Study of the molecular effects of isoflavones on inflammatory gene expression

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Abbreviations

Ab  antibody
AF  activation function
Ag  antigen
AhR aryl hydrocarbon receptor
AP-1 activator protein-1
APC antigen-presenting cell
ASK apoptosis signal-regulating kinase
BAFF B cell-activating factor
C   carboxy
cAMP cyclic AMP
CBP CREB-binding protein
CD40L CD40 ligand
C/EBP CCAAT enhancer-binding protein
COX cyclooxygenase
CRE cAMP-responsive element
CREB CRE-binding protein
CTL cytotoxic T lymphocyte
DBD DNA-binding domain
DC dendritic cell
DD death domain
DNMT DNA methyltransferase
ER estrogen receptor
ERR estrogen-related receptor
ERE estrogen-recognition element
ERK extracellular signal-regulated kinase
FDA Food and Drug Administration
GAPDH glyceraldehyde-3-phosphate dehydrogenase
GC glucocorticoid
GM-CSF granulocyte monocyte-colony stimulating factor
GR glucocorticoid receptor
GSH reduced glutathione
H1/2A/2B/3/4  histone 1/2A/2B/3/4
H12 helix 12
HAT histone acetyltransferase
HDAC histone deacetylase
HMT histone methyltransferase
HRT hormone replacement therapy
IFN interferon
<table>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IkB</td>
<td>inhibitor of NF-κB</td>
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<td>IKK</td>
<td>IkB kinase</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<td>IL-1R</td>
<td>IL-1 receptor</td>
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<td>IRAK</td>
<td>IL-1-associated protein kinase</td>
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<td>IRF</td>
<td>interferon regulatory factor</td>
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<td>ISRE</td>
<td>IFN-stimulated responsive element</td>
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<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>LBD</td>
<td>ligand-binding domain</td>
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<td>LBP</td>
<td>LPS-binding protein</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>LT-β</td>
<td>lymphotoxin-β</td>
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<tr>
<td>Mal</td>
<td>MyD88-adaptor-like</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>MEK=MMK=MAPKK=MAP kinase</td>
<td></td>
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<tr>
<td>MEKK=MAPKKK=MAP3K=MAPKK kinase</td>
<td></td>
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<tr>
<td>MBP</td>
<td>methyl-binding protein</td>
</tr>
<tr>
<td>M-DC</td>
<td>myeloid DC</td>
</tr>
<tr>
<td>Mdm2</td>
<td>Mouse double minute 2</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>MSK</td>
<td>mitogen- and stress-activated protein kinase</td>
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<tr>
<td>N</td>
<td>amino</td>
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<tr>
<td>NAC</td>
<td>N-acetyl-L-cystein</td>
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<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
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<td>NES</td>
<td>nuclear export sequence</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
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<tr>
<td>NLS</td>
<td>nuclear localization sequence</td>
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<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
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<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>P-DC</td>
<td>plasmacytoid DC</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
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<tr>
<td>PGK</td>
<td>phosphoglycerate kinase</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKA/B/C</td>
<td>protein kinase A/B/C</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PR</td>
<td>progesteron receptor</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern-recognition receptor</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RBP-Jκ</td>
<td>recombination signal sequence-binding protein-Jκ</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>redox</td>
<td>reduction-oxidation</td>
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<td>RHD</td>
<td>Rel homology domain</td>
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<tr>
<td>RIP</td>
<td>receptor-interacting protein</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RSK</td>
<td>ribosomal S6 kinase</td>
</tr>
<tr>
<td>SDR</td>
<td>specificity-determining region</td>
</tr>
<tr>
<td>SERM</td>
<td>selective estrogen receptor modulator</td>
</tr>
<tr>
<td>SHBG</td>
<td>sex hormone-binding globulin</td>
</tr>
<tr>
<td>sIL-6R</td>
<td>soluble IL-6 receptor</td>
</tr>
<tr>
<td>SIR</td>
<td>silent information regulator</td>
</tr>
<tr>
<td>SNP</td>
<td>single-nucleotide polymorphism</td>
</tr>
<tr>
<td>SOCS</td>
<td>suppressor of cytokine signaling</td>
</tr>
<tr>
<td>SODD</td>
<td>silencer of death domains</td>
</tr>
<tr>
<td>Sp1</td>
<td>specificity protein 1</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TA</td>
<td>transactivation domain</td>
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<td>TAB</td>
<td>TAK1-binding protein</td>
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<td>TAK</td>
<td>TGF-β-activated kinase</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>Th</td>
<td>T helper</td>
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<tr>
<td>TICAM</td>
<td>TIR domain-containing adapter molecule</td>
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<td>TIR</td>
<td>Toll/interleukin (IL)-1 receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR domain-containing adaptor protein</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR-associated death domain</td>
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<tr>
<td>TRAF</td>
<td>TNF receptor-activated factor</td>
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<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
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<tr>
<td>TRIF</td>
<td>TIR-related adaptor protein inducing IFN-β</td>
</tr>
<tr>
<td>TSA</td>
<td>trichostatin A</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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<td>WHI</td>
<td>Women's Health Initiative</td>
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Objectives & Introduction
I. INTRODUCTION

1. Inflammation

1.1. The inflammatory process

The inflammatory process can be interpreted as a localized protective response, elicited by the notification of trauma, which includes physical injury, tissue destruction and an infection with a threatening agent or organism. In fact, inflammation is designed to ward off invasion by pathogens, such as bacteria, viruses, parasites, etc. The subsequent activation of the intrinsic defence system causes the cardinal symptoms of inflammation, which were first described by Aulus Cornelius Celsus nearly 2000 years ago as 'rubor et tumor cum calore et dolore' or 'redness and swelling, accompanied by heat and pain' (Figure 1). Therefore, this term is coined by the ancients, based on the latin verb 'inflammare', which means 'to set on fire' (Scott et al, 2004).

Figure 1: This cartoon depicts the 5 cardinal signs of inflammation. From left to right: heat, redness, swelling, pain, loss of function. Drawn by P. Cull.

Histologically, acute tissue inflammation involves a complex series of events, starting with the encountering of pathogens by phagocytic cells, such as macrophages and dendritic cells (DCs). Upon stimulation, these crucial players of the innate immune response are triggered to release biologically active molecules, including inflammatory cytokines. These cytokines orchestrate the immune response further, as they stimulate the dilatation and permeability of local blood capillary vessels, leading to a local increase in blood flow, decrease in blood velocity and exudation of fluids, including plasma proteins. In addition, cytokine expression affects the adhesive properties of the endothelium, so that leukocytes stick to the inner walls. Attracted by chemokines and other cytokines, the leukocytes migrate through the endothelial junctions of the blood vessel into the inflammatory focus. These inflammatory cells, mainly macrophages and neutrophils, with potent antimicrobial activity, represent the effector phase of 'innate resistance'. Besides killing the pathogens by acidifying the phagosomes in which the invaders are captured, they also...
produce and release enzymes, chemical mediators and toxic molecules. As these toxic products, including superoxide anions ($O_2^-$), hydrogen peroxide ($H_2O_2$) and nitric oxide (NO), affect host cells as well, they may cause additional or extensive tissue damage by necrosis. After this initial phase, constituting the first line of defence, other types of activated immune cells, such as T and B lymphocytes, contribute to inflammation in a process that is called ‘adaptive immune response’. Thus, the process of inflammation and the general immune response are interwoven.

Apart from the role of inflammation in combating infection by delivery of effector molecules and cells at the local site, its second function is providing a physical barrier in the form of a microvascular fibrin clot by coagulation to prevent the spread of the infection into the blood stream. Finally, inflammation needs to promote the repair of injured tissue. Beyond their role in inflammation and host defence, immune cells have an integral function in regulation of wound healing. By secretion of signaling molecules, such as cytokines and growth factors, they stimulate fibroblastic proliferation and angiogenesis.

1.2. Immune response

The effective development of the overall immune response depends on regulation of the two main branches of immunity, termed ‘innate’ and ‘adaptive’ immunity. Innate immunity is based on the genetic memory of germline-encoded receptors or pattern-recognition receptors (PRR) to recognize pathogen-associated molecular patterns (PAMPs), that are conserved between and shared by large classes of pathogens. Although innate resistance is efficient at either preventing an infection or greatly reducing the pathogen load, sterile cure or control of an infection is achieved only when adaptive immunity is induced. Adaptive or acquired immunity, akin to somatic memory, is a complex process initiated by the recognition of specific pathogenic antigens (Ags), leading to expansion of Ag-specific T and B cell populations with clonally distributed receptors. Therefore, an efficient adaptive response is reached only several days after primary infection. In fact, innate and adaptive immunity are not simply sequential and complementary processes, as they mutually regulate each other in a careful interplay, through cellular contacts and secretion of soluble mediators.

1.2.1. Ag presentation

An essential step to achieve cross-talk between innate and adaptive immune responses is covered by Ag presentation. After detection of intracellular intruders or uptake of extracellular pathogens, peptides of foreign proteins are presented on Major Histocompatibility Complexes (MHC)I and II at the cell surface. MHCI molecules are wide-
Introduction

spread among all nucleated cells, with highest expression levels in hematopoietic cells, to which MHCII expression is normally restricted. This distinct distribution reflects their functional capacities, as MHCI or MHCII are recognized by CD8⁺ or by CD4⁺ T lymphocytes, which results in generation of cytotoxic T lymphocytes (CTLs) or T helper (Th) cells, respectively. From a classical point of view, MHCI molecules present antigenic peptides derived from intracellular proteins, whereas MHCII molecules do so for exogenous and membrane proteins. This phenomenon is reflected in the two major cellular breakdown pathways for proteins, namely proteasomal degradation, particularly relevant to the generation of MHCI peptides, and degradation by the endosomal/lysosomal system, which is responsible for the processing of MHCII peptides. However, the separation of these distinct pools of source proteins is less stringent than originally believed. It is now well established that MHCI molecules are able to present peptides derived from exogenous Ags by a process known as cross-presentation. On the other hand, intracellular proteins can be presented by MHCII molecules through autophagy (Schmid & Munz, 2005).

1.2.2. Ag-presenting cells

Ag presentation by non-immune endothelial cells is becoming increasingly interesting, as this enhances T cell responsiveness and cytokine production and also directs migration of Ag-specific T subsets into the site of inflammation (Marelli-Berg & Jarmin, 2004). In addition, intestinal epithelial cells also function as Ag-presenting cells (APCs), capable of regulating T cell responses in the intestinal mucosa. However, the typical APC characteristic refers to B cells, macrophages and DCs. The latter are strategically positioned on the boundaries between the inner and outside world, thereby bridging innate and adaptive immunity.

1.2.2.1. B cells and macrophages

B cells function as APCs primarily in secondary lymphoid tissues, where they use the Ag-specific B cell receptor to effectively capture and concentrate Ags in late endosome/lysosome-like compartments, called MHCII compartments. This process enables the cognate T-B cell interactions, required to elicit T cell-dependent humoral immunity. In contrast to B cells, macrophages reside in peripheral tissues to engulf pathogens. This capture activates the anti-microbial killing mechanisms of macrophages and stimulates the production of inflammatory mediators, that initiate acquired T cell immunity. The development of macrophages into potent APCs requires their activation by cytokines, such as interferon (IFN)-γ and granulocyte monocyte-colony stimulating factor (GM-CSF).
1.2.2.2. DCs

The development of DCs is considered to occur in several distinct stages out of hematopoietic pluripotent stem cells. These continuously generate, under as yet unknown influences, DC progenitors in the bone marrow, which give rise to circulating DC precursors in the blood. Based on the expression of the β2 integrin CD11c, two subsets of DCs were identified in human blood, which both represent a small fraction (~ 0.3%) of the entire circulating blood leukocyte population. These groups are defined as belonging to the myeloid and lymphoid lineage and therefore named myeloid (M-DCs) and plasmacytoid DCs (P-DCs), respectively. M-DCs are characterized by a monocytic morphology, possessing an irregular outline and a hyperlobulated nucleus. Peripheral blood mononuclear cells rise to immature M-DCs after culturing with GM-CSF and interleukin (IL)-4 in vitro. P-DCs, in contrast, have a plasma cell-resembling morphology with an oval or intended nucleus and a prominent perinuclear pale zone. As this subtype DC is inefficient in Ag capture and is mainly located within the thymic medulla and lymph node T cell areas, their activity relates to the acquisition of immune tolerance by recognizing self-Ags. M-DCs, however, home to a diverse range of peripheral tissues at portals of pathogen entry, such as the skin or the airway and gastrointestinal mucosae. There, they reside as immature sentinel cells, which constitutively macropinocytose extracellular fluid. Immature DCs also have a high phagocytic, pinocytic and receptor-mediated endocytic capacity (Brode & Macary, 2004). C-type lectin receptors (mannose receptor, DEC205), as well as receptors for heat-shock proteins and the Fc domain of immunoglobulins, facilitate pathogen internalization.

Upon pathogen processing, DCs enter the lymphatic system and migrate to T cell-containing areas in secondary lymphoid organs, such as peri-arteriolar sheaths of the splenic white pulp and the paracortex of lymph nodes (Figure 2). This translocation is tightly regulated as a function of phenotypical and functional changes in the process of maturation, triggered by specific maturation signals, such as lipopolysaccharide (LPS), CD40 ligand (CD40L), etc. Maturation is characterized by a decreased Ag-processing capacity, an increased cell surface expression of MHCII and costimulatory molecules, in addition to rearrangement of cytoskeleton, change in adhesion molecules, cytokine and chemokine receptors and cytokine expression. In the lymphoid niches, mature DCs attract T cells by releasing chemokines and also maintain viability of recirculating T lymphocytes. In the T cell zone, they present their Ags from extracellular pathogens to naïve CD4+ T cells. T cell activation is fully accomplished by the engagement of the T cell receptor/CD3 complex with the antigenic peptide presented by MHCII molecules (signal 1), as well as the binding of appropriate costimulatory receptors CD28 by B7 ligands (signal 2) on the APC. This T cell
triggering results in induction of clonal expansion and differentiation into effector Th cells or regulatory T cells, which prevent autoimmune responses by specifically suppressing activation and proliferation of effector T cells. Two subtypes Th cells are distinguished by characterization of their distinct cytokine expression profiles and immune functions that they mediate. Th1 cells primarily release IFN-γ and tumor necrosis factor (TNF)-β, which are strong inducers of cell-mediated immunity by activating cytotoxic and phagocytic functions in effector cells, such as CTLs, natural killer cells and macrophages. Furthermore, Th1 cells are responsible for the support of CD8+ antiviral effector T cells and the induction of immunoglobulin (Ig)G antibodies (Abs) that mediate opsonization and phagocytosis. In contrast, Th2 cells produce cytokines, such as IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, which provide signals for B cell isotype switching to IgG1, IgA and IgE activation and Ab production. In addition, Th2 cells stimulate the growth and differentiation of mast cells and eosinophils. The importance of a correct Th choice is stressed by the fact that, depending on the nature of the invading microorganism, the effective elimination of an infection requires a specific type of Th response (cell-mediated versus humoral immune response). In general, efficient clearance of intracellular pathogens is based on innate immune cell activation, whereas Ab responses are best suited for extracellular infections.

Polarization into Th1 or Th2 cells is influenced by several factors, including the amount of Ag to which DCs have been exposed (high Ag doses are usually associated with the generation of Th1 responses) (Boonstra et al, 2003), the ratio of DCs to T cells (low ratios favor Th1 development) (Tanaka et al, 2000) and the costimulatory molecules preferentially expressed by DCs (expression of OX40L or a high B7.2/B7.1 ratio generally promote Th2 responses) (Akiba et al, 2000; Kuchroo et al, 1995). However, cytokines, present in the microenvironment during the initial phases of T cell activation (signal 3), represent one of the principal factors that defines the balance of Th1/Th2 in a positive and negative way, which has been termed cross-regulation. Mature DCs secrete multiple cytokines and express membrane-bound molecules, such as OX40 ligand, which determine the character of the ensuing immune response (Kalinski et al, 1999). Prototypically, IL-12 p70 drives polarization of T cells towards a Th1 phenotype. Furthermore, type I IFNs, such as IFN-γ, are also instrumental in inducing Th1 cells. Conversely, exposure of naïve T cells to IL-4 results in the induction of Th2 responses. While IL-12 and IFN-γ are produced by stimulated macrophages and DCs, these cells do not represent the initial source of IL-4. IL-6, however, has been found to indirectly stimulate IL-4 production in T cells (Rincon et al, 1997) via induction and activation of the transcription factor NFAT (Diehl et al, 2002). In addition to positive effects on differentiation, IL-6 also exerts negative regulation on the differentiation of
Introduction

Th0 cells. The molecular mechanism involves activation of ‘signal transducer and activator of transcription’ (STAT)3 in response to IL-6 signaling, which leads to subsequent ‘suppressor of cytokine signaling’ (SOCS)-1 gene expression and inhibition of IFN-γ signaling in T cells (Diehl et al, 2000). Likewise, IL-6 negatively regulates IL-12 production of pulmonary DCs, leading to inhibition of Th1 polarization (Dodge et al, 2003).

Figure 2: Priming of distinct Th responses by DCs, upon encountering pathogens in the peripheral tissues by internalization of extracellular pathogens. The different signals for naïve T cell activation and polarization are indicated by numbers, corresponding to the text.

Apart from the role of DCs in priming effector T cells upon first encounter of an infectious organism, DCs are also responsible for initiating immune responses by memory T cells. In addition, central and peripheral tolerance is controlled and maintained by DCs. For the self/non-self discrimination, the pattern recognition model and the danger model have been proposed. The former implies that the PRRs, recognizing conserved pathogenic motifs, but not self-Ags, serve as cues to upregulate stimulatory functions of DCs (Janeway, 1992). The danger model does not focus on recognition of self/non-self, but rather suggests that the release of self-molecules, such as heat-shock proteins by necrotic cells or mitochondrial products, would serve as danger signals to instruct DC maturation (Matzinger, 2002). Without complete maturation, T cell engagement by recognition of self-Ags induces
Introduction

Peripheral tolerance by T cell deletion, anergy or by promoting naïve CD4<sup>+</sup> and CD8<sup>+</sup> subsets to differentiate into IL-10-producing regulatory T cells (Dhodapkar et al, 2001; Jonuleit et al, 2000), which inhibit Th1 differentiation (Jonuleit et al, 2000) and suppress CD8<sup>+</sup> T cell responses (Dhodapkar et al, 2001). In this way, the immune system overcomes at least some of the risks of developing autoimmunity and chronic inflammation.

1.3. Chronic inflammation

As immune cells contribute to the process of inflammation, the inflammatory response is an immune-mediated physiological process of paramount importance to the organism. Without these reactions, a host would quickly succumb to invading pathogens or damaging stimuli, whereas excessive or inappropriate activation of these responses causes tissue and cell damage, and even death. Despite its self-amplifying nature, under normal circumstances, the acute inflammatory response is self-limiting as well, so that inflammation is resolved after a few days. Although the mechanisms of inflammation resolution are not well known, pro-inflammatory cells receive apoptotic stimuli and subsequently are phagocytosed by macrophages. As macrophages block pro-inflammatory cytokine production in an auto- and paracrine manner, they play a vital role in this resolution process as well (Fadok et al, 1998). In addition, gene disruption studies and pharmacological inhibition have identified a role for a number of endogenous mediators, including the cytokines IL-10 and transforming growth factor (TGF)-β, and lipid mediators, such as lipoxins and cyclopentenone prostaglandins, in negative regulation of inflammation and inflammatory diseases. Furthermore, several receptors on macrophages and DCs (CD200, STK) have been described to evoke anti-inflammatory responses after ligand engagement. Therefore, maintaining immune homeostasis by both pro- and anti-inflammatory mechanisms is critical to health and survival.

Apart from lack of appropriate anti-inflammatory mechanisms, chronic inflammation can also ensue from ineffectual elimination of the pathogen in primary infection or from prolonged exposure to potentially toxic agents. In addition, failure of conferring protective immunity against reacquisition, due to the various pathogen mechanisms for evading the immune responses, participates in the progression of acute to chronic inflammation. Finally, chronic inflammation is a hallmark of autoimmunity, such as in rheumatoid arthritis (RA).

1.3.1. Prerequisite for aging

Chronic inflammatory diseases, such as RA, exhibit an increasing incidence with age. Progressively degenerative diseases, associated with aging, include Alzheimer’s disease,
Parkinson disease, amyotrophic lateral sclerosis, atherosclerosis, type 2 diabetes, etc. These disorders embody a chronic inflammatory state, as evidenced by local infiltration of inflammatory cells, high deposition of complement components, free radical-induced injuries and increased circulatory levels of pro-inflammatory cytokines. The pro-inflammatory condition might function as a primary causal event or as a secondary concomitant condition of aging. Although this remains unknown, inflammation profoundly influences the pathogenesis, progression and prognosis of age-related diseases. Large studies have demonstrated that high plasma levels of IL-6 predict disability onset in the elderly (Ferrucci et al, 1999). Similarly, serum TNF-α levels are considered a strong predictor of mortality in both old people (Bruunsgaard et al, 2003b) and centenarians (Bruunsgaard et al, 2003a). Moreover, high levels of IL-6, IL-1Rα and C-reactive protein are significantly associated with poor physical performance and muscle strength in older people (Cesari et al, 2004).

Consequently, treatment with anti-inflammatory agents provides symptomatic relief to several aging-associated diseases.

A unified concept that explains the pathogenesis of diverse degenerative conditions in different organs is the free radical theory of aging. The basic premise is that, firstly, aging and its related disease processes are the net consequence of free radical-induced damage during lifetime and, secondly, the inability to counterbalance these changes by anti-oxidative defences. Reduction-oxidation (redox) reactions occur as part of normal biological processes, such as respiration or inflammation, during which toxic intermediates, such as H₂O₂, O₂⁻ and OH⁻, are generated. Other sources of radicals include transition metals, NO, radiation energy and enzymatic metabolism. Anti-oxidants (vitamins A/E/C, glutathione, etc) and scavenging enzymes (catalase, superoxide dismutase, thioredoxin reductase, etc) constitute the major defence mechanisms against free radical-mediated damage. The oxidative stress by remaining reactive species activates redox-sensitive transcription factors, leading to generation of pro-inflammatory molecules and a state of low-grade chronic inflammation, as inflammation itself amplifies the process by a positive feed-back loop on radical formation. In this sense, the term ‘inflamm-aging’ is coined to indicate the shift towards a pro-inflammatory profile at systemic level when getting older (Franceschi et al, 2000). Serum levels of inflammatory markers, such as C-reactive protein and serum amyloid A, are 2-4-fold more elevated in aged individuals than in young-middle-aged people. However, inflamm-aging is not only related to the immunological history of individuals, but also to energy metabolism and mitochondrial activity, which undergo profound dysfunction during the aging process (Salvioli et al, 2006). As dysregulation of cytokine expression has also been observed for healthy old people, inflamm-aging by itself may not be sufficient to
Introduction

trigger age-related diseases and to reduce survival, despite being the inescapable result of long lasting exposure to acute and chronic infections and the consequent life-long antigenic burden. It is predicted that a second hint is necessary, including a genetic predisposition, such as polymorphisms, to the onset of specific age-related diseases (Franceschi et al, 2000).

1.3.2. Prerequisite for cancer

In 1863, Rudolf Virchow noted leukocytes in neoplastic tissues and speculated that the chronic inflammatory infiltrate, observed in tumors, reflects the origins of cancer (Balkwill & Mantovani, 2001). It has been estimated that more than 15 percent of all cancers are initiated by chronic inflammatory diseases. This is exemplified by hepatitis or inflammatory bowel disease that predispose to liver cancer or colon cancer, respectively (Coussens & Werb, 2002; Li et al, 2005a). Chronic inflammation constitutes a risk factor for a variety of epithelial cancers, such as malignancies of prostate, cervix, esophagus, stomach, liver, colon, pancreas and bladder. Cigarette smoking and gastroesophageal reflux usually leads to persistent inflammation and are associated with cancer of the lungs and esophagus, respectively. However, for many of these inflammation-associated cancers, the initiating influence remains obscure, although some infectious etiologies have been described, including colonization by hepatitis B/C virus or subtypes of human papilloma virus that can lead to hepatocellular (Perz et al, 2006) or cervical cancer (DiMaio & Liao, 2006), respectively. Bacterial pathogens too, such as Helicobacter pylori, may cause gastric cancer (Konturek et al, 2006). The epidemiological finding that long-term use of non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, markedly decreases colorectal cancer risk by 40-50% strengthens the proposed link between inflammation and cancer (Smalley & Dubois, 1997).

In addition to the secretion of cytokines and chemokines, the activation of inflammatory cells leads to a respiratory burst that releases superoxide free radicals via NADPH oxidase, which contribute to malignant transformation by peroxidating lipids in cellular and organellar membranes. Other reactions by free radicals with amino acids or DNA result in protein fragmentation or single-stranded breaks and genetic mutations, respectively (Hussain et al, 2003). Such cellular damage stimulates apoptotic cell death and compensating reactive hyperproliferation, that promotes further mutation under this selection pressure. Ultimately, populations of cells bearing survival advantages constitute the phase of malignant transformation and cancer progression, which is further enhanced by cytokine-mediated angiogenesis and growth. Another way by which inflammation affects
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tumorigenesis is by induction of CD11b⁺ Gr1⁺ immature myeloid cells in lymphoid organs, which inhibit activation of CD4⁺ and CD8⁺ T cells (Bunt et al, 2006). In turn, cancer cells avoid immunosurveillance through outgrowth of poorly immunogenic tumor cell variants (immunoselection) and through subversion of the immune system, by actively suppressing the immune response (Zitvogel et al, 2006). One such mechanism is skewing the balance of immune responses towards a Th2 type, which reduces the available anti-tumor activity of CTLs. In addition, the Ras pathway in tumors stimulates IL-8 chemokine production, leading to more inflammatory cell recruitment and therefore contributes to inflammation-associated carcinogenesis (Sparmann & Bar-Sagi, 2004). Tumor cells also re-program macrophages to produce pro-tumorigenic factors, such as angiogenic factors, matrix metalloproteases, reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Pollard, 2004). The association between inflammation and cancer, which is discussed throughout this paragraph, is schematically shown in Figure 3.

![Figure 3](image)

**Figure 3:** If genetic damage is the match that lights the fire of cancer, inflammation may provide the fuel that feeds the flames. Adapted from (Moss & Blaser, 2005).

2. Transcription factor NF-κB

2.1. Rel family

Discovery and early characterization of nuclear factor-κB (NF-κB) are rooted in understanding B cell biology. In 1986, NF-κB was discovered biochemically as a nuclear DNA-binding activity in activated B cells, with affinity for the transcriptional enhancer of the Ig κ light chain gene (Sen & Baltimore, 1986). Later on, however, it turned out that none of the information that the name implies is fully correct, as NF-κB is not a critical regulator of Ig κ light chain transcription, nor is it B cell-specific, nor truly a nuclear factor per se (Hoffmann &
In fact, among numerous eukaryotic transcription factors, NF-κB is an essential and critical regulator, which exists in virtually all cell types.

Based on structural homology, the NF-κB or Rel family is a collective designation for a group of protein transcription factors, sharing a highly conserved stretch of 300 amino acids, named the Rel homology domain (RHD). In mammals, five Rel members have been described so far: p50/p105 (NFKB1), p52/p100 (NFKB2), c-Rel, RelB and RelA (p65). Their amino (N)-terminal RHD contains a nuclear localization sequence (NLS), which regulates NF-κB subcellular localization through interaction with specific ‘inhibitor of NF-κB’ (IκB) proteins. Furthermore, the RHD is involved in sequence-specific DNA-binding and dimerization. To obtain functional competence, the NF-κB proteins pair to form hetero- or homodimers, which are associated with specific biological responses that stem from their ability to regulate target gene transcription differently. Most commonly encountered in mammalian cells is the p50/p65 heterodimer, despite the diversity of other members. Only RelA, RelB and c-Rel contain a distinct carboxy (C)-terminal transactivation region. In this region, p65 consists of 2 transactivation domains (TAs), namely TA1, which is confined to the terminal 30 amino acids and TA2, within the N-terminally adjacent 90 amino acids (Schmitz & Baeuerle, 1991). RelB even has additional transactivation sequences in its N-terminus (Dobrzanski et al, 1993). Out of these five NF-κB members, 15 combinatorial associations offer a dynamic transcriptional regulation over a range of physiological conditions. Among them, most are transcriptionally active, except for p52 homodimers, p50 homodimers and p50/p52 dimers, which can repress transcription (Hoffmann & Baltimore, 2006). Nevertheless, p50 homodimers have been recently identified in promoting IL-10 transcription (Cao et al, 2006). Furthermore, RelB displays a greater regulatory flexibility, as it can be both an activator and repressor. In a given cell, a subset of dimers may be present, depending on the cell type, stage and conditioning by environmental cues. Generally, RelA is ubiquitously expressed, whereas c-Rel is constitutively present in mature monocytic and lymphocytic lineages, while in other cells only following stimulation (Hoffmann & Baltimore, 2006).

### 2.2. IκB family

In unstimulated cells, NF-κB dimers are retained in a latent condition by binding of IκB proteins, as the latter prevent NF-κB-DNA binding (Baeuerle & Baltimore, 1988). This multigene family comprizes several members containing a region of multiple 30-33 amino acid motifs for protein-protein interaction, called ankyrin repeats. The canonical IκBα, IκBβ and IκBε belong to this family, as well as IκBζ, IκBNS, oncogene Bcl-3 and the precursor proteins p105 and p100, the C-terminal portions of which have also been termed IκBγ and
IκBα, respectively. However, it remains unclear whether these precursor proteins and/or C-termini actually function as bona fide IκB proteins. Although Bcl-3, IκBNS and IκBζ harbour characteristic IκB ankyrin repeats, they are constitutively nuclear, fulfilling a role in transcription modulation (Kuwata et al, 2006; Yamamoto et al, 2004; Yamazaki et al, 2001).

Under basal conditions, IκB proteins specifically interact via their ankyrin repeat domain with the NF-κB RHD, due to which they interfere with the NLS of NF-κB in a physical manner and also avoid NF-κB-DNA binding. However, X-ray crystallographic structures of IκBα and IκBβ, bound to p50/p65 dimers revealed that only the NLS of p65 is masked, whereas the NLS of p50 remains accessible in the case of IκBα (Malek et al, 2001). Coupled with nuclear export sequences (NES) on both NF-κB (Harhaj & Sun, 1999) and IκBα, the partial masking results in a constant active shuttling of NF-κB/IκBα complexes between the nucleus and the cytoplasm, although a predominant cytoplasmic localization of NF-κB is kept (Huang et al, 2000; Tam et al, 2000). However, upon stimulation and IκBα degradation, the dynamic balance between cytoplasm and nucleus is altered in favour of higher nuclear abundance of NF-κB molecules. Because IκBβ, in contrast to IκBα and IκBε, contains no functional NES, it cannot efficiently remove Rel proteins from the nucleus (Huang & Miyamoto, 2001). However, IκBβ represses NF-κB-driven transcription, possibly by forming a ternary complex with NF-κB and DNA (Tran et al, 1997). Cytoplasmic retention of p65 has been classically associated with IκB proteins, although in cells lacking isoforms α, β and ε, p65 is still mainly localized in the cytoplasm (Tergaonkar et al, 2005). Therefore, other proteins, such as 14-3-3 proteins, could be involved. These form ternary complexes with both p65 and IκBα, and are required for the appropriate regulation of NF-κB activity. In more detail, 14-3-3 proteins alter the subcellular distribution of NF-κB by facilitating the nuclear export of IκBα-p65 complexes in basal or TNF-α-stimulated conditions (Aguilera et al, 2006).

Besides its function as a chaperone, IκBα participates in basal promoter repression as well. Efficient recruitment of IκBα, in association with histone deacetylase (HDAC)1/5, is detected on the regulatory regions of the Notch-target gene Hes1, whereas this mechanism is not operating on classical NF-κB-regulated genes, such as RANTES or IL-6 (Aguilera et al, 2004). Other nuclear roles of IκB proteins are described in 2.3.1.

2.3. Signaling pathways to NF-κB activation

NF-κB stands out as an exceptionally important factor due to its pleiotropic effects, implicated in several physiological processes. Mouse knock-out studies reveal that NF-κB is involved in cell growth, proliferation, cell cycle regulation, cell-cell communication, differentiation, apoptosis, neuronal learning, inflammation and innate/acquired immune
responses. Therefore, more than 150 substances and physiological conditions are known to activate NF-κB (Pahl, 1999), which can be grouped into 3 types of stimuli: firstly, bacterial, fungal, protozoan, parasite-derived or viral products (endotoxin, peptidoglycan, Tax, etc) and pro-inflammatory cytokines (IL-1, TNF-α, etc); secondly, non-inflammatory signaling mediators (lymphotoxin (LT)-β, B cell-activating factor (BAFF), etc) and thirdly, metabolic, environmental or genotoxic stress (free radicals, radiation, oxidants, DNA damage, etc). Accordingly, 3 distinct NF-κB-activating pathways have been described, which all rely on sequentially activated kinases (Figure 4).

![Figure 4: Illustration of the classical/canonical, alternative/non-canonical and atypical NF-κB-activating pathways. Adapted from (Viatour et al, 2005).](image)

### 2.3.1. Canonical pathway

The evolutionarily conserved classical pathway is strongly induced by binding of pro-inflammatory molecules, such as LPS or cytokines like TNF-α, to their recognition receptors on the cellular plasma membrane of many cell types. Although the proximal-receptor adaptor proteins differ for both stimuli (see 3.1 and 3.2), the recruitment of those signaling proteins eventually leads to the activation of the classical IκB kinase (IKK) complex, comprised of scaffold protein ‘NF-κB essential modulator’ (NEMO)/IKKγ, and the kinases IKKα and IKKβ. IKKβ is responsible for phosphorylation of IκBα/β/ε on two specific N-terminal serine residues (for IκBα on Ser32 and Ser36 (Brown et al, 1995), for IκBβ on Ser19 and Ser23 (McKinsey et al, 1996), and for IκBε on Ser18 and Ser22 (Whiteside et al, 1997)), which then act as a docking platform for a WD repeat- and F box-containing protein, called β-TRCP. The subsequent ubiquitination at Lys21 and Lys22 of IκB proteins takes place by a specific ubiquitin ligase, belonging to the Skp-1/Cul/F box family. These modifications induce the 26S proteasome-mediated degradation of IκB proteins without affecting the integrity of the bound NF-κB dimers. This results in liberation of the transcription factor NF-κB, of which the NLS directs its translocation to the nuclear compartment, where it can bind to promoter
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recognition sequences and activate target gene transcription. Additionally, Src tyrosine kinases are directly implied in the LPS- or TNF-α-induced tyrosine phosphorylation of IκBα proteins and necessary for NF-κB release (Abu-Amer et al, 1998; Kang et al, 2005). They may indirectly contribute to serine phosphorylation of IκBα (Abu-Amer et al, 1998).

Depending on the cell type and stimulus, IκBα and IκBβ proteins display different kinetics, as rapid degradation (within 15 min) is observed for IκBα, in contrast to IκBβ (30-60 min), because IKKβ phosphorylates IκBβ less efficiently (Wu & Ghosh, 2003). In this way, overall activation of NF-κB consists of two overlapping phases, an IκBα-mediated, transient phase and a persistent phase, through IκBβ (Thompson et al, 1995). As the IκBα promoter itself contains 3 NF-κB-binding sites (Ito et al, 1994), IκBα drives a powerful negative feedback mechanism, resulting in post-induction repression of NF-κB activity upon stimulus removal (Hoffmann et al, 2002; Scott et al, 1993). In this way, reestablishing the cytoplasmic pool of inactive NF-κB-IκBα complexes allows priming for additional rounds of NF-κB activation. Furthermore, this negative loop may give rise to an oscillatory temporal NF-κB activation profile during chronic stimulation (Hoffmann et al, 2002; Nelson et al, 2004). The IκBα-mediated oscillatory propensity in signaling is counteracted by a second negative feedback system, controlled by IκBε, which is delayed and in anti-phase to IκBα (Kearns et al, 2006). IκBβ, by contrast, undergoes no substantial post-NF-κB activation, due to variance at the level of gene regulation (Griffin & Moynagh, 2006).

