 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) (**Figure 22**).

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 (**Figure 22**).

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 (**Figure 24**), 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 (**Figure 26-30**).

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 (**Figure 25, 30-31).**

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.

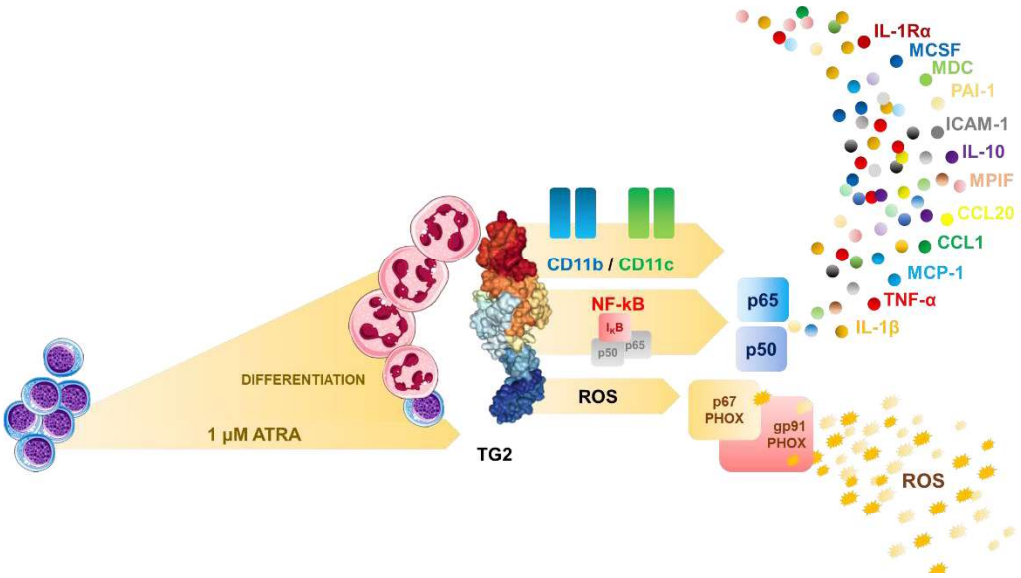


Figure 40. *TG2 facilitates the production of inflammatory cytokines/chemokines and expression of CD11 surface markers during ATRA induced differentiation in NB4 cells*

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 (**Figure 36**). 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 (**Figure 41**).

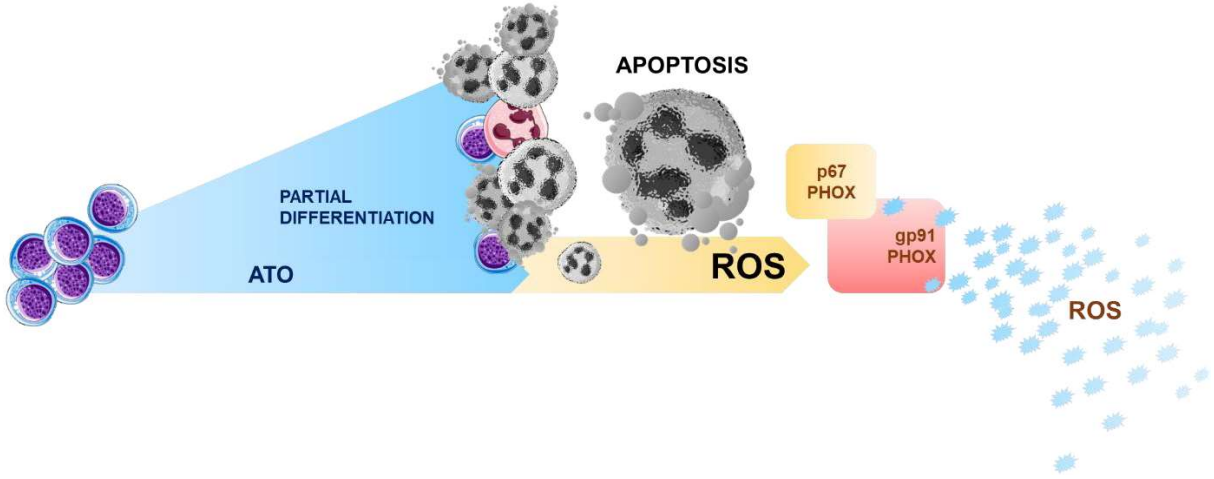


Figure 41. *ATO facilitates the production ROS via the NADPH-oxidase system, which could lead to the degradation of the mitochondrial membrane, followed by the apoptosis of the NB4 cells without induction of the TG2.*

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 (**Figure 35**). 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 (**Figure 35**).

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 (**Figure 35**). 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 (**Figure 36**).

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 (**Figure 42**).

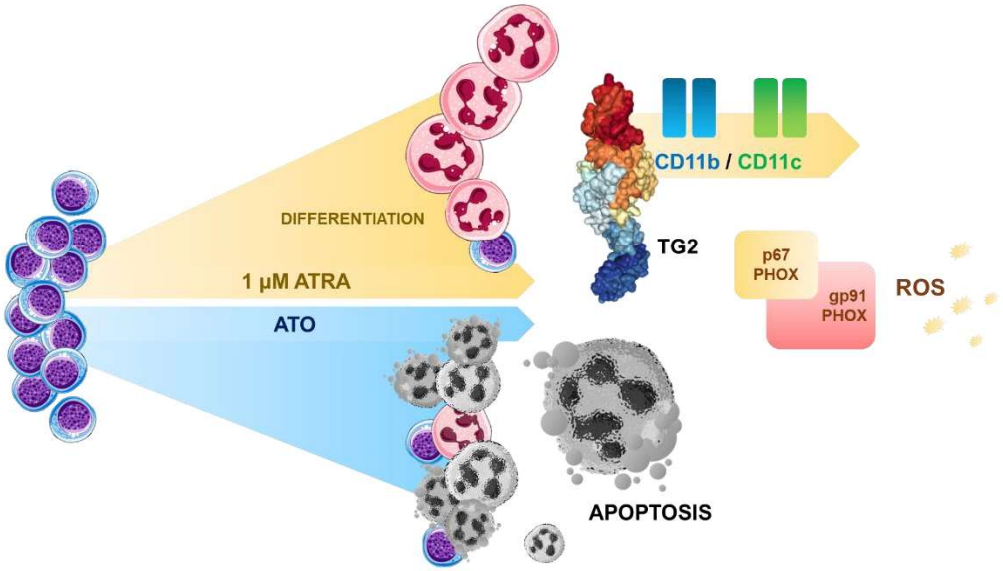


Figure 42. Combined treatment of NB4 cells with ATRA + ATO

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 alone (**Figure 38**).

The pro-inflammatory and inflammatory protein production was significantly lower in the decreased (TG2-KD) or in the absence of TG2 (TG2-KO) cases. 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 (**Figure 25**).

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 (**Figure 31-32**). 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 (**Figure 32**).

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 (**Figure 25**). As a result, the production of inflammatory cytokines/chemokines were inhibited (**Figure 24., 27-30**).

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, could demolish the expression and secretion of the three inflammatory markers and the production of ROS very effectively (**Figure 37, 39**).

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 (**Figure 43**).

