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Interaction of *Allium sativum* L. and *Leontopodium alpinum* Cass. with mediators of inflammation involved in the molecular mechanisms of atherosclerosis

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Erklärung

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Ehrenwörtliche Versicherung

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Wissenschaft basiert auf
Zufall, Versuch und Irtum.
(Marcel Asper, Spiegel 19;2003)

I. Contents

II. Introduction

1. Background and aim of the work

Fundamentally inflammation is a protective response to injury. The ultimate goal of this process is to rid the organism of both the initial cause of cell injury (e.g. microbes, toxins) and the consequences of such injury (e.g. necrotic cells and tissue). Further on it sets up, as far as possible, the stage for healing and reconstitution of the injured tissue. Without inflammation, infections would go unchecked, wounds would never heal, and injured organs might remain permanent festering sores. However, chronic inflammatory diseases (e.g. atherosclerosis, arthritis, colitis, multiple sclerosis or lung fibrosis) are the number one cause for morbidity and mortality in humans.

An important process during the onset of the inflammatory response is the activation of the nuclear factor kappa B (NF-κB). NF-κB is an ubiquitous transcription factor, which is among others involved in the transcription of cytokines, chemokines, other transcription factors, adhesion molecules and growth factors. Adhesion molecules like E-selectin and intercellular adhesion molecule-1 (ICAM-1) are important for leukocyte recruitment and transmigration across the endothelium to the interstitial tissue.

Garlic (*Allium sativum* L.) is one of the eldest medical plants known and was used as a treatment for heart disorders, tumors, worms, bites and other illnesses (Rahman, 2001). Since the 1960´s garlic attracted increasing attention by investigators around the world. It is proposed, that garlic extracts mediate anti-atherosclerotic properties by lowered cholesterol synthesis, LDL oxidation and fibrinolytic activity (Gebhardt, 1993; Lau, 2001; Legnani et al., 1993). Further studies revealed anti-inflammatory properties of garlic (Geng et al., 1997). However, the described *in vitro* effects could not be conclusively reproduced in *in vivo* studies (Steiner et al., 1996a; Yeh and Liu, 2001; Silagy and Neil, 1994). So far the anti-atherosclerotic and anti-inflammatory effects and the underlying molecular mechanisms of garlic remain elusive.

Edelweiss (*Leontopodium alpinum* Cass.) was used by inhabitants of the Alps as traditional folk medicine against inflammatory diseases of the gastrointestinal tract, diarrhea in human and animals (Pickl-Kerk, 1995; Kiene, 1992). However, up to now pharmacological studies of the anti-inflammatory properties of edelweiss constituents are missing completely.

Aim of the present work was the investigation of molecular mechanisms involved in the proposed anti-inflammatory properties of *A. sativum* and *L. alpinum*. In the course of the present thesis work, the following questions should be answered:

1. Are compounds and extracts of *A. sativum* capable to inhibit NF-κB activation and subsequently the expression of adhesion molecules?

2. Is garlic able to modify cytokine release from human blood cells?

3. Is sulfur-fertilization of garlic plants a reasonable approach in order to improve the biological effects of garlic?

4. Are constituents of *L. alpinum* able to inhibit NF-κB activation and the succeeding expression of adhesion molecules and what pathways are involved?

The present studies about garlic were part of the EU Garlic & Health project supported by the European Community.
(QLK1-CT-1999-00498; www.plant.wag-ur.nl/projects/garlicandhealth).

2. Pathologic basis of inflammation

Inflammation is the reaction of vascularized living tissue to local injury. The course of inflammation can be divided in acute and chronic conditions. Acute inflammation can last up to a few days and shows typical events like exudation of fluid and plasma proteins and the emigration of leukocytes. Chronic inflammation is of longer duration and is associated histologically with the presence of lymphocytes and macrophages at the site of inflammation, destruction of tissue by proteolytic enzymes and deranged healing by replacing damaged tissue against connective tissue (Cotran et al., 1999).

2.1 Short overview of processes during acute inflammation

After injury of tissue by microbial infections, chemicals and physical agents, increased blood flow in the injured area can be detected. In combination with an increased vascular permeability protein-rich extravascular fluid leaves the venous vessel through widened inter-endothelial cell junctions. Attracted by chemokines, like monocyte chemoattractant protein-1 (MCP-1), leukocytes adhere to the endothelium by binding to adhesion molecules and transmigrate across the endothelium to the site of inflammation. Recruited leukocytes eliminate the harmful substance and set the stage for repair and healing (Cotran et al., 1999).

2.2 Atherosclerosis, an inflammatory disease

Atherosclerosis and its clinical manifestations of heart attack, stroke and peripheral vascular insufficiency contribute to a higher mortality and serious morbidity in the western world than any other disease. Commonly, atherosclerosis is characterized by the formation of intimal fibrous plaques that often have a central necrotic core rich in lipids (Lusis, 2000). Risk factors for the development of atherosclerosis are hyperlipidemia, hypertension, diabetes and cigarette smoking but also gender, age and genetic predisposition. The development of an atherosclerotic plaque starts with an injury of the endothelial lining in large and medium sized arteries (Gimbrone, 1999). A major cause of injury to the endothelium are elevated levels of low-density lipoprotein (LDL). LDL particles are trapped in the artery by specific interactions with apolipoprotein B (ApoB) containing lipoproteins and are oxidized to oxLDL (Watson et al., 1997). Oxidation of LDL can be inhibited by high-density lipoprotein (HDL) (Lin et al., 2002). Oxidized LDL stimulates the endothelium to produce pro-inflammatory substances, like adhesion molecules, chemokines and growth factors (Yla-Herttuala et

al., 1991; Dwivedi et al., 2001). The recruitment of leukocytes (especially monocytes, but not neutrophils) is mediated by adhesion molecules (e.g. P- and E-selectin, ICAM-1 and VCAM-1) (Dong et al., 1998; Poston et al., 1992) and chemotactic factors (e.g. MCP-1) (Gerszten et al., 1999) as depicted in figure 1A. Monocytes are differentiated to macrophages, which start to take up oxLDL *via* scavenger receptors (SR-A and CD36) and transform into foam cells (Suzuki et al., 1997).

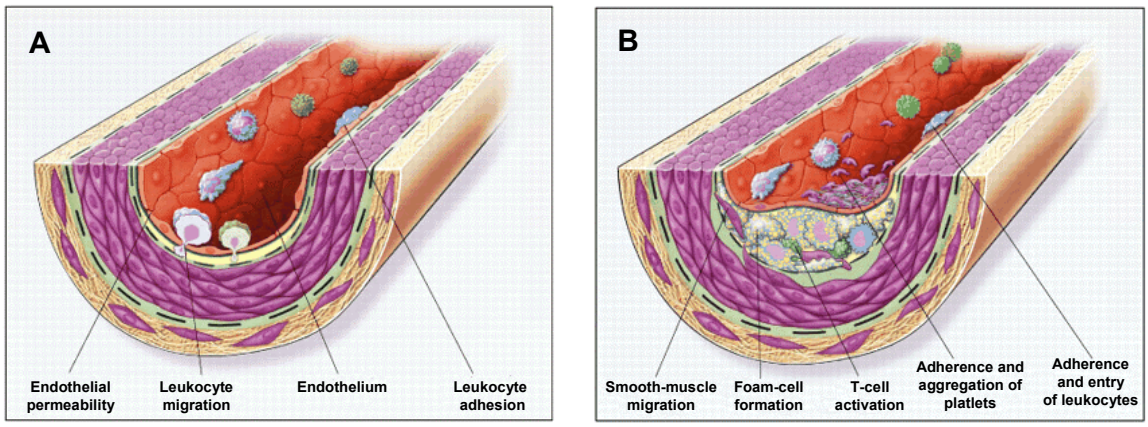


Figure 1: Endothelial dysfunction and fatty streak formation in atherosclerosis

A: The earliest changes in the endothelium include increased permeability for LDL, leading to recruitment and transmigration of leukocytes by up-regulation of adhesion molecules.
B: During the fatty streak formation monocytes and macrophages take up oxLDL and transform into foam cells. Smooth muscle cells are stimulated by various growth factors, including platelet derived growth factor and transforming growth factor, to migrate in the fatty streak. T-cells become activated by a series of cytokines, such as TNF-α, IL-2 and GM-CSF.
Pictures are adopted from the homepage of the University of Arizona Biology Learning Center (http://www.blc.arizona.edu/courses/181gh/Lectures_WJG.01/LDL_F.01/atherosclerosis.html)

T-cells and macrophages are activated by interaction of CD40 and CD40 ligand (CD40L) (Hansson et al., 2002). Activated macrophages and T-cells secrete cytokines, (like TNF-α, IL-1, interferon-γ (INF-γ) and transforming growth factor β (TGF-β)), proteolytic enzymes (especially metalloproteinases) and growth factors (platelet derived growth factor or insulin-like growth factor I). The secretion of cytokine and growth factors results in migration and proliferation of smooth muscle cells (SMC) (figure 1B). Progressive proliferation of SMC and macrophages, and recruitment of leukocytes leads to the formation of more complex lesions (Lusis, 2000). In this type of lesion foam cells die, leaving behind a growing mass of cell debris and extra-cellular lipids, which form the necrotic core. The necrotic core is walled off the lumen by a fibrous cap consisting of smooth muscle cells (Jonasson et al., 1986). Continuous leukocyte recruitment causes

the expansion of the lesion (figure 2A). Due to constant secretion of collagenases or gelatinases by macrophages and T-cells, the structure of the atherosclerotic plaque becomes vulnerable (Galis et al., 1994). Once the structure of the plaque becomes too unstable it breaks open and leads to thrombosis which can result in occlusion of the artery, leading to acute coronary events like stroke and myocardial infarction (figure 2B).

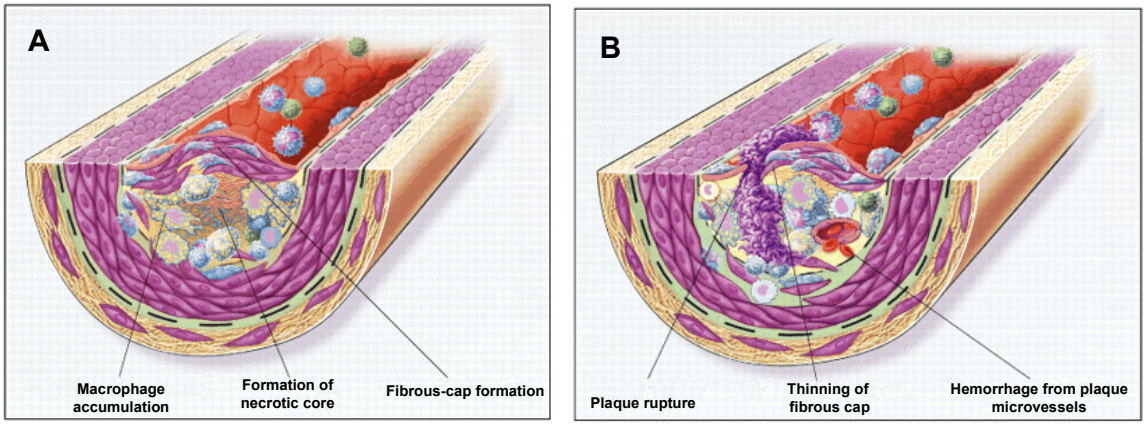


Figure 2: Formation of advanced lesion and unstable fibrous plaques in atherosclerosis

A: Formation of advanced atherosclerotic lesion is characterized by formation of a fibrous cap consisting of smooth muscle cells and underlying necrotic core. The necrotic core consists of a mixture of lipids, cell debris and leukocytes.
B: Rupture of the atherosclerotic plaque occurs mainly at the lesion edges, which are rich in foam cells, as a result of constant thinning of the fibrous cap. Proteolytic enzymes, like collagenases, gelatinases and cathepsin are responsible for the degradation of the fibrous cap.
Pictures are adopted from the homepage of the University of Arizona Biology Learning Center (http://www.blc.arizona.edu/courses/181gh/Lectures_WJG.01/LDL_F.01/atherosclerosis.html)

A central mediator of atherogenesis is the transcription factor nuclear factor-kappa B. It was shown by Brand et al. that activated NF-κB is present in SMC, macrophages and endothelial cells in atherosclerotic lesions. However, in healthy vessels little activity has been measured (Brand et al., 1996). Activated NF-κB is associated with the pathophysiology of the vessel wall, including cytokine and chemokine production, expression of adhesion molecules and genes that regulate cell proliferation and cell survival (Collins and Cybulsky, 2001).

2.3 Activation of the nuclear factor - kappa B

The nuclear factor - kappa B (NF-κB) is a ubiquitous dimeric transcription factor, involved in the activation of a large number of genes in response to infections, inflammation, and other stressful situations. Since the discovery in 1986 (Sen and Baltimore, 1986) the nuclear factor kappa B has been intensively investigated and is now one of the best understood transcription factors. It is composed of members of the REL family of DNA binding proteins including REL A (p65), NF-κB1 (p50;p105), NF-κB2 (p52; p100), c-REL and REL B (Ghosh et al., 1998). They all recognize with high affinity a specific decameric DNA sequence motif, i.e. 5′-GGGPuNNPyPyCC-3′ (Grimm and Baeuerle, 1993). The nuclear factor kappa B can be rapidly activated by a wide range of stimuli, e.g. pro-inflammatory cytokines like tumor-necrosis factor (TNF) or interleukin-1 (IL-1), pathogens like lipopolysaccharide (LPS), viruses and physical and chemical stress, including oxidized lipids, oxidative stress and shear stress (Ghosh et al., 1998; Michiels et al., 2002; Li and Karin, 1999; Weber et al., 1999; Imberti et al., 2000; Robbesyn et al., 2003). Under normal conditions active NF-κB is sequestered in the cytoplasm by specific inhibitors called IκB proteins (e.g. IκB-α, IκB-β, IκB-ε). Recent studies showed that IκB-NF-κB complexes may also shuttle from the cytosol to the nucleus and back (Malek et al., 2001; Huang et al., 2000; Lee and Hannink, 2002). Most known stimuli, except UV-radiation and H_2O_2, lead to the phosphorylation of the IκB-kinase complex (IKK) (Karin and Ben Neriah, 2000). IKK mediates phosphorylation of IκB at specific amino-terminal serine residues. For example, IκBα is phosphorylated at Ser32 and Ser36 (Traenckner et al., 1995).The phosphorylated IκB is then poly-ubiquitinylated at Lys21 and Lys22, marking it for degradation by the 26S proteasome (Baldi et al., 1996). After degradation of IκB free NF-κB dimers translocate to the nucleus. Activity of NF-κB is not exclusively regulated by signal-induced IκB degradation, but also by direct modification of NF-κB proteins through phosphorylation and acetylation (Sizemore et al., 1999; Wang and Baldwin, 1998; Chen et al., 2001). It has been demonstrated that various kinases (protein kinase A, casein kinase II and IKK2) phosphorylate p65 at a specific serine residue leading to enhanced transcriptional activity (Sakurai et al., 1999; Sizemore et al., 2002; Zhong et al., 1997). Phosphorylation of p65 is required for further modification by acetylation (Li and Verma, 2002). Recent reports described that a p50-p50-HDAC1 complex represses NF-κB transcription in unstimulated cells After stimulation of cells, phosphorylated p65 contacts with the co-activator CREB binding protein (CBP) and becomes acetylated. The complex of acetylated p65-CBP displaces the p50-p50-HDAC1 complex from the NF-κB binding site (Ashburner et al., 2001; Zhong et al., 2002)..