2.3.2. Non-canonical pathway

In contrast to the canonical pathway, which is largely responsible for regulating inflammation, as well as for the control of proliferation and apoptosis of lymphoid cells during the immune response, the non-canonical or alternative pathway for NF-κB activation is more associated with the lymphoid organogenesis, lymphocyte development and activation, particularly of B cells. Therefore, this pathway provides the link between innate and adaptive immunity. This functional distinction is echoed by the related biochemical characteristics of these pathways as the canonical signaling is fast (responding in minutes) and transiently acting (negative feed-back loop), while the non-canonical pathway provides a long-lasting NF-κB activity without dynamic control. Cytokines, such as LT-β, BAFF, CD40L, and viruses, such as human T-cell leukaemia virus or Epstein-Barr virus, trigger this alternative pathway, relying on IKKα-dependent p100 cleavage to generate p52. Together with RelB, p52 translocates to the nucleus to initiate target gene transcription.
2.3.3. Atypical pathway

The last NF-κB activation mechanism is classified as atypical, because it is independent of the IKK complex, although the proteasome can be required, relying on the stimulus. The type of stress stimulus (UV radiation, oxidative stress, hypoxia, etc) determines which specific kinases are implicated in the release of NF-κB (CK2, Syk, etc).

3. Inflammatory stimuli and gene expression

3.1. LPS

3.1.1. Structure

As essential and major component of the outer leaflet in the outer membrane of Gram-negative bacteria, LPS is a complex glycolipid composed of 3 distinct regions: a hydrophilic, short, non-repeating core oligosaccharide, a distal polysaccharide (O-Ag) and a hydrophobic domain, known as lipid A (endotoxin). The lipid A structure varies between bacteria of different species and is responsible for the biological activity of LPS in infection. As such, LPS represents one of the conserved microbial structures or PAMPs responsible for activation of the innate immune system. The evolutionarily conserved innate immune system is regarded as the first line of defence mechanisms for protecting the host against invading pathogenic organisms.

3.1.2. LPS receptor TLR4

The plasma membrane-bound recognition receptor for LPS was identified through analysis of a strain of mice, C3H/HeJ, that is hyporesponsive to LPS. This strain carries a point mutation within the Toll-like receptor 4 (TLR4) gene region encoding the cytoplasmic tail (Poltorak et al, 1998). Generation of TLR4−/− mice has confirmed that TLR4 is required for LPS signaling (Hoshino et al, 1999). TLR4 mutations are also associated with endotoxin hyporesponsiveness in man (Arbour et al, 2000) and certain TLR4 polymorphisms may be associated with increased risk of septic shock (Lorenz et al, 2002). Stimulation of TLR4 by microbial components triggers expression of several genes that are involved in the immune response, such as inflammatory cytokines, costimulatory molecules, recognition receptors, type I interferons, etc. However, accessory molecules are needed for efficient LPS recognition by TLR4. The secreted co-receptor MD-2 is directly involved in high-affinity ligand binding and subsequent receptor activation, whereas serum LPS-binding protein (LBP) and membrane-bound CD14 control ligand presentation to the receptor complex and influence
the amplitude of LPS responses. CD14 even coordinates the assembling of signaling adaptor complexes downstream of TLR4 (Jiang et al, 2005). Additionally, another ancillary protein, CD11b/CD18, must be engaged to deliver optimal signaling (Perera et al, 2001).

TLR4 belongs to the mammalian TLR family, which consists of 10 human and 13 murine members, homologous to the Drosophila type I transmembrane Toll family. The founding member herefrom, Toll, was first identified as an essential player in embryonic patterning in Drosophila and was subsequently shown to have a role in anti-fungal immunity. Besides LPS, TLR4 also recognizes viral (F protein) and plant products, such as taxol, a diterpene purified from the bark of the western yew (Taxus brevifolia), as well as endogenous host-derived proteins (hsp60, hsp70, extra domain A of fibronectine, etc). However, in contrast to LPS, which is a potent immuno-activator, very high concentrations are needed for endogenous ligands. In addition, it has been shown that LPS contaminations in the hsp70 preparation confers ability to activate TLR4, which warrants further investigation on putative endogenous TLR4 agonists.

TLR4 contains an extracellular domain bearing leucine-rich repeats, a transmembrane domain and cytoplasmic Toll/interleukin (IL)-1 receptor (TIR) domain. Although IL-1 receptors possess 3 Ig-like structures in their extracellular domain, a high sequence similarity in the cytoplasmic domain of the TLR4 and IL-1 receptor (IL-1R) family is observed. This TIR domain is indispensable for signal transduction, as the proximal events of TLR4-mediated signaling are triggered by receptor homodimerization (Saitoh et al, 2004) and TIR domain-dependent heterophilic interaction with TIR domain-containing cytosolic adapters. These transfer signaling to IKK via two distinct pathways, the MyD88-dependent (see 3.1.3.) and TRIF-dependent pathway (see 3.1.4.).

3.1.3. LPS-induced signaling pathway via MyD88 (Figure 5)

The MyD88-dependent pathway is analogous to signaling pathways through IL-1Rs. MyD88 harbours a C-terminal TIR domain, by which it presumably associates to the TLR4 TIR domain. The N-terminal death domain (DD) of MyD88 recruits and anchors the serine/threonine kinase IL-1-associated protein kinase (IRAK)-4 via its DD, which leads to IRAK-4 activation. IRAK-4 phosphorylates and activates IRAK-1, resulting in association with TNF receptor-activated factor (TRAF)6. TRAF6 contains a N-terminal RING domain that is found in a number of E3 ubiquitin ligases and forms a complex with Ubc13 and Uev1A to promote synthesis of polyubiquitin chains linked through Lys63 of ubiquitin. This modification in turn activates TGF-β-activated kinase (TAK)1, which indirectly activates the IKK complex, culminating in NF-κB activation.
Simultaneously with NF-κB activation, a second signaling cascade evolves in mitogen-activated protein kinase (MAPK) activation. LPS-induced ROS production is shown to induce complex formation between TRAF6 and apoptosis signal-regulating kinase (ASK)1, which activates MAPK kinase (MKK)3/6 and subsequently, p38 MAPK (Matsuzawa et al, 2005). In addition, TRAF6-MKK kinase (MEKK)3 association regulates both NF-κB activation and p38/JNK MAPK activation (Huang et al, 2004). Activated TAK1 phosphorylates MKK6, which activates JNK and p38 MAPK (Wang et al, 2001). Protein kinase (PK)Cζ induces MEK-ERK signaling (Monick et al, 2000), whereas Raf1-ERK regulates tyrosine phosphorylation of phosphatidylinositol 3-kinase (PI3K) in other cells (Nakayama et al, 2003). The importance of all identified kinases, involved in signaling after LPS stimulation, needs to be considered in relation to potentiation of transcription factor activity or specific gene transcription responses.

**Figure 5**: Overview on the TLR4-dependent, intracellular signaling pathways towards NF-κB-driven inflammatory gene transcription. Adapted from (Kawai & Akira, 2006).

Apart from adaptor molecule MyD88, a structurally related protein with a TIR domain was identified in a database search and named ‘TIR domain-containing adaptor protein’ (TIRAP) or MyD88-adaptor-like (Mal). Similar to MyD88 null mice, TIRAP/Mal-deficient mice show defective inflammatory responses to TLR4 ligands, indicating TIRAP/Mal is a linking adaptor for TLR4 signaling.

### 3.1.4. LPS-induced signaling pathway via TRIF (Figure 5)

The discovery of a second TLR4-initiated pathway was based on the observation that MyD88-deficient cells were still able to produce IFN-β and capable to induce late NF-κB and MAPK activation peaks (Kawai et al, 1999). So, another TLR4-adaptor, ‘TIR-related adaptor
protein inducing IFN-β' (TRIF)/'TIR domain-containing adapter molecule' (TICAM)1, was identified to be involved in this MyD88-independent pathway. As inflammatory cytokine production is reduced in TRIF-deficient mice, both pathways seem required for maximal induction of inflammatory cytokines in response to LPS. In analogy to the MyD88 requirement of TIRAP/Mal, TLR4 does not interact with TRIF directly but via TRIF-related adaptor molecule (TRAM)/TICAM2 as a bridging adaptor protein.

The C-terminal region of TRIF contains a RIP homotypic interaction motif (RHIM), which mediates interaction with receptor-interacting protein (RIP)1, culminating in NF-κB activation. A different pathway may also be involved in TRIF-mediated NF-κB activation, as TRIF possesses three typical TRAF6-binding domains in the N-terminal region. Both pathways might converge at the IKK complex to induce maximal activation of NF-κB. Besides NF-κB, the TRIF-dependent signaling leads to activation of the interferon regulatory factor (IRF)-3 transcription factor, essential for type I IFN production, particularly IFN-β. Key molecules for IRF3 activation have been revealed to be non-canonical IKKs, TBK1 and IKKι/IKKe (Sharma et al, 2003), which were shown to associate with the N-terminal portion of TRIF. Another IRF, IRF-5, is found downstream of TLR4 and has an integral role in the induction of pro-inflammatory genes, such as IL-6. IRF-5 forms a trimeric complex with MyD88 and TRAF6 (Takaoka et al, 2005).

3.1.5. TLR4 biological function

Belonging to the evolutionarily conserved PRRs, TLRs have emerged as the key sensors for PAMPs. They are mainly expressed on sentinel cells of the innate immune system, such as DCs, macrophages, monocytes, etc. These cells not only stimulate appropriate gene expression (cytokines, chemokines) in response to infection, mounting an efficient immune response, but also engulf and lyse pathogens. TLR4/MyD88 proteins are reported to be involved in this phagocytosis process, and more detailed, in the maturation of phagosomes (Blander & Medzhitov, 2004). Further studies indicate that p38 activation is specifically implied (Doyle et al, 2004). Inside the phagosomal/lysosomal compartments, pathogens are broken down and the resulting peptide fragments are presented to the T cell receptor of CD4+ T cells as Ags on specific MHCII molecules. Together with the TLR4-induced costimulatory molecule expression (CD80, CD86), these T cells are instructed by the APCs to proliferate and differentiate into effector cells. Depending on the type of cytokine production in the local cellular environment, naïve T cells are polarized to a type 1 or type 2 phenotype, which distinguish in their cytokine secretion pattern and immunomodulatory effects, provoking cell-based or humoral immune responses, respectively (Constant &
Bottomly, 1997) (see 1.2.2.2). So, TLR4 and the TLR family in general play an instructive role in innate immune responses against pathogenic insults, as well as in the subsequent induction of adaptive immune responses.

3.2. TNF-α

3.2.1 Structure and function

TNF-α was identified in 1975 as an endotoxin-induced serum factor, that causes necrosis of certain murine tumors in vivo (Carswell et al, 1975). This research effort was based on the work of a pioneering surgeon, who used bacterial vaccins to treat inoperable sarcoma in patients (Wiemann & Starnes, 1994). After isolation in 1984, TNF-α was characterized as a pleiotropic pro-inflammatory cytokine, encoded within the MHC. This homotrimeric protein is primarily produced by activated macrophages, as a 26 kDa transmembrane protein, that can be cleaved to release a 17 kDa soluble TNF-α form. It is also referred to as cachectin or differentiation-inducing factor, pointing to its role in differentiation, as well as in proliferation and cell death. However, another main function of TNF-α concerns the regulation of immune cells and inflammatory reactions. Therefore, TNF-α is involved in the pathogenesis and severity of many diseases and inflammatory disorders, such as RA, psoriasis, inflammatory bowel disease, asthma, cancer, septic shock, osteoporosis, etc.

3.2.2. TNF-α receptors

The TNF-α ligand achieves physiological and pathological effects by binding to 2 TNF receptor (TNFR) isotypes, TNFR1 (also known as p55, p60, CD120α) and TNFR2 (also known as p75, p80, CD120β), with apparent molecular masses of 55-60 and 75-80 kDa, respectively. TNFR1 expression is constitutive on all nucleated cells, whereas TNFR2 is primarily restricted to cells of the hematopoietic lineage. These single transmembrane glycoproteins contain 4 tandemly repeated Cys-rich motifs in their extracellular domain. Their intracellular region differs significantly, as only TNFR1 but not TNFR2 bears a DD. The unrelated structure and the more restricted tissue distribution of TNFR2 suggests delineation of their signaling and biological functions. Interestingly, the observation that human TNF-α displays a high affinity for mouse TNFR1 but not for TNFR2 (Lewis et al, 1991), led to in vivo studies on the role of both receptors. These revealed a decisive in vivo function of TNFR1 in host defence against intracellular pathogens (Pfeffer et al, 1993), whereas TNFR2 plays a role in TNF-α-induced necrosis (Erickson et al, 1994). However, TNFR2-mediated activation
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of NF-κB has been reported in some cell lines (Laegreid et al, 1994), possibly by enhancing or synergizing TNFR1 signaling, e.g. by ligand passing (Tartaglia et al, 1993).

Upon binding the trimerized ligand TNF-α, the TNFR homotrimerizes, which allows the signal to pass to downstream adaptors. However, this model is challenged by the discovery of a preformed ligand-binding assembly domain in the extracellular region of TNFRs, which is required for ligand-independent trimerization and more importantly, for efficient signal transduction (Chan et al, 2000). This theory implies certain conformational changes upon TNF-α binding. As this work is mainly focusing on inflammation and immunity, we will only discuss the signaling pathway orchestrating these physiological processes.

3.2.3. TNFR signaling pathway (Figure 6)

The silencer of death domains (SODD) protein constitutively associates intracellularly with the DD of TNFR1 (Jiang et al, 1999), which is shown to be critical for regulation of the TNF response (Takada et al, 2003). Upon TNF-α treatment, SODD is released from TNFR1, permitting the recruitment of adapter proteins (Jiang et al, 1999). TNFR-associated death domain (TRADD) interacts with the TNFR via the DD as a protein-protein interaction domain. Subsequently, the DD-containing RIP1 associates with TRADD and recruits TRAF2 via its intermediate domain (Hsu et al, 1996). Whereas both TRAF2 and RIP1 play an important role in IKK complex recruitment (Devin et al, 2000; Zhang et al, 2000), RIP1 mediates IKK activation as a scaffold protein, since RIP1 interacts with TAK1-MEKK3 (Blonska et al, 2005). In this functional complex, TAK1 regulates auto-phosphorylation of MEKK3, which directly phosphorylates the IKK complex (Yang et al, 2001). Alternatively, signal-mediated RIP1 ubiquitination on Lys63 facilitates recruitment of TAK1-binding protein (TAB)2, which is necessary for activating TAK1 (Kanayama et al, 2004). TAK1, in turn, is critical for IKK activation (Takaesu et al, 2003). In this way, RIP1 may indirectly activate the IKK complex. Alternatively, TRAF2-associated NIK is required for TNF-α-induced NF-κB activation (Malinin et al, 1997) via IKK (Woronicz et al, 1997). Apart from this TRAF2-linked pathway, an additional but non-redundant kinase cascade towards IKK activation has been observed. The sequential activation of phospholipase C-γ2, PKCα and c-Src leads to tyrosine phosphorylation of IKKβ on Ser188 and Ser199 and is also linked to NIK, which mediates the classical serine phosphorylations of IKKβ (Huang et al, 2003a; Huang et al, 2003b). For TNF-α signaling, this IKK complex requires additional proteins, such as cdc37, Hsp90 and ELKS. Heterocomplex formation of IKK with cdc37 and Hsp90 is a prerequisite for IKK
trafficking from the cytoplasm to the membrane (Chen et al, 2002a) and ELKS likely functions in the recruitment of IκBα to the IKK complex (Ducut Sigala et al, 2004).

**Figure 6:** Overview on the TNF-α-induced intracellular signaling pathways towards downstream players for NF-κB activation.

After TNF receptor stimulation, the signaling cascade targets the IKK complex, leading to NF-κB activation, but also activates p38, JNK and ERK MAPKs (Boone et al, 1998). The MAPK pathways employ a central three-tiered module of protein kinases, characterized by MAP3Ks and dual-specificity MAPK kinases (MAP2K). The bifurcation of these 2 signaling pathways occurs at the MAP3K level. The TNF-α-induced p38 activation and IL-6 production was impaired in RIP1−/− MEF cells and RIP1 association with MEKK3 is necessary for p38 activation (Lee et al, 2003b). In addition, MEKK1, as well as TAK1 (via MKK3/6), have been described to activate JNK and p38 MAPK (Lu et al, 1997; Moriguchi et al, 1996). Although the ERK MAPKs are activated after TNF-α stimulation, their upstream kinase partners remain unknown. Possibly, a similar sequence as after growth factor stimulation, the Ras-Raf-MEK1/2-ERK pathway, is involved. Most kinases implicated in TNF-α-stimulated MAPK activation signaling have been identified in different cell types, context and by distinct methods, ranging from *in vitro* assays, overexpression to the use of knock-out systems. This hampers a clear overview on the actual signaling cascades. Furthermore, the outcome of each player in TNF-α-signaling differs, as not all MAPK types affect the same transcription factors and gene promoters.
3.3. NF-κB regulation

While the proximal cytoplasmic signaling events control the activation of NF-κB, subsequent enzymatic events provide an additional level of regulation, as they influence the initiation, strength and duration of NF-κB action. Many reports demonstrate that both p50 and p65 are subject to posttranslational modifications, which regulate different functions, including NF-κB transactivation, DNA-binding affinity, subcellular localization and affinity for IκBα assembly.

3.3.1. Phosphorylation

As stimulus-induced phosphorylation of NF-κB is extensively studied, many different kinases and signal transduction pathways have been reported to target p65 on distinct serine residues in response to TNF-α or LPS, underscoring the complexity and specificity of gene regulation (Figure 7). In the RHD of p65, phosphorylation of Ser276 occurs by the catalytical subunit of PKA in response to LPS (Zhong et al, 1997) or by mitogen- and stress-activated protein kinase (MSK)1 in response to TNF-α (Vermeulen et al, 2003b). This promotes interaction of p65 with the co-activator CBP (Zhong et al, 2002; Zhong et al, 1998), which is required for TNF-α-induced IL-6 gene expression (Okazaki et al, 2003). Phosphorylation on Ser311 is mediated by PKCζ (Anrather et al, 1999) and correspondingly, TNF-α-induced p65 phosphorylation is inhibited in PKCζ-/- MEF cells (Leitges et al, 2001). In contrast to Ser276 phosphorylation, IKKβ-dependent phosphorylation of Ser468 in the TA2 of p65 does not contribute to its transactivation in response to TNF-α (Schwabe & Sakurai, 2005). However, Ser529 seems necessary for efficient p65 transactivation (Duran et al, 2003). In the TA1 of p65, Ser529 of p65 is phosphorylated by casein kinase II in response to TNF-α (Wang & Baldwin, 1998; Wang et al, 2000). In experiments using reconstituted p65 null cells, this serine residue contributes to p65 transactivation (O'Mahony et al, 2004). However, others have found no role for Ser529 in p65 activity in response to TNF-α treatment or IKKβ overexpression (Okazaki et al, 2003; Sakurai et al, 1999a; Yang et al, 2003). Lastly, regulated Ser536 phosphorylation in the TA1 of p65 is suggested to be due to IKKβ (Sakurai et al, 1999b), which is confirmed by later reports showing that both IKKs phosphorylate Ser536 in vitro (Sizemore et al, 2002). TRAF2, TRAF5, TAK1 and IKKα/β are shown to be important mediators for phosphorylation of Ser536 in response to TNF-α (Sakurai et al, 2003). In contrast, a further study demonstrates that the LPS- and TNF-α-induced phosphorylation of endogenous p65 at Ser536 is unaffected in IKKα null fibroblasts. Only the LPS-induced, but not the TNF-α-induced phosphorylation is lost in IKKβ null cells (Yang et al, 2003). Finally, TBK1, in complex with NAP1, effectively phosphorylates p65 on Ser536 and
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this activity is stimulated by TNF-α (Fujita et al, 2003). Stimulus-induced Ser536 phosphorylation has been proposed to enhance the nuclear translocation rate of the RelA-containing dimer (Mattioli et al, 2004) and to regulate specific acetylation that is correlated with transcriptional activity (Chen et al, 2005).

Figure 7: Overview of the LPS- and TNF-α-induced phosphorylation sites in the p65 protein and the corresponding responsible kinases.

The Ser536 phosphorylation site has been implicated in the regulation of NF-κB activity, in response to different stimuli. Among these, p53 expression stimulates the ribosomal S6 kinase (RSK)1 to phosphorylate Ser536 on p65, which negatively affects its affinity for IκBα and, as such, promotes target gene binding (Bohuslav et al, 2004). After IL-1 stimulation, at least 5 kinases, including IKKa/β and IKK-related kinases TBK1 and IKKe, converge on Ser536 of p65. Phosphorylation subsequently enhances efficient coupling of p65 to the basal transcription machinery. However, mutations of this residue do not render the p65 protein inactive, suggesting that this phosphorylation site plays a modulatory rather than an essential role and acts in concert with other p65 phosphorylations (Buss et al, 2004b). T cell costimulation induces phosphorylation of p65 Ser536 by IKKβ, which serves to prolong the residence time of p65 in the cytoplasm. However, the predominant cytosolic occurrence and short duration of p65 phosphorylation argue against a prominent role of this posttranslational modification in NF-κB-dependent gene expression (Mattioli et al, 2004). Furthermore, evidence exists for a crucial role of the NIK-IKKα cascade in p65 activation by phosphorylation on Ser536 in LT-βR signaling (Jiang et al, 2003). In conclusion, the responsible pathways and function of this phosphorylation site on p65 have not been conclusively unraveled, although it is conceivable that these vary in a cell type- and stimulus-dependent way and that other modifications on NF-κB proteins contribute to the in vivo effect of a specific phosphorylation.

In contrast to the transcription-enhancing effect of the above-mentioned phosphorylations, the transactivation potential of p65 is repressed by phosphorylation at Thr502 by p14ARF (Rocha et al, 2003; Rocha et al, 2005) or by glycogen synthase kinase 3β-induced phosphorylation of p65 at Ser468 (Buss et al, 2004a). However, T cell costimulation-induced phosphorylation at this latter residue by IKKe has been reported transactivation-
enhancing (Mattioli et al, 2006). Nevertheless, phosphorylation within the TA1 or RHD domain of p65 has generally been associated with enhanced transactivation function. For p65, phosphorylation in the RHD governs an exchange between CBP and HDAC1 (Zhong et al, 2002). Similarly, IKKα-induced phosphorylation of p65 is a critical determinant for active derepression of the SMRT-HDAC3 complex from the p50/p65 heterodimer, allowing p300 to acetylate p65 at Lys310 for full transcriptional activity (Hoberg et al, 2006).

3.3.2. Acetylation

Co-regulators with an intrinsic histone acetyltransferase (HAT) activity have emerged as co-activators for various DNA-binding transcription factors. As such, the p300/CREB-binding protein (CBP) HATs play a major role in the in vivo acetylation of RelA, principally targeting Lys218, Lys221 and Lys310. Acetylation of Lys221 enhances DNA binding and impairs assembly with IκBα. Therefore, this modification impedes IκBα-mediated nuclear export of the NF-κB complex, thereby allowing the prolongation of the NF-κB response. In contrast, acetylation of Lys310 is required for full transcriptional activity of RelA, independent of changes in DNA or IκBα binding (Chen et al, 2001; Chen et al, 2002b). However, another group reported that pCAF/p300-mediated acetylation of p65 on Lys122 and Lys123 control post-activation turn-off of NF-κB, by affecting its DNA-binding affinity (Kiernan et al, 2003). In turn, acetylated RelA is deacetylated by HDAC3. Overexpression of p300 augments p50 acetylation on Lys431, Lys440, and Lys441, which correlates with higher DNA binding in vitro, compared to its unacetylated form (Furia et al, 2002). In agreement with this, enhanced p50 acetylation leads to increased p50 binding to two NF-κB-dependent promoters in vivo (Deng & Wu, 2003; Deng et al, 2003).

3.4. NF-κB-regulated genes

Molecular biological studies over the past 20 years have led to the identification of functional 10 bp DNA elements, called κB sites, in about 150 promoters of NF-κB-responsive genes. To these motifs, NF-κB dimers bind with varying affinities. These reflect the function of NF-κB in regulation of proliferation, apoptosis (see 3.4.2.), inflammation or immunological processes (see 3.4.1.).

3.4.1. Inflammation and immunity

These genes code for cytokines (TNF-α, IL-6, GM-CSF,...), chemotactic proteins (IL-8, MCP-1, RANTES,...), adhesion molecules (ICAM-1, VCAM-1, E-selectin, ELAM...), pro-angiogenic factors (VEGF), inducible enzymes (iNOS, COX-2, MMP-9,...), anti-microbial
peptides (β-defensine,…), cell surface (co-)receptors (MHCII, CD80, CD86,…), acute phase reactants (C-reactive protein,…), etc. Cytokines, such as IL-1β and TNF-α, can also directly activate NF-κB, thus establishing a positive auto-regulatory loop that in this way amplifies the inflammatory response and increases the duration of chronic inflammation. However, to avoid serious systemic disorders due to excess production of cytokines and other inflammatory mediators, mechanisms to modulate inflammatory and immune responses have evolved. The phenomenon of endotoxin tolerance exemplifies this, since exposure to microbial components, such as LPS, results in a severely reduced response to a subsequent LPS challenge. Several observations of negative regulation may explain the model. These range from extracellular decoy receptors (soluble TLR4) to intracellular inhibitors primarily on the MyD88-dependent pathway (MyD88s, IRAKM, SOCS-1, NOD2, PI3K, TOLLIP and A20), membrane-bound suppressors (ST2, SIGIRR, TRAILR and RP105), downregulation of TLRs (degradation, block of transcription) and TLR-induced apoptosis (Liew et al, 2005). Although the role of NF-κB in mediating these aspects of negative regulation is not fully understood, promoter analyses reveal that NF-κB plays a key role in induction of a certain microRNA (miRNA), miR-146a, in myeloid cells stimulated with IL-1β, TNF-α or LPS. As TRAF6 and IRAK-1 may be potential molecular targets via their 3'-untranslated region (UTR), this miR-146a could function as a novel negative regulator in fine-tuning the inflammatory and immune responses (Taganov et al, 2006).

3.4.2. Apoptosis

NF-κB activates genes that promote proliferation (cyclins, c-myc, etc) and cell survival via inhibition of apoptosis (c-FLIP, c-IAP1/2, XIAP, Bcl-XL, p53, etc). The essential role for RelA as anti-apoptotic regulator is exemplified by RelA knock-out cells, which are highly susceptible to apoptotic stimuli, such as TNF-α (Beg & Baltimore, 1996). Chromosomal amplification, overexpression and rearrangement of genes coding for Rel factors have been noted in many human hematopoietic and solid tumors. In line with its anti-apoptotic activity, aberrant constitutively activated NF-κB has been reported in various stages of numerous cancers, such as breast, colon, ovarian, liver cancer, among others. This persistent NF-κB activation is mediated by overexpression of viral homologues of proteins involved in NF-κB signaling, by (in)activating mutations in NF-κB proteins or signaling mediators, or by activation of upstream signaling molecules (Rayet & Gelinas, 1999). Besides implicating NF-κB in the control of the apoptotic response in established tumors and metastasis, a role in the resistance of tumor cells to therapy has also been attributed to NF-κB, since the action mechanism of most radio- and chemotherapies is based on apoptosis induction (Baldwin, 2005).
Genetic proof for the crucial involvement of NF-κB activation in early stages of cancer development, consistent with the acute oncogenicity of the viral oncoprotein v-Rel (Hannink & Temin, 1991), has been provided by several mice models. IKKβ deficiency in intestinal epithelial cells leads to a decrease in incidence of colitis-associated colon carcinoma, accompanied by an increased epithelial apoptosis during the tumor-promoting phase (Greten et al, 2004). Similarly, two distinct model systems of inflammation-induced cancer propose that NF-κB is not involved in tumor initiation, but rather has a role in the promotion phase by preventing apoptosis of pre-malignant cells (Luo et al, 2004a; Pikarsky et al, 2004). Additionally, NF-κB activation in tumor-associated inflammatory cells contributes to tumor growth by inducing synthesis of tumor-promoting pro-inflammatory mediators (Greten et al, 2004; Luo et al, 2004a; Pikarsky et al, 2004).

However, in some mouse models, NF-κB inhibition induces cancer. Blockade of NF-κB in keratinocytes predisposes murine skin to squamous cell carcinoma, and in normal human epidermal cells, NF-κB triggers cell cycle arrest (Dajee et al, 2003). Similarly, inhibiting NF-κB in the liver in the course of a chemical carcinogenesis model, involving acute injury, accelerates tumorigenesis (Maeda et al, 2005). A possible explanation for this enigma could be context specificity, as extensive investigation of the model systems proposes that the pro-tumorigenic effect of NF-κB inhibition in skin could be due to an indirect upregulation of TNF-α, which mediates carcinogenesis, rather than to a direct anti-neoplastic effect of NF-κB itself (Pikarsky & Ben-Neriah, 2006). However, under certain circumstances, NF-κB confers pro-apoptotic activities. In addition to differences due to the cell type involved, the function of NF-κB as an anti-apoptotic or pro-apoptotic factor likely depends on the nature of the inducing stimulus. In the same cells, NF-κB antagonizes TNF-α-activated apoptosis, yet promotes H2O2-induced apoptosis (Kaltschmidt et al, 2000). The timing of modulation of NF-κB activity relative to the death stimulus may play a key role in determining which of these cellular responses occurs (Clemens et al, 1998; Lin et al, 1998).

### 3.4.3. Promoter specificity

The NF-κB recognition site displays a remarkably loose consensus sequence, often described as 5′-GGGRNNYYCC-3′, where R indicates purine (A or G), N any nucleotide and Y pyrimidine (C or T) (Ghosh et al, 1998). This heterogeneity has been thought to confer specificity of regulation, as mice deficient in a single NF-κB gene show specific phenotypes. RelA−/− animals are embryonic lethal with massive hepatocyte apoptosis (Beg et al, 1995), while c-Rel−/− mice develop normally but have defects in lymphocyte proliferation (Kontgen et al, 1995). Mice homozygous for the disrupted RelB locus have phenotypic abnormalities,
including inflammatory cell infiltration in several organs and an impaired cellular immunity, as observed in contact sensitivity experiments (Weih et al, 1995). These mouse models indicate that absence of a specific NF-κB member cannot be functionally compensated by any other member of the Rel family, suggesting unique functional features. Indeed, a comparative analysis of single and double NF-κB knock-out cells did reveal promoter-specific requirements for specific NF-κB proteins (Hoffmann et al, 2003). However, attempts to elucidate a general specificity code for NF-κB dimers versus κB site sequences have failed so far, both biochemically and genetically. With the possible exception of a few RelB-p52 selective sites (Bonizzi et al, 2004), no obvious dimer-site pairs exist in the NF-κB system, although different dimers display measurable differences in the affinity for any given κB site (Udalova et al, 2002). The molecular basis for c-Rel specificity of the IL-12 p40 promoter in mouse macrophages is explained by a short sequence stretch of 46 amino acids in the RHD, called specificity-determining region (SDR) (Sanjabi et al, 2005). Rather than mediating critical protein-protein interactions, it is assumed that the c-Rel SDR affects binding to selective κB sites indirectly, i.e. via promoting specific conformations that endow c-Rel with the ability to recognize deviant κB sequences at high affinity.

However, specificity in NF-κB-regulated gene expression is generated at multiple levels, also extrinsic to the NF-κB system (Natoli & De Santa, 2006). Synergistic interactions between NF-κB and partner transcription factors are critical to shape specific aspects of the NF-κB response, such as stimulus- and tissue-specific expression of target genes. Some IRF3-dependent genes, which do not contain an interferon-stimulated response element (ISRE), require IRF3 as a co-activator for κB site-bound p65. Similarly, a transcriptional synergism, involving NF-κB and p38-activated cAMP-responsive element binding protein (CREB), is described as a key component of the macrophage anti-apoptotic response (Park et al, 2005). This gene-specific control by partner factors is also exemplified by the finding that the restriction for a subset of dimers, imposed by a κB site, pertains to one stimulus. More detailed, the sequence imposes which co-activator will form productive interactions with the bound NF-κB dimer (Leung et al, 2004). In addition, the specificity mechanisms are operating in a very dynamic manner, as during DC maturation rapidly activated p50/p65 dimers, bound to a subset of target promoters, are gradually displaced by slowly activated p52/RelB dimers (Saccani et al, 2003). This exchange allows for fine-tuning of the response over time and rather points to a more complex regulatory mechanism than to redundancy.

At the molecular level, the NF-κB-dependent gene promoters can be divided into 2 groups based on their stimulus-induced activation kinetics. Comparison of the expression properties of macrophages after LPS induction reveals rapidly (after 30 min) or rather slowly
(after 2 hours) increased mRNA levels, which classifies genes into respectively, primary (Cxcl-2, TNF-α) or secondary (IL-12β, IL-6) response genes (Ramirez-Carrozzi et al, 2006). Recruitment of NF-κB to these target genes is asynchronous (Saccani et al, 2001), as an increase in histone acetylation precedes NF-κB binding to genes activated with slow kinetics (Saccani et al, 2002). Furthermore, NF-κB recruitment to the same target genes in different cell types occurs with distinct kinetics, which points to a cell type-specific regulation (Natoli et al, 2005). These reports indicate the existence of an epigenetic control of the NF-κB response in which chromatin imposes some restrictions and may represent the link between temporal control of NF-κB kinetics and alternative induction of gene expression. Since simple wrapping of κB sites around the histone octamer is not sufficient to prevent NF-κB-DNA binding (Angelov et al, 2004), repressive histone modifications or the incorporation of interfering histone variants, such as macroH2A (Angelov et al, 2003), may play a critical role in generating nucleosomes with different affinities for NF-κB. Additionally, nucleosomes may mediate transcription factor cooperativity as multiple factors act collaboratively to exclude nucleosomes and gain access to target sites in the yeast DNA (Bernstein et al, 2004).

**3.4.4. Stimulus specificity**

By the use of a mathematic model and time-lapse single cell analysis to study kinetics of NF-κB activity in response to TNF-α stimulation, it has been shown that stimulus duration generates specificity in NF-κB-driven gene expression. A direct link between temporal control of NF-κB activity and selective gene induction has been demonstrated, as mutation of the negative feed-back, mediated by IκBα, results in aberrant gene expression (Hoffmann et al, 2002; Nelson et al, 2004). Some NF-κB-dependent genes in TNF-α-stimulated fibroblasts require the presence of NF-κB in the nucleus for a relatively long time (more than 2 hours), while many other genes are induced even in response to a single and short cycle of NF-κB activity.

Furthermore, the temporal profile of NF-κB activity is largely independent of the dosis (Cheong et al, 2006), but is rather determined by the type of stimulus. Although TNF-α-induced IKK activity is rapidly attenuated by negative feed-back, LPS signaling and LPS-specific gene expression programs are dependent on a cytokine-mediated positive feed-back mechanism (Werner et al, 2005). NF-κB activity, induced by a pulse of LPS in fibroblasts, is sustained due to an autocrine loop, dependent on IRF3-mediated TNF-α induction and secretion (Covert et al, 2005). However, not all differences between TNF-α- and LPS-induced gene expression programs can be reduced to differential NF-κB activation profiles, as a significant fraction of the early induced genes are stimulus-specific (Werner et al, 2005).
4. p53 protein

4.1. Tumor suppressor p53

p53, first described in 1979, was the first tumor suppressor gene to be identified, and its importance to human health is underlined by the high frequency of incorrect functioning in tumor cells. Almost half of all cancers display mutations or deletions in the p53 gene and in many other cancers, the p53 protein is indirectly inactivated through binding to viral proteins, or as a result of alterations in genes, the products of which interact with p53 or transmit information to or from p53 (Vogelstein et al, 2000). The dominantly inherited cancer predisposing syndrome in humans, called Li-Fraumeni, is due to germ line mutations in the p53 gene (Malkin et al, 1990). Similarly, mice that lack p53 develop normally but are prone to neoplastic malignancy and as such, they rapidly succumb to cancers within 6 months of age (most commonly lymphomas for homozygous mutants and sarcomas for heterozygous mutants) (Jacks et al, 1994). This implies that, at the whole organism level, p53 acts purely as a tumor suppressor. Conversely, mice engineered to have high p53 activity are resistant to tumors but age prematurely, which presents another aspect of p53 biology (Tyner et al, 2002). These observations explain why the activity of this safeguard protein is tightly regulated, principally at the level of protein stability.

