Effect of retinoic acid on DNA damage-induced apoptosis in lymphoid cells.

Master of science thesis

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♥ To my nephew and nieces

Oslo, April 2008

Randi Vassbotn

Abstract

Conventional cancer therapy, such as γ-irradiation and cytotoxic drugs, acts via DNA damage-induced apoptosis and inhibition of proliferation. Major problems with conventional cancer therapy are that only subgroups of patients respond favourably to a given treatment, and that side effects often limit the dose efficiency of the treatment. The immune cells of the patient may for instance commit to apoptosis as a result of the therapy. It is therefore a constant search for modulators of DNA damage responses to improve such cancer therapies. To identify potential modulators it is important to have good read out systems. One of the key proteins down stream of the DNA damage checkpoint is the tumour suppressor p53. Upon DNA damage, the transcription factor p53 is stabilized and activated to affect target genes involved in growth arrest, DNA repair and apoptosis. Testing the efficacy of a given modulator of DNA damage responses may therefore include analysis of p53 expression and activity.

Vitamin A plays an important role in the immune system to protect against infection, and deficiency of this vitamin affects more than 100 million children throughout the world. One of the important metabolites of vitamin A is retinoic acid (RA), and RA is a well known regulator of cell proliferation and differentiation of numerous cell types, including lymphoid cells. The purpose of the present study was to explore a possible role of RA as a modulator of DNA damage responses in lymphocytes, and in particular we wished to elucidate the effects of RA on regulation of p53 expression in this process. Freshly isolated lymphocytes from healthy blood donors were stimulated with mitogens and treated with RA prior to induction of DNA damage by γ-irradiation. The amount of apoptotic cells was measured by flow cytometry, and Western blot analysis was performed to identify p53 expression. We were able to show that although RA stimulates and inhibits the proliferation of normal T - and B cells, respectively, DNA damage-induced apoptosis was inhibited in both cell types. The effect of RA was particularly strong in TPA-stimulated T cells, and we could show that apoptosis induced by both γ-irradiation and the cytotoxic drug doxorubicin

was inhibited. Importantly, we also showed that the RA-mediated inhibition of DNA damage-induced apoptosis was associated with reduced expression of p53, indicating that RA affects the DNA damage checkpoint. The physiological implications of the present results in that the vitamin A status of a cancer patient receiving chemotherapy or irradiation might influence the effect of the treatment, both by affecting the killing of malignant lymphocytes, as well as to protect the immune system from unwanted damage during irradiation or chemotherapy.

Abbreviations

Aa	Amino acid
ACAD	Activated T cell autonomous death
AICD	Activation-induced cell death
AML	Acute myeloid leukemia
Apaf-1	Apoptotic protease activating factor 1
APL	Acute promyelocytic leukemia
APS	Ammonium persulphate
ARAT	Acyl CoA:retinol acyltransferase
A-T	Ataxia-telangiectasia
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia mutated and RDS related
at-RA	All-trans retinoic acid
Bcl-2	B cell lymphoma
BID	BH3-interacting domain death agonist
Bis	N,N- methylene bisacrylamide
BRAC-1	Breast cancer susceptibility protein 1
C	Carboxy
cAMP	Cyclic adenosine monophosphate
Cdk	Cyclin dependent kinase
CHK	Checkpoint kinase
CI	Confidence interval (in statistics)
CIP	Members of CKI
CKI	Cyclin dependent kinase inhibitor
Con A	Concanvalin A
COP-1	Coat protein complex 1
CRBP	Cellular retinol binding protein
CREBP	cAMP response element binding protein
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DR	Direct repeat

DSB	Double strand break
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra acetic acid
FACS	Fluorescense activated cell sorter
FADD	Fas associated death domain protein
FBS	Fetal bovine serum
FSC	Forward scatter
G0	Gap 0 (in cell cycle)
G1	Gap 1 (in cell cycle)
G2	Gap 2 (in cell cycle)
GADD45	Growth arrest and DNA damage
GALT	Gut-associated lymphoid tissue
Gy	Gray, unit of absorbed radiation
h	Hour/hours
HLA	Human leukocyte antigen
HRE	Hormone responsive element
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IR	Ionizing radiation
kDa	Kilo Dalton
LI	Lower intake level
LRAT	Lecitin:retinol acyltransferase
M	Mitosis (in the cell cycle)
MAC	Mitochondrial apoptosis-induced channel
MALT	Mucosa-associated lymphoid tissue
MDM-2	Murine double minute 2
MHC	Major histocompability complex
Min	Minute(s)
MOMP	Mitochondrial outer membrane permeabilization
n	Number (of trials)
N	Acidic amino
NES	Nuclear export signal
NLS	Nuclear localization signal

p	P value (in statistics)
PBS	Phosphate buffered saline solution
PCD	Programmed cell death
PEM	Protein-energy malnutrition
pH	Potentia Hydrogenii
PHA	Phytohemagglutinin
PI	Propidium iodide
PKA	Protein kinase A
PKC	Protein kinase C
PML	Promyelocytic leukemia
PMSF	Phenylmethylsulphonyl fluoride
pRB	Retinoblastoma protein
PS	Penicillin and Streptomycin
p53RE	p53 response element
R	Restriction point
RA	Retinoic acid
RAE	Retinol activity equivalent
RAR	Retinoic acid receptor
RARE	Retinoic acid receptor element
Rb	Retinoblastoma gene
RE	Retinyl ester
RIPA	Radio Immuno Precipitation Assay
ROH	Retinol
ROS	Reactive oxygen species
RT	Room temperature
RXR	Retinoid X receptor
S	Synthesis (in the cell cycle)
SAC	Staphylococcus Aureus Cowan
SD	Standard deviation (in statistics)
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
SEM	Standard error of the mean (in statistics)
Ser1981	Serine 1981

SSC	Side scatter
TCR	T cell receptor
TEMED	N,N,N’,N’-tetramethylethylenediamine
Th	T helper cell
TLR	Toll like receptor
TPA	12-O-tetradecanoylphorbol-13-acetate
TNF	Tumor necrosis factor
TRADD	TNF receptor associated death domain
TTR	Transtyretin
TUNEL	TdT-mediated dUTP nick end-labeling
UL	Upper intake level
UV	Ultra violet
V	Volt
VAD	Vitamin A deficiency

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1. Introduction

1.1 Vitamin A

Vitamin A plays an important role in vision, bone growth, reproduction, cell division and -differentiation, as well as in the immune system (1;2). The major form of vitamin A is retinol (ROH), but vitamin A is defined as ROH and other retinoids with the same biological activity as ROH.

1.1.1 Vitamin A metabolism

There are two categories of vitamin A, depending on whether the food source is animal-derived (retinyl ester), or plant-derived (carotenoids). Retinyl esters (RE) are absorbed in the form of ROH after being hydrolyzed in the intestine by the pancreatic enzyme triglyceride lipase, and by the intestinal brush-border enzyme phospholipase B (3). Sources include liver, egg, fatty fish, whole milk, margarine and fish-liver oil. Carotenoids are found in colourful fruits and vegetables such as carrots and spinach, and include α- β- and λ-carotene, as well as cryptoxanthin. β-carotene is efficiently metabolized into ROH, providing two molecules of ROH from one molecule of β-carotene (4).

After absorption into the enterocytes, carotenoids are either metabolized to retinal or absorbed intact into the lymph. Retinal reductase reduces retinal to ROH, and ROH is bound to cellular retinol-binding protein type II (CRBP II) in the enterocytes. ROH in complex with CRBP II is estrified to RE by the two enzymes lecitin:retinol acyltransferase (LRAT) and acyl-CoA:retinol acyltransferase (ARAT). RE is incorporated into chylomicrons together with other neutral lipid esters, and secreted into the lymph (3).

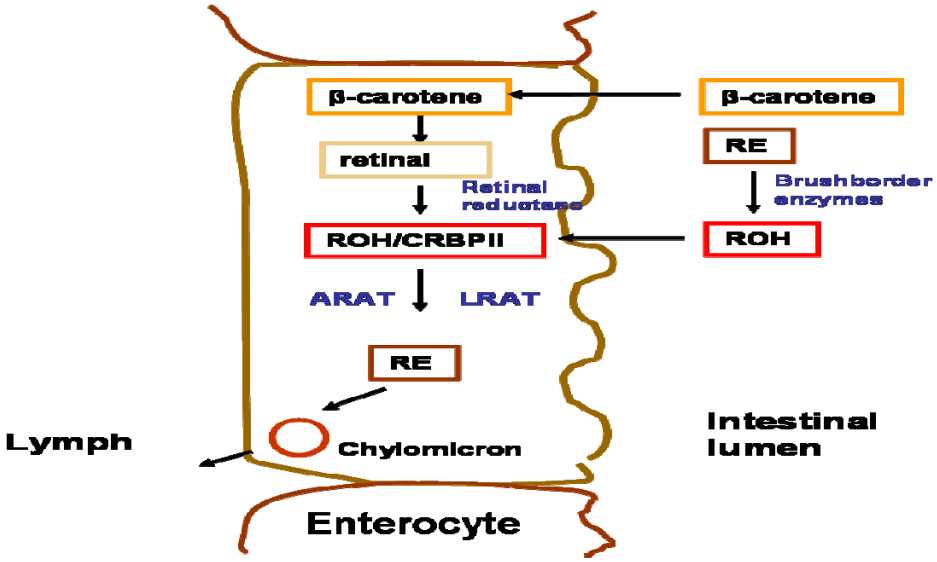


Figure 1: The absorption of vitamin A. Absorbed ROH is estrified to RE, which is incorporated into chylomicrons together with other neutral lipid esters, and secreted into the lymph.

The absorbed RE is circulated to several types of tissues, but most of the RE is metabolized to ROH in parenchymal liver cells. In these cells retinol binding protein (RBP) forms a complex with ROH, and transports ROH to nonparenchymal stellate cells in the liver. Here, ROH can be stored in the form of RE. Prior to mobilization from the liver, RE is hydrolyzed, and RBP once again binds to ROH. The ROH-RBP complex binds to transtyretin (TTR) in plasma, resulting in reduced glomerular filtration since the complex alone is too small to prevent renal filtration. The ROH-RBP-TTR complex circulates, providing vitamin A to extrahepatic tissue when intake of the vitamin is low, ensuring the plasma concentration to be maintained at approximately 2 µM (4;5).

1.1.2 Retinoids

Retinoids include naturally occurring forms of vitamin A but also synthetic derivatives of ROH, with or without biological activity (6).

A number of retinoids are thought to be bound to albumin, like *all-trans* retinoic acid (*at*RA) and 13-cis retinoic acid, as such they are present at nanomolar concentrations in plasma (7). While the visual function of vitamin A depends on its metabolite 11-cis retinal, most of the other functions of vitamin A including immunological functions, depend on *at*RA (8). Retinal can be reversibly reduced to produce ROH or it can be irreversibly oxidized to produce RA (9).

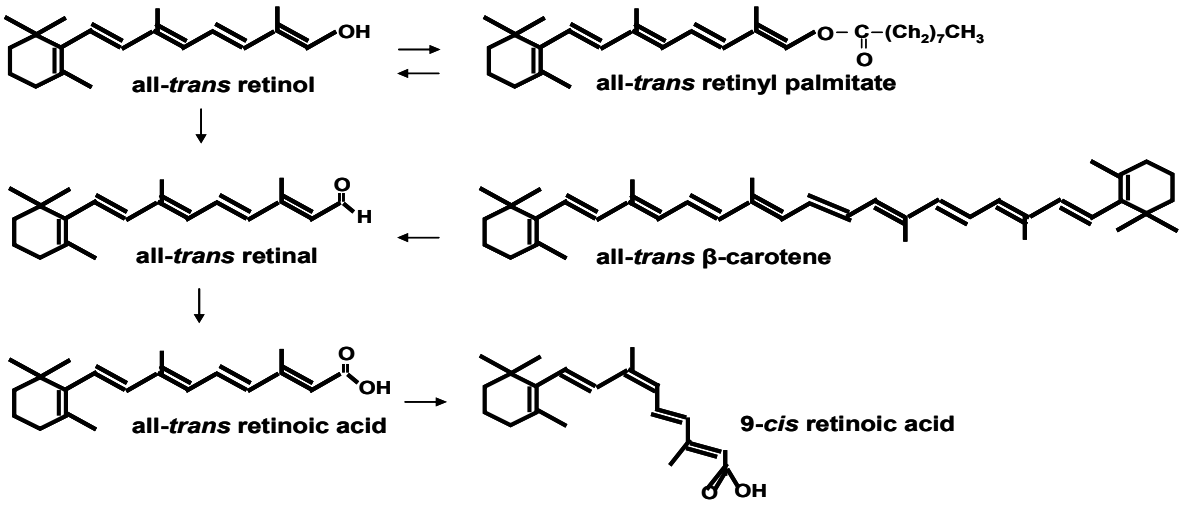


Figure 2: Molecular structure of some retinoids.

Retinoic acid receptors

Fat-soluble vitamins serve as precursors for ligands that bind to receptors in the nucleus. Nuclear receptors bind to specific DNA sequences known as hormone response elements (HREs). Each receptor recognizes unique HREs, typically direct repeats (DR) of the core AGGTCA half-site (10). Two important families of nuclear receptors include retinoic acid receptors (RARs), which bind both *at*RA and 9-cis RA, and retinoid X receptors (RXRs), which binds 9-cis RA (10;11). Each receptor exist in three isoforms; α, β and γ (12). Retinoic acid response element (RARE) are typically composed of DRs spaced by 2-5 nucleotides (DR2-DR5 respectively) and RXRs bind to DR1 where one nucleotide separate the repeated cores. RXR can bind

to DR1 both as a homodimer and as a heterodimer with RAR (10). In the absence of the ligand RA, RAR/RXR binding to RAREs recruits nuclear co-repressor to silence target gene by altering chromatin structure. Upon RA ligation, the co-repressor complex is released and the RAR/RXR complex associates with transcriptional co activators. This leads to transcription of RA-responsive genes (13;14).

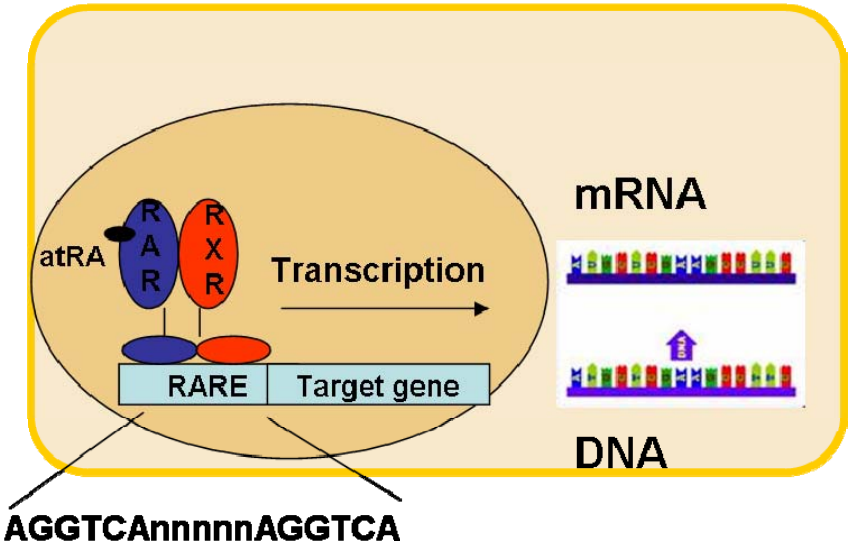


Figure 3: Retinoic acid receptors and its response element.

1.2 Proliferation and cell death

1.2.1 Regulation of the cell cycle

The cell cycle consists of four phases: G1-phase, S-phase, G2-phase (collectively known as interphase) and M-phase. To produce two genetically identical daughter cells, the DNA must be replicated to two complete copies, and this occurs in the S-phase (S for synthesis) of the cell cycle. M-phase is composed of two processes: mitosis, in which the cell's DNA is divided between the two daughter cells, and cytokinesis, in which the cell's cytoplasm divides forming distinct cells. G1 and G2

are gap phases between the M and S phases. The two gap phases allow the cell to grow and double the mass of proteins and organelles, and to monitor the environment to ensure that conditions are suitable and preparations are complete before a new phase of the cell cycle starts (15).

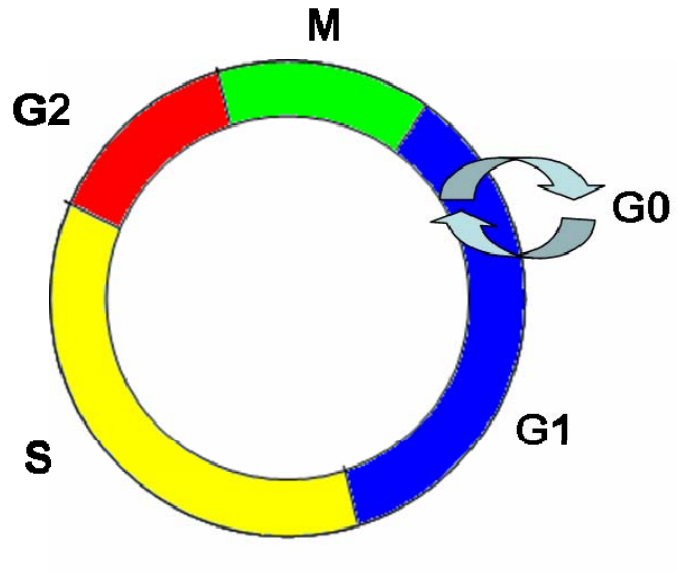


Figure 4: The cell cycle. The cell cycle consists of four distinct phases: G1, S phase, G2 (collectively known as interphase) and M phase. During interphase the cell grows continuously, and during M phase the cell divides. G0 is a resting state for quiescent cells, either temporarily or permanently.

The cell cycle control system is imperative for regulating cell numbers in the tissues of the body. At certain points during the cycle, biochemical pathways delay or arrest the cell cycle progression if the internal or external conditions are not favorable. These points are called checkpoints. Cells that have temporarily or reversibly stopped dividing enter a state of quiescence called G0 phase. If everything is functioning normally, and any damage to the DNA has been corrected, the cell moves on through the cycle. If DNA damage cannot be corrected, the cell halts its progression through the cycle and may initiate apoptosis and close down. When the system malfunctions, excessive cell divisions can result in cancer (16).

A group of protein kinases, the cyclin dependent kinases (Cdks), are at the center of the cell cycle engine, phosphorylating the retinoblastoma protein (pRB). Cell proliferation moves forward as phosphorylated pRB dissociates with the transcription factor E2F, and transcription of specific S-phase genes can take place. As a monomer, the Cdk subunit is not active; it must associate with a cyclin subunit forming a heterodimeric complex. (17). There are several different Cdks and cyclins expressed in the different phases of the cell cycle, and specific cyclin dependent kinase inhibitors (CKIs) are also involved in cell cycle regulation. CKI bind to Cdk complexes, and inhibits the activity of the cells. Growth factors are normally required for a cell to pass from G1 to S-phase, and the factors stimulate the cell cycle machinery by activating Cdks to phosphorylate pRB (18). Upon phosphorylation, pRB will release the transcription factor E2F, and E2F will in turn promote transcription of genes required for S-phase entry, such as DNA polymerase, thymidine kinase, cyclin E, cyclin A etc. When pRB is phosphorylated and E2F is released, the cells have passed the important restriction point (R) in the G1 phase of the cell cycle (18).

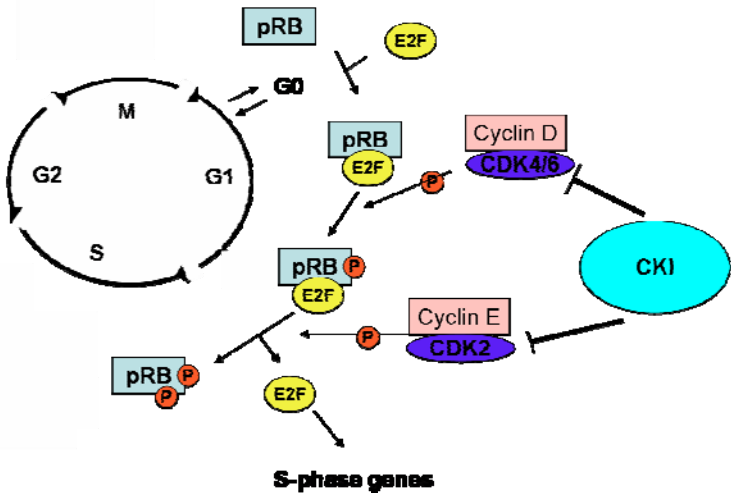


Figure 5: Cell cycle arrest. See text for details.

1.2.2 Cell death

The process of cell death is important for both embryonic development and for control of cell number in tissues. Essentially, two types of cell death exist, necrosis and programmed cell death. The most common type of programmed cell death is apoptosis. In general, apoptosis confers advantages to an organism. For example, the differentiation of fingers and toes in a developing human embryo occurs because cells between the fingers undergo apoptosis (15). Necrosis, on the other hand, is accidental death of cells, which provokes an inflammatory response (19).

Apoptosis

Programmed cell death (PCD) is death of a cell in any form, mediated by an intracellular program. The process requires ATP, and no inflammation is induced in surrounding tissues due to the fact that the membrane is never disrupted. Autophagic cell-death is one type of PCD, characterized by the formation of large vacuoles within the cell which eat away organelles in a specific sequence prior to the nucleus being destroyed (20).