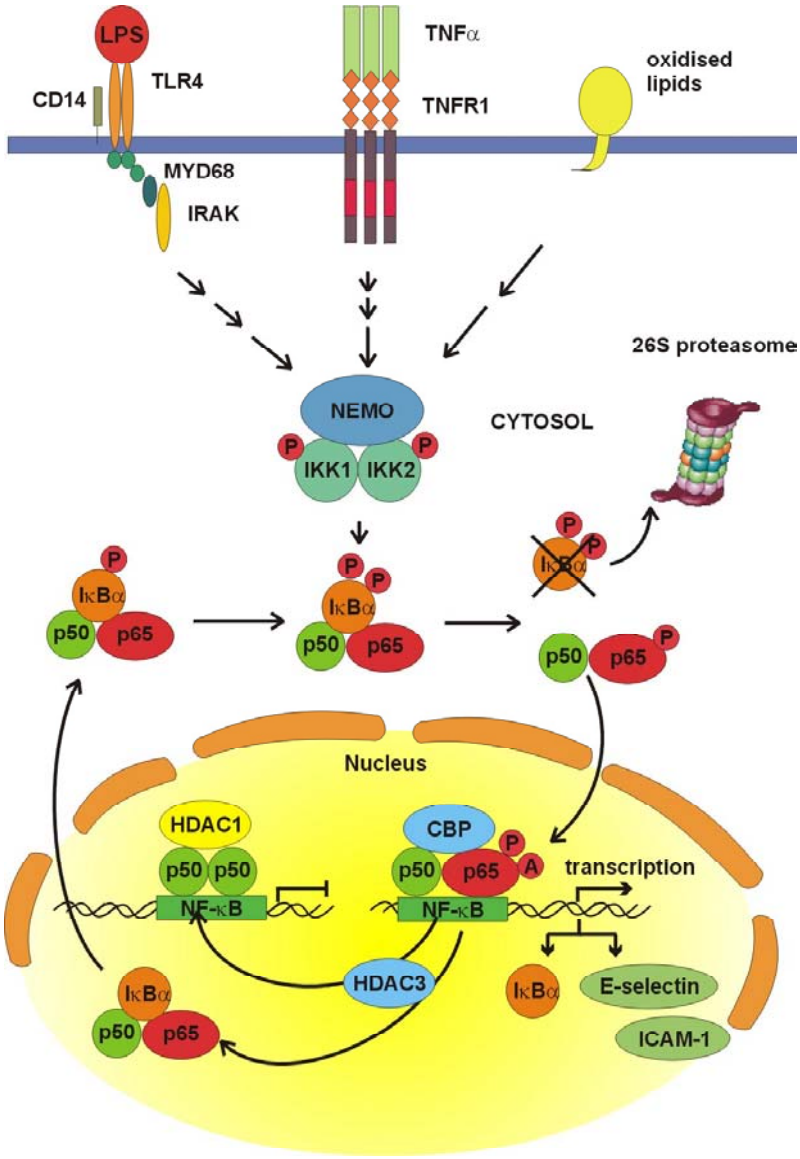


Figure 3: Scheme of NF-κB activation pathways

Nuclear factor-κB (NF-κB) is activated by a wide range of stimuli, including lipopolysaccharide (LPS), tumor necrosis factor (TNF) and oxidized lipids. Ligation of diverse stimuli to the various receptors launches a cascade leading to the phosphorylation of the IκB-kinase complex (IKK): The IKK is composed of IKK-1 and IKK-2 and the regulatory subunit NF-κB essential modulator (NEMO). Activated IKK phosphorylates IκB, which leads to its degradation by the 26S proteasome, and allows NF-κB dimers to translocate to the nucleus. After IκB degradation NF-κB is subject to further phosphorylation and acetylation in order to control its activity. Possible target genes of NF-κB are intercellular adhesion molecule-1 (ICAM-1), E-selectin or IκB. Newly synthesized IκB displaces NF-κB from its DNA binding sites and subsequently the NF-κB-IκB complex translocates to the cytoplasm.

Depending on the cell type and circumstance of its activation NF-κB can regulate different genes. NF-κB-regulated genes are cytokines (e.g. TNF, IL-1, IL-2), chemokines (monocyte chemoattractant protein (MCP-1, IL-8), transcription factors

(e.g. p50, cRel), NF-κB inhibitor IκBα, growth factors (M-CSF; G-CSF, GM-CSF) and many others (Ledebur and Parks, 1995; Albrecht et al., 1995; Quentmeier et al., 2000; Auphan et al., 1995; Baeuerle and Henkel, 1994; Guha and Mackman, 2001). The duration of NF-κB activity is regulated by histone deacetylase 3 (HDAC3). HDAC3 deacetylates p65 and thus enhances the binding activity to IκB (Chen et al., 2001). Newly synthesized IκB terminates NF-κB activity by displacing NF-κB from its DNA binding sites. The nuclear export signal (NES) on the IκB protein marks the IκB-NF-κB complex for export to the cytosol (Huang et al., 2000). A simplified scheme of NF-κB activation pathways is depicted in figure 3.

2.4 Leukocyte adherence and transmigration across the endothelium

A critical function of inflammation is the proper delivery of leukocytes to the site of injury, in order to challenge intruding pathogens. However, unbalanced extravasation of leukocytes may prolong inflammation and induce tissue damage by releasing proteolytic enzymes (e.g. phosphatases, collagenases), chemical mediators (e.g. TNF, INF-γ) or toxic oxygen radicals. The coordinated interaction between the endothelium and leukocytes is divided into four sequential steps leading to leukocyte transmigration (figure 4). The hemoconcentration that follows vascular leakage in the early phase of the inflammatory response slows blood flow so that leukocytes can make contact with the endothelium. The initial contact is mainly mediated by the selectin family of adhesion molecules (e.g. P-selectin, L-selectin and E-selectin) and serves to attach and slow down the trespassing leukocyte, also referred to as rolling (Lorenzon et al., 1998). To stop rolling, the low affinity transient interactions of rolling must be replaced by high affinity adhesion between leukocyte and endothelium. Such strong adhesions are triggered by stimuli (e.g. chemokines, cell surface proteins) presented by the endothelium (Baggiolini, 1998). After leukocyte activation integrins bind to their counterparts on the endothelium, namely adhesion molecules of the immunoglobuline family (e.g. ICAM-1, VCAM-1) (Reilly et al., 1995; Muller, 2002). Now the leukocyte stops rolling and begins to crawl on the endothelial cell surface. Although the preceding steps in leukocyte migration are potentially reversible, the transmigration of leukocytes (diapedesis) is the “point of no return”. The leukocyte undergoes a dramatic change in cell shape and extensive remodeling of cyto-skeleton, enabling it to squeeze through the endothelial junction (Hahn et al., 1997; Burns et al., 1997; Schenkel et al., 2002).

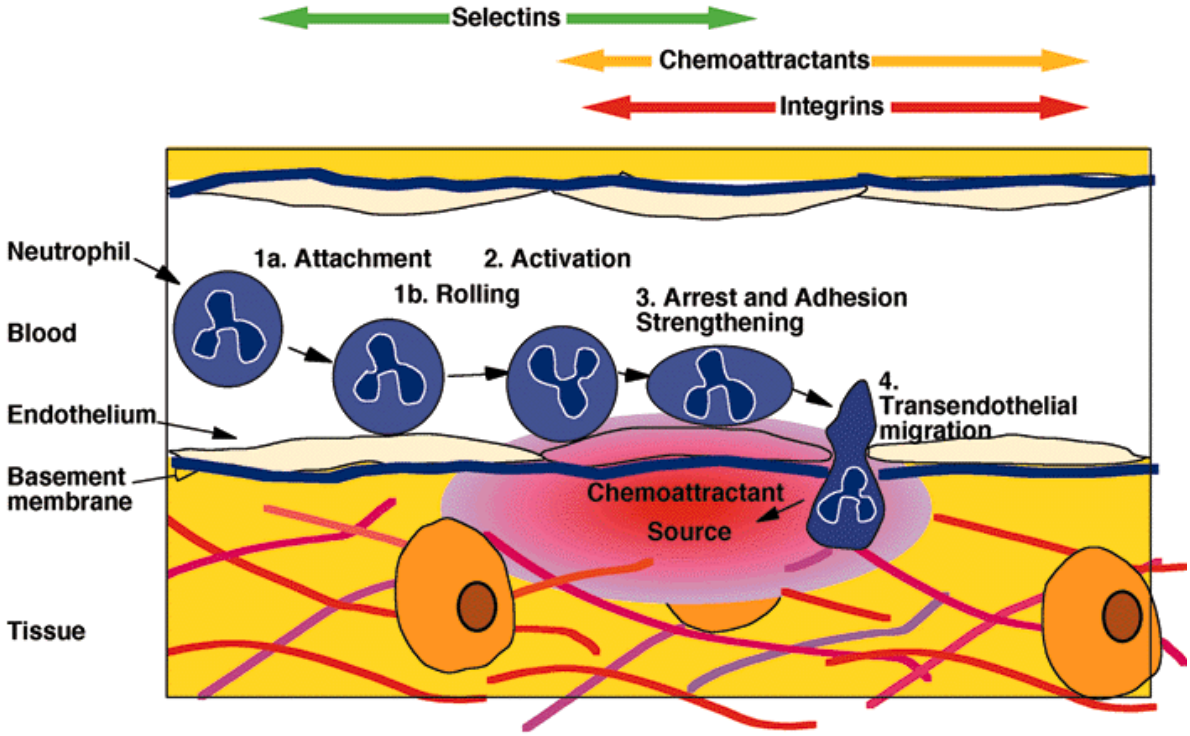


Figure 4: Recruitment of leukocytes to the site of inflammation
At least four steps of the adhesion cascade are involved in leukocyte adhesion to and migration out of the vascular endothelium: (1) Attachment (tethering) and rolling, in which selectins and glyco-conjugated selectin-ligands are involved, (2) activation, mediated by soluble and/or membrane-bound chemokines, (3) firm adhesion and (4) transmigration, both mediated by integrins and their ligands, e.g. intercellular adhesion molecules etc.. Each of these four steps appears to be necessary for effective leukocyte recruitment, because blocking any step can severely reduce leukocyte accumulation in the tissue
Adapted from the Weizmann Institute of Science
(www. bioinfo.weizmann.ac.il/_ls/ ronen_alon/ronen_alon.html)

Newer investigations showed, that adhesion molecules are not only docking-stations for leukocytes. Furthermore, ligation of ICAM-1 can induce VCAM-1 expression through activation of extra-cellular signal-regulated kinase (ERK) and AP-1. (Lawson et al., 1999; Takahashi et al., 1996; Wang and Doerschuk, 2002). Adhesion molecules important for the recruitment of leukocytes are listed in table 1 (Aplin et al., 1998).

Endothelial molecule	Leukocyte counter-ligand	Major role
P-selectin	PSGL-1	Tethering, rolling
Sialyl-Lewis X	L-selectin	Tethering, rolling
E-selectin	ESL-1, CLA bearing sLeX,	Tethering, rolling
VCAM-1	VLA4	Tethering, rolling, firm adhesion
ICAM-1	LFA-1, MAC-1	firm adhesion
PECAM-1	PECAM-1	Diapedesis
CD99	CD99	Diapedesis

Table 1: Common leukocyte-endothelial cell adhesion molecules in inflammation

PSGL-1, P-selectin glycoprotein 1; VLA-4, very late antigen 4; VCAM-1, vascular cell adhesion molecule 1; LFA-1, leukocyte function antigen-1; ICAM-1, -2, intercellular adhesion molecule-1, -2; CR3, complement receptor 3; PECAM-1, platelet/endothelial cell adhesion molecule-1, ESL-1, E-selectin ligand, CLA, cutaneous lymphocyte antigen.
(Adapted from Muller, 2002)

2.5 Cytokines involved in inflammation

Cytokines are proteins, mainly produced by activated lymphocytes and macrophages, but also by endothelial and epithelial cells or the connective tissue. They modulate the function of other cell types. Initially they were associated with cellular immune response. Nowadays it has been demonstrated that they also play a critical role in inflammation. Lipopolysaccharide (LPS) induces the liberation of cytokine (e.g. TNF-α, IL-1, IL-6, GM-CSF, IL-10) in monocytes and macrophages. Involved in this process are various mitogen activated protein kinases, like ERK, c-Jun N-terminal kinase (JNK) and p38 pathways and transcription factors, such as activator protein-1 (AP-1), serum response element (SRE) and cAMP-responsive enhancer-binding protein (CRE). Further the NF-κB and the PI3-kinase-AKT pathway plays an important role for the expression of cytokines in response to injuring stimuli (Guha and Mackman, 2001).

2.5.1 Tumor necrosis factor α (TNF-α)

Human TNF-α is a non-glycosylated protein with a weight of 17 kDa and a length of 157 amino acids. TNF-α is a pro-inflammatory cytokine and is mainly produced by activated monocytes and macrophages. Exposure of cells to TNF-α can either lead to apoptosis, activation of AP-1 and NF-κB or even to expression of anti-apoptotic genes (Baetu and Hiscott, 2002; Baud and Karin, 2001; Poulaki et al., 2002). TNF-α enhances activation of T-cells and induces proliferation of T-cells and B-cells. Tumor necrosis factor attracts macrophages and granulocytes to sites of inflammation. This attraction stimulates further macrophages and other immune cells to release tissue damaging, oxygen containing substances and prostaglandins to promote further inflammation.

2.5.2 Interleukin 1 (IL-1)

Interleukin-1 is the prototype of a "multifunctional" cytokine, since it affects nearly all cells. IL-1 gene family consists of three members IL-1α, IL-1β and IL-1 receptor antagonist (IL-1ra). The effects mediated by IL-1α and IL-1β are biologically more or less equivalent.
IL-1 stimulates T-helper cells, B-cells, NK-cells and fibroblasts, promoting their proliferation and activation (Fauteux and Osmond, 1996; Collins and Oldham, 1995). IL-1 causes many alterations of endothelial functions *in vivo*. It promotes thrombotic processes and attenuates anti-coagulatory mechanisms (Joseph et al., 2002). IL-1 therefore plays an important role in pathological processes such as venous thrombosis, arteriosclerosis, and vasculitis. Further IL-1 promotes the adhesion of neutrophils, monocytes, T-cells, and B-cells by enhancing the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin (McDouall et al., 2001). IL-1 is also a strong chemoattractant for leukocytes; in vivo the injection of IL1- leads to the local accumulation of neutrophils at the site of injection. IL-1 also activates oxidative metabolism in neutrophils (Dinarello, 1996).

2.5.3 Interleukin 10 (IL-10)

IL-10 is a homodimeric protein with subunits having a length of 160 amino acids, belonging to the group of anti-inflammatory cytokines. IL-10 inhibits the synthesis of a number of cytokines, such as interferon-γ (INF-γ), interleukin-1 and TNF-β in subpopulations of T-cells (Chung, 2001). In humans, IL-10 is mainly produced by T-cells and monocytes, and it down-regulates the function of Th1 and Th2 T-cells, macrophages, monocytes. In macrophages stimulated by bacterial LPS, IL-10 inhibits the synthesis of IL-1, IL-6 and TNF-α mainly by promoting the degradation of cytokine mRNA (Lisinski and Furie, 2002; Denys et al., 2002). It also leads to an inhibition of antigen presentation. In human monocytes INF-γ and IL-10 antagonize each other's production and function (Moore et al., 2001).

3. Plants as anti-inflammatory drugs

For thousands of years mankind treated maladies and ailments with plant extracts. The knowledge of the medical benefit of plants was not restricted to advanced cultures, like the Egyptians, as the glacier mummy in the Oetztal alps proofed (Spindler, 1995). Knowledge of the therapeutic use of plants and their extracts increased over centuries, leading to the discovery of drugs like acetylsalicylic acid, morphine or quinine. The quest nowadays is to set the assumed therapeutical effects of traditional medical plants on a scientific basis. Therefore it is important to elucidate the molecular mechanisms how plants mediate their therapeutic effects.

3.1 Garlic (*Allium sativum* L.)

Garlic is one of the most quoted herbs with medical potentials in literature. It was first mentioned in the ancient *Codex Ebers* 3500 years ago. Garlic was used all over the world by Chinese, Greeks, Egyptians, Indians, Koreans or Romans. Astonishingly garlic was used by the various cultures for treatment of similar symptoms. It was mainly given in order to cure ailments of the heart, gastrointestinal tract, infections and abnormal growth (Rivlin, 2001). These days it is assumed that garlic can mediate beneficial effects as an anti-thrombotic, anti-tumor and anti-atherogenic agent.

3.1.1 Sulfur compounds and metabolites of garlic

Dried garlic contains approximately 1 % sulfur. The main sulfur constituents of whole garlic are alliin, methiin, isoalliin and two γ-glutamylcysteins. When garlic tissue is harmed alliin, isoalliin and methiin undergo a rapid enzymatic transformation, forming mainly allicin. The enzymatic reaction is catalyzed by alliinase, a pyridoxal 5´-phosphate dependent glycoprotein, normally stored in the vacuoles (Lawson, 1996; Kuettner et al., 2002; Lawson et al., 1991). The chemical reaction for the transformation is depicted in figure 5.

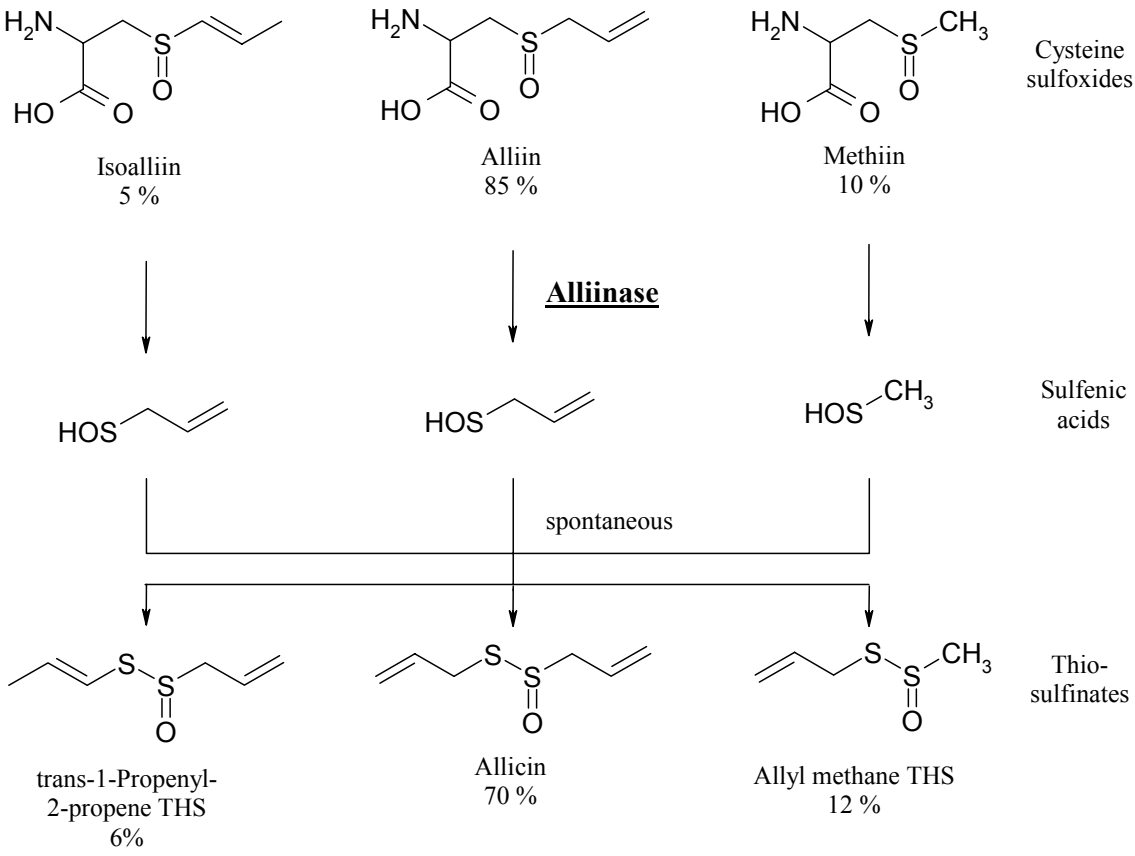


Figure 5: Transformation of S-alk(en)ylcysteine sulfoxides to thiosulfinates .