Under normal circumstances, the low intracellular amount of p53 protein is mainly determined by its fast degradation rate, through ubiquitin-mediated proteolysis by the 26S proteasome. This process is triggered by the ubiquitin ligase Mouse double minute 2 (Mdm2) and subject to a feed-back loop, as p53 acts as a transcription factor for Mdm2 expression (Kubbutat et al, 1997). The interval between p53 activity and consequent Mdm2 accumulation defines a time window during which p53 exerts its effects. Besides targeting p53 for degradation, this negative regulator of p53 also directly inhibits p53-mediated transcriptional activity by concealing its activation domain (Oliner et al, 1993) and facilitates the nuclear export of p53 as well.

4.2. Nuclear functions and regulation of p53 (Figure 8)

Besides inducing the Mdm2 gene, p53 directly controls transcription of genes implicated in cell cycle arrest (senescence) and cell death (apoptosis), as p53 stimulates the expression of e.g. p21^{WAF1/CIP1}, an inhibitor of various cyclin-dependent kinases, and Bax, respectively (among other proteins). This exemplifies its central role in halting the growth of tumor cells. Furthermore, p53 may be involved in maintaining genetic stability by induction of regulators of nucleotide-excision DNA repair, chromosomal recombination and segregation.
In addition, p53 stimulates the expression of genes that inhibit neovascularization. In tumor, inflamed or damaged tissues, p53 levels are profoundly elevated.

![Figure 8: Outline of the regulation and different functions of the p53 protein.](image)

p53 binds in the promoter region of target genes as a homotetrameric transcription factor on the consensus sequence 5'-RRRC(A/T)(T/A)GY,Y-3', where R indicates purine (A or G), N any nucleotide and Y pyrimidine (C or T). In p53-inducible promoters, the p53 recognition sequence is usually present as a repeat of this 10 bp motif, separated by 0-13 bp. Functionally, the p53 protein is divided in 3 domains, including a N-terminal activation domain (amino acids 1-43), a core sequence-specific DNA-binding domain (amino acids 100-300), which mostly harbors point mutations in malignant cells, and a multifunctional C-terminal domain (amino acids 300-393). The C-terminus holds a tetramerization region and a regulatory domain that negatively affects DNA binding. In addition to degradation, the p53 protein is regulated at distinct levels, including posttranslational modification (acetylation and phosphorylation) and protein-protein interaction (transcription factors, co-factors, viral proteins, ankyrin repeat proteins, such as 53 BP1/2).

Various types of stress signals lengthen the intracellular half-life of p53 (stabilization), resulting in p53 accumulation. DNA-damaging agents (chemotherapeutica, oxygen radicals, ionizing irradiation, UV light, etc), hypoxia and oncogenes activate p53, albeit via different mechanisms. In case of DNA damage, checkpoint proteins, such as ATM, ATR, Chk1/2, phosphorylate p53 at N-terminal sites that are close to the Mdm2-binding region, thereby blocking the interaction between Mdm2 and p53, leading to stabilization of p53. The hypoxic induction of p53, however, is attributable to the downregulation of Mdm2 protein levels. In contrast, oncogenes stimulate the transcription of the p14ARF gene or stabilize the corresponding protein, which subsequently binds and inhibits Mdm2 or sequesters it in the nucleolus.

Aside from its transcriptional activation function, p53 also acts as a transcriptional repressor, mainly on cell cycle regulators, which may be important for its ability to carry out
its tumor suppressor role (Spurgers et al, 2006). Proposed mechanisms include site-specific DNA binding (Lee et al, 1999) and interference with transcription initiation (Farmer et al, 1996). An additional model involves p53 interaction with transcriptional activators at the promoter level and with the basal transcription machinery (Dhar et al, 2006; Ho & Benchimol, 2003). Furthermore, recruitment of co-repressors (Ho et al, 2005) or histone/chromatin modifying factors (Van Bodegom et al, 2006) to reduce promoter accessibility has been described as well. However, also indirect gene repression is reported, as a consequence of p53-mediated p21 induction (Lohr et al, 2003; Shats et al, 2004).

4.3. p53 and NF-κB

As stimulation of NF-κB has been shown to promote resistance to programmed cell death (see 3.4.2.), in contrast to activation of p53, which is associated with the induction of apoptosis, a regulatory mechanism must exist to integrate these opposing outcomes and coordinate this critical cellular decision-making event. It is conceivable that these transcription factors mutually regulate each others transactivation. As such, the complete or partial repression of p53, observed in many tumors, may be the result of constitutive activation of NF-κB (Gurova et al, 2005). In case of chemotherapeutic drugs, which simultaneously activate p53 and NF-κB, experiments with IKKβ−/− MEF cells reveal a role for NF-κB in the acquisition of resistance to chemotherapy and p53-mediated cell death (Tergaonkar et al, 2002). Conversely, in response to UV radiation, p53 has a suppressive activity on the cell signaling pathways leading to activation of activator protein (AP)-1 and NF-κB. The anti-cancer mechanism of p53 is mediated via upregulation of PTEN, a tumor suppressor and negative modulator of Akt by dephosphorylation of PIP3 (Wang et al, 2005). Reporter gene studies with p53 overexpression show a direct effect on the transcriptional potential of NF-κB in various cell lines. The basal activity levels of different NF-κB-dependent promoter constructs increase when mutant p53 instead of wild-type p53 is transfected (Komarova et al, 2005), and UV light-induced p53 can block the activity of a synthetic NF-κB-driven reporter gene (Webster & Perkins, 1999).

Many possible mechanistic explanations have been proposed for this reciprocal inhibition, including competition due to the common use of a limiting pool of co-activators (Ravi et al, 1998; Wadgaonkar et al, 1999; Webster & Perkins, 1999). A direct association between p53 and p65, which mutually compromises their activation potential, has been described too (Ikeda et al, 2000; Jeong et al, 2004). Another mechanism is an indirect effect of p53 on the cytoplasmic IκBα expression, which sequesters NF-κB in the cytoplasm, correlating with a decrease in NF-κB translocation and DNA binding (Shao et al, 2000).
However, other ones report on a positive association between p53 and NF-κB, as the p53-induced NF-κB activity, based on MEK1 and RSK1 activation, is required for p53-mediated apoptosis (Ryan et al, 2000). Similarly, the topoisomerase inhibitors etoposide and doxorubicin, which induce p53 due to DNA breaks, activate p65 in a p53-dependent manner as well, via RSK1-mediated Ser536 phosphorylation, which subsequently decreases the affinity of p65 for IκBα (Bohuslav et al, 2004).

4.4. p53 and inflammation

The capacity of p53 to prevent cancer is usually viewed as a result of its surveillance of genomic integrity and stability, whereby p53 acts through elimination of genetically damaged cells by anti-proliferative and apoptotic events. Interestingly, p53 also plays the role of general buffer of innate immune responses in vivo, which is well consistent with its tumor suppressor function, as chronic inflammation promotes cancer development. More specifically, molecular analysis of p53 null mice clearly points to a repressive effect of p53 on basal and LPS-induced cytokine expression (IL-6, TNF-α, IL-1) by modulating the activity of NF-κB (Komarova et al, 2005). In agreement with this negative p65-p53 association, a significant proportion of early deaths of p53−/− mice is not tumor-related, but results from unresolved spontaneous inflammation (Donehower et al, 1992). p53 expression is upregulated in a number of conditions, not directly related to oncogenesis, such as inflammation, trauma, infection, etc. Additionally, p53 mutations have been detected in synovial cells of patients with RA (Firestein et al, 1997), while p53 gene transfer ameliorates autoimmune arthritis in vivo by inducing synovial apoptosis and reducing the inflammatory infiltrate (Yao et al, 2001). Synovial regions with high p53 mutation rates contain greater amounts of IL-6 mRNA, suggesting that p53 somatic mutations may exacerbate arthritis by production of inflammatory cytokines (Yamanishi et al, 2002). Furthermore, the use of mice disease models (autoimmune encephalomyelitis, collagen-induced arthritis, autoimmune diabetes) establishes the general importance of p53 in autoimmune diseases by controlling cytokine production (Okuda et al, 2003; Yamanishi et al, 2002; Zheng et al, 2005).

Although the direct influence of p53 on IL-6 expression levels is shown by comparing murine p53 wild-type with p53 null macrophages (Zheng et al, 2005) and thymus cells (Komarova et al, 2005), the underlying mechanisms have not been conclusively proven. Earlier in vitro approaches with p53 overexpression suggest that p53 decreases binding of CEBP, CREB, AP-1 and NF-κB to the IL-6 promoter (Angelo et al, 2002; Asschert et al, 1999; Margulies & Sehgal, 1993). No direct binding of p53 has been observed, in
accordance with the lack of a known p53 recognition site in the IL-6 promoter (Santhanam et al, 1991).

5. IL-6

5.1. IL-6 signaling

IL-6 is known as a helix bundle glycoprotein, comprising 4 long, straight $\alpha$-helices, which are arranged in an up-up-down-down topology. Unlike other cytokines, major effects of IL-6 occur at sites distinct from its origin and are consequent upon its circulating concentrations. Therefore, IL-6 is also called an endocrine cytokine, with pleiotropic functions, which underlie some of its alternative names, including B cell-stimulatory factor-2, B cell differentiation factor, cytotoxic T cell differentiation factor, IFN-β2, 26 kDa protein, hybridoma/plastocytoma growth factor and hepatocyte stimulating factor. In 1986, the region coding for the 26 kDa protein was established, as well as the neighbouring sequences, including the inducible promoter area, revealing that the 26 kDa protein gene and the so-called IFN-β2 gene are identical (Haegeman et al, 1986).

IL-6 exerts its actions via signaling, mediated by a functional IL-6 receptor, which consists of two components, a 80 kDa IL-6 binding protein ($\alpha$-chain, IL-6R) and a 130 kDa signal-transducing membrane glycoprotein, known as gp130 ($\beta$-chain). On target cells, IL-6 directly binds to the non-signaling IL-6R. This complex subsequently associates with gp130, thereby promoting its homodimerization and the initiation of intracellular signaling. gp130 is expressed by most, if not all, cells in the body, whereas IL-6R is mainly expressed by hepatocytes, neutrophils, monocytes/macrophages and some lymphocytes. Moreover, IL-6R expression is tightly regulated. The function of the IL-6R to render target cells sensitive to the IL-6 cytokine, however, can also be taken over by a naturally occurring, soluble form of the IL-6R (sIL-6R). This type of IL-6R, which lacks the transmembrane and cytoplasmic part, has been found in various extracellular body fluids, such as serum, urine, cerebrospinal fluid and synovial fluid (Frieling et al, 1994), and is generated by two independent mechanisms: limited proteolysis of the membrane protein (shedding) (Mullberg et al, 1993) and translation from an alternatively spliced mRNA (Holub et al, 1999). In contrast to most soluble receptors, the sIL-6R functions in an agonistic way, as it is able to stimulate cells, which only express gp130, a process that is termed trans-signaling (Jones et al, 2005).

Although gp130s possess no intrinsic tyrosine kinase domain, the dimerization of gp130 leads to activation of associated cytoplasmic tyrosine kinases of the Janus kinase (JAK) family. Of these, JAK1 plays an essential role, as IL-6 signal transduction is severely
impaired in JAK1-deficient cells (Guschin et al, 1995). Tyrosine phosphorylation on distinct sites of the gp130 leads to recruitment of STAT transcription factors. Subsequent to receptor binding by their SH2 domain, STAT3 and, to a minor extent, STAT1 are potently activated by phosphorylation on a single tyrosine residue, which initiates their dimerization and translocation to the nucleus. Besides the activation of the JAK/STAT pathway, IL-6 receptor triggering also leads to induction of the MAPK cascade. Tyrosine phosphatase SHP2 is rapidly recruited to tyrosine-phosphorylated gp130 and becomes phosphorylated in a JAK1-dependent manner. This attracts the Grb2-Sos complex, allowing for subsequent Ras-Raf-MAPK activation.

5.2. IL-6 production

The expression of IL-6 is tightly regulated and normally kept at very low, even undetectable, plasma concentrations, which follow a circadian rhythm (Vgontzas et al, 2005). Cells of the immune system (T cells, macrophages/monocytes, mast cells, DCs), as well as fibroblasts, vascular endothelial cells, microglial cells, adipocytes and various cancer types, represent the main source of IL-6 (Naka et al, 2002), in response to a variety of stimuli, such as TNF-α, LPS, PDGF, phorbol esters, cAMP, interferons, etc. Upon infection, IL-6 levels abruptly increase to nanogram amounts. Similarly, with advancing age, IL-6 concentrations rise for both sexes (Maggio et al, 2006) and a clear difference can be observed between pre- and post-menopausal women (Giuliani et al, 2001). This phenomenon, which inspires the alternative name for IL-6, ‘cytokine for gerontologists’, is proposed to be multi-factorial, but cannot be explained by IL-6 gene polymorphisms (Walston et al, 2005). In time, the tight regulation of IL-6 gene expression may become less effective. In addition, age-associated loss of T cell immunoregulatory functions, as well as menopausal loss of estrogen, could play a role. Furthermore, the continuous load of stress reactions throughout life-time, which causes a concomitant increase in radicals might be important in the context of inflamm-aging (Franceschi et al, 2000) and forms the conceptual basis for the free radical theory of aging (Sarkar & Fisher, 2006) (see 1.3.1.).

5.3. IL-6 function in (patho)physiology

IL-6 was discovered as an Ag-nonspecific human B cell differentiation factor or B cell stimulatory factor in the culture supernatants of mitogen- or Ag-stimulated peripheral blood mononuclear cells, that could induce the final maturation of B cells into Ig-secreting plasma cells (Hirano et al, 1985). This observation has exponentially increased scientific interest in this cytokine, which led to improvement of the knowledge on the biological activities of IL-6
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(Figure 9). In acute phase reactions, IL-6 stimulates hepatocytes to produce acute phase response proteins, such as C-reactive protein, fibrinogen, α1-anti-trypsin and serum amyloid A (Castell et al, 1988; Gauldie et al, 1987). As an endocrine cytokine, IL-6 potently stimulates the hypothalamic-pituitary-adrenal axis in response to stress or inflammation, leading to release of adrenocorticotropic hormone and cortisol (Turnbull & Rivier, 1999). In addition to its earlier demonstrated ability to promote B cell differentiation and growth, IL-6 also acts as a growth regulator in cells of the T lymphocyte lineage. IL-6 augments CTL generation from mature and immature human T cells in vitro (Okada et al, 1988) and mature T cell proliferation by increasing the expression of the IL-2 receptor (Noma et al, 1987) and production of IL-2 (Garman et al, 1987). Furthermore, the IL-3-mediated proliferation of multipotential hematopoietic progenitors is enhanced by IL-6 (Ikebuchi et al, 1987), as well as the maturation of megakaryocytes (Ishibashi et al, 1989). Concerning T cell polarization, IL-6 promotes Th2 differentiation by activation of NFAT in naive T cells, resulting in early IL-4 production (Diehl et al, 2002; Rincon et al, 1997). Independent of this effect, IL-6 inhibits IFN-γ signaling in CD4+ T cells by upregulating SOCS-1 transcription, culminating in blockage of Th1 differentiation (Diehl et al, 2000; Diehl & Rincon, 2002). Moreover, DC-derived IL-6 renders pathogen-specific T cells refractory to the repressive activity of CD4+ CD25+ regulatory T cells (Pasare & Medzhitov, 2003) and determines the generation balance between regulatory T cells and T H17 cells, which is critical to auto-immune reactions (Bettelli et al, 2006). The above mentioned activities of IL-6, among others, are necessary for resolving innate immunity and promoting acquired immunity (Jones, 2005). Transition from a neutrophil to a mononuclear cell population defines the switch between innate and acquired immunity as a central event in the resolution of any inflammatory condition. Disruption of this immunological switch may potentially distort the immune response and affect the onset of autoimmune or chronic inflammatory disorders (Hoebe et al, 2004).

The appreciation of all these IL-6-mediated processes emphasizes a prominent and central role for IL-6 in inflammation, immunity and host defence. Furthermore, IL-6 is also a growth or survival factor for certain cell types, such as epidermal keratinocytes, renal mesangial cells, and for a variety of tumor types, e.g. multiple myeloma, prostate and ovarian cancer. A possible mechanism to accomplish this feature is maintenance of promoter methylation of the tumor suppressor p53, by increasing the expression levels of DNA methyltransferase (DNMT)-1 (Hodge et al, 2005). Furthermore, IL-6 promotes osteoclast differentiation (Udagawa et al, 1995) and is negatively associated with bone mineral density, which leads to osteoporosis (Moffett et al, 2004). Concerning reproduction, IL-6 affects fertility (testicular development, function and spermatogenesis) (Potashnik et al, 2005).
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As such, dysregulation of IL-6 is associated with a range of pathologies (reviewed in (Maggio et al, 2006)) (Figure 9). Observational studies show that IL-6 is a risk factor for type 2 diabetes and correlates with disease severity in Crohn’s disease and RA. Increased IL-6 levels are also a feature of physical frailty and disorders, such as osteoporosis, atherosclerosis and psoriasis. Higher serum IL-6 levels positively correlate to decline in cognitive function and dementia (Alzheimer’s disease) (Engelhart et al, 2004; Weaver et al, 2002). Patients with renal cell carcinoma have significantly higher serum IL-6 levels (median 8.3 ng/l) compared with controls (median 0.5 ng/l) and these values correlate to cancer state and adverse survival (Ljungberg et al, 1997). Similarly, colorectal cancer tissue expression of IL-6 correlates with an aggressive cancer phenotype (Chung et al, 2006). Additionally, higher serum IL-6 levels predict a poorer response to chemo-endocrine therapy and represent a bad prognosis in metastatic breast cancer. So, IL-6 may function as a prognostic marker for survival in several types of cancer, including gastric cancer (Ashizawa et al, 2005), in advanced CLL (Lai et al, 2002), metastatic malignant melanoma (Tas et al, 2005) and hormone-refractory breast cancer (Bachelot et al, 2003).

Altogether, this causal or associative relation between IL-6 and chronic inflammatory pathologies of advanced age highlights the therapeutical potential of targeting IL-6 activities. Experiments with androgen-dependent prostate cancer xenografts in castrated mice reveal that a monoclonal Ab against IL-6 inhibits the malignant conversion to an androgen-independent phenotype (Wallner et al, 2006). Similarly, an anti-IL-6 monoclonal Ab induces apoptosis and regression of human prostate cancer xenografts in nude mice (Smith & Keller, 2001). Inhibition of IL-6 trans-signaling, by TGF-β-signaling, inhibits tumor progression in colon cancer (Becker et al, 2004). Besides modulation of IL-6 in combating cancer

Figure 9: The role of the multifunctional cytokine IL-6 in physiological and pathophysiological conditions.
progression, applications for IL-6 therapy exist in other clinical fields as well. Significantly, IL-6-deficient mice remain resistant to the induction of experimental autoimmune conditions (Ohshima et al, 1998) and myosin-induced experimental myositis (Scuderi et al, 2006). In an Ag-induced arthritis model, IL-6 deficiency results in a mild, transient inflammation instead of developing a chronic, destructive synovitis (de Hooge et al, 2000). Accordingly, humanized recombinant monoclonal Abs against the IL-6R have shown considerable promise in clinical trials for diverse chronic inflammatory diseases, including arthritis and Crohn’s disease by inhibiting the function of IL-6 proteins (Choy et al, 2002; Ito et al, 2004; Yokota et al, 2005). These therapies are based on the crucial role of IL-6 production for pathogen-specific T cell activation (Pasare & Medzhitov, 2003). However, the detrimental consequences of IL-6 in chronic inflammatory diseases must be balanced by its capacity to protect against septic shock and to direct resolution of local acute inflammation by controlling the level of pro-inflammatory cytokines (Xing et al, 1998) and by inducing IL-1R antagonist synthesis and the release of soluble TNFR1 (Tilg et al, 1994). Thus, in contrast to pro-inflammatory cytokines, such as TNF-α and IL-1α/β, IL-6 has anti-inflammatory properties as well.

5.4. IL-6 promoter regulation

5.4.1. Promoter polymorphisms

The IL-6 gene maps to the proximal region of murine chromosome 5 (Mock et al, 1989) and is localized on the short arm of human chromosome 7 (Ferguson-Smith et al, 1988). In the promoter region of the IL-6 gene, a single-nucleotide polymorphism (SNP) of G exchange to C at position -174 has been reported (Fishman et al, 1998). The frequency of the IL-6 174C allele is 40% in the general population. This nucleotide exchange polymorphism has a functional role as healthy persons with the IL-6 174C allele have significantly lower plasma concentrations of IL-6 (Fishman et al, 1998) and IL-6 measurements after vaccination confirm the association of the G allele with higher plasma IL-6 values (Bennermo et al, 2004). In accordance, in vitro transfection of this genetic variant into a cell line results in reduced production of IL-6, compared to the IL-6 174G allele, suggesting that this polymorphism affects the transcription of the IL-6 gene. However, additional common polymorphisms have been identified in the 5’-flanking region of the IL-6 gene, including −597G>A, −572G>C and a polymorphic tract of A and T residues (AnTn) at position −373 (Kelberman et al, 2004). All these polymorphisms are located adjacent to cis-acting regulatory elements involved in controlling IL-6 expression at the level of transcription, suggesting that they may influence the interaction of proteins with the DNA at these sites. In
transcriptional regulation, they do not act independently as one base difference influences the functional effect of variation at other polymorphic sites in vitro (Terry et al, 2000). Also in vivo, the combination of several SNPs regulates IL-6 activity, as shown by corresponding biochemical marker levels (Ferrari et al, 2003). These results show that genetic polymorphisms in the promoter influence IL-6 transcription not by a simple additive mechanism, but rather through complex interactions determined by the haplotype (Terry et al, 2000).

As genetic polymorphisms influence the IL-6 gene transcription levels and considering that IL-6 is associated with several inflammatory pathologies and disorders of advanced age, patient genotyping studies have investigated the frequency of certain haplotypes of allelic variations in these IL-6-correlated diseases. The homozygous IL-6 174G,G genotype is more prevalent in type 1 diabetes patients compared with normal controls (50.6% versus 33.3%), while the C,C genotype is less occurring in patients compared with controls (12.5% versus 24.2%), suggesting that the IL-6 gene polymorphisms may contribute to the genetic susceptibility to type 1 diabetes (Jahromi et al, 2000). A cohort study on healthy post-menopausal women finds a significant increase in the level of C-reactive protein (79%) and bone resorption markers (32%) with a decreasing number (from 4 to 1) of IL-6 protective alleles -572G and -174C, indicating that these functional polymorphisms in the IL-6 gene regulatory region influence the risk of osteoporosis (Ferrari et al, 2003). The reduced frequency of the potentially protective IL-6 174C,C genotype in young systemic-onset juvenile chronic arthritis patients may contribute to the pathogenesis of their disease (Fishman et al, 1998). In RA patients with an IL-6 174G,G genotype, the active form of RA is more frequently diagnosed and the parameters of disease activity score are significantly increased compared with homozygous CC and GC patients, suggesting that the IL-6 promoter polymorphism may be a genetic risk factor for RA activity (Fishman et al, 1998). This IL-6 174C>G polymorphism has been associated with the prevalence, incidence and/or prognosis of a variety of disease states, such as Alzheimer’s disease (Licastro et al, 2003), cardiovascular disease (Flex et al, 2002; Humphries et al, 2001), young adult Hodgkin lymphoma, breast cancer (Cozen et al, 2004; DeMichele et al, 2003), type 2 diabetes (Vozarova et al, 2003) and sepsis (Schluter et al, 2002).

5.4.2. Transcription factor-binding sites

A 1,2 kB fragment of the 5’-flanking region of the IL-6 gene contains multiple cis-acting elements, necessary for induction of the gene by all known inducers. Comparison of the human and mouse IL-6 genes shows 60% sequence similarity in the coding regions and
high homology in the 3′-UTR and the proximal promoter up to 300 bp upstream of the transcription start site (Tanabe et al, 1988) (Figure 10). This conserved control region contains most, if not all, of the elements necessary for IL-6 induction. Electrophoretic mobility shift assays, as well as promoter deletion and point mutation analysis, revealed the presence and functional involvement of a NF-κB-binding element between positions −73 and −63, a multiple response element consisting of cAMP-responsive element (CRE), followed by a binding site for the CCAAT enhancer-binding protein (C/EBPβ or NF-IL6) between −173 and −145, and an AP-1 site located between −283 and −277 (Vanden Berghe et al, 1999; Vanden Berghe et al, 1998). Also, 2 GC-rich recognition sequences for specificity protein (Sp)1 are detected in the IL-6-proximal promoter region (Kang et al, 1996).

**Figure 10:** Schematic drawing of the relative positions of transcription factor binding sites in the promoter region of the human (A) and mouse (B) IL-6 gene. The exon-intron structure of the IL-6 gene is presented as well, with indications of the translation initiation and stop site.

Depending on the cell type or stimulus, certain transcription factor combinations may regulate IL-6 gene induction. In TNF-α-stimulated fibroblasts, NF-κB is identified as the main trigger for IL-6 transcription (Vanden Berghe et al, 1998). Although the *in vivo* regulation is not known, the *in vitro* binding of NF-κB to DNA is inducible, whereas other transcription factors (CREB, AP-1, C/EBP) form constitutive DNA-binding complexes (Vanden Berghe et
al, 1999). Important in TNF-α-induced IL-6 regulation is the p38/ERK-regulated nuclear kinase MSK1, which associates with p65 and phosphorylates this transcription factor at Ser276, leading to its transactivation (Vermeulen et al, 2003b). Recently, LPS-induced IκBζ is found to associate with p50 and as such to be recruited to the IL-6 promoter, of which the production is severely impaired in IκBζ-deficient cells (Yamamoto et al, 2004). In contrast to this indirect effect, LPS-activated Jak2 and STAT5 proteins are required for IL-6 expression, as STAT5 interacts with p50 and binds the IL-6 promoter (Kimura et al, 2005).

Several lines of evidence indicate that the intracellular redox status participates in modulating NF-κB activation (Flohe et al, 1997). This is exemplified by the range of agents that not only activate NF-κB, but also result in increased intracellular formation of ROS. These include superoxide anions, hydroxyl radicals and hydrogen peroxide as major components of the endogenous oxidants. The importance of generating ROS after TNF-α stimulation is substantiated by the inhibition of NF-κB activation and IL-6 expression, when the mitochondrial oxidative mechanism is depleted in fibroblast cells (Schulze-Osthoff et al, 1993). In accordance, anti-oxidant treatment of different cell types, such as neutrophils, confirms the dependence of LPS- and TNF-α-induced NF-κB activation on ROS production and signaling (Asehnoune et al, 2004). Similarly, removal of superoxide by a Mn-containing superoxide dismutase mimetic inhibits LPS-induced production of TNF-α and IL-6 in alveolar macrophages by a mechanism involving suppression of redox-sensitive IκBα degradation (Ndengele et al, 2005). Not only NF-κB is redox-regulated, as LPS-induced IL-6 expression is conferred by the differential activation of C/EBPβ (Su et al, 2003).

5.4.3. Interference with transcription factors leads to decreased IL-6 transcription

SOCS-1 suppresses LPS-induced IL-6 transcription by specifically targeting the activation of Jak2 and STAT5 (Kimura et al, 2005). However, no interference of TLR-induced SOCS proteins with NF-κB or MAPK activation is found (Baetz et al, 2004). An IκB family member that almost entirely consists of ankyrin repeats, IκBNS, associates with p50 and is recruited to the IL-6 promoter, resulting in selective inhibition of LPS-induced IL-6 production in macrophages of the colonic lamina propria (Hirotani et al, 2005). As such, the regulatory role of IκBNS resembles the function of the structurally related molecule Bcl-3, which specifically represses LPS-induced TNF-α-production, but not IL-6, via similar mechanisms (Kuwata et al, 2003). A recent report demonstrates the crucial function of IκBNS in controlling the cytokine response, as IκBNS-deficient macrophages and DCs show increased TLR-mediated expression of genes, such as IL-6 and IL-12p40, and a prolonged NF-κB activity at the promoter level (Kuwata et al, 2006). As discussed in 4.3. and 4.4., also the
transcription factor p53 downregulates IL-6 transcription, possibly by affecting critical transcriptional regulators.

5.4.4. Chromatin modifications

Apart from transcription factor binding to the IL-6 promoter, additional levels of promoter regulation exist, since IL-6 is classified as a secondary response gene (Ramirez-Carrozzi et al., 2006), in analogy to an earlier report on regulated and late accessibility of IL-6 (Sacca et al., 2001). This means that in basal conditions, the IL-6-proximal promoter chromatin resides in a non-permissive state for efficient binding of the transcription machinery. To facilitate IL-6 gene activation, the repressive chromatin configuration must be altered, allowing accessibility to nucleosomal DNA (Ramirez-Carrozzi et al., 2006). This counterbalance is accomplished by stimulus-induced covalent modifications of histone tails (see 5.4.4.2.) and chromatin remodeling by ATP-dependent enzyme complexes (see 5.4.4.1.). Additionally, the state of promoter DNA methylation controls transcriptional regulation (Armenante et al., 1999a) (see 5.4.4.3.).

5.4.4.1. ATP-dependent nucleosome-remodeling complexes

These protein complexes utilise energy from ATP hydrolysis to modify the spatial relationship between nucleosomes and DNA, either through changing the position of specific nucleosomes (sliding) or through altering their three-dimensional structure. In mammalian cells, two of the most studied families of ATP-remodeling complexes are SWI/SNF and Mi-2/NuRD complexes. The former contain either of two ATPase subunits BRG1 and BRM, along with a number of BRG1-associated factors (BAFs) (Becker & Horz, 2002). The latter consist of the Mi-2α or Mi-2β ATPase subunit, complexed with associated factors, such as histone deacetylases (Feng & Zhang, 2003). For LPS-induced IL-6 activation, the catalytical subunit BRG1/BRM of the SWI/SNF class of ATP-dependent nucleosome-remodeling complexes is required. However, the antagonistically acting Mi-2β complex is selectively recruited to the control region of IL-6, resulting in restricted gene transcription (Ramirez-Carrozzi et al., 2006).

5.4.4.2. Posttranslational histone modifications

Besides physical chromatin remodeling, covalent posttranslational modifications of the N-terminal histone tails or globular core domains also influence the chromatin configuration. Among them, phosphorylation, acetylation, methylation, ADP-ribosylation, ubiquitination and sumoylation are implicated in transcriptional regulation. As the relatively
unstructured N-termini of histones protrude out of the nucleosomes, they provide an extended interaction surface for other nucleosomes as well as for enzymes implicated in signal transduction, which contain specific targeting modules. Bromodomains and chromodomains contribute to the recognition of specific patterns of modifications, as they mainly bind to acetylated and methylated Lys, respectively. These protein modules, as well as SANT domains, that interact primarily with unmodified histone tails, are found in different chromatin-modifying enzymes, such as HATs, histone methyltransferases (HMTs) and ATP-remodeling enzymes (de la Cruz et al., 2005). Therefore, histone modifications can affect both chromatin folding (and consequently accessibility) and recruitment of proteins for transcriptional regulation. More specifically, modifications located on the solute accessible face of the nucleosome have the ability to alter higher-order chromatin structure and chromatin-protein interactions. Histone lateral surface modifications are uniquely capable of affecting histone-DNA interaction, and modifications on the histone-histone interface have the exclusive ability to disrupt intranucleosomal interactions, thereby altering nucleosome stability (Mersfelder & Parthun, 2006). Thus, apart from the genetic information encoded within the DNA, a second, more dynamic histone code comprizes the combination of interdependent histone modifications, which specify unique downstream functions (Mellor, 2006). This histone code hypothesis has even been extended to the nucleosome code hypothesis, by proposing that high-order chromatin is largely dependent on the local concentration and combination of differentially modified nucleosomes.

Among all possible chemical modifications, phosphorylation and acetylation are mostly considered as positively acting on transcriptional activation. For methylation, the outcome on transcription differs depending on the histone residue, the surrounding modifications and degree of methylation (mono-, di-, tri-methylation). As such, it has been reported that late recruitment of NF-κB to IL-6 is preceded by LPS-induced histone (H)4 hyperacetylation (Saccani et al., 2001). Inflammatory stimuli induce p38-dependent histone phosphorylation and phosphoacetylation of a subset of cytokine and chemokine gene promoters, which is required to enhance the accessibility of the cryptic NF-κB binding site (Saccani et al., 2002). The rapid and transient phosphorylation on H3 Ser10 subsequently promotes the acetylation reaction on H3 Lys14, which correlates with transcriptional activation (Cheung et al., 2000; Lo et al., 2000). In fibroblasts, the TNF-α-induced H3 Ser10 phosphorylation on the IL-6 promoter may depend on the recruitment of MSK1, which not only transactivates p65, but also helps to modify the promoter into a more permissive configuration (Vermeulen et al., 2003a).
5.4.4.3. Promoter methylation

An additional level of transcriptional regulation is imposed by DNA methylation, occurring on cytosine bases within the sequence context cytosine-guanine (CpG). These are mostly enriched in short stretches of CpG-dense DNA, known as CpG islands (Bird, 1986), that frequently surround the 5'-end of genes. As an epigenetic modification of the DNA sequence, this mark does not change the genetic code but affects chromosomal stability and gene expression. Aberrant de novo DNA methylation in cancer cells is accompanied by local changes in histone modifications and chromatin structure, so that the CpG island and its embedded promoter take on a repressed conformation, which is incompatible with gene transcription (Antequera et al, 1990). The resulting gene silencing, together with point mutations and deletions, serves as one mechanism contributing to the inactivation of tumor suppressors and other critical genes in human cancers (Baylin, 2005). Several de novo (DNMT3a/b/l) and maintenance methyltransferases (DNMT1) constitute the core enzymatic components of the DNA methylation system in mammals. DNA- or chromatin-targeting motifs, transcription factors, as well as RNAi-based systems, function in targeting these methylating enzymes. Two basic models underpinning the relation between methylation and silencing have evolved: in the first, DNA methylation can directly repress transcription by blocking transcriptional activators from binding to cognate DNA sequences; in the second, methyl-binding proteins (MBPs), recognizing methylated DNA, recruit co-repressors to silence gene expression directly. Furthermore, MBPs might be involved in inhibiting elongation, either directly or by their effects on the surrounding chromatin structure. In addition to their DNA methyltransferase activities, DNMT enzymes are also physically linked to HDAC and HMT activities. Besides in the promoter region, DNA methylation within the body of genes can also have a dampening effect on transcriptional elongation (Klose & Bird, 2006).

For breast cancer cell lines, the remarkable difference in basal and TNF-α/IL-1β-stimulated IL-6 expression between benign MCF7 cells and malignant MDA-MB231 cells relies on distinct chromatin configurations. Treatment of less IL-6-producing MCF7 cells with 5-aza-2'-deoxycytidine results in elevated TNF-α-induced IL-6 transcription and hypomethylation, as assessed by restriction digests with a methylation-sensitive enzyme. This indicates that the repression of the IL-6 gene is associated with hypermethylation in the 5'-flanking region of the gene (Armenante et al, 1999a). Moreover, the silencing mechanism involves a modification of the chromatin structure and additional binding of cell-specific proteins, as revealed by a decreased sensitivity to DNaseI and by in vivo footprinting, respectively (Armenante et al, 1999b).
6. Inhibitors of NF-κB-dependent gene expression

As excess or inappropriate activation of NF-κB has been observed in a host of inflammatory diseases and types of cancer, in which the NF-κB signaling pathway plays a key role in disease pathophysiology, many different levels in the NF-κB signaling cascade are a potential target for pharmacological intervention. Over 750 inhibitors of the NF-κB pathway have been identified, including a variety of natural and synthetic molecules (Gilmore & Herscovitch, 2006).

6.1. NSAIDs

Non-steroidal anti-inflammatory drugs (NSAIDs) are synthetic molecules, widely used in the treatment of chronic inflammatory states. Regular intake of different NSAIDs causes regression of intestinal polyps, reduces the incidence of colorectal cancer development (Thun et al, 2002) and lowers the risk on breast cancer (Harris et al, 2003). The putative action of NSAIDs in reducing inflammation is to inhibit cyclooxygenase (COX) activity, which in turn results in a lowering of prostaglandin levels. These prostaglandins are believed to contribute to the development of cancer by multiple mechanisms, including stimulation of cell proliferation and cytokine synthesis induction. Furthermore, their production is accompanied by the formation of mutagenic by-products.