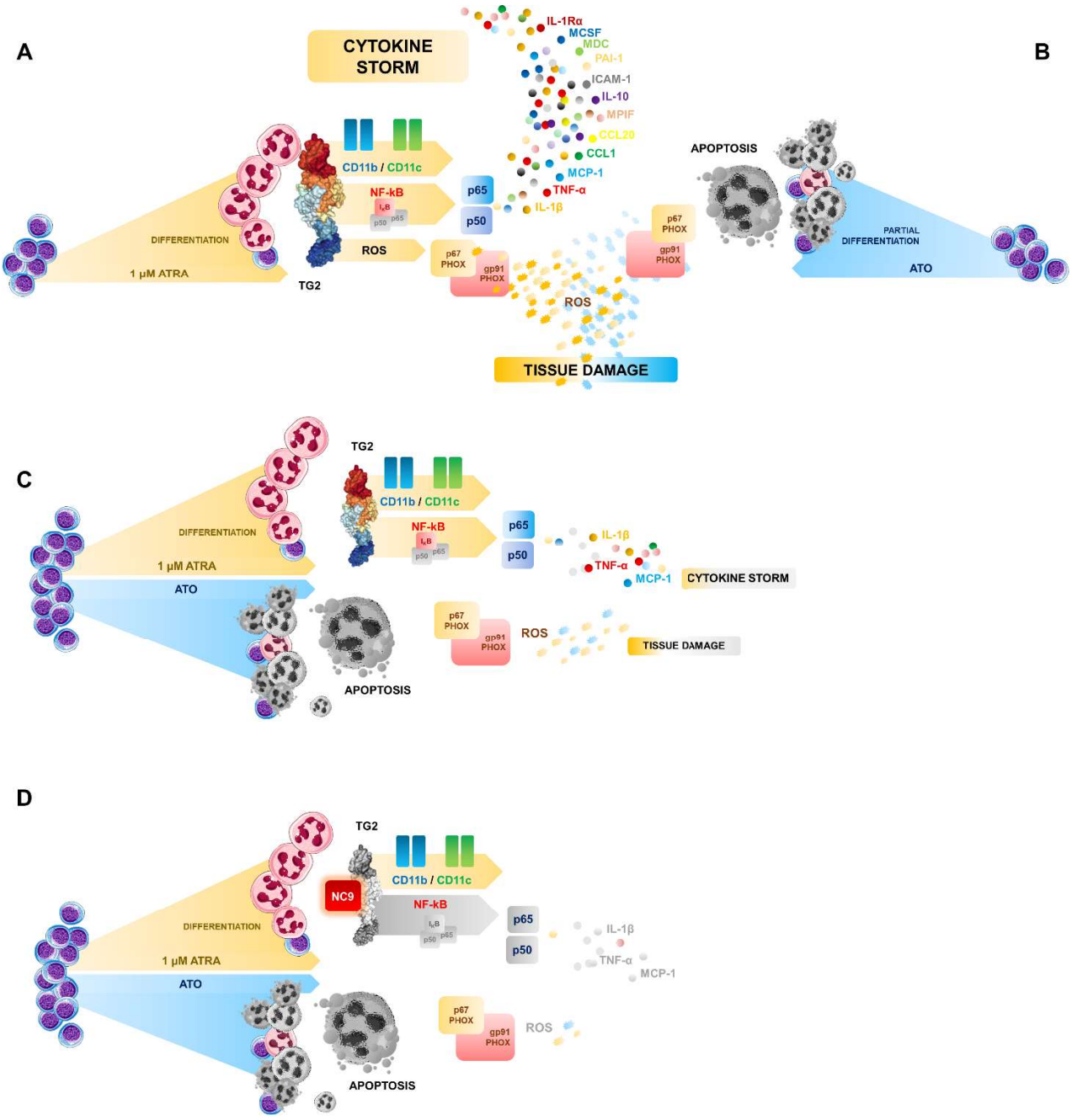


Figure 43. *Role of Tissue transglutaminase 2 (TG2) in the differentiation, death and cytokine production of all-trans retinoic acid (ATRA) and arsenic-trioxide (ATO) treated NB4 leukaemic cells (A) ATRA treatment (B) ATO treatment (C) ATRA+ ATO treatment (D) ATRA +ATO + NC9*

6. Summary

6.1. Morphological changes

As a result of the ATRA treatment, the NB4 cells undergo terminal differentiation towards neutrophil granulocytes, the shape of the nucleus changes, the size of the cytoplasm increases. ATO induces a partial differentiation and a cytotoxic effect in a time and concentration-dependent manner. In the presence of 2.0 μM ATO, the cell membrane is damaged, and the NB4 cells were showing apoptotic features in the early stages of the five days treatment. The ATRA induced TG2 expression in NB4 WT, and TG2-C cells reduced the number of apoptotic features, while the lack of tissue transglutaminase generated a significantly higher amount of dead cells in the case of TG2-KD and TG2-KO cell lines (**Figure 35**).

6.2. Changes in gene expression

Tissue transglutaminase (*TGM2*)

Upon ATRA treatment, the *TG2* mRNA expression was increased significantly in NB4 WT and TG2-C cell lines and partially in the case of the TG2-KD. The 11 days of ATRA treatment did not induce *TG2* mRNA expression in the TG2-KO cells. Since the NC9, an irreversible TG2 inhibitor, it did not influence the mRNA expression pattern of the TG2 (**Figure 21**).

NCF2/GP91PHOX

ATRA treatment alone induced high mRNA expression of the two key elements of the NADPH-oxidase system: *NCF2/p67PHOX* and *GP91PHOX* genes. The same results were detectable in the case of ATO treatment alone. ATRA induced TG2 facilitates the production of ROS via the NADPH-oxidase system in the NB4 WT and TG2-C cell line; however, the lack of TG2 generates a lack of mRNA expression regarding the two major components. Surprisingly the combined treatment of ATRA + ATO significantly decrease the mRNA expression of these genes and as a consequence the ROS production respectively. Applying the NC9, TG2 inhibitor, the ROS production was inhibited, which was further reduced if we used as ATRA +ATO +NC9 (**Figure 23, 36**).

Inflammatory cytokines/chemokine mRNA expression patterns

Alone the ATRA treatment induces high mRNA expression of the MCP-1, IL-1β and TNF-α genes. This phenomenon was not detectable in the case of single ATO treatment. The ATRA induced TG2 supports the NFkB pathway, which can be reduced by combined treatment of ATRA + ATO or in the case of ATRA +NC9 treatment. The decreased or lacking expression

of TG2 in NB4 TG2-KD, and TG2-KO cell lines resulted in lower mRNA expression and NF-κB pathway activity. The induction of the inflammatory cytokine/chemokine production can be further reduced using NC9 treatment in combination with ATRA or with ATRA + ATO (**Figure 24., 25., 38., 39.**).

6.3. Changes at the level of the proteins

CD11b/CD11c

The protein expression of CD11c/CD11b was detectable at the surface of NB4 cells, TG2 independently. All the cell lines express the activated form of the integrins, which is induced by ATRA treatment alone. However, the expression can not be further enhanced by the addition of phorbol myristate acetate (PMA) (**Figure 22**).

TG2

At the level of the mRNA expression, we saw that the ATRA treatment increases the TG2, which was detectable at protein levels, respectively. In the presence of NC9, the level of the TG2 was lower compared to the ATRA treatment ones, which shows a facilitated degradation of the tissue transglutaminase by NC9 (**Figure 33**).