Alliinase catalyzed transformation of isoalliin, alliin and methiin to allicin, trans-1-propenyl-2-propene thiosulfinates and allyl-methane-thiosulfinates. Typical amounts of compounds found in whole or crushed cloves are given in percent (w/w). Thiosulfinates are abbreviated as THS. (adopted from Lawson, 1996)

Although garlic is one of the top supplements in the U.S. (Amagase et al., 2001), studies of the metabolic fate of garlic and the formation of metabolites are scarce. Analysis of garlic compounds in breath, plasma or gastric fluids revealed diallyl-disulfide (DADS), allyl-mercaptane (AM), methyl-allyl-sulfide (AMS) as possible metabolites in humans (Rosen et al., 2001; Egen-Schwind et al., 1992; Taucher et al., 1996). In another study allyl-methyl-sulfone ($AMSO_2$) and allyl-methyl-sulfoxide (AMSO) were found in the stomach, plasma, liver tissue and urine of rats fed with DADS (figure 6). Peak levels of $AMSO_2$ were found 24 – 48 h after administration of DADS (Germain et al., 2002).

Diallyl-disulfide Allyl-mercaptane Allyl-methyl-sulfone Allyl-methyl-sulfoxide

Figure 6: Structures of selected garlic metabolites

3.1.2 Therapeutic actions of garlic

Around the world garlic has been intensively investigated during the last 100 years and has revealed its possible therapeutic effects against cancer, hyperlipidemia and atherosclerosis.

Many *in vitro* and *in vivo* studies demonstrated the favorable effect of garlic on various forms of cancer. It is known that garlic reduces the carcinogenicity of nitrosamines, aflatoxin, dimethylbenz[a]anthracene or benzopyrene by affecting cytochrome P enzymes (Milner, 2001). Other groups showed that garlic is able to mediate apoptosis on several cancer cell lines (Antlsperger et al., 2003; Dirsch et al., 1998a; Dong et al., 2001; Robert et al., 2001). Epidemiological studies revealed that garlic consumption can reduce the incidence of malignant diseases (Robert et al., 2001).

Besides anti-tumoral properties, anti-atherogenic effects are associated with the usage of garlic. The anti-atherogenic effects can be divided in lipid-lowering, anti-oxidative and anti-inflammatory effects.
In vitro studies demonstrated, that garlic constituents are able to reduce cholesterol biosynthesis by inhibiting hydroxymethylglutary-CoA reductase (HMG-CoA reductase) and squalene monooxygenase (Gebhardt, 1993; Liu and Yeh, 2002; Gupta and Porter, 2001), whereupon higher concentrations of garlic are needed for squalene monooxygenase than for HMG-CoA reductase inhibition (Gebhardt, 1993). Further inhibitory effects on fatty acid and triglyceride synthesis by water-soluble organosulfur compounds of garlic have been reported recently (Liu and Yeh, 2001). Although garlic showed strong inhibitory effects on cholesterol synthesis *in vitro*, *in vivo* studies where controversial. Many studies observed that garlic reduces the levels of blood lipids (Steiner et al., 1996b; Yeh et al., 1997; Yeh and Yeh, 1994), however, other studies

demonstrated that blood lipid levels remain unaffected by garlic consumption (Isaacsohn et al., 1998). The differences of the observed effects may be due to the various garlic preparations used in each individual study. Furthermore the quality of the garlic used was not addressed in previous studies (Agarwal, 1996; Silagy and Neil, 1994).

Additionally, it has been shown that garlic attenuates oxidative stress by inhibiting hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) production *via* an augmented activity of the anti-oxidative enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (Wei and Lau, 1998). Also it has been shown that garlic preparations are able to reduce copper-induced (Cu^{2+}) oxidation of LDL (Imai et al., 1994; Numagami and Ohnishi, 2001; Munday et al., 1999). Further it has been revealed that garlic interacts with nitric oxide (NO) production. It was demonstrated by Dirsch et. al. that allicin and ajoene are able to inhibit LPS-induced expression of inducible NO-synthase (iNOS), similar results were shown recently for S-allyl cysteine (Dirsch et al., 1998b; Kim et al., 2001). Lately published *in vivo* studies showed, that garlic preparations increase NO production in mice and rats (Pedraza-Chaverri et al., 1998; Morihara et al., 2002). However, recent *in vitro* studies demonstrated that the endothelial NO-synthase (eNOS) is not affected by garlic compounds (Kim et al., 2001; Leikert, 2002). This indicates that the observed increase of NO-production by garlic preparations is most likely not due to specific interactions with NO-synthetases.

Furthermore, increasing evidence exists that garlic mediates immunomodulatory effects. It was shown that alliin and aged garlic extract increased phagocytosis activity of peripheral blood cells (PBC). Further, treatment of PBC with garlic extract modulated LPS-induced expression of cytokines (Kyo et al., 2001; Salman et al., 1999b; Hodge et al., 2002).

It was demonstrated that aged garlic extracts are able to inhibit NF-κB activation after TNF-α and H_2O_2 treatment and subsequently the expression of adhesion molecules (Geng et al., 1997; Campbell et al., 2001; Ho et al., 2001).

Another possible mechanism how garlic may suppress atherosclerosis consists in a reduced proliferation of smooth muscle cells and endothelial cells after treatment with garlic extracts (Efendy et al., 1997; Lee et al., 1994; Campbell et al., 2001).

3.2 Phytoestrogens

Phytoestrogens are naturally occurring, plant-based diphenolic compounds that are similar in structure and function to estradiol. There are many types of phytoestrogens, but the major categories include isoflavones, lignans and coumestans. The estrogen-like activity of these compounds were identified in the 1940s, when an epidemy of infertility among sheep was investigated. The animals were grazing on clover (*Trifolium sp*) containing large amounts of formononetin, which is converted by bacteria in the rumina to daidzein and afterwards to equol. It has been demonstrated that the estrogenic effect was correlated with formononetin levels of various clover varieties, and not with genistein levels (Shutt, 1976).

3.2.1 Isoflavones

Isoflavones, the predominant type of phytoestrogens, are present in large amounts in soy beans and soy products. Peak levels of isoflavones (e.g. genistein and daidzein) can be detected 6 h after intake of soy products. Due to their phenolic nature they are rapidly transformed by mammalian UDP-glucuronosyltransferases and sulfotransferases and subsequently secreted (Hendrich, 2002). Epidemiologic studies have shown, that populations with high intake of soy products (south-east Asia) have a significant lower incidence of atherosclerosis than in countries with low soy consumption (Barnes, 1998). It is estimated that isoflavones mediate this effect mainly by reducing plasma cholesterol and LDL, as well as LDL oxidation (Kirk et al., 1998; Lovati et al., 1987; Clarkson, 2002; Samman et al., 1999). Pre-treatment with genistein inhibited NF-κB activation by blocking the degradation of IκB and the nuclear translocation of p65. As a result gene expression of adhesion molecules was reduced (Natarajan et al., 1998; Weber et al., 1995). These results were confirmed in an human *ex vivo* model, showing that soy isoflavone supplementation in healthy men prevented TNF-α induced NF-κB activation in blood lymphocytes (Davis et al., 2001).

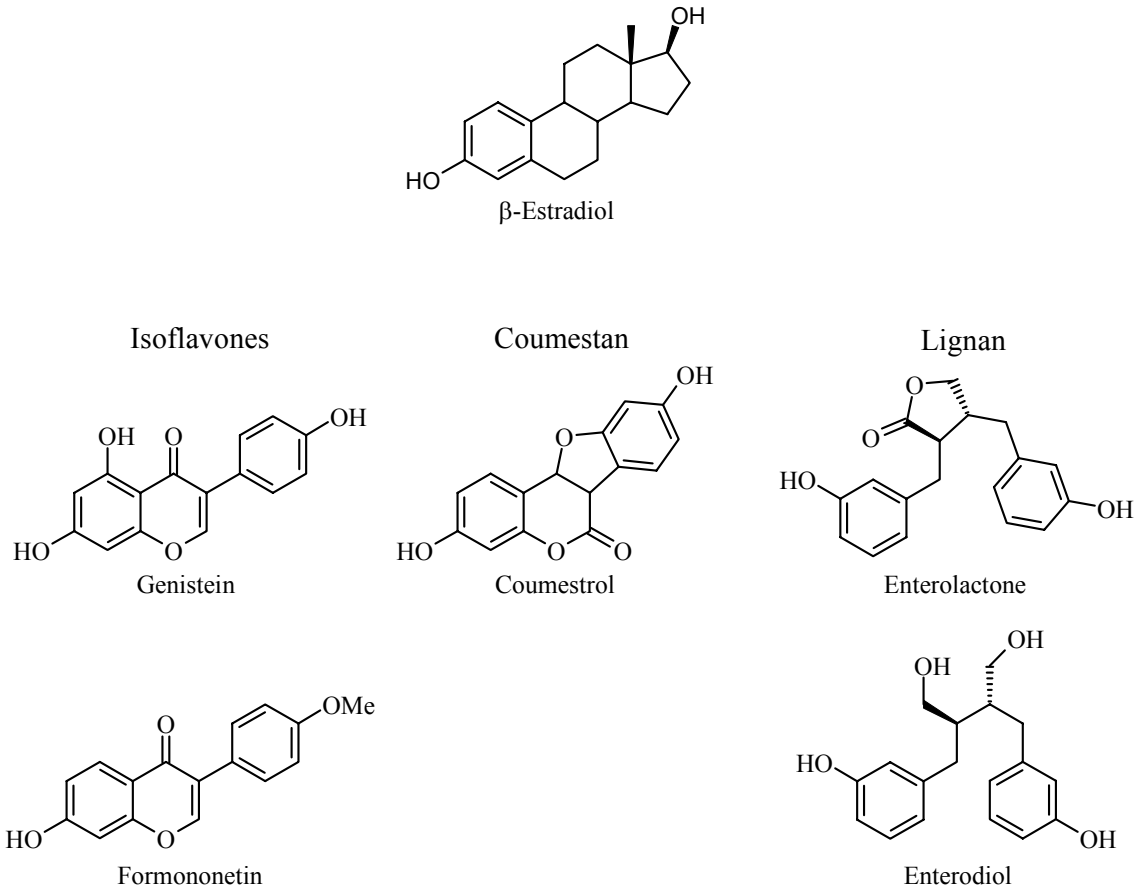
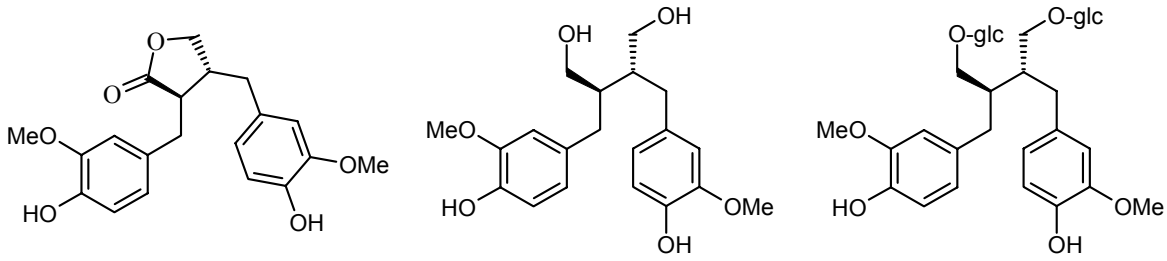


Figure 7: Structures of β-estradiol and some selected phytoestrogens

3.2.2 Lignans

Lignans are chemically related to polymeric lignins of the plant cell wall and are found mainly in whole grain cereals, seeds, nuts and vegetables. Besides the group of plant lignans, two mammalian lignans (enterolactone and enterodiol; figure 7) were discovered as major compounds in serum, urine, bile and seminal fluids (Setchell et al., 1981; Setchell et al., 1980). The mammalian-derived lignans differ from plant-derived (dietary) lignans in possessing phenolic hydroxyl groups only in the *meta* position of the aromatic rings. Two of the dietary precursors of these lignans are secoisolariciresinol (SECO) and matairesinol (MAT), which are present in garlic and sweet potatoes. A main source of plant lignans, however, is flaxseed, containing up to 25 mg/g of SECO-diglucoside (figure 8) (Meagher et al., 1999).



Matairesinol | Secoisolariciresinol | Secoisolariciresinol-diglucoside

Figure 8: Structures of lignan-type phytoestrogens

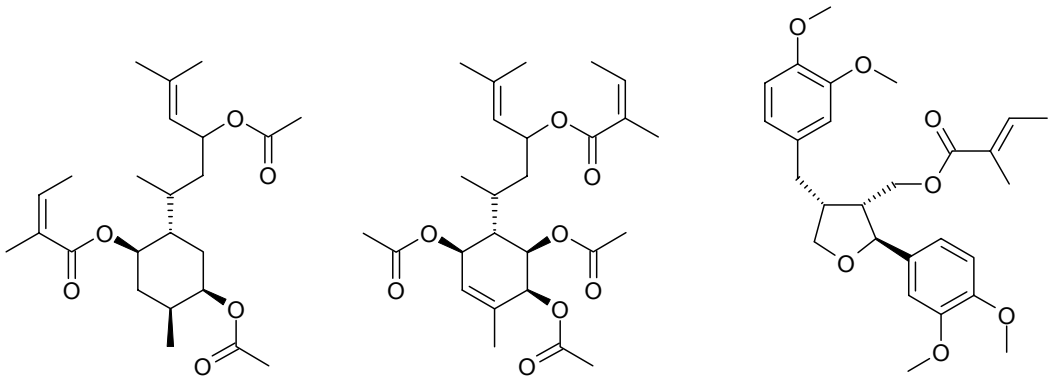
The production of mammalian lignans depends on the presence of bacteria in the intestinal tract. Metabolism studies showed that peak levels of enterodiol (END) and enterolactone (ENL) are found 9 h after flaxseed consumption (Nesbitt et al., 1999).
END and ENL received much attention during the last 20 years because of their estrogen-like biological properties. It was demonstrated that highest urine and plasma levels of ENL and END were found in countries with low cancer and coronary heart disease incidence. Lowest levels of mammalian lignans were found in breast cancer patients or women with high risk for breast cancer (reviewed by Adlercreutz and Mazur, 1997). Although a critical review of 18 studies addressing the influence of phytoestrogens on breast cancer development found that there is no evidence for reduced cancer risk by consumption of phytoestrogens (Peeters et al., 2003).
In recent years many biological activities of different lignans were revealed by scientists around the world. It was demonstrated that lignans are able to inhibit proliferation of T-cells and various cancer cell lines (Cho et al., 2001a; Bohnenstengel et al., 1999; Filleur et al., 2001; Cho et al., 2001b). Furthermore it was shown that NF-κB could be effectively inhibited by plant lignans (Baumann et al., 2002; Cho et al., 2002), as well as expression of adhesion molecules and the liberation of pro-inflammatory cytokines (Ahn et al., 2001; Yesilada et al., 2001; Cho et al., 2001b).
Additionally, it has been published that some plant lignans are able to interfere with the replication of the human immunodeficiency virus type 1 (HIV-1) (Gnabre et al., 1995; Eich et al., 1996).

3.3 Edelweiss (*Leontopodium alpinum* Cass.)

Edelweiss is a small, ornamental and protected flower that can be found in the mountains of Europe and Asia. In the Alps, edelweiss plays an important role in the traditional life of people. Taking into account how well known this plant is it is very astonishing that so far only very few phytochemical investigations have been performed. Up to now flavonoids, phenolic acids, terpens, a chromane derivate and some bisabolane derivates have been isolated from edelweiss (Stuppner H. et al., 2002; Comey N. et al., 1997; Grey A.I. et al., 1999).

It is reported that *Leontopodium alpinum* was used in traditional medicine for treatment against cancer (Hartwell, 1968). Further it was used to cure maladies of the gastro-intestinal tract, like abdominal cramping or pain, diarrhea, poisoning, toxemia as well as bronchitis (Knechtl, 1992; Pickl-Kerk, 1995; Kiene, 1992). However, pharmacological studies are missing completely.

For our investigations of the anti-inflammatory effects of *Leontopodium alpinum* Prof. Dr. H. Stuppner (University of Innsbruck) provided us several constituents isolated from the roots of edelweiss, namely two bisabolane sesquiterpenoids called bisabolane A and bisabolane CD and one plant-lignan called MAB F7 (figure 9).