Apoptosis is an evolutionarily conserved and highly controlled form of PCD that plays a crucial role in development, maintenance of homeostasis, and immunological responses in multicellular organisms. Apoptosis involves a series of biochemical events eventually leading to cell death. A variety of morphological changes are performed, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Dysregulation of apoptosis is implicated in a range of diseases, including cancer, neurodegenerative disorders and autoimmune disease (21).

The intrinsic and extrinsic pathway

Cells undergo apoptosis through two major pathways, the intrinsic and the extrinsic pathway (15). The extrinsic pathway requires the interaction of death receptors and

their ligands in the cell membrane, for example the tumour necrosis factor (TNF) binding to its receptor TNF-R1, or Fas ligand binding to Fas-receptor. This binding initiate the pathway that leads to caspase activation via the intermediate membrane proteins TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD); caspases are recruited and activated, resulting in cell death. In general, caspases are proteases that cleave their substrates at Aspartate, and have cystein in the active site. These caspases can also cleave and activate BH3-interacting-domain death agonist (BID) enabling it to co-engage the intrinsic pathway (22).

The intrinsic pathway is engaged by cellular stress, for example DNA damage. It is controlled by proteins of the B cell lymphoma (Bcl-2) family. Bcl-2 proteins are able to promote (Bax/Bim) or inhibit (Bcl-2/Bcl-x) apoptosis as they govern mitochondrial outer membrane permeabilization (MOMP), either direct on MAC (mitochondrial apoptosis-induced channel) or indirectly through other proteins. Cytochrome C is released from mitochondria due to formation of the channel, MAC, in the outer mitochondrial membrane, and serves a regulatory function as it precedes morphological change associated with apoptosis (23). Once cytochrome C is released it binds to apoptotic protease activating factor 1 (Apaf-1) and ATP, which then bind to pro-caspase-9 to create a protein complex known as an apoptosome. The apoptosome cleaves the pro-caspase to its active form caspase-9, which activates caspase-3, leading to apoptosis (22).

1.3 DNA damage

During the life of a cell, DNA damage can be induced by environmental or endogenous genotoxic insults such as radiation, chemicals, drugs and reactive cellular metabolites. DNA damage may lead to mutations, cancer and cellular death.

1.3.1 DNA damage checkpoints

Depending on the type of damage, different responses may occur, involving DNA damage checkpoint pathways leading to cell cycle inhibition or cell death. The DNA damage checkpoints are signal transduction pathways consisting of sensors, transducers and effectors. One class of signal transducers is composed of phospho-inositide kinase (PIK)-related proteins which include ATM (ataxia telangiectasia mutated) and ATR (ATM-RAD3-related), regulating the downstream checkpoint kinases (CHK) CHK1 and CHK2. The effectors that execute the functions of the DNA damage response are substrates of both PIK and CHK, and include proteins involved in DNA repair, transcription regulation and cell cycle control, such as the Cdk inhibitor $p21^{CIP}$, breast cancer susceptibility protein-1 (BRCA1) and p53 (24).

ATM and ATR activation

In response to double strand breaks (DSB) induced by ionizing radiation (IR), the ATM kinase activation is global and rapid whereas the enhanced backup activation of ATR is local and delayed (25;26).

Mutations in the gene that encodes the critical checkpoint-signaling protein kinase ATM causes the cancer-predisposing genetic disorder ataxia telangiectasia (A-T) (25). When IR causes DSB, the local change in chromatin topology initiates autophosphorylation at serine 1981 (Ser1981) on the ATM dimers, dissociating the dimeric ATM into monomers competent to bind and phosphorylate their targets, amongst them CHK2 and p53 (26)**.** As an effector kinase in response to DSB, CHK1 is partly redundant with CHK2, but ATR activity is required to keep the downstream effector mechanisms (which eventually inhibit DNA replication), poised to respond to the acutely activated ATM. In this way, ATR and ATM are both required to the complete checkpoint response to IR (16).

CHK1 and CHK2

Activation of ATM and ATR activates CHK2 and CHK1 respectively, which in turn phosphorylate Cdc25A (CHK2) or Cdc25C (CHK1). In a phosphorylated state, Cdc25 associates with the p53 target molecule 14-3-3 and is exported from the nucleus, resulting in CDK1 inactivation and establishment of G2 arrest. When Cdc25A is phosphorylated, CDK2 phosphorylation is inhibited and the cell is unable to initiate DNA replication in S-phase (27).

1.3.2 The p53 pathway

In 1979, the tumor suppressor gene p53 was described in a complex with SV40 large T antigen, but was initially presumed to be an oncogene. Its character as a tumor suppressor gene was finally revealed in 1989, when wild-type p53 was detected (28).

p53 is a 53 kDa protein with anticancer functions. It plays a role in apoptosis, genetic stability, and in inhibition of angiogenesis. Being a nuclear transcription factor, it activates the transcription of target genes involved in these processes (28). In normal cells the level of p53 is usually low, and it is inactive, bound to the protein murine double minute 2 (MDM-2, HDM-2 in humans), which prevents its action and promotes its degradation by acting as ubiquitin ligase. In addition to being regulated at the level of stability, the activity of p53 can also be post-translationally regulated in response to stress signals. Thus, both the N- and C terminal of p53 can be modified by phosphorylation, acetylation, O-glycosylation, methylation, ubiquination and sumolation (27).

Structure

The p53 protein consists of 393 amino acid (aa) residues, divided into five structurally and functionally different domains. The acidic amino (N)-terminal end consists of the first 42 aa, and is responsible of the transactivating properties of the protein. Without this domain, the induction of target genes cannot occur. Interaction with MDM-2 also occurs via this domain.

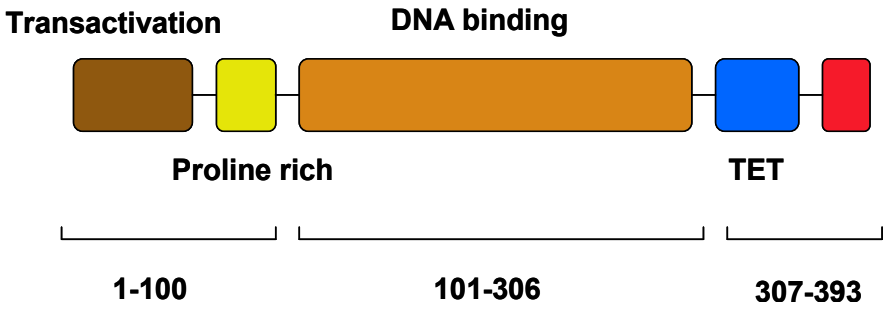


Figure 6: The p53 protein. See text for details.

The central region (aa 101-306) contains the DNA binding domain, and is the site of 90 % of the p53 mutations found in human cancers. Between these regions, a novel, proline rich domain has been identified. The oligomerization domain TET (aa 307-355) consists of a beta-strand, followed by an alpha-helix necessary for dimerization, as p53 is composed of two dimers. A nuclear export signal (NES) is localized in this oligomerization domain. The carboxy (C)-terminus of p53 (aa 356-393) contains three nuclear localization signals (NLS) and a non-specific DNA binding domain that binds to damaged DNA.

p53 – regulator of cell growth and apoptosis

Active p53 is induced in response to a myriad of stress types, which include DNA damage (induced by radiation or chemical agents), oxidative stress, osmotic shock, ribonucleotide depletion and deregulated oncogene expression. Although there are multiple DNA damage detection and repair systems in the cell, every type of DNA damage is reported to the p53 protein and its pathway. If DNA damage can be repaired, p53 activates genes to fix the damage. If the damage cannot be repaired, the p53 tumor protein prevents the cell from dividing and signals it to undergo apoptosis (27).

These processes prevent cells with mutated or damaged DNA from dividing, which in turn helps to prevent the formation of new mutations and development of tumors. The advantage of this arrangement is that one gene can act as an efficient integrator of

information about all the different types of stress that can act upon the cell. On the other hand, giving one single gene and its products such a crucial role also makes the system vulnerable. If the p53 gene is damaged, tumor suppression is severely reduced. More than 50 percent of human tumors contain a mutation or deletion of the p53 gene (29).

In unstressed cells, p53 levels are kept low through a continuous degradation of p53. MDM-2 binds to p53 and transports it from the nucleus to cytosol where it is degraded in proteasomes. Under normal conditions the p53 protein has a half life of 6-20 min (28). Activation is marked by two major events. Firstly, the half-life of the p53 protein is increased drastically, leading to a quick accumulation of p53 in stressed cells. Secondly, a conformational change forces p53 to take on an active role as a transcription regulator in these cells. The increased concentration and activity of the p53 protein leads to transcription of a set of genes that have a p53 response element (p53RE) in their promotors (30).

P53 is an important downstream effector in the DNA damage checkpoint. DNA damage causes ATR, ATM, CHK1 or CHK2 to phosphorylate p53 at sites that are close to or within the MDM-2-binding region. Phosphorylation of the N-terminal end of p53 by the protein kinases disrupts MDM-2-binding (27). Other proteins, such as Pin1, are recruited to p53 and induce a conformational change in p53 which prevents MDM-2-binding even more. Recently, two other ubiquitin ligases, Coat protein complex 1 (COP-1) and Pirh-2, were shown to act upon the stability of the p53 protein (28;31).

p53 target genes

Once activated, p53 leads to expression of several genes. In a negative feedback loop MDM-2 is itself induced by the p53 protein. To induce cell cycle arrest, the $p21^{CIP1}$ gene is induced. $p21^{CIP1}$ inhibits Cdk/cyclin complexes that normally phosphorylate pRB causing it to dissociate from E2F, thus inhibiting the transcription of S-phase genes required for G1/S transition. p53 also induces GADD45 (Growth arrest and DNA damage), which is a nuclear protein participating in cell cycle arrest and DNA

repair (32). If DNA repair is not successful, stabilized p53 increases the expression of numerous pro-apoptotic genes (BAX, NOXA, PUMA etc) causing damaged cells to undergo apoptosis. Recently it has also been revealed that p53 has extra-nuclear apoptotic functions independent of transcription. Thus p53 binds the anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-x) and activates the pro-apoptotic multi domain proteins (Bax and Bak) to induce cytochrome C release and subsequent apoptosis (33). Cells that have engaged p53-dependent apoptosis typically follow the intrinsic pathway, but it is also demonstrated that p53 can promote Fas-mediated (extrinsic) apoptosis by increasing the expression of Fas receptor, enabling BID to co-engage with the intrinsic pathway (33).

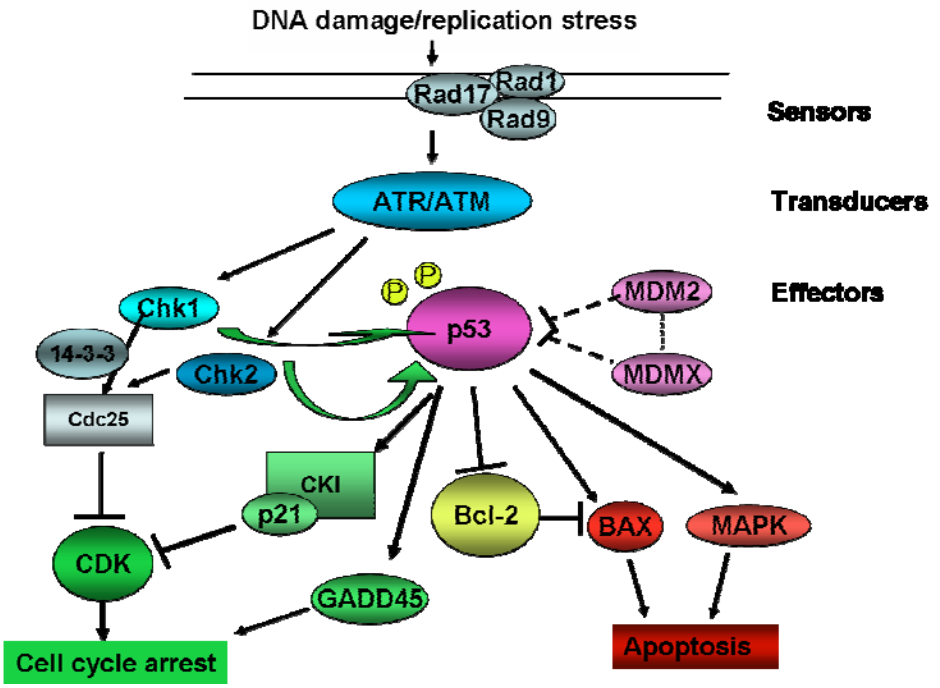


Figure 7: p53 induces both cell cycle arrest and apoptosis in response to DNA damage.

p53 has been described as "the guardian of the genome", referring to its role in conserving stability by preventing genome mutations (28). Tumor suppressor genes are often inactivated in cancer cells, resulting in the loss of normal functions of the

cells. Mutations that deactivate p53 in cancer usually occur in the central DNA-binding core domain. Most of these mutations destroy the ability of the protein to bind to its target DNA sequences, and thus prevents transcriptional activation of these genes.

If the p53 gene is damaged, tumor suppression is severely reduced. People who inherit only one functional copy of the p53 gene will most likely develop tumors in early adulthood, a disease known as Li-Fraumeni syndrome (29).

1.3.3 DNA damage induction

DNA damage can be induced by environmental or endogenous genotoxic insults such as ionizing or ultraviolet radiation (IR or UV, respectively), chemicals, drugs and reactive cellular metabolites.

Radiation

Radiation, as used in physics, is energy in the form of waves or moving subatomic particles. Radiation can be classified as ionizing or non-ionizing radiation, depending on its effect on atomic matter. UV radiation is an example of non-ionizing radiation, being electromagnetic radiation with a wavelength shorter than that of visible light, but longer than soft X-rays.

Gamma rays, used in IR, are generally characterized as electro-magnetic radiation having the highest frequency and energy, and also the shortest wavelength within the electromagnetic spectrum, i.e. high energy photons. Double-strand breaks (DSB) are considered the typical form of DNA damage resulting from IR, as one DNA DBS is sufficient to trigger apoptosis. A variety of other DNA lesions are also induced, among them single-strand breaks, base and sugar damage (27).

Cytotoxic agents - doxorubicin

Various cytotoxic agents can be used to induce DNA damage. Most chemotherapeutic drugs act by impairing mitosis, effectively targeting fast-dividing

cells, or cause cells to undergo apoptosis. Many of the commonly used chemotherapeutic drugs can be classified as either alkylating agents, antimetabolites, anthracyclines, plant alkaloids or topoisomerase inhibitors, and all of these drugs affect cell division or DNA synthesis. The class of anthracyclines includes the chemical doxorubicin. Activation of p53, which in turn promotes apoptosis of tumor cells, is considered to be a key mechanism of action of doxorubicin (34;35), but it also inhibits topoisomerase II, which unwinds DNA for transcription. Doxorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication (36). Doxorubicin is widely used in chemotherapy of cancer. A side effect of doxorubicin-treatment is induction of apoptosis also in normal cells. ROS (Reactive oxygen species) generation during intracellular metabolism of doxorubicin and subsequent oxidative stress has been proposed to be important mechanism(s) for its cardiac toxic side effects (37).

1.4 The immune system

The immune system protects an organism against diseases by identifying and killing pathogens and tumour cells. It detects a wide variety of agents, from viruses to parasitic worms, and needs to distinguish them from the organism's own healthy cells and tissues in order to function properly. Physical barriers like the skin and mucous membranes, surface linings of the eyes and the respiratory, urinary, and intestinal tracts prevent pathogens such as bacteria and viruses from entering the body. If a pathogen breaks these barriers, the innate immune system provides an immediate, but non-specific response. However, if pathogens successfully evade the innate response, vertebrates possess a third layer of protection, the adaptive immune system. Here, the immune system adapts its response during an infection to improve its recognition of the pathogen. This improved response is then retained after the pathogen has been eliminated, in the form of an immunological memory, and allows the adaptive immune system to mount faster and stronger attacks each time this pathogen is encountered. As the innate immune system is essential in activating the adaptive immune system, the two systems do not function as two separate entities (15).

An important role of the immune system is to identify and eliminate tumor cells, which express antigens not found on normal cells. As the antigens appear foreign to the immune system, T cells attack the tumor cells. Tumor antigens are presented on major histocompatability complex (MHC) class I molecules in a similar way to viral antigens, but as tumor cells often have a reduced number of MHC class I molecules on their surface, detection by T cells may be avoided (38). In addition, immunological tolerance may develop against tumor antigens, resulting in the immune system no longer attacking the tumor cells (39).

1.4.1 The lymphoid organs

The immune system includes the primary/central lymphoid organs (the bone marrow and the thymus), the secondary/peripheral lymphoid organs (lymph nodes, spleen,

mucosa-associated lymphoid tissue (MALT) and gut-associated lymphoid tissue (GALT)), and circulating immune cells. The immune cells are generated and developed in the primary lymphoid organs, whilst the secondary lymphoid organs are the major sites of immune activation (15).

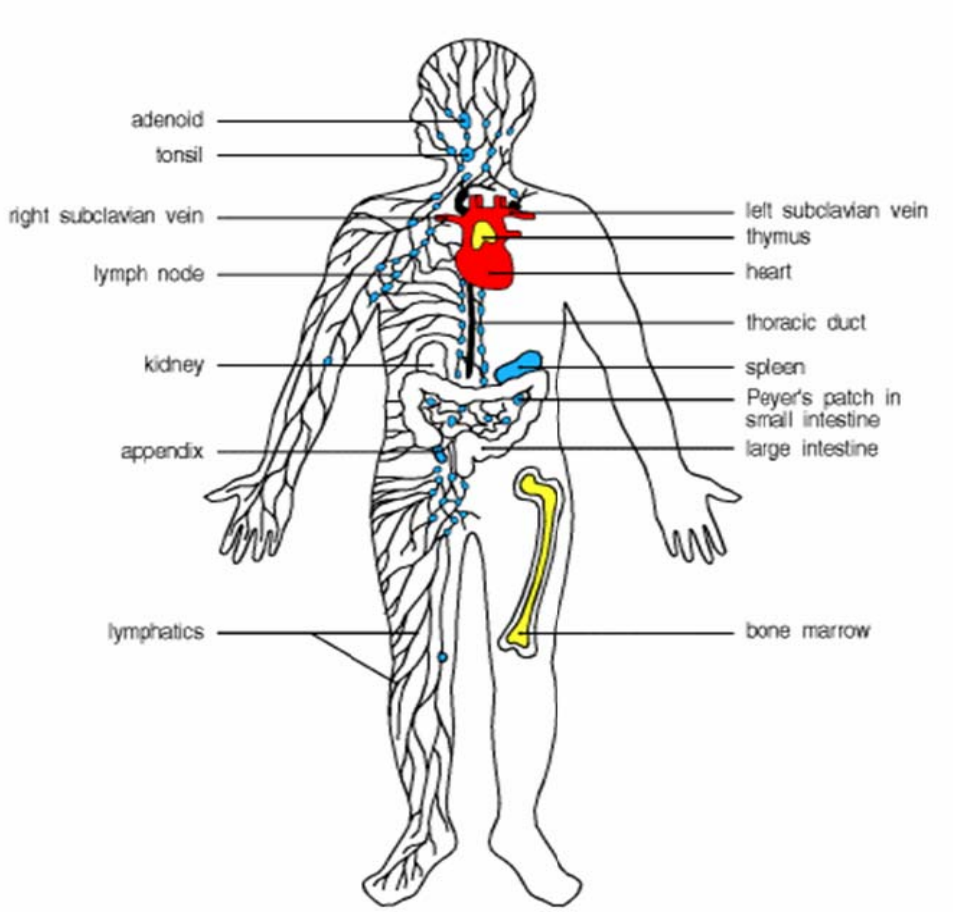


Figure 8: Schematic illustration of the immune system (15). The bone marrow and the thymus are the central lymphoid organs (shown in yellow), while the peripheral lymphoid organs (lymph nodes, spleen, MALT and GALT) are shown in blue. Lymphatic vessels drain fluid (lymph) containing antigen from the tissues through the lymph nodes where the antigen is trapped to interact with T cells.

1.4.2 The adaptive immune defense

The cells of the adaptive immune system are special types of leukocytes, called lymphocytes (primarily B cells and T cells), and the lymphocytes are derived from haematopoietic stem cells in the bone marrow. B cells are involved in the antibody

response, whereas T cells are involved in cell-mediated immune response. After microbes are engulfed by dendritic cells of the innate immune system, they are presented at the surface together with MHC. The dendritic cells will migrate to a nearby lymphoid organ where they present the agents to T cells. Some T cells then migrate to the site of infection where they destroy the microbes, while others remain in the lymphoid organ to activate B cells. B cells secrete antibodies that circulate in the body, coating the microbes and targeting them for efficient phagocytosis. Some B cells are stimulated to multiply and differentiate into memory cells to be able to react to the same antigen later in a system called secondary immune response, or immunological memory (15).

B cells and T cells are generated in the bone marrow in adults, and while B cells also develop and mature here, T cells migrate to the thymus in order to develop. Both cell types rearrange their receptors, and when T cell receptor is expressed at the surface of T cells, it is subjected to negative and positive selection before they exit as naïve T cells into the blood stream. B cells express IgM before negative selection, where potentially autoreactive cells are eliminated before completing development with expression of IgD, CD19 and CD38. When these matured, naïve B cells leave the bone marrow for the periphery; they can undergo activation and differentiation towards plasma cells or differentiate towards memory B cells. T cells are activated by foreign antigen to proliferate and differentiate into effector cells, termed T-helper (Th) 1 cells and Th2 cells. These cells activate macrophages and B cells, respectively. B cells can be activated by antigens with or without help from Th2 cells. Before activation both B and T cells are in the resting phase G0 in peripheral blood or lymphoid organ (15), and in this state T cells are very resistant to apoptosis.