NF-κB pathway

We have shown that the mRNA expression of inflammatory cytokines and chemokines were upregulated after ATRA treatment and can be reduced by ATRA + ATO; ATRA + ATO + NC9 or ATRA + NC9 treatments. The protein expression of the NFkB dimer: p65 and p50 were attenuated by irreversible TG2 inhibitor, which exhibits the supporting role of the TG2 in the case of the translocation of p65 into the nucleus, consequently the expression of inflammatory genes (**Figure 25., 27-29., 31-33.**).

Inflammatory cytokines/chemokine secretion

The secretion of the inflammatory cytokines and chemokines was determined by ELISA method, from which our results show that the ATRA treatment alone significantly increases the production of MCP-1, IL-1β and TNF-α, following parallel the TG2 expression. From the Human 200 cytokine profiler measurement, we could identify 17 cytokines which were TG2 and NF-κB pathway-dependent.

Production of ROS

After ATRA treatment alone via the NADPH-oxidase system is active, resulting in a high amount of ROS production in NB4 cells. The presence of TG2 facilitates the process, while

the lack of the TG2 generates less amount of ROS in NB4 TG2-KD and TG2-KO cells. ATO alone can enhance the production of the superoxide form as we described previously through the NADPH system, whereas the expression of the p67phox and gp91phox genes was significantly high. Combination treatments like ATRA + ATO, ATRA + NC9 or ATRA + ATO +NC9 could reduce/withdraw the production of the ROS in NB4 cell lines significantly (**Figure 36., 42.**).

6. Összefoglalás – Magyar nyelven

6.1. Morfológiai változások

Az ATRA kezelés eredményeként az NB4 sejtek terminálisan differenciálódnak a neutrofil granulocita irányba, megváltozik a sejtmag alakja, növekszik a citoplazma mérete. Az ATO részleges differenciálódást és citotoxikus hatást vált ki idő és koncentráció függvényében. 2.0 μM ATO jelenlétében a sejtmembrán megsérül, és az NB4 sejtek apoptotikus tulajdonságokat mutattak az öt napos kezelés korai szakaszában is. Az ATRA indukálta TG2 expresszió az NB4 WT-ben és a TG2-C sejtek esetében csökkentette az apoptotikus formák megjelenését/számát, míg a szöveti transzglutamináz hiánya a TG2-KD és a TG2-KO sejtvonalak esetében szignifikánsan nagyobb apoptótikus sejtet eredményezett (**35. ábra**).

6.2. A gén expressziójának változásai

Szöveti transzglutamináz (*TGM2*)

Az ATRA kezelés után a TG2 mRNS expressziója szignifikánsan megnövekedett az NB4 WT és TG2-C sejtvonalakban, kisebb mértékben a TG2-KD esetében. Az ATRA kezelés 11 napja nem indukálta a TG2 mRNS expresszióját a TG2-KO sejtekben. Az NC9, egy irreverzibilis TG2 inhibitor, mely nem befolyásolta a TG2 mRNS expressziós mintázatát (**21. ábra**).

NCF2/GP91PHOX

Az ATRA kezelés önmagában indukálta a NADPH-oxidáz rendszer két kulcsfontosságú elemének, az *NCF2/p67PHOX* és a *GP91PHOX* gének magas mRNS expresszióját. Ugyanezek az eredmények voltak kimutathatók egyedül az ATO-kezelés esetén. Az ATRA által indukált TG2 támogatja a ROS termelését a NADPH-oxidáz rendszer segítségével az NB4 WT és TG2-C sejtvonalban; azonban a TG2 hiánya a ROS termelésért felelős gének mRNS expressziójának hiányát okozza. Meglepő módon az ATRA + ATO kombinált kezelése szignifikánsan csökkenti ezen gének mRNS expresszióját, és ennek következtében a ROS termelést is. Az NC9 alkalmazásával gátoltuk a ROS-termelést, amely tovább csökkent, ha ATRA + ATO + NC9-ként használtuk (**23., 36. ábra).**

Gyulladásos citokinek/kemokin mRNS expressziós minták

Az ATRA kezelés önmagában indukálja az *MCP-1, IL-1β* és *TNF-α* gének magas mRNS expresszióját. Ez a jelenség nem volt kimutatható csupán ATO-kezelés esetén. Az ATRA által indukált TG2 támogatja az NF-κB útvonalat, amelyet csökkenthetünk az ATRA + ATO kombinált kezelésével vagy az ATRA + NC9 kezeléssel. A TG2 csökkent vagy hiányos expressziója az NB4 TG2-KD és TG2-KO sejtvonalakban alacsonyabb mRNS expressziót és NF-κB útvonal aktivitást eredményezett. A gyulladásos citokin / kemokin termelés indukcióját

tovább csökkenthetjük NC9 + ATRA-val vagy ATRA + ATO-val kombinálva (**24., 25., 38., 39. ábra).**

6.3. Változások a fehérjék szintjén

CD11b/CD11

A CD11c/CD11b fehérje expressziója kimutatható volt az NB4 sejtek felszínén, TG2 független módon. Az összes sejtvonal expresszálja az integrinek aktivált formáját, amelyet önmagában az ATRA kezelés indukál (**22. ábra**).

TG2

NC9 jelenlétében a TG2 szintje alacsonyabb volt, mint az ATRA kezeléssel, ami azt mutatja, hogy a szöveti transzglutamináz degradációjáért felelős lehet az NC9 kezelés (**33. ábra**).

NF-κB útvonal

A gyulladásos citokinek és kemokinek mRNS expressziója az ATRA kezelés után magas volt, és csökkenthető az ATRA + ATO-val; ATRA + ATO + NC9 vagy ATRA + NC9 kezelésekkel. Az NF-κB dimer fehérje expresszióját: a p65-et és a p50-et a TG2-inhibitor csökkentette, amely a TG2 kulcsfontosságú szerepét mutatja a magba történő transzlokáció során, következésképpen a gyulladásgének expressziójában (**25., 27-29., 31-33. ábra**).

Gyulladásos citokinek/kemokin szekréció

A gyulladásos citokinek és kemokinek szekrécióját ELISA módszerrel határoztuk meg, amelynek eredményei azt mutatják, hogy önmagában az ATRA kezelés szignifikánsan növeli az MCP-1, IL-1β és TNF-α termelését, TG2 expresszió függő módon. A Humán 200 citokinprofil méréséből 17 citokint tudtunk azonosítani, amelyek TG2 és NF-κB függőek voltak.

ROS termelés

Az ATRA kezelés után önmagában a NADPH-oxidáz rendszert aktívan tartja, ami nagy mennyiségű ROS termelést eredményez az NB4 sejtekben. A TG2 jelenléte segíti a folyamatot, míg a TG2 hiánya kevesebb mennyiségű ROS-t generál az NB4 TG2-KD és TG2-KO sejtekben. Az ATO önmagában fokozhatja a szuperoxid formák termelődését, amint azt korábban már a NADPH rendszeren keresztül leírtuk, míg a p67phox és gp91phox gének expressziója szignifikánsan magas volt. Az olyan kombinált kezelések, mint az ATRA + ATO, ATRA + NC9 vagy ATRA + ATO + NC9, jelentősen csökkenthetik az NB4 sejtvonalakban a ROS képződését (**36., 42. ábra**).