However, the COX enzymes may not be the exclusive target of NSAIDs, as NF-κB inhibition contributes considerably to their anti-inflammatory effects. The benefits of mesalazine and sulfasalazine in therapy for ulcerative colitis presumably rely on their ability to inhibit NF-κB, as revealed by patient biopsies (Bantel et al, 2000; Gan et al, 2005). Aspirin (acetylsalicylate), ibuprofen, sulindac, phenylbutazone, naproxen, diclofenac and indomethacin inhibit TNF-α-induced NF-κB activation in cell cultures (Takada et al, 2004). The molecular mechanism of the most common NSAID aspirin is inhibition of ATP binding to IKKβ, thereby abrogating its kinase activity (Yin et al, 1998). Recent findings show that aspirin impairs the proteasome function and causes severe mitochondrial abnormalities, leading to apoptosis induction (Dikshit et al, 2006). Although aspirin and other NSAIDS suppress NF-κB activation by blocking IKK activation and IκBα degradation, mesalazine prevents IL-1-stimulated p65 phosphorylation (Egan et al, 1999).

Unfortunately, the potential usefulness of NSAIDs is hampered by the fact that they cause gastrointestinal irritation and ulceration. Therefore, specific COX-2 inhibitors, such as celecoxib and rofecoxib, which do not target constitutively expressed COX-1, have been developed. Although promising in their attenuation of NF-κB activation (Callejas et al, 2003; Lavagno et al, 2004; Shishodia et al, 2004), they elicit more serious cardiovascular events.
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(Fosslien, 2005), which stresses the need to weigh the risks against any potential benefit in inflammation and inflammation-associated cancers.

6.2. Glucocorticoids

Synthetic glucocorticoids (GCs), such as dexamethasone and prednisone, belong to the most commonly and effectively used drugs in the clinic, to relieve inflammation and various immune disorders. These steroidal hormone agents interact with the glucocorticoid receptor (GR) to downregulate the expression of specific genes that regulate the inflammatory response. As such, the beneficial potential of the GR primarily resides in its ability to negatively modulate pro-inflammatory cytokines. Endogenous glucocorticoids, such as cortisol, produced by adrenal glands as part of the inflammatory response, act to enhance some IL-6 effects in specific systems, such as acute phase protein synthesis in liver cells. However, in general, they downregulate IL-6 expression, providing a negative feed-back pathway on the inflammatory response in vivo.

The repressor function of steroid hormone receptors often occurs on promoters that lack a steroid response element, indicating no requirement for direct receptor-DNA interaction. Instead, ligand-activated nuclear receptors, GR (Caldenhoven et al, 1995; Scheinman et al, 1995; Uht et al, 1997) and progesteron receptor (PR) (Kalkhoven et al, 1996), interfere with gene transcription via NF-κB or AP-1. To explain their anti-inflammatory capacity, the role of GCs and GRs in inhibiting the activity of the transcription factor NF-κB has been widely investigated and several mechanisms have been proposed (De Bosscher et al, 2006). One of these is consistent with a function of GCs in inducing IκBα expression; other ones claim that direct protein-protein interactions between GR and p65 could prevent NF-κB activation. Competition between those two transcription factors for co-activators in limiting amounts could also explain the GR-mediated NF-κB inhibition. Lastly, GR may directly disrupt p65 interactions with the basal transcription machinery (De Bosscher et al, 2003; De Bosscher et al, 2000).

However, chronic steroid use elicits adverse side effects, including osteoporosis, muscle wasting, hypertension, behavioral alterations and disorders of glucose and lipid metabolism (Rosen & Miner, 2005), mainly as the consequence of gene-transactivating capacities of GR (Reichardt et al, 2000).
6.3. Estrogens

6.3.1. Definition

Estrogens, such as 17β-estradiol (E$_2$), estron and estriol, are steroidal molecules, mainly produced by the ovaries and named for their importance in the oestrus cycle, functioning as the primary female sex hormones. These molecules mediate the endocrine balance in the hypothalamus-pituitary-gonadal axis and are active in the development of the mammary glands and the uterus, in the maintenance of pregnancy and bone density, in protection from cardiovascular diseases and in the relief of menopausal symptoms. However, estrogens can also stimulate malignant growth and hence contribute to the development of estrogen-dependent tumors, such as breast or endometrium cancer.

6.3.2. Estrogen receptors

As a ligand-inducible transcription factor, the estrogen receptor (ER) belongs to the superfamily of steroid/thyroid hormone nuclear receptors. A decade after the description of the classical ER$_\alpha$, a second ER isoform was discovered in 1996 and named ER$_\beta$ (Kuiper et al, 1996). Like the majority of other members of their family, ER proteins have a modular architecture in functional domains (Figure 11). Their central structure consists of a highly conserved DNA-binding domain (DBD) in between a hypervariable N-terminal region and a C-terminal ligand-binding domain (LBD) and dimerization region. In the absence of ligands, ERs are complexed with heat-shock Hsp90 and Hsp70 by a multi-protein chaperone machinery, formed specifically with the LBD, to open the steroid-binding cleft for access of a steroid. The hinge region contains the NLS, which mediates the translocation to the nucleus. In addition, two transcriptional activation function (AF) domains contribute to its transactivation, necessary for transcription initiation, including a ligand-independent AF-1 in the N-terminus and a ligand-dependent AF-2 in the C-terminus (Ascenzi et al, 2006).

![Figure 11: Modular structure of the ER protein, with indications of the domain function and of the percentages domain homology between hER$\alpha$ and hER$\beta$. Based on (Enmark et al, 1997).](image)

Figure 11: Modular structure of the ER protein, with indications of the domain function and of the percentages domain homology between hER$\alpha$ and hER$\beta$. Based on (Enmark et al, 1997).

Estrogen effects are mediated by binding of these steroid hormones in the hydrophobic cavity of the LBD. This high affinity interaction induces a ligand-dependent
conformational change of the receptor, followed by the separation of the receptor from its cytoplasmic chaperone proteins. A prerequisite for ER to modulate target gene expression is homo- or heterodimerization of the ligand-bound receptors and interaction with co-factors (co-activators, co-repressors). The latter is specifically triggered by the extent of exposure of AF-1 and AF-2 on the protein surface. After binding to estrogen-sensitive promoters via an estrogen recognition element (ERE), interaction with the transcription machinery drives gene transcription. Besides this classical model, an indirect mode of target gene regulation by estrogens has also been discovered, in which ER tethers onto other transcription factors bound on their specific recognition sites. Positive influence has been described for AP-1 (Cheng et al, 2003; Jones et al, 2002) and Sp1 (Kim et al, 2003; Li et al, 2001; Safe, 2001) (Figure 12).

However, effects elicited by estrogens within seconds to minutes, which are too rapid to be mediated by enhancement of RNA and protein synthesis, also exist. These are initiated from the cell membrane and designated ‘non-conventional’ or alternatively, ‘non-genomic’ (Figure 12). Probably via formation of a protein complex with many signaling molecules, cytosolic fractions of liganded ERα and ERβ proteins lead to activation of MAPK (Watters et al, 1997) and Akt signaling pathways and/or increases in cAMP (Aronica et al, 1994), etc. As an example, 17β-estradiol has beneficial atheroprotective properties by stimulation of NO production via activation of eNOS, which is mediated by membrane-associated ERα (localized to caveolae) and dependent on interaction between ERα and Gαi, MAPK activation and calcium release (Chambliss & Shaul, 2002). Functionally, estrogen-initiated non-genomic events have important physiological consequences, leading to DNA synthesis, cell proliferation and protection against cell death (Song et al, 2005).

**Figure 12:** Estrogen elicits cellular responses by 2 different modes: genomic actions via ERE-binding (direct) or by tethering to transcription factors (indirect) (1) and non-genomic mechanisms (2). The repression of NF-κB-dependent gene expression is also depicted.
6.3.3. Estrogen-mediated repression of NF-κB-driven gene expression

Besides positive regulation of gene activation in a direct or indirect manner, estrogens are also implicated in repression of specific genes via the ER, similar to ligand-activated GR. Several groups have identified the potential for a reciprocal transcriptional inhibition between agonist-bound ER and activated NF-κB. The cell type-dependent mechanisms of this cross-talk involve direct protein-protein interactions (Stein & Yang, 1995), inhibition of NF-κB-DNA binding (Deshpande et al, 1997; Galien & Garcia, 1997; Ray et al, 1997), disruption of NF-κB transactivation (Liu et al, 2005), blockage of IκBα protein degradation (Sun et al, 1998) or competition for co-activators, such as CBP and p300 (Harnish et al, 2000; Speir et al, 2000). Indirectly, ER also inhibits NF-κB by increasing the IκB-like precursor protein p105 (Hsu et al, 2000).

This reciprocal transcriptional interference occurs in vivo as well. Subcutaneous treatment of ovariectomized C57BL/6 mice with an estrogen, 17α-ethinylestradiol, results in an ER-dependent inhibition of NF-κB-directed gene expression, induced in the liver by an atherogenic (high-fat) diet (Evans et al, 2001). Indirect evidence is provided by the observation in retrospective studies that serum levels of IL-6 in post-menopausal women, receiving classical hormone replacement therapy (HRT) (different types included), were significantly lower, compared to women without HRT (Straub et al, 2000). In addition, loss of ER function in cancer is often associated with constitutive NF-κB and MAPK activation, which culminates in an aggressive, metastatic, hormone-independent cancer type (Nakshatri et al, 1997). However, this phenomenon could be correlated with the increased level of epidermal growth factor family receptors in ER-deficient cells (Biswas et al, 2000).

6.3.4. HRT

As estrogen constitutes a crucial molecule in keeping an endocrine homeostasis, many studies persue to improve the life quality of estrogen-deprived women, such as post-menopausal women or those who have had a total hysterectomy. They frequently suffer from symptoms, such as reduced bone density or osteoporosis, vaginal atrophy, mood swings, climacteric changes, particularly hot flushes, night sweats and other discomforts. ‘Classical’ HRTs with 17β-estradiol esters, conjugated equine estrogens and/or progestins, such as medroxyprogesterone acetate, have been intensively propagated in the last 25 years, for relieving these symptoms. The results of a well designed, randomized, controlled, long-term endpoint study (WHI) and a large-scale observational study (Million Women Study) in the UK strongly suggest that progestins, combined with estrogens, increase the risk of breast cancer above that associated with estrogen alone (Beral, 2003). The Women’s Health Initiative
(WHI) safety committee has decided to interrupt one arm of the study after a mean of 5.2 years follow-up of healthy post-menopausal US women, because those on the combined estrogen-progestin therapy had an increased risk for cardiovascular events (blood clots, heart attacks, strokes) and breast cancer, though with a lesser risk of osteoporotic fractures and colon cancer (Rossouw et al, 2002). The net harm is markedly accentuated by a subsequent report of a significant 2-fold increase in dementia among WHI women older than 65 years (Shumaker et al, 2003). The extra cancer occurrence of women in an unopposed estrogen HRT is predominantly localized to the uterus (Grady et al, 1995) and ovaries (Lacey et al, 2002). According to the data from the WHI trial, estrogen alone therapy also has adverse effects on strokes, dementia (Shumaker et al, 2003) and probably pulmonary embolism (Anderson et al, 2004). However, both regimens have the important benefit of decreasing fracture risk (Hulley & Grady, 2004), which is associated with the bone mineral density state and osteoporosis. In relation to the adverse outcomes, conventional HRTs are contra-indicated, although controversy still exists about the trial set-ups, the reliability and the public generalization of the results. Nevertheless, the net negative effects has lead many women to gain interest in more natural alternatives to HRT, preferring the so-called phyto-estrogens. The identification of these non-steroidal plant estrogens and first evidence for their estrogen-like biological activities is based on the observation in the mid-1940s that sheep grazing on clover-rich pastures in Western Australia became infertile (Bennetts et al, 1946). Unfortunately, the name phyto-estrogen does not fully describe the action of these compounds (see 6.4.4.2.).

6.4. Phyto-estrogens

6.4.1. Classification

Broadly defined, phyto-estrogens include terpenoids, saponins and phenolics, such as coumestans, lignans, stilbenes, flavonoids and isoflavonoids (Cos et al, 2003). Of these, resveratrol (trans-3,5,4’-trihydroxystilbene), belonging to the stilbenes, is one of the most common phyto-estrogens in the Western world, as it is present in the skin of grapes and consequently in red wine. Much interest centers on its perceived effects on cardiovascular health, postulating that resveratrol may be a crucial factor in explaining the so-called ‘French paradox’. This refers to the phenomenon that the French with a relatively high dietary fat intake (due to the renowned soft cheese consumption) have a lower than average incidence of cardiovascular diseases. In this study, however, we focus on the effects of isoflavonoids, which we will discuss further in more detail.
6.4.2. Isoflavones

Isoflavones enjoy a rather restricted distribution in the plant kingdom, being mostly limited to the family Fabaceae (Leguminosae), exemplified by soybean (Glycine max), red clover (Trifolium pratense) and various beans and sprouts. However, increasingly more plant species of diverse classes are reported to contain isoflavones (Mackova et al, 2006). In plants, isoflavones are synthesized from flavanone intermediates, either naringenin (4',5,7-trihydroxyflavanone) or liquiritigenin (4',7-dihydroxyflavanone), by an unusual aryl migration reaction, catalysed by the enzyme p450 cytochrome isoflavone synthase (Steele et al, 1999). They play a role in plant-microorganism interaction, as part of the host’s defence mechanism. As such, isoflavonoids exhibit broad-spectrum anti-microbial activity in vitro (Ulanowska et al, 2006) and are considered precursors for phytoanticipins or inducible phytoalexins (Dixon & Ferreira, 2002). The function of isoflavonoids in plant disease resistance has been confirmed by genetic approaches (Dixon, 2001). Furthermore, isoflavones serve as secreted nodulation-inducing signals from nitrogen-fixating plants to symbiotic bacteria (rhizobia) (Rolfe, 1988).

The most abundant isoflavones of soybean include genistein (4',5,7-trihydroxyisoflavone), daidzein (4',7-dihydroxyisoflavone) and to a lesser extent, glycitein (4',7-dihydroxy-6-methoxyisoflavone) (Delmonte et al, 2006). Red clover, however, contains mainly the 4'-methyl esters of genistein and daidzein, biochanin A and formononetin, respectively (Delmonte et al, 2006) (Figure 13). In plants, isoflavones predominantly exist in a soluble and inactive form as glycoside malonate conjugates (6''-O-malonyl-7-O-β-D-glucoside) (Figure 13). After ingestion and passage through the gastrointestinal tract, gastric hydrochloric acid and bacterial or intestinal enzymes (β-glucosidases) hydrolyse and, as such, allow absorption of the aglycones by non-ionic passive diffusion through the intestinal wall. Before absorption, extensive metabolism by the microflora in the gut includes demethylation of biochanin A and formononetin and partial conversion of isoflavones to equol (7-hydroxy-3-(4-hydroxyphenyl)-chroman), O-desmethylangolensin and other metabolites. Only about 35% of the adult population is able to make this biotransformation to equol, which is influenced by the overall composition of the diet as well (Lampe et al, 1998; Rowland et al, 2000). Interestingly, only one diastereoisomer, S-equol, is selectively produced by the intestinal microflora, which has important clinical relevance, as only this enantiomeric form shows a relatively high affinity for ERβ (Setchell et al, 2005). Isoflavone aglycones or metabolites become extensively metabolized and reconjugated during first-pass uptake by enterocytes or, later on, by liver cells. This accounts for their abundance in blood as glucuronides and to a lesser extent, sulfates (D’Alessandro et al, 2005). Apart from the
reductive and conjugative metabolism, oxidative biotransformation of isoflavones by liver microsomes has also been reported in man (Kulling et al, 2002). Complete metabolization is proposed to occur locally within target tissue, as supernatant analysis of breast cancer cell cultures reveals the presence of sulfated, hydroxylated and methylated isoflavone metabolites (Peterson et al, 1998). If excreted via bile, these compounds undergo enterohepatic circulation (D’Alessandro et al, 2005). In contrast to xeno-estrogens, a heterogenous group of synthetic chemicals as pesticides (DDT), preservatives (BHT), plasticizers (bisphenol-A), solvents (hydrocarbons), which behave as endocrine disruptors, phyto-estrogens are not stored in the fat tissue but excreted via bile or urine. In many studies, the fractional recoveries of isoflavones were low and highly variable among persons, ranging from 18-95% for daidzein and 5-42% for genistein (Setchell et al, 2003). This suggests marked individual differences in the intestinal metabolism and the existence of a range of metabolites.

Figure 13: Chemical structures of naturally occurring isoflavones, from soybean and red clover. Their plant-derived glucosylated forms are also shown.
Concerning pharmacokinetics, isoflavones normally display a fast serum clearance rate, resulting in the majority appearing in the urine within the first 24 hours after ingestion, compatible with their short serum half-life (genistein 7.13 hours and daidzein 9.34 hours) (Setchell et al, 2001). The mean time to attain peak plasma concentrations ($t_{\text{max}}$) for the aglycones varies between 4-7 hours after ingestion, depending on the food matrix (de Pascual-Teresa et al, 2006). However, the profile of $\beta$-glucosides shows a delay, with the $t_{\text{max}}$ shifted to 8-11 hours, consistent with the resident time for cleavage of the glycoside moiety for bioavailability (Setchell et al, 2001). There is no evidence to support absorption of the conjugated forms of isoflavones in humans (Setchell et al, 2002).

Besides in blood and urine, isoflavones are detected in other biological matrices, such as prostatic fluid (Morton et al, 1997), breast milk (Franke et al, 1998), faeces (Adlercreutz et al, 1995), amniotic fluid and umbilical cord plasma (Adlercreutz et al, 1999). The tissue distribution of isoflavones is not thoroughly investigated, although a HPLC method, combined with UV detection, reveals the presence of genistein and equol in breast tissue homogenates after intake of soy-based isoflavone preparations (Maubach et al, 2003). A 2-fold higher concentration of isoflavones is detected in prostate tissue, compared to plasma levels, indicating that the prostate can concentrate circulating isoflavones (Hong et al, 2002; Rannikko et al, 2006). In general, the isoflavone content of human plasma or urine may serve as a biological marker of soy intake because isoflavones are unique constituents of soybeans. Daidzein (<5 ng/ml), genistein (2-30 ng/ml) and mainly equol (45-293 ng/ml) have been detected in cow’s milk (King et al, 1998) and in certain vegetables (legume sprouts, beans, peas, lentils, such as alfalfa, black bean, chickpeas, etc), albeit in low concentrations. Equol excretion has been associated with meat intake, as it is presumed that animals are capable of similar isoflavone metabolism (Lampe et al, 1999) when on soy-, alfalfa-, or clover-supplemented feed. Thus, besides soy products, no other commonly consumed human foods contain appreciable amounts of isoflavones. However, care has to be taken in extrapolating measured isoflavone concentrations to its bioavailability, as the existence of many different metabolites confound quantitative results. As only about 1.5% of the total genistein plasma pool was unconjugated during the first 2 hours after a single oral dosis (50 mg) of glycoside genistin (Setchell et al, 2001), this presumably poses further limitation to intracellular access. Furthermore, their biological potential cannot be assessed by measuring plasma concentrations only, as isoflavones bind plasma carrier proteins, such as sex hormone-binding globulin (SHBG) (Dechaud et al, 1999), which may modulate their bioavailability to tissues. An in vitro assay on breast cancer cells shows differences in the
uptake levels of genistein compared to equol and biochanin A, corresponding with 45.8%, 49.7% and 2.4% effective free fraction in serum, respectively (Nagel et al, 1998).

Phyto-estrogen-containing soybeans are a basic ingredient of many soy foods, comprising tofu, tempeh, soy milk, miso (bean paste), natto, soy flour, etc. So, these soy products provide a significant dietary source of phytochemicals, containing 1.0-3.0 mg/g of total isoflavone after normalization to aglycone levels (Song et al, 1998). Although in soybeans the isoflavones are predominantly sugar-conjugated, processing techniques to soy foods alter the ratio of different chemical forms, including aglycones, glycoside malonate conjugates, glycosides (7-O-β-D glucosides) or glycoside acetate conjugates (6"-O-acetyl-7-O-β-D-glucosides) (Coward et al, 1998). Fermentation (for production of miso and natto) leads to a greater proportion of aglycones, whereas heating reduces the concentration of malonylglucosides (Song et al, 1998). Because isoflavones migrate with the protein fraction of the soybean during its processing, some soy products, such as soy oil, are devoid of isoflavones. Soy protein is rarely a normal component in the average Western diet, which accounts for the negligible dietary intake of isoflavones in Western populations (less than 3 mg/day), in contrast to the 15-50 mg/day for Japanese people (Arai et al, 2000). Accordingly, Asiatic plasma concentrations within the high nanomolar range have been observed, varying from 200 to 500 nM for daidzein and genistein, independently of the sexes. The plasma isoflavone concentrations of British people is approximately 15 times lower (Morton et al, 2002; Pumford et al, 2002). By isoflavone supplementation in a single or long-term administration, plasma concentrations in the low micromolar range can be reached (up to 25 µM total genistein) (Bloedon et al, 2002; Busby et al, 2002; Takimoto et al, 2003; Vedrine et al, 2006). In Western countries, infant formulas, manufactured from soy protein isolates, have been in use for more than 30 years and contain significant amounts of isoflavones. So, young children, fed with soy-based formulas, are therefore exposed to 22-45 mg isoflavones per day, whereas the intake of these phyto-estrogens from human milk is negligible (<0.01 mg/day) (Setchell et al, 1998).

6.4.3. Isoflavone effects on diseases and disorders

Epidemiological studies have observed large ethnic differences in breast and prostate cancer rates, among others. Relatively to US people, a significantly lower incidence and mortality of these hormone-dependent cancers exist in Asian populations (Althuis et al, 2005; Hsing & Devesa, 2001). In Japan, the mortality rate for breast cancer is approximately 4-fold lower (Ursin et al, 1994). Population-based studies have been examining Asian migrant populations, to determine the extent of genetic versus environmental factors. Emigration
towards the West substantially increases the risk on breast cancer with 60% (Ziegler et al, 1993) and leads to cancer risks tending towards the levels of the native people in the destination country (Luo et al, 2004b). These findings suggest that rather environmental, behavioral and life-style factors influence the cancer frequency between populations (Parkin, 1989). Smoking, alcohol consumption, exposure to noxious agents (fumes, radioactivity, dust, fossil fuel combustion products, etc) and diet belong to these factors. Concerning nutritional intake, the western and eastern diet vary widely with the source of protein as a striking distinction. Historically, the kitchen in the western world generally relies on animal protein, whereas eastern dishes are based on soybeans as protein source. However, the recent adoption of a more westernized diet correlates with a 2-fold increased breast cancer incidence and mortality in urban areas of Japan and Singapore between 1960 and 1985 (Ursin et al, 1994).

In this sense, case-control studies associate the reduced risk of prostate cancer and endometrium cancer with the intake of soy food and isoflavones (Goodman et al, 1997; Horn-Ross et al, 2003; Lee et al, 2003a). For breast cancer, a retrospective study reveals an association between decreased soy food consumption during adolescence and breast cancer in a cohort of Chinese patients (Shu et al, 2001). A prospective nested case-control study confirms the association between high genistein circulation levels and reduced breast cancer risk in Dutch pre- and post-menopausal women, with relatively low overall plasma isoflavone levels (Verheus et al, 2007). Similarly, a recent prospective cohort study of approximately 75000 Chinese women provides direct evidence that soy food consumption reduces the risk of cardiovascular diseases (Zhang et al, 2003b). In 1999, the US Food and Drug Administration (FDA) authorized health claims on food labels, about the association of soy protein and the reduced risk of coronary heart disease. Moreover, the prevalence of menopausal vasomotor symptoms and osteoporosis-related bone fractures is reported to be lower among Asian women, relative to women in the western world (Boulet et al, 1994; Ho et al, 1993; Shea, 2006). A prospective study suggests that soy intake has a protective effect against hot flashes (Nagata et al, 2001).

In view of the beneficial effect of isoflavones on these disorders in case-controlled observational, retro- or prospective studies, randomized supplementation trials and animal studies are needed for confirmation. Several studies on orchidectomized or ovariectomized rats and mice provide convincing data on isoflavone-mediated preservation of bone volume, bone mineral density and other bone markers, despite androgen or estrogen deficiency (Chanawirat et al, 2006; Ishimi et al, 2002; Kim & Lee, 2005). Daily oral consumption prevents bone loss, both by depressing bone resorption and stimulating osteoblast activity
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(Picherit et al, 2001). Supplementation trials of relatively short duration (1 year) on post-menopausal women confirms the protective effect of isoflavones against bone loss (Chen et al, 2003b; Huang et al, 2006). However, further research on the optimal dose for the favorable effect and on the exact mechanism of action is warranted, as well as larger and longer duration studies to draw clear conclusions in regard to the role of isoflavones on bone (Arjmandi & Smith, 2002). Also hot flushes, another menopausal marker, are reduced after a daily 90 mg genistein supplementation of post-menopausal women during 6 weeks (Albertazzi et al, 2005) or after 3 months with 54 mg genistein a day (Crisafulli et al, 2004). However, some studies show no relief of frequency and/or severity of hot flushes (reviewed in (Usui, 2006)), which may question the value of isoflavones as therapeutic alternatives in the management of the menopausal syndrome. In view of the beneficial effects of estrogens on the cardiovascular system, studies with isoflavones show improved endothelial function (Squadrito et al, 2003) and reduced total and low-density lipoprotein cholesterol (Allen et al, 2006) in post-menopausal women. Furthermore, isoflavones may improve insulin resistance and reduce adipogenesis, suggesting favorable effects on type 2 diabetes and obesity (reviewed in (Usui, 2006)).

Although isoflavones exert estrogen-like effects under some conditions (see 6.4.4.1.), which may affect the above-mentioned hormone-associated disorders, they also possess non-hormonal properties (see 6.4.4.2.). So, their health benefits may be attributable to a variety of potential molecular mechanisms, which possibly constitute the strength of isoflavones.

6.4.4. Molecular effects of isoflavones

6.4.4.1. Estrogenic effects

The isoflavone diphenolic structure of 2-phenynaphtalene-type strikingly resembles the planar chemical form of endogenous estrogen 17β-estradiol (Figure 14). Even the distance of 11-12 Å (1,1-1,2 nm) between the hydroxyl groups at the end of both molecules is virtually identical. Not surprisingly, these characteristics account for their potential to bind to the ligand binding pocket of the ER (Barnes et al, 2000). In contrast to 17β-estradiol, the affinity of genistein and daidzein is 20-fold higher for ERβ, compared to ERα, which is reflected in the values of half-maximal transcriptional activity: the EC50 value for genistein on ERα/β is 20 nM/6 nM (Kuiper et al, 1998). Although, based on these values, genistein’s estrogenic potency for ERβ emerges as 3-fold higher than its potency for ERα, isoflavones have a greater ERα-selective efficacy (Barkhem et al, 1998). Compared to estrogens,
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Isoflavones exhibit weak estrogenic activity in the order of 100 or 1000 times less than that of estrogens (Kuiper et al, 1998; Zava et al, 1997), but this is compensated as they may reach plasma concentrations of 100-fold higher than endogenous estrogens: 50-800 ng/ml (if consuming modest quantities of soy foods) versus 40-80 pg/ml, respectively (Setchell & Cassidy, 1999). Even for western women, phyto-estrogen levels are more than 50-fold higher than that of endogenous estrogens as mean genistein plasma values of 4 ng/ml are measured in Dutch women (Verheus et al, 2007).

![Figure 14: Chemical structures of endogenous estrogen 17β-estradiol and the isoflavone skeleton.](image)

Although both classic estrogens, as well as isoflavones, act as ER ligands, this does not necessarily implicate ER agonism as crystallization studies of activated receptors unambiguously reveal distinct ER conformations. In the complex ER-17β-estradiol, helix 12 (H12) orients itself to form a lid over the ligand-binding cavity and complete a hydrophobic cleft together with amino acid residues of other helices. This agonistic positioning of H12 is crucial for the formation of an interaction surface for ligand-dependent AF-2 co-activator recruitment. In contrast, despite the fact that genistein is completely burried into the core of the ERβ LBD, H12 adopts a quasi-agonist/antagonist position with a sub-optimal alignment, which accounts most likely for co-repressor binding and suggests that co-activators must displace H12 prior to binding (Pike et al, 1999) (Figure 15). As the ligand-dependent conformation impacts the selectivity and kinetics of co-activator/co-repressor interactions with the ER, it dictates the estrogenic/anti-estrogenic gene-regulatory activity of the ligand. The outcome of ligand effects also depends on the relative importance of AF-1 to AF-2 and the ratio and type of co-regulators for modulation of gene transcriptional activity, which are cell type- and promoter-selective. This might explain the tissue-specific effect of isoflavones, together with the different tissue and organ distribution of both ERs (Nilsson & Koehler, 2005), which is originally discovered in rats. Human ERβ is highly expressed in testis, ovary, small intestine, kidney and thymus, whereas ERα is predominantly localized in ovary and
uterus (Enmark et al, 1997). As such, isoflavones may act as natural selective estrogen receptor modulators (SERMs). The term SERM implies ER-agonistic activities in some tissues and -antagonistic effects in others. Regarding antagonism, depending on the endogenous estrogen levels, isoflavones may compete for binding to the ER, thereby mediating anti-estrogenic activities.

**Figure 15**: Representation of the differential conformational states of H12 of the ER LBD. The conformations of the ERα-LBD, bound to 17β-estradiol (agonist), as well as the ERβ-LBD, complexed with genistein (partial agonist), are depicted. Derived from (Pike et al, 2000).

Not only competition with estrogen determines the outcome of genistein effects, also the concentration of this compound plays a substantial role. Genistein concurrently activates ER and peroxisome proliferator-activated receptor (PPAR)γ, which have opposite effects on osteogenesis and adipogenesis. High genistein concentrations (more than 1 µM) provoke anti-estrogenic effects via PPARγ, as osteogenesis is decreased in favor of adipogenesis. Conversely, lower genistein concentrations, in the physiological range, shift the balance to ER-mediated effects (Dang et al, 2003). Genistein and other isoflavones are shown to transactivate other receptor types too, such as estrogen-related receptor (ERR)α and aryl hydrocarbon receptor (AhR), although no physiological function has been revealed (Han et al, 2006; Suetsugi et al, 2003; Zhang et al, 2003a).

Based on the estrogenic effects of isoflavones, some research groups describe that genistein stimulates tumor growth *in vivo*. Athymic mice, subcutaneously implanted with estrogen-dependent tumors, show increased cell proliferation when fed with varying genistein concentrations (Allred et al, 2001; Hsieh et al, 1998). In a post-menopausal breast cancer mouse model as well, genistein exerts adverse effects on tumor progression (Power et al, 2006). However, the number of reports on estrogen-like, growth-promoting effects on cancer cells *in vivo* remains limited, whereas increasingly more evidence on the anti-carcinogenic role of genistein accumulates.
### 6.4.4.2. Other activities of isoflavones

Several animal studies support the anti-carcinogenic role of isoflavones, as neonatal treatment with genistein increases latency, decreases incidence and multiplicity of chemical-induced mammary cancer by enhancing mammary gland differentiation (fewer terminal end buds and more lobules) (Lamartiniere et al, 1995). A similar effect of genistein is observed in a nude mouse xenograft model of mammary cancer, in which both ER-positive and ER-negative cells become differentiated by genistein treatment, resulting in a lower tumorigenic potential after implantation (Constantinou et al, 1998).

However, the anti-carcinogenic and even chemopreventive activities of isoflavones may also be mediated via their anti-proliferative and apoptosis-inducing effects. As such, genistein-fed rats show an enhanced apoptotic index in mammary glands due to increased expression of the tumor suppressor PTEN, as well as of the pro-apoptotic genes Bok, Bax and p21 (Dave et al, 2005). Another mechanism of genistein includes phosphorylation of Smad2 and Smad3, associated with increased TGF-β1 expression (Yu et al, 2005). Genistein-mediated inhibition of the proteasomal chymotrypsin-like activity in prostate, breast and SV40-transformed cancer cells, but not normal cells, may also account for its cancer-preventive activity (Kazi et al, 2003). Alternatively, genistein-mediated apoptosis induction or cell cycle stop occurs via ATM-dependent p53 and Chk2 activation (Ye et al, 2001). This DNA-damage response pathway is probably the result of inhibition of topoisomerase II enzymes by genistein via stabilization of the covalent topo II-DNA cleavage complex, inhibiting religation and resulting in DNA strand breaks (Markovits et al, 1989). However, in bacterial systems and in vivo assays, genistein is generally negative for genotoxicity (Michael McClain et al, 2006). In a clinical trial on prostate cancer patients, unconjugated genistein administration results in no genetic damage, although peak plasma concentrations of 27 µM total genistein were reached. These findings refute a clastogenic effect of genistein in vivo (Miltyk et al, 2003). The G2/M cell cycle arrest is one mechanism by which genistein exerts its anti-proliferative effects and the proposed underlying causes encompass the transcriptional repression of cyclin B1, increase of p21\(^{WAF1/CIP1}\), ATM-mediated p53 and Chk2 activation, which result in cdc-2 inactivation (Chang et al, 2004; Choi et al, 2000; Frey et al, 2001). However, Myt-1 and Wee-1 are identified as necessary mediators in p21-independent inactivation of the cyclin B1/cdc-2 complex and concomitant G2/M arrest by genistein (Touny & Banerjee, 2006).

Furthermore, genistein is reported as an inhibitor of a variety of enzymes, involved in endogenous hormone metabolism, including aromatase (Edmunds et al, 2005; Rice et al, 2006), 3α-hydroxysteroid dehydrogenase (Blomquist et al, 2005), 3β-hydroxysteroid
dehydrogenase (Ohno et al, 2004) and 17β-hydroxysteroid dehydrogenase (Brooks & Thompson, 2005). Together with the induction of SHBG plasma levels (Pino et al, 2000), this inhibition may account for attenuated endogenous hormone activity, which positively affects hormone-dependent cancer proliferation. Genistein, as well as other isoflavones, is shown to inhibit cAMP/cGMP phosphodiesterase activity (Nichols & Morimoto, 2000). Genistein is a broad inhibitor of tyrosine protein kinase activity (Akiyama et al, 1987), which is associated with many cellular growth receptors (EGFR, PDGF, IGF) and intracellular signal transduction. As such, this property of genistein may play an important role in inhibition of cell proliferation, transformation, among other processes. In prostate cancer cells, genistein represses telomerase hTERT transcription via the downregulation of c-Myc expression and by post-translational modification of hTERT via Akt (Jagadeesh et al, 2006). The inhibition of Akt activity by genistein is described to modulate survival of prostate cancer cells by cross-talk with NF-κB (Li & Sarkar, 2002).

Apart from promoting differentiation and inhibiting growth, isoflavones reduce the risk on cancer by diminishing oxidative stress via different ways. As they react with superoxide anions, hydroxyl and peroxyl radicals, genistein and other isoflavones acts as ROS scavengers (Kruk et al, 2005). In addition, they inhibit RNS (peroxynitrite, NO) activity by scavenging (Yen & Lai, 2003) and inhibition of iNOS activity and expression (Sheu et al, 2001). Their anti-oxidant activities are closely associated with the presence of hydroxyl groups at positions 4’ and 5’ and with the position of the aromatic ring. Furthermore, the ability of genistein and daidzein to increase cellular levels of GSH (reduced glutathione) and alter the redox status might be important for their action in biological systems (Guo et al, 2002). Genistein-mediated transcriptional induction of glutathione reductase, microsomal glutathione S-transferase-1 and metallothionein 1X constitutes an important anti-oxidative defence system, which in turn maintains DNA integrity (Raschke et al, 2006). Improved vascular reactivity in rats, fed with a soy protein-rich diet, is paralleled by increased mitochondrial glutathione and mRNA levels for the anti-oxidant enzymes manganese superoxide dismutase and cytochrome c oxidase (Mahn et al, 2005). In general, the modulation of cellular anti-oxidant capacities by redox-regulating phyto-chemicals provides a rational and pragmatic strategy for chemoprevention (Surh et al, 2005).

Apart from direct influence of isoflavones on cancer cells, isoflavones elicit anti-angiogenic effects as well, partially by downregulating cell adhesion-related genes (Piao et al, 2006), by inhibiting expression of angiogene growth factors and matrix proteases (Su et al, 2005). This substantially precludes neovascularization, cancer expansion and metastasis.
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6.4.4.3. Molecular effects of genistein on NF-κB-mediated gene expression

The described apoptosis-inducing, anti-angiogenic and anti-metastatic effects of isoflavones are based on their influence on many regulatory systems, in which NF-κB plays a crucial and central role.