In vitro, T cell activation can be mimicked by antibodies, and T cell receptor activation can be bypassed by using chemicals such as 12-0-tetradecanoylphorbol 13-acetate (TPA), or by lectins such as phytohemagglutinin (PHA). TPA activates PKC, whereas PHA crosslink surface receptors (40) resulting in activation of T cells. In B cells, this activation can be mimicked *in vitro* by stimulating the B cells with anti-

immunoglobulin or various lectins known to crosslink the surface immunoglobulin. Staphylococcus Aureus Cowan (SAC) is a crude bacterial cell suspension that activates B cells via lectins at the bacterial surface (41). Memory B cells can persist for decades after initial activation, whereas when T cells are activated, they become more susceptible to die. This is because T cells are destined to die at some point either because they are not needed, or they must be deleted if they are repeatedly activated by self-antigen. The former process occurs through the intrinsic pathway and has been termed activated T cell autonomous death (ACAD), whereas the latter is called activation-induced cell death (AICD), and is to a large extend mediated by the death receptor pathway (42).

1.4.3 Epstein-Barr virus

Epstein-Barr virus is a double stranded DNA virus genome of 172 kb. It belongs to the γ herpes virus family, and was the first candidate of human tumor virus, identified in 1964. It targets lymphoid cells, mainly B cells, but is also known to infect other cells, like epithelial cell. The EBV virus has a unique ability to transform resting B cells into permanent, latently infected lymphoblastoid cell lines, and persist in resting nonproliferating memory cells. There is a delicate balance between the virus and the host immune system, where disruption of the balance may lead to the development of EBV-associated disease like mononucleosis, lymphoproliferative disease or Burkitt's lymphoma. Although 90 % of the human population is infected, the EBV virus generally persists in a host for life without causing disease (43).

1.5 Vitamin A and the immune system

Vitamin A plays an important role in the immune system to protect against infection, and deficiency of this vitamin affects more than 100 million children throughout the world (2;44-46). Approximately 250 000 to 500 000 malnourished children in developing countries become blind each year, and each year more than one million childhood deaths are associated with vitamin A deficiency (VAD), mainly infectious

diseases (47)**.** VAD significantly impairs the mucosal epithelial barriers in the eye, respiratory, gastrointestinal and urogenital tracts, and it has been demonstrated that deficiency affect the innate immune system by enhancing macrophage-mediated inflammation, but impairing their ability to ingest and kill bacteria (48). Hence, it may lead to increased pathogen replication, increased pathology, inflammation and secondary immune responses (49). On the other hand, a too large intake of this fat-soluble vitamin can result in acute or chronic toxicity (4).

1.5.1 Regulation of B cells and T cells by Vitamin A

The reported effects of VAD on immunity in human populations can be difficult to interpret due to concurrent effects of protein-energy malnutrition (PEM) and infections, but by animal studies it is possible to bypass some of these obstacles. Beneficial effects of retinoids on antibody responses in animal studies are generally reflected also in human studies (49;50). Semba and coworkers found that children with mild VAD had relative lower IgG responses to tetanus immunization than children who had been supplemented to normal vitamin A levels, and later the same group concluded that vitamin A intervention enhanced the relevant humoral responses in terms of the IgG1 subclass (50). VAD has been associated with impaired mucosal immunity due to selective loss of IgA producing cells (51), and RA has, in the presence of IL-5, the ability to induce isotype switching to IgA (51;52). It has been demonstrated that dendritic cells (DC) from GALT were able to induce IgA-secretion in murine B cells, concomitant with induction of gut-homing receptors at the B cell surface. The GALT-derived DC are known to express the enzymes required to convert dietary vitamin A to retinoic acid (53;54), and the IgA-secretion induced by RA was shown to require cooperation with GALT-DC-derived IL-5 or IL-6 (54).

In vitro, our group have demonstrated that physiological as well as pharmacological doses of RA inhibits mature B cell proliferation as well as growth of B cell precursors (55;56), and we have demonstrated that this inhibition by RA is because RA prevent

phosphorylation of pRB, due to an up regulated level of the CKI $p21^{CIP}$ at the level of transcription (57). Recently, however, our group could show that whereas naïve cells are inhibited by RA, memory B cells stimulated via toll like receptor (TLR) 9 are stimulated (58). We have also demonstrated that RA treatment of normal B cells can inhibit spontaneous apoptosis (59).

In addition to affecting antibody responses, VAD also changes the pattern of Th1/Th2 cytokine production in animal studies (49). It seems clear that vitamin A is an important regulator of the Th1/Th2 balance in the immune system, as VAD skews the Th1/Th2-balance in a Th1-direction (49).*In vitro*, a number of studies has shown conflicting data as to whether RA enhanced, reduced or had no effect on lymphoid proliferation (60-62). This inconsistency may be due to the use of different concentrations of RA; high concentrations of RA (above 10 μM) were inhibitory, whereas lower concentrations (0.1-1 μM) were stimulatory on PHA-mediated proliferation (63). Most reports indicate, however, that vitamin A stimulates T cells (62-64). Our lab has demonstrated that RA enhances the proliferation of human T cells by stimulating the production of IL-2 (65) and we have also shown that RA protects normal T cells from apoptosis due to enhanced secretion of IL-2 (66).

1.5.2 Recommendations for intake of vitamin A

Norwegian recommendations for intake of vitamin A are provided by Sosial og helsedirektoratet, based on The Nordic Nutrition Recommendations (NNR). Different forms of vitamin A are metabolized to different quantities of retinol, and the recommendations are therefore given as retinol activity equivalent (RAE). One RAE is equal to either 1 μg ROH, 12 μg β-carotene, 24 μg α-carotene or 24 μg cryptoxanthin (4;67).

The NNR is guidelines for the nutritional composition of a diet which provides a basis for good health, based on the current nutritional situation in the Nordic countries. To prevent deficiency but also avoid toxicity, a lower and upper level of intake is established. In addition, an intake level (RI) is recommended to maintain

good nutritional status among practically all healthy individuals. RI is based on an average requirement, and is 900 RAE for men, and 700 RAE for women, unless the woman is pregnant or lactating in which case the RI is higher; 800 and 1100 RAE respectively. The Upper Intake Level (UL) for vitamin A is the maximum daily intake unlikely to result in adverse health effects**,** and is set to 3000 µg of preformed vitamin A for both men and women, as toxic effects primarily have been linked to ROH or RE. The Lower intake level (LI) of vitamin A is defined as an intake below a level that can lead to deficiency symptoms in some individuals. The LI for women is 400 RAE and 500 RAE for men (68).

Vitamin A deficiency diminishes the ability to fight infections, but also mild degree of VAD may increase children's risk of developing respiratory and diarrheal infections, decrease growth rate, slow bone development, and decrease likelihood of survival from serious illnesses (44;46;49). Xerophthalamia is an ocular disease caused by VAD, often associated with a concurrent or recent history of infection like diarrhea, pneumonia or measles (49). It has been reported that vitamin A supplements have failed to improve vitamin A status after various infections, and animal studies have shown that vitamin A stores are depleted after acute viral infection, indicating that severe or recurrent infections can lead to serious VAD (49).

Severe zinc deficiency often accompanies VAD, associated with strict dietary limitations or anorexia. Zinc is required to produce RBP, and therefore a deficiency in zinc limits the body's ability to transport vitamin A from stores in the liver to body tissues (67). In addition, the level of the transport protein RBP can be lower than normal during disease due to impaired tubular reabsorption of low-molecular weight proteins like RBP, leading to a higher loss of retinol through urine (49). Iron deficiency can also affect vitamin A metabolism, and iron supplements provided to iron-deficient individuals may improve body stores of vitamin A (67). In a interesting recent study it was suggested that high intake of vitamin A in childhood in industrial countries may be the cause of the high incidence of asthma in this part of the world (69). This is due to the skewing of the immune response to Th2- with IgA responses.

1.6 Cancer and cancer treatment

Malignant tumors are characterized by deregulated cell growth and apoptosis, and their ability to invade and destroy adjacent tissues. Malignant tumors may also spread to other locations in the body (metastasise). All cancers are caused by DNA mutations due to DNA damaging agents (tobacco smoke, radiation, chemicals, or infectious agents) or through errors in DNA replication (15). Lymphoma is the general term for a group of cancers that originates from cells in the lymphatic system, and it is the result of a lymphocyte undergoing a malignant change and multiplying, eventually crowding out healthy cells and creating tumors which enlarge the lymph nodes or other sites in the body. Non-Hodgkin lymphoma represents a diverse group of cancers, with the distinctions between types based on the characteristics of the cancerous cells. Hodgkin lymphoma is a specialized form of lymphoma, with characteristics that distinguish it from all other cancers of the lymphatic system: including the presence of an abnormal cell called the Reed-Sternberg cell (a large, malignant cell found in Hodgkin lymphoma tissues). Malignant cells originating from lymphoid precursors in the bone marrow are called leukemia, and occasionally also lymphoma cells will leave the solid tumors and populate the blood or lymph as leukemia.

There are several strategies for treating cancer, such as surgery, chemotherapy, radiation therapy and immunotherapy, and often several treatments are combined. Many of the treatment strategies depend on DNA damage responses to kill cancer cells. Inducing DNA damage in dividing cells is an efficient way of killing cancer cells, although a side effect is that also rapidly dividing normal cells are affected by the treatment.

1.6.1 Vitamin A and cancer treatment

From the early 1900s, vitamin A deficiency has been suggested to be associated with tumor development, and the anticancer activity of RA and other retinoids has been demonstrated in animal cancer models (46). RA can both induce the differentiation of undifferentiated stem cells, and inhibit the proliferation of activated or transformed cells, and a lot of effort has been put in research to use RA in prevention and treatment human cancers (70). The M3 subtype of acute myeloid leukemia (AML), also known as acute promyelocytic leukemia (APL), is almost universally treated with *at*RA in addition to chemotherapy. APL is characterized by a chromosomal translocation (t [15; 17]), involving a fusion between the RARα gene and a previously unknown locus named promyelocytic leukemia (PML). A consequence of this gene rearrangement is expression of the PML-RARα chimeric oncoprotein, which is responsible for the cellular transformation as well as the RA response observed in APL (71).

2. Aims of the study

A major problem with conventional cancer therapy is that only a subgroup of patients responds to a given treatment, and the side effects are often the limitation for the dose efficiency of the treatment. DNA damage responses are the basis for common anti-cancer treatments like irradiation and chemotherapy (24;25;34;36). In order to increase the number of patients that respond to a given treatment, and to increase the therapeutic index, there is a constant search for modulators of DNA damage responses as targets in cancer therapy.

We have previously demonstrated that elevation of intracellular cAMP in fibroblasts and lymphoid cells lead to inhibition of DNA-replication, and in the same paper we demonstrated that the reduced DNA replication resulted in inhibition of DNA damage-induced apoptosis (72). Based on our previous demonstrations that RA has profound effects on proliferation of various populations of human B- and T cells, we anticipated that RA also might modulate DNA damage responses in these cells. The tumor suppressor p53 is an important downstream mediator of DNA damage responses (27;28). Thus upon DNA damage, p53 will be stabilized and activated and thereby induce transcription of a number of genes involved in growth arrest, DNA repair and apoptosis (31).

The overall purpose of the present study is to determine if retinoic acid can be a modulator of DNA damage responses in lymphoid cells. The specific aims are:

1. Reveal the effects of RA on DNA damage-induced apoptosis in
   I. normal B cells
   II. EBV-transformed B cells
   III. normal T cells
2. Elucidate the effects of RA on regulation of p53 expression upon induction of DNA damage in lymphoid cells

3. Materials and methods

Materials

Chemical	Producer
[^{3}H] thymidine (1000mCi/ml)	Amersham Biosciences
Acidic acid	Merck
Acrylamide/Bis	Sigma
Ammonium persulfate (APS)	Shelton scientific
Aprotinin	Sigma
Bio-Rad Protein assay dye	Bio-Rad
Bromphenol Blue	Bio-Rad
Doxorubicin (3.45 mM)	Sigma
ECL+ Western blotting detection kit	Amersham Biosciences
EDTA (1 mM)	Prolabo
Ethanol	Arcus
Fetal Bovine serum FBS	Gibco
Forskolin (20mM)	Calbiochem
Gamma-globulin	Sigma
Glycerol	Prolabo
Glycine	Sigma
HCl	Merck
Leupeptine	Sigma
L-Glutamine	Gibco
Lymphoprep™	Fresenius Kabi Norge
Methanol	BDH
Microscint™	Packard
Molecular weight standard	Bio-Rad
Non-fat dried milk	Nestlé Molico
Penicillin Streptomycin	Gibco
PHA (2 mg/ml)	Sigma

PMSF	Sigma
Ponceau S	Sigma
Propidium Iodide (1,5 mM)	Sigma
Retinoic acid, all-trans (3.3 mM)	Department of nutrition
RPMI 1640 medium	Gibco
SAC (10 %)	Sigma
Sodium Azide (NaN_3)	Merck
Sodium Chloride (NaCl)	BDH labratories
Sodium dihydrogenphosphate(NaH_2PO_4)	Prolabo
Sodium deoxycholate ($C_{24}H_{39}NaO_4$)	Sigma
Sodium dodecyl sulphate (SDS)	Sigma
Sodium Fluoride (NaF)	Merck
Sodium orthovanadate (Na_3VO_4)	Sigma
TEMED	Bio-Rad
TPA (10^{-3} M)	Sigma
Tris Hcl	Angus Buffers & Biochemicals
Triton X-100	Sigma
Tween 20	Sigma
β-mercaptoethanol	Sigma
β-glycerophosphate	Sigma

Equipment

Bio-Rad Mini Protean Π gel apparatus	Bio-Rad
Buffy coat	Ullevål University Hospital
Cell culture flasks	Costat
Cell culture plates (6,12,24 and 96-wells)	Cellstar
Biofuge fresco table top centrifuge	Haraeus
Sigma 4K15 centrifuge	Sigma
Beckman J2-21 centrifuge	Beckman
CO_2 incubator	Nuaire™
Coulter® Microdiff 18 (Cell counter)	Dan Meszantsky
Cryo tubes	Cell star

Eppendorf tubes (micro-tubes 1.5 ml)	Axygen Sientific
FACSCalibur (Flowcytometer)	Becton Dickinson
Falcon tubes (15 and 50 ml)	Becton Dickinson
Film cassette	Kodak
Filtermate 196 harvester	Packard
Gamma-cell®3000 Elan (Irradiator)	MDS Nordion
Heating block	Dan Meszantsky
Hybond ECL™Nitrocellulose membrane	Amersham Bioscience
Hyperfilm™	Amersham Bioscience
Kodak X-omat 1000 processor	Kodak
MACS® LS Separation Column	Miltenyi Biotech
MACS® Separator magnet	Miltenyi Biotech
Magnet for cell culture flask	Self-made
Magnetic stirrer	Framo Gerätetechnik
Microscint™	Packard
Microlitre glass syringe (Hamilton)	Kebolab
Penicillin Streptomycin	Gibco
Plastic wrap	Andvord
Platform shaker	Edmund Blücher
Rock-n-roller	Labinco
Rotator mixer	Stuart Scientific
Semimicro disposable cuvettes (1.5 ml)	Plastibrand
Sterile scissors	Rocket Medical
TopCount™	Packard
Transblot®SD-semi dry transfer cell	Bio-Rad
Ultrospec 3100 pro spectrophotometer	Amersham Biosciences
Unifilter®-96, GF/C®	Laborel
Versi-dry™Protective paper	Nalgene
Vortexer	Heidolph
Water bath	Julabo
Whatman chromatography paper 3 MM	Whatman

Antibodies

Anti actin primary antibody (C-2)	Sigma
Anti p53 primary antibody (DO-1)	Sigma
Goat-anti mouse IgG	Bio-Rad
DETACHaBEAD®CD19	Dynal
Dynabeads® CD19 (Pan-B)	Dynal
MACS® CD4 MicroBeads	Miltenyi Biotech

Stock solutions

RPMI/PS medium
480 ml RPMI 1640 medium
10 ml PS
10 ml glutathione

0.01 M EDTA in RPMI/ PS medium
490 ml RPMI/PS medium
10 ml EDTA (1 mM)

10 % FBS in RPMI/ PS medium
450 ml RPMI/PS medium
50 ml FBS

1 % FBS in PBS
9.9 ml PBS
100 µl FBS

0.5 % FBS/ 2 mM EDTA in PBS
198 ml PBS
1 ml FBS
0.8 ml EDTA (1 mM)

PBS (20 x)

4.8 g KH_2PO_4

28.8 NaH_2PO_4

4 g KCl

160 g NaCl

800 ml H_2O

Adjust pH to 7.4. Distilled water to 1 l. The buffer is stored at 4-8ºC.

TBS-Tween:

24.2 g Tris

80 g NaCl

0.1% Tween 20

Distilled water to 10 litre, pH is adjusted to 7.6 with HCl

Primary antibody solution p53 :

1 µg/ml anti p53 antibody in 5 % milk with TBS-Tween

0.03 % NaN_3

Primary antibody solution actin:

1 µg/ml anti actin antibody in 0.5 % milk with TBS-Tween

0.03 % NaN_3

Secondary antibody solution:

Goat Anti-Mouse IgG HRP (1:4000) in 2 % milk with TBS-Tween

Doxorubicin
Doxorubicin (3.45 mM) in RPMI/PS medium

Forskolin
Forskolin (20 mM) in RPMI/ PS medium containing 10 % FBS

PHA
PHA (2 mg/ml) in RPMI/ PS medium containing 10 % FBS

Retinoic acid (RA)
All-trans Retinoic acid (3.3 mM) in RPMI/ PS medium containing 10 % FBS

SAC
SAC (10 %) in RPMI/ PS medium containing 10 % FBS

TPA
TPA (10^{-3} M) in RPMI/ PS medium containing 10 % FBS

[^{3}H] thymidine
[^{3}H] thymidine (1000mCi/ml) in RPMI/ PS medium

3.1 General techniques for culturing cells

To avoid contamination of microorganisms, all solutions and equipment that are in contact with the cells are autoclaved, and as an extra precaution, antibiotics are included in the culture medium to prevent bacterial growth. Gloves treated with ethanol are worn while handling the cells. To simulate an *in vivo* situation, the cells are cultured in an incubator at 37˚C, with 5 % CO_2 and humidified air.

3.1.1 Culturing of EBV cells

The lymphoid cell line EBV is cultured in 10 % fetal bovine serum (FBS) in complete RPMI medium containing inorganic salts, amino acids, vitamins, glucose, glutathione and pH-indicator, and growth factors that all cells need to grow and proliferate. To prevent contamination and to avoid glutathione loss due to a short half-time, penicillin and glutathione are added to the RPMI medium.

The cell cultures are diluted every 2-3 day to keep the density between 0.1-1.5 x 10^6 cells/ml, as the cells have a doubling time of 24 hours (h).

3.1.2 Culturing of B- and T cells

Freshly isolated lymphoid cells are cultured in RPMI medium containing 10 % FBS. B cells are cultured at a density of 1.5 x 10^6 cells/ml, whereas T cells are cultured at a density of 2 x 10^6 cells/ml.

3.2 Isolation of lymphocytes

Lymphocytes are isolated from buffycoats. Buffycoats are end products from blood after erythrocytes and plasma is removed. The buffycoats are derived from volunteer blood donors at Ullevål University Hospital.

3.2.1 Isolation of B cells

All B cells, except plasma cells, express the CD19 antigen on the surface (73). Positive selection by the use of magnetic beads coupled to CD19 antibody (Dynabeads® CD19 (Pan-B)) is used to isolate B cells from buffycoats, as the antibodies have high affinity to the antigen. To separate the cells from the Dynabeads, two different methods are available; one which allow the cells to detach over night, the other method using DETACHaBEAD®CD19. DETACHaBEAD®CD19 are beads coated with polyclonal antibody produced by immunising sheep with Fab fragments of mouse immunoglobulin. DETACHaBEAD®CD19 detaches B cells that have been positively selected using Dynabeads® CD19 (Pan-B). This method is considered a gentle way of removing the beads from the isolated B cells, providing cells with >99% purity and >98% viability. Typically 10-40 x 10^6 B cells are isolated from one buffycoat.

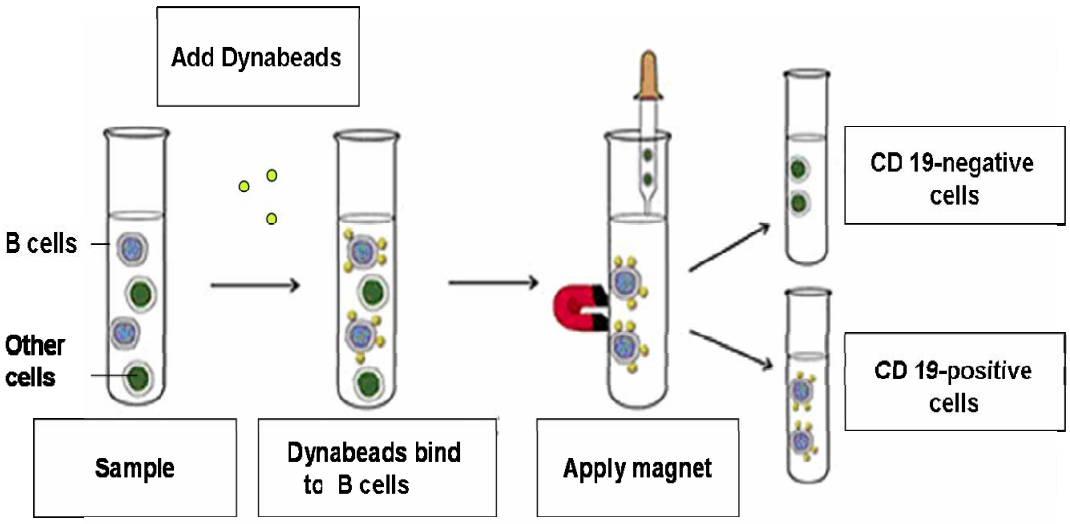


Figure 9: Isolation of B cells by the use of CD19 Dynabeads. B cells are isolated by letting CD19 beads adhere to the cells, followed by using a magnet to separate CD19- positive and -negative cells.