7. References

1. Abaza, Y.; Kantarjian, H.; Garcia-Manero, G.; Estey, E.; Borthakur, G.; Jabbour, E.; Faderl, S.; O'Brien, S.; Wierda, W.; Pierce, S. Long-term outcome of acute promyelocytic leukemia treated with all-trans-retinoic acid, arsenic trioxide, and gemtuzumab. Blood, 2017, 129, 1275–1283.
2. Ablain, J., & de The, H. Revisiting the differentiation paradigm in acute promyelocytic leukemia. Blood, The Journal of the American Society of Hematology, 2011, 117(22), 5795-5802.
3. Adhikary, G.; Grun, D.; Alexander, H.R.; Friedberg, J.S.; Xu, W.; Keillor, J.W.; Kandasamy, S.; Eckert, R.L. Transglutaminase is a mesothelioma cancer stem cell survival protein that is required for tumor formation. Oncotarget, 2018, 9, 34495.
4. Akbar, A.; McNeil, N.M.; Albert, M.R.; Ta, V.; Adhikary, G.; Bourgeois, K.; Eckert, R.L.; Keillor, J.W. Structure–activity relationships of potent, targeted covalent inhibitors that abolish both the transamidation and GTP binding activities of human tissue transglutaminase. J. Med. Chem. 2017, 60, 7910–7927.
5. Akimov, S. S., & Belkin, A. M. Cell surface tissue transglutaminase is involved in adhesion and migration of monocytic cells on fibronectin. Blood, 2001, 98(5), 1567-1576.
6. Arber, D. A. Realistic pathologic classification of acute myeloid leukemias. American journal of clinical pathology, 2001, 115(4), 552-560.
7. Bainton DF, Miller LJ, Kishimoto T, et al. Leukocyte adhesion receptors are stored in peroxidase-negative granules of human neutrophils. J Exp Med. 1987;166(6):1641-1653.
8. Balajthy, Z.; Csomós, K.; Vámosi, G.; Szántó, A.; Lanotte, M.; Fésüs, L. Tissue-transglutaminase contributes to neutrophil granulocyte differentiation and functions. Blood 2006, 108, 2045–2054.
9. Belkin, A. M., Akimov, S. S., Zaritskaya, L. S., Ratnikov, B. I., Deryugina, E. I., & Strongin, A. Y. Matrix-dependent proteolysis of surface transglutaminase by membrane-type metalloproteinase regulates cancer cell adhesion and locomotion. Journal of Biological Chemistry, 2001, 276(21), 18415-18422.
10. Bennet J. Proposed revised criteria for the classification of acute leukemia: A report from the French-American-British Cooperative Group. Ann Intern Med. 1985; 3:620-625.
11. Bennett J, Catovsky D, Daniel M, et al. A variant form of hypergranular promyelocytic leukemia (M3). Ann Intern Med. 1980; 92:261.
12. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the Classification of the Acute Leukaemias French-American-British (FAB) Co-operative Group. Br J Haematol. 1976; 33(4):451-458.
13. Borden, K. L. Pondering the puzzle of PML (promyelocytic leukemia) nuclear bodies: can we fit the pieces together using an RNA regulon?. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2008, 1783(11), 2145-2154.
14. Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood. 1997; 89(10):3503-3521.
15. Boston University Biology. 2018. Target Genes of NF-κB. [ONLINE] Available at: https://www.bu.edu/NF-κB /gene-resources/target-genes/. [last Accessed 15 May 2020]
16. Boukhenouna, S.; Wilson, M.A.; Bahmed, K.; Kosmider, B. Reactive oxygen species in chronic obstructive pulmonary disease. Oxid. Med. Cell. Longev. 2018, 5730395.
17. Breitman T, Collins SJ, Keene B. Terminal differentiation of human promyelocytic leukemic cells in primary culture in response to retinoic acid. Blood. 1981; 57(6):1000-1004.
18. Budillon, A.; Carbone, C.; Di Gennaro, E. Tissue transglutaminase: A new target to reverse cancer drug resistance. Amino Acids 2013, 44, 63–72.
19. Camacho, L. H., Soignet, S. L., Chanel, S., Ho, R., Heller, G., Scheinberg, D. A., ... & Warrell Jr, R. P. Leukocytosis and the retinoic acid syndrome in patients with acute promyelocytic leukemia treated with arsenic trioxide. Journal of clinical oncology, 18(13), 2000, 2620-2625.
20. Candi, E., Oddi, S., Terrinoni, A., Paradisi, A., Ranalli, M., Finazzi-Agró, A., & Melino, G. Transglutaminase 5 cross-links loricrin, involucrin, and small proline-rich proteins in vitro. Journal of Biological Chemistry, 2001, 276(37), 35014-35023.
21. Caron NS, Munsie LN, Keillor JW, et al. Using FLIM-FRET to measure conformational changes of transglutaminase type 2 in live cells. PloS One. 2012;7(8): e44159.
22. Chen, G. Q., Shi, X. G., Tang, W., Xiong, S. M., Zhu, J., Cai, X., ... & Liu, M. M. Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): I. As2O3 exerts dose-dependent dual effects on APL cells. Blood, The Journal of the American Society of Hematology, 1997, 89(9), 3345-3353.