Bisabolane A Bisabolane CD MAB F7

Figure 9: Structures of provided constituents of Leontopodium alpinum Cass.

III. Materials and Methods

1. Materials

Agarose was purchased from BMA. 10 % non-fat dry milk (Blotto) and Bradford reagent were from BioRad. HEPES, TRIS, TRIS·HCl and Triton X-100 were purchased from Roth. Folin & Ciocalteu's phenol reagent, bovine serum albumin, Na_2EDTA and Na_2EGTA were from SIGMA. Collagenase A was purchased from Roche. Restriction enzymes (BamHI, HindIII, AatII and SspI) were purchased from MBI Fermentas. Medium 199, RPMI 1640, penicillin/streptomycin and tumor necrosis factor-α were from PAN-Biotech. Endothelial cell growth medium was from Promocell. Phenolred-free DMEM was purchased from Bio Whittaker. Foetal calf serum and Collagen G was from Biochrom. Tryptone, yeast extract, Lennox Broth base and Lennox Broth agar were purchased from GibcoBRL. All other chemicals were from Roth if not stated otherwise.

2. Plant materials and single constituents

2.1 Garlic (*Allium sativum* L.)

2.1.1 Garlic constituents

The garlic constituents diallyl-disulfide (DADS), allyl-mercaptane (AM), allicin (DADSO), allyl-methyl-sulfoxide (AMSO) and allyl-methyl-sulfide (AMS) were obtained from Prof. Dr. J. Auger (Université de F. Rabelais, Tours, France). γ-glutamylcystein (γ-GC) was obtained from Dr. T. Haffner (Lichtwer Phama, Berlin, Germany). S-allylcysteine (SAC) was obtained from Wakanuga (Wakanuga of America Co., Madero, USA). Purity of DADS, AM, AMS, γ-GC and SAC was >95 %, purity of allicin was ~ 80% judged by HPLC.
The garlic constituents were solved either in medium or DMSO at the concentrations needed and stored at –87°C until use.

2.1.2 Garlic powders

French garlic powders from *Allium sativum* Morado, *Allium sativum* Morasol and *Allium sativum* Printanor in different fertilization stages were obtained from Dr. R. Kahane (COOP D´OR, Dijon, France)
Spanish garlic powders from *Allium sativum* Morado, *Allium sativum* Morasol and *Allium sativum* Printanor in different fertilization stages were obtained from Prof. J. Martin Sanchez (Universidad de Cordoba, Cordoba, Spain)

Variety	**Fertilization (SO_4^{2-} U/ha)**	**Origin**	**Year of harvest**
Morasol	0	France	2000
Morasol	50	France	2000
Morasol	100	France	2000
Morasol	200	France	2000
Morasol	0	Spain	2000
Morasol	50	Spain	2000
Morasol	100	Spain	2000
Morasol	200	Spain	2000
Morado	0	France	2000
Morado	50	France	2000
Morado	100	France	2000
Morado	200	France	2000
Morado	0	Spain	2000
Morado	50	Spain	2000
Morado	100	Spain	2000
Morado	200	Spain	2000
Printanor	0	France	2001
Printanor	50	France	2001
Printanor	100	France	2001
Printanor	200	France	2001
Printanor	0	Spain	2001
Printanor	50	Spain	2001
Printanor	100	Spain	2001
Printanor	200	Spain	2001
Printanor	0	France	2001
Printanor	100	France	2001
Printanor	200	France	2001
Printanor	400	France	2001
Printanor	0	Spain	2001
Printanor	100	Spain	2001
Printanor	200	Spain	2001
Printanor	400	Spain	2001
Printanor	2000	Spain	2001

Table 2: Used garlic powders

All garlic powders used for our experiments are listed in table 2. Cultivation, processing and analysis of garlic powders are described in detail by Arnault et al. (Arnault I. et al., 2003). For extraction garlic powders were suspended in either RPMI 1640 or DMSO and shaken vigorously for 30 minutes. Thereafter the unsolvable parts were separated by centrifugation (24,000xg; 5 min) and the supernatant was sterilized by filtration. The filtrate was either used directly or was diluted with medium or DMSO to the concentration needed. Garlic extracts were prepared fresh for each experiment.

2.2 Constituents from *Leontopodium alpinum* Cass. (Edelweiss)

The constituents bisabolane A, bisabolane CD and MAB F7 from *L. alpinum* were obtained from Prof. Dr. H. Stuppner (University of Innsbruck, Innsbruck, Austria). Purity of substances was >95 % judged by HPLC.
The edelweiss constituents were solved in DMSO at the concentrations needed and stored at –20°C until use.

3. Cell culture

Solutions:

PBS:

Na_2HPO_4	8.0 mM
KH_2PO_4	1.5 mM
NaCl	160 mM
H_2O	

PBS^+ :

NaCl	137 mM
KCl	2.6 mM
Na_2HPO_4 x 2 H2O	8.1 mM
KH_2PO_4	1.5 mM
$MgCl_2$ x 6 H_2O	0.5 mM
$CaCl_2$ x 2 H_2O	0.9 mM
H_2O	

Trypsin/EDTA (T/E):

Trypsin (0,05%)	0.5 g
EDTA (0,02%)	0.2 g
PBS	ad 1000 ml

PBS + Collagen:

Collagen G (Biochrom)	1.25 ml
PBS	ad 500 ml

3.1 Human umbilical vein endothelial cells

Human endothelial cells were isolated from umbilical veins (HUVECs). The vein was washed with 50 ml PBS^+ then filled with 0.1 g/l collagenase A (Roche) in PBS^+ and incubated at 37°C for 40 min. Subsequently the vein was washed with 50 ml M 199 (PAN Biotech) containing 10% FCS (Biochrom). The eluate was centrifuged at ~220xg, 20°C for 10 min and the pellet was resuspended in 5 ml ECGM (Promocell) and grown in 25 cm^2 tissue culture flasks (TPP AG) pre-coated with 0.25 % Collagen G (Biochrom) at 37°C and 5% CO_2. When cells reached confluence they were washed with PBS and subsequently detached with trypsin/EDTA. The digestion was stopped by addition of M 199 + 10 % FCS. After centrifugation at ~220xg cells were resuspended in ECGM (Promocell) and plated in pre-coated 75 cm^2 tissue culture flask (TPP AG). HUVECs were sub-cultured 1:3 in culture flasks or plates and grown until confluence. Experiments were performed with cells of passage number three grown in 6-, 12-, or 24-well plates (TPP AG). HUVECs were found > 95% pure as judged by FACS analysis, using an antiserum against the "von Willebrand"-protein (Serotec).

3.2 Human embryonic kidney cell line 293

Cells of the human embryonic kidney cell line 293 (HEK 293) were grown in phenol red-free DMEM (Biowitthaker) supplemented with 10 % FCS (Biochrom), 2 mM glutamine and penicillin/streptomycin (PAN Biotech) at 37°C and 5 % CO_2 in 75 cm^2 tissue culture flasks. Cells were split when reaching ~85-90 % confluency. Cells were washed with phosphate buffered saline (PBS) and subsequently detached with trypsin/EDTA in PBS and spilt 1:10.

3.3 Human leukemic T-cell line

Human akute leukemic T-cell line (Jurkat) was grown in RPMI 1640 (PAN Biotech) supplemented with 10 % FCS (PAA Laboratories) and 1 mM pyruvate (Sigma) at 37°C and 5 % CO_2 in 75 cm^2 tissue culture flasks. Cells were split when reaching a concentration of ~ $1x10^6$ cell/ml.

4. Flow cytometric analysis of cell adhesion molecules

FACS-buffer:

NaCl	8.12 g
KH_2PO_4	0.26 g
Na_2HPO_4	2.35 g
KCl	0.28 g
Na_2EDTA	0.36 g
LiCl	0.43 g
Na-azide	0.20 g
H_2O	ad 1000 ml pH 7.37

Antibodies for flow cytometry:

immunogen	conjugate	isotype	vendor
human-CD106 (VCAM-1)	FITC	mouse IgG_1	Biosource
human-CD54 (ICAM-1)	FITC	mouse IgG_1	Biosource
human-CD62E (E-selectin)	FITC	mouse IgG_1	Calbiochem
isotype control	FITC	mouse $IgG_{1,\kappa}$	PharMingen
human-"von-Willebrand"-factor	FITC	sheep IgG	Serotec

Table 3: Antibodies used for flow-cytometry

Experimental procedure:

Analysis of cell adhesion molecules was carried out as described before (Weber et al., 1999). HUVECs were grown in 12 well-plates until confluence and were left either untreated or were treated with 5 ng/ml TNF-α (PAN Biotech). The effect of the following substances on the surface expression of cell adhesion molecules was determined: diallyl disulfide, allyl mercaptane, MAB F7. Substances were added to the cells 2 h before TNF-α. After 4 h (E-selectin) or 16 h (ICAM-1, VCAM-1), surface expression of adhesion molecules was measured by flow cytometry using FITC-labeled antibodies against ICAM-1, VCAM-1, and E-selectin. Briefly, cells were washed with PBS three times and trypsinized. The digestion was stopped by addition of M 199 containing 10% FCS. After scraping the cells with a rubber policeman and transferring them into FACS tubes, cells were centrifuged at 220xg (20°C, 10 min). The cell pellet was washed with PBS three times. Cells were incubated with 1 % bovine serum albumin (BSA) for 30 min at room temperature. Subsequently the adequate antibody was added and incubated for 2 h at room temperature in the dark. After incubation cells

were re-suspended in a volume of 300 µl PBS for flow cytometric analysis. For analysis 10,000 cells/sample were counted and evaluated. Specific binding of antibodies was controlled with a FITC-labeled mouse IgG_1 isotype control (PharMingen). Surface expression of adhesion molecules was quantified by flow cytometry (Becton Dickinson) at λ_{em} 530 nm (green fluorescence, FL-1) after excitation at 488 nm with an argon laser.

5. Electrophoretic mobility shift assay

5.1 Isolation of nuclear and cytosolic proteins

Solutions:

Buffer A:		**Buffer B:**	
HEPES pH 7.9	10 mM	HEPES pH 7.9	20 mM
KCl	10 mM	NaCl	0.4 M
EDTA	0.1 mM	EDTA	1.0 mM
EGTA	0.1 mM	EGTA	1.0 mM
DTT	1.0 mM	DTT	1.0 mM
PMSF	0.5 mM	PMSF	1.0 mM
H_2O		Glycerol (100%)	25 %
		H_2O	

Experimental procedure:

HUVECs were cultured in 6-well plates until confluence and were either left untreated or stimulated with TNF-α (10 ng/ml) for 30 min to 1 h in the presence or absence of various substances as indicated. Nuclear and cytosolic extracts were prepared as described in (Schreiber et al. 1989). Briefly, HUVECs were washed with PBS, scraped, and resuspended in 400 µl of hypotonic buffer A. Cells were allowed to swell on ice for 15 min. Nonidet P-40 (10%, 25 µl) was added followed by 10 sec of vigorous vortexing and centrifugation at 12,000 x g for 30 sec. The supernatant (containing the cytosolic proteins) was removed. The nuclear pellet was extracted with 50 µl of hypertonic buffer B by shaking at 4°C for 15 min. The extract was centrifuged at 12,000xg and the supernatant was frozen at -85°C. Protein concentrations were determined by the method of Lowry (Lowry et al., 1951)

5.2 Radioactive labeling of oligonucleotides

Solutions:

5 x binding buffer:

Glycerol		20 % (v/v)
$MgCl_2$		5.0 mM
EDTA		2.5 mM
NaCl		250.0 mM
Tris-HCl	pH 7,5	50.0 mM
DTT		2.5 mM
H_2O		

Loading buffer:

Tris-HCl pH 7,5	250 mM
Bromphenolblue	0.2 % (m/v)
Glycerol	40 % (v/v)

10 x TBE buffer:

Tris-Base	900 mM
Boric acid	25 mM
H_2O	ad 1000 ml
TEMED	10 µl
APS 10% (m/v)	150 µl
H_2O	15.5 ml

PAGE-non denaturing 4.5% gel:

10 x TBE	1 ml
Acrylamide-bis 30 %	3 ml
Glycerol (100%)	0.5 µl

Experimental procedure:

A 22-mer double-stranded oligonucleotide probe containing a consensus binding-sequence for NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3', Promega), AP-1 (5`-CGC TTG ATG AGT CAG CCG GAA-3`, Promega) or AP-2 (5`-GAT CGA ACT GAC CGC CCG CGG CCC GT-3`, Promega) was 5' end-labeled with 10 µCi [^{32}P]-ATP (Amersham) using T4 polynucleotide kinase (USB).

5.3 Binding reaction and electrophoretic separation

Equal amounts (i.e.10 µg) of nuclear protein were incubated (20 min, RT) in a 15 µl reaction volume containing 10 mM Tris-HCl pH 7.5, 5 x 10^6 cpm radiolabeled oligonucleotide probe, 2 µg polydeoxyinosinic-deoxycytidylic-acid (ICN), 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaCl, and 0.5 mM DTT. Nucleoprotein-oligonucleotide complexes were resolved by gel-electrophoresis (4.5% non-denaturing polyacrylamide gel, 100 V) and bands were visualized by phosphoimaging (Packard). Specificity of the DNA-protein complex was confirmed by competition with a 100-fold excess of unlabeled NF-κB, AP-1, and AP-2 oligo nucleotides, respectively.

6. Determination of cell viability by propidium-iodide staining

The viability of cells was determined by propidium-iodide (PI) staining. Cells were grown in 24-well plates and treated with various substances for up to 24 hours. Then cells were trypsinized, washed with PBS and incubated with 8 ng/well PI for 30 min at room temperature in the dark. PI positive (= dead cells) were quantified by flow cytometry (Becton Dickinson) at λ_{em} 585 nm (red fluorescence, FL-2) after excitation at 488 nm with an argon laser.

7. Dual luciferase assay

For the dual luciferase reporter gene assay (DLR) the following plasmids were used:

name	**promoter**	**reporter gene**	**vendor**
pNF-κBluc	NF-κB (5x)	firefly - luciferase	Stratagene
pFC-Mekk	Positive control for pNF-κBluc		Stratagene
pEGFP-N1	CMV	green fluorescent protein	Clontech
pRL-TK	HSV-TK	renilla luciferase	Promega

Table 4: List of used plasmids

Solutions:

SOB-Media:

Tryptone	20 g
Yeast extract	5 g
NaCl	0.5 g
KCl	2.5 mM
$MgCl_2$	20 mM
H_2O	ad 1000 ml

Adjust pH to 7.0

SOB-Agar:

Agarose	15 g
SOB – Media	ad 1000 ml

SOC-Media:

Glucose	20 mM
SOB – Media	ad 1000 ml

Lennox Broth-Media:

LB Broth Base	20 g
Glucose	1 g
H_2O	ad 1000 ml

Adjust pH to 7.2

Lennox Broth -Agar:

LB-agar	32 g
Glucose	1 g
H_20	ad 1000 ml

TFB I:

MOPS	10 mM
$RbCl_2$	10 mM
$CaCl_2$	75 mM
Glycerol	15 % (v/v)
H_20	
Adjust pH to 7.0	

TFB II:

$RbCl_2$	100 mM
$MnCl_2$	50 mM
K^+-acetate	30 mM
$CaCl_2$	10 mM
Glycerol	15 % (v/v)
H_2O	
Adjust pH to 5.8	

Alkaline lysis buffer I (ALP I)

Glucose		50 mM
Tris-HCL	pH 8.0	25 mM
EDTA		10 mM
H_20		

Alkaline lysis buffer II (ALP II)

NaOH	0.2 N
SDS	1 % (w/v)
H_2O	

Alkaline lysis buffer III (ALP III)

K^+-acetate (5M)	60 ml
Glacial acetic acid	11.5 ml
H_2O	28.5 ml

Tris/EDTA (TE)

Tris-HCl pH7.5	10 mM
Na-EDTA	1 mM
RNAse	50 µg/ml
H_2O	

7.1 Preparation of competent *E.coli* DH5α

Bacteria (*E.coli* strain DH5α) from a frozen stock were plated on SOB agar plate and grown at 37°C for 16 h. Several single colonies were suspended in 30 ml of SOB medium. Bacteria were grown at 37°C (200 rpm). When reaching an OD_{600} of ~ 0.4 bacteria were sedimented at 2,700xg (10 min at 4°C). The pellet was resuspended in 30 ml ice-cold TFB I buffer and incubated for 30 min on ice. Afterwards bacteria were pelleted at 2,700xg (10 min at 4°C) and then resuspended in 4 ml ice-cold TFB II buffer. 140 µl DMSO were added and incubated for 15 min on ice. Afterwards again 140 µl DMSO were added and then aliquots of competent *E.coli* were shock-frozen in liquid nitrogen and stored at –87°C.

7.2 Transformation of DH5α

50 µl of competent DH5α bacteria were incubated for 30 minutes with 1 ng plasmid DNA on ice. Then the bacteria were heat-shocked for 90 seconds at 42°C and afterwards put on ice for 2 minutes. 800 µl SOC-Media were added and the bacteria were incubated at 37°C (200 rpm for 45 minutes). After incubation, up to 200 µl of the bacteria suspension were plated on selective (ampicillin 50 µg/ml or kanamycin 30 µg/ml) SOB-agar and incubated overnight at 37°C.