Equipment and solutions:	
Versi-dry™ protective paper	Magnet for eppendorf tubes
Buffycoat	Magnet for cell culture flasks
Sterile scissors	RPMI/ PS medium
Cell culture flask 75 cm^2	0.01 M EDTA in RPMI/ PS medium
Dynabeads® CD19 (Pan-B)	10 % FBS in RPMI/ PS medium
DETACHaBEAD®CD19	1 % FBS in PBS
Eppendorf tubes	CO_2 incubator (Nuaire™)
15 ml Falcon tubes	Cell counter (Coulter® Microdiff 18)
Rotator mixer	Rock-n-roller

Procedure:

1. Isolation of B cells by the use of Dynabeads® CD19 and overnight detachment

The blood from the buffycoat is drained into a 75 cm^2 flask containing 25 ml RPMI medium with penicillin and streptomycin (PS) and 0.01 M EDTA. 350 µl Dynabeads® CD19 (Pan-B) is added, and to let the B cells bind to the CD19 antibody on the beads, the flask is put to rotate at 4˚C for about 40 minutes (min). After rotation, the blood is separated from the CD19 beads by letting the beads adhere to a magnet. The blood is removed, and the B cell-bound beads are washed 6 times with RPMI/PS before they are resuspended in 20 ml medium containing 10 % FBS, and left over night in a humidified CO_2 incubator. The beads are then removed by a magnet, and the cells are subjected to centrifugation at 500 x g for 10 min at 4ºC. The pellet is resuspended in 2 ml medium containing 10 % FBS, and the cells are counted by the use of Coulter® Microdiff 18 cell counter according to the manufacturer's manual.

2. Removing beads by the use of DETACHaBEAD®CD19

The cells are isolated by the use of Dynabeads® CD19 (Pan-B) as described in the previous section, but instead of leaving the cells in a CO_2 incubator over night in 20 ml medium containing 10 % FBS, the final pellet is resuspended in 200 µl medium containing 10 % FBS. 200 µl DETACHaBEAD®CD19 is added, and after 45 min of incubation on a rock-n-roller, the tube is put on a magnet rack for 1.5 min. The supernatant is transferred to a Falcon tube, and to make sure all the beads have been removed, the cells are washed three times with PBS containing 1 % FBS. After collecting the cells by centrifugation at 300 x g for 10 min, the cells are washed two more times before resuspended in medium containing 10% FBS. The cells are counted by the use of Coulter® Microdiff 18 cell counter, and the cells are kept in a humidified CO_2 incubator for further use.

3.2.2 Isolation of T cells

Isolation of T cells from buffycoat is performed by a two-step procedure. In the first step, lymphocytes are isolated by density centrifugation using Lymphoprep™, and in the second step, T cells are positively isolated by magnetic beads conjugated to monoclonal anti-human CD4 antibodies. Typically 50-100 x 10^6 $CD4^+$ T cells are isolated from one buffycoat.

1. Lymphoprep™ separation

Lymphoprep™ contains sodium-metrizoat and polysaccharides, and therefore has higher density than lymphocytes. Due to the density distinction, blood cells are separated during centrifugation, placing cells with the highest density at the bottom of the tube i.e. erythrocytes and granulocytes. Due to its lower density, lymphocytes will accumulate as a layer on top of the Lymphoprep™.

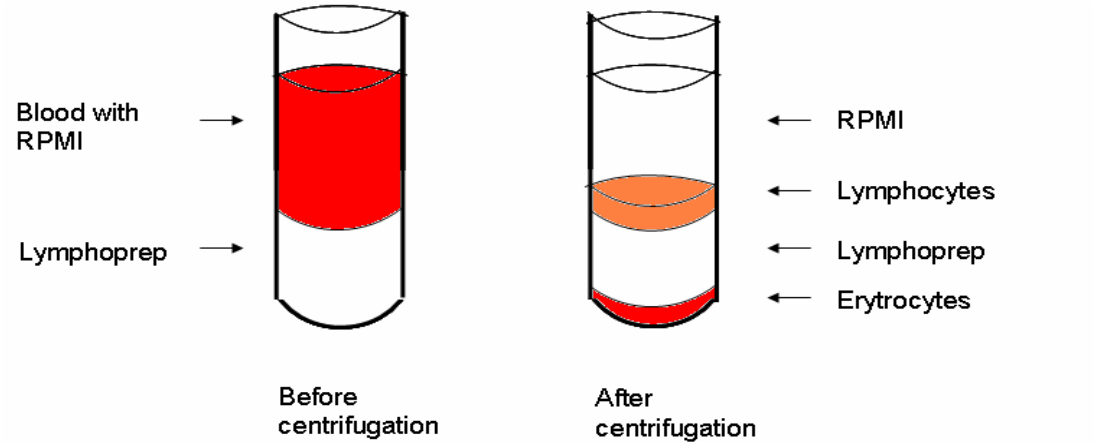


Figure 10: Separation of lymphocytes. See text for details.

2. Isolation of T cells by the use of CD4 antibodies

CD4 is an accessory molecule involved in the recognition of MHC class II/peptide complexes by the TCR heterodimers, and it is expressed on T helper cells and at a lower level on monocytes and dendritic cells. CD4 is expressed on most thymocytes and about 65% of all peripheral blood T cells. After letting the lymphocytes adhere to CD4 MicroBeads, the $CD4^+$ cells are separated from the negative cells by the use of a LS MACS® Separation Column and a MACS® Separator magnet. Thus, after removal of the column from the magnetic field, the magnetically retained $CD4^+$ cells can be eluted as the positively selected cell fraction.

Equipment and solutions:	
Versi-dry ™ protective paper	RPMI/ PS medium
Buffycoat	0.01 M EDTA in RPMI/ PS medium
Sterile scissors	0.5 % FBS/ 2 mM EDTA in PBS
Cell culture flask 75 cm^2	10 % FBS in RPMI/ PS medium
Lymphoprep ™	MACS® CD4 MicroBeads
50 ml Falcon tubes	LS MACS® Separation Column
Rotator mixer	MACS® Separator magnet
Cell counter (Coulter® Microdiff 18)	CO_2 incubator (Nuaire™)

Procedure:

The blood is mixed with 160 ml 0.01 M EDTA solution in a 75 cm^2 flask, followed by carefully distribution into 50 ml tubes each containing 15 ml Lymphoprep™. The tubes are subjected to centrifugation at 800 x g for 20 min at room temperature (RT). The layers of lymphocytes are aspirated and transferred to new 50 ml Falcon tubes before centrifugation at 300 x g for 15 min at 4˚C. To remove the layer of platelets on top of the lymphocytes the tubes are carefully inverted, as the platelets then loosen and can be removed. After washing the remaining cells, the pellet is resuspended in 10 ml medium containing 10 % FBS and the cells are counted by the use of Coulter® Microdiff 18 cell counter. After centrifugation at 300 x g for 10 min at room temperature, the cell pellet is resuspended in 1280 µl PBS containing 0.5 % FBS and 2 mM EDTA. Before incubation for 15 min at 4-8˚C, 320 µl MACS® CD4 MicroBeads are added. 25 ml PBS containing 0.5 % FBS and 2 mM EDTA is added before centrifugation at 300 x g for 10 min at 4˚C and the pellet is resuspended in 800 µl PBS containing 0.5 % FBS and 2 mM EDTA. A LS MACS® Separation Column is placed on the magnetic field and the column is calibrated with 3 ml PBS containing 0.5 % FBS and 2 mM EDTA before cells are added. The column is washed 3 times in 3 ml PBS containing 0.5 % FBS and 2 mM EDTA, before 5 ml of the same solution is pressed through the column with the stamp and the cells are collected in a 15 ml Falcon tube. The cells are subjected to centrifugation for 10 min at 300 x g and resuspended in 2 ml medium containing 10 % FBS before the cells are counted in a Coulter® Microdiff 18 cell counter. The cells are kept in a humidified CO_2 incubator for further use.

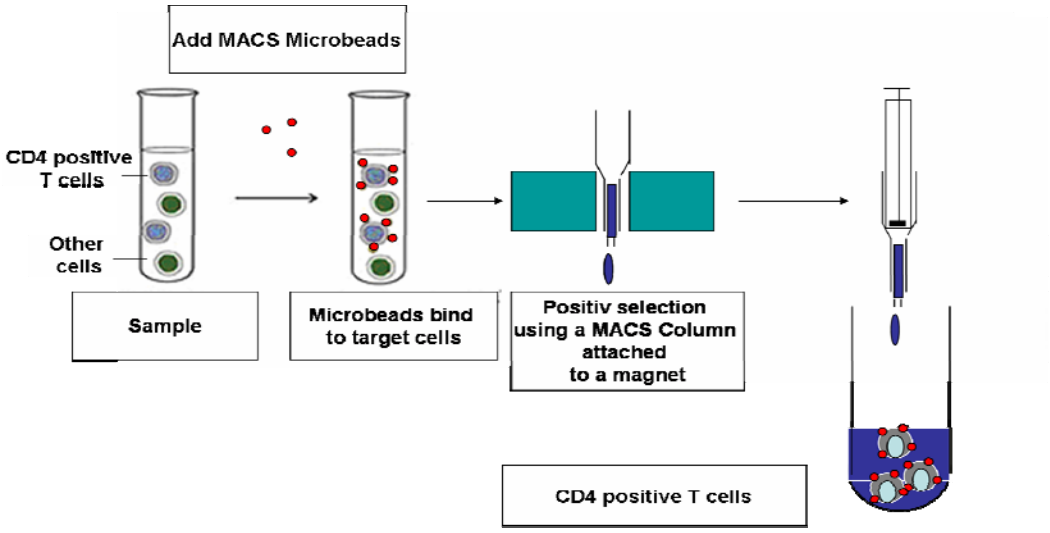


Figure 11: Isolation of CD4$^+$ T cells. MACS® CD4 MicroBeads are added to the lymphocyte fraction, positively selecting CD4$^+$ T cells. A LS MACS® Separation Column is placed on the magnetic field and buffer is pressed through the column with the stamp collecting the CD4$^+$ T cells.

3.3 Analysis of cell proliferation

Different methods can be used to assess proliferation. The number of cells can be directly measured by using a Bürcker chamber or a cell counter. Alternatively, proliferation can be indirectly measured by analyzing DNA synthesis.

3.3.1 Cell counting

An automatic cell counter (Coulter® Microdiff 18) determines the cell concentration by detecting and measuring changes in electrical resistance when a particle in a conductive liquid phase passes through a small aperture. As each cell goes through the aperture, it disrupts the current and causes a measurable pulse. The number of pulses equals the numbers of cells.

3.3.2 Measuring DNA synthesis

Cells incorporate thymidine into DNA when they are in S-phase, and uptake of radio-labelled thymidine can then be used to assess the percentage of cells in S-phase. Since cells in S-phase usually proceed to mitosis and divide, measuring uptake of radio-labelled thymidine in cells gives a good estimation of the extent of proliferation.

Equipment and solutions:	
96 well culture plate	[^{3}H] thymidine in RPMI solution
10 % FBS in RPMI/PS	Packard filtermate 196 harvester
Unifilter®-96, GF/C®	Scintillation counter (Packard TopCount™)
Microscint™	CO_2 incubator (Nuaire™)

Procedure:

Medium and stimulants are distributed in a 96 well plate before the cells are added to each well to a total volume of 200 µl. After incubation for 72 h, 50 µl of the [^{3}H] thymidine solution is added to the wells, and the cells are incubated for another 24 h. The cells are then harvested onto a filter by using a cell harvester and according to the manufacturer's manual. DNA synthesis is measured in the scintillation counter Packard TopCount™.

3.4 Induction of DNA damage

Double strand DNA damage can be induced in different ways. We have used two different methods, γ-irradiation and chemotherapeutic agents, respectively.

3.4.1 *γ* –irradiation of cells

To induce double strand breaks the cells are exposed to ionizing radiation (IR). When IR causes double strand breaks (DSB), the local change in chromatin topology initiate

autophosphorylation at serine 1981 (Ser1981) of the ATM dimers, dissociating the dimeric ATM into monomers competent to bind and phosphorylate their targets, amongst them CHK2 and p53. IR is induced by radiation from a 137 Cs source.

Equipment and solutions:	
Gamma-cell® 3000 Elan irradiator	12-well culture plate
15 ml Falcon tubes	CO_2 incubator (Nuaire™)

Procedure:

The EBV, B or T cells (0.7-2 x 10^6 cells/ml) are exposed to 10 Gray (Gy) in the 137 Cs Gamma-cell® 3000 Elan-apparatus, each sample in a 15 ml Falcon tube. Following γ-irradiation, 0.5-1 ml of each sample is transferred to a 12-well culture plate, before the cells are incubated for 18-20 h in a humidified CO_2 incubator (Nuaire ™).

3.5 Cell death

3.5.1 PI staining of cells

To distinguish between dead and viable cells, the DNA-binding dye propidium iodide (PI) can be used. Viable and early apoptotic cells exclude PI due to an intact cell membrane, whereas necrotic and apoptotic cells with leaky membranes take up PI and will stain the DNA. PI-staining of the cells is detected as red fluorescence in a flow cytometer equipped with an argon laser.

Equipment and solutions:
Cryo tubes
Propidium iodide (1.5 mM) in dH_2O
FACSCalibur (Flow cytometer)

Procedure:

All handling of PI should be done under protection from light.

Cells are transferred to polystyrene round bottom tubes, PI is added to a final concentration of 20 µg/ml, and the tubes are kept dark in room temperature for 10 min. By using the flow cytometer according to the manufacturers' manual, the samples can be analysed for red fluorescence (PI-staining).

3.5.2 Scatter analysis

A fluorescence activated cell sorter (FACS) can be used to measure the distribution of viable and dead cells. FACS analyses are based on optical properties of single cells passing through a focused laser beam. As the cells pass through the laser beam, they disrupt and scatter the laser light, detected as forward- and side scatter (FSC and SSC respectively). The side scatter is an indicator of the cell's granularity, whereas the forward scatter is related to cell size. Combined, these parameters can distinguish viable cells from apoptotic or necrotic cells. Apoptotic cells are smaller and more granular than viable cells due to shrinkage of the cells, chromatin condensation, fragmentation of nuclei and the formation of apoptotic bodies. Necrotic cells will be larger than viable cells due to swelling. Analysis of five hundred cells counted per second, with a total of 10 000 cell counted from each sample provides a statistically significant picture of the physical and biochemical state of the sample. The flowcytometer is used according to the manufacturer's manual.

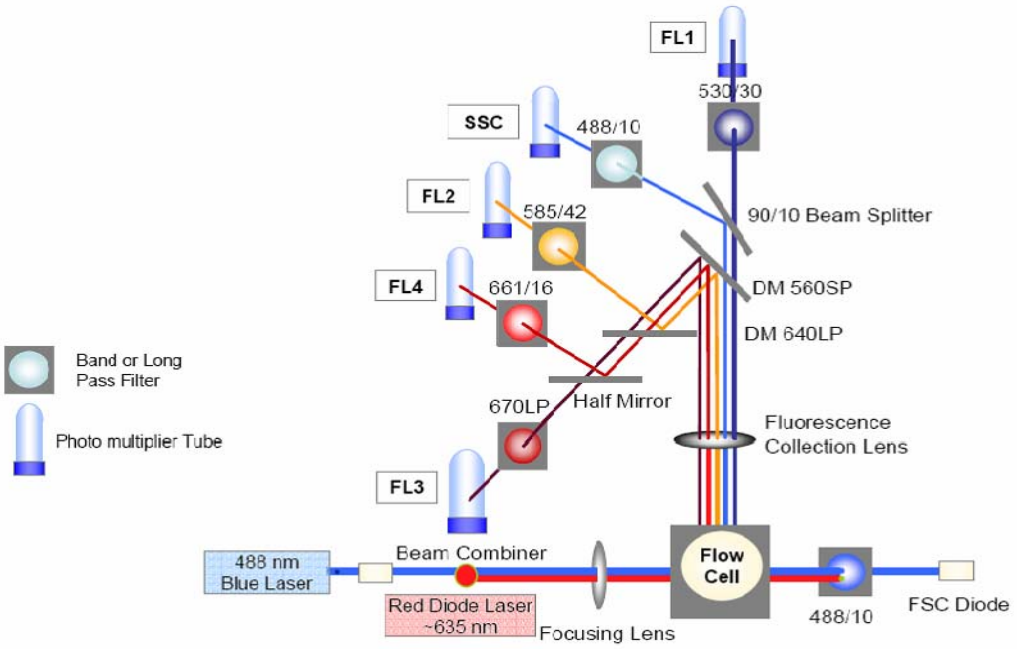


Figure 13: Flow cytometry, **FACSCalibur.** Single cells pass through a focused laser beam, and as they pass through the laser beam, they disrupt and scatter the laser light, detected as forward- and side scatter Picture from manufacturer's manual.

3.6 Analysis of protein expression by Western Blot

Western blot analysis detects and quantifies specific proteins in a sample of tissue homogenate or extract. After the protein samples are solubilised by the use of detergents, it is denatured by boiling. Following separation of the proteins by electrophoresis on a polyacrylamide gel, the proteins are transferred from the gel and onto a nitrocellulose membrane where they are hybridised using antibodies specific to the antigenetic epitopes of the protein. By adding a secondary antibody conjugated to horseradish peroxidase (HRP) that catalysis the oxidation of lumniol, the primary antibody bound to the specific protein can be detected by chemiluminescence and autoradiography.

3.6.1 Preparation of cell lysates for electrophoresis

The purpose of the lysis is to solubilize all of the target antigens in a form that is immunoreactive and undegraded. Several methods of solubilization lead to the release of intracellular proteases that may digest the target protein. Therefore, inhibitors of proteases are included in the detergent lysis buffer, and the extract is to be kept on ice to prevent protein degradation.

Equipment and solutions:
Vortexer
1 % FBS in PBS
RIPA buffer

RIPA buffer:

1.25 ml Tris Hcl pH 8.0 (1 M)	2.5 ml NaF (0.5 M)
750 µl NaCl (5 M)	250 µl β-glycerophosphate (1 M)
50 µl Triton	250 µl Na_3VO_4
250 µl NaDeoxycholate (1 %)	Distilled water to 20 ml
250 µl SDS (10 %)	Stored at -20˚C without aprotinin
50 µl PMSF (0.1 M)	5 µl Aprotinin added pr ml RIPA
25 µl Leupeptin (10 µg/ml)	The buffer is kept on ice when in use.

Procedure:

Cells are collected by centrifugation and washed with 500 µl PBS containing 1 % FBS. The cells are resuspended in the appropriate amount of Radio Immuno Precipitation Assay (RIPA) buffer (depending on the number of cells and size of the pellet), and incubated on ice for 20 min, while vortexed every 5 min. The cell membranes are collected by centrifugation at 13 800 x g for 20 min at 4˚C. The supernatant is transferred to a new tube, flash frozen in liquid N and stored at -70˚C.

3.6.2 Determination of protein concentration

The Bio-Rad assay is used to quantify the concentration of proteins in the cell lysate. Binding of the protein to Coomassie brilliant blue G250 results in a shift in the absorption maximum of the dye from 465 to 595 nm, and this can be monitored by using a spectrophotometer. The absorbance of the sample is compared to a standard curve where the known protein concentration is set by gamma globulin (γ-globulin).

Equipment and solutions:	
Eppendorf tubes	Disposable cuvettes (1.5 ml)
Bio-Rad protein dye: dH_2O (1:1)	Spectrophotometer, Ultrospec 3000
1 mg γ-globulin in 1ml dH_2O	

Procedure:

Preparation of standards:

RIPA buffer (2.5 µl) is added to standards of γ-globulin (2-20 µl) in a total volume of 600 µl water. Two parallels of each standard are made.

Preparation of samples:

The samples are prepared in parallels by adding 2.5 µl of lysate to a total volume of 600 µl water.

400 µl of Bio-Rad protein dye is added to all the standards and samples, before they are transferred to disposable cuvettes. The absorbance at 595 nm is measured in a spectrophotometer according to the manufacturer, within 30 min. The protein concentrations in the lysate are then estimated from an obtained standard curve.

3.6.3 Preparing a polyacrylamide gel

Acrylamide is the basic element of the gel, and when ammonium persulfate (APS) is added, the free radicals generated initiate a chain reaction that results in the formation

of long chains of acrylamide from the initial monomers. As N,N-methylenebisacrylamide (Bis) cross-bind the chains, the physical properties of the gel and its pore size are controlled by the proportion of polyacrylamide in the gel and its degree of cross linking.

Equipment and solutions:	
SDS polyacrylamide gel (10 %)	Bio-Rad Mini Protean Π gel apparatus

SDS polyacrylamide gel (10 %):	
Separating gel:	**Stacking gel:**
3.3 ml acrylamide	1.3 ml acrylamide
4 ml dH_2O	6.1 ml dH_2O
2.5 ml Tris-Hcl pH 8.8 (1.5 M)	2.5 ml Tris-Hcl pH 6.8 (0.5 M)
100 µl SDS (10%)	100 µl SDS (10%)
50 µl APS	50 µl APS
5 µl TEMED	10 µl TEMED

Procedure:

Polyacrylamide gels were prepared according to the manual from Bio-Rad. For identification of p53, 10 % separating gels are used.