23. Chen, S.-J.; Zhou, G.-B.; Zhang, X.-W.; Mao, J.-H.; de Thé, H.; Chen, Z. From an old remedy to a magic bullet: Molecular mechanisms underlying the therapeutic effects of arsenic in fighting leukemia. Blood 2011, 117, 6425–6437.
24. Cornic, M., Delva, L., Guidez, F., Balitrand, N., Degos, L., & Chomienne, C. Induction of retinoic acid-binding protein in normal and malignant human myeloid cells by retinoic acid in acute promyelocytic leukemia patients. Cancer research, 1992, 52(12), 3329-3334.
25. Csomós K., Német I., Fésüs L., Balajthy Z. Tissue transglutaminase contributes to the all trans retinoic acid–induced differentiation syndrome phenotype in the NB4 model of acute promyelocytic leukemia. Blood. 2010;116(19):3933-3943.
26. Csomós K., Kristóf E., Jakob B., Csomós I., Kovács G., Rotem O., Hodrea J., Bagoly Z., Muszbek L., Balajthy Z., Csősz É,. Fésüs L. Protein cross-linking by chlorinated polyamines and transglutamylation stabilizes neutrophil extracellular traps. Cell Death Dis., 2016 Aug 11;7(8):e2332
27. De Botton S., Dombret H., Sanz M., Miguel JS., Caillot D., Zittoun R., Gardembas M., Stamatoulas A., Condé E., Guerci A., Gardin C., Geiser K., Makhoul DC., Reman O., de la Serna J., Lefrere F., Chomienne C., Chastang C., Degos L., Fenaux P. Incidence, clinical features, and outcome of all trans-retinoic acid syndrome in 413 cases of newly diagnosed acute promyelocytic leukemia. The European APL Group. Blood. 1998 Oct 15;92(8):2712-8.
28. De Thé, H., Pandolfi, P. P., & Chen, Z. Acute promyelocytic leukemia: a paradigm for oncoprotein-targeted cure. Cancer Cell, 2017, 32(5), 552-560.
29. Detmers PA., Lo SK., Olsen-Egbert E., Walz A., Baggiolini M., Cohn ZA. Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. J Exp Med., 1990 Apr 1;171(4):1155-62.
30. Dos Santos, G. A., Kats, L., & Pandolfi, P. P. Synergy against PML-RARa: targeting transcription, proteolysis, differentiation, and self-renewal in acute promyelocytic leukemia. Journal of Experimental Medicine, 2013, 210(13), 2793-2802.
31. Downey, G. P., Gumbay, R. S., Doherty, D. E., LaBrecque, J. F., Henson, J. E., Henson, P. M., & Worthen, G. S. Enhancement of pulmonary inflammation by PGE2: evidence for a vasodilator effect. Journal of Applied Physiology, 1998 64(2), 728-741.
32. Dubbink, H. J., de Waal, L., van Haperen, R., Verkaik, N. S., Trapman, J., & Romijn, J. C. The human prostate-specific transglutaminase gene (TGM4): genomic organization, tissue-specific expression, and promoter characterization. Genomics, 1998, 51(3), 434-444.
33. Dubois, C., Schlageter, M. H., De Gentile, A., Balitrand, N., Toubert, M. E., Krawice, I., Degos, L. Modulation of IL-8, IL-1 beta, and G-CSF secretion by all-trans retinoic acid in acute promyelocytic leukemia. Leukemia, 1994, 8(10), 1750.
34. Duester, G. Retinoic acid synthesis and signaling during early organogenesis. Cell, 2008, 134(6), 921-931.
35. Eckert RL, Fisher ML, Grun D, et al. Transglutaminase is a tumor cell and cancer stem cell survival factor. Mol Carcinog. 2015;54(10):947-958.
36. Eckert RL., Sturniolo MT., Broome AM., Ruse M., Rorke EA "Transglutaminase function in epidermis". The Journal of Investigative Dermatology. 2005, 124 (3): 481–92.
37. Eckert, R.L.; Kaartinen, M.T.; Nurminskaya, M.; Belkin, A.M.; Colak, G.; Johnson, G.V.; Mehta, K. Transglutaminase regulation of cell function. Physiol. Rev. 2014, 94, 383–417.
38. Emi, N. Arsenic Trioxide: Clinical Pharmacology and Therapeutic Results. Chemotherapy for Leukemia, 2017, 221–238.
39. Estey, E.; Garcia-Manero, G.; Ferrajoli, A.; Faderl, S.; Verstovsek, S.; Jones, D.; Kantarjian, H. Use of all-trans retinoic acid plus arsenic trioxide as an alternative to chemotherapy in untreated acute promyelocytic leukemia. Blood 2006, 107, 3469–3473.
40. Falasca, L., Iadevaia, V., Ciccosanti, F., Melino, G., Serafino, A., & Piacentini, M. Transglutaminase type II is a key element in the regulation of the anti-inflammatory response elicited by apoptotic cell engulfment. The Journal of Immunology, 2005, 174(11), 7330-7340.
41. Fenaux P., Wang Z., Degos L. Treatment of acute promyelocytic leukemia by retinoids. Acute Promyelocytic Leukemia: Springer, 2007:101-128.
42. Ferrero-Miliani L., Nielsen OH., Andersen PS., Girardin SE.; Nielsen; Andersen; Girardin "Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation". Clin. Exp. Immunol., 2007, 147 (2): 227–35
43. Fesus L, Piacentini M. Transglutaminase 2: an enigmatic enzyme with diverse functions. Trends Biochem Sci. 2002;27(10):534-539.

44. Flombaum CD, Isaacs M., Reich L. Acute renal failure associated with the retinoic acid syndrome in acute promyelocytic leukemia. Am J Kidney Dis. 1996;27(1):134-137.
45. Frankel SR., Eardley A., Lauwers G. The “retinoic acid syndrome” in acute promyelocytic leukemia. Ann Intern Med. 1992;117(4):292-296.
46. Gallagher R, Collins S, Trujillo J, McCredie K, Ahearn M, Tsai S, Metzgar R, Aulakh G, Ting R, Ruscetti F, Gallo R. Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. Blood. 1979; 54(3):713-33.
47. Goldman, J. M. Acute promyelocytic leukaemia. Br Med J, 1974, 1(5904), 380-382.
48. Grenard, P., Bates, M. K., & Aeschlimann, D. Evolution of Transglutaminase Genes: Identification of a Transglutaminase Gene Cluster on Human Chromosome 15q15 structure of the gene encoding transglutaminase x and a novel gene family member, transglutaminase Z. Journal of Biological Chemistry, 2001, 276(35), 33066-33078.
49. Grignani F., Ferrucci PF., Testa U. The acute promyelocytic leukemia-specific PML-RARα fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. Cell. 1993;74(3):423-431.
50. Grignani, F.; Testa, U.; Rogaia, D.; Ferrucci, P.F.; Samoggia, P.; Pinto, A.; Aldinucci, D.; Gelmetti, V.; Fagioli, M.; Alcalay, M. Effects on differentiation by the promyelocytic leukemia PML/RARalpha protein depend on the fusion of the PML protein dimerization and RARalpha DNA binding domains. EMBO J. 1996, 15, 4949–4958.
51. Guidez, F., Ivins, S., Zhu, J., Söderström, M., Waxman, S., & Zelent, A. Reduced retinoic acid-sensitivities of nuclear receptor corepressor binding to PML-and PLZF-RARα underlie molecular pathogenesis and treatment of acute promyelocytic leukemia. Blood, The Journal of the American Society of Hematology, 1998, 91(8), 2634-2642.
52. Gundemir, S., Colak, G., Tucholski, J., & Johnson, G. V. Transglutaminase 2: a molecular Swiss army knife. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2012, 1823(2), 406-419.
53. Hang, J., Zemskov, E. A., Lorand, L., & Belkin, A. M. Identification of a novel recognition sequence for fibronectin within the NH2-terminal β-sandwich domain of tissue transglutaminase. Journal of Biological Chemistry, 2005, 280(25), 23675-23683.
54. Harada A, Sekido N, Akahoshi T. Essential involvement of interleukin-8 (IL-8) in acute inflammation. J Leukoc Biol. 1994;56(5):559-564.
55. Hietikko, M., Koskinen, O., Kurppa, K. Small-intestinal TG2-specific plasma cells at different stages of coeliac disease. BMC Immunol, 2018, 19, 36.
56. Hodrea, J., Demény, M. Á., Majai, G., Sarang, Z., Korponay-Szabó, I. R., & Fésüs, L.. Transglutaminase 2 is expressed and active on the surface of human monocyte-derived dendritic cells and macrophages. Immunology letters, 2010, 130(1-2), 74-81.
57. Hsieh, Y. F., Liu, G. Y., Lee, Y. J., Yang, J. J., Sandor, K., Sarang, Z., Tsay, G. J. Transglutaminase 2 contributes to apoptosis induction in Jurkat T cells by modulating Ca2+ homeostasis via cross-linking RAP1GDS1. PLoS One, 2013, 8(12), e81516.
58. Huynh, T.T.; Sultan, M.; Vidovic, D.; Dean, C.A.; Cruickshank, B.M.; Lee, K.; Loung, C.-Y.; Holloway, R.W.; Hoskin, D.W.; Waisman, D.M. Retinoic acid and arsenic trioxide induce lasting differentiation and demethylation of target genes in APL cells. Sci. Rep. 2019, 9, 1–13.
59. Jeanne, M., Lallemand-Breitenbach, V., Ferhi, O., Koken, M., Le Bras, M., Duffort, S., & de Thé, H. PML/RARA oxidation and arsenic binding initiate the antileukemia response of As2O3. Cancer cell, 2013, 18(1), 88-98.
60. Jensen, K., Shiels, C., & Freemont, P. S. PML protein isoforms and the RBCC/TRIM motif. Oncogene, 2001, 20(49), 7223-7233.
61. Jing, Y., Dai, J., Chalmers-Redman, R. M., Tatton, W. G., & Waxman, S. Arsenic trioxide selectively induces acute promyelocytic leukemia cell apoptosis via a hydrogen peroxide-dependent pathway. Blood, The Journal of the American Society of Hematology, 1999, 94(6), 2102-2111.
62. Johnson, T. S., Griffin, M., Thomas, G. L., Skill, J., Cox, A., Yang, B., El Nahas, A. M. The role of transglutaminase in the rat subtotal nephrectomy model of renal fibrosis. The Journal of clinical investigation, 1997, 99(12), 2950-2960.
63. Kakizuka, W.H. Miller, K. Umesono, R.P. Warrell, S.R. Frankel, V.V.V.S. Murty, E. Dmitrovsky, R.M. Evans, Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RARα with a novel putative transcription factor, PML, Cell, 1991, Volume 66, Issue 4, Pages 663-674
64. Kalinin, A. E., Kajava, A. V., & Steinert, P. M. Epithelial barrier function: assembly and structural features of the cornified cell envelope. Bioessays, 2002, 24(9), 789-800.