7.3 Mini preparation of plasmids

Single colonies of transformed DH5α were picked and each colony incubated in 2 ml selective LB-Media at 37°C (300 rpm for 16-20 h). 1.5 ml of cultured bacteria were centrifuged at 24,000xg, 4°C for 30 seconds and the pellet was resuspended in ALP I. Thereafter ALP II was added and the mixture was heavily shaken. Subsequently ice cold ALP III was added. The lysate was incubated on ice and then centrifuged at 24,000xg for 5 minutes at 4°C. 400 µl from the supernatant were separated and the DNA was precipitated from the supernatant with 800 µl of ice-cold ethanol 100%. DNA was isolated by centrifugation at 24,000xg at 4°C for 5 minutes and subsequently washed twice with 70% ethanol. The air dried pellet was dissolved in TE. Plasmids were cut with appropriate restriction enzyme and then identified by size with agarose gel electrophoresis after ethidium bromide staining.

7.4 Maxi preparation of plasmids

After successful mini preparation 0.5 ml of the transformed bacteria were incubated in 100 ml selective LB-Media and incubated overnight at 37°C and 300 rpm in a water bath. The suspension was centrifuged at 6,000xg for 15 minutes at 4°C. The isolation was carried out with EndoFree™ Plasmid Maxi-Kit (Qiagen) according to the manufacturer's description.

7.5 Transfection of HEK 293 cells

$1x10^6$ HEK 293 cells were seeded in a 60 mm dish the night before transfection and grown at 37°C, 5% CO_2. On the following day 1 µg pNF-κBluc, 100 ng pRL-TK and 1.1 µg salmon sperm DNA were mixed and 250 µl of sterile 250 mM $CaCl_2$ and 250 µl sterile 2xHBS pH 7.06 (280 mM NaCl; 10 mM KCl; 1.5 mM $Na_2HPO_4*2H_2O$; 12 mM Glucose; 50 mM HEPES) were added, mixed carefully and incubated for 45 minutes at room temperature. The precipitate was added to the cells and then incubated for 8 hours at 37°C, 5 % CO_2. Afterwards the medium was aspirated and the cells were washed twice with PBS^+ and then fed with fresh medium. On the next day, transfected cells were seeded in 96-well plates at a concentration of $1.5x10^4$ cells/well and grown for additional 16 h. Transfection efficiency was judged by fluorescence microscopy evaluating GFP-transfected cells.

In order to rule out interactions between different plasmids control cells were transfected with pNF-κB, pRL-TK or salmon-sperm alone. Cells co-transfected with pNF-κB, pRL-TK, pFC-MEKK and salmon-sperm were used as positive control.

7.6 Dual luciferase reporter assay

Cells were pre-incubated for 2 h with various substances and subsequently stimulated with 1 ng/ml TNFα for 6 h. Then cells were washed twice with PBS^+ and afterwards lysed. NF-κB activity was measured by the Dual Luciferase Reporter Assay (Promega) according to manufacturer's description with a AutoLumat plus (Berthold).

8. Cytokine liberation assay

Solutions

Wash solution		**Blocking solution**	
Tween 20	0.05 %	BSA	3 %
PBS		PBS	

Coating Buffer:		**Streptavidin-peroxidase:**	
$NaHCO_3$	0.1 M	Streptavidin-peroxidase	1 µg/ml
H_2O		BSA	3%
Adjust pH to 8.2		PBS	

immunogen	**isotype**	**usage**	**vendor**
hIL-1β	mouse $IgG_{2bκ}$	capture	Endogen
hIL-1β	recombinant protein	standard	Endogen
hIL-1β biotinylated	mouse $IgG_{1κ}$	detection	Endogen
hIL-6	goat IgG	capture	R & D
hIL-6	recombinant protein	standard	R & D
hIL-6 biotinylated	goat IgG	detection	R & D
hIL-10	rat IgG_1	capture	PharMingen
hIL-10	recombinant protein	standard	PharMingen
hIL-10 biotinylated	rat IgG_{2a}	detection	PharMingen
hTNF-α	mouse $IgG_{1κ}$	capture	Endogen
hTNF-α	recombinant protein	standard	Endogen
hTNF-α biotinylated	mouse $IgG_{1κ}$	detection	Endogen
hINF-γ		capture	Endogen
hINF-γ	recombinant protein	standard	Endogen
hINF-γ biotinylated		detection	Endogen

Table 5: Used antibodies for ELISA

8.1 Incubation of human whole blood with garlic powder extracts

To study the LPS-induced cytokine release from human whole blood, 1,600 µl of RPMI 1640 (Biochrom) supplemented with 100 IU/ml penicillin/streptomycin (Biochrom) was pipetted into a polypropylene reaction tube, and 10 ng/ml LPS from Salmonella abortus equi (Sigma) and either a garlic constituent or garlic powder extract was added. Finally, 400 µl of heparinized whole blood (withdrawn in lithium-heparin-S-monovettes from Sarstedt) from healthy volunteers were added (final assay volume, 2 ml). The tubes were incubated at 37°C and 5% CO_2 for 16-20 h. After incubation, the tubes were shaken, and blood cells were sedimented by centrifugation (16,000xg, 2 min). The cell-free supernatants were stored at -80°C until cytokine measurement.

8.2 Cytokine measurement

Cytokines in the cell-free supernatants were quantified by sandwich ELISA. First ELISA plates (Greiner) were coated overnight at 4°C with 50 µl/well coat antibody in 0.1 M $NaHCO_3$, pH 8.2. After blocking with 200 µl/well PBS supplemented with 3% bovine serum albumin (Serva), pH 7.0, for 2 h at room temperature the plates were washed twice with PBS/0.05% Tween 20. Sample (50 µl/well) and tracer antibody (50 µl/well) in PBS/bovine serum albumin 3% were added and incubated for 2 h for all ELISA from Endogen. For measuring IL-6 and IL-10 100 µl of sample was incubated for 3 h, then the plate was washed four times, and then 100 µl of tracer antibody was incubated for 2 h. After six wash cycles, plates were incubated for 30 min with streptavidin-peroxidase (Dianova). After eight washes, 100 µl/well 3,3',5,5'-tetramethyl-benzidine liquid substrate solution (Sigma) was added and incubated at room temperature for 5 to 30 min. After addition of 50 µl/well stop solution (1 M H_2SO_4), absorption was measured at 450 nm using a reference wavelength of 690 nm.

9. Real-time RT-PCR

9.1 RNA isolation and reverse transcription

HUVECS were grown in 6 cm dishes to confluency. Cells were pre-incubated with MAB F7 or MG-132 at the indicated concentrations or with DMSO alone for 2 h. Afterwards 5 ng/ml TNF-α was added and cultured for additional 4 h. mRNA was isolated with RNeasy KIT (Qiagen) according to manufactures description. Total RNA

concentration was determined from spectrophotometric optical density measurement (260 and 280 nm). Reverse transcription was carried out using the RNA PCR Core Kit (PE Applied Biosystems). Each reaction tube contained 2 µg of total RNA in a volume of 100 µl containing 5.5 mM $MgCl_2$, 1x RT Buffer, 500 µM of each dNTP, 2.5 µM of random hexamers, 0.4 U/µl RNAse inhibitor, 1.25 U/µl MultiScribe reverse transcriptase and water to volume. RT-reactions were carried out in a GeneAmp PCR system 9700 (Applied Biosystems) at 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. cDNA was stored at –20°C.

9.2 Real-time PCR

The cDNA sequence for ICAM-1 was obtained from NCBI. PCR primers and TaqMan fluorogenic probes were designed using the Primer Express 2.0 software program (PE Applied Biosystems). TaqMan TAMARA probes and primers were purchased from PE Applied Biosystems; forward primer: 5'-GCAGACAGTGACCATCTACAGCTT-3'; reverse primer: 5'-CTTCTGAGACCTCTGGCTTCGT-3' ; TaqMan probe : 5'-CCGGCGCCCAACGTGATTCT-3'. Real-time PCR was performed using the TaqMan Universal PCR Master Mix, 900 nM of forward primer, 300 nM of reverse primer, 200 nM of probe and 200 ng of ICAM-1 total RNA. All samples were run in duplicate. Standard curves were constructed on a 1:2 serial dilution of total RNA. As internal standard the TaqMan Pre-Developed Assay Reagents (Applied Biosystems) against 18s RNA was used. Amplification conditions included 50°C 2 min, 95°C 10 min and run for 40 cycles at 95°C 15 sec and 60°C 1 min on the GeneAmp 5700 (Applied Biosystems). The threshold cycle was determined by the software, based on the generated standard curves.

10. Western blot analysis

Solutions:

Lysis buffer	
Tris-HCl pH 7.5	30 mM
NaCl	150 mM
EDTA	2 mM
Triton X-100	1 % (v/v)
Complete™ (25x)	
H_2O	

SDS Sample buffer (5x)	
Tris-HCl (pH 6.8)	0.3 M
Glycerol	50 %
SDS	5 %
DTT	2 %
Pyronin Y	0.025 %

Resolving gel (7.5%)	
PAA solution 30%	5.6 ml
1.5 M Tris, pH 8.8	5.6 ml
SDS 10%	0.3 ml
TEMED	23.0 µl
APS	112.5 µl
H_2O	11.0 ml

Stacking gel	
PAA solution 30%	2.6 ml
1.25 M Tris, pH 6.8	1.5 ml
SDS 10%	0.2 ml
TEMED	30.0 µl
APS	150.0 µl
H_2O	10.5 ml

Electrophoresis buffer	
Tris Base	3.0 g
Glycin	14.4 g
SDS	1.0 g
H_2O	ad 1000 ml

Blotting buffer	
Tris Base	3.0 g
Glycin	14.4 g
Methanol	20.0 %
H_2O	ad 1000 ml

TBS-T (pH 8.0)	
Tris-Base	3.0 g
NaCl	11.1 g
Tween 20	1.0 ml
H_2O	ad 1000 ml
Adjust pH to 8.0	

10.1 Preparation of samples

HUVECs cells were grown in 60 mm dishes until reaching confluency. Then cells were pre-incubated with MAB F7 for 2 h and subsequently stimulated with TNF-α (5 ng/ml) for additional 16 h. After incubation cells were washed with PBS and incubated with 300 µl lysis buffer for 30 min on ice. Thereafter cells were scraped with a rubber policeman and transferred to a pre-cooled reaction tube and sedimented for 10 min at 14,000xg, 4°C. After determination of protein concentration by the method of Bradford (Bradford, 1976), an appropriate volume of sample buffer was added to the probes. After boiling at 95°C for 5 min, the samples were either stored at –20°C or immediately used for protein electrophoresis.

10.2 Electrophoresis, blotting and detection of proteins

Separation of proteins was performed by denaturating SDS-polyacrylamide gel (PAGE) electrophoresis (Laemmli, 1970). Samples with equal amount of protein and the molecular weight marker were loaded into the slots of the prepared polyacrylamid gels. Electrophoresis was run at 100 V for 21 min for stacking and at 200 V for 36 min for resolving of the proteins. After electrophoresis proteins were transferred to a nitro-cellulose membrane (Amersham) at 150 V for 1 h. Then membranes were blocked with

10 % nonfat dry milk in TBS-T for 2 h at RT. Membranes were washed several times with TBS-T and incubated with primary polyclonal rabbit antibody against human ICAM-1 (Santa Cruz; 1:500 in 1% nonfat dry milk in TBS-T) overnight at 4°C. On the next day membranes were washed several times with TBS-T and subsequently incubated with secondary horseradish peroxidase (HRPO)-labeled goat anti-rabbit IgG antibody (Jackson Laboratories; 1:10,000. in 1% nonfat dry milk in TBS-T) for 1 h. Chemiluminescence detection was performed using Western Blot Chemiluminescence Reagent Plus (NEN Life Science) and imaged by a Kodak Digital Science^{TM} Image station 440CF (NEN Life Science). Band intensity was quantified by densitometry of immunoblots using KODAK 1D software, version 3.5.4.

10.3 Coomassie blue staining

Coomassie blue staining was used for control of equal protein loading and effective blotting of PAGE-gels.

Staining solution		**Destaining solution**	
Coomassie blue	0.3 %	Glacial acetic acid	10% (v/v)
Glacial acetic acid	10.0 % (v/v)	Ethanol 96%	30% (v/v)
Ethanol 96%	45.0 % (v/v)	H_2O	
H_2O			

After Western blotting, gels were shaken with staining solution for 45 min and subsequently destained with destaining solution three times for 15 min. Proteins remaining in the gel became visible as blue bands.

11. Analysis of protein synthesis

11.1 Expression of GFP-protein

HEK 293 cells were transfected with pEGFP-N1 as described before (see II.7.5). After transfection cells were incubated with MAB F7 or CHX for 24 h. Expression of GFP was determined using fluorescent microscopy and subsequently quantified by flow cytometry. For FACS analysis cells were detached with 250 µl trypsin/EDTA. Digestion was stopped by addition of 750 µl DMEM + 10 % FCS. Cells were pelleted at 220 g, 10 min, the cell pellet was washed twice with PBS. Afterwards cells were re-suspended in 400 µl PBS and analyzed by flow cytometry. GFP positive cells were

quantified at λ_{em} 530 nm (green fluorescence, FL-1) and λ_{em} 585 nm (red fluorescence, FL-2), after excitation at 488 nm with an argon laser.

11.2 [^{3}H]-Leucin incorporation

HUVECs were seeded at a concentration of $5x10^4$ cells/well in a 6-well plate and were grown for 24 h. Cells were incubated with various concentrations of MAB F7 or cycloheximide (CHX, Sigma) and 1µCi [^{3}H]-leucine (Amersham) for 24 h. To test whether inhibitory effects are reversible cells were incubated with MAB F7 or CHX for 24 h. Thereafter growth medium was changed and cells were incubated for additional 24 h with 1µCi [^{3}H]-leucine. After incubation with [^{3}H]-leucine cells were washed twice with ice-cold PBS and subsequently protein was precipitated for 5 min with 5 % -trichloroacetic acid (Riedel-de-Haen). Afterwards cells were washed twice with ice-cold PBS and lysed with 0.4 N NaOH. 800 µl of the lysate were transferred to a scintillation tube and acidified with 200 µl 1N HCl. Then 5 ml scintillation liquid (Roth) were added. [^{3}H]-leucine incorporation was quantified using a Beckman LS 6500 (Beckman).

12. Statistics

All data are expressed as mean and standard error, unless stated otherwise. The significance of differences between experimental groups was determined by one-way ANOVA with Bonferroni post-test or paired t-test. $P<0.05$ was considered statistically significant. Analysis of data was performed using the software GraphPad PRISM®, Version 3.02 (GraphPad Software Inc.).

IV. Results

1. Garlic (*Allium sativum* L.)

1.1 Synthesis of allyl-methylsulfone

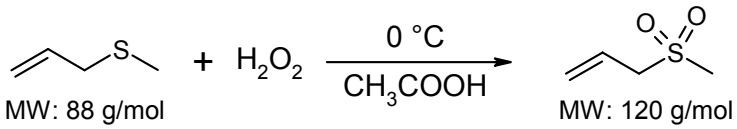


Figure 10: Synthesis of allyl-methylsulfone

9.1 mmol of allyl-methylsulfide (Acros Organics) was oxidized with 18.2 mmol hydrogenperoxide (H_2O_2) in 20 ml glacial acetic acid by stirring at 0 °C for 18 h. Afterwards the whole preparation was neutralized with saturated sodium bicarbonate solution ($NaHCO_3$) and the products were extracted with ethyl-ether. The extracted products were identified by thin layer chromatography (solvent hexane:ethyl-ether 1:1 and detected with potassium manganate (K_2MnO_4) in 1 N sulfuric acid (H_2SO_4)) and further separated by flash chromatography as described before (Still et al., 1978). As solvent a mixture of hexane:ethyl ether 1:1 was used. The purified substance was identified by mass spectrometry (120 U), ^{1}H-NMR and ^{13}C-NMR (see figure 11 & table 6).