3.6.4 Separation of proteins by SDS-PAGE

SDS-PAGE (polyacrylamide gel electrophoresis) is a technique used to separate proteins on the basis of their size, as they migrate differently through a polyacrylamide gel in an electric field. Before loading the proteins onto a gel, 30 µg of protein from each lysate is transferred to eppendorf tubes. To denaturise the proteins they are boiled with the strong anionic detergent SDS in combination with the reducing agent β-mercaptoethanol. The denaturised polypeptides bind SDS and become negatively charged. The proteins then can be separated according to size.

Equipment and solutions:	3 x SDS sample buffer
Electrophoresis buffer	Heat block
Molecular weight standard	Syringe

10 X Electrophoresis buffer:	50 g SDS
4 g Tris (Base)	Distilled water to 5 litre
7 g Glycine	Diluted 1:10 when used

3X SDS sample buffer:	
583 µl Tris-HCl pH 6.8 (1.5 M)	125 µl β-mercaptoethanol
2.57 ml SDS (10%)	818 µl dH_2O
904 µl Glycerol (80%)	5-10 µg Bromphenol Blue

Procedure:

The samples were prepared by dissolving equal amounts of proteins (20-40 µl cell lysate) in 3 x SDS sample buffer to a final concentration of 1 x SDS, boiled for 5 min and applied to the gel with a syringe. A molecular weight standard is applied to the gel to visualize the migration of proteins of various sizes. The gel is run at 200 V, constant voltage, and the separation time depends on the size of the protein. For p53 analysis (53 kDa) the gel was run for approximately 45 min.

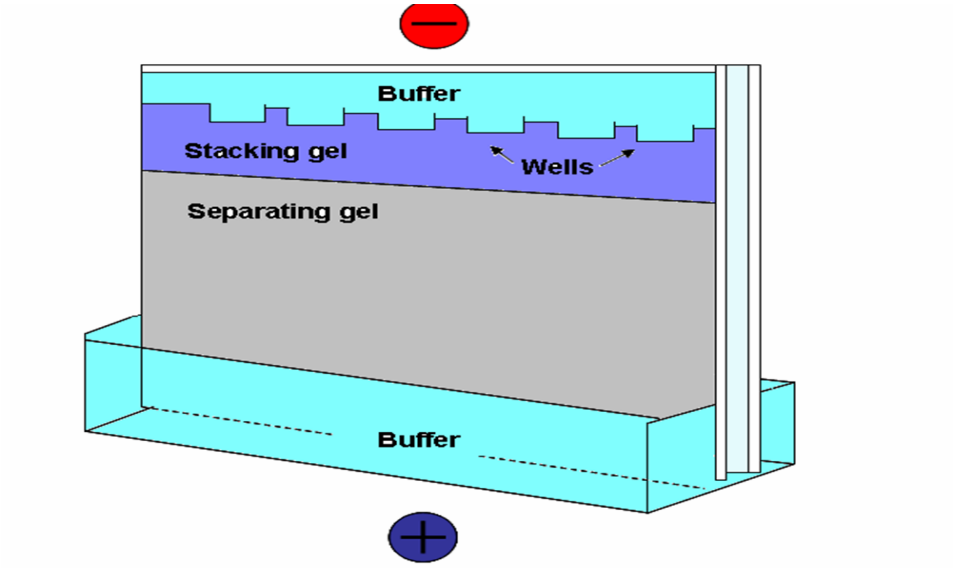


Figure 14: SDS Polyacrylamide gel electrophoresis. See text for details.

3.6.5 Transfer of proteins from gel to nitrocellulose membranes

The western blot analyses were carried out by direct electrophoretic transfer of proteins from the gel to a nitrocellulose membrane. Nitrocellulose bind proteins strongly and non-specifically, and the specific proteins can be visualized using highly selective antibodies. In our experiments we used a semi-dry transfer apparatus with plate electrodes in a horizontal configuration.

Equipment and solutions:	
Hybond ECL Nitrocellulose membrane	Trans-Blot ® SD Semi-Dry Transfer cell
Whatman 3 MM paper	Transfer buffer

Transfer buffer:	3.75 ml SDS (10 %)
5.82 g Tris Hcl	200 ml methanol
2.93 g Glycine	Distilled water to 1 litre

Procedure:

Following electrophoresis, the stacking gel is removed, and the gel is equilibrated in transfer buffer. Six pieces of Whatman paper and one piece of nitrocellulose filter is cut at the exact size of the SDS-gel and soaked in transfer buffer. 3 pieces of Whatman paper is placed in the transfer cell, the membrane is put on top of the papers, and the gel is put on top of the nitrocellulose membrane. Finally, the last 3 Whatman papers are placed on top of the gel. Hence, the gel and the membrane are placed between the buffer-soaked filter papers, which serve as the ion reservoir and replace the buffer tank. Air bubbles are removed by rolling a glass tube over the sandwich. The cathode is placed onto the stack, and the proteins are transferred from the gel to the membrane at 17 V for 25 min.

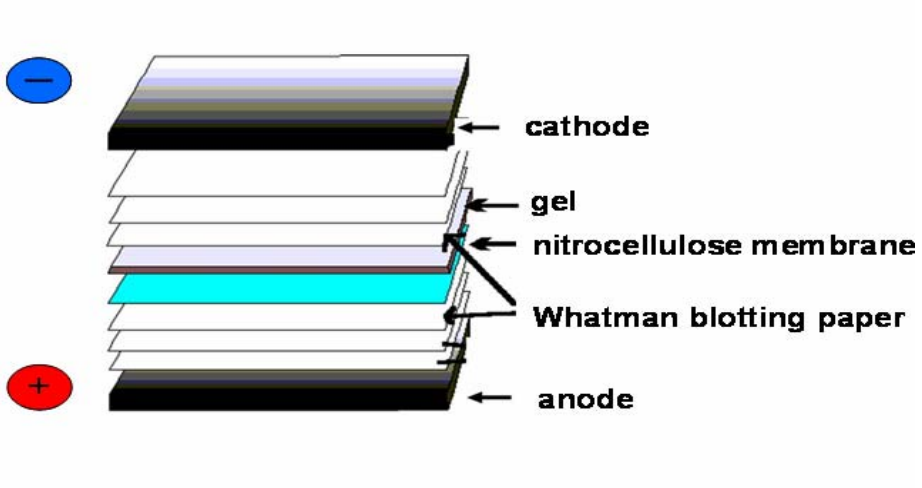


Figure 15: Semi-dry transfer of proteins from gel to nitrocellulose membrane.

3.6.6 Staining of proteins immobilised on nitrocellulose filters

As control of equal loading, the amount of total protein in each lane is visualized by incubating the membrane in Ponceau solution. The red dye binds to the proteins and visualizes the amount of protein added to each lane.

Equipment and solutions:	TBS-Tween
Ponceau red solution	dH_2O

Ponceau red solution:	5% acidic acid
0.1% Ponceau S	dH_2O

Procedure:

The nitrocellulose membrane is incubated in Ponceau solution for 2 min before washing with distilled water to visualize the amount of protein in each lane. Before further processing of the membrane, the colour is removed by incubating the membrane in TBS-Tween for 2 x 2 min.

Figure 16: Ponceau-staining of proteins.

3.6.7 Blocking unspecific binding of antibodies to the nitrocellulose membrane

The sensitivity of the Western Blot analysis depends on reducing the background of non-specific binding of antibodies, by blocking potential binding sites with irrelevant proteins. The best and least expensive blocking-solution is based on non-fat dried milk.

Equipment and solutions:
8 % Nestle Molico non-fat dried milk in TBS-Tween
Platform shaker

Procedure:

The membrane is incubated in 8 % non-fat dried milk in TBS-Tween for 45 min at room temperature on a platform shaker, or in 4˚C over night with gentle agitation. Excess blocking solution is washed away with TBS-Tween.

3.6.8 Hybridisation of the filter with antibodies

The proteins on the nitrocellulose membrane are hybridised in two stages. First, the blot is incubated for 45 min in a solution of an unlabeled antibody specific to the target protein in the presence of the milk/TBS-Tween reagent. After washing the blot with TBS-Tween for 3 x 10 min, the blot is incubated with a solution of anti-

immunoglobulin coupled to HRP. After further washing, the antigen-antibody complex is identified by chemiluminescence and autoradiography.

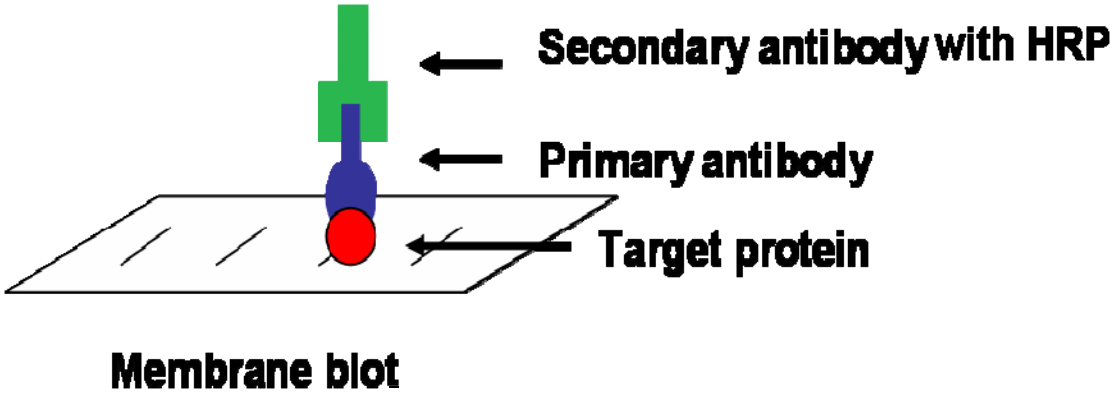


Figure 17: Antibodies are used to detect target protein.

Equipment and solutions:	TBS-Tween
Primary antibody solution	Rock-n-roller
Secondary antibody solution	Platform shaker

Procedure:

The blot is incubated with the primary antibody solution for 1.5 h at room temperature, or at 4˚C over night on a rock-n-roller. After washing the blot for 1 x 10 min and 2 x 5 min in TBS-Tween on a platform shaker, the blot is incubated with the secondary antibody solution for 45 min at room temperature. After washing the membrane for 1 x 10 min and 3 x 5 min in TBS-Tween, the protein is visualised by chemiluminescence and autoradiography.

3.6.9 Visualisation of specific proteins by chemiluminescence and autoradiography

The secondary antibodies are coupled to HRP. The "ECL Plus™ Western blotting detection" system from Amersham Pharmacia Biotech provides a method for detection of immobilized antigens conjugated to HRP-labelled antibodies. HRP catalyses the oxidation of Lumigen PS-3 acridan substrate, generating thousands of

acridinium ester intermediates per minute. These intermediates interact with peroxide under slight alkaline conditions to produce a sustained, high intensity chemiluminescence with maximum emission at 430 nm.

Equipment and solutions:	Hyperfilm™
ECL Plus™ Western blotting detection system	Film cassette
Kodak X-omat 1000 processor	plastic wrap

ECL Plus™ Western blotting detection system:
Reagent A (ECL plus substrate solution containing Tris buffer)
Reagent B (Stock Arcidan solution in Dioxane and Ethanol)
Reagent A and B are mixed (1:40), 1 ml reaction fluid is used for each membrane.

Procedure:

After draining excess TBS-Tween, the membrane is placed with the protein side upwards on a piece of plastic wrap. The blot is incubated in the mixture of A and B reagents for 5 min at room temperature before excess detection fluid is drained off. The membrane is wrapped in plastic wrap and is in the dark room placed in a film cassette with the protein side against a sheet of Hyperfilm™. The film is exposed to the membrane for 1 second up to 30 min depending on the strength of the signal. The exposed film is run through an automatic film developer, and the protein recognized by the primary antibody will appear as black lines on the film.

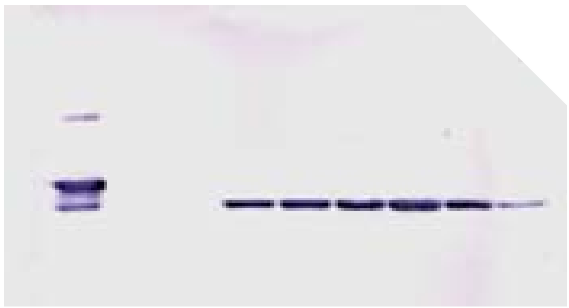


Figure 18: The proteins appear on the film after chemiluminescence and autoradiography.

4. Results

4.1 Regulation of proliferation of lymphoid cells by RA

Conventional cancer treatments like γ-irradiation and chemotherapy is based on DNA damage responses, and it is well established that many chemotherapeutic agents depend on ongoing DNA synthesis to be effective (34;35;37). We have previously shown that elevation of cAMP inhibits DNA regulation and thereby also inhibits DNA damage induced by γ-irradiation and chemotherapy (72;74). Since our lab previously also has shown that retinoic acid stimulates and inhibits DNA synthesis and proliferation of normal T- and B cells, respectively (65;74), we anticipated that retinoic acid might regulate DNA damage responses differently in B- and T cells. However, we first had to confirm that retinoic acid indeed regulated the DNA synthesis in these cells.

4.1.1 RA reduces proliferation of SAC stimulated B cells in a dose-dependent manner

B lymphocytes in peripheral blood exist in a resting phase (G0), and they remain in this phase of the cell cycle also after isolation by the use of magnetic Dynabeads (73) *In vivo*, B cells can be activated by specific antigens, and together with growth factors the cells will enter S-phase, proliferate and finally mature into plasma cells (41). *In vitro*, this activation can be mimicked by stimulating the B cells with anti-immunoglobulins or various lectins known to crosslink the surface immunoglobulins. Staphylococcus Aureus Cowan (SAC) is a crude bacterial cell suspension that activates B cells via lectins at the bacterial surface (41). We wished to see how RA affected the proliferation of B cells, and we used forskolin to compare the effect of RA. Proliferation was indirectly measured as DNA synthesis based on incorporation of [^{3}H] thymidine into DNA in S-phase.

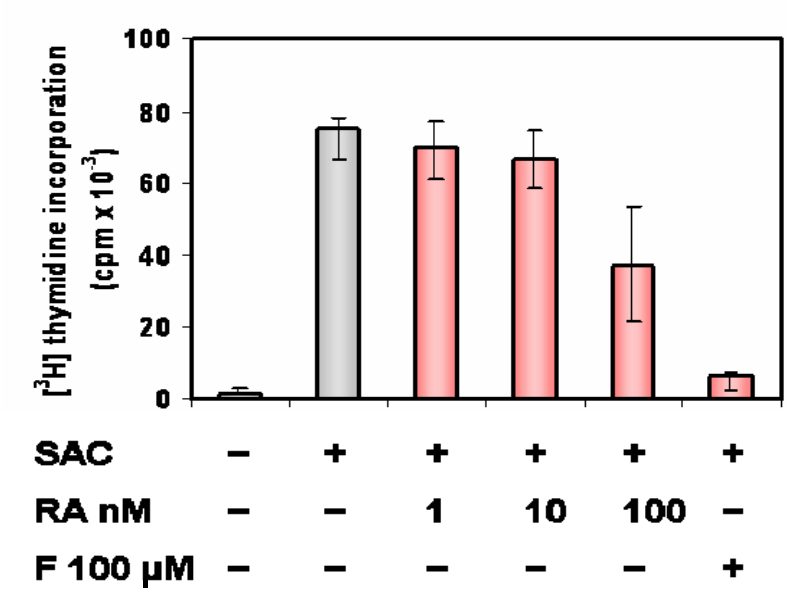


Figure 19: Effect of RA and forskolin on proliferation of B cells

Normal human B cells were isolated from buffycoats, as described in Materials and Methods. Cells (100 000/well) were stimulated with SAC (1:20000), RA and forskolin as indicated for 72 h. [^{3}H] thymidine was added, and DNA synthesis was measured as described in Materials and Methods. The values represent the median of triplicates, and the vertical bars represent the range of the triplicates in the experiment.

As can be seen in figure 19, RA reduced the proliferation of SAC-stimulated B cells in a concentration-dependent manner. As compared to the 50 % reduced DNA synthesis in the presence of 100 nM RA, forskolin reduced the proliferation of the cells by 90 %.

4.1.2 RA does not inhibit DNA damage-induced apoptosis in normal B cells isolated by overnight detachment

Based on the inhibiting effect of RA on DNA synthesis in B cells, we wished to examine if RA would reduce apoptosis induced by DNA damage. To induce DNA damage in B cells, we exposed the cells to γ-irradiation at 10 Gy.

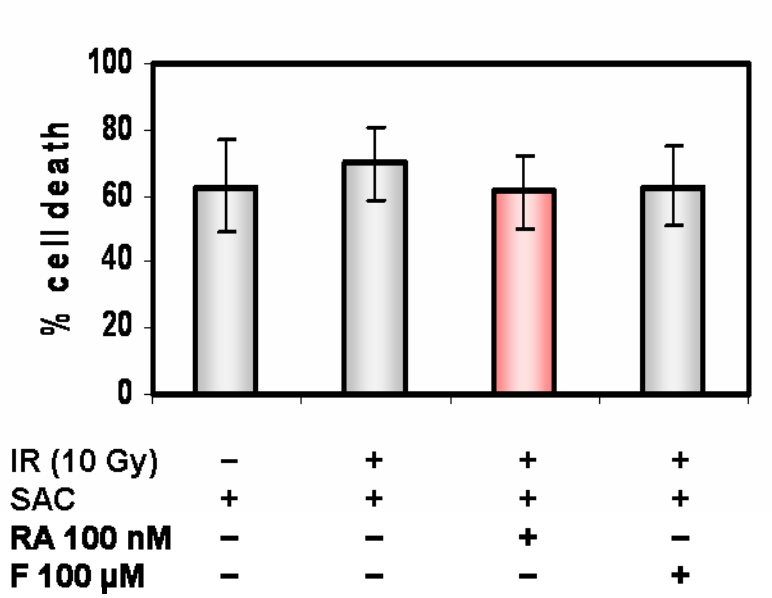


Figure 20: Effect of RA on DNA damage-induced apoptosis in normal B cells isolated by overnight detachment

Normal B cells were isolated from buffycoats by overnight detachment of the cells from the Dynabeads as described in Materials and Methods. Cells (1.5 x 10^6 cells/ml) were stimulated with SAC (1:20000) for 22 h, and RA (100 nM) or forskolin (100 µM) was added 2 h before IR (10 Gy). 20 h after IR, apoptosis was analyzed by uptake of propidium iodine as described in Materials and Methods (n = 2, mean ± range).

As shown in figure 20, the viability of B cells isolated by detachment from Dynabeads over night was low. After culturing the cells for 20 h, 63 % of the cells were dead. γ-irradiation did not markedly increase the percentage of apoptotic cells, and RA or forskolin did not reduce this cell death. We could conclude that overnight detachment of the cells was not a suitable method for isolating B cells for analysing cell death.

4.1.3 Effect of RA on DNA damage-induced apoptosis in normal B cells isolated by Detachabeads

Since B cells isolated by allowing the cells to detach from the Dynabeads over night had low viability, we wished to isolate the cells by an alternative method based on DETACHaBEAD®CD19. This method rapidly detaches the beads from the cells.

Again we analyzed the viability of the cells before and after induction of DNA damage by γ-irradiation, and the effect of RA on this process was assessed.

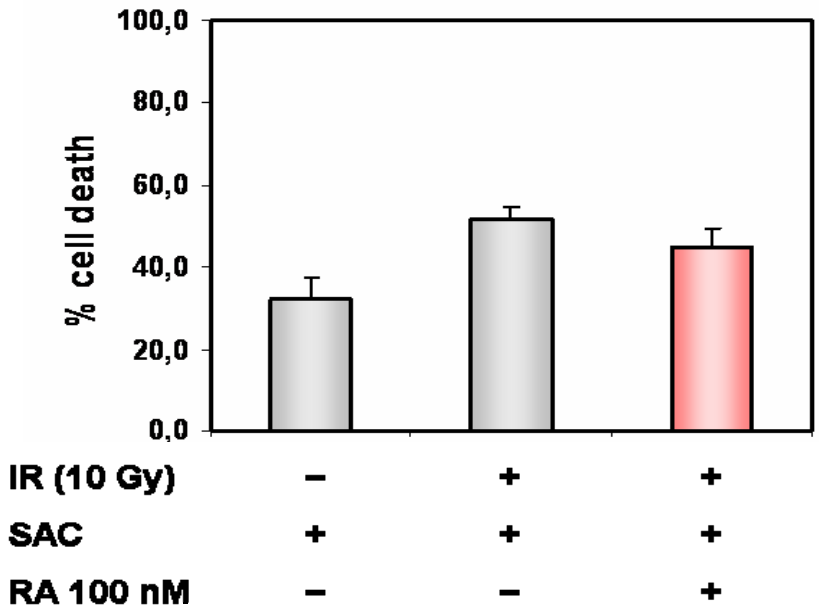


Figure 21: Effect of RA on DNA damage-induced apoptosis in normal B cells isolated by the detachabead method

Normal B cells were isolated from buffycoats by the use of the detachabead method as described in Materials and Methods. Cells (1.5 x 10^6 cells/ml) were stimulated with SAC (1:20000) for 22 h. and RA was added 2 h before IR (10 Gy). 20 h after IR, apoptosis was analyzed by uptake of propidium iodine as described in Materials and Methods (n= 4, mean ± SEM).