In MCF-7 cells, genistein treatment leads to an ER-dependent increase in MnSOD anti-oxidant expression and reduction in peroxide levels. Molecular analysis reveals a rapid phosphorylation of ERK1/2 and nuclear translocation of the p50 subunit of NF-κB (Borras et al, 2006). Another study on glioblastoma cells found that genistein sustains NF-κB activity, induced by IL-1α stimulation, and suggests that an increase in tyrosine kinase-regulated proteasome activity accounts for the observed prolonged degradation of ΙκBα (Tago et al, 2001).

In contrast, most reports of genistein effects on NF-κB-mediated gene expression demonstrate repression of the signaling cascade, albeit by distinct mechanisms and on different targets. Genistein blocks the LPS-induced serine and tyrosine phosphorylation and degradation of ΙκBα in alveolar macrophages, which results in blockage of NF-κB activity (Kang et al, 2005). In peripheral blood monocytes, genistein is reported to decrease tyrosine phosphorylation of the LPS-stimulated TLR4, which presumably leads to lower NF-κB-DNA-binding levels (Chen et al, 2003a). Concerning pro-apoptotic effects by genistein specifically on prostate cancer cells, abrogated NF-κB-DNA binding by genistein treatment is mediated by blockage of Akt activation (Li & Sarkar, 2002). Similarly, decrease of NF-κB-DNA binding by genistein in MDA-MB-231 breast cancer cells is mediated via inhibition of the Akt pathway (Gong et al, 2003). In T lymphoma cells, marked reduction in nuclear NF-κB levels is caused by the genistein-mediated caspase-3 activation and subsequent generation of an ΙκBα cleavage form, ΔΙκBα (Baxa & Yoshimura, 2003). Even more strikingly, genistein exerts inhibitory effects on p65 expression and abrogates the chemotherapeutic agents-mediated increase in p65 expression and activation (Li et al, 2005b). Related to its anti-oxidative property, genistein attenuates intracellular oxidative stress by direct scavenging of peroxide and NO radicals, by delaying the consumption of cellular GSH and by activating anti-oxidant enzymes, which may result in suppression of NF-κB activation (Choi et al, 2003).

6.4.4.4. Molecular effects of isoflavones on chromatin regulation and epigenetics

The ‘developmental origins hypothesis’ postulates that nutrition and other environmental factors during prenatal and early postnatal development influence developmental plasticity, enabling a single genotype to produce a broad range of adult phenotypes (Bateson et al, 2004). External factors lead to persistent epigenetic changes and
as such, alter susceptibility to cancer and other adult-onset chronic diseases (Waterland & Jirtle, 2004). Methylation of cytosines in CpG dinucleotides represents a critical DNA modification as a type of epigenetic alteration, affecting gene expression and cellular function. Limited evidence suggests that postnatal exposure to phyto-estrogens alters the epigenome. Neonatal exposure to high doses equol is correlated with hypermethylation of a proto-oncogene in the rat pancreas (Lyn-Cook et al, 1995). In adult mice, dietary genistein causes changes in CpG island methylation of specific genes in the prostate gland (Day et al, 2002). However, the genome is likely the most vulnerable to environmental factors during embryogenesis, due to high DNA synthesis rates and establishment of DNA methylation patterning. In this regard, maternal dietary genistein supplementation during gestation results in permanent changes of the offspring phenotype due to hypermethylation of multiple CpG sites (Dolinoy et al, 2006).

Unlike other genomic alterations that occur during carcinogenesis, abnormal DNA hypermethylation and the subsequent gene silencing is potentially reversible via the use of preventive or therapeutic agents, such as 5-aza-2'-deoxycytidine and nucleoside analogue inhibitors of DNMTs. In cancer therapy, attempts to restore normal gene expression are focused on tumor suppressor genes, DNA repair genes, cell cycle checkpoint genes, etc. Genistein reactivates methylation-silenced retinoic acid receptor \( \beta \) and p16\(^{INK4a} \), partially through direct inhibition of DNMTs, which may contribute to its chemopreventive activity (Fang et al, 2005).

### 6.4.5. Commercial availability of isoflavones

In health shops, pharmacies and on the internet, a broad range of food supplements are available in powder, liquid, tablet or gel capsule form, manufactured and marketed by the health food industry for sale without prescription. Traditionally, dietary supplements refer to products made of one or more of the essential nutrients, such as vitamins, minerals, amino acids and certain lipid acids. This definition has been broadened to include herbal or plant-derived extracts or concentrated and purified constituents thereof. Some of these dietary supplements that intend to relieve menopausal symptoms are based on phyto-estrogens derived from soy, red clover, hop, among other plants. In pharmacies, soy-derived products of many different formulas and manufacturers exist, including Vitafytea Isopro, Vitafytea Isoflavon, Arkopharma Phyto Soya, Bio-Fyt Pharma Estro-Fyt, Prevalon Iso, FuncioMed Isomex, Ymea plus, etc (Figure 16).
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Figure 16: Commercially available products at pharmacies, to attenuate the menopausal transition phase. Isomex and Ymea plus are examples of preparations, based on isoflavones. In contrast, menohop contains 8-prenylnaringenin (hopeïn), extracted from hop (Humulus lupulus L.).

All these supplements vary widely in composition and dosis of isoflavones, showing no consensus on the optimal or appropriate intake. In general, phyto-supplements contain different blends of constituents from different times of their growth cycle, which are furthermore affected by the weather, soil quality, time of harvest, crop years and genetics. As the quantity of active ingredients varies between batches of raw material, standardization ensures stated levels of active phyto-chemicals in each dosis. However, analyses of dietary supplements have found differences between the label listings and the real content, especially for products sold in health shops and ware-houses. Approximately half (16/31) of the evaluated products have isoflavone levels that deviate by more than 10% from the claimed value (Setchell et al, 2001). Moreover, standardization is no guarantee for product quality, since no legal definition of ‘certified’, ‘qualified’ or ‘standardized’ for food supplements exists. In fact, food supplements are regulated as a special category of foods by the US FDA or European Medicines Evaluation Agency (EMEA), founded by the European Commission in 1995 as equivalent for the FDA. This means that they do not have to meet the same standards as drugs or medicines, in terms of quality control, proof of safety, efficacy and good manufacturing practices (GMP). As such, there are virtually no data on the bioavailability, pharmacokinetics or pharmacology of these phyto-estrogen supplements. Because they fall under the scrutiny of the Dietary Supplement Health and Education Act (DSHEA) of 1994, the manufacturer is responsible for ensuring that a dietary supplement is safe, before it is marketed and that the product label information is truthful and not misleading. However, the supplement industry does not need to register their products with FDA nor get FDA approval before producing or selling dietary supplements. FDA's post-marketing responsibilities include monitoring safety, e.g. voluntary dietary supplement adverse event reporting and product information, such as labeling, claims, package inserts, and accompanying literature. Many of the health claims that drive supplement sales are based on clinical and nutritional data from phyto-estrogen-rich foods, rather than from supplements. One of the most widely held beliefs of consumers concerning plant-derived nutrient supplements is that their natural origin infers safety, which is clearly a misconception, as poisonous plants also do exist and as products may be enriched for
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certain substances in relatively high concentrations. Although diets rich in phyto-estrogens have been consumed by millions of people since a long time, the amounts ingested daily, estimated 15-50 mg, are below the dose for supplementation and lower than in some fortified functional foods. Furthermore, pharmacokinetics studies on pre- and post-menopausal women, after a single bolus ingestion of soy nuts, corresponding to 16,4-65,5 mg isoflavones, reveal that the bioavailability of isoflavones from a soy food matrix, such as soy nuts, is non-linear. This means that the systemic availability is reduced with higher isoflavone intake, which is most likely explained by a decrease in absorption (Setchell et al, 2003). In this sense, steady-state serum levels are more likely to be achieved by multiple intakes of soy foods during the day. Although further research is warranted before generalizing these results, some criticism may already be raised against nutraceutical companies, which persist in flooding the market with products, containing isoflavone levels that exceed maximal dietary intakes.

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II. OBJECTIVES

The gradual decline in endogenous hormone levels around the average age of 50, which is called the menopause, is unavoidable and related to natural aging for women. As the endocrine balance is shifting, this life phase is mostly accompanied by symptoms, such as mental and emotional confusion, climacteric changes, osteoporosis, etc. Efforts to substitute for the loss with estrogen-like hormones have recently turned out to cause serious adverse health effects. Therefore, a quest for alternative therapies was started and led to phytoestrogens. Their initial discovery was based on the observation of estrogen-like activities in animals. Moreover, geographical differences in menopausal complaints as well as other diseases of advanced age (cardiovascular diseases, hormone-dependent cancers, etc), which relate to the common intake of soy, strengthen the claim of isoflavones as health beneficial molecules. Nowadays, many shops and pharmacies sell food supplements based on isoflavones, which presume to improve menopausal health. As these nutritional products are not registered as medicines, they are only subjected to short-term evaluation, mainly to assure safety. However, a lot of controversy exists on the clinical effects of isoflavones, especially on the long-term, concerning relief of menopausal disorders and disease prevention. Furthermore, the targets at the molecular level, by which isoflavones mediate their health effects are not well known, as many different properties of isoflavones have been described. Therefore, our objective was to deal with this issue and characterize the molecular effects of soy isoflavones, specifically on inflammatory systems, as inflammation plays a key role in many diseases and is also tightly connected with the aging process (‘inflamm-aging’). One of the cytokines involved, IL-6, has many functions in inflammatory and immune processes; so, its deregulation is correlated with a range of illnesses and disorders, from osteoporosis to Alzheimer disease and cancers. The signal-activated transcription factor NF-κB, among other regulators, controls pro-inflammatory gene expression and IL-6 transcription. In addition, many genes implicated in triggering the immune response and regulating inflammation are NF-κB-dependent.

The first goal was to investigate by which mechanisms and intracellular targets isoflavones affect NF-κB-dependent inflammatory gene expression and regulate IL-6 in particular. For this part, L929sA fibroblast cancer cells were used as a model system, as our lab has previously described the signal pathways, mediating TNF-α-induced IL-6 gene transcription. Related herewith, we wanted to characterize which activities of these isoflavone molecules are required for the observed effects. Ideally, this would allow
production and use of designer molecules, with the essential structure to effectiveness. Another interesting issue relates to the potential specificity of isoflavones, in a cell type- and gene-dependent way. Therefore, breast cancer cell lines and other NF-κB-driven genes, besides IL-6, were investigated. In regard to the use of estrogens in HRT, it would also be interesting to analyse at what point isoflavones differ from classical estrogens. To deal with this, we made use of ER-dependent and -independent breast cancer cell lines.

Additionally, in view of the systemic spread of isoflavones after oral (soy) intake, the second objective of this study was to explore whether or not genistein, as prototype isoflavone, harbors cell- or stimulus-specific effects and mechanisms. To compare with fibroblast cells, we used a functionally distinct cell type, namely primary monocyte-derived DCs, which have an important regulatory role in the immune system. Based on the observed effects on this physiological cell system, we might be able to draw conclusions about the impact of isoflavones on DC cell function and immune-regulated processes. Therefore, we not only studied the molecular mechanism of genistein on IL-6 transcription, but read out the effects of this molecule on other cytokines and molecular maturation markers of DCs as well.
Results & Discussion
III. RESULTS

In this part, we will first handle the results obtained in the study of soy isoflavone effects on NF-κB-dependent gene expression in TNF-α-stimulated fibrosarcoma cells. The signal transduction pathways from the TNFR towards NF-κB activation and transactivation have been intensively studied in this cell type. Furthermore, the IL-6 promoter structure and regulation are well known, which allows us to focus on the effects and molecular impact of isoflavones on NF-κB-mediated gene transcription and on IL-6 in particular.

Thereafter, the molecular effects of isoflavones, obtained in this former cell type, will be revisited in a totally different cell system to explore possible cell type specificity and stimulus-dependent effects. Monocyte-derived DCs are considered crucial players in immunity, at the boundaries between the innate and adaptive immune system. Stimulated by PAMPs, such as LPS, these cells mature and excrete cytokines, like IL-6, IL-12, TNF-α, etc. Most induced gene expression depends on the transcription factor NF-κB. We will describe the molecular effects of genistein, a soy isoflavone, on NF-κB-dependent gene expression. Furthermore, as we work with a primary cell type, these observations allow us to discuss the potential of genistein to affect physiological processes.

Part I: Attenuation of MSK1-driven NF-κB-dependent gene expression by soy isoflavones does not require estrogenic activity.

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Due to space restrictions, we apologize for having replaced various original references by more extensive review articles referring to the original work.

Footnote: TNF in part I means TNF-α.
Abstract

We have analyzed in molecular detail how soy isoflavones (genistein, daidzein, and biochanin A) suppress nuclear factor-κB (NF-κB)-driven interleukin-6 (IL-6) expression. In addition to its physiologic immune function as an acute stress cytokine, sustained elevated expression levels of IL-6 promote chronic inflammatory disorders, aging frailty, and tumorigenesis. Our results in estrogen-unresponsive fibroblasts, mitogen- and stress-activated protein kinase (MSK) knockout cells, and estrogen receptor (ER)-deficient breast tumor cells show that phyto-estrogenic isoflavones can selectively block nuclear NF-κB transactivation of specific target genes (in particular IL-6), independently of their estrogenic activity. This occurs via attenuation of mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK) and ERK activity, which further downregulates MSK-dependent NF-κB p65 and histone H3 phosphorylation. As constitutive NF-κB and MSK activity are hallmarks of aggressive metastatic ER-deficient breast cancer, the MSK signaling pathway may become an attractive target for chemotherapy.

Introduction

The inflammatory response is a tightly controlled process that is critically important to homeostasis. The pleiotropic cytokine interleukin-6 (IL-6) affects inflammatory reactions, hematopoiesis, bone metabolism, reproduction (spermatogenesis and menstrual cycle), and aging frailty. In addition to its role in inflammation, IL-6 acts as a paracrine/autocrine growth factor of many tumor cells, which establishes a functional link between both types of affections. IL-6-mediated signaling pathways have been implicated in tumor progression, invasion, motility, and chemoresistance in solid and hematopoietic tumors (renal cell carcinoma, breast, lung, colon, ovarian, and gut cancer and multiple myeloma; reviewed in ref. 1). In this respect, serum IL-6 levels are considered as a diagnostic and prognostic marker for tumor progression. Underscoring the potential value of targeted anti-IL-6 therapy in cancer, anti-IL-6 monoclonal antibodies were found to induce apoptosis and regression of xenografted human prostate cancer cells in a nude mouse model. Similarly, inhibition of IL-6 trans-signaling inhibits tumor progression in colon cancer.

IL-6 is normally expressed at low levels, except during infection, trauma, aging, or other stress conditions. Tumor necrosis factor (TNF)-induced IL-6 gene expression is primarily controlled at the transcriptional level by the transcription factor nuclear factor-κB (NF-κB) and requires in addition to IκB kinase (IKK) activity, activation of the mitogen-activated protein kinase (MAPK)/MSK kinase pathway, which phosphorylates NF-κB p65 and histone H3, to establish a transcription-competent promoter complex (enhanceosome; ref. 2).
After menopause or andropause, IL-6 levels are increasing with age as a consequence of a rapid decline in circulating estrogen or testosterone hormones. This altered regulation may certainly account for several disease-associated inflammatory pathologies, phenotypical changes of advanced age, and accelerated tumorigenesis (1, 3). With the aging of our population, prevention of these types of complaints and maintenance of the important physiologic inflammatory balance has attained paramount importance. Until recently, conventional hormone replacement therapy (HRT) was thought of as a cornerstone in that process (4, 5). The ground for estrogen supplementation following menopause was based on the clinical observations that elderly women without circulating sex steroids had a higher incidence of osteoporotic fractures, coronary heart disease, hot flashes, and mood fluctuations. However, as conventional HRT has recently been associated with an increased incidence of trombosis and breast and endometrial cancer (4, 5), there is a renewed interest in using dietary natural plant estrogens (phyto-estrogens), fueled by observational studies showing a lower incidence of menopausal symptoms, osteoporosis, cardiovascular disease, and breast and endometrial cancers in Asian women who have a diet rich in soy products (6, 7). The isoflavones genistein, daidzein, and biochanin A, which are abundant in soybeans and available as herbal tablets, gained enormous attention as structure-function studies have revealed a stable, strong binding to the estrogen receptor (ER), which raised assumptions ranging from mimicking normal estrogenic actions to competitive inhibitory effects (8). The predominant biological effects of estrogen hormones are mediated through two distinct intracellular receptors, ER\textsubscript{α} and ER\textsubscript{β} (9). The functional interaction or “cross-talk” between the ER and NF-κB has been suggested to play a key role in estrogen prevention of age-related inflammatory pathologies and tumorigenesis in vivo (reviewed in refs. 1, 10, 11). Of special note, loss of ER function has been associated with constitutive NF-κB activity and hyperactive MAPK in response to constitutive secretion of cytokines and growth factors, which culminates in aggressive, metastatic, hormone-resistant cancers.

Besides its ER-dependent activities, several other estrogen-independent properties of isoflavones may contribute to their actions as well, as antiosteoporotic activities by isoflavones were also described in ovariectomized rats. Other features observed include binding to other (nuclear) receptors [estrogen-related receptor, peroxisome proliferator-activated receptor (PPAR), aryl hydrocarbon receptor, etc.], antioxidant effects due to their polyphenolic nature, modulation of detoxification and of steroid metabolism, interference with Ca transport, Na*/K*ATPases, favorable effects on lipid and lipoprotein profiles, inhibition of tyrosine protein kinases, phosphatidylinositol 3-kinase (PI3K)/Akt kinase, topoisomerase II and of cyclic AMP (cAMP)-phosphodiesterase-4 enzymes, and interference with cell cycle...
Results

Phyto-estrogenic isoflavones genistein, daidzein, and biochanin A but not synthetic estrogen 17β-estradiol inhibit endogenous IL-6 gene induction in response to TNF.

Because estrogens can elicit stimulatory as well as repressive effects on NF-κB-dependent gene expression in a cell type- and gene-specific way (11), we measured the effects of various (phyto-)estrogens (i.e., genistein, daidzein, and biochanin A versus 17β-estradiol) on TNF-induced IL-6 gene expression in L929sA mouse fibroblasts. As we and others detect at least 100-fold weaker estrogenic activity of isoflavones than 17β-estradiol to elicit comparable estrogenic activities (refs. 20, 21; data not shown), phyto-estrogen concentrations were increased ~ 100-fold compared with 17β-estradiol to reach a similar ER hormone efficacy in subsequent experiments. Secreted IL-6 protein levels present in the supernatants were quantified by IL-6 ELISA. L929sA cells were treated with TNF alone or in combination with different concentrations of soy isoflavones compared with the synthetic estrogen 17β-estradiol or the reference glucocorticoid hormone dexamethasone (22). In Fig. 1A, strongly elevated levels of IL-6 protein are detected after TNF treatment as expected. Interestingly, whereas low micromolar concentrations of the reference hormone dexamethasone potently inhibit TNF-induced IL-6 gene expression, and phyto-estrogenic isoflavones are weakly repressing, 17β-estradiol hormone seems completely ineffective (Fig. 1A; data not shown). Clearly, a specific and dose-dependent inhibition of IL-6 production can be observed upon cotreatment of the various isoflavones, genistein, daidzein, and biochanin A in the micromolar range (Fig. 1A), whereas high micromolar concentrations of pure dexamethasone or 17β-estradiol were found to be cytotoxic at these supraphysiologic hormone doses (data not shown).

Briefly, although synthetic estrogens may fail to specifically transrepress NF-κB in L929sA fibroblasts in a hormone-dependent way, soy isoflavones can dose-dependently...
repress IL-6 gene expression. These results are confirmed at the mRNA level because IL-6 RTPCR and blot assays reveal a similar dose-dependent (range, 2-200 µmol/L) and specific decrease in IL-6 mRNA levels upon exposure to genistein but not in the presence of 17β-estradiol (Fig. 1B). The lack of estrogen-dependent NF-κB transrepression is remarkable and will be discussed further throughout the results, as ERα and ERβ mRNA can both be detected in L929sA fibroblasts (Fig. 1B).

Figure 1. Regulation of NF-κB-driven gene expression by soy isoflavones.
A, L929sA cells were pretreated for 2 hours with reference hormone compounds dexamethasone (2 µmol/L) or 17β-estradiol (2 and 20 µmol/L), or various doses of the soy isoflavones genistein, daidzein, biochanin A (200, 100, 50, and 20 µmol/L) followed by 6 hours of treatment with 2,000 IU/mL TNF. Corresponding levels of secreted IL-6 protein were quantified by mIL-6 ELISA.
B, L929sA cells were pretreated for 2 hours with genistein (200 µmol/L) or 17β-estradiol (2 µmol/L) followed by 6 hours of treatment with 2,000 IU/mL TNF. Total RNA was isolated and semiquantitative RT-PCR was done with mouse-specific IL-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer sets. Alternatively, dose response of genistein (micromolar concentrations as indicated) on TNF-induced IL-6 mRNA levels was revealed by dot blot analysis upon mIL-6 cDNA probe hybridization to total RNA of various treatments, spotted as a 1:3 dilution series on a Hybond
nylon filter. Presence of ERα/β mRNA was revealed by RT-PCR on total RNA of L929sA cells with isoform-specific mouse primers, negative RT-PCR control without cDNA was done in the same experiment.

C, L929sA fibroblasts were stably transfected with reporter gene plasmids controlled by the natural IL-6 promoter (p1168hu.IL-6P-luc+), a synthetic NF-κB-responsive promoter [p(IL-6-κB)350hu.IL-6P-luc+] or the constitutive phosho-glycerokinase promoter (pPGKβgeobpA), referred to as IL-6, 3xFN-κB, and PGK, respectively. L929sA transfectants were pretreated for 2 hours with genistein, daidzein, or biochanin A (200 µmol/L) followed by 6 hours of treatment with 2,000 IU/mL TNF upon which lysates were prepared for quantification of luciferase and/or galactosidase reporter gene levels. After normalization as described previously (17), the induction factor is defined as the amount of luciferase produced in treated cells compared with untreated cells (the latter is arbitrarily set to 1).

D, L929sA cells, stably transfected with the synthetic NF-κB-responsive promoter construct p(IL-6-κB)350hu.IL-6P-luc+, were pretreated for 2 hours with either genistein (200 µmol/L), 17β-estradiol (1 µmol/L), OH-tamoxifen (1 µmol/L), ICI182780 (1 µmol/L), genistein+ICI182780, ciglitazone (30 µmol/L), tyrphostin A23 (50 µmol/L), rolipram (200 µmol/L), NAC (30 mmol/L), BHA (200 µmol/L), wortmannin (100 nmol/L), or LY294002 (20 µmol/L) followed by 6 hours of treatment with 2,000 IU/mL TNF, upon which lysates were prepared for quantification of luciferase levels. Corresponding luciferase expression levels are represented as bar graphs.

**Isoflavones genistein, daidzein, and biochanin A inhibit NF-κB-driven reporter gene expression in response to TNF.**

To verify whether IL-6 gene repression by isoflavones is reflected at the transcriptional level, various promoter reporter gene constructs [i.e., p1168hu.IL-6P-luc+ or p(IL-6-κB)350hu.IL-6P-luc+, and pPGKβGeobpA], containing, respectively, the natural IL-6 promoter, a synthetic promoter with multimerized NF-κB-responsive elements and the housekeeping promoter phosphoglycerate kinase (PGK), were stably transfected into L929sA cells (or Sertoli TM4 as indicated in figure legends). The resulting stable cell pools were identically treated, and the lysates were assayed for corresponding reporter gene activity (Fig. 1C-D; Supplementary Fig. S1). Enhanced luciferase expression levels were measured in response to TNF, whereas cotreatment with various phyto-estrogens consistently decreased IL-6 promoter activity, and more specifically, NF-κB-driven reporter gene activity, thus mimicking endogenous IL-6 gene regulation. The promoter specificity of the observed regulatory effects is further shown by the housekeeping promoter PGK, which remained unaffected by the different stimulating agents used. In line with our results obtained in Fig. 1A, 17β-estradiol does not inhibit NF-κB-driven gene expression in L929sA fibroblasts, although dexamethasone is strongly repressing NF-κB at the same hormone concentrations. Interestingly, in Sertoli TM4 cells, significant transrepression can be observed with both estrogen and glucocorticoid hormones, pointing to cell type-dependent regulation of ER transrepression (Supplementary Fig. S1). Upon Western blot analysis of
ERα/β expression levels in both cell types, it becomes clear that lack of ER-dependent NF-κB effects in L929sA fibroblasts may originate from the limiting trace amounts of ERα/β present compared with the corresponding protein levels observed in TM4 (Supplementary Fig. S1). In contrast, soy isoflavones can potently inhibit NF-κB-dependent reporter gene activity in both cell types (Fig. 1C; data not shown).

To further evaluate if genistein-dependent NF-κB repression is a superposition of multiple effects, ranging from hormone-like activities (ERα/β and PPARγ), antioxidant properties, inhibition of tyrosine kinases, of PI3K/Akt kinases, and/or of phosphodiesterases (PDE4; see Introduction), we have measured NF-κB effects in presence of a panel of reference compounds related to these activities. Inhibitor doses presented in Fig. 1D reflect optimized doses that give specific effects (but leave PGK housekeeping promoter activity unaffected). Upon further exploring hormone-like activities, no significant NF-κB repression could be measured in the presence of an ER agonist (17β-estradiol), a SERM with mixed agonist/antagonist properties (OH-tamoxifen), or a PPARγ agonist (ciglitazone). In addition, an ER antagonist (ICI182780) was not able to reverse genistein-dependent NF-κB repression.

Furthermore, as wortmannin, LY294002, and rolipram also fail to potently repress NF-κB at optimal doses tested, PI3K and PDE4 are presumably no major players involved in NF-κB-driven gene expression in mouse fibroblasts either. In contrast, the tyrosine kinase inhibitor tyrphostin A23 and the antioxidants NAC and BHA are able to potently inhibit NF-κB-driven reporter gene expression to the same extent as genistein. Whether the antioxidant and tyrosine kinase inhibitor properties of genistein may mimic tyrphostin and antioxidant effects on NF-κB by a common repression mechanism, will be addressed in further experiments.

**Isoflavones inhibit NF-κB-driven gene expression by attenuation of the ERK-MAPK/MSK1 cascade.**

As NF-κB-driven gene expression requires a coordinated interplay of IKK activation and MAPK (MSK)-dependent transactivation mechanisms (2, 23-26), we evaluated to which extent genistein-dependent IL-6 gene repression relies on inhibition of either pathway in L929 mouse fibroblasts. The first control level concerns the cytoplasmic regulatory event, in which NF-κB is released from its physiologic inhibitor IκB upon its degradation. The second level affects MAPK signaling-dependent enhanceosome dynamics in relation to the surrounding chromatin environment.
In this respect, L929sA cells were treated with TNF alone or in combination with genistein for the indicated time points and cell extracts were subsequently analyzed by Western blot for protein expression levels of IκB, or the activation status of various kinases (IKKα/β, p38, ERK, MEK1, and MSK) by use of phospho-specific antibodies. Furthermore, complementary to IκB Western analysis, release of NF-κB followed by nuclear DNA binding is revealed by EMSA with a NF-κB-specific oligonucleotide probe. From Fig. 2A, it seems that in mouse fibroblasts, isoflavones do not affect NF-κB activation by the IKK pathway, as the P-IKK activation pattern, and IκB degradation and resynthesis kinetics upon TNF stimulation are similar in presence or absence of genistein. These results are completely in line with the EMSA data, which reveal induced NF-κB p50-p65/DNA-binding upon TNF stimulation that remains unaffected in presence of genistein (Fig. 2B). The amount of constitutively binding factor RBP-Jκ remained unchanged under the various conditions evaluated. Characterization of the various inducible and constitutive transcription factor binding complexes at the IL-6 NF-κB site has previously been shown by extensive supershift analysis and binding competition experiments (17, 27). Interestingly, in contrast to results obtained in L929sA, we and others found significant suppression of NF-κB/DNA binding in other cell types pointing to cell specific thresholds for IKK inhibition in response to anti-inflammatory phyto-chemicals (data not shown).

However, although genistein effects are absent in cytoplasmic NF-κB activation and DNA binding in L929sA cells, important effects can be observed on the NF-κB transactivation pathways. We previously established that the TNF-activated p38 and ERK MAPK pathways contribute to the transactivation capacity of NF-κB (17), by driving MSK1-mediated phosphorylation of p65 Ser276 (2). We now found that genistein attenuates TNF-induced ERK activation, whereas p38 activation remains unaffected (Fig. 2C). Consequently, MSK1 activation is hampered upon cotreatment of TNF and genistein in a time-dependent fashion (Fig. 2D and E). Upstream of ERK MAPK, we also observe inhibition of MEK1 activation by genistein, which may contribute to the reduction in ERK activity (Fig. 2D). Of particular interest is the observation that the MAPK/MSK pathways are also decreased by isoflavones in ERα/β-deficient SKBR3 breast cancer cells (Fig. 2D), which again suggests that attenuation of MSK1 activation by phyto-estrogens does not require classic estrogen (receptor) activity (Fig. 2D). This is further corroborated by the observation that in analogy to genistein, P-MSK levels can be reduced with antioxidant compounds or tyrphostins (i.e., NAC and A23; Fig. 2G), in line with the reporter gene results (Fig. 1D). Finally, upon further testing different doses of the most potent isoflavones, significant repression of MSK1 activation could be measured with a minimal dose of 12.5 µmol/L biochanin A and 50 µmol/L
Results
genistein (Fig. 2F), suggesting subtle differences in structure requirements to elicit inhibition of MSK signaling pathways, this being in line with the different immunosuppressive potencies of biochanin A > genistein > daidzein isoflavones.

Figure 2. Effect of soy isoflavones on NF-κB/DNA binding and MAPK-MSK activation.
A, L929sA cells were either or not pretreated for 2 hours with genistein followed by treatment with 2,000 IU/mL TNF for the indicated times. Total cell lysates were analyzed for P-IKKα/β and IκBα levels by Western blot analysis.
Results

B, L929sA cells were either or not pretreated for 2 hours with genistein followed by 30 min of treatment with 2,000 IU/mL TNF. Nuclear cell lysates were incubated with a \(^{32}\)P-labeled IL-6 κB site-containing probe. Binding complexes formed were analyzed by EMSA. Loading of equal amounts of protein was verified by comparison with the binding activity of the repressor molecule RBP-Jκ (27).

C to G, Serum-starved L929sA fibroblasts or D, SKBR3 (ERα/β) breast cancer cells, were either or not pretreated for 2 hours with 200 µmol/L genistein or biochanin A, 2 µmol/L 17β-estradiol, 50 µmol/L A23, or 30 mmol/L NAC followed by treatment with 2,000 IU/mL TNF for the indicated times. Cell lysates were analyzed for P-p38 and P-ERK MAPK, P-MEK1/2, and P-MSK1 by phospho-specific Western blot analysis. As a control for equal protein loading, blots were developed against constitutive p38, ERK kinase levels.

E, Similarly, P-MSK levels, detected in a prolonged time kinetics experiment of L929sA cells exposed to TNF alone or in combination with genistein, were revealed by Western blot analysis and quantified by Image J software (open source Image J software available at http://rsb.info.nih.gov/ij/). Signal intensities are plotted in function of time and corrected for protein loading by normalisation with the constitutive MSK signal.

F, Along the same line, P-MSK Western signals detected after 15 min TNF treatment, in the absence or presence of different doses of genistein or biochanin A, were again quantified by Image J software and normalized for loaded protein levels.

G, Similar experiments were done in presence of 2 µmol/L 17β-estradiol, 30 mmol/L NAC, or 50 µmol/L A23 followed by treatment with 2000 IU/mL TNF for the indicated times in serum-starved L929sA fibroblasts. Cell lysates were again analyzed for P-MSK1 by phospho-specific Western blot analysis. As a control for equal protein loading, blots were developed against p65 (data not shown).

**Isoflavones decrease NF-κB p65 transactivation by interfering with MSK1 kinase activity and histone acetyltransferase/histone deacetylase cofactor activities.**

Further proof for soy isoflavones in targeting NF-κB transactivation via the MSK1 pathway comes from an immunoprecipitation MSK kinase assay and the Gal4 one hybrid technique. Effects of TNF and/or genistein on endogenous MSK1 activity can be measured by a kinase assay of MSK1 immunoprecipitates from cell lysates (i.e., of cells treated with TNF alone, or in combination with genistein or 17β-estradiol). The Gal4 one-hybrid system is a read-out assay for NF-κB p65 transactivation activity, independent of IκB. Before, we have established the crucial link between MSK-dependent NF-κB p65 S276 phosphorylation and TNF-induced pGal4-p65\(^{1-551}\) transactivation driving the reporter gene construct p(GAL4)\(^{2-50hu.IL-6-luc+}\) (2). Upon comparison of MSK1 kinase activity (Fig. 3A) and Gal4-p65 transactivation potency (Fig. 3B), a tight correlation in responses can be observed in line with previous findings (2). TNF treatment clearly up-regulates MSK1 kinase activity and p65 transactivation, which can be completely abrogated in presence of the MAPK inhibitor cocktail SB205380 and U0126. As genistein only blocks ERK, but not p38 pathways (Fig. 2C and D), only partial reduction of MSK kinase activity can be observed (Fig. 2D and E), but to a similar extent as treatment with the ERK inhibitor U0126 alone, whereas 17β-estradiol is
Results

completely ineffective (Fig. 3A). These results perfectly mirror the pattern obtained in the Gal4-p65 transactivation results.

Figure 3. Soy isoflavones affect MSK activity, NF-κB transactivation, and cofactor activity.

A, L929sA cells were starved for 48 hours in serum-free medium, and cells were left untreated or were pretreated for 2 hours with the MAPK inhibitors SB203580 (10 µmol/L) and/or U0126 (10 µmol/L), genistein (200 µmol/L), or 17β-estradiol (2 µmol/L) followed by 30 min of treatment with 2,000 IU/mL TNF. Cells were lysed, and endogenous MSK1 was isolated by immunoprecipitation. The corresponding activity of MSK1 was assessed by an in vitro kinase assay on p65-S276-peptide.

B, Pools of L929sA cells stably expressing Gal4-p65 were transiently transfected with p(Gal4)2-50hu.IL-6-luc+. At 48 hours after transfection, cells were left untreated or were pretreated for 2 hours with the MAPK inhibitors SB203580 (10 µmol/L) and/or U0126 (10 µmol/L), genistein (200 µmol/L), or 17β-estradiol (2 µmol/L) followed by 6 hours of treatment with 2,000 IU/mL TNF. After normalization, the corresponding induction factors are represented as bar graphs.

C, HEK293T cells were transiently transfected with a combination of expression plasmids (i.e., 300 ng p1168hu.IL-6-luc+, 20 ng pPGK(lgeobpA, 20 ng pRcRSVp65, and/or 80 ng CBP expression plasmid). The total amount of DNA was kept constant in all setups by supplementing empty vector DNA. Cells were either or not treated for 16 hours with genistein (200 µmol/l), starting at 32 hours after
transfection, and all transfected setups were lysed at time point 48 hours. Corresponding luciferase expression levels in lysates are represented as bar graphs and have been normalized for protein concentrations and transfection efficiency.

D, L929sA were stably transfected with a reporter gene plasmid controlled by the natural IL-6 promoter (p1168hu.IL-6P-luc+). Transfectants were untreated or pretreated for 2 hours with genistein (200 µmol/L) followed by 6 hours of treatment with 2,000 IU/mL TNF, trichostatin A (TSA; 100 nmol/L), or both together. Corresponding normalized induction factors are represented as bar graphs.