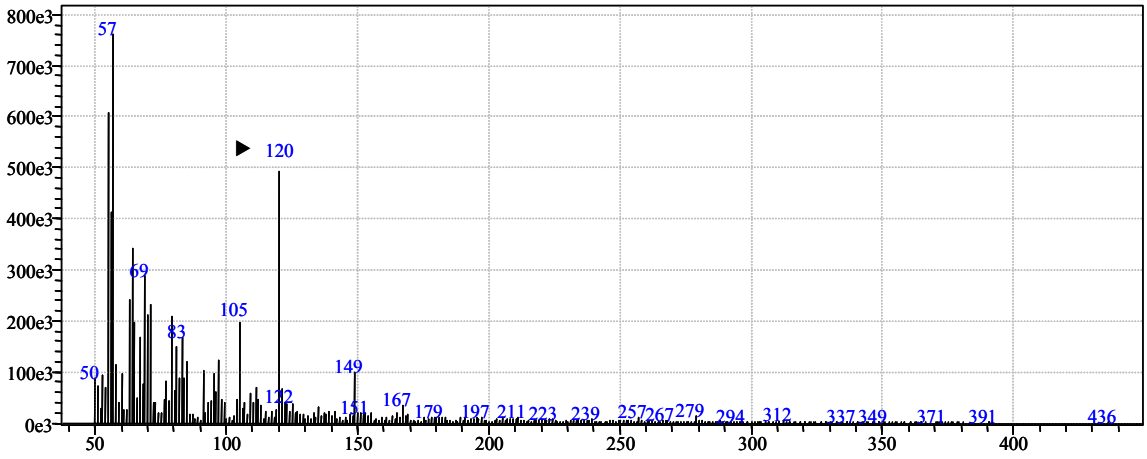


Figure 11: Mass-spectrogram of allyl-methy-sulfone

Calculated molar weight of $AMSO_2$ MG: 120 g/mol

Group	^{13}C (ppm)	signal	^{1}H (ppm)	signal
$CH_2=$	124	singulet	3,75	duplet
$=CH—$	125	singulet	5,98	multiplet
$—CH_2—$	59	singulet	5,54	double-doublet
$—CH_3$	39	singulet	2,88	singulet

Table 6: NMR-signals of allyl-methyl-sulfone

1.2 Effect of sulfur-fertilization on total sulfur content and the content of sulfur compounds in dried garlic powder

The total sulfur content in unfertilized garlic was 3091 ppm. With sulfur fertilization ($CaSO_4$, 320 kg/ha) the amount of sulfur in garlic powders rose to 6097 ppm. Analysis of total sulfur content was performed by Dr. Laurence Trueman (HRI, Wellesbourne, U.K.). The distribution of sulfur in alliin, allicin, allyl-cysteine and γ-glutamyl-cysteines in dried garlic powders was assessed by HPLC analysis of the same powders. HPLC analysis was performed by T. Haffner at Lichtwer AG (Berlin, Germany). As can be seen in figure 12 sulfur-fertilization significantly increased detected allicin, alliin, γ-glutamyl-1-propyl-cysteine and γ-glutamyl-phenylalanin levels. In contrast, the amount

of γ-glutamyl-allyl-cysteine remained unchanged and the level of allyl-cysteine was slightly reduced.

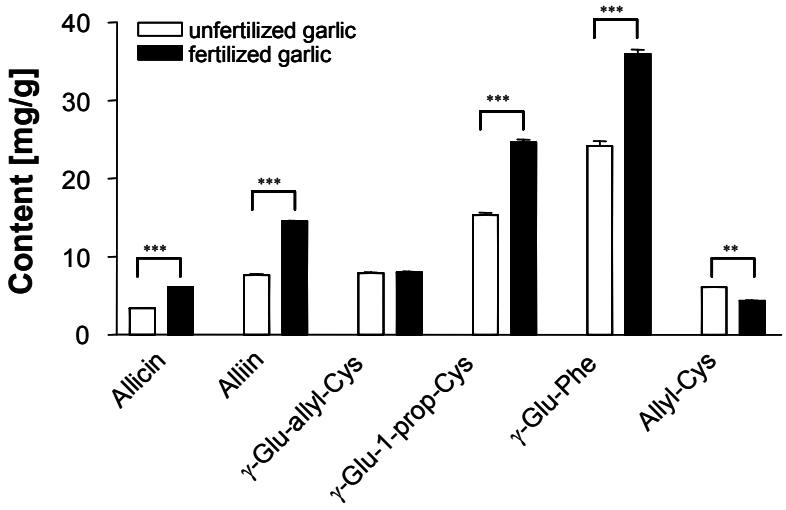


Figure 12: Sulfur fertilization increases the content of sulfur compounds in dried garlic powder.

*The relative content of the following compounds in garlic powders of unfertilized or sulfur fertilized garlic, as indicated, was analyzed by HPLC-UV: allicin, alliin, γ-glutamyl-allyl-cysteine (γ-Glu-allyl-Cys), γ-glutamyl-1-propyl-cysteine (γ-Glu-1-prop-Cys), γ-glutamyl-phenylalanin (γ-Glu-Phe), allyl-cysteine (Allyl-Cys). Bars represent the mean ± SEM of two independent experiments. **P<0.01, ***P<0.001 (paired t-test)*

1.3 Interaction of garlic with nuclear factor-kappa B (NF-κB)

In order to investigate possible inhibitory effects of garlic on TNF-α-induced NF-κB activation, we investigated NF-κB DNA binding by electrophoretic mobility shift assay (EMSA) and NF-κB transactivation activity with a NF-κB-driven firefly-luciferase reporter gene assay. We used single garlic constituents, i.e. diallyl-disulfide, allyl-mercaptane, S-allylcysteine, allyl-methylsulfone and γ-glutamyl-cysteine. Further we prepared hydrophilic and lipophilic extracts of garlic powders as described under methods. Solvent for hydrophilic extraction was RPMI-media and for lipophilic extraction DMSO.

1.3.1 Garlic metabolites do not inhibit TNF-α-activated NF-κB binding activity.

Figure 13 demonstrates that TNF-α (10 ng/ml) activates DNA binding activity of NF-κB, and that this binding activity is not considerably decreased in cells pre-treated with diallyl-disulfide (DADS) or allyl-mercaptane (AM) (5-20 µM).

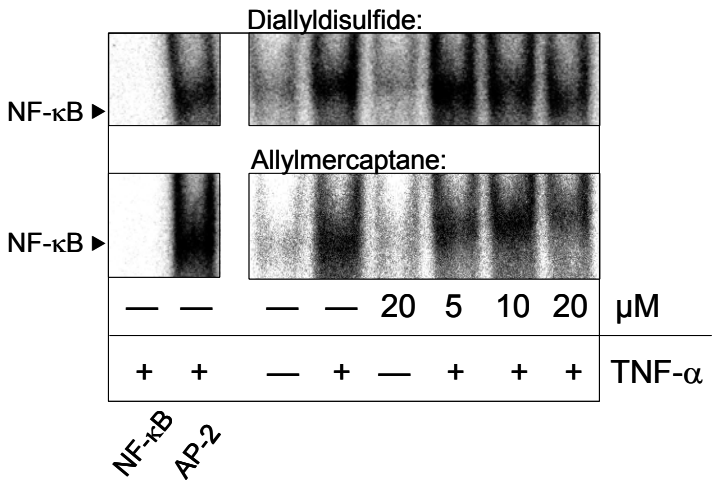


Figure 13: Allyl-mercaptane and diallyl-disulfide do not inhibit TNF-α-induced NF-κB DNA binding activity.

HUVECSs were either left untreated or were treated with DADS or AM as indicated. After 2 h TNF-α (10 ng/ml) was added for an additional hour. Equal amounts of nuclear extracts were employed to EMSA as described under Methods*. The first two lanes of each gel show DNA binding of nuclear extracts of TNF-α-activated cells incubated with a 100-fold excess of an unlabeled NF-κB (unlabeled competitor, line 1) or AP-2 (unlabeled noncompetitor, line 2) consensus oligonucleotide. All experiments were performed at least three times. Representative gel shift experiments are shown.*

Similar results were obtained investigating γ-glutamylcysteine (1 nM-1 mM), a major constituent of whole and crushed garlic gloves (Lawson, 1996) and SAC (0.1-10 mM), a major sulfur constituent of aged garlic extract (Borek, 2001) as depicted in figure 14.

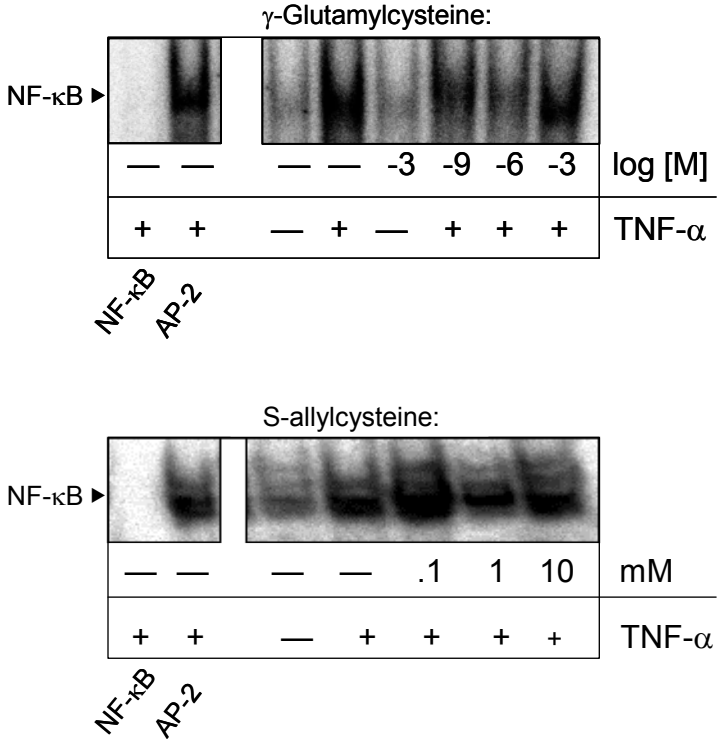


Figure 14: γ-Glutamylcysteine (γGC) and S-allylcysteine (SAC) do not inhibit TNF-α-induced NF-κB DNA binding activity.

HUVECSs were either left untreated or were treated with γGC and SAC as indicated. After 2 h TNF-α (10 ng/ml) was added for an additional hour. Equal amounts of nuclear extracts were employed to EMSA as described under Methods*. The first two lanes of each gel show DNA binding of nuclear extracts of TNF-α-activated cells incubated with a 100-fold excess of an unlabeled NF-κB (unlabeled competitor, line 1) or AP-2 (unlabeled noncompetitor, line 2) consensus oligonucleotide. All experiments were performed at least three times. Representative gel shift experiments are shown.*

In order to show the functionality of our test system we tested a known NF-κB inhibitor. HUVECSs were treated with the sesquiterpenlactone parthenolide, a well-established NF-κB inhibitor (Hehner et al., 1999). Parthenolide concentration-dependently (0.1-10 μM) inhibited NF-κB binding activity (figure 15).

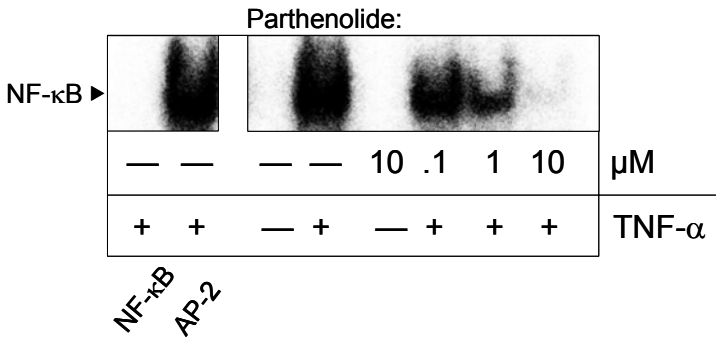


Figure 15: Parthenolide inhibits TNF-induced NF-κB DNA binding activity

HUVECSs were pre-treated with parthenolide for 2 h. Then cells were stimulated with 10 ng/ml TNF for 1 h. Cells pre-treated with parthenolide showed strongly impaired NF-κB binding. Equal amounts of nuclear extracts were employed to EMSA as described under Methods*. The first two lanes of each gel show DNA binding of nuclear extracts of TNF-α-activated cells incubated with a 100-fold excess of an unlabeled NF-κB (unlabeled competitor, line 1) or AP-2 (unlabeled noncompetitor, line 2) consensus oligonucleotide. All experiments were performed at least three times. A representative gel shift experiment is shown.*

1.3.2 Garlic metabolites do not inhibit TNF-α-activated NF-κB transactivation activity.

Accumulating evidence indicate that NF-κB activity is not only dependent on translocation of p50/RelA into the nucleus, but also on direct modification of NF-κB proteins through phosphorylation and acetylation (Zhong et al., 1998; Zhong et al., 2002; Chen et al., 2001). Therefore we established a NF-κB-driven firefly luciferase reporter gene assay to determine the actual transactivation activity of NF-κB.
First, a time- and concentration-course (figure 16, figure 17) for TNF-α-induced NF-κB-dependent transcriptional activation was performed to establish the experimental procedure for further reporter gene assays.

TNF-α induced NF-κB transactivation activity was monitored over 24 h. As depicted in figure 16, maximal luciferase activity was measured at 16 h. For our experiments we chose a stimulation time of 6 h to minimize possible cytotoxic effects that may emerge from test compounds in the presence of TNF-α.

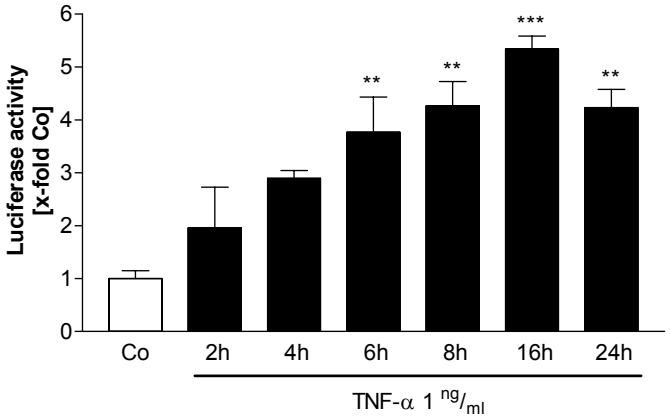


Figure 16: Time course TNF-α

Transfected HEK 293 cells were stimulated with 1 $^{ng}/_{ml}$ TNF-α for the indicated times. HEK 293 cells were transfected as described under Methods*. Co represents luciferase activity of untreated cells. Bars represent mean ± SEM of three independent experiments performed in triplicate.**p<0.01 and ***p<0.001 (ANOVA/Bonferroni).*

Luciferase activity increased dose-dependently with a maximum at 10 ng/ml TNF-α (figure 17). For our further experiments we chose 1 ng/ml TNF-α, in order to minimize cytotoxic effects of TNF-α.

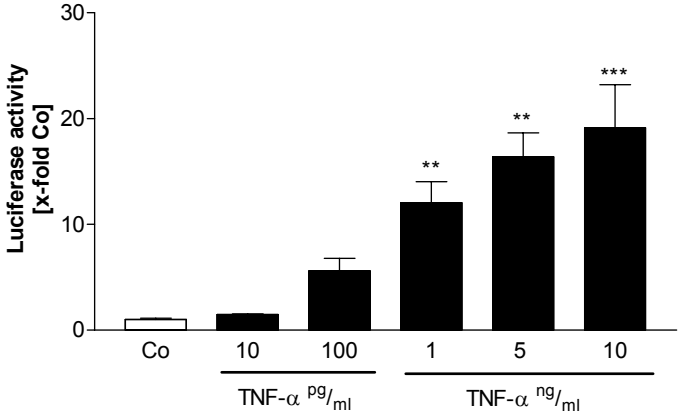


Figure 17: Concentration course TNF-α

Transfected HEK 293 cells were stimulated with the indicated concentrations of TNF-α for 6h. HEK 293 cells were transfected as described under Methods*. Co represents luciferase activity of untreated cells. Bars represent mean ± SEM of three independent experiments performed in triplicate.**p<0.01 and ***p<0.001 (ANOVA/Bonferroni).*

Of further interest was whether TNF-α-induced NF-κB transactivation activity could be inhibited by known NF-κB inhibitors. Therefore we investigated whether the known proteasome-inhibitor carbobenzoxyl-leucinyl-leucynil-leucynal (MG-132), and the sesquiterpenelactone parthenolide inhibit TNF-α-induced luciferase activity (Palombella et al., 1994; Hehner et al., 1999).
Pre-treatment with the peptide-aldehyde MG-132 dose-dependently inhibited NF-κB transactivation at concentrations of 1 and 10 μM. Treatment with 0.1 μM showed no effect on TNF-α-induced NF-κB activity (figure 18).

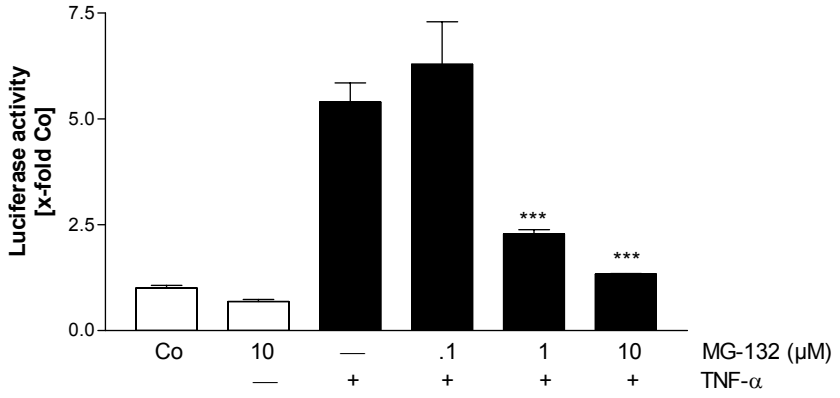


Figure 18: MG-132 inhibits NF-κB transcriptional activity

*Transfected HEK 293 cells were pre-incubated with various concentrations of MG-132 for 2 h. Then cells were activated with 1 ng/ml TNF-α for 6h. Co represents luciferase activity of untreated cells. Bars represent mean ± SEM of three independent experiments performed in triplicates. ***p<0.001 (ANOVA/Bonferroni).*

Pre-treatment with the sesquiterpenelactone parthenolide in concentrations of 0.1 μM to 10 μ M also led to a dose-dependent inhibition of NF-κB (figure 19).