As shown in figure 21, the Detachabead method improved the viability of the isolated B cells as compared with cells isolated by overnight detachment. Thus, the percentage of apoptotic cells after 20 h of culturing was reduced to 32 %. DNA damage induced by γ-irradiation increased the percentage of the apoptotic cells to 50 %, and RA had a small, but reproducible inhibiting effect on the DNA damage-induced apoptosis. However, since the effect was only marginal, we concluded that we did not wish to further study the effect of RA in this cell system.

4.1.4 RA has no effect on DNA damage-induced cell death in Epstein-Barr virus transformed B cells

It has been shown that RA inhibits the proliferation of Epstein-Barr virus (EBV) transformed B cells (75). EBV transformation of B cells is a common method for making cell lines of normal B cells, and in our lab we have recently shown that forskolin inhibits DNA damage-induced apoptosis of EBV^+ B cells (Kloster et al 2008, journal in press). We used one of the cell lines established in our lab to measure the effects of RA on DNA damage-induced apoptosis. DNA damage was again induced by γ-irradiation.

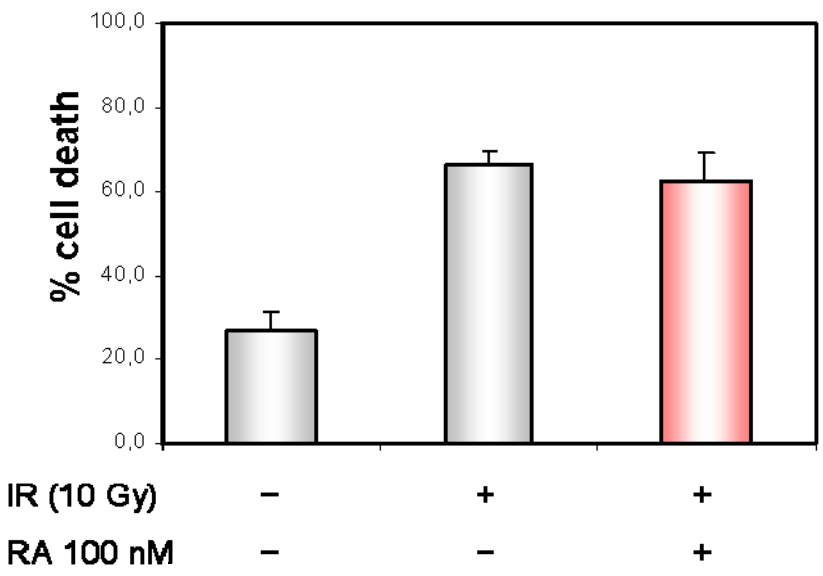


Figure 22: Effect of RA on DNA damage-induced apoptosis in EBV^+ B cells

RA was added to EBV^+ B cells (0.7 x 10^6 cells/ml) 24 h prior to IR (10 Gy). 20 h after IR, apoptosis was analyzed by uptake of propidium iodine as described in Materials and Methods (n = 3, mean ± SEM).

As shown in figure 22, γ-irradiation of the cells enhanced the cell death from 27 % to 66 %. RA had only a marginal effect on the DNA damage-induced cell death. From the results on normal B cells and EBV^+ B cells we could conclude that RA did not have profound effect on DNA damage-induced apoptosis in B cells.

4.1.5 RA potentiates T cell proliferation

We now wished to elucidate the effects of RA on DNA damage-induced cell death in T cells. Since previous results from our lab has shown that RA enhances proliferation of T cells (65), we anticipated that RA might enhance DNA damage-induced apoptosis. First, however we had to confirm prior results in our lab on the effect of RA on DNA synthesis in normal human T cells. Phytohemagglutinin (PHA) is a plant lectin, especially abundant in beans, and it is known to stimulate T cells to proliferate by crosslinking the T cell receptor (40). Normal T cells were isolated from peripheral blood, and the effect of RA was measured in PHA-stimulated cells.

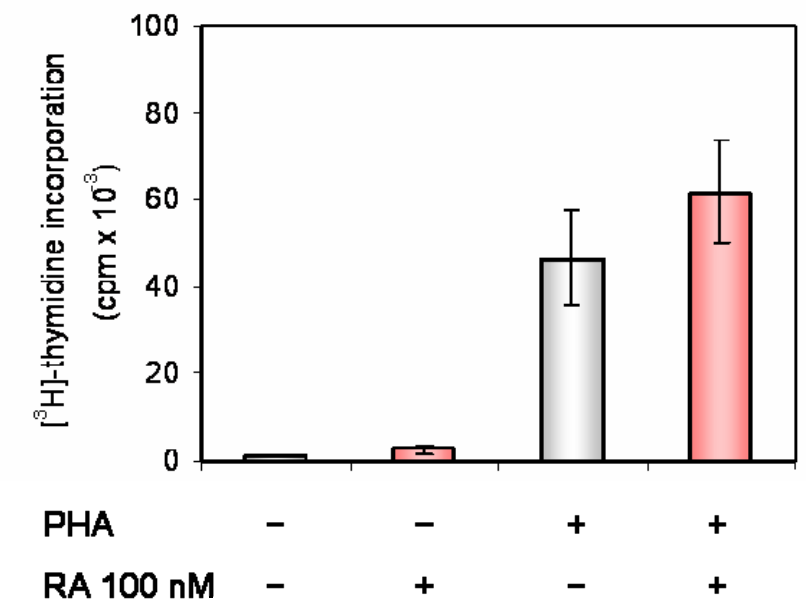


Figure 23: Effect of RA on T cells stimulated with PHA

Normal human T cells were isolated from buffycoats, as described in Materials and Methods. Cells (100 000/well) were stimulated with PHA (5µg/ml) in the presence or absence of RA for 72 h. [^{3}H] thymidine was added, and incorporation was measured as described in Materials and Methods. The values represent the median of triplicates (± range).

As shown in figure 23, RA enhanced the DNA synthesis of PHA-stimulated T cells by 32 % as compared to untreated cells. The stimulatory effect of RA on proliferation of human T cells was therefore confirmed.

4.1.6 RA reduces DNA damage-induced apoptosis in PHA-stimulated T cells

Having confirmed that RA potentiates proliferation of PHA-treated T cells, we now elucidated the effect of RA on DNA damage-induced apoptosis. To induce DNA damage, the cells were γ-irradiated. Since we had previously shown that forskolin inhibits DNA damage-induced apoptosis (Kloster et al 2008, journal in press), we included forskolin as a control.

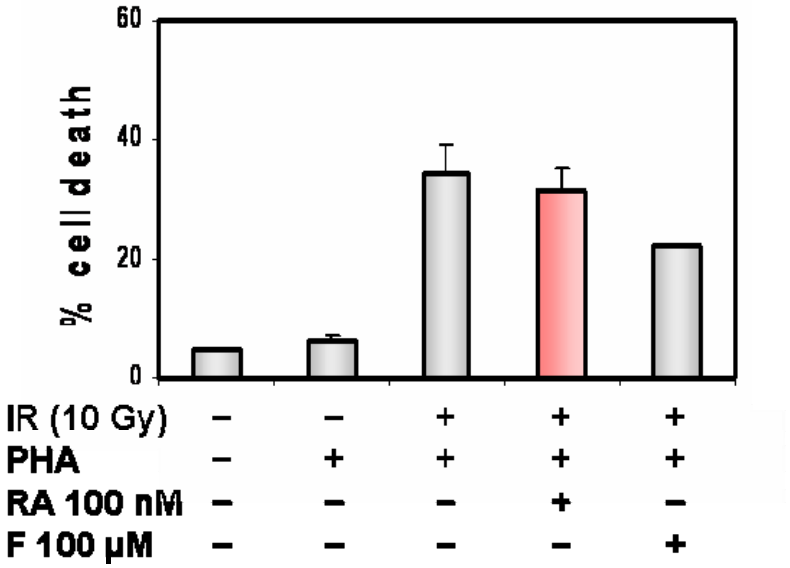


Figure 24: Effect of RA and forskolin on DNA damage-induced apoptosis in T cells stimulated with PHA

Normal human T cells were isolated from buffycoats, as described in Materials and Methods. Cells (2.0 x 10^6 cells/ml) were stimulated with PHA (5µg/ml) for 22 h, and RA or forskolin was added 2 h before IR (10 Gy). 20 h after IR, apoptosis was analyzed by uptake of propidium iodine as described in Materials and Methods (n =5, mean ± SEM).

As shown in figure 24, the viability of the isolated T cells was high, and γ-irradiation enhanced the apoptosis from 5 to 38 %. There was a small, but reproducible inhibitory effect of RA on DNA damage-induced apoptosis. Since we had expected that RA would enhance rather than inhibit DNA damage-induced apoptosis, the findings were unexpected. We therefore decided to proceed the results.

4.1.7 The effect of RA on DNA damage-induce apoptosis is time-dependent

Previous work in our lab had proven that RA had to be added to the T cells early during stimulation in order to enhance the proliferation (65). We therefore investigated if this was the case for RA on DNA damage-induced apoptosis in T cells. Thus, we added RA to T cells 2 h or 24 h prior to γ-irradiation.

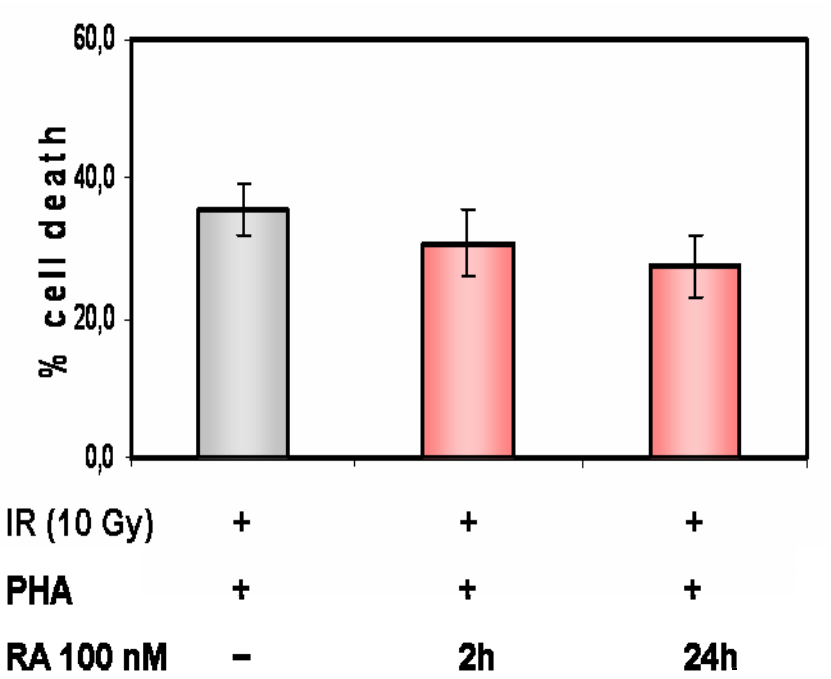


Figure 25: The kinetics of the effect of RA on DNA damage-induced apoptosis in PHA stimulated T cells

Normal human T cells were isolated from buffycoats, as described in Materials and Methods. Cells (2.0 x 10^6 cells/ml) were stimulated with PHA (5µg/ml) for 22 h, and RA or forskolin was added 2 h or 24 h prior to IR (10 Gy). 20 h after IR apoptosis was analyzed by uptake of propidium iodine as described in Materials and Methods (n = 2, ± range).

As shown in figure 25, there was a tendency that the effect of RA on DNA damage-induced apoptosis was stronger when RA was added to the cells 24 h before γ-irradiation as compared to 2 h prior to γ-irradiation. Based on this result, RA was added 24 h before γ-irradiation in the remaining experiments.

4.1.8 The phorbol ester TPA potentiates T cell proliferation

Previous results in our lab had indicated that RA had a stronger stimulatory effect on T cells stimulated with the phorbol ester tumor 12-O-tetradecanoylphorbol 13-acetate (TPA), than cells stimulated with PHA (Å. Ertesvåg, personal communication). TPA induces proliferation of many cell types by mimicking diacylglycerol to stimulate protein kinase C (PKC) (76). We wished to examine if RA would affect DNA damage-induced apoptosis differently in cells stimulated with TPA than cells stimulated by PHA. Again we had to confirm prior results in our lab on effects of RA on DNA synthesis of the cells before DNA damage could be analyzed. Thus, normal T cells were isolated from peripheral blood, and the effect of RA on proliferation was measured in TPA-stimulated cells.

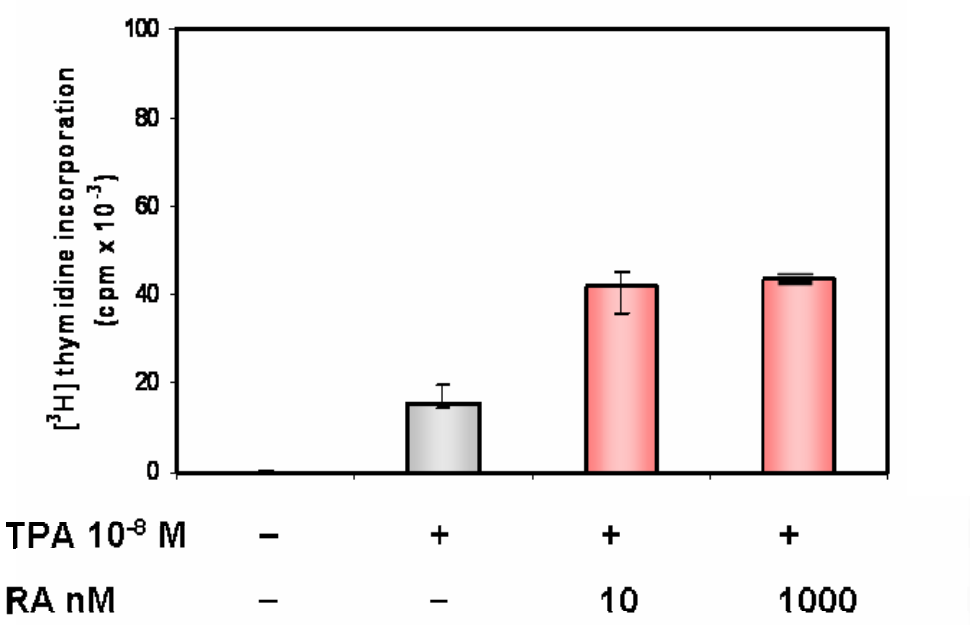


Figure 26: Effect of RA on proliferation of T cells stimulated with TPA

T cells were isolated from buffycoats, as described in Materials and Methods. Cells (100 000/well) were stimulated with or without TPA, in the presence or absence of different concentrations of RA for 24 h. [^{3}H] thymidine was added, and DNA synthesis was measured as described in Materials and Methods. The values represent the median of triplicates (± range).

As shown in figure 26, RA enhanced the DNA synthesis of TPA-stimulated T cells. The stimulatory effect of RA on proliferation of TPA-stimulated T cells was therefore confirmed.

4.1.9 RA inhibit DNA damage-induced apoptosis in TPA stimulated T cells

Having confirmed that RA potentiates proliferation of TPA-treated T cells, we now wished to investigate how RA affected DNA damage-induced apoptosis.

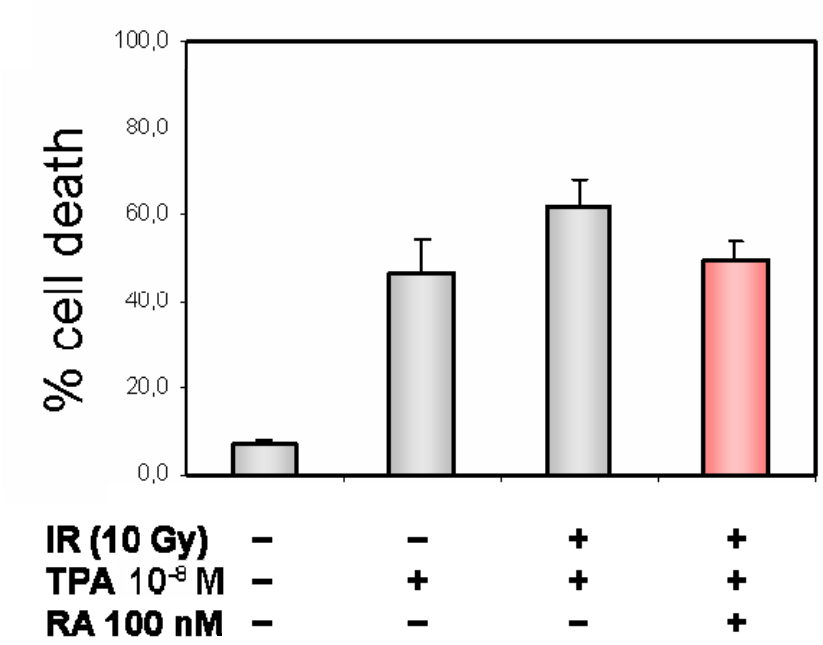


Figure 27: Effect of RA on DNA damage-induced apoptosis in T cells stimulated with TPA

Normal human T cells were isolated from buffycoats, as described in Materials and Methods. Cells (2.0 x 10^6 cells/ml) were stimulated with TPA and RA for 24 h prior to IR (10 Gy). 20 h after IR, apoptosis was analyzed by uptake of propidium iodine as described in Materials and Methods (n = 9, mean ± SEM).

As shown in figure 27, TPA alone induced apoptosis in 45 % of the cells. Irradiation enhanced this apoptosis to 60 %, and RA reduced the cell death. However, the high percentage of cell death in TPA-treated cells was a problem, and we therefore wished to test lower concentrations of TPA.

4.1.10 Dose-respond of TPA

We now wished to examine how RA affects cell death induced in γ-irradiated T cells stimulated with different concentrations of TPA.

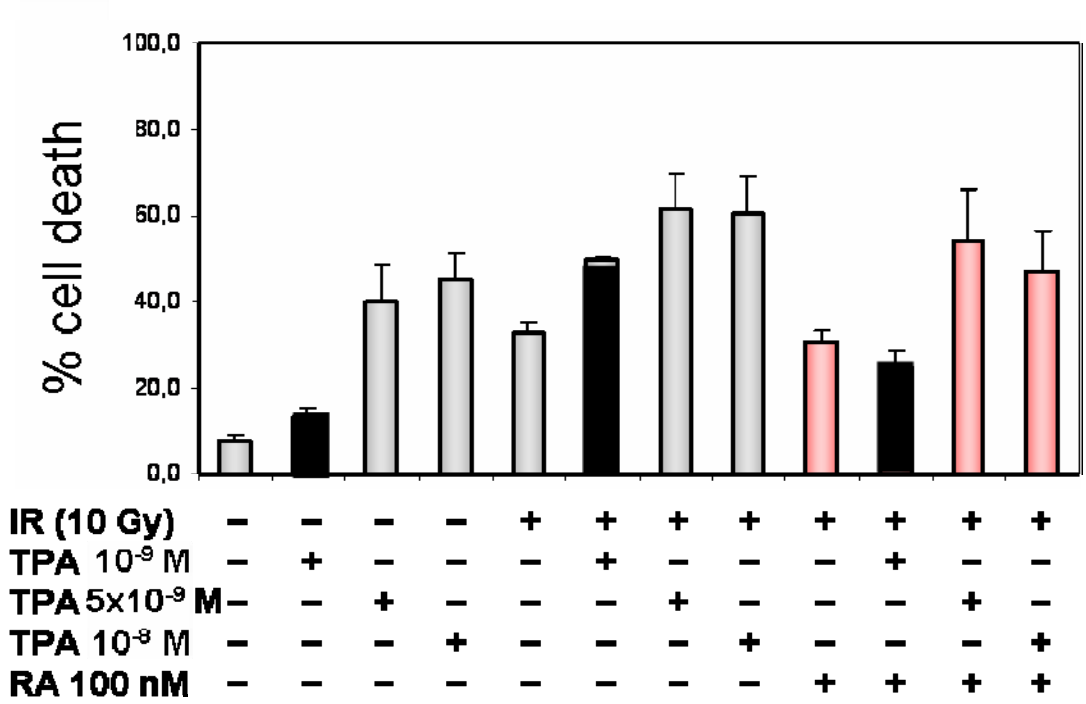


Figure 28: Effect of RA on DNA damage-induced cell death in T cells stimulated with different concentrations of TPA

Normal human T cells were isolated from buffycoats, as described in Materials and Methods. Cells (2.0x 10^6 cells/ml) were stimulated with or without different concentrations of TPA in the presence or absence of RA for 24 h prior to IR (10 Gy). 20 h after IR, apoptosis was analyzed by uptake of propidium iodine as described in Materials and Methods (n = 4, mean ± SEM). The black bars represent cells stimulated with 10^{-9} M TPA.

As shown in figure 28, only 15 % of the cells were dead when cultured in 10^{-9} M TPA alone, and the strongest effect of RA on DNA damage-induced apoptosis was obtained at this concentration of TPA. Thus, the cell death was reduced by approximately 50 % when RA was added to γ-irradiated cells stimulated with 10^{-9} M TPA as compared with only 12 % and 22 % reduction in γ-irradiated cells stimulated with TPA $5x10^{-9}$ M or 10^{-8} M respectively. In the remaining experiments, the cells were therefore stimulated with 10^{-9} M TPA.

4.1.11 Dose-respond and kinetics of RA

The concentration of *all-trans* RA in peripheral blood is ~ 10 nM (77). Previous work in our lab had shown that RA potentiate DNA synthesis of TPA-stimulated T cells in a concentration- and time dependent manner (65). First we therefore wished to elucidate how different concentrations of RA affected DNA damage-induced apoptosis in T cells stimulated with TPA.