Another aspect of nuclear regulation of NF-κB activity relies on its interactions with chromatin-modifying cofactor complexes (23, 28-30). Transcription factors mediate gene activation or repression via recruitment of “tandem” cofactor complexes of CBP/p300, histone acetyltransferases (HAT), and RSK2/MSK1 kinases (31, 32), or histone deacetylases (HDAC) and phosphatases PP1, respectively (33). Previously, we have shown the crucial role of the NF-κB p65 subunit in engaging CBP/p300 and HAT activity for transcriptional activation of the IL-6 promoter (18), which strongly depends on MSK-dependent p65 Ser276 phosphorylation (2). In this respect, we have investigated the ability of CBP to synergize with NF-κB p65 in absence or presence of genistein by means of transient cofactor transfection assays or reporter gene experiments with stable chromatinized promoters in presence of the HDAC inhibitor trichostatin A. Subsaturating amounts of expression plasmids of p65 and/or CBP were transiently transfected to HEK293T cells together with the IL-6 reporter gene construct p1168hu.IL-6P-luc+. As is clear from Fig. 3C, NF-κB-dependent gene expression is strongly increased if CBP is coexpressed with p65 but reversed upon prior addition of genistein. Expression levels of p65 or CBP remained unaffected under the tested conditions as confirmed by Western analysis (data not shown). In a more physiologic approach with trichostatin A on a chromatinized IL-6 promoter, trichostatin A augments NF-κB-driven but not basal gene expression in response to TNF, as expected (ref. 18; Fig. 3D). Interestingly, genistein is able to completely counteract this synergy. These results indicate that genistein is able to block NF-κB-triggered CBP/p300 HAT activity by inhibiting MSK1-dependent phosphorylation of NF-κB p65 S276 (2). Similar results were obtained with other isoflavones (data not shown).

Isoflavones interfere with MSK1 recruitment and consequent factor phosphorylation and chromatin dynamics at IL-6 gene promoters.

Considering that genistein attenuates the TNF-activated MSK1 pathway and that MSK1 kinase substrates include transcription factors (i.e., NF-κB p65 Ser276 and cAMP-responsive element binding protein Ser133) as well as chromatin protein histone H3 Ser10
(2, 34), we have investigated isoflavone effects on NF-κB or H3 phosphorylation by phospho-specific Western analysis. Interestingly, in serum-starved L929sA fibroblasts treated with genistein, a significant reduction of basal and TNF-induced NF-κB Ser276 and H3 Ser10 phosphorylation can be observed (Fig. 4A and B). A similar reduction can be detected with biochanin A and to a lesser extent daidzein but not with 17β-estradiol (Fig. 4B; data not shown). Total cellular levels of NF-κB p65 and H3 Lys9 dimethylation remained unaffected under the various conditions tested, pointing to the specificity of the isoflavone effect.

**Figure 4. Soy isoflavones strongly reduce MSK1-effects at the IL-6 gene promoter.**
A to B, L929sA cells were serum starved and left untreated or pretreated for 2 hours with genistein, daidzein, biochanin A (200 µmol/L), or 17β-estradiol (2 µmol/L) followed by 30 min of treatment with 2,000 IU/mL TNF. P-NF-κB Ser276 (A) and P-histone H3 (B) levels are revealed by phospho-specific Western analysis. Equal protein loading is shown by Western detection of corresponding NF-κB p65 or methylated histone H3 levels.

C to D, Serum-starved L929sA mouse fibroblasts were treated for 30 min with 2,000 IU/mL TNF alone, or following 2 hours of pretreatment with the inhibitors SB203580 + PD98059 (10 µmol/L) or H89 (10 µmol/L) or genistein (200 µmol/L).
immunoprecipitation analysis was done against phosphorylated (S10) or phospho-acetylated (K9-S10) histone H3 (C) or MSK1 (D). After reversal of cross-linking, coimmunoprecipitated genomic DNA fragments were analyzed by quantitative PCR for 27 cycles with IL-6 or H4 promoter-specific primer sets and is revealed by gel electrophoresis. Input reflects the relative amounts of sonicated DNA fragments present before immunoprecipitation and revealed by quantitative PCR with either IL-6- or H4-specific primers.

To further confirm our results on an IL-6 gene-specific basis, we did chromatin immunoprecipitation against various histone H3 modifications and/or MSK1 at the level of the IL-6 gene promoter. As chromatin immunoprecipitation grade P-NF-κB S276 antibodies are currently not available, we were unable to include this setup in our further chromatin immunoprecipitation experiments, although a similar pattern can be expected as with the P-H3 S10 chromatin immunoprecipitations. More particularly, we found increased phospho(acetylated) H3 levels at the endogenous IL-6 promoter following TNF stimulation, but these effects are completely lost in the presence of genistein (Fig. 4C). The complete inhibition of histone phospho(acetyl) modifications is remarkable because MSK1 kinase activity could only be partially inhibited by isoflavones (Fig. 3A). However, upon investigating MSK1 recruitment at the IL-6 promoter under the same conditions, we did observe a complete loss of TNF-induced MSK1 recruitment at the IL-6 promoter, explaining complete disappearance of histone H3 (phospho)acetylation in the presence of genistein, comparable with treatment with the MAPK inhibitor cocktail SB205380+PD98059 (Fig. 4D). Similar results were obtained with other isoflavones (Supplementary Fig. S2). This suggests that genistein may have cumulative effects on MSK-driven gene expression by interfering with MSK1 activation and kinase activity, as well as with MSK recruitment on chromatinized promoters. As the spatial context in which MAPK/MSK kinases operate in transcription complexes is poorly understood, future studies of subcellular dynamics of MAPK/MSK regulation will be of high interest (35).

**Isoflavones selectively inhibit NF-κB-dependent gene expression.**

To obtain final proof for involvement of the MSK pathway in isoflavone effects on NF-κB-driven gene expression, we compared gene expression patterns of TNF-treated wild-type and MSK1/2 double-knockout mouse embryonal fibroblasts (2, 36, 37), in comparison with TNF- and TNF+genistein-treated L929sA fibroblast cells (Fig. 5). Upon analysis of various NF-κB superarray filter hybridizations, a remarkable similarity in gene expression regulation can be observed between IL-6 or VCAM mRNA expression, affected by genistein treatment (Fig. 5B and D), or by knocking out MSK1/2 (Fig. 5A and C). In contrast, the target gene
NFKB2 that is also strongly induced by TNF seems to be insensitive to genistein treatment and in parallel to genetic disruption of MSK1/2. Finally, 17\(\beta\)-estradiol treatment has only minor effects on NF-\(\kappa\)B gene expression in fibroblasts in agreement with our results discussed above.

Figure 5. Similarities in NF-\(\kappa\)B-driven gene expression pattern in MSK KO (A and C) and genistein-treated cells (B and D).

Serum-starved MSK1\(^{-/-}\) MSK2\(^{-/-}\) and wild-type MEF cells were treated with 2,000 IU/mL TNF for 4 hours (A and C). Alternatively, serum-starved L929sA cells were treated for 4 hours with 2,000 IU/mL TNF alone, or following a 2-hour pretreatment with genistein (200 \(\mu\)mol/L) or 17\(\beta\)-estradiol (2 \(\mu\)mol/L; B and D). Total RNA was isolated and analyzed using NF-\(\kappa\)B GEArray technology according to the manufacturer’s instructions. Specific mRNA expression was normalized for loading differences with housekeeping gene signals. Spot intensities of IL-6, VCAM1, and NFKB2 are marked in the array figures (A and B), and the corresponding signal intensities quantified by phosphor-imager analysis are represented as bar graphs (C and D).

Genistein interferes with MSK1 signaling in ER\(\alpha\)-deficient breast cancer cells.

Because soybean-derived isoflavones have been associated with reduced rates of breast cancer (1) and are recommended as HRT alternative for people with increased breast cancer risk or breast cancer survivors (5, 38), we next investigated whether isoflavones may
have similar effects in a breast cancer model. Upon reinvestigation of raw array data sets of breast cancer patients among which 44 with good and 34 with worse prognosis signature (19), a remarkable increase can be observed in MSK1, IL-6, and IL-8 but not in NFKB2 gene expression levels in the worse prognosis population. Remarkably, these data coincide with loss of ERα (Supplementary Fig. S3) and are in line with previous reports that classify IL-6 and IL-8 as metastasis-promoting cytokines in advanced breast cancer (1). Similarly, we and others have observed a strong increase in IL-6 gene expression in the invasive metastatic breast cancer cells MDA-MB231 (ERα/β⁺) compared with non-invasive MCF7 (ERα⁺/β⁺) cells (Fig. 6A; refs. 39, 40).
Results

Figure 6. Genistein affects MSK-dependent IL-6 gene expression in ERα-negative breast cancer cells.

A, To compare IL-6 gene expression levels in MCF7 and MDA-MB231 cells, both cell types were left untreated or TNF-treated (2,000 IU/mL) for 6 hours. Corresponding levels of secreted IL-6 protein were quantified by hIL-6 ELISA.

B and C, Similarly, MCF7 and MDA-MB231 cells were left untreated or TNF-treated (2,000 IU/mL) for 30 min, and nuclear lysates were analyzed for NF-κB/DNA-binding activity (B) and P-MSK levels (C) by means of EMSA or Western analysis, respectively. For the latter, equal loading was detected by an anti-actin antibody.

D, In addition, the constitutive occurrence of NF-κB and MSK1 in untreated MDA-MB231 or MCF7 cells were revealed by chromatin immunoprecipitation analysis against NF-κB p65 and MSK1 at the endogenous IL-6 gene promoter.

E, Finally, we measured the effects of 200 µmol/L genistein on TNF-stimulated H3 phosphorylation and MSK recruitment on the IL-6 gene promoter by ChIP analysis in serum-starved MDA-MB231 cells, treated with 2,000 IU/mL TNF for 30 min, either or not after a 2-hour pretreatment with genistein (200 µmol/L).

F, To determine genistein effects at IL-6 mRNA gene expression in MDA-MB231, cells were TNF treated with 2,000 IU/mL for 4 hours either or not following a 2-hour pretreatment with genistein (200 µmol/L) or 17β-estradiol (2 µmol/L). Total RNA was isolated and analyzed using GEArray technology according to the manufacturer’s instructions. Specific mRNA expression was normalized for loading differences, quantified by phosphor-imager, and represented as bar graphs according to hybridization intensity.

Besides a very strong TNF-induced NF-κB activation in MDA-MB231 cells compared with MCF7 cells (Fig. 6B), we do also observe a constitutive MSK phosphorylation, NF-κB/DNA-binding activity (Fig. 6B and C), and occupation of the IL-6 gene promoter by p65 and MSK1 in the basal state in MDA-MB231, as revealed by IL-6 promoter-specific chromatin immunoprecipitation analysis (Fig. 6D). Finally, in line with our results obtained in fibroblasts, genistein is able to significantly reduce IL-6 mRNA gene expression levels, MSK1 recruitment, and H3 phosphorylation at the IL-6 promoter in the metastatic MDA-MB231 cells (Fig. 6E and F). This suggests that soy isoflavones may protect against aggressive breast cancer progression by attenuation of MSK1 activities, irrespective of the cellular ER status.

Discussion

The isoflavones genistein, daidzein, and biochanin A, which are abundant in soybeans and widely available as herbal tablets, are especially popular among postmenopausal women. Only lately, many studies have been undertaken to unveil the mode by which phyto-estrogens mediate their NF-κB-suppressive effects. Our results in estrogen-unresponsive fibroblasts, MSK knockout cells, and ER-deficient breast tumor cells show that phyto-estrogenic isoflavones but not 17β-estradiol selectively block nuclear NF-κB
transactivation of particular NF-κB target genes, including IL-6. This occurs via attenuation of MEK1 and ERK activity, which slows down MSK1-dependent NF-κB p65 and histone H3 phosphorylation.

Glucocorticoids are able but 17β-estradiol fails to inhibit NF-κB-driven IL-6 gene expression in L929sA fibroblasts at the same hormone concentrations. Along the same line, glucocorticoids but not 17β-estradiol are able to stimulate GR- or ER-driven reporter gene activity, respectively, in L929sA cells. This suggests that L929sA mouse fibroblasts may lack functional ER, which is required to mediate NF-κB cross-talk. Nowadays, constitutive growth factor signaling and kinase cascades, as well as elevated threshold levels of NF-κB, have all been shown to drastically affect ER functionality by interfering with its ligand sensitivity, localization, and turnover rate (11). As ERα/β mRNA transcription can clearly be detected by RT-PCR in L929sA fibroblasts (Fig. 1B), but corresponding protein levels are at the detection limit (Supplementary Fig. S1), we assume that high turnover rates of ERα/β in these cells may prevent dose-dependent responses (either transactivation or transrepression) by agonists, antagonists, or mixed SERMS, as can also be observed in other cell types, that result in an estrogen-resistant cell phenotype (5). In this respect, the isoflavone-dependent IL-6 gene inhibition in L929sA mouse fibroblasts is most probably independent of classic estrogenic properties. This hypothesis is further strengthened by the observation that the ER antagonist ICI182780 is unable to modulate NF-κB-dependent gene expression or reverse the isoflavone effects. Although PPARγ too has been proposed as a hormone receptor for soy isoflavones (41), we found no evidence for PPARγ involvement in mediating isoflavone effects because IL-6 promoter activity could not be significantly downregulated by ciglitazone. Interestingly, comparison of other possible properties of isoflavones (12) with small-molecule inhibitors as reference compounds rather suggests potential involvement of the tyrosine kinase inhibitory and/or antioxidant activities by genistein because A23 or NAC were found to inhibit NF-κB-dependent gene expression to a similar extent as genistein and as such may affect similar regulatory targets.

Further experiments clearly established that isoflavones rather attenuate the NF-κB transactivation potency than cytoplasmic NF-κB activation by blocking the ERK/MSK pathway, as shown in estrogen-unresponsive fibroblasts and in ERα/β-deficient SKBR3 breast cancer cells. In analogy to genistein, the tyrosine kinase inhibitor A23 and antioxidant NAC were also found to inhibit the ERK and MSK1 pathway. Various receptor tyrosine kinases with an intrinsic, ligand-dependent tyrosine kinase activity stimulate the Ras/Raf-MEK-ERK pathway, which controls fundamental cellular processes, including proliferation, differentiation, survival, and NF-κB transactivation (42, 43). Furthermore, TNF has also been
shown to activate various non-receptor tyrosine kinases (i.e., c-Src, lyn, Pyk2, Eth/Bm, and Syk; refs. 43, 44). In addition, previous studies in L929 cells have also revealed that formation of reactive oxygen intermediates is essential for the IL-6 gene-inductive effects of TNF (45), whereas depletion of mitochondrial oxidative metabolism results in inhibition of IL-6 gene induction by TNF. Interestingly, the mitochondrial antioxidant system controlling the cellular redox balance is sensitive to growth factor tyrosine kinase activity (46). Altogether, MSK1 may integrate upstream tyrosine kinase and ROS activities via the MAPK signaling cascades, leading towards IL-6 gene induction, which itself may thus be sensitive to the inhibitory effects of soy isoflavones at multiple levels.

Although it has been proposed that antioxidants may inhibit TNF-induced NF-κB activation by lowering the affinity of TNF for its receptor resulting in a general reduction in magnitude of all TNF signaling events (47), this can definitely not be generalized for all kind of antioxidants in every cell type. More particularly, genistein was found to only affect ERK/MSK1 pathways, leaving TNF-induced p38 and IKK activity unaffected. Furthermore, genistein only inhibits particular NF-κB target genes, such as IL-6 and VCAM, whereas other TNF-responsive genes remain unaffected (such as NFKB2). Alternatively, depending on cellular redox changes in the cell, distinct redox forms of TNFRI could be identified with distinct ligand binding, clustering, and signaling ability (48).

At the level of the IL-6 promoter enhanceosome, it seems that genistein totally blocks recruitment of the histone H3 kinase MSK1, coinciding with a complete loss of H3 phosphorylation and acetylation, although MSK activity itself is only partially inhibited. This suggests that genistein may have cumulative effects on MAPK/MSK-driven gene expression by interfering with MSK kinase activity as well as with its recruitment on chromatinized promoters. Of particular interest, RSK (and in many cases also MSK) interactions with the acetylase CBP, the phosphatase PP2C, or 14-3-3β proteins have been found to regulate its subcellular localization and restrict its activities in time and space (31, 49–51). Further experiments will be required to unravel genistein effects on spatiotemporal dynamics of MSK(RSK)-cofactor complexes in relation to selective NF-κB-dependent gene expression.

Because isoflavone-rich soy diets have been associated with reduced rates of breast cancer (1), we also investigated genistein effects in a breast cancer model. Publically available array data sets from breast cancer patients with good or worse prognosis (19) already illustrate a significant increase in IL-6 and IL8 but not NFKB2 gene expression coinciding with higher MSK expression levels and loss of ERα in breast cancer patients with bad prognosis signature (Supplementary Fig. S3). Along the same line, we observed strongly elevated (but still TNF-inducible) NF-κB and MSK1 activity coinciding with elevated IL-6 gene
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expression in the aggressive metastatic breast tumor cells MDA-MB231, lacking ERα, which could still be reversed in presence of genistein. The fact that in breast cancer cells, IL-6 gene expression has been associated with mitogenic, angiogenic, metastatic, and invasive responses, whereas phyto-estrogens have been found to inhibit these activities may offer interesting therapeutic opportunities for isoflavone compounds. Indeed, as constitutive NF-κB and MSK activity are hallmarks of aggressive metastatic ER-deficient breast cancer, the MSK pathway may be a relevant therapeutic target for soy phyto-estrogens, in case classic hormone therapy fails.

Of special note, genistein plasma concentrations of 3.4 µmol/L in mice or 10 to 25 µmol/L among humans were measured upon oral feeding of genistein (52), which could be considered as a physiologic isoflavone concentration range. Because we observe weak inhibition of endogenous IL-6 gene expression in presence of 10 to 50 µmol/L soy isoflavones in L929sA fibroblasts (Fig. 1A and B) and significant inhibition of P-MSK levels with the most potent soy isoflavone biochanin at 12.5 µmol/L, or mild isoflavone genistein around 50 µmol/L, the experimental concentration range applied is within reach of a physiologic frame. In a real life diet, responses to physiologic-dose phyto-chemicals might reveal synergistic effects, in combination with other classes of functional foods (53). To limit excessive IL-6 production to a health-beneficial concentration range, a partial decrease of MSK activity at physiologic isoflavone concentrations may be sufficient to elicit chemopreventive effects, whereas a complete block of IL-6 gene expression at elevated isoflavone concentrations may be detrimental for immune homeostasis.

Furthermore, long-term (time frame of various months or years) chemopreventive effects, upon daily exposure to an isoflavone-rich diet, may be difficult to mimic in short-term (timeframe of a few hours) tissue culture experiments at similar concentrations and may require higher doses of individual compounds to reveal molecular black and white effects in the latter case. Further pharmacologic studies are required with respect to bioavailability and metabolism of soy isoflavones to determine health beneficial doses at short-term/long-term periods, which may interfere with pathologic MSK1 activity in various cell types in vivo. Finally, chemopreventive (daily diet) or chemotherapeutic use (medicinal application to hormone-resistant tumors) of isoflavone preparations may require distinct dose ranges to fine-tune MSK activities.

In summary, our results show that soy phyto-estrogens in contrast to 17β-estradiol, can counteract MSK-dependent NF-κB transactivation on specific NF-κB target genes in estrogen-unresponsive fibroblasts and ER-defective breast tumor cells, presumably via inhibition of tyrosine kinases or by their antioxidant capacity. Structure/function analysis of
different isoflavone metabolites could reveal minimal core structure domains involved in its antioxidant, tyrosine kinase inhibitor, or hormone ligand properties. This may finally allow metabolic engineering of superior isoflavone drugs as “selective MSK pathway modulators” with therapeutic benefit in chronic inflammatory disorders, longevity, and/or cancer biology.

Supplementary figures

S1. 17β-estradiol represses the NF-κB-driven reporter gene expression in TM4 Sertoli cells.
As a control set-up, L929sA and TM4 cells, stably transfected with the synthetic NF-κB-responsive promoter p(IL-6-κB)350hu.IL-6P-luc+, were treated with 1 µM reference hormones dexamethasone or 17β-estradiol, followed by 6h treatment with 2,000 IU/ml TNF, before cell lysis. Normalized luciferase expression levels are represented as bar graphs. Corresponding ERα and ERβ protein expression levels in both cell types are revealed by Western blot analysis (aspecific bands reflect equal protein loading).

S2. Modulation of histone modifications on the endogenous IL-6 promoter by isoflavones.
Serum-starved L929sA mouse fibroblasts were treated for 30 min with 2,000 IU/ml TNF alone, or following 2h pretreatment with genistein, daidzein or biochanin A (200 µM). ChIP analysis was performed against phosphorylated (S10) or phospho-acetylated (K9-S10) histone H3. After reversal of cross-linking, coimmunoprecipitated genomic DNA fragments were analysed by quantitative PCR for 27 cycles with IL-6-specific primer sets. Band intensities were quantified by Phosphor-Imager and normalized for input variation. Results are represented as bar graphs; in the latter case, error bars reflect standard deviation of triplicate PCR measurements of a representative ChIP experiment.
Results

S3. Gene expression profiling of breast cancer groups with different survival prognosis.
From raw data sets of 78 breast cancer patients with good (44) or bad (34) prognosis signature (19, 54) (data sets freely available at http://www.rii.com/publications/2002/ vantveer.htm), we re-analysed gene expression patterns of various cytokines, NF-κB signaling players, hormone receptors and kinases. Data sets were extracted and Log_{10}(ratio) (the mean ratio of the intensities of the red and green fluorescent channels which reflect the extent of induction or repression of a given gene) of the gene of interest of the various patients are represented in scatter column graph plots with indication of the calculated means of good (represented as green dots) and worse (represented as red dots) prognosis patient populations.

Materials and Methods

Cell culture assays.
Mouse fibroblast L929sA cells, primary fibroblasts from wild-type and MSK1\(^{+/+}\) MSK2\(^{-/-}\) mice, human embryonic kidney HEK293T cells, MDA-MB231, SKBR3, and MCF7 breast cancer cells were regularly cultured in DMEM supplemented with 5% FCS and 5% newborn calf serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. TM4 cells were grown in
DMEM/Nut Mix F12 supplemented with 5% horse serum, 2.5% FCS, and 1% penicillin/streptomycin (all reagents from Invitrogen, San Diego, CA).

**Cytokines, kits, and inhibitors.**

Recombinant TNF has been described previously (2). Secreted IL-6 levels were determined by IL-6 immunoassay kit purchased from R&D Systems, Inc. (Minneapolis, MN). SB203580, PD98059, tyrphostin A23, rolipram, LY294002, and wortmannin were purchased from Alexis (Lausen, Switzerland) and H89 was obtained from Calbiochem-Novabiochem International (San Diego, CA). Trichostatin A, genistein, daidzein, biochanin A, 17β-estradiol, OH-tamoxifen, BHA, ciglitazone, and N-acetyl-L-cystein were purchased from Sigma (St. Louis, MO). ICI182780 was obtained from Tocris (Ellisville, MO) and U0126 from Promega (Madison, WI).

**Western blotting and antibodies.**

L929sA cells were grown until subconfluency in six-well plates and were treated as indicated in the figure legends. For phospho-specific Western analysis [p38, extracellular signal-regulated kinase (ERK), MAP/ERK kinase (MEK), MSK], cells were serum starved for at least 24 hours. After induction, total lysates were prepared with SDS-Laemmli buffer, containing 62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 1 mmol/L DTT, and bromophenol blue. Cell lysates were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Schleicher & Schuell Bioscience, Keene, NH). For Western detection of p65 and ERα, sc-372 (C20) and sc-7202 (H184) were used, respectively (Santa Cruz Biotechnology, Santa Cruz, CA). Phospho-specific polyclonal rabbit antibodies to p38 (T180/Y182), p42/44 ERK (T202/Y204), MAPK, MEK1 (S217/S221), MSK1 (S376), IKKα/β (S180/S181), and NF-κB p65 (S276) were used to detect the respective phosphorylated forms and purchased from Cell Signaling (Beverly, CA). Dimethyl Lys9 H3 antibodies were purchased from Upstate (Lake Placid, NY). Anti-phosphorylated-H3 (P-H3) and anti-phospho-acetyl-H3 antibodies were kindly provided by A. Clayton (15). ERβ antibody was a kind gift of M. Warner (16). Anti-actin antibody was obtained from ICN (Irvine, CA).

**Promoter analysis and transactivation assays, electrophoretic mobility shift assay.**

The plasmids p1168hu.IL-6P-luc+, p(IL-6-κB)350hu.IL-6P-luc+, pPGKβgeobpA, and pCMV-CBP were described previously (17, 18). L929sA cells or HEK293T cells were transiently or stably transfected with the plasmids indicated in the figure legends, by the
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DEAE dextrane method or the calcium phosphate precipitation procedure, respectively, as described previously (17). Reporter gene assays were carried out essentially as described elsewhere (17). Luciferase activity, expressed in arbitrary light units, was corrected for the protein concentration and transient transfection efficiency by normalization for coexpressed β-galactosidase levels. The latter were quantified with a chemiluminescent reporter assay Galactostar kit from TROPIX (San Francisco, CA).

L929sA cells constitutively expressing Gal4-p651-551 were transiently transfected with the reporter gene construct p(GAL4)2-50hu.IL-6-luc+ using the DEAE dextrane method (2). The reporter plasmid containing two sites for the yeast transcription factor Gal4 in front of an IL-6-TATA box-containing minimal promoter was described previously (17). Electrophoretic mobility shift assay (EMSA) has been done as described elsewhere (17).

**Immunoprecipitation-MSK1 kinase assay.**

L929sA cells were seeded at 1 x 10^6 per dish and grown until subconfluence. After 48 hours of starvation, cells were treated as indicated in figure legends. Endogenous MSK1 was immunoprecipitated, as described elsewhere (2). Immunoprecipitated MSK1 was incubated with 30 μmol/L p65-tide (CMQLRRPSDRELSE) for 20 min at 30°C in the phosphorylation assay buffer [50 mmol/L Tris-HCl (pH 7.5), 0.1 mmol/L EGTA, 0.1% β-mercaptoethanol, 2.5 μmol/L PKI, 1 μmol/L microcystin, 10 mmol/L Mg(Ac)₂, 0.1 mmol/L γ[^32P]ATP (100-200 cpm/pmol)]. Incorporation of phosphate into peptides was determined using p81 phosphocellulose paper.

**Chromatin immunoprecipitation assay.**

Chromatin immunoprecipitation analysis and semiquantitative PCR has been previously described (2). The following promoter-specific primers were used: hIL-6 sense, 5'-GCGCTAGCCTCAATGACGACCTAAG-3' and hIL-6 antisense, 5'-GAGCCTCAGACATCTCCAGTCTAT-3'; mIL-6 sense, 5'-TGACTTCAGCTTTACTCTTGT-3' and mIL-6 antisense, 5'-CTGATTGGAAACCTTATTAAG-3'; H4 sense, 5'-GACACCGCATGCAAAGAATAGCTG-3' and H4 antisense, 5'-CTTTCCAAGGCTTTACCACC-3'. One tenth of the immunoprecipitated DNA and one fiftieth of the input DNA was used for each PCR. Histone cell extraction and acid-urea gel electrophoresis has been described elsewhere (2, 15).
Reverse transcription-PCR and dot blot analysis.

Subconfluent L929sA fibroblasts were treated as indicated, and total cellular RNA was isolated with the acid-guanidinium-thiocyanate-phenol chloroform method using the Trizol reagent (Invitrogen). Reverse transcription was done on 5 µg total RNA to prepare cDNA for a conventional reverse transcription-PCR on IL-6 (sense, 5'-GGAGTACCATACTACCTGG-3' and antisense, 5'-GACCACAGTGAGGAATGTCC-3'; amplicon, 331 bp) and glyceraldehyde-3-phosphate dehydrogenase (sense, 5'-GTCCATGCCATCACTGCCA-3' and antisense, 5'-GTGGGAGTTGCTGTTGAAG-3'; amplicon, 342 bp). PCR conditions applied were as follows: at 94°C for 4 minutes, 29 cycles at 62.5°C for 30 seconds, at 72°C for 30 seconds, and at 94°C for 45 seconds; final annealing for 1 minute; final elongation for 5 minutes. For dot blot analysis, the obtained total RNA was denatured in formaldehyde mix at 60°C and as a dilution series spotted on nylon filters (Hybond membranes, Amersham Pharmacia Biotech, Piscataway, NJ). After fixation by UV cross-linking, the filter was hybridized with a specific $^{32}$P-IL-6 probe at 42°C for 24 hours.

Superarray analysis.

The mouse NF-κB signaling pathway gene array and human breast cancer and ER signaling gene array (GEA array) kits were obtained from SuperArray, Inc. (Bethesda, MD) and used according to the manufacturer's instructions (2). Quantification and normalization of the obtained hybridization signals was done using Phosphor-Imager and SuperArray software. Comparison of the GEAarray and RT-PCR or Northern results revealed good correlation and confirmed signal specificity.

Supplementary array data analysis.

We analyzed published raw data sets of 78 breast cancer patients, of which 44 have good prognosis signature and 34 have bad prognosis signature (ref. 19; data sets and details regarding sample selection, preparation, and expression profiling are freely available at http://www.rii.com/publications/2002/vantveer.htm). The accompanying Microsoft Excel spreadsheet ArrayData_less_than_5yr.xls and ArrayData_greater_than_5yr.xls contains pages with actual results of ~24,500 gene measurements. Data sets for various cytokines, NF-κB signaling players, hormone receptors, and kinases were extracted and log$_{10}$(ratio), the mean ratio of the intensities of the red and green channels that reflect the extent of induction or repression of a given gene, of the gene of interest of the various patients are represented in scatter column graph plots with indication of the calculated means of good and worse
Results

prognosis patient populations. Increasing or decreasing trends are indicated by arrows, whereas unaffected targets are indicated with an equation sign.

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Part II: A critical role for p53 in the control of NF-κB-dependent gene expression in TLR4-stimulated dendritic cells exposed to genistein.

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Abbreviations used in this paper: DC, dendritic cell; MoDC, monocyte-derived DC; BMDC, bone marrow-derived DC; Gen, genistein

Abstract

Considerable research has focused on the anti-inflammatory and anti-proliferative activities exhibited by the soy isoflavone genistein. We previously demonstrated that genistein suppresses TNF-α-induced NF-κB-dependent IL-6 gene expression in cancer cells...
by interfering with the mitogen- and stress-activated protein kinase 1 (MSK1) activation pathway. However, effects of isoflavones on immune cells, such as dendritic cells (DC), remain largely unknown. Here we show that genistein markedly reduces IL-6 cytokine production and transcription in LPS-stimulated human monocyte-derived DC. More particularly, we observe that genistein inhibits IL-6 gene expression by modulating the transcription factor NF-κB. Examination of NF-κB-related events downstream of TLR4 demonstrates that genistein affects NF-κB subcellular localization and DNA binding, although we observe only minor inhibitory impact of genistein on the classical LPS-induced signaling steps. Interestingly, we find that genistein significantly increases p53 protein levels. We also show that overexpression of p53 in TLR4/MD2 HEK293T cells blocks LPS-induced NF-κB-dependent gene transcription, indicating the occurrence of functional cross-talk between p53 and NF-κB. Moreover, analysis of IL-6 mRNA levels in bone marrow-derived p53 null versus wild-type dendritic cells confirms a role for p53 in the reduction of NF-κB-dependent gene expression, mediated by genistein.

Introduction

Genistein belongs to the category of phenolic non-steroidal isoflavonoids, which can mainly be found in soy beans and other plants of the Leguminosae family (1). Based on its phyto-estrogen characteristic, genistein is pharmaceutically recommended as dietary supplement for use in alternative hormone replacement therapy to relieve menopausal symptoms (2). Besides that, genistein behaves as an inhibitor of several enzymes or kinases (topoisomerase II, cAMP-phosphodiesterase, Akt, tyrosine protein kinase), as an anti-oxidant due to its phenolic nature, and it affects a host of other intracellular processes (3). We have previously studied the molecular effects of genistein on the TNF-α-stimulated signal transduction towards IL-6 transcription in L2929sA fibroblasts and have shown that genistein affects the kinase cascade towards mitogen- and stress-activated protein kinase 1 (MSK1) activation, which accounts for NF-κB p65 transactivation via serine 276 phosphorylation and for histone H3 serine 10 phosphorylation on the IL-6 promoter (4). Other research groups have demonstrated that isoflavones have inhibitory effects on classical NF-κB activation (5, 6) and subsequently on NF-κB-dependent anti-apoptotic and inflammatory processes, which may contribute to their anti-carcinogenic and anti-inflammatory properties. Potential inhibitory effects of genistein on the acquired immune system have been reported in some rodent studies, but the data are inconsistent and even conflicting (7). However, the role of genistein in modulating normal primary cells of the innate immune system and in particular its effect on DCs, have received little attention.
It is well established that DCs, as professional APCs of the innate immune system, reside at the host-pathogen interface and play a key role in directing adaptive immune responses by initializing T cell activity. When immature DCs are triggered by microbial compounds such as LPS, the expression of Ag-presenting MHC class II, accessory molecules and cytokines is upregulated. Furthermore, depending on the DC maturation status and on the type of cytokines produced by the DC and by the environmental innate immune cells, naive CD4+ Th cells are instructed to differentiate into Th1, Th2 effector cells or regulatory T cells (8). The role of the prototype cytokines IL-12 and IL-4 in promoting respectively Th1- and Th2-polarized immune responses is well established (9, 10).

However, DC-derived IL-6 is postulated to influence the nature of the immune response too, as this cytokine induces the initial IL-4 production by naive T cells (11, 12). Even more recently is demonstrated that IL-6 shifts the TGF-β-driven regulatory T cell generation into pathogenic Th17 cell differentiation (13). Apart from this, IL-6 is involved in a myriad of cancer-, inflammation- and immunity-related events, as this pleiotropic cytokine induces tumor growth in an autocrine manner, synthesis of acute phase response proteins in hepatocytes, growth of hematopoietic stem cells, terminal differentiation of B cells into plasma cells and monocytes into macrophages (14) and directs the transition from innate to acquired immunity (15). Therefore, aberrant IL-6 expression has been associated with various chronic inflammatory disorders and auto-immune diseases, such as rheumatoid arthritis, Crohn's disease, psoriasis, etc (16, 17).

The pro-inflammatory gene transcriptional program of DCs challenged with LPS is activated through TLR4-induced signal transduction (18), that targets various downstream effectors such as NF-κB. This transcription factor is a dimer composed of Rel protein family members. p52 and p50 precursor molecules (respectively p100 and p105) and several Rel proteins with a transactivation domain such as p65, c-Rel and RelB belong to this family. The prototype NF-κB heterodimer, p50-p65, is kept inactive through binding to its inhibitor IκBα. The TLR4-signaling pathway operates via 2 adaptor-dependent mechanisms, which converge by recruitment of TNF receptor-associated factor 6 (TRAF6) and TGF-β-activated protein kinase 1 (TAK1). The latter is responsible for MEK, MKK 4/7 and MKK3/6 (and respectively ERK, JNK and p38) activation and, moreover, indirectly activates the IκB kinase (IKK) complex (reviewed in (19)). Subsequently, this complex phosphorylates IκBα on serines 32 and 36 causing its ubiquitinylation and proteasomal degradation. This elimination liberates NF-κB and permits its translocation into the nucleus, where it can bind to κB promoter elements and induce related gene transcription of cytokines, chemokines, etc (20).
In this study, we show that LPS-induced IL-6 production by human MoDCs is profoundly downregulated by genistein. However, unlike previous data obtained on TNF-α-treated fibroblast cancer cells, MAPK signaling is only slightly affected, suggesting that genistein acts through different mechanisms in DCs. We have therefore analysed the impact of this phyto-chemical on NF-κB activation and localization. Furthermore, we have explored potential p53-NF-κB cross-talk in response to genistein, which may be responsible for downregulation of NF-κB-dependent gene expression. On the whole, our results suggest that genistein acts as a potent modulator of DC functions and these findings highlight the anti-inflammatory and immunomodulatory properties of genistein, since DCs are critical players in the initiation and regulation of immune responses. In this view, genistein may represent an attractive dietary tool to dampen unwanted cellular immune response and excessive cytokine production after transplantation or in auto-immune diseases.

**Results**

**Genistein strongly downregulates TLR-dependent IL-6 production in MoDC.**

In a first series of experiments we examined whether or not genistein influences IL-6 cytokine production in MoDCs, in response to TLR2, TLR3 and TLR4 agonists. Investigation of IL-6 levels in the supernatant samples of 2 donors shows increased secreted IL-6 protein amounts after FSL1, Poly (I:C) and LPS stimulation, which are significantly reduced when pretreated with genistein (Table I).