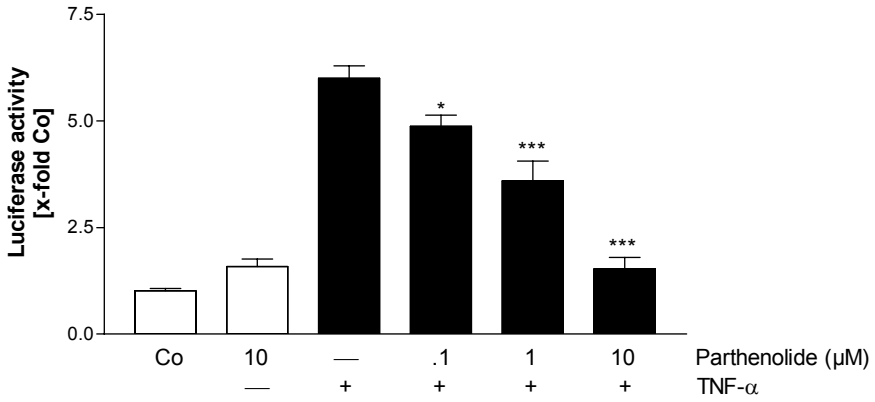


Figure 19: Parthenolide inhibits NF-κB transcriptional activity

*Transfected HEK 293 cells were pre-incubated with various concentrations of parthenolide for 2 h. Then cells were activated with 1 ng/ml TNF-α for 6h. Co represents luciferase activity of untreated cells. Bars represent mean ± SEM of three independent experiments performed in triplicate. *p<0.05, **<p0.01, ***p<0.001 (ANOVA/Bonferroni).*

After finding optimal assay settings we investigated whether the garlic metabolites DADS, AM, $AMSO_2$ and allicin influence the transactivation activity of NF-κB.
Neither DADS (1-100 µM) nor AM (1-100 µM) affected TNF-α (1 ng/ml)-induced firefly-luciferase expression (figure 20, figure 21). $AMSO_2$ and allicin showed similar results as DADS and AM (data not shown).

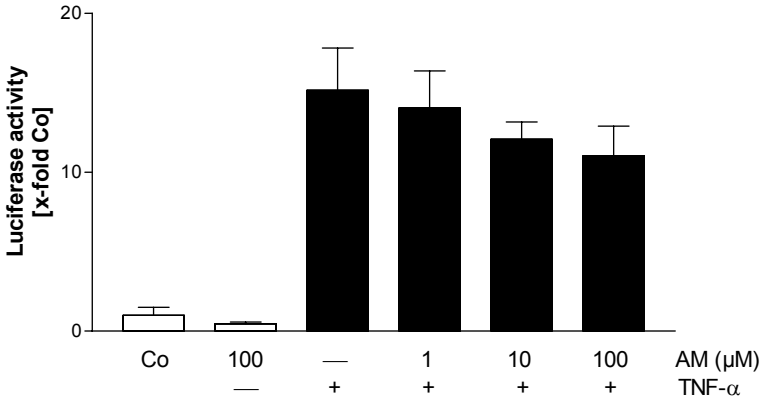


Figure 20: Allylmercaptane (AM) does not inhibit NF-κB transactivation activity

HEK 293 cells were either left untreated or were pre-treated with AM as indicated. After 2 h TNF-α (1 ng/ml) was added for additional 6 h. Co represents luciferase activity of untreated cells. Bars represent the mean ± SEM of at least three independent experiments performed in triplicate.

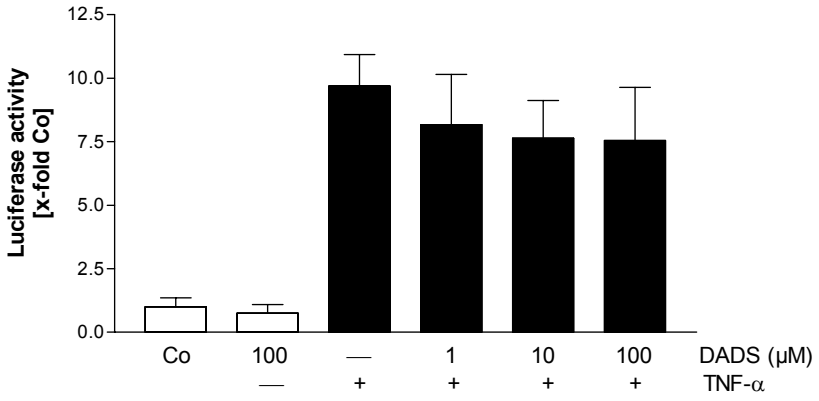


Figure 21: Diallyl-disulfide (DADS) does not inhibit NF-κB transactivation activity

HEK 293 cells were either left untreated or were pre-treated with DADS as indicated. After 2 h TNF-α (1 ng/ml) was added for additional 6 h. Co represents luciferase activity of untreated cells. Bars represent the mean ± SEM of at least three independent experiments performed in triplicate.

Additionally we tested whether various garlic powders-extracts have an impact on TNF-α-induced NF-κB transactivation. In detail we examined extracts of Morasol 0 & 100, Morado 200, Printanor 0 & 200 (year 2000) and Printanor 0, 200, 400 & 2000 (year 2001) from France and Spain (see page 18). Concentrations used for aqueous extracts were from 1 µg/ml to 1000 µg/ml, for DMSO extracts from 1 µg/ml to 500 µg/ml.

None of the tested extracts showed an inhibitory effect on TNF-α-induced NF-κB transcriptional activity. Depicted are two representative graphs in figure 22 and figure 23.

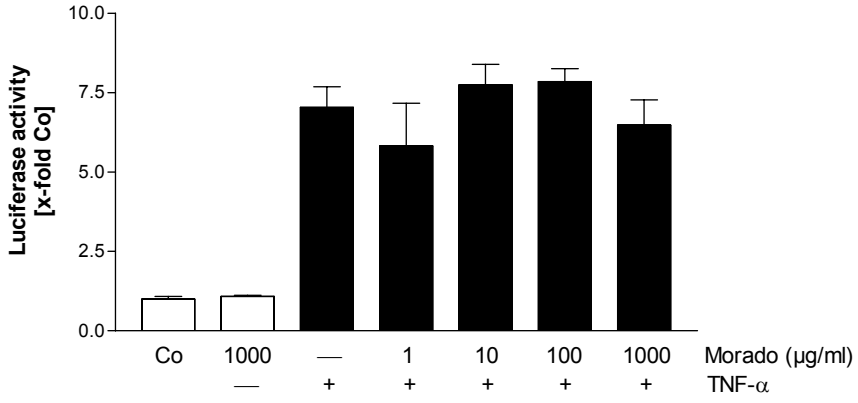


Figure 22: Aqueous extract of Morado 200 does not inhibit NF-κB transactivation activity

HEK 293 cells were either left untreated or were pre-treated with aqueous extract of Morado 200 as indicated. After 2 h TNF-α (1 ng/ml) was added for additional 6 h. Co represents luciferase activity of untreated cells. Bars represent the mean ± SEM of at least three independent experiments performed in triplicate.

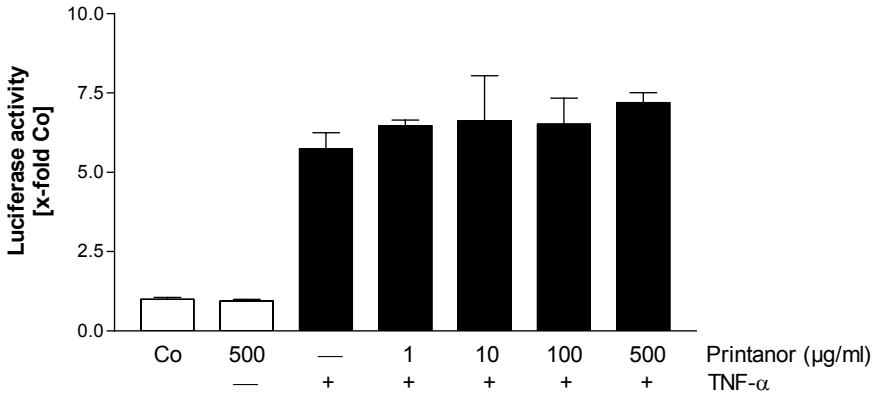


Figure 23: DMSO extract of Printanor 200 does not inhibit NF-κB transactivation activity

HEK 293 cells were either left untreated or were pre-treated with DMSO extract of Printanor 200 as indicated. After 2 h TNF-α (1 ng/ml) was added for additional 6 h. Co represents luciferase activity of untreated cells. Bars represent the mean ± SEM of at least three independent experiments performed in triplicate.

1.4 Interaction of garlic with adhesion molecules

Of interest was further whether the main garlic metabolites DADS and AM mediate an inhibitory effect on the TNF-α-induced expression of adhesion molecules ICAM-1 and E-selectin. Analysis of cell-surface proteins was carried out by flow-cytometry.

1.4.1 Garlic metabolites do not affect TNF-α-activated E-selectin expression.

2 h pre-treatment of HUVECSs with DADS (5-20 µM) and AM (5-20 µM) does not inhibit TNF-α-induced E-selectin expression. DADS rather slightly increased E-selectin levels (figure 24).

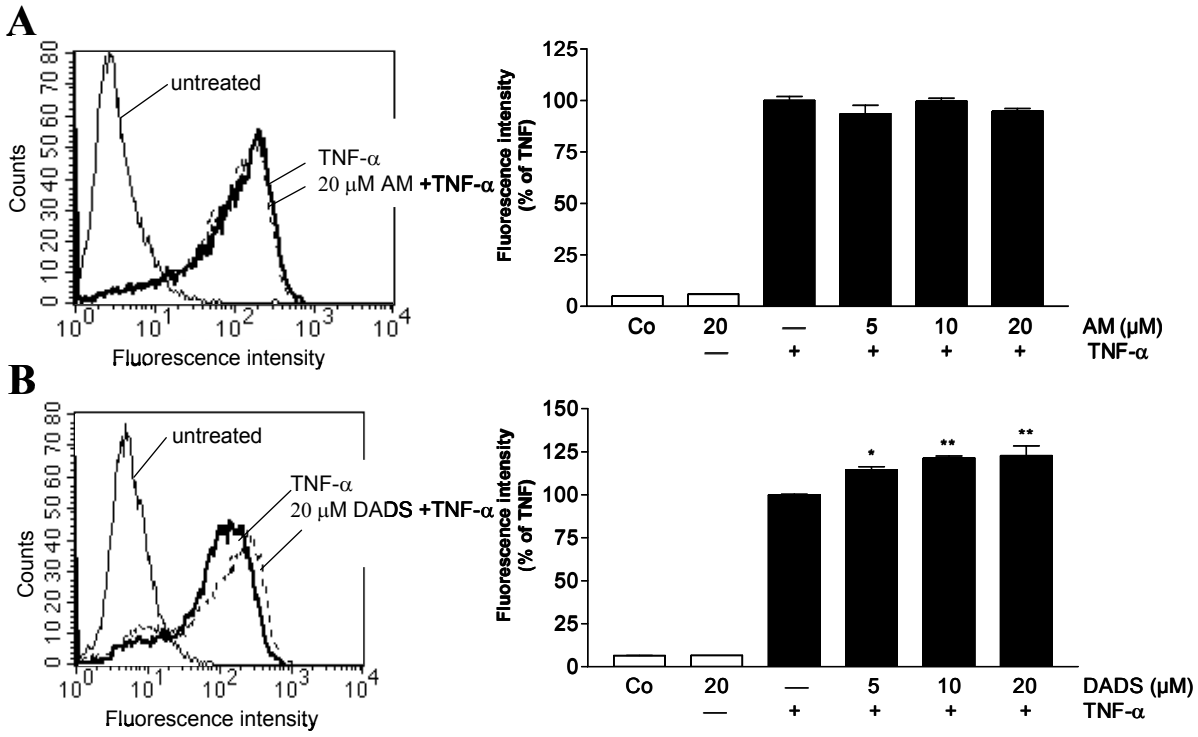


Figure 24: DADS and AM do not inhibit the induction of the adhesion molecule E-selectin

HUVECSs were either left untreated or were pre-treated with AM (A) or DADS (B) as indicated. After 2 h TNF-α (10 ng/ml) was added for additional 4 h. Expression of E-selectin was quantified by flow cytometry as described under Methods*. Data in the left panels show representative histograms demonstrating numbers of cells* vs. *fluorescence intensity at 520 nm. Co represents E-selectin expression of untreated cells. Bars represent the mean ± SEM of at least three independent experiments performed in triplicate. * P < 0.05, ** P < 0.01 (ANOVA/Bonferroni).*

It was reported by Ide and Lau, 2001 that SAC pre-incubation for 24 h inhibited TNF-α-induced NF-κB translocation. Therefore, we decided to extend the pre-incubation period to 24 h and to increase the amount of DADS and AM. Pre-treatment with DADS or AM up to 100 μM for 24 h also showed no effect on the expression E-selectin (figure 25)

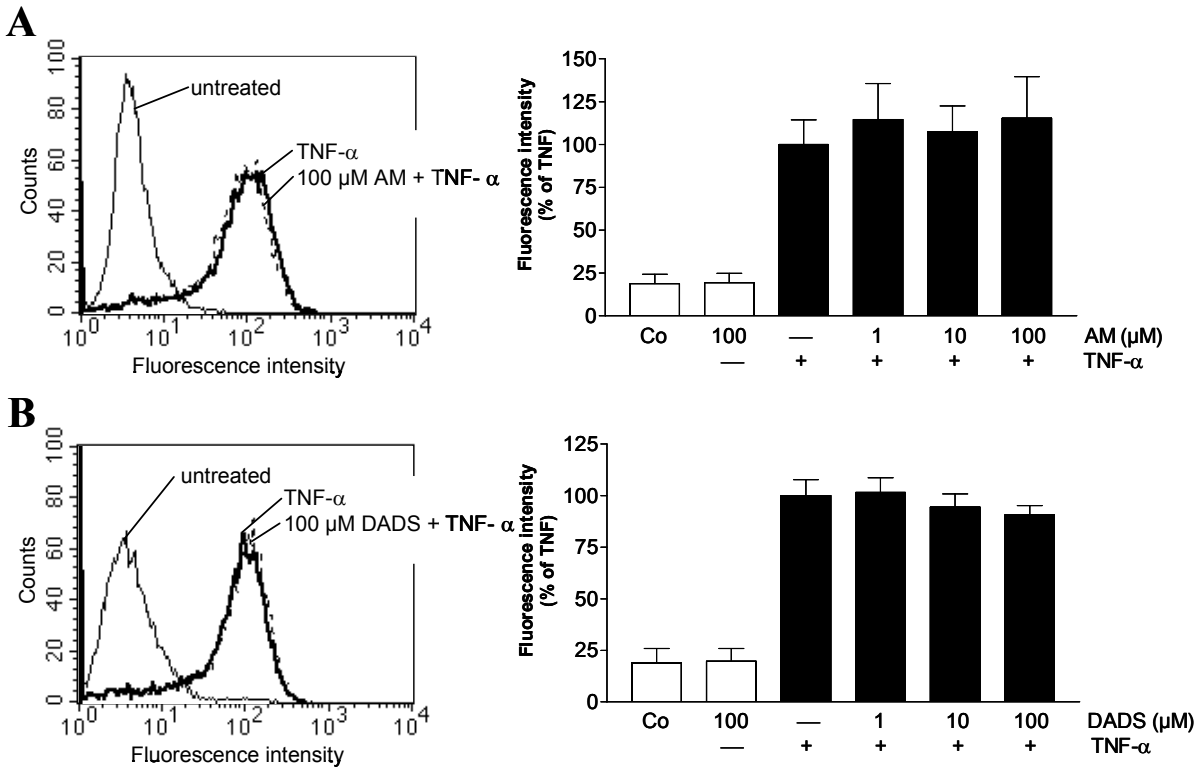


Figure 25: 24 h pre-incubation with DADS and AM do not inhibit the induction of E-selectin

HUVECSs were either left untreated or were pre-treated with AM (A) or DADS (B) as indicated for 24 h. Afterwards TNF-α (10 ng/ml) was added for additional 4 h. Expression of E-selectin was quantified by flow cytometry as described under Methods*. Data in the left panels show representative histograms demonstrating numbers of cells* vs. *fluorescence intensity at 520 nm. Co represents E-selectin expression of untreated cells. Bars represent the mean ± SEM of at least three independent experiments performed in triplicate.*

To demonstrate the functionality of our assay we performed experiments with the known NF-κB inhibitor parthenolide (0.1 – 10 µM). TNF-α-mediated expression of E-selectin was significantly inhibited with 10 µM parthenolide (figure 26).