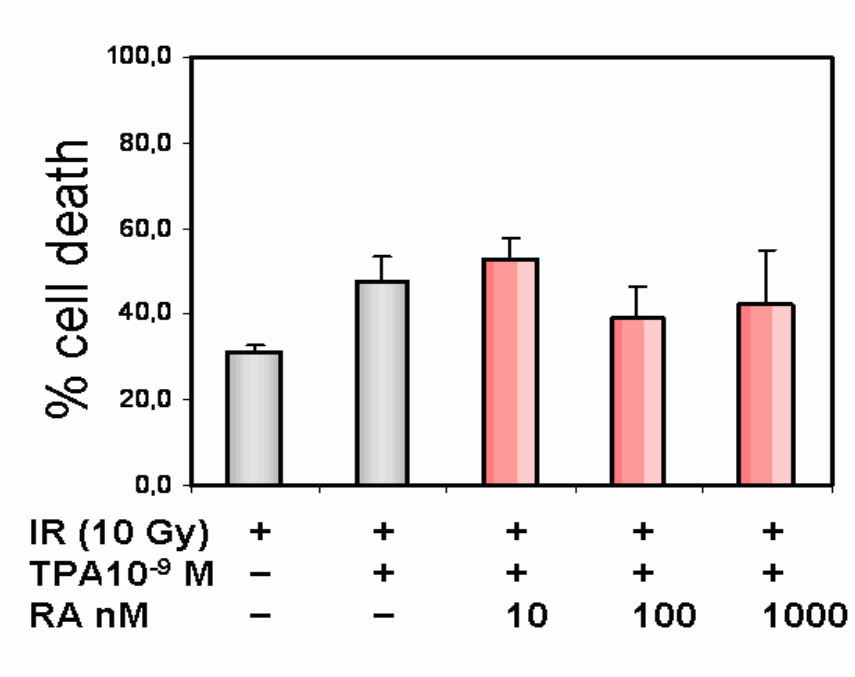


Figure 29: The effect of concentration of RA on DNA damage-induced cell death in T cells stimulated with TPA

Normal human T cells were isolated from buffycoats, as described in Materials and Methods. Cells (2.0 x 10^6 cells/ml) were stimulated with or without TPA in the presence or absence of different concentrations of RA 24 h prior to IR (10 Gy). 20 h after IR, apoptosis was analyzed by uptake of propidium iodine as described in Materials and Methods (n = 3, mean ± SEM).

As shown in figure 29, RA reduced DNA damage-induced cell death in a concentration dependent manner, the effect being optimal at 100 nM. This concentration was therefore used in the remaining trials.

4.1.12 The effect of RA on DNA damage-induced apoptosis is time-dependent

To elucidate if the time of addition of RA was critical for its inhibitory effect on DNA damage-induced apoptosis, RA was added to the cells 1-24 h prior to γ-irradiation.

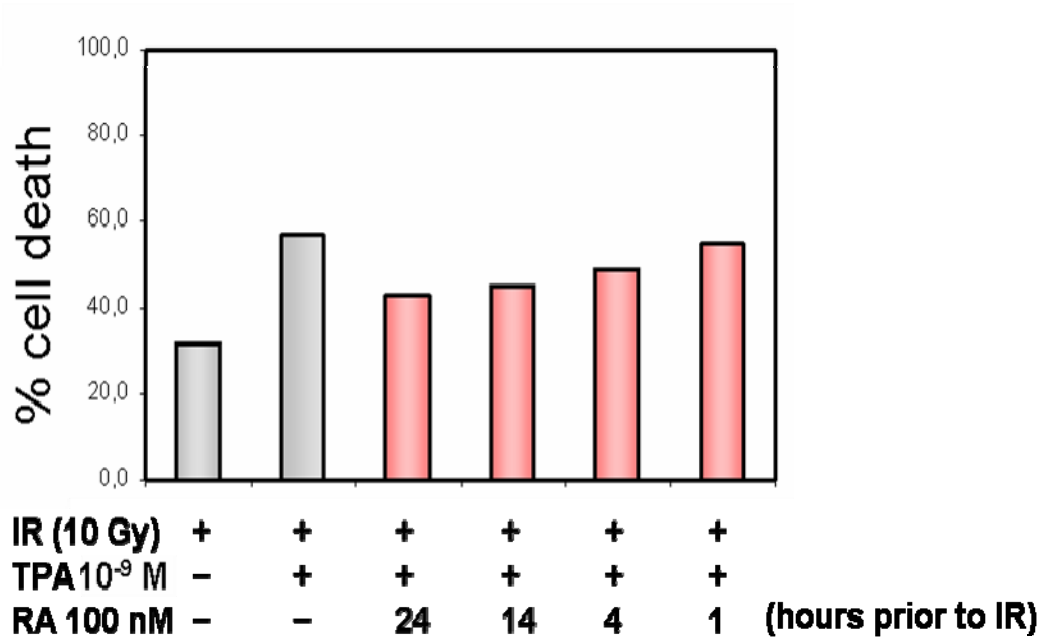


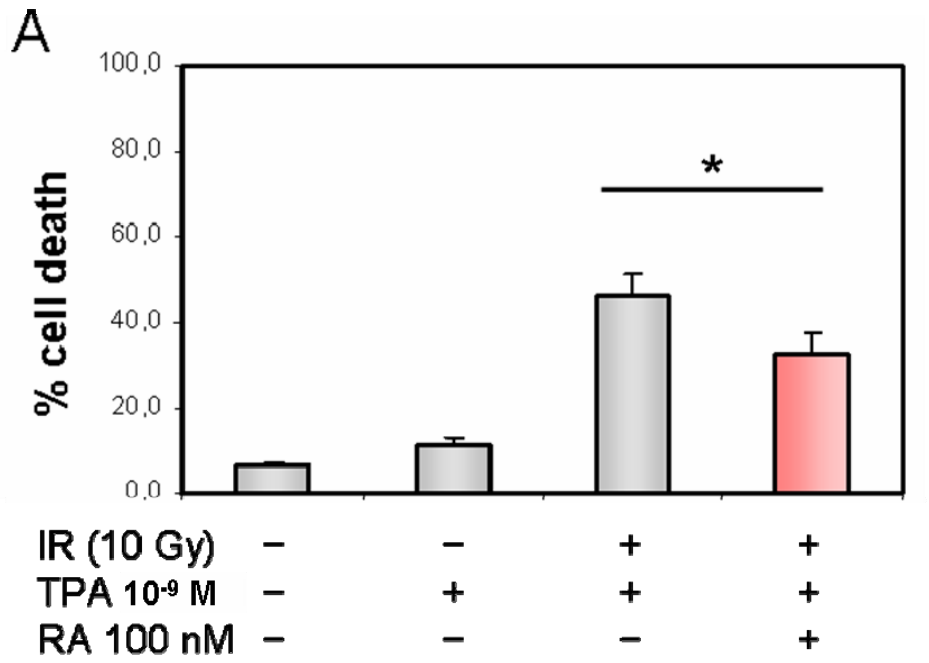
Figure 30: The effect of time of RA addition on DNA damage-induced cell death in T cells stimulated with TPA

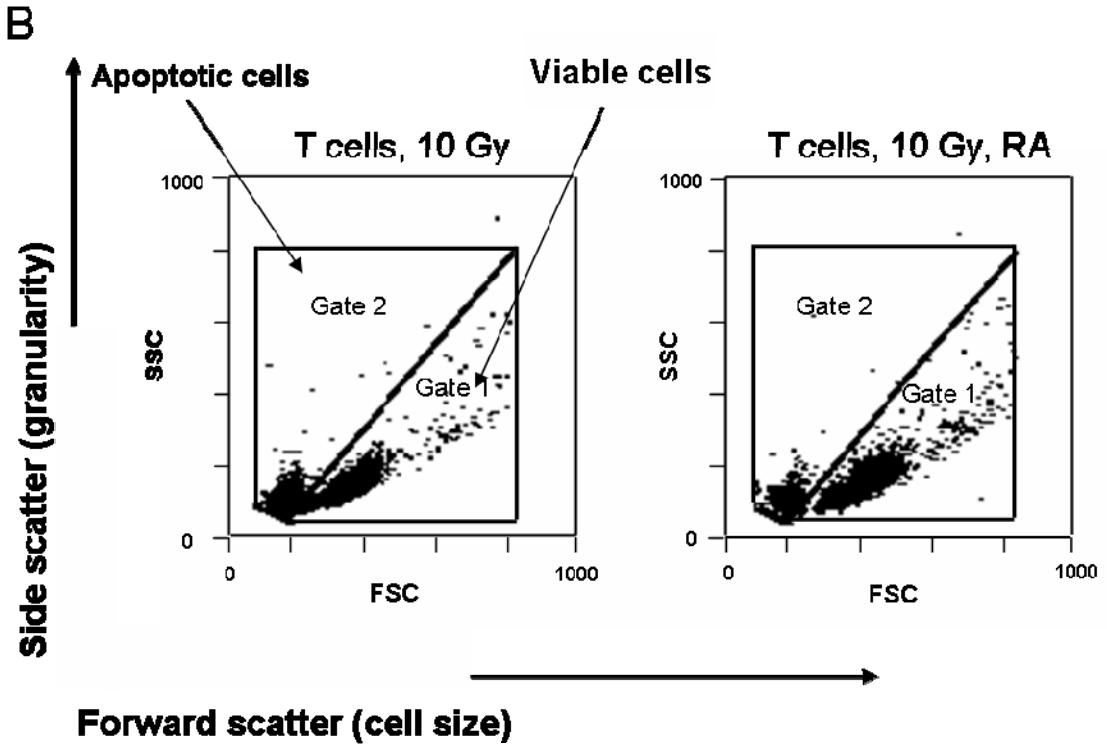
Normal human T cells were isolated from buffycoats, as described in Materials and Methods. Cells (2.0 x 10^6 cells/ml) were stimulated with or without TPA in the presence or absence of RA added 1-24 h prior to IR (10 Gy). 20 h after IR, apoptosis was analyzed by uptake of propidium iodine as described in Materials and Methods.

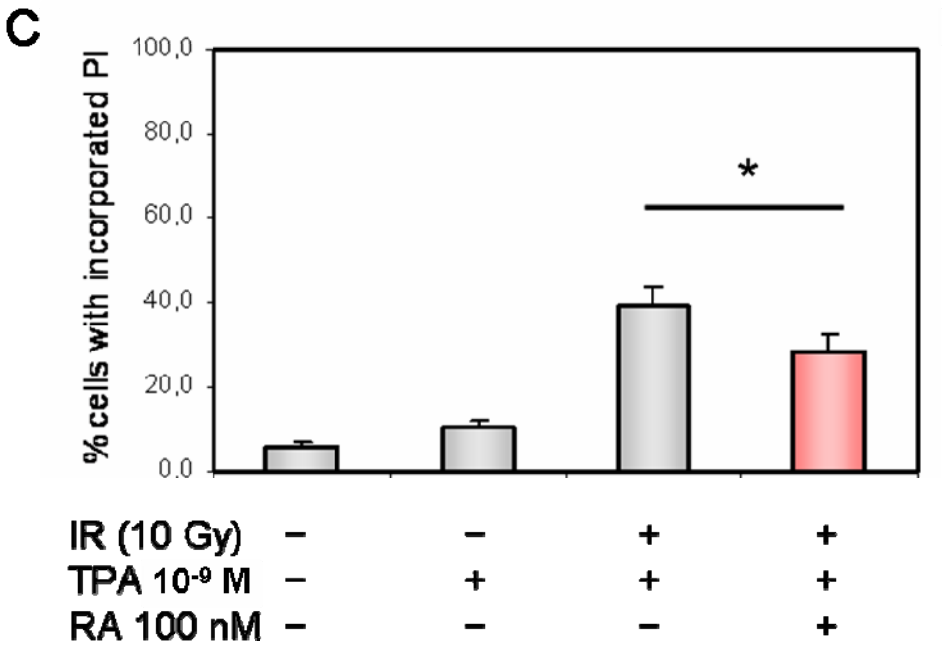
As shown in figure 30, RA inhibited cell death in a time-dependent manner. Best effects were obtained when RA was added 24 h prior to γ-irradiation.

4.1.13 The DNA damage-induced cell death in normal T cells is apoptotic and not necrotic

In viable cells, the cell membrane is intact and excludes charged dyes like propidium iodine (PI). In contrast, the membrane in dead or dying cells are leaky, and this result in uptake of PI and staining of DNA. Dead cells are therefore stained by PI, whereas viable cells have no or minimal uptake of the dye (78). However, PI-staining of cells does not discriminate between cells dead by necrosis or by apoptosis. In order to verify that DNA damage induced apoptosis and not necrosis, we also analyzed the cells by flow cytometric scatter analysis. Side scatter indicates the granularity of the cells, whereas forward scatter measures the cell size. Apoptotic cells are smaller and more granular than viable cells, whereas necrotic cells are larger than viable cells (78). We therefore analyzed the effect of RA on irradiation-induced cell death by scatter analysis.




B
Apoptotic cells
Viable cells
T cells, 10 Gy
T cells, 10 Gy, RA
1000
0
SSC
Gate 2
Gate 1
FSC
Side scatter (granularity)
Forward scatter (cell size)


C
% cells with incorporated PI
100,0
80,0
60,0
40,0
20,0
0,0
*
IR (10 Gy) – – + +
TPA 10-9 M – + + +
RA 100 nM – – – +

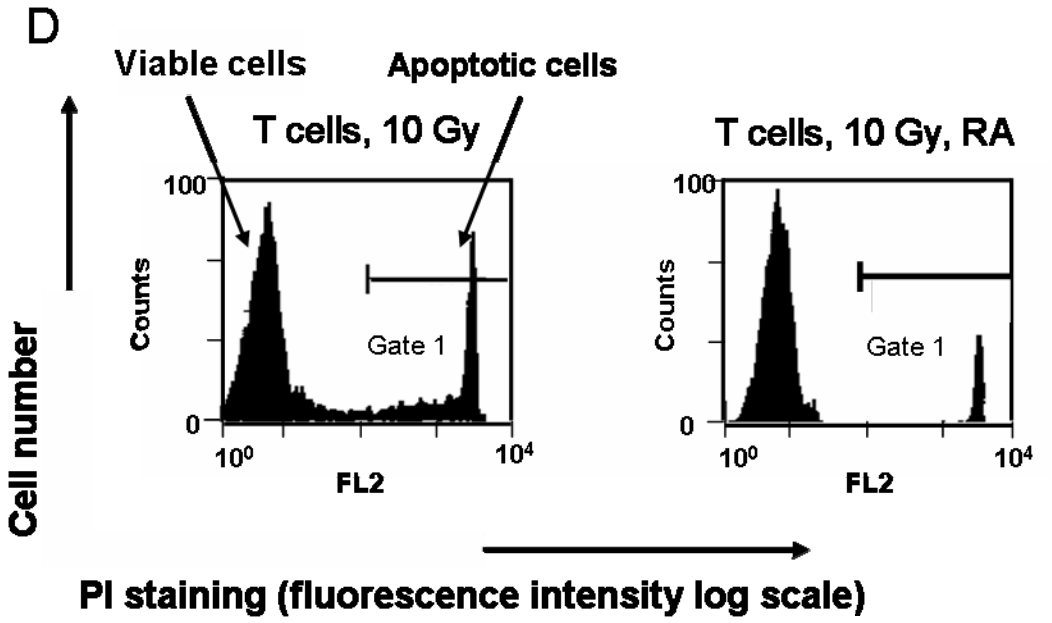


Figure 31: **DNA damage-induced cell death in T cells measured by scatter analysis and incorporation of PI**

Normal human T cells were isolated from buffycoats, as described in Materials and Methods. Cells (2.0 x 10^6 cells/ml) were stimulated with or without TPA, in the presence or absence of RA 24 h prior to IR (10 Gy). 20 h after IR, apoptosis was analyzed by scatter analysis (panel A and B), or by uptake of propidium iodine (panel C and D) as described in Materials and Methods (n = 15, mean ± SEM).

As shown in figure 31 A, there was a significant reduction in DNA damage-induced cell death measured by a shift in scatter when RA (100 nM) was added 24 h prior to γ-irradiation (n = 15, ± SEM, Paired samples T-test: * $p < 0.001$, mean (13.55), 95 % CI [7.87-19.22], SD (10.24)). The same results were obtained when cell death was analyzed by uptake of PI (figure 31 C). Thus, when PI-incorporation was measured, there was a significant reduction in DNA damage-induced cell death when RA was added 24 h prior to γ-irradiation (n = 15, ± SEM, Paired samples T-Test: * $p = 0.001$, mean (10.56), 95 % CI [5.44, 16.88], SD (8.86)). Figure B and D show the flowcytometry histograms of one of the experiments presented in figure A and C. Thus, figure B represent scatter analysis of the cells, whereas figure D represent uptake of PI. We could conclude that γ-irradiation of TPA-treated cells induced apoptosis. The PI-uptake and scatter analysis gave similar results, and RA clearly reduced the DNA damage-induced apoptosis as measured by both methods.

4.1.14 RA reduces DNA damage-induced cell death in T cells treated with doxorubicin

Having demonstrated that RA reduced irradiation-induced apoptosis in stimulated T cells, we wished to investigate the effects of RA on other DNA-damaging agents. Doxorubicin (DOX) is a chemotherapeutic agent used in treatment of a broad spectrum of human cancers including lymphomas (35;79). It induces apoptosis by inhibiting topoisomerase II, which unwinds DNA for transcription. Doxorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication (36). For comparison of cell death we used γ-irradiated cells.

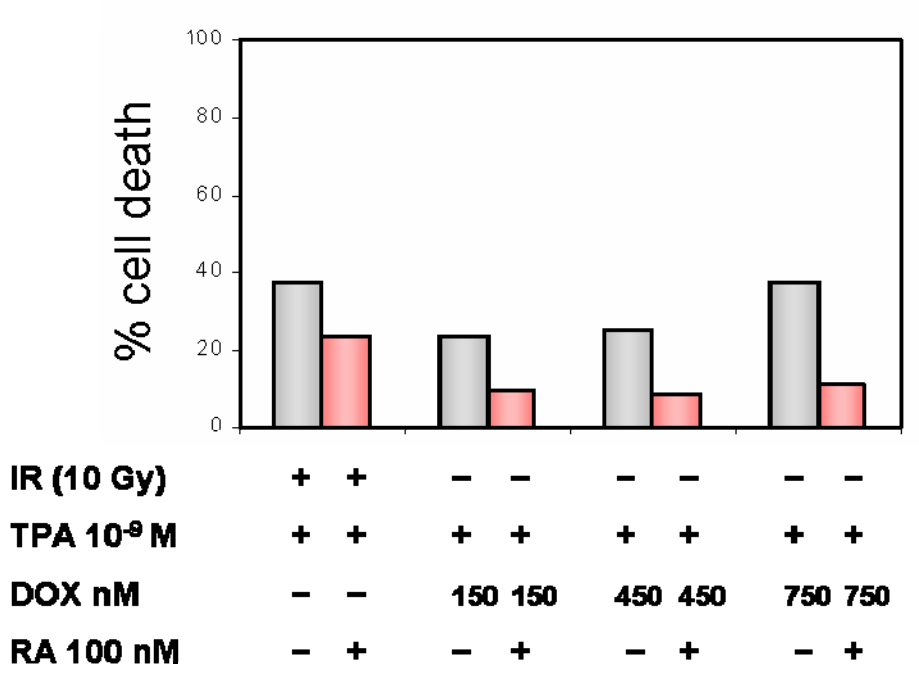


Figure 32: Effect of RA on cell death induced by doxorubicin or IR

Normal human T cells were isolated from buffycoats, as described in Materials and Methods. Cells (2.0 x 10^6 cells/ml) were stimulated with TPA in presence or the absence of RA for 24 h prior to IR (10 Gy) or treatment with different concentrations of doxorubicin. 20 h after IR or treatment with doxorubicin, apoptosis was analyzed by uptake of propidium iodine as described in Materials and Methods.

As shown in figure 32, RA reduced DNA damage-induced apoptosis also when DNA damage was enforced by doxorubicin. The effect of RA was strongest (70 % reduction) at 750 nM doxorubicin, and at this concentration of doxorubicin, the inhibitory effect of RA was even stronger than its effect on γ-irradiated cells.

4.2 Determination of p53 expression downstream of DNA damage in T cells

Expression of active p53 is induced in response to DNA damage (27). Recent studies in our lab have shown that cAMP inhibits apoptosis induced by DNA damaging apoptosis like γ-irradiation, and that this inhibition involves down-regulation of p53 (E. Hallan, unpublished data).

4.2.1 RA reduces p53 expression in γ-irradiated T cells

In order to assess the levels of p53 upon induction of DNA-damage, we performed Western Blot analysis of the cells after γ-irradiation. We used the same conditions as we had previously found to be optimal for assessing the effect of RA on DNA damage-induced apoptosis, i.e. stimulation of the cells with 10^{-9} M TPA and 24 h pretreatment with RA prior to γ-irradiation.