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Since LPS elicits the highest IL-6 response, we further focused on this stimulus. A time kinetics experiment with LPS with/without genistein pretreatment reveals that LPS stimulation increases IL-6 levels and that genistein has a potent inhibitory effect on IL-6 cytokine production (Fig 1A). An additional dose-response experiment, covering genistein concentrations from 200 μM to 6,25 μM confirms its repressive effect on LPS-stimulated IL-6 production.
levels (Fig 1B). A dose of 200 µM genistein, which was shown not to affect MoDC viability after 6 hours treatment, as assessed by PI/annexin V-Alexa Fluor 488 staining (data not shown), was used in all subsequent experiments.

**Figure 1. Genistein decreases IL-6 production in LPS-stimulated MoDC.**

DCs were left untreated, treated with genistein (200 µM or as indicated) or DMSO for 1-2 hours, then stimulated with LPS (1 µg/ml) as indicated (A) or during 24 hours (B). IL-6 cytokine concentration was measured in the supernatants by ELISA.

A, Time kinetics data are expressed as concentration IL-6 in pg/ml.

B, A 1:2 dilution series for genistein from 200 µM to 6.25 µM final concentration was made. ELISA results are expressed as percentage IL-6 induction relative to the LPS condition. A sigmoidal dose-response curve is used for curve fitting. The calculated IC50 value is 52.07 µM (R² = 0.9731). The LPS+DMSO value (110%) is not included in the graph.

**Genistein significantly reduces LPS-stimulated IL-6 transcription in MoDC.**

To verify if genistein affects IL-6 at the (post-)translational level or acts at the transcriptional level, we performed quantitative real-time RT-PCR on IL-6 mRNA. A time kinetics experiment with LPS was performed to examine the rate and extent of IL-6 mRNA production in MoDC (Fig 2A). This assay shows detectable IL-6 mRNA amounts already after 1 hour, clearly abundant levels after 3 hours, which rise up to 6 hours or later. At 24 hours IL-6 signals are almost basal again. mRNA analysis after 6 hours LPS stimulation shows that IL-6 levels of MoDC pretreated with genistein are strongly reduced (Fig 2B), whereas the β-actin mRNA levels remain unaffected.

To strengthen the indications that genistein negatively affects IL-6 mRNA production, we performed a restriction enzyme accessibility assay on the IL-6 gene promoter in MoDC as
Results

a read-out for chromatin relaxation and transcriptional activity (31) (Ndlovu et al., submitted for publication). By the use of selected restriction enzymes that cut on specific positions in the IL-6 promoter, the local chromatin accessibility can be unveiled. As the chromatin configuration and level of chromatin opening is tightly controlled and related to transcription initiation in general (32-35), this test allows us to investigate possible effects of genistein on IL-6 transcription. As shown in Fig 2C, after 3 hours of LPS treatment we can clearly observe a higher accessibility in the IL-6 proximal promoter region of MoDC, as revealed by NheI or BsrBI digestion. In contrast, genistein pretreatment efficiently abrogates the LPS-stimulated chromatin opening. Interestingly, a more upstream promoter region containing an additional recognition site for the BsrBI enzyme was not remodelled upon LPS stimulation. The corresponding 3021 bp fragments can be considered as an internal control, showing equal loading of gDNA.
**Results**

*Figure 2. Genistein represses IL-6 transcription in LPS-stimulated MoDCs.*

A, DCs were stimulated with LPS (1 µg/ml) for different times. IL-6 mRNA levels were normalized to β-actine mRNA values.

B, DCs were left untreated, pretreated with genistein (200 µM) or DMSO for 1 hour and stimulated with LPS (1 µg/ml) during 6 hours. IL-6 mRNA results were normalized to β-actine mRNA levels and mean values of 4 different donors were calculated (±SD). The results are expressed relatively to the control set-up (untreated). ***, vs LPS or LPS+DMSO. The LPS value is not significantly different from the LPS+DMSO value.

C, DCs were pretreated with genistein (200 µM) or DMSO for 2 hours and then stimulated with LPS (1 µg/ml) during 3 hours. REA was performed as described in Material and Methods. After treatment, DCs of 4 different donors were pooled per treatment condition. Of each, 15 µg control and BsrBI-treated genomic DNA and 7 µg Nhel-treated samples were used for southern blotting. Control samples of each condition were included in the experiment and treated totally similarly, except for the *in vivo* enzyme digestion. One representative experiment out of 3 is shown. *, PstI-PstI band 7710 bp; **, BsrBI-PstI band 3021 bp; ***, Nhel-PstI band 2674 bp; ****, BsrBI-PstI band 2565 bp; NS, non-specific band. The IL-6 gDNA is visualized by a straight line, on which the position of the IL-6 proximal promoter and the NF-κB site are indicated by an open rectangle and a filled circle, respectively. The probe, restriction enzyme cutting sites and transcriptional start position are also drawn on the figure.

**Proximal TLR4-initiated kinase signaling pathways in MoDC are not significantly influenced by genistein.**

The clear reduction of IL-6 transcription roused us to explore if genistein affects the related molecular signaling cascade towards the IL-6 gene promoter. MoDCs were either or not pretreated with genistein and stimulated with LPS in a 10 to 60 min kinetics (Fig 3A). Western analysis of lysates shows LPS-induced IKK, MAPK (ERK, p38, JNK) and MSK1 activation in a time-dependent manner, with peak phosphorylations at 30 min. Consequently, the NF-κB inhibitor IκBα is degraded at this time point and is even not resynthesized at 60 min. In contrast to the profound effects in fibroblast L929sA cells (4), genistein treatment only slightly lowers the phosphorylation peaks of MAPKs and MSK1, but does not affect the IKK phosphorylation pattern. However, statistical analysis of the band intensities of at least 2 western blots (by Image J software) reveals that the observed differences at either time point do not reach significance (data not shown).

In line with the lack of effect on proximal receptor signaling, we still observe a significant reduction of IL-6 mRNA levels if genistein is added 30 min after LPS stimulation instead of 1-2 hours in advance (Fig 3B).
Results

A

![Figure 3. TLR4-dependent signaling cascades towards IL-6 gene transcription in MoDCs are not affected by genistein.](image)

A, DCs were either or not pretreated with genistein (200 μM) for 2 hours, followed by stimulation with LPS (1 μg/ml) for the indicated times. Total cellular lysates were prepared, blotted and analysed with the respective (phospho-specific) antibodies to visualize IκBα degradation and the amount of phosphorylated kinases in a time kinetics. The occurrence of aspecific bands and the control

B

![Bar graph.](image)

with anti-p65 for the different membranes (not shown) show equal protein loading in each lane. The presented pattern is a representative result of western blotting experiments on minimum 2 donors.

B, DCs were left untreated, treated with genistein (200 μM) or DMSO as indicated: 2 hours, 1 hour, 30 min before, simultaneously or 30 min after adding LPS (1 μg/ml). The LPS stimulation lasted for 3 hours. IL-6 mRNA levels were normalized to β-actine mRNA values.

NF-κB-dependent promoter activity in TLR4/MD2 HEK293T cells is significantly reduced by genistein.

As genistein potently inhibits TLR4-mediated IL-6 gene expression without profoundly affecting upstream signaling events such as MAPK and IKK activation, we continued by investigating further downstream events of these signal cascades. We performed reporter studies on a HEK293T cell line, which stably expresses TLR4/MD2 proteins (23). Of all known transcription factors binding to the IL-6 promoter region, we focused on NF-κB because of its crucial role in triggering IL-6 transcription (36) and also because genistein and other isoflavones were previously reported to inhibit NF-κB-mediated processes (5, 6, 37-40). In accordance with the dose-dependent effect of genistein on endogenous IL-6 levels in MoDCs, genistein significantly lowers the LPS-stimulated NF-κB-dependent promoter activity.
in these HEK293T cells (Fig 4A). Moreover, in line with the results obtained with the synthetic NF-κB reporter construct containing multimerized κB sites, a similar dose-dependent genistein repression could be observed with another reporter construct, driven by the endogenous hIL-6 promoter sequence (results not shown). Furthermore, a severely impaired LPS response was measured in reporter experiments with a NF-κB-mutated IL-6 promoter construct (data not shown).

**Figure 4.** NF-κB-dependent promoter activity in TLR4/MD2 HEK293T cells is dose-dependently reduced by genistein.

A. TLR4/MD2 HEK293T cells were transiently transfected with the p(IL-6-κB)350hu.IL-6P+ reporter construct. Inductions were performed in triplicate. Cells were left untreated, treated with genistein (200, 100, 50 μM) or DMSO during 2 hours and overnight stimulated with LPS (1 μg/ml). Equal amounts of protein lysates were analysed in luciferase assays and values are expressed as fold induction (±SD) relatively to the control (untreated). Two other independent experiments gave similar results. ***, vs LPS or LPS+DMSO. The LPS value is not significantly different from the LPS+DMSO value.

B. DCs were either or not pretreated with genistein (200 μM) for 1 hour, then stimulated with LPS (1 μg/ml) during 12 hours. Expression of immunostimulatory molecules for DC function (CD40, HLA-DR, CD80, CD86) was analysed by flow cytometry. DMSO vehicle control-treated cells did not show significant differences with LPS-treated cells. Results are plotted as percentage of expression relative to LPS values and are the mean (±SD) of 3 different donors, using mean values of histograms. ***, vs LPS+DMSO.

Interestingly, since NF-κB is generally involved in the LPS-triggered maturation process of DCs (41), we also studied some additional NF-κB-driven cytokines and
maturation markers in MoDCs (Fig 4B). As such, we observe a significant repression of protein production of IL-12 p40, TNF-α and IL-10. The induction of LPS+Gen relative to LPS+DMSO (arbitrarily set at 100%), after 6 hours stimulation using 2 donors, reaches 5.9% and non-detectable values for IL-12 p40; 13.4% and 6.5% for TNF-α and 14.9% and non-detectable values for IL-10. Even so, flow cytometric analysis shows reduced cell surface expression of Ag-presenting molecules and other maturation markers (CD40, HLA-DR, CD80, CD86).

Genistein attenuates LPS-induced NF-κB-DNA binding in MoDCs.

Mechanistic insight into the molecular effects of genistein on NF-κB needs investigation of NF-κB-related events after release from IκBα, the degradation of which is not influenced by genistein (see Fig 3A). Thus, NF-κB should be able to translocate to the nucleus. Therefore, we analysed the capacity of NF-κB to bind its recognition site in vitro following LPS with or without genistein pretreatment in DCs. For all conditions, nuclear extracts were subjected to gelshift (Fig 5A). LPS treatment increases the DNA binding of NF-κB, mainly p65-p50 heterodimers and p50 homodimers, as demonstrated by supershifting the corresponding bands with the respective antibodies. Genistein pretreatment significantly diminishes both LPS-stimulated NF-κB-DNA binding bands. In contrast, the binding of the constitutive complex RBP-Jκ remains unaffected, which indicates specificity of the genistein effect and, at the same time, shows equal protein loading in the different set-ups (25).

Genistein influences NF-κB subcellular localization in MoDCs.

From Fig 5A, it appears that genistein affects NF-κB-DNA binding, although the upstream signaling to activate NF-κB is intact (see Fig 3A); so, either the binding capacity of NF-κB is changed or the nuclear abundance of NF-κB is altered. We addressed this question by analysing subcellular fractions of treated MoDCs for the abundance of the NF-κB subunits identified in EMSA (see Fig 5A). As shown in Fig 5B, western blotting results reveal a clear cytoplasmatic-nuclear shift of p65 and p50 after 40 min of LPS stimulation, in accordance with the full degradation of the cytoplasmic inhibitor IκBα at 30 min, shown in western blotting of total lysates (see Fig 3A). However, p65 and p50 subunits are less abundant in nuclear extracts, when DCs are pretreated with genistein as compared to the LPS-alone condition at the same time point. Confocal microscopy unambiguously reveals that genistein diminishes LPS-stimulated p65 nuclear localization in the majority of the cell population (Fig 5C).
Results

Figure 5. Genistein affects NF-κB-DNA binding, localization and p53 protein levels in MoDCs.
DCs were left untreated, pretreated with genistein (200 μM) or DMSO during 2 hours, and then stimulated with LPS (1 μg/ml) during 50 min for EMSA (A), during 40 min for western analysis (B) or during 30 min for immunofluorescence assay (C).

A, Nuclear extracts were prepared and analysed in EMSA. Supershift was performed using 2 μg anti-p50 and anti-p65 antibodies in the reaction mixture. *, p65-p50 heterodimer; **, p50 homodimer; ***, RBP-Jκ; NS, non-specific band. One representative EMSA out of 3 independent experiments on 5 different donors is shown.

B, Equal amounts of nuclear and cytoplasmic extracts (10 μg) were analysed by western blotting. Detection of PARP and Grb2 indicates equal loading and purity of the fractionated extracts. One representative experiment out of independently performed assays on 4 different donors is shown.

C, Immunofluorescent staining using anti-p65 (a) was performed as described in Material and Methods. One representative field of each condition is shown. Phase-contrast microscopy images give an overview on the whole cells (b). This experiment was repeated on 4 different donors, giving similar results.
Genistein upregulates p53 protein expression in MoDC and p53 negatively affects NF-κB-dependent gene expression in TLR4/MD2 HEK293T cells.

So far, no molecular targets or activities of genistein have been elucidated in MoDCs, which could explain a decrease of nuclear NF-κB, independently of IκBα. In literature, genistein is commonly known as a topoisomerase I- (42) and II-inhibitor (43, 44). In addition and concurrent with this activity, genistein is shown in cell lines to induce ATM kinase activity and to stabilize p53 proteins by increased phosphorylation on serine 15 (45, 46). Based on these reports, we proceeded by evaluating p53 protein levels of MoDCs in response to LPS with/without genistein in western blotting (Fig 5B). Basal p53 levels are rather low and mainly nuclear, as p53 is a very unstable transcription factor in non-cancerous cells. Interestingly, treatment with genistein alone or in combination with LPS is able to upregulate p53 protein levels.

**Figure 6. p53 affects NF-κB-mediated promoter activity in TLR4/MD2 HEK293T cells.**

TLR4/MD2 HEK293T cells were transiently transfected with the full-length p1168hu.IL-6P-luc+ reporter, combined with 1 ng pRcRSV-p65 expression vector (A) or the p(IL-6-κB)350hu.IL-6P-luc+ reporter (B). The pCMV-HA-p53 expression vector, with or without pSUPER-p53 added in similar amounts, was cotransfected in various concentrations (10, 50, 100 ng as indicated in the figure legend). Inductions were performed in triplicate and luciferase values were measured in cell lysates after overnight LPS induction (1 μg/ml). Protein concentrations of lysates were determined and showed no significant differences. Values are plotted as arbitrary light units for each condition. For both reporter gene assays, one representative out of 2 independently performed experiments is shown. ***, for p53
Results

overexpression: vs the basal condition; for p53 RNAi: vs similar conditions in p53 overexpression without p53 RNAi.

Furthermore, p53 seems to have a repressive effect on the basal and LPS-stimulated IL-6 promoter activity as shown by transient transfection of increasing amounts of p53 expression vector (10-100 ng) in TLR4/MD2 HEK293T cells (Fig 6A).

Conversely, eliminating p53 by RNAi approaches totally reverses this reduction and even increases promoter activity levels above the levels of the corresponding set-ups without p53 RNAi, probably because HEK293T cells have high endogenous p53 levels, which are also affected by the RNAi system (data not shown). Finally, similar effects were observed on the NF-κB-dependent synthetic (IL-6-κB)_3 promoter (Fig 6B) and endogenous IL-8 promoter reporter gene constructs (data not shown) upon overexpression of p53, further confirming that p53 affects NF-κB-dependent transcription.

Genistein acts via the repressor p53 on LPS-stimulated IL-6 transcription in BMDCs.

To further investigate whether a similar p53-dependent NF-κB regulation in DCs exists and to verify if genistein acts via p53 on NF-κB-dependent IL-6 expression in vivo, we used BMDCs from p53+/+ and p53−/− mice. As shown in Fig 7A, immunofluorescence analysis of LPS-treated BMDCs shows a global cellular localization of p65 and an essentially predominant nuclear staining compared to the basal conditions. Of special note, genistein pretreatment clearly reduces the p65 nuclear abundance in the majority of p53+/+ BMDCs, but not in p53−/− cells. Next, we investigated whether these effects of genistein are also reflected at the level of endogenous IL-6 expression. Quantitative real-time RT-PCR of p53+/+ BMDCs reveals a median reduction of 70% in LPS-stimulated IL-6 mRNA levels, when cells were pretreated with genistein. However, genistein has no significant repressive effect on LPS-stimulated IL-6 transcription in p53−/− BMDCs (Fig 7B). In summary, these data clearly indicate that genistein-caused immunosuppression and attenuation of NF-κB-mediated IL-6 gene transcription involve p53.
Results

Figure 7. Genistein acts via p53 on NF-κB-mediated IL-6 transcription in murine BMDCs.
A, p53+/+ and p53−/− BMDCs were pretreated with Gen (200 μM) or DMSO for 1 hour, followed by LPS stimulation (1 μg/ml) for 40 min. Immunofluorescent staining using anti-p65 antibody was done as described in Material and Methods on BMDCs from 3 mice of each genotype. Representative pictures of each cell genotype and condition are shown. a, DMSO; b, LPS+DMSO; c, Gen; d, LPS+Gen for p65 detection, DAPI staining and overlay (Merge).

B, p53+/+ and p53−/− BMDCs were pretreated with Gen (50 μM) or DMSO (0.125% v/v) for 1-2 hour and stimulated with LPS (1 μg/ml) for 5-6 hours. RNA was quantified and equal amounts of all conditions were used in RT-PCR. IL-6 mRNA levels were determined by real-time qPCR. As a control, cDNA concentrations were measured with a Nanodrop ND-1000 spectrophotometer and IL-6 mRNA levels were corrected to these values. Induction percentages of LPS+Gen relative to LPS+DMSO conditions for the BMDCs of each mouse are plotted in a scatter diagram and the median is represented by a horizontal line. *, vs p53+/+ BMDC.

Discussion

In this study we show that the isoflavone genistein profoundly represses LPS-stimulated IL-6 expression in MoDCs, as well as IL-6 production in response to other TLR
Results

Genistein dose-dependently reduces secreted IL-6 protein levels (Table I, Fig 1), presumably via its effects on IL-6 transcription (Fig 2B). Although we cannot exclude additional post-transcriptional or (post-)translational effects of genistein, the fact that complete repression of NF-κB-driven IL-6 promoter constructs was observed after genistein pretreatment in reporter gene experiments in TLR4/MD2 HEK293T cells (Fig 4A) strongly argues for IL-6 transcription as a primary genistein target. In addition, previous studies have correlated transcriptional activity with accessibility of the IL-6 core promoter region (32-34) (Ndlovu et al., submitted for publication). Therefore, the observation that genistein keeps the chromatin structure surrounding the proximal IL-6 promoter in a closed conformation (Fig 2C) corroborates that genistein inhibits IL-6 gene expression at the transcriptional level.

At the conserved proximal promoter, various transcription factors such as AP-1, CREB, C/EBP-β, Sp-1 and NF-κB may regulate endogenous IL-6 chromatin transcription (31, 47, 48 and references herein). However, as we and others found that NF-κB is a crucial mediator in IL-6 expression in response to (pro-)inflammatory stimuli (e.g. LPS, TNF, etc) (36), we have further focused on the impact of genistein on the activation of this transcription factor. Essentially, we found that genistein interferes with NF-κB subcellular localization and subsequently NF-κB-DNA binding (Fig 5). Effects of genistein on IL-6 promoter regulation were previously investigated in a murine fibrosarcoma cell line, showing blockage of the TNF-α-induced MEK1-ERK-MSK1 kinase cascade and, consequently, of p65 transactivation and IL-6 chromatin modifications (4). In contrast, we did not find significant effects of genistein on the classical signaling steps leading to NF-κB activation in MoDCs (Fig 3A). Instead, we observed that in MoDCs, genistein influences the expression level of the transcription factor p53 (Fig 5B). Interestingly, p53 has the ability to decrease (TNF-α-induced) NF-κB reporter gene activity (49, 50). Accordingly, we noticed that p53 acts as a repressor of basal and LPS-induced NF-κB transactivation in TLR4/MD2 HEK293T reporter gene assays (Fig 6). Moreover, based on our results with p53+/+ and p53−/− BMDCs, we suggest that genistein effects depend on p53 activity, which counteracts NF-κB-mediated IL-6 transcription (Fig 7B). Interestingly, these observations argue for cell type-specific (fibroblast vs MoDC) and/or stimulus-specific (TNF-α vs LPS) molecular effects of genistein. It would be interesting to ascertain if other cells of the innate or adaptive immune system show a similar p53-dependent inhibition of LPS-stimulated NF-κB-driven gene expression in response to genistein.

The genistein-mediated increase in p53 protein levels is presumably due to protein stabilization, as we have noticed serine 15 phosphorylation of p53 only after genistein

stimuli (Poly (I:C) and FSL1).
Results

treatment (data not shown) (51). This residue among others confers p53 stability by regulating association with Mdm2, an oncogenic inhibitor that possesses ubiquitin ligase activities on itself and its target p53 (51). However, other related mechanisms of genistein such as Mdm2 downregulation by ubiquitinylation cannot be excluded (52).

Apart from in vitro studies on p53-NF-κB cross-talk, p53 also appears to act as a repressor of NF-κB in vivo, as NF-κB-dependent cytokines are elevated in LPS-treated p53−/− macrophages (53) and thymus/spleen tissue (50). Furthermore, p53 mutations have been found to elicit hyperinflammatory conditions, which increase the severity of chronic diseases (54) and promote cancer progression (55). However, how exactly p53 affects NF-κB remains an open question. Some research groups suggest that their reciprocal inhibition is due to the common use of a limiting pool of co-activators (49, 56, 57). Others reported a p53-mediated increase in IκBα expression, which sequesters NF-κB in the cytoplasm (58). A direct association between p53 and p65, which mutually compromises their activation potential, has been reported too (59, 60). So far, we have not been able to detect remaining endogenous IκBα levels upon LPS+genistein cotreatment of MoDC, which could have explained the reduced nuclear abundance of NF-κB (Fig 3A). Actually, it seems more plausible that p53 affects NF-κB nucleocytoplasmic shuttling by blocking nuclear import of NF-κB or by stimulating its nuclear export (61, 62). Alternatively, genistein may promote nuclear degradation of NF-κB. Elucidation of the precise mechanism of mutual cross-talk between p53 and NF-κB needs further investigation.

Interestingly, p53-dependent suppression of IL-6 promoter activity has been attributed to interference with C/EBP, CREB and AP-1 activities as well (55, 63, 64). Thus, inhibition of IL-6 transcription by p53 may be a cumulative effect of multiple regulatory effects on transcription factors, cofactors (CBP/p300), or chromatin remodelling factors (Brg1, SWI/SNF), as the reduction in NF-κB-DNA binding in response to genistein (Fig 5A) is less pronounced than the effects on IL-6 transcription (Fig 2B, Fig 4) and promoter accessibility (Fig 2C) in MoDCs.

Although our analysis of genistein-mediated effects on NF-κB has essentially focused on p65 and p50, we cannot exclude p53 effects on other NF-κB family members such as p52, c-Rel or RelB, which are also considered important in regulating cytokine expression in immune cells (65, 66). However, we have not been able to detect significant binding of these NF-κB proteins as revealed by supershift analysis of nuclear extracts from LPS-induced MoDCs (67).

In conclusion, we found that the soy isoflavone genistein significantly reduces expression of various NF-κB-mediated genes (cytokines, Ag-presenting HLA-DR, (co-
Results

Stimulatory molecules, see results text and Fig 4B) and suppresses global DC maturation in a p53-dependent manner. In that sense, these findings open up new perspectives for dietary (by phyto-chemicals) or therapeutic (by p53-activators such as nutlins (68)) interventions in transplantation or immune disorders such as allergy, asthma or auto-immunity, because it could dampen unwanted or excessive immune responses. On the other hand, these indications warrant further investigation of possible health-disturbing effects of genistein-containing products, such as soy milk, on infants, whose immune system development is still in a premature state.

Materials and Methods

Mice

p53\(^{-/-}\) and p53\(^{+/+}\) mice on C57/BL6 background were described by Jacks (21). Female and male mice were used for bone marrow extraction at the age of 4-7 weeks. The mice were bred and maintained in specific pathogen-free conditions according to the institutional guidelines.

Generation of DCs

MoDCs were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml geomycine, 1% non-essential amino acids, 50 μM 2-mercaptoethanol. MoDCs were generated from PBMCs from healthy donors. Briefly, PBMCs in medium were allowed to adhere onto 75 cm\(^2\) flasks. After 2 hours at 37°C, non-adherent cells were removed and after extensive washing, adherent cells were cultured in complete medium containing recombinant hGM-CSF (800 U/ml, Schering-Plough, Kenilworth, NJ) and hIL-4 (200 U/ml, R&D Systems, Minneapolis, MN). Every 2 days, 800 U hGM-CSF and 200 U hIL-4 were added. On day 6 of culture, non-adherent cells, which correspond to the MoDC-enriched fraction, were harvested and used for experiments.

BMDCs were cultured in RPMI 1640 glutamax (Invitrogen, San Diego, CA) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 55 μM 2-mercaptoethanol and non-essential amino acids (Invitrogen, San Diego, CA). BMDC were flushed with sterile PBS from the femur and tibia of mice. 3.5x10\(^6\) cells/10 cm dish were plated out in complete medium supplemented with 10 ng/ml murine recombinant GM-CSF (Biosource, Camarillo, CA and Peprotech, Rocky Hill, NJ). New medium with mGM-CSF was added on day 3 and primary culture was replated on day 7 similarly as on day 0, followed by addition of fresh medium with mGM-CSF on day 10. Cells were used in subsequent
Results on day 12. The BMDC population obtained with this protocol (originally described by Lutz (22)) contained routinely greater than 70% of CD11c+ DCs as assessed by FACS analysis.

Stable cell lines, plasmid constructs and reagents

The human embryonic kidney (HEK)293T cells stably expressing TLR4/MD2 have been described previously (23). The luciferase reporter constructs p1168hu.IL-6P-luc+ and p(IL-6-κB)350hu.IL-6P-luc+ have been used before (24, 25). The expression vector pCMV-HA-p53 and RNAi construct pSUPER-p53 were described by Unger (26) and Brummelkamp (27), respectively. The pRcRSV-p65 expression vector was described earlier (28). LPS from Salmonella enterica (serotype abortus equi, used for HEK293T and BMDC) and from Escherichia coli (serotype 0128:B12, used for MoDC), DMSO and genistein were from Sigma-Aldrich (Irvine, UK). Both LPS types were dissolved in sterile water and used at a final concentration of 1 μg/ml. FSL1 and Poly (I:C) are from Invivogen (San Diego, CA) and Amersham (Rainham, UK), respectively. Genistein was dissolved in DMSO to a stock concentration of 80 mM. DMSO was used as solvent control in an equal volume to the highest concentration of genistein treatment (0.25% v/v unless differently indicated in the figure legends). In none of the experiments did DMSO treatment show significant effects. Therefore, no-solvent conditions or DMSO controls were not shown in some experiments.

Transient transfection and luciferase assays

HEK293T cells were seeded in 24-well dishes and transiently transfected using the lipofectamine transfection methods according to the manufacturer’s instructions (Invitrogen, San Diego, CA). After 18 hours cells were treated as indicated. Promoter activities were analysed as described elsewhere (24).

EMSA

Nuclear and cytoplasmic extracts were prepared as described previously (25). Following quantification of protein amounts by the Bradford assay, 10 μg of nuclear extracts were analysed for their binding activity to an IL-6-derived κB sequence-containing probe essentially as described previously (29). The NF-κB oligonucleotide 5’-AGCTATGTGGGATTTTCCATGAGC-3’ was labeled with Klenow enzyme using [α-32P]-dCTP and electrophoresis was carried out on a 6% native polyacrylamide gel. For supershift assays, anti-p65 C20 and anti-p50 NLS were included in the reaction mixture. The gels were
dried and exposed to phosphorimager screens, which were scanned by StormScan Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

**Western blotting**

Total cellular extracts were made in Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT). Equal amounts of cytoplasmic/nuclear extracts or equal volumes of total lysates from each condition were resolved by 10% SDS-PAGE, transferred onto nitrocellulose membranes and analysed by western blotting. Chemiluminescent detection was performed using HRP-coupled secondary antibodies and western lightning chemiluminescent reagent plus (Perkin Elmer Life Sciences, Boston, MA) on the Kodak image station 440CF. Anti-P-IKK, anti-P-ERK, anti-P-p38, anti-P-JNK and anti-P-MSK1 were obtained from Cell Signaling (Danvers, MA). Anti-IκBα C21, anti-PARP H250, anti-Grb2 C23, anti-p53 DO1, anti-p65 C20 and anti-p50 C19 were purchased from Santa-Cruz Biotechnology (Santa Cruz, CA).

**Flow cytometry**

DCs were analysed for the expression of cell surface molecules by flow cytometry. The following mouse anti-human IgG1 fluorochrome-coupled antibodies were used: CD80-PE, CD86-PE, HLA-DR-FITC, CD40-FITC (BD Biosciences, Franklin Lakes, NJ).

**Quantification of cytokine production**

All cytokine levels in cell-free culture supernatants were determined using specific ELISA kits (Biosource, Camarillo, CA) with detection limits of 15 pg/ml, according to the manufacturer’s instructions.

**RNA isolation and RT-PCR**

Total RNA was extracted with the acid guanidinium thiocyanate-phenol-chloroform method using the Trizol-Reagent (Invitrogen, San Diego, CA). Reverse transcription was performed on 0.5 µg total RNA in 30 µl total volume. Following 1:5 dilution, quantitative real-time PCR was performed on 5 µl of each condition using Biorad iQ Supermix (for probe assay) or Invitrogen Sybr green platinum supermix-UDG on a iCycler apparatus (Biorad, Richmond, CA). The probe and primer sets were designed by primer3 software:

- hIL-6 fw : GACAGCCTCCTCCTTCCTCA
- hIL-6 rv : AGTGCCCTTTTGCTGCTTTC
- hIL-6 probe : (6-Fam)CCTCGACGGCATCTCAGCCC(Tamra)(phosphate)
Results

hβ-actine fw : GGATGCAGAAGGAGATCACTG
hβ-actine rv : CGATCCACACGGAGTACTTG
hβ-actine probe : (6-Fam)CCCTGGCACCAGCACAATG(Tamra)(phosphate)
mIL-6 fw: GAGGATACCACTCCCCAACAGACC
mIL-6 rv: AAGTGCATCCTGTTTTCATACA

A serial dilution of a cDNA mix standard was used to determine the efficiency of the PCR reaction. All amplifications were performed in duplicate or triplicate and data were analysed using Genex software (Biorad, Richmond, CA) taking primer set efficiency into account.

Immunofluorescence assay

DC were fixed with 2% paraformaldehyde-PBS, washed in PBS and stored in methanol in -20°C. For the staining, DCs were attached to cytoslides by cytospin in PBS or to coverslips and permeabilized with 0.1% Triton X-100 followed by blocking with 2% bovine serum albumin. For MoDC, coverslips were then incubated with rabbit polyclonal NF-κB p65 antibody A, from Santa-Cruz Biotechnology (Santa Cruz, CA) for 1 h at room temperature and after washing extensively, samples were incubated with the anti-rabbit Alexa 568 from Molecular Probes (Invitrogen, San Diego, CA). Then the slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Specimens were analysed with the Leica confocal SP2 laser scanning microscope. For BMDCs, cytoslides were incubated overnight with rabbit polyclonal p65 antibody C20 (Santa-Cruz Biotechnology, Santa Cruz, CA) at 4°C, washed and incubated with anti-rabbit Alexa 488 (Molecular Probes, Invitrogen, San Diego, CA). Nuclei were stained using DAPI and coverslips were mounted on cytoslides with vectashield (Vector Laboratories, Burlingame, CA). Analysis was performed on the Zeiss Axiosver 200M immunofluorescence microscope.

Restriction enzyme accessibility assay (REA)

The REA technique was performed essentially as described earlier (30) with some modifications. In brief, nuclei were extracted using buffer A (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.3 M sucrose) supplemented with 1 mM sodium butyrate and 0.5% NP-40. 5x10⁶ purified nuclei in buffer A with 1 mM sodium butyrate, 100 μg/ml BSA and 0.1 mM PMSF were partially digested by 40 U Nhel (Promega, Madison, WI) or BsrBI (New England Biolabs, Beverly, MA) restriction enzymes for 30 min at 37°C. Reactions were stopped by addition of 2x proteinase K buffer (100 mM Tris-HCl pH 7.5; 1% SDS, 200 mM NaCl, 2 mM EDTA). Following proteinase K and RNase A treatment, genomic DNA was twice phenol/chloroform and once chloroform extracted and dissolved in sterile water after ethanol
precipitation. Purified gDNA (7-15 μg) was digested overnight with 30 U PstI enzyme (Promega, Madison, WI). Samples were analysed by electrophoresis on a 1.5% agarose gel. After denaturation of the gel, capillary transfer to Hybond N’ membrane (Amersham, Rainham, UK) and UV-cross-linking, hybridization was performed with a 32P- labelled probe (HindIII-PstI) spanning nt +1756 to nt +2448 from the hIL-6 gene. The obtained bands Nhel-PstI and BsrBI-PstI are 2674 and 2565/3021 bp respectively. The restriction enzyme sites and band lengths are based on the human IL-6 mRNA sequence with accession number NM_000600, blasted to the human genome on www.ensembl.org.

Statistics

All statistical calculations were done in Graphprism v. 3.0. according to One-way ANOVA (Bonferroni’s Multiple Comparison Test) except for the data of Fig 7B, which has been analysed using unpaired two-tailed student t-test. The degree of significance is indicated in the figures by *, p<0.05; **, p<0.01, ***, p<0.001.

Acknowledgments

The authors thank M. Nguyen for generating DCs out of blood buffy coats; K. Van Wesemael, J. Claes and P. Faes for technical support; S. De Koker for assistance in flow cytometry of BMDC and other members of both labs for their practical help, suggestions and critical comments.

References


Results


Results


IV. ADDITIONAL DATA

1. In support of results part I:

**Attenuation of MSK1-driven NF-κB-dependent gene expression by soy isoflavones does not require estrogenic activity.**

![Figure 1. Comparison of genistein and 17β-estradiol effects on NF-κB-driven promoter activity in fibroblast and Sertoli cells.](image)

L929sA and TM4 cells, stably transfected with pPGKβGeobpA and with the synthetic NF-κB-responsive promoter p(IL6-κB)350hu.IL6P-luc+, were pretreated with 17β-estradiol (1 µM) or genistein (200 µM) and then treated with 2000 IU/ml TNF for 6 hours, before cell lysis. Normalized luciferase expression levels are represented as bar graphs, expressed as percent induction compared to the TNF alone condition (arbitrarily set at 100%) for both cell lines.

In the ERα/β-expressing TM4 cell line, estrogen reduces the TNF-α-induced NF-κB-driven promoter activity, while genistein exerts even a stronger downregulation, presumably through additive effects on ER and MSK1.

2. In support of results part II:

**A critical role for p53 in the control of NF-κB-dependent gene expression in TLR4-stimulated dendritic cells exposed to genistein.**

<table>
<thead>
<tr>
<th></th>
<th>IL-12 p40 (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>donor 1</td>
<td>donor 2</td>
<td>donor 1</td>
</tr>
<tr>
<td>untreated</td>
<td>ND</td>
<td>8,0</td>
<td>ND</td>
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<tr>
<td>LPS</td>
<td>1666,7</td>
<td>1097,2</td>
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<tr>
<td>DMSO</td>
<td>ND</td>
<td>ND</td>
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<td>805,6</td>
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<td>LPS+Gen</td>
<td>ND</td>
<td>47,2</td>
<td>128,8</td>
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</table>

**Table 1. Genistein represses LPS-stimulated production of different cytokines in MoDC.**

MoDCs were left untreated or pretreated with genistein (200 µM) or DMSO for 1 hour and stimulated with LPS (1 µg/ml) for 6 hours. IL-12 p40, TNF-α and IL-10 protein levels in culture supernatants were
analysed by ELISA. Data represent absolute values from 2 donors, expressed in pg/ml. IL-6 protein concentrations, in Table I, are derived from the same donors.