65. Kanchan K, Fuxreiter M, Fésüs L. "Physiological, pathological, and structural implications of non-enzymatic protein-protein interactions of the multifunctional human transglutaminase 2". Cellular and Molecular Life Sciences. 2015, 72 (16): 3009–35
66. Keillor JW, Chica RA, Chabot N. The bioorganic chemistry of transglutaminase—from mechanism to inhibition and engineering. Can J Chem. 2008; 86(4):271-276.
67. Kerr C, Szmacinski H, Fisher ML Transamidase site-targeted agents alter the conformation of the transglutaminase cancer stem cell survival protein to reduce GTP binding activity and cancer stem cell survival. Oncogene. 2017; 36(21):2981-2990.
68. Kim, D. S., Park, K. S., & Kim, S. Y. Silencing of TGase 2 sensitizes breast cancer cells to apoptosis by regulation of survival factors. Front Biosci, 2009, 14, 2514-2521.
69. Kim, H.-J.; Bae, S.-C. Histone deacetylase inhibitors: Molecular mechanisms of action and clinical trials as anti-cancer drugs. Am. J. Transl. Res. 2011, 3, 166.
70. Kogan SC, Hong S-h, Shultz DB, Leukemia initiated by PMLRARα: the PML domain plays a critical role while retinoic acid–mediated transactivation is dispensable. Blood. 2000;95(5):1541-1550.
71. Kojima, S., Nara, K., & Rifkin, D. B. Requirement for transglutaminase in the activation of latent transforming growth factor-beta in bovine endothelial cells. The Journal of cell biology, 1993, 121(2), 439-448.
72. Koyama, T.; Hirosawa, S.; Kawamata, N.; Tohda, S.; Aoki, N. All-trans retinoic acid upregulates thrombomodulin and downregulates tissue-factor expression in acute promyelocytic leukemia cells: Distinct expression of thrombomodulin and tissue factor in human leukemic cells. Blood, 1994, 84, 3001–3009.
73. Kühnl A, Grimwade D. Molecular markers in acute myeloid leukaemia. Int J Hematol. 2012;96(2):153-163.
74. Kumar S, Mehta K. Tissue transglutaminase constitutively activates HIF-1α promoter and nuclear factor-κB via a non-canonical pathway. PloS One. 2012;7(11):e49321.
75. Kumar S., Mehta K. Tissue transglutaminase, inflammation, and cancer: How intimate is the relationship? Amino Acids. 2013;44:81–88.
76. Kumar, Rukmini; Clermont, Gilles; Vodovotz, Yoram; Chow, Carson C. "The dynamics of acute inflammation". Journal of Theoretical Biology, 2004, 230 (2): 145–155.
77. Kuo, T. F., Tatsukawa, H., & Kojima, S. New insights into the functions and localization of nuclear transglutaminase 2. The FEBS journal, 2011, 278(24), 4756-4767.
78. Kurokawa, R., DiRenzo, J., Boehm, M., Sugarman, J., Gloss, B., Rosenfeld, M. G., ... & Glass, C. K. Regulation of retinoid signalling by receptor polarity and allosteric control of ligand binding. Nature, 1994, 371(6497), 528-531.
79. Lanotte M, Martin-Thouvenin V, Najman S, et al. NB4, a maturation inducible cell line with t (15; 17) marker isolated from a human acute promyelocytic leukemia (M3). Blood. 1991;77(5):1080-1086.
80. Larsen GL, Henson PM. "Mediators of inflammation". Annu. Rev. Immunol. 1983, 1: 335–59.
81. Larson, R.S.; Tallman, M.S. Retinoic acid syndrome: Manifestations, pathogenesis, and treatment. Best Pract. Res. Clin. Haematol. 2003, 16, 453–461.
82. Liang JX, Liang Y, Gao W. Clinicopathological and prognostic significance of sialyl Lewis X overexpression in patients with cancer: a meta-analysis. Onco Targets Ther. 2016; 9:3113-3125.
83. Lin, R. J., & Evans, R. M. Acquisition of oncogenic potential by RAR chimeras in acute promyelocytic leukemia through formation of homodimers. Molecular cell, 2000, 5(5), 821-830.
84. Liu, P., & Han, Z. C. Treatment of acute promyelocytic leukemia and other hematologic malignancies with arsenic trioxide: review of clinical and basic studies. International journal of hematology, 2003, 78(1), 32-39.
85. Loike JD, Sodeik B, Cao L, et al. CD11c/CD18 on neutrophils recognizes a domain at the N terminus of the A alpha chain of fibrinogen. Proc Natl Acad Sci. 1991;88(3):1044-1048.
86. Lorand L, Graham RM. Transglutaminases: crosslinking enzymes with pleiotropic functions. Nat Rev Mol Cell Biol. 2003;4(2):140-156.
87. Luesink, M.; Pennings, J.L.; Wissink, W.M.; Linssen, P.C.; Muus, P.; Pfundt, R.; de Witte, T.J.; van der Reijden, B.A.; Jansen, J.H. Chemokine induction by all-trans retinoic acid and arsenic trioxide in acute promyelocytic leukemia: Triggering the differentiation syndrome. Blood 2009, 114, 5512–5521.
88. Mangala, L. S., Fok, J. Y., Zorrilla-Calancha, I. R., Verma, A., & Mehta, K. Tissue transglutaminase expression promotes cell attachment, invasion and survival in breast cancer cells. Oncogene, 2007, 26(17), 2459-2470.
89. Martucciello, S., Paolella, G., Esposito, C., Lepretti, M., & Caputo, I. Anti-type 2 transglutaminase antibodies as modulators of type 2 transglutaminase functions: a possible pathological role in celiac disease. Cellular and Molecular Life Sciences, 2018, 75(22), 4107-4124.