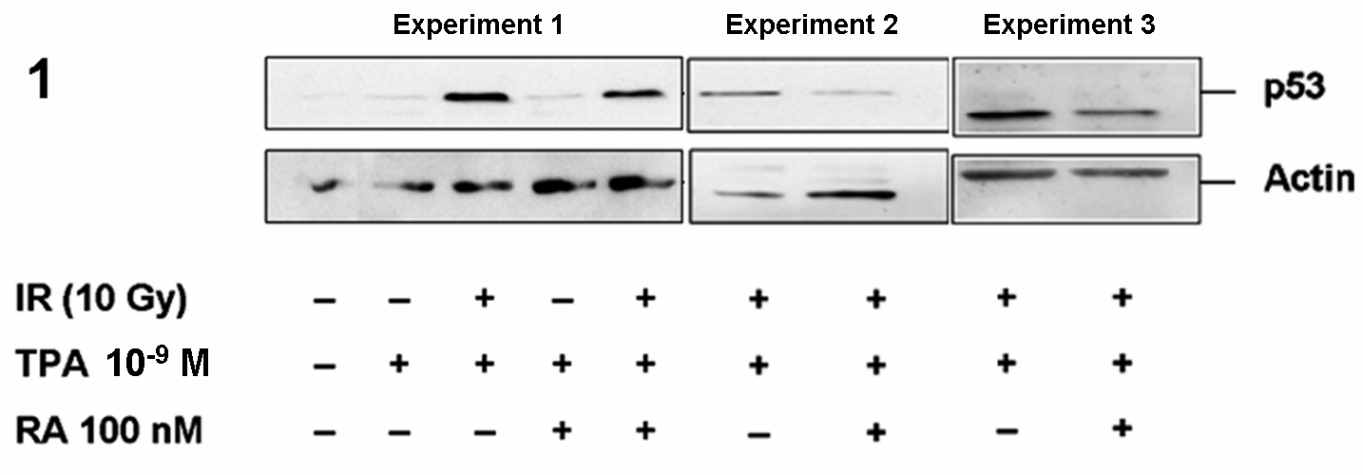


Figure 33 panel 1: Effect of RA on irradiated-induced p53 in T cells stimulated with TPA

T cells were incubated with or without TPA, in the presence or absence of RA for 24 h prior to exposure to IR (10 Gy). The cells were harvested 20 h after IR, and equal amounts of protein (30 μg) were separated on a 10 % polyacrylamide gel before performing Western blot analysis using antibodies against p53. For visualization of loading, the blot was rehybridized with antibodies against actin. Three representative blots are shown.

TPA alone did not induce p53, but as expected γ-irradiation induced a pronounced elevated expression of the p53 protein in the TPA-treated cells. RA treatment reduced the p53-expression as demonstrated in all three Western blots depicted in figure 33, panel 1.

In order to verify the inhibitory effects of RA on irradiation-induced p53 expression, the three Western blots in figure 33, panel 1 were scanned using Scion Image software.

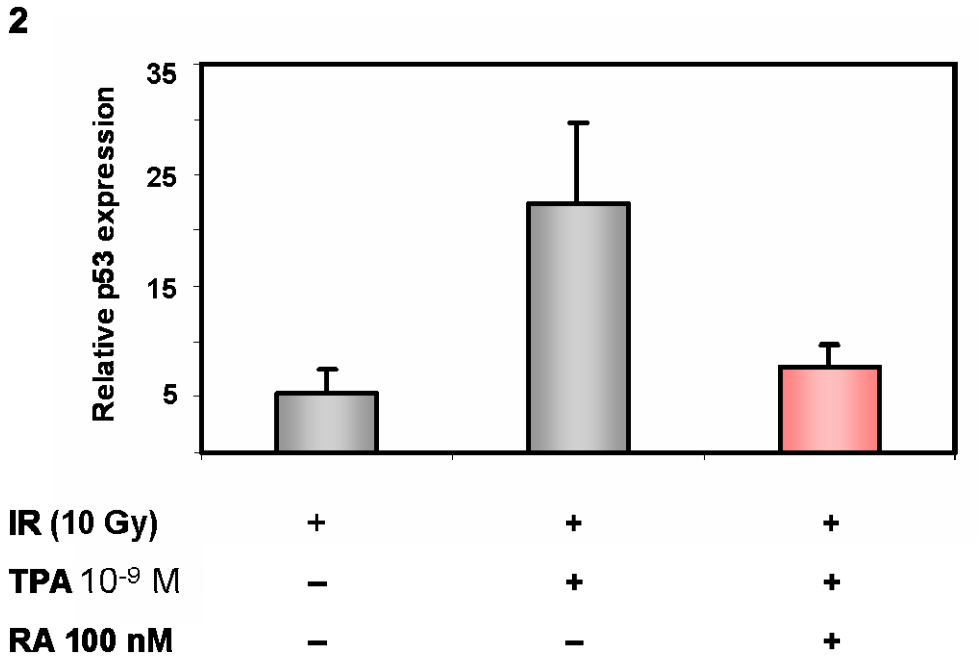


Figure 33, panel 2: Relative expression of p53

Western blots of p53 and actin expression were scanned by Scion Image software. The p53 levels were normalized against actin, and the relative expression of p53 was measured (n=3, mean ± SEM).

As shown in figure 33, panel 2, it was clear that γ-irradiation induced p53 expression in TPA-treated T cells. RA reduced the expression of p53 by 65 %.

5. Discussion

Deregulation of the cell cycle may lead to tumor formation. When genes encoding components of the programmed cell death pathway mutate, cells with damaged DNA may continue to proliferate. This will in turn result in new mutations and eventually tumor formation. Conventional cancer treatment such as γ-irradiation and use of chemotherapeutic agents act via DNA damage-induced apoptosis. The treatment is an effective way of reducing the amount of tumor cells, but at the same time the side effects of the treatment can be that our immune cells respond to the DNA damage and may commit to apoptosis. Vitamin A strengthen the immune system by preventing and fighting infection (46;49), but the mechanisms involved are not yet fully understood. In our lab we have previously shown that RA inhibits the proliferation of naïve B cells, but stimulates the proliferation of memory B cells as well as T cells (56;58;65;80).

Upon DNA damage, the transcription factor p53 is stabilized and activated to induce target genes involved in growth arrest, DNA repair and apoptosis. p53 is a tumour suppressor and an important downstream mediator of DNA damage responses (27). The aims of this paper were to reveal the effects of RA on DNA damage-induced apoptosis in human B- and T cells. In particular we wished to elucidate the effects of RA on regulation of p53 expression upon induction of DNA damage in lymphoid cells. But firstly, we discuss some methods used in this thesis.

5.1 Methodological considerations

5.1.1 Cell systems

Normal lymphocytes have limited lifespan and the isolation from peripheral blood is time-consuming, expensive, and gives a low yield of cells. As cell lines derived from malignant lymphocytes are easy to culture, and it is easy to obtain a large number of

cells, they have become valuable tools in the study of lymphoid cell biology. However, in order to understand relevant mechanisms in a physiological context, it is better to use normal cells compared to cell lines. This is because cell lines usually possess numerous genetic alterations as they often are derived from cancer cells. Another advantage of using normal lymphocytes is that the majority of the cells are naturally synchronized in the G0 phase of the cell cycle, thereby avoiding procedures to synchronize the cells. The data obtained from normal cells are therefore often easier to interpret than results from cell lines. However, when it comes to doing research on normal human cells, it is of importance to consider human variety. It is generally accepted that the cellular responses of lymphoid cells can vary substantially between different blood donors (65).

In this thesis we have primarily studied normal human B- and T cells, but a few experiments were performed on EBV-transformed B cells. Epstein Barr virus has a unique ability to transform resting B cells into permanent, latently infected lymphoblastoid cell lines. We isolated B lymphoid cells from peripheral blood of human blood donors (buffycoats) using antibody-coated Dynadeads (anti-CD19 antibodies). The yield of B cells is usually 10-40 x 10^6 B cells per buffycoat. Based on the cell death analysis done by flow cytometry, a high amount of cells died when cells were left over night to detach from the beads. Therefore, we removed the beads by adding Detachabeads to the cells, which gave a higher percentage of viable cells.

T cells can be negatively or positively isolated; the first method we used was based on removing contaminating cells, whereas the method we finally chose (and present in the thesis) is based on positive isolation of the T cells. We isolated T cells from buffycoats by positive selection, using $CD4^+$ beads. This method gave high viability of the cells, and provided a pure $CD4^+$ T cell population. We typically obtained 50-100 x 10^6 $CD4^+$ T cells from one buffycoat, and the purity was approximately 98 %. The advantage of using peripheral blood lymphocytes compared to cells from other lymphoid tissue is both that it is easy to obtain a large number of lymphocytes from peripheral blood, and that peripheral blood contain fewer *in vivo* activated cells than

cells isolated from for instance tonsils. This eliminates the problems of different responses in activated and resting cells.

5.1.2 Activation of lymphoid cells

Mounting an appropriate immune response depends on the careful regulation of lymphocyte activation. Stimulation of lymphocytes by antigen or mitogen results in macromolecular synthesis (DNA, RNA and protein) and production of lymphokines followed by proliferation and differentiation of the progeny into various effector and memory cells. To study immune responses *in vitro*, we activated the lymphoid cells. To stimulate B cells we used Staphylococcus Aureus Cowan (SAC). SAC mimics antigen stimulation by cross linking surface receptors of B cells and driving them into S-phase. With this stimulus approximately 30 % of the cells enter S-phase (41). T cells can be activated by components that interact with the T cell receptor, or by the use of pharmacological agents which circumvent receptor activation. Mitogenic lectins such as concanavalin A (Con A) or PHA interact with cell surface T cell receptor (81). TPA, a chemical tumor-promoting agent, is an effective mitogen for peripheral blood lymphocytes. It activates PKC by mimicking diacylglycerol (DAG) (76). Another chemical agent for activation of T cells is the calcium ionophore ionomycin which enhances the calcium levels in the cells (82). In this thesis, we used TPA or PHA to stimulate T cells. The advantage of using polyclonal activators like SAC, PHA, Con A or TPA to stimulate lymphocytes is that large percentages of the cells are driven into the cell cycle. This makes it easier to measure downstream effects.

5.1.3 Determination of cell proliferation

Incorporation of radioactive thymidine into DNA is a commonly used method for determining cell proliferation. The amount of radioactivity incorporated in the cells, reflects DNA synthesis in S-phase of the cell cycle. There is however some limitations related to this method. It has been reported a lack of correlation between

thymidine incorporation and cell proliferation in lectin-stimulated lymphocytes (83). Furthermore, the results give no accurate information about the amount of cells that are present in S-phase. Nevertheless, the advantage of determining DNA synthesis rather than for example direct cell counting is that the procedure is easy to perform and many samples can be analyzed simultaneously.

5.1.4 Determination of cell death

A common method for measuring cell death is based on propidium iodine (PI) uptake by dead and dying cells, as viable cells exclude charged dyes like PI because of the intact membrane. The PI uptake is analyzed by flow cytometry (78). The advantage of this method is that it is simple and of low cost, but it does not distinguish between necrotic and apoptotic cells. An alternative method for discriminating between viable and dead cells is scatter analysis by flow cytometry (84). In contrast to necrotic cells, which typically swell (due to plasma membrane dysfunction), apoptotic cells typically shrink and become more granular. However, caution should be made when studying apoptosis by this method, because decreased light scattering properties are not specific to apoptosis since both mechanically broken cells, isolated cell nuclei and necrotic cells can have low light scatter properties. Cell death can also be measured by the TUNEL method which detects DNA strand breaks by TdT-mediated dUTP nick end-labeling (TUNEL) of apoptotic cells, or by measuring changes in the membrane potential of dying cells (66). Since previous work in our lab (66) has proved that these two latter methods to assess cell death give the same results as scatter light analysis, we analyzed cell death by measuring changes in scatter light in addition to uptake of PI.

5.1.5 Retinoic acid

At the end of an immune response, most activated T cells die through activated T cell autonomous death (ACAD), a process in which activated T cells commit to apoptosis because survival signals fade some time after T cell activation (85). Previous work in

our lab has proven that retinoic acid (RA) prevents this process (66), and that it at the same time enhances the T cell proliferation (65). *In vivo*, retinol is transported in plasma bound to retinol-binding protein (RBP), and it is taken up by target cells via RBP-receptors (77). The plasma concentration of retinol-RBP is strictly regulated, and it is normally between 1 and 3 μM. Newly absorbed retinol is also present as retinyl esters in chylomicrons and their remnants. Retinyl esters in these particles can be taken up by the cells via LDL-receptor-related proteins (77). The intake of pharmacological concentration of retinol will result in plasma concentrations of retinyl esters in chylomicrons remnants of 10-30 μM (86). Only small amounts (~ 5-10 nM) of retinoic acid are normally found in plasma. During retinoic acid therapy, however, the concentration may increase considerably. For example, the intake of 50 mg RA will result in peak plasma concentrations of about 0.5 μM (86). In most *in vitro* studies retinoids are administered to the cells solubilised in ethanol, and it is reported that in such studies concentrations of RA above 1 μM generally are considered to be toxic (65). It has been shown that RA can be directly taken up by cells both *in vitro* and *in vivo* (87;88), and in previous paper our group demonstrated that similar effects were obtained on B cell growth by RA solubilised in ethanol as with retinol bound to RBP and by retinol esters (55). To study the effect of retinoids on B- and T cell proliferation as well as on DNA damage-induced apoptosis, retinoic acid, the major active retinoid metabolite, was used in this thesis. We could generally detect effects of RA at concentrations as low as 10 nM, but in most experiments we used the optimal concentration of 100 nM.

5.1.6 Protein expression of p53

In the present thesis we studied RA-mediated regulation of p53 expression in γ-irradiated T cells by Western Blot analysis. To ensure that an equivalent amount of proteins was present in each of the samples, we used actin as a control. Actin is a cytoplasmic protein present at high constitutive levels, and this protein is commonly used as a loading control. We also measured the relative expression of p53 by the use

of the software Scion Image, where we normalized the p53 labeling with the actin labeling.

5.2 Discussion of the results

5.2.1 The effect of RA on DNA damage-induced apoptosis

The important role of retinoids in the immune system has been underlined by numerous studies (49). A part of the increased resistance to infection has been ascribed to improved epithelial integrity, but a direct effect on the immune system is well established (48;89). The key regulatory event in the G1/S-phase transition of the cell cycle is the phosphorylation of pRB (90), and previous work in our lab has proven that RA markedly enhanced TPA-mediated pRB phosphorylation in T cells (65), whereas SAC-mediated pRB-phosphorylation of B cells was inhibited (57;65). Consistent with these previous findings we found that the proliferation of B cells was inhibited by physiological levels of retinoids, whereas the T cells were stimulated by RA. This cannot be ascribed to the fact that different mitogens were used to stimulate T- and B cells, as previous work in our lab has shown that RA also inhibits the proliferation of TPA-stimulated B cells (Å. Ertesvåg, personal communication).

Many DNA-damaging agents rely on DNA-replication to be effective (72). Previous work in our lab has shown that inhibition of lymphocyte-proliferation by elevated levels of cAMP also prevented DNA damage-induced apoptosis (72). Since RA enhanced and inhibited the proliferation of T- and B cells respectively, we anticipated that DNA damage-induced apoptosis would be enhanced and reduced, respectively in the two cell types. However, in contrast to what we expected, DNA damage-induced apoptosis was inhibited in both normal B cells, EBV-transformed B cells and in normal T cells. Interestingly, the inhibitory effect was particularly strong in T cells. When doing research on normal human lymphocytes, it is of importance to consider human variety. It is generally accepted that the cellular responses of T cells can vary substantially between different blood donors (66). Of the 5 first donors examined for

T cell apoptosis, we noticed that T cells from one of the donors responded much stronger to RA than the four others. We therefore extended the results by including a total of 15 donors. In the initial studies the T cells were stimulated with PHA, but since previous results in our lab had shown that TPA is a stronger stimulus than PHA (Å. Ertesvåg, personal communication), we used TPA to stimulate the T cells isolated from the 15 donors. It has previously been shown that concentrations above 3 x 10^{-9} M TPA inhibits proliferation of certain cell types (91). We therefore assessed different concentrations of TPA, and found that 10^{-9} M TPA was the strongest inducer of T cell proliferation. From a total of 15 donors, we found that RA significantly ($p < 0.001$) reduced the apoptosis of γ-irradiated T cells stimulated with 10^{-9} M TPA. TPA mimics diacylglycerol in activation of PKC (76), and it has been shown that retinoids in certain cell types can regulate PKC activity (92). However, previous work in our lab has shown that there was no potentiation of PKC-activity in RA-treated T cells (65).

It has, however, been shown that RA induces IL-2 production in TPA-stimulated T cells (65;66) and it is possible that this IL-2 production might prevent apoptosis induced by γ-irradiation of RA-treated cells. Consistent with a role of IL-2 in this process, we observed that RA had a stronger inhibitory effect on DNA damage-induced apoptosis when it was added to TPA-treated T cells early during stimulation (24 h before γ-irradiation) compared to 2 h before γ-irradiation.

In addition to reducing apoptosis in lymphoid cells after exposure to γ-irradiation, we showed that RA also reduces DNA damage-induced apoptosis in T cells exposed to cytotoxic chemical agents. Doxorubicin is used in treatment of a broad spectrum of human cancers. The chemical has been shown to stimulate both intrinsic (mitochondria-mediated) and extrinsic (Fas ligand-mediated) pathways of apoptosis in cellular and *in vivo* models (34). In tumor cells, doxorubicin causes early activation of p53 followed by caspase-3 activation and DNA fragmentation, resulting in apoptosis (34). A side effect is that doxorubicin induces apoptosis in normal cell types, for example endothelial cells, cardiomyocytes and peripheral blood

lymphocytes (34;79). However, doxorubicin induces apoptosis in normal and tumor cells via distinctly different mechanisms. In normal cells, doxorubicin induces apoptosis by a H_2O_2-mediated mechanism and is largely independent of p53 activation (34). To be able to compare the effects of RA on doxorubicin-treated T cells and irradiated T cells, we aimed for the same degree of apoptosis after both treatments. We found that 750 nM doxorubicin gave approximately the same percentage of apoptotic cells as cells exposed to γ-irradiation at 10 Gy. When using this concentration of doxorubicin, the inhibitory effect of RA was even stronger than its effect on γ-irradiated cells. Thus, doxorubicin-induced apoptosis was reduced by 70 %, whereas RA inhibited apoptosis induced by γ-irradiation by approximately 35 %.

5.2.2 The effects of RA on regulation of p53 expression upon induction of DNA damage in lymphoid cells

In order to understand how RA inhibits apoptosis induced by DNA-damaging agents like γ-irradiation and doxorubicin in normal T cells, we investigated the events downstream of DNA damage. The DNA damage checkpoint is characterized by the ATM/ATR-CHK1/CHK2 pathway (16;26). The activation of CHK1/CHK2 by ATM/ATR leads to phosphorylation, stabilization, and activation of p53, and this will in turn lead to p53-mediated induction of genes involved in growth arrest, DNA repair and apoptosis (27). Also transcription-independent effects of p53 are induced (28). As p53 is such a critical mediator of the DNA-damage checkpoint, we studied the effect of RA on p53-expression. We showed that γ-irradiation of the TPA-stimulated T cells induced p53 expression, and that RA inhibited this induction by 65%. The present study is the first to assess the effect of RA on DNA damage-induced apoptosis in any cell type. In our lab, we have previously shown that RA prevents spontaneous apoptosis of normal B cells (59), and in melanoma cells RA has been shown to increase apoptosis (93). In the melanoma cells the induced apoptosis was accompanied by enhanced expression of p53 (93).

5.3 Future perspective

How RA inhibits the level of p53 in γ-irradiated T cells will be further studied by our lab. The effects on upstream mediators like activation of ATM/ATR or CHK1/2 will be studied by phospho-specific antibodies to active forms of these proteins. We will also examine at which level p53-expression is inhibited by RA. Generally, the expression of p53 is mainly regulated at the level of stability, with HDM-2-mediated degradation of p53 in proteasomes as the main mechanisms (31). We can not, however, exclude the possibility that RA inhibits the transcription of p53. Several hundred genes are directly or indirectly regulated by RA (94), but p53 is not one of the genes directly regulated. Thus, if RA inhibits the expression of p53 at the transcriptional level, the effect must be indirect. The C terminal region of p53 forms a region that has key regulatory properties. Modification of this region by acetylation, phosphorylation, O-glycosylation, and RNA binding have been reported, but the physiological significance of these post-translational modifications remains uncertain (95). It has been shown that RA can alter the glycosylation pattern of various proteins, and glycosylation is known to affect, for instance, the protein stability (65).

The physiological implications of the present thesis results are that the vitamin A status of a cancer patient receiving chemotherapy or γ-irradiation might influence the effect of the treatment. Thus, if the effects of RA on T cells mimic the effects on tumor cells, i.e. T cell lymphomas or T cell leukaemia, then it should not be recommended to have high vitamin A levels in plasma during the treatment. In fact, perhaps retinoic acid receptor antagonists could be used to enhance the effect of DNA damaging agents on apoptosis of the cancer cells. On the other hand, apoptosis of cells of the immune system is a serious side effect of most cancer treatments, leading to enhanced susceptibility to infections. Thus, in terms of protecting the patient's T cells during cancer treatment, a high level of vitamin A should be recommended. More research is therefore clearly needed before justified recommendations as to vitamin A status and cancer treatments like irradiation and chemotherapy can be given.

6. Conclusion

In the present study, we have shown that although RA markedly induces proliferation of normal T cells and inhibits B cells, DNA damage-induced apoptosis is inhibited in both cell types. This effect was particularly studied in T cells, demonstrating that DNA damage induced by both γ-irradiation and the cytotoxic agent doxorubicin was inhibited. One of the most important effector proteins in the DNA damage checkpoint is p53, and we showed that RA inhibited the DNA damage-induced p53 expression in stimulated T cells. Our result may have implications for cancer treatment, both by influencing the killing of malignant lymphocytes, as well as to protecting the immune system from unwanted damage during γ-irradiation or chemotherapy.

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