LPS-stimulation in MoDC induces production of other cytokines, besides IL-6. Moderate levels of pro-inflammatory IL-12 p40 and very high levels of TNF-α are reached, as well as low levels of anti-inflammatory IL-10. Genistein pretreatment profoundly reduces these protein levels.

**Figure 1.** TLR3/TLR4-dependent NF-κB-driven promoter activity is downregulated by genistein in HEK393T cells.

TLR4/MD2 HEK293T cells were transiently transfected with the p(IL6-κB)350hu.IL6P+ reporter construct in combination with the pPGKβgeoβpA plasmid. Inductions were performed in triplicate. Cells were left untreated, treated with genistein (200, 100, 50 µM) or DMSO during 2 hours and then stimulated for 6 hours with PamCSK (500 ng/ml) (A) or with Poly I:C (PIC) (50 µg/ml) (B). Luciferase activity in the lysates, expressed in arbitrary light units, was corrected for transient transfection efficiency and protein concentration by normalization with coexpressed β-galactosidase levels. Mean values of 3 experiments are expressed as fold induction (±SD) relatively to the PamCSK value (arbitrarily set at 100%). **, vs PamCSK+DMSO; ***, vs PIC+DMSO. The absolute PamCSK value is not significantly different from the PamCSK+DMSO value.

Besides LPS, other TLR types initiate signaling pathways that induce IL-6 expression. Stimulation of MoDC with TLR3 ligand poly I:C and TLR2 ligand FSL1 leads to high IL-6 production, which is significantly repressed by genistein pretreatment. The extent of inhibition for both stimuli is similar to LPS (more than 90%). Triggering of TLR2 and TLR3 in Hek293T
with respectively, PamCSK and Poly I:C, induces NF-κB-driven promoter activation, which is also dose-dependently abrogated by genistein.

Mixed lymphocyte reaction (MLR) assay: MoDCs from 2 donors were either or not pretreated with genistein (200 µM) or DMSO for 1 hour, then stimulated with LPS (1 µg/ml). After 12 hours incubation, MoDCs were thoroughly washed with medium and mixed with CD4⁺ T cells, extracted from 2 different human peripheral blood mononuclear cell (PBMC) populations. Distinct ranges of MoDC:T cell were used, including 1:100, 1:30 and 1:10, in triplicate.

Figure 2. Genistein does not affect T cell proliferation in MLR.
After 5 days of incubation, thymidine-methyl-³H (5 µCi/ml) was added and 24 hours later cells were harvested and T cell proliferation was measured. The scintillation results are shown as the mean value of 2 combinations with each T cell donor (±SD), expressed as counts per minute (c.p.m.).

Table 2. Cytokine response of T cells in MLR is not influenced by genistein.
After 6 days of incubation, culture supernatants of triplicate set-ups were pooled and cytokine levels were measured by ELISA. For the 1:10 DC:T cell range, IFN-γ and IL-5 protein levels are presented, expressed in pg/ml, for the 4 combinations.

<table>
<thead>
<tr>
<th>Combinations</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-5 (pg/ml)</th>
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<tbody>
<tr>
<td>DC2/T4</td>
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<td>161,0</td>
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<td>DC3/T4</td>
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<td>untreated</td>
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</table>

The T cell proliferation measurement in a MLR assay reveals no significant downregulation by genistein treatment of DCs. Also the DC-stimulated cytokine expression profile of T cells was unchanged by genistein. IL-5 and IFN-γ protein
expression levels were chosen because they constitute typical Th2 and Th1 cytokines, respectively. These results do not fit with our previous observations on cytokine production and maturation markers expression, within a short time span (Figure 4B). However, genistein was washed away after 12 hours of DC treatment, before mixing DCs with T cells. This procedure, necessary to avoid direct effects of genistein on the T cell population, puts some experimental limitations on this approach as a time period of 5 days may overcome the genistein effects.

Figure 3. Genistein effects on phosphorylated protein levels. MoDCs were pretreated with genistein (200µM) or DMSO during 2 hours and then either or not stimulated with LPS (1µg/ml) for 40 min. Nuclear extracts were prepared and analysed by Western blotting. Similar PARP protein levels reflect equal loading between conditions.

A, Nuclear protein levels of p53 and phosphorylated p53 (on Ser15) are shown for both tested donors.
B, Phosphorylated p65 (S276) levels are shown for both tested donors.

In MoDCs, genistein is able to upregulate nuclear p53 protein levels (see also Figure 5B) and to induce phosphorylation of p53 on S15, irrespective of LPS. Furthermore, in accordance to the the minimal effect on the extent of LPS-induced MSK1 phosphorylation on S376 (Figure 3A), genistein has no influence on the level of LPS-stimulated p65 phosphorylation on S276. In this cell type, MSK1 may not play a crucial role as H89, a non-specific inhibitor of MSK1 and PKA, does not affect LPS-stimulated IL-6 production (data not shown).

Figure 4. Genistein inhibits LPS-stimulated Akt phosphorylation. MoDCs were either or not pretreated with genistein (200 µM) for 2 hours and then stimulated with LPS (1 µg/ml) for the indicated times. Western blot analysis was performed on total lysates, with anti-P-Akt (T308) and control anti-Akt antibodies.
Additional data

In accordance to reports on abrogation of survival pathways in cancer cells (Gong et al, 2003; Li & Sarkar, 2002), genistein blocks the LPS-induced phosphorylation of Akt in MoDC (Figure 4). Since it has been shown before that Akt positively regulates NF-κB activity, this observation could explain the repressive effects of genistein on NF-κB-driven gene expression. However, synthetic PI3K inhibitors such as wortmannin increase IL-6 protein production after LPS stimulation (data not shown) and has a slightly positive effect on the LPS-induced chromatin accessibility of the IL-6 promoter in MoDC (Figure 5). In addition, wortmannin enhances the LPS-stimulated NF-κB-DNA-binding activity and nuclear translocation in MoDC (Aksoy et al, 2005). Thus, Akt plays no substantial role in genistein-mediated inhibition of NF-κB-dependent transcription.

3. Investigation of the implication of SIR2 in the regulation of IL-6 transcription.

An additional topic that was investigated in this study of isoflavone effects on inflammatory gene expression was the potential influence of isoflavones on sirtuin proteins, discovered due to their involvement in yeast longevity. Members of the silent information regulator (SIR)2 family of genes encode highly conserved nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylases. In mammals, SIRT1 (human) and SIR2 (mouse) are the presumed yeast Sir2 orthologs due to sequence similarity. Calorie restriction promotes cell survival by inducing SIRT1 (Cohen et al, 2004) and SIRT1 expression levels progressively drop as normal cells or animals age in culture (Michishita et al, 2005; Sasaki et al, 2006). Previous report have shown a functional link between NF-κB and sirtuin activity. The phytochemical resveratrol induces human SIRT1 activity, which attenuates the NF-κB transactivation potential (by deacetylating Lys 310) and augments apoptosis in response to TNF-α (Yeung et al, 2004). In a similar manner, SIRT1 interferes with amyloid β-stimulated

Figure 5. Wortmannin does not negatively influence the chromatin configuration of the IL-6 promoter.

MoDCs of 2 donors were pretreated with genistein (200 µM), DMSO (as a solvent control for genistein) or wortmannin (1 µM). Thereafter, LPS was added (1 µg/ml) for stimulation during 3 hours and a restriction enzyme accessibility (REA) assay was performed with Nhel as outlined in Materials and Methods of results part II. As a control, samples without intranuclear Nhel digestion were included for each treatment.
NF-κB activity in microglial cells (Chen et al, 2005) and mediates transcriptional repression of NF-κB-driven genes in cooperation with neddylated breast cancer-associated protein 3 (BCA3) (Gao et al, 2006). As we did not observe any difference in effects of isoflavones on TNF-α-induced IL-6 mRNA levels between SIR2 wt and null MEF cells (described in (McBurney et al, 2003)), we focused on the potential role of sirtuins in regulation of TNF-α-induced IL-6 expression. Due to difficulties in characterizing the MEF cells genotypes, we only obtained some preliminary data, which seem intriguing and controversial. Analysis of IL-6 mRNA levels demonstrates a lower basal and TNF-α-stimulated transcriptional activity at the IL-6 promoter in absence of SIR2. In accordance with this result, p65 is less detected in the nucleus of untreated SIR2 null cells, relative to SIR2 wt cells. After TNF-α-stimulation, IκBα degradation does not occur in SIR2 null cells. The role of sirtuins in modulating the NF-κB activation pathway needs further verification at the molecular level.

4. References


IV. SUMMARY

1. General discussion

Experiments in different cell types, including fibrosarcoma cells, epithelial breast cancer cell lines, TM4 Sertoli cells and DCs, disclosed that isoflavones are able to potently reduce NF-κB-driven gene expression. More particularly, the pro-inflammatory cytokine IL-6 is clearly affected on the protein and mRNA level by various isoflavones, including genistein, daidzein and biochanin A, albeit to a different extent. As we were mainly interested in revealing the molecular targets of isoflavones in the stimulus-induced signaling pathway towards IL-6, we focused on isoflavone effects at the transcriptional level. Many different stimuli activate IL-6 transcription, ranging from growth factors, pro-inflammatory cytokines to PAMPs. In view of the role of IL-6 in inflammation and immunity, we studied the molecular mechanism of isoflavones after TNF-α and LPS induction. We principally investigated the signaling pathway towards NF-κB, because this transcription factor plays a central role in triggering IL-6 transcription in response to TNF-α (Vanden Berghe et al, 1999) as well as LPS (data not shown).

Isoflavones strongly downregulate TNF-α-stimulated NF-κB-mediated gene transcription by interfering with the MEK1 kinase and subsequently the ERK MAPK activation pattern. This partial blockage eventually leads to a lower activation profile of downstream MSK1. The seemingly paradoxal difference in extent of inhibition of IL-6 transcription versus MSK1 inhibition explained by the additive effect of isoflavones on MSK1 recruitment to the IL-6 promoter. As MSK1 is known to regulate chromatin dynamics and transcription factor activation by phosphorylation, the isoflavone-mediated MSK1 inhibition accounts for the reduction in intracellular NF-κB p65 Ser276 and histone H3 phosphorylation. Specifically on the IL-6 promoter, a lack of phosphorylation and phospho-acetylation of H3 is observed, which harmonizes with the influence of isoflavones on cofactor activities. More detailed, genistein interferes with CBP-mediated HAT effects and precludes TSA-induced transcriptional activation, presumably by its effects on NF-κB transactivation. As both crucial transcriptional elements, such as NF-κB and a permissive chromatin configuration, are kept silent in this way, necessary components for effective transcriptional activation are compromised.

Interestingly, the mechanism by which genistein reduces IL-6 transcription in response to LPS is totally different from TNF-α, as the LPS-induced MSK1 phosphorylation kinetics is only slightly affected, consistent with the lack of effect on P-p65 Ser276 levels. In
contrast, genistein affects IL-6 transcription by modulating p53 Ser15 phosphorylation and, correspondingly, stability. Also the NF-κB-DNA binding and NF-κB subcellular localization are changed by genistein, although the LPS-induced IKKα/β activation (measured by phosphorylation level analysis) and IκBα degradation kinetics are intact. Here, genistein-mediated interference of p53 with NF-κB localization and NF-κB-dependent transcriptional activity plays a role in the substantial reduction of IL-6 transcription. Based on these observations, we can conclude that isoflavones have pathway- and/or cell type-specific molecular targets in the regulation of IL-6 transcription.

In our study, approaches to identify the characteristics or mechanisms by which isoflavones operate, remain limited to comparison with prototype molecules for most of the known isoflavone properties, including ligands for ER/PPARγ, inhibitors for PDE/PI3K/tyrosine kinases and anti-oxidants. As only tyrosine kinase inhibitors and anti-oxidant molecules inhibit the TNF-α-induced IL-6 gene expression to a similar extent as isoflavones and diminish P-MSK1 levels as well (for NAC also described in Sury et al, 2006), isoflavones may act as tyrosine kinase inhibitors or ROS scavengers, although conclusive evidence for these activities is difficult to obtain. Counteracting the effects of isoflavones by experimental tools, such as ROS overproduction, may affect viability and other cellular systems. Also the exact role and implication of those potential players in TNF-α-stimulated IL-6 transcription and the cross-talk with MEK1, MSK1 or NF-κB remains elusive. Ideally, identification of the exact crucial players would allow pharmacological modeling of target-specific molecules. However, the diverse characteristics of isoflavones may work in a synergistic manner, so that selecting one mechanism as target for modulation of inflammation would be disadvantageous. In contrast, of several tested compounds in MoDCs, only anti-oxidants and the PI3K inhibitor wortmannin influence LPS-stimulated IL-6 protein production, in a negative and positive way, respectively (data not shown). This indicates that oxidative stress contributes to LPS signaling, but whether this is an additional target of genistein or not, or coupled to p53 effects, remains unknown.

Besides insight into the stimulus-specific effect of isoflavones, we now have indications for gene selectivity as well, related to the molecular target of isoflavones in the TNF-α-signaling pathway, MSK1. The induction pattern of MSK1/2-deficient MEF cells corresponds to the expression profile of genistein-treated fibroblasts, revealing that NF-κB2 is differently regulated than IL-6 or VCAM1. However, further research needs to be performed to verify whether this gene specificity applies to all MSK1-regulated genes and whether a similar dependency exists in MoDCs for p53-regulated genes. So far, we haven't found any LPS-stimulated NF-κB-dependent gene that is not affected by genistein in
MoDCs, although no statistically significant difference can be observed in genistein-mediated repression of IL-12 p35 and p40 transcription between p53 wt and null BMDCs (data not shown). This possibly indicates that, besides p53, genistein acts on additional targets. Alternatively, IL-12 promoters may not be p53-regulated.

Concerning the impact of isoflavones on health and physiology, our data extend to other LPS-induced NF-κB-regulated cytokines and maturation markers, showing that genistein also downregulates these but to different extents, pointing to gene-specific regulatory programs. Furthermore, genistein represses signaling of other TLR types towards IL-6 expression too, in accordance with its modulation of a downstream central player, NF-κB, in IL-6 regulation. In view of the function of primary DCs in controlling T cell activation and adaptive immune responses, these results point to interference of isoflavones with immunomodulatory cytokine production, as well as with the costimulatory and Ag-presenting capacity of DCs. Based on these effects, genistein may inhibit efficient priming and polarization of naïve T cells, thereby affecting mounting of an appropriate immune response. We presume that genistein elicits acute, not persistent responses in DCs, which do affect their functionality. Therefore, continuous high exposure to isoflavones may hamper the initiation of acquired immune responses and subsequently lead to inefficient pathogen removal and to chronic pathologies. However, care has to be taken in drawing systemic conclusions, as the in vivo immune system consists of diverse cell types in a network, which may all be differentially influenced by genistein. Also, traditional intake of soy food is rather considered health-protecting, based on epidemiological data. In mice studies, genistein exposure enhances immune responses, although the effects vary, depending on the exposure duration, gender and litter order (Guo et al, 2005). However, other ones report that genistein attenuates cellular and humoral immune function in rodent models and induces thymic atrophy (Kogiso et al, 2006; Yellayi et al, 2003). In healthy post-menopausal women, soy milk and supplemental isoflavones increase the B cell population (Ryan-Borchers et al, 2006). Study of human subjects, using different end point markers and after an immunological challenge, would elucidate this issue further. Whereas the potential impairment of the immune system would be considered deleterious, this could be applied in a beneficial manner under certain conditions, such as organ transplantation, so that allograft rejection could be delayed or weakened, similarly to genistein and biochanin A supplementation in rat cardiac transplantation models (O’Connor et al, 2002; Schrepfer et al, 2005).

In contrast to the general presumption that isoflavones direct ER-dependent modulation of gene expression, because they harbour estrogen-like properties, we have
Summary

found in our cell-based system that isoflavones can mediate ER-independent repression of NF-κB-driven gene transcription. ER agonists and antagonists (with or without genistein) show no effect on the TNF-α-induced NF-κB-dependent promoter activity in fibroblasts, although they do in TM4 Sertoli cells, which express high levels of ERα/β. Moreover, similar isoflavone effects are observed in an ER-independent (SKBR3) breast cancer cell line. Since IL-6 is an important growth factor for cancer cells and is associated with lower survival rates, isoflavones may become an attractive means in cancer therapies, even for hormone-insensitive phenotypes, which generally correlate with a worse prognosis. In the same group of worse prognosis breast cancers, we could detect an increased expression level of MSK1, relative to the group of good prognosis cancers, in line with the reports on constitutive MAPK activity in aggressive cancers. In this view, MSK1 may be an important target in chemotherapy, independent of the ER status.

As IL-6 deregulation is implicated in the progression of several pathologies of advanced age and chronic inflammatory diseases, the use of isoflavones seems promising to combat or prevent aberrantly high IL-6 levels. However, to validate their therapeutical or preventive value, we cannot merely extrapolate our observations in an in vitro system to gain insight into the in vivo situation, since the latter is an extremely complex system, which warrants further investigation. Important in drawing conclusions is considered the isoflavone dose, since it has been revealed before that low genistein concentrations (less than 1 µM) stimulate in vitro cancer proliferation, whereas high concentrations inhibit cancer growth (Hsieh et al, 1998; Wang et al, 1996). A similar phenomenon has been found for osteogenesis and adipogenesis, in which the genistein concentration determines the balance between ER- and PPARγ-mediated effects (Dang et al, 2003). In this regard, we need to know what intracellular concentration of isoflavones, and in which chemical form, is reached in our experiments in a time-dependent manner, when a pharmacological dosis of 200 µM is applied. The direct contact and the high dosis allow us to visualize black-and-white effects, which may be less obvious in vivo but therefore not non-existing. Lower concentrations, down to 20 µM genistein, still affect NF-κB-dependent gene expression in vitro and elevate intracellular p53 protein levels in MoDCs (data not shown). Likewise, 50 µM genistein halves MSK1 phosphorylation in TNF-α-stimulated fibroblasts. Additionally, a continuous daily intake of isoflavones mounts up a certain plasma concentration of different isoflavone metabolites with differential potencies, which might be even more effective than giving single shots in a short time frame in in vitro experiments. Moreover, to limit an excessive inflammatory or immune response, partial molecular effects on p53 and MSK1 by physiologically reachable concentrations may be sufficient, while an almost complete block of
NF-κB-driven gene expression at elevated isoflavone concentrations may hamper immune homeostasis. However, the required dosis may also vary depending on the specific application, namely on a daily basis under normal conditions or as a therapy in case of infection or cancer.

2. Future directions

Nowadays many efforts pursue to resolve how nutrition affects gene expression (alternatively called "nutrigenomics"). However, recent evidence suggests that exposure to nutrients also affects epigenetics, as e.g. maternal genistein diet was found to protect mouse offspring from obesity by modifying the fetal epigenome in utero (Dolinoy et al, 2006). As we have noticed that genistein tremendously influences chromatin accessibility in the IL-6 promoter in human primary cells, it is conceivable that soy diets may modulate gene expression profiles not only by interfering with signaling factors, but by scrambling the chromatin settings as well. In this thesis, we elucidated the effects of isoflavones on upstream signaling. Nevertheless, additional modulation at the level of chromatin modifiers, such as DNMTs or remodelling complexes, cannot be excluded. With regard to reports of genistein-triggered changes in DNA methylation (Dang et al, 2003; Dolinoy et al, 2006; Fang et al, 2005), the relation between isoflavones and epigenomics may be an interesting aspect to focus on in the future.

3. References


IV. SAMENVATTING

1. Algemene discussie

Experimenten in verschillende celtypes, waaronder fibroblastcellen, epitheliale borstkankercellinen, TM4 Sertoli-cell en dendritische cellen onthulden dat isoflavonen in staat zijn om NF-κB-gedreven genexpressie sterk te verminderen. Meer bepaald het pro-inflammatoir cytokine IL-6 is duidelijk beïnvloed door meerdere isoflavonen (waaronder genisteïne, daïdzeïne en biochanine A) op het niveau van proteïne en mRNA, hoewel in verschillende mate. Aangezien we voornamelijk geïnteresseerd waren om de moleculaire aangrijpingspunten van isoflavonen op de stimulus-geïnduceerde signaalweg naar IL-6 op te helderen, hebben we ons toegelegd op isoflavoneffecten op het transcriptieniveau. Veel verschillende stimuli, welke variëren van groeifactoren, pro-inflammatoire cytokines tot PAMPs, activeren IL-6 transcriptie. Gezien de rol van IL-6 in ontsteking en immunititeit, bestudeerden we het mechanisme van isoflavonen na TNF-α- of LPS-inductie. Wij onderzochten de signaalweg naar NF-κB, omdat deze transcriptiefactor een centrale rol speelt in het uitlokken van IL-6 transcriptie na zowel TNF-α (Vanden Berghe et al, 1999) als LPS (data niet getoond).

Isoflavonen halen de TNF-α-gestimuleerde NF-κB-gemedieerde gentranscriptie sterk onderuit door te interfereren met het activatiepatroon van MEK1 kinase en vervolgens ook dat van ERK MAPK. Deze gedeeltelijke blokkade leidt uiteindelijk tot een lager activatieprofiel van het stroomafwaartse MSK1. Het ogenschijnlijk paradoxaal verschil in de sterkte van inhibitie van IL-6 transcriptie tegenover MSK1-inhibitie wordt verklaard door het bijkomende effect van isoflavonen op MSK1-recrutering naar de IL-6 promotor. Aangezien het geweten is dat MSK1 transcriptiefactor-activatie door fosforylatie regelt, draagt de door isoflavon gemedieerde MSK1-inhibitie bij tot de vermindering van intracellulaire NF-κB p65 Ser276- en histon H3-fosforylatie. Een gebrek aan H3-fosforylatie en -fosfoacetylatie is specifiek waargenomen op de IL-6 promotor, wat overeenstemt met de invloed van isoflavonen op de activiteit van cofactoren. Meer bepaald interfereert genisteïne met CBP-gemedieerde HAT-effecten en voorkomt TSA-geïnduceerde transcriptionele activatie, waarschijnlijk door zijn effecten op NF-κB-transactivatie. Aangezien zowel cruciale transcriptionele elementen, zoals NF-κB en een toegankelijke chromatineconfiguratie, op deze manier onderdrukt worden, zijn noodzakelijke componenten voor effectieve transcriptie-activatie gecompromiteerd.
Het is interessant dat het mechanisme waarmee genisteïne IL-6-transcriptie na LPS reduceert totaal verschillend is van TNF-\(\alpha\), aangezien de LPS-geïnduceerde MSK1-fosforylation kinetiek enkel lichtjes beïnvloed wordt, wat overeenkomt met het gebrek aan effect op fosfo-p65 Ser276-niveaus. In tegenstelling hiermee, beïnvloedt genisteïne IL-6-transcriptie door Ser15-fosforylation te veranderen, en hierdoor ook de stabilité van het p53. Ook de NF-\(\kappa\)B-DNA-binding en subcellulaire NF-\(\kappa\)B-localisatie worden door genisteïne veranderd, hoewel de LPS-geïnduceerde IKK\(\alpha/\beta\)-activatie (gemeten door analyse van fosforylationen niveaus) en I\(\kappa\)B\(\alpha\)-degradatie intact zijn. Hier speelt de door genisteïne gemedieerde interferentie van p53 met NF-\(\kappa\)B-localisatie en NF-\(\kappa\)B-affhankelijke transcriptionele activiteit een rol in de aanzienlijke reductie van IL-6-transcriptie. Gebaseerd op deze observaties kunnen we besluiten dat isoflavonen signaalweg- en/of celtype-specificieke moleculaire aangrijpingspunten hebben in de regulatie van IL-6 transcriptie.

In onze studie blijven benaderingen om de eigenschappen of mechanismen, waarmee isoflavonen inwerken, beperkt tot het vergelijken met prototype-molecules voor de meeste van de gekende isoflavoneigenschappen, waaronder ER/PPAR\(\gamma\)-liganden, inhibitoren voor PDE/PI3K/tyrosinekinases en anti-oxidanten. Aangezien enkel tyrosinekinase-inhibitoren en anti-oxidant molecules de TNF-\(\alpha\)-geïnduceerde IL-6-geneexpressie in dezelfde mate als isoflavonen inhiberen en eveneens fosfo-MSK1 niveaus verminderen (voor NAC ook beschreven in Sury et al, 2006), zouden isoflavonen kunnen inwerken als inhibitoren van tyrosinekinases of als ROS ‘scavengers’, hoewel afdoende bewijzen voor deze activiteiten moeilijk te verkrijgen zijn. De effecten van isoflavonen teniet doen door experimentele middelen zoals ROS-overproductie, zou leefbaarheid en andere cellulaire systemen beïnvloeden. Ook de exacte rol en betrokkenheid van die mogelijke spelers in TNF-\(\alpha\)-gestimuleerde IL-6-transcriptie en de ‘cross-talk’ met MEK1, MSK1 of NF-\(\kappa\)B blijft onbekend. Ideaal gezien zou de identificatie van de exacte cruciale spelers farmacologische modelering van doelwit-specifieke moleculen toelaten. Nochtans zouden de diverse eigenschappen van isoflavonen op een synergistische manier kunnen werken, zodat selectie van één mechanisme als doel voor ontstekingsmodulatie nadelig zou kunnen uitvallen. Tegenstrijdig hiermee beïnvloeden uit allerlei geteste stoffen enkel anti-oxidanten en de PI3K-inhibitor wortmannine de LPS-gestimuleerde IL-6 proteïneproduktion in MoDCs, respectievelijk op een negatieve en positieve manier (data niet getoond). Dit toont aan dat oxidatieve stress bijdraagt aan LPS-signalisatie, maar of dit een bijkomend aangrijpingspunt van genisteïne is of niet, of gekoppeld is aan p53-effecten, blijft onbekend.

Naast inzicht in de stimulus-specifieke effecten van isoflavonen hebben we nu eveneens aanwijzingen van gen-specificiteit, gerelateerd aan het moleculaire
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aangrijpingspunt van isoflavonen in de TNF-α-signalweg, nl. MSK1. Het inductiepatroon van MEF-cellen zonder MSK1/2 komt overeen met het expressieprofiel van met genisteïne behandelde fibroblasten, waaruit blijkt dat NF-κB2 verschillend gereguleerd is ten opzichte van IL-6 of VCAM1. Nochtans moet verder onderzoek gebeuren om te verifiëren of deze genspecificiteit voor alle MSK1-gereguleerde genen telt en of een gelijkaardige afhankelijkheid voor p53-gereguleerde genen bestaat in MoDCs. Tot dusver hebben we geen enkel LPS-gestimuleerd NF-κB-afhankelijk gen gevonden dat niet beïnvloed wordt door genisteïne in MoDCs, hoewel geen statistisch significant verschil gezien kan worden in de door genisteïne gemedieerde repressie van IL-12 p35- en p40-transcriptie tussen p53 'wt' en 'null' BMDCs (data niet getoond). Dit wijst er mogelijk op dat genisteïne andere doelwitten heeft naast p53, ofwel dat IL-12 promotors niet p53-gereguleerd zouden zijn.

Met betrekking tot de invloed van isoflavonen op gezondheid en fysiologie reiken onze data naar andere LPS-geïnduceerde NF-κB-gereguleerde cytokines en maturatiemerkers. Hieruit blijkt dat genisteïne ook deze vermindert, maar in verschillende mate, wat wijst op genspecifieke regulatorische programma’s. Verder represseert genisteïne ook signalisatie naar IL-6-expressie vanuit andere TLR-types, wat overeenkomt met zijn modulatie van een stroomafwaartse centrale speler in IL-6 regulatie, nl. NF-κB. Gezien de functie van primaire DCs in controle van T cel-activatie en adaptieve immuunresponsen, wijzen deze resultaten op interferentie van isoflavonen met immunomodulatoire cytokineproductie, evenals met de costimulatoire en antigen-presenterende capaciteit van DCs. Gebaseerd op deze effecten zou genisteïne de efficiënte ‘priming’ en polarisatie van naïeve T cellen inhiberen, waardoor het opzetten van een passende immuunrespons beïnvloed zou worden. We nemen aan dat genisteïne acute, niet-blijvende responsen in DCs uitlokt, welke hun functionaliteit wel beïnvloeden. Daarom zou een continu hoge blootstelling van isoflavonen het begin van verworven immuunresponsen belemmeren en vervolgens leiden tot inefficiënte verwijdering van pathogenen en tot chronische pathologieën. Nochtans is voorzichtigheid geboden bij het trekken van systemische conclusies, aangezien het in vivo immuunsysteem uit een netwerk van diverse celtypes bestaat, die allemaal verschillend beïnvloed kunnen zijn door genisteïne. Ook wordt de traditionele sojavoedselinname eerder als ‘gezondheidsbeschermend’ beschouwd, gebaseerd op epidemiologische data. In muisstudies verhoogt genisteïne-blootstelling immuunresponsen, hoewel de effecten variëren, afhankelijk van de duur van blootstelling, geslacht en generatie van nakomelingen (Guo et al, 2005). Nochtans rapporteren anderen dat genisteïne de cellulaire en humorale immuunfunctie in knaagdiermodellen vermindert en thymische atrofie induceert (Kogiso et al, 2006; Yellayi et al, 2003). In gezonde post-menopausale vrouwen verhogen sojamelk en
supplementaire isoflavonen de B cel-populatie (Ryan-Borchers et al, 2006). Studie van
personen, gebruik makende van verschillende eindpuntmerkers en na een immunologische
opstoot, zou deze kwestie meer ophelderen. Terwijl de mogelijke verslechtering van het
immuunsysteem als schadelijk zou kunnen aanzien worden, zou dit op een gunstige manier
kunnen toegepast worden in bepaalde omstandigheden, zoals orgaantransplantatie, zodat
transplantaat-afstoting zou kunnen worden vertraagd of afgezwakt, op een gelijkaardige
wijze als supplementatie met genisteïne en biochanine A in rat harttransplantatiemodellen
(O’Connor et al, 2002; Schrepfer et al, 2005).

In tegenstelling tot de algemene opvatting dat isoflavonen genexpressiemodulatie
dirigeren via ER, omdat ze estrogenachtige eigenschappen bezitten, hebben wij in ons
celsysteem gevonden dat isoflavonen NF-κB-gedreven genexpressie kunnen represseren op
ER-onafhankelijke wijze. ER-agonisten en -antagonisten (met of zonder genisteïne) vertonen
geen effect op de TNF-α-geïnduceerde NF-κB-afhankelijke promotoractiviteit in fibroblasten,
hoewel ze dat wel doen in TM4 Sertolicellen, welke hoge niveaus van ERα/β expresseren.
Meer nog, gelijkaardige isoflavoneffecten zijn opgemerkt in een ER-onafhankelijke (SKBR3)
borstkankercellijn. Aangezien IL-6 een belangrijke groeifactor voor kancercellen is en
geassocieerd wordt met lagere overlevingskansen, zouden isoflavonen een aantrekkelijke
middel kunnen worden in kancertherapieën, zelfs voor hormoon-ongevoelige fenotypes die
meestal overeenstemmen met een slechtere prognosis. In dezelfde groep van slechtere
prognosis borstkancers konden we een gestegen MSK1-expressieniveau waarnemen,
relatief ten opzichte van de groep van goede prognosis kancers, overeenkomstig met de
rapporten over constitutieve MAPK-activiteit in agressieve kancers. In dit opzicht zou MSK1
een belangrijk doelwit in chemotherapie kunnen zijn, onafhankelijk van de ER-status.

Aangezien IL-6-deregulatie betrokken is in de voortgang van verscheidene
pathologieën van hogere leeftijd en chronische ontstekingsziekten, lijkt het gebruik van
isoflavonen om abnormaal hoge IL-6 niveaus te bestrijden of te voorkomen veelbelovend.
Nochtans kunnen we, om hun therapeutisch of preventief potentieel naar waarde te schatten,
niet louter onze observaties in een in vitro systeem extrapoleren om inzicht te verwerven in
de in vivo situatie, aangezien dit een extreem complex systeem is, welke verder onderzoek
rechtvaardigt. De isoflavon-dosis wordt als belangrijk beschouwd in het trekken van
conclusies, omdat het voordien aangetoond is dat lage genisteïne-concentraties (minder dan
1 µM) in vitro kancerproliferatie stimuleren, terwijl hoge concentraties kankergroei inhiberen
(Hsieh et al, 1998; Wang et al, 1996). Een gelijkaardig fenomeen is gevonden voor
osteogenese en adipogenese, waarin de genisteïne-concentratie de balans tussen ER- en
PPAR-gemedieerde effecten bepaalt (Dang et al, 2003). In dit opzicht, moeten we weten
welke intracellulaire concentratie isoflavonen, en dan ook nog in welke chemische vorm, bereikt wordt in onze experimenten op een tijdsafhankelijke manier, wanneer een farmacologische dosis van 200 µM toegepast wordt. Het direct contact en de hoge dosis van 200 µM laten ons toe zwart-wit-effecten te visualiseren, welke minder duidelijk kunnen zijn in vivo, maar daarom niet onbestaande. Lagere concentraties, tot 20 µM genisteïne beïnvloeden nog steeds NF-κB-afhankelijke genexpressie in vitro en verhogen intracellulaire p53 proteïne-niveaus in MoDCs (data niet getoond). Op een gelijkaardige manier halveert 50 µM genisteïne MSK1-fosforylatie in TNF-α-gestimuleerde fibroblasten. Daarbij komt nog dat een continue dagelijkse inname van isoflavonen een bepaalde plasmaconcentratie van verschillende isoflavonmetabolieten met differentiële sterktes veroorzaakt, wat zelfs meer effectief kan zijn dan aparte injecties over korte tijdspenpen in in vitro experimenten. Meer nog, om een overdadige inflammatoire respons of immuunrespon te beperken, zouden gedeeltelijke moleculaire effecten op p53 en MSK1 door fysiologisch bereikbare concentraties voldoende kunnen zijn, terwijl een bijna volledige blokkade van NF-κB-gedreven genexpressie bij verhoogde isoflavon concentraties immuunhomeostase zou kunnen verhinderen. Toch zou de nodige dosis ook kunnen variëren, afhankelijk van de specifieke toepassing, namelijk op een dagelijkse basis onder normale omstandigheden of als een therapie in geval van infectie of kanker.

2. Toekomstperspectieven

Tegenwoordig doelen veel pogingen op het ophelder hoe voeding genexpressie beïnvloedt (alternatief “nutrigenomica” genoemd). Nochtans suggereert recent bewijs dat blootstelling aan nutriënten ook epigenetica beïnvloedt, aangezien gevonden werd dat een genisteïne-dieet van de moedermuis de nakomelingen beschermt tegen obesiteit door het foetaal epigenoom in de baarmoeder te veranderen (Dolinoy et al, 2006). Aangezien we opgemerkt hebben dat genisteïne de chromatine-toegankelijkheid in de IL-6 promotor van menselijke primaire cellen in sterke mate wijzigt, is het mogelijk dat sojavoeding genexpressieprofielen niet alleen door interferentie met signalisatiefactoren zou kunnen moduleren, maar ook door chromatine-opstellingen te beïnvloeden. In deze thesis wierpen we een licht op de effecten van isoflavonen op stroomopwaartse signalisatie. Niettemin kunnen bijkomende modulaties op het niveau van chromatine-modificeerders, zoals DNMTs of remodelleringscomplexen, niet uitgesloten worden. Ten aanzien van rapporten over veranderingen in DNA-methylatie door genisteïne (Dang et al, 2003; Dolinoy et al, 2006;
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Fang et al, 2005), zou de relatie tussen isoflavonen en epigenomica een interessant aspect kunnen zijn om in de toekomst op te concentreren.

3. Referenties


Allereerst wil ik een woord van dank richten tot mijn promotor, Prof. Dr. Guy Haegeman, die me de mogelijkheid heeft geboden om in het verlengde van mijn licentiaatsthesis bij LEGEST een doctoraatsonderzoek aan te vangen. Zijn goedkeuring en jarenlange steun liggen aan de basis van dit wetenschappelijk werk.

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Nathalie
Addendum

Education

1998-2000

Bachelor (candidate) in Biology, University of Hasselt, Belgium
Succeeded with great distinction

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Master (licentiate) in Biotechnology, University of Gent (Ugent), Belgium
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Master thesis: ‘Investigation of the effect of estrogenic molecules on inflammatory systems.’
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The Journal of Immunology, accepted
Research article
Thesis submitted in partial fulfillment of the requirements for the degree of Doctor (PhD) in Sciences: Biotechnology

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