90. Marzari, R., Sblattero, D., Florian, F., Tongiorgi, E., Not, T., Tommasini, A., Bradbury, A. Molecular dissection of the tissue transglutaminase autoantibody response in celiac disease. The Journal of Immunology, 2001, 166(6), 4170-4176.
91. Mehta, K.; Kumar, A.; Im Kim, H. Transglutaminase 2: A multi-tasking protein in the complex circuitry of inflammation and cancer. Biochem. Pharmacol. 2010, 80, 1921–1929.
92. Martin, A., De Vivo, G., & Gentile, V. Possible role of the transglutaminases in the pathogenesis of Alzheimer's disease and other neurodegenerative diseases. International journal of Alzheimer's disease, 2011, 865432. https://doi.org/10.4061/2011/865432
93. Mitrovic M, Suvajdzic N, Elezovic I. Thrombotic events in acute promyelocytic leukemia. Thromb Res. 2015; 135(4):588-593.
94. Montesinos, P.; Bergua, J.M.; Vellenga, E.; Rayón, C.; Parody, R.; de la Serna, J.; León, A.; Esteve, J.; Milone, G.; Debén, G. Differentiation syndrome in patients with acute promyelocytic leukemia treated with all-trans retinoic acid and anthracycline chemotherapy: Characteristics, outcome, and prognostic factors. Blood, 2009, 113, 775–783.
95. Morré, D. J., & Brightman, A. O. NADH oxidase of plasma membranes. Journal of bioenergetics and biomembranes, 1991, 23(3), 469-489.
96. Muszbek L., Bereczky Z., Bagoly Z., Komáromi I., Katona É. "Factor XIII: a coagulation factor with multiple plasmatic and cellular functions". Physiological Reviews. 2001, 91 (3): 931–72.
97. Nagy, L., Kao, H. Y., Chakravarti, D., Lin, R. J., Hassig, C. A., Ayer, D. E., ... & Evans, R. M. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell, 1997, 89(3), 373-380.
98. Ninomiya, M., Kiyoi, H., Ito, M., Hirose, Y., & Naoe, T. Retinoic acid syndrome in NOD/scid mice induced by injecting an acute promyelocytic leukemia cell line. Leukemia, 2004, 18(3), 442-448.
99. Niu, C.; Yan, H.; Yu, T.; Sun, H.-P.; Liu, J.-X.; Li, X.-S.; Wu, W.; Zhang, F.-Q.; Chen, Y.; Zhou, L. Studies on treatment of acute promyelocytic leukemia with arsenic trioxide: Remission induction, follow-up, and molecular monitoring in 11 newly diagnosed and 47 relapsed acute promyelocytic leukemia patients. Blood 1999, 94, 3315–3324.
100. Nupponen I, Andersson S, Järvenpää A-L. Neutrophil CD11b expression and circulating interleukin-8 as diagnostic markers for early-onset neonatal sepsis. Pediatrics. 2001;108(1): E12.
101. Nurminskaya, M.V.; Belkin, A.M. Cellular functions of tissue transglutaminase. Int. Rev. Cell Mol. Biol. 2012, 294, 1–97.
102. Park, K. S., Kim, D. S., Jeong, K. C., & Kim, S. Y. Increase in transglutaminase 2 expression is associated with NF-kappaB activation in breast cancer tissues. Front Biosci, 2009, 14, 1945-1951.
103. Park, K.-S.; Kim, H.-K.; Lee, J.-H.; Choi, Y.-B.; Park, S.-Y.; Yang, S.-H.; Kim, S.-Y.; Hong, K.-M. Transglutaminase 2 as a cisplatin resistance marker in non-small cell lung cancer. J. Cancer Res. Clin. Oncol. 2010, 136, 493–502.
104. Patatanian E, Thompson D. Retinoic acid syndrome: a review. J Clin Pharm Ther. 2008; 33(4):331-338.
105. Peng, X., Zhang, Y., Zhang, H., Graner, S., Williams, J. F., Levitt, M. L., & Lokshin, A Interaction of tissue transglutaminase with nuclear transport protein importin-α3. FEBS letters, 1999, 446(1), 35-39.
106. Piacentini, M., Amendola, A., Ciccosanti, F., Falasca, L., Farrace, M. G., Mastroberardino, P. G., Autuori, F. Type 2 transglutaminase and cell death. Progress in experimental tumor research, 2005, 38, 58-74.
107. Powell, B. L. Acute promyelocytic leukemia: progress far and wide. Blood, The Journal of the American Society of Hematology, 2013, 121(11), 1925-1926.
108. Raelson JV., Nervi C., Rosenauer A. The PML/RAR alpha oncoprotein is a direct molecular target of retinoic acid in acute promyelocytic leukemia cells. Blood, 1996; 88(8):2826-2832.
109. Rego, E. M., He, L. Z., Warrell, R. P., Wang, Z. G., Pandolfi, P. P. Retinoic acid (RA) and As2O3 treatment in transgenic models of acute promyelocytic leukemia (APL) unravel the distinct nature of the leukemogenic process induced by the PML-RARα and PLZF-RARα oncoproteins. Proceedings of the National Academy of Sciences, 2000, 97(18), 10173-10178.
110. Rodolfo, C., Mormone, E., Matarrese, P., Ciccosanti, F., Farrace, M. G., Garofano, E., Piacentini, M. Tissue transglutaminase is a multifunctional BH3-only protein. Journal of Biological Chemistry, 2004, 279(52), 54783-54792.
111. Rosenthal, A. K., Gohr, C. M., Henry, L. A., & Le, M. Participation of transglutaminase in the activation of latent transforming growth factor β1 in aging articular cartilage. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology, , 2000, 43(8), 1729-1733.

112. Rowley, J., Golomb, H., & Dougherty, C. 15/17 translocation, a consistent chromosomal change in acute promyelocytic leukaemia, 1997, Lancet (London, England), 1(8010), 549-550.
113. Sadhu C, Hendrickson L, Dick KO Novel Tools for Functional Analysis of CD11c: Activation-Specific, Activation-Independent, and Activating Antibodies. J Immunoassay Immunochem. 2007;29(1):42-57.
114. Sanz, M. A., Martín, G., González, M., León, A., Rayón, C., Rivas, C., De La Serna, J. . Risk-adapted treatment of acute promyelocytic leukemia with all-trans-retinoic acid and anthracycline monochemotherapy: a multicenter study by the PETHEMA group. Blood, 2004, 103(4), 1237-1243.
115. Schröder, K. NADPH oxidase-derived reactive oxygen species: Dosis facit venenum. Exp. Physiol. 2019, 104, 447–452.
116. Seale, J., Delva, L., Renesto, P., Balitrand, N., Dombret, H., Scrobohaci, M. L., Chomienne, C. All-trans retinoic acid rapidly decreases cathepsin G synthesis and mRNA expression in acute promyelocytic leukemia. Leukemia, 1996, 10(1), 95-101.
117. Shibakura M, Niiya K, Niiya M, et al. Induction of CXC and CC chemokines by all trans retinoic acid in acute promyelocytic leukemia cells. Leuk Res. 2005; 29(7):755-759.
118. Stewart M, Thiel M, Hogg N. "Leukocyte integrins". Curr. Opin. Cell Biol. 1996, 7 (5): 690–6.
119. Sumi, D.; Shinkai, Y.; Kumagai, Y. Signal transduction pathways and transcription factors triggered by arsenic trioxide in leukemia cells. Toxicol. Appl. Pharmacol. 2010, 244, 385–392.
120. Sumimoto, H. Structure, regulation and evolution of Nox-family NADPH oxidases that produce reactive oxygen species. The FEBS journal, 2008, 275(13), 3249-3277.
121. Szondy, Z., Korponay-Szabó, I., Király, R., Sarang, Z., & Tsay, G. J. Transglutaminase 2 in human diseases. BioMedicine, 2017, 7(3).
122. Tabolacci, C., De Martino, A., Mischiati, C., Feriotto, G., & Beninati, S. The Role of Tissue Transglutaminase in Cancer Cell Initiation, Survival and Progression. Medical sciences (Basel, Switzerland), 2019, 7(2), 19.
123. Takami M1, Terry V, Petruzzelli L. Signaling pathways involved in IL-8-dependent activation of adhesion through Mac-1. J Immunol. 2002;168(9):4559-4566.
124. Tallman MS, Andersen JW, Schiffer CA, et al. Clinical description of 44 patients with acute promyelocytic leukemia who developed the retinoic acid syndrome. Blood. 2000;95(1):90-95.
125. Tallmann, M. S. Curative therapeutic approaches to APL. Annals of hematology, 2004, 83, S81-2.
126. Tang L, Chai W, Ye F, et al. HMGB1 promotes differentiation syndrome by inducing hyperinflammation via MEK/ERK signaling in acute promyelocytic leukemia cells. Oncotarget. 2017;8(16):27314-27327.
127. Tatsukawa, H.; Furutani, Y.; Hitomi, K.; Kojima, S. Transglutaminase 2 has opposing roles in the regulation of cellular functions as well as cell growth and death. Cell Death Dis. 2016, 7, e2244.
128. Thangaraju, K., Biri, B., Schlosser, G., Kiss, B., Nyitray, L., Fésüs, L., & Király, R. Real-time kinetic method to monitor isopeptidase activity of transglutaminase 2 on protein substrate. Analytical Biochemistry, 2016, 505, 36-42.
129. Tsai W-H, Hsu H-C, Lin C-C et, al. Role of interleukin-8 and growth-regulated oncogene-α in the chemotactic migration of all-trans retinoic acid-treated promyelocytic leukemic cells toward alveolar epithelial cells. Crit Care Med. 2007;35(3):879-885.
130. Vardiman, J. W., Harris, N. L., & Brunning, R. D. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood, 2002, 100(7), 2292-2302.
131. vDubbink HJ., de Waal L., van Haperen R. "The human prostate-specific transglutaminase gene (TGM4): genomic organization, tissue-specific expression, and promoter characterization". Genomics, 1994, 51 (3): 434–44.
132. Wang, Z.-Y.; Chen, Z. Acute promyelocytic leukemia: From highly fatal to highly curable. Blood, 2008, 111, 2505–2515.
133. Wiernik PH., Gallagher RE., Tallman MS. Acute promyelocytic leukemia. Neoplastic Diseases of the Blood: Springer, 2013:403-453.
134. Yoshida H., Kitamura K., Tanaka K. Accelerated degradation of PML-retinoic acid receptor α (PML-RARA) oncoprotein by all-trans-retinoic acid in acute promyelocytic leukemia: possible role of the proteasome pathway. Cancer Res. 1996; 56(13):2945-2948.
135. Zhang, X.-W.; Yan, X.-J.; Zhou, Z.-R.; Yang, F.-F.; Wu, Z.-Y.; Sun, H.-B.; Liang, W.-X.; Song, A.-X.; Lallemand-Breitenbach, V.; Jeanne, M. Arsenic trioxide controls the fate of the PML-RARα oncoprotein by directly binding PML. Science 2010, 328, 240–243.
136. Zhong, S., Müller, S., Ronchetti, S., Freemont, P. S., Dejean, A., & Pandolfi, P. P. Role of SUMO-1–modified PML in nuclear body formation. Blood, The Journal of the American Society of Hematology, 2000, 95(9), 2748-2752.

137. Lu, J., Chew, E. H., & Holmgren, A. Targeting thioredoxin reductase is a basis for cancer therapy by arsenic trioxide. Proceedings of the national academy of sciences, 2007, 104(30), 12288-12293.
138. Zhu, J., Chen, Z., Lallemand-Breitenbach, V., & de Thé, H. How acute promyelocytic leukaemia revived arsenic. Nature Reviews Cancer, 2002, 2(9), 705-714.

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/237/2020.PL
Subject: PhD Publikációs Lista

Candidate: Károly Jambrovics
Neptun ID: B0LXWU
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

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.
DOI: http://dx.doi.org/10.3324/haematol.2018.192823
IF: 7.116

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

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.
   Proc. Natl. Acad. Sci. U. S. A. 116 (42), 21120-21130, 2019.
   DOI: http://dx.doi.org/10.1073/pnas.1901382116
   IF: 9.412

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
   IF: 3.449

Total IF of journals (all publications): 26,103
Total IF of journals (publications related to the dissertation): 13,242

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

17 August, 2020

DEBRECENI EGYETEM
Egyetemi és Nemzeti Könyvtár

8. Keywords

Acute promyelocytic leukemia (APL)
All-trans retinoic acid (ATRA)
Tissue transglutaminase (TG2)
Arsenic trioxide (ATO)
Differentiation syndrome (DS)
Apoptosis, cell death
ATRA and ATO combination treatment
Reactive superoxide species (ROS)
Nuclear factor-kappa B (NF-κB)
Inflammatory cytokine production

8. Kulcsszavak

Akut promielocita leukémia (APL)
Csupa transz-retinsav (ATRA)
Szöveti transzglutamináz (TG2)
Arzén-trioxid (ATO)
Differenciációs szindróma (DS)
Apoptózis, sejthalál
ATRA és ATO kombinált kezelés
Reaktív szuperoxid formák (ROS)
Nukleáris faktor kappa-B (NF-κB)
Gyulladásos citokintermelés

9. Acknowledgement

I would like to express my biggest gratitude to my supervisor, Dr Zoltán Balajthy for allowing me to start the research work and the professional guidance, useful advice, ideas and support from the very beginning since I was a master student.

I would like to thank the current and former head of the Institute of Biochemistry and Molecular Biology, Prof. Dr László Fésüs and Prof. Dr József Tőzsér to allow me to work in the department.

I would like to thank Jeffrey Keillor for his professional "inhibitor" support and helpful advice in our collaborations. Without his contribution, this work could not be done.

Due to the help of Dr Jujcsi, my "going to develop in white" funniest colleague from the Institute of Biophysics and Cell Biology, with whom Olaf had learnt how to deal with the esoteric membrane fractions on colourful weekdays and those funny bands on busy weekends too. A great help was Máté Demény also, who was reading through the entire work and gave useful advice.

To "Erzsike", and "Dzsinni" our coolest assistants/magicians of our group, whom I learned the basics of laboratory work, and whom I can count on to those days, I am really lucky to have such a kind/patient and professional/crazy people while working. To our dear "Pipi" and "Rasimusi" who were almost late from everywhere, but were there on time when it needed.

Without the stable pack of the most original characters in the back, like "Rinito de la blott", "Pingu & Zuzi Holiday", "Vamos A.T. isolator" and "Főtörzsasszony" working on this project would be boring. Our constant misunderstanding each other helps us to make these days more fun. I am so grateful to had/have my two little minions "Mókus" and "Csibe" who made all my days more funny and proud with their "spicer sequence" and with the famous "CEFU". The accurate way they worked and learnt all the skills made me proud as a"scientific dad". Thank you to my friends/colleagues from the department in and out who kept saying just keep going.

This work is also dedicated to my family, especially to my mom, to the best grandma: Marika néni, Jutka & Dani, Beni, Petya and my beloved pack of the Kerékgyártó's.