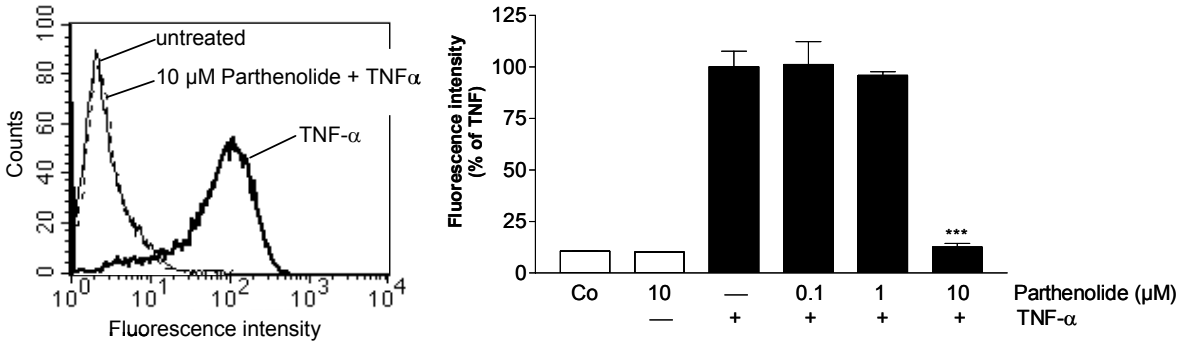


Figure 26: Parthenolide significantly inhibits induction of E-selectin

HUVECSs were either left untreated or were pre-treated with parthenolide as indicated. After 2 h TNF-α (10 ng/ml) was added for additional 4 h. Expression of E-selectin was quantified by flow cytometry as described under Methods*. Data in the left panel show a representative histogram demonstrating numbers of cells* vs. *fluorescence intensity at 520 nm. Co represents E-selectin expression of untreated cells. Bars represent the mean ± SEM of at least three independent experiments performed in triplicate. *** P < 0.001 (ANOVA/Bonferroni).*

1.4.2 Garlic metabolites do not affect TNF-α-activated ICAM-1 expression

Pre-incubation with AM (5-20 µM) for 2 h (figure 27) was as ineffective in inhibiting TNF-α-induced expression of ICAM-1 as pre-treatment with DADS (1 – 100 µM) and AM (1 – 100 µM) for 24 h (figure 28).

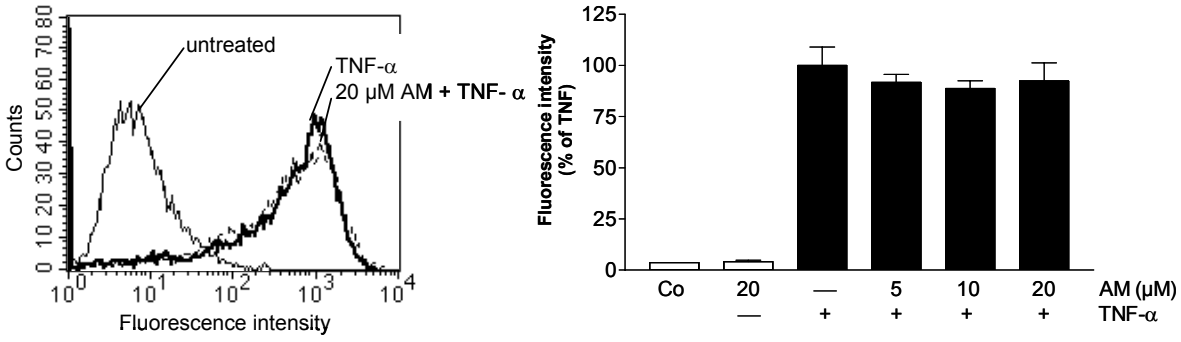


Figure 27: AM does not inhibit the induction of ICAM-1

HUVECSs were either left untreated or were pre-treated with AM as indicated. After 2 h TNF-α (10 ng/ml) was added for additional 4 h. Expression of E-selectin was quantified by flow cytometry as described under Methods*. Co represents ICAM-1 expression of untreated cells. Data in the left panel show a representative histogram demonstrating numbers of cells* vs. *fluorescence intensity at 520 nm.*

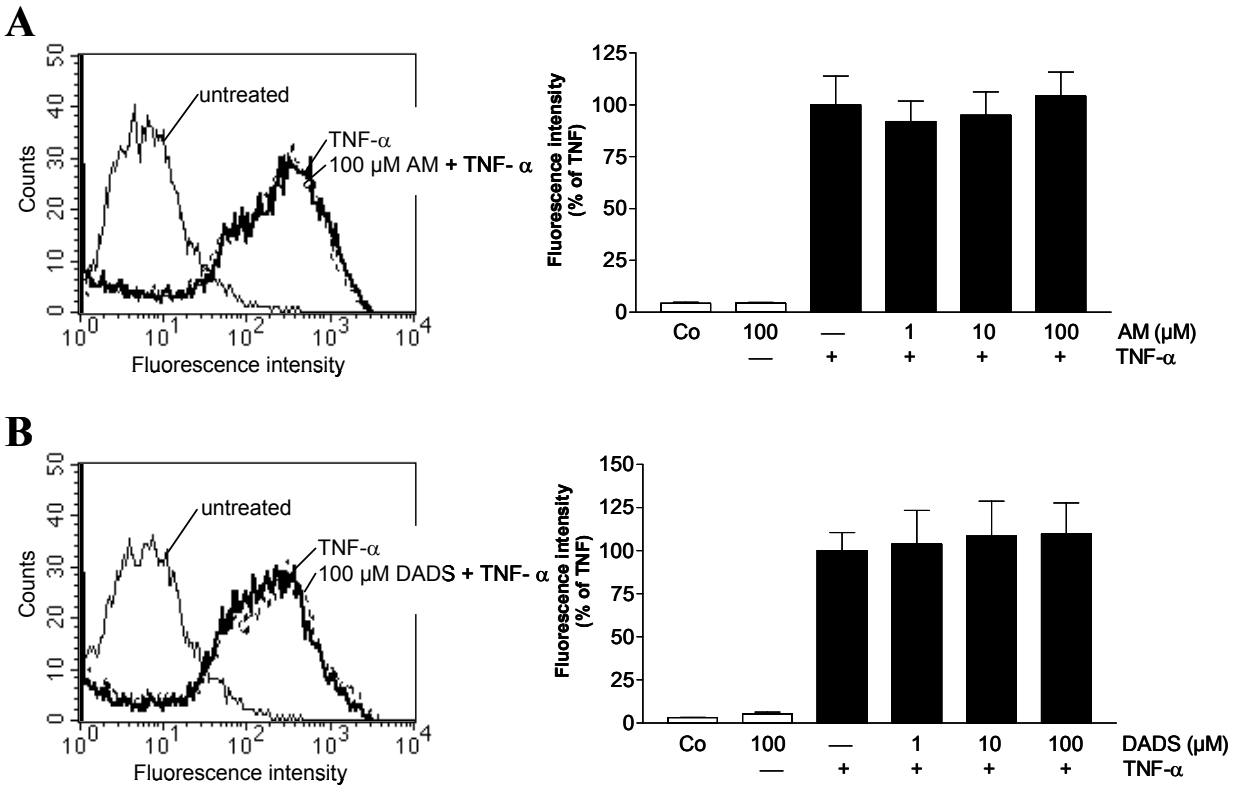


Figure 28: 24 h pre-incubation with DADS and AM do not inhibit the induction of ICAM-1

HUVECSs were either left untreated or were pre-treated with AM (A) or DADS (B) as indicated for 24 h. Afterwards TNF-α (10 ng/ml) was added for additional 16 h. Expression of ICAM-1 was quantified by flow cytometry as described under Methods*. Data in the left panels show representative histograms demonstrating numbers of cells* vs. *fluorescence intensity at 520 nm. Co represents ICAM-1 expression of untreated cells. Bars represent the mean ± SEM of at least three independent experiments performed in triplicate.*

1.5 Interaction of garlic with cytokines

The human immune system (e.g. leucocytes, monocytes and macrophages) reacts in the presence of even small amounts of LPS with massive liberation of cytokines, which can cause severe inflammatory response leading to sepsis, septic-shock or multi-organ failure (Ginsburg, 2002). Additionally, it has been shown that the transcription factor NF-κB is also involved in the LPS-induced liberation of cytokines (Guha and Mackman, 2001). Since garlic mediates anti-inflammatory effects, we were interested how garlic constituents or garlic extracts would influence cytokine liberation in an *ex*

vivo human whole blood model. We used garlic powders from fertilized and unfertilized garlic plants and prepared two different kinds of extracts. The solvents used were RPMI medium on the one hand as aqueous solvent and DMSO as lipophilic solvent on the other hand.

1.5.1 Interaction of aqueous dry garlic powder extracts with cytokines

We examined aqueous extracts made from fertilized or unfertilized garlic dry powders (aGPE) in final concentrations of 0.1 $^{mg}/_{ml}$ and 1 $^{mg}/_{ml}$ (garlic powder/ml). Treatment of whole blood with 10 ng/ml LPS led to a strong liberation of the pro-inflammatory cytokines IL-1β and TNF-α (figure 29 A&B). Stimulation of human whole blood with aqueous garlic powder extract alone led to a dose-dependent liberation of the pro-inflammatory cytokines IL-1β and TNF-α. Co-treatment with aGPE and LPS resulted in an additive increase of liberated pro-inflammatory IL-1β (figure 29A) and TNF-α (figure 29B). Similarly, anti-inflammatory cytokine IL-10 was liberated by garlic extracts alone. Blood samples co-treated with LPS and garlic showed higher amounts of IL-10, than samples treated with LPS alone (figure 29C). The liberation of cytokines by aqueous garlic extracts point to an endotoxin-like activity of aGPE. Interestingly, we found that treatment with an extract from sulfur-fertilized garlic showed lower stimulating effects alone or with LPS as an extract from unfertilized garlic.

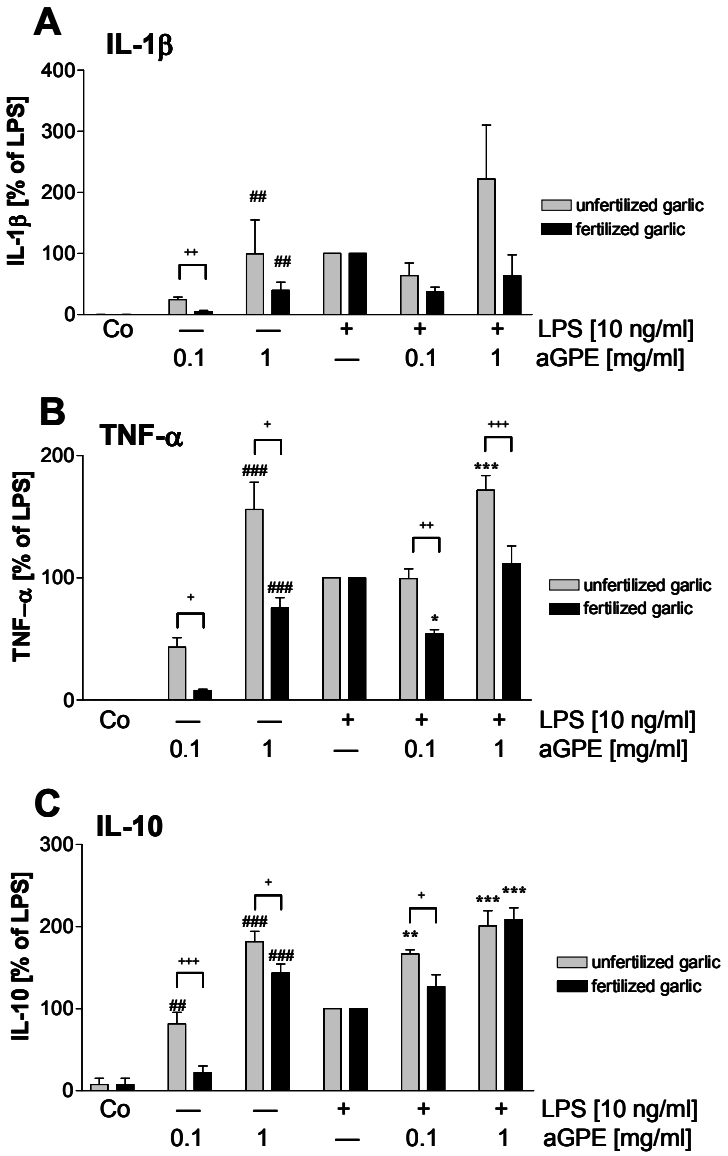


Figure 29: Effect of aqueous garlic extracts on the liberation of cytokines.

Whole blood was incubated with the indicated concentrations of either aGPE alone or where indicated with 10 ng/ml LPS and subsequently incubated for 16 h. The levels of IL-1β (A), TNF-α (B) and IL-10 (C) were measured by ELISA as described under Methods*. Co represents cytokine levels of untreated cells. Bars represent the mean ± SEM of values obtained from four healthy blood donors. ##P<0.01, ###P<0.001 compared to Co; *P<0.05, ***P<0.001 compared to LPS (ANOVA/Bonferroni); +P<0.05, ++P<0.01, +++P<0.001 compared to unfertilized garlic (paired t-test).*

1.5.2 Interaction of DMSO dry garlic powder extract with cytokines

Treatment of whole blood with 10 ng/ml LPS led to a strong liberation of the pro-inflammatory cytokines IL-1β and TNF-α (figure 30A&B). Co-treatment with DMSO garlic powder extract (dGPE 10 and 100 µg/ml) resulted in a significant reduction of liberated IL-1β (figure 30A). Similarly, the amount of liberated TNF-α was significantly impaired compared to human whole blood treated with LPS only (figure 30B). dGPE alone, in the absence of LPS, did not influence cytokine levels in human whole blood indicating that it possesses a low endotoxin-like activity. Additionally we found that an extract from sulfur-fertilized garlic inhibited LPS-induced TNF-α and IL-1β release better than the extract of unfertilized garlic.

LPS alone increased the expression of IL-10. Co-incubation with 10 or 100 µg/ml of dGPE showed no inhibitory effect on LPS-induced liberation of IL-10. In contrast, dGPE tended to increase IL-10 release (figure 30C). Since expression of IL-10 peaks later than the expression of TNF-α or IL-1β (Boneberg and Hartung, 2001; Boneberg and Hartung, 2001), it seems unlikely, that a cytotoxic effect of dGPE is responsible for the decreased TNF-α and IL-1β levels. Again dGPE alone did not affect IL-10 production in the absence of LPS. Fertilization seems not to have a effect on IL-10 liberation.

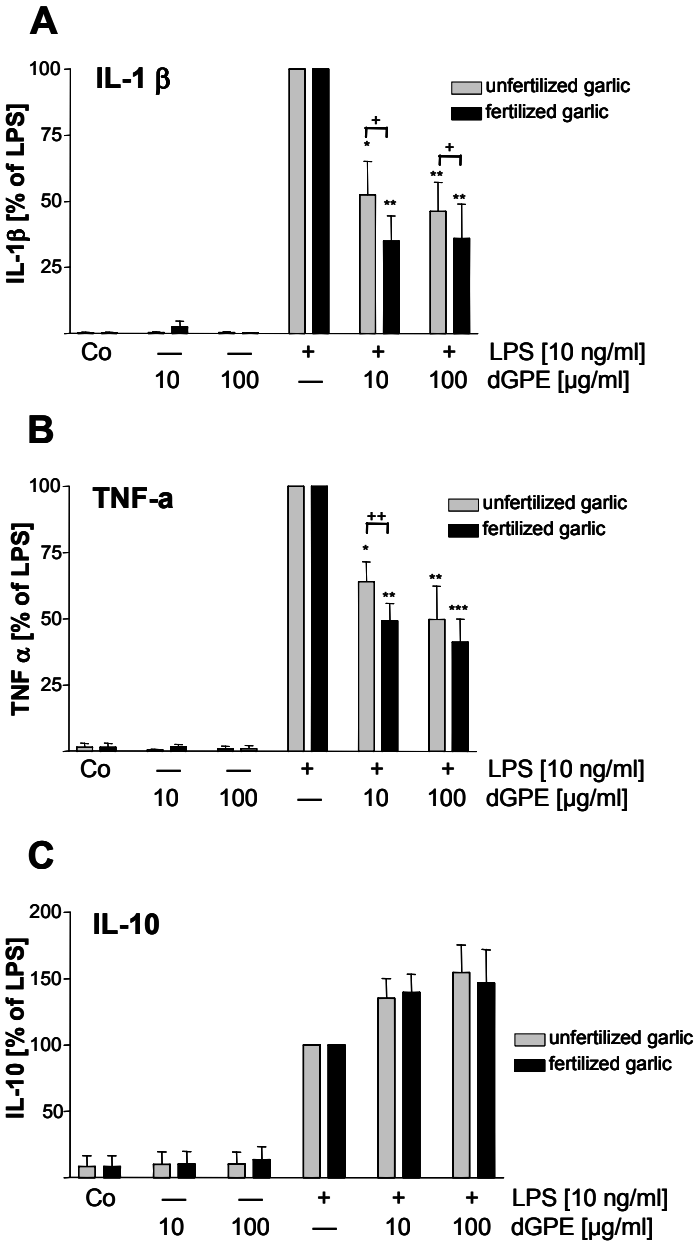


Figure 30: Effect of DMSO garlic extracts on the liberation of cytokines.

Whole blood was incubated with the indicated concentrations of either dGPE alone or where indicated with 10 ng/ml LPS and subsequently incubated for 16 h. The levels of IL-1β (A), TNF-α (B) and IL-10 (C) were measured by ELISA as described under Methods*. Co represents cytokine levels of untreated cells. Bars represent the mean ± SEM of values obtained from four healthy blood donors. *P<0.05, **P<0.01, ***P<0.001 compared to LPS (ANOVA/Bonferroni); +P<0.05, ++P<0.01 compared to unfertilized garlic (paired t-test).*

1.5.3 Interaction of garlic constituents with cytokines

In order to examine whether garlic constituents allicin, DADS, γ-Glu-Cys and $AMSO_2$, contribute to the observed effects of GPE we employed all of the compounds in the whole blood assay system.

Incubation of blood samples with LPS in the presence of DADS (1-100 µM) led to a significant reduction of Il-1β and TNF-α. Yet DADS showed no influence on LPS-induced IL-10 release (figure 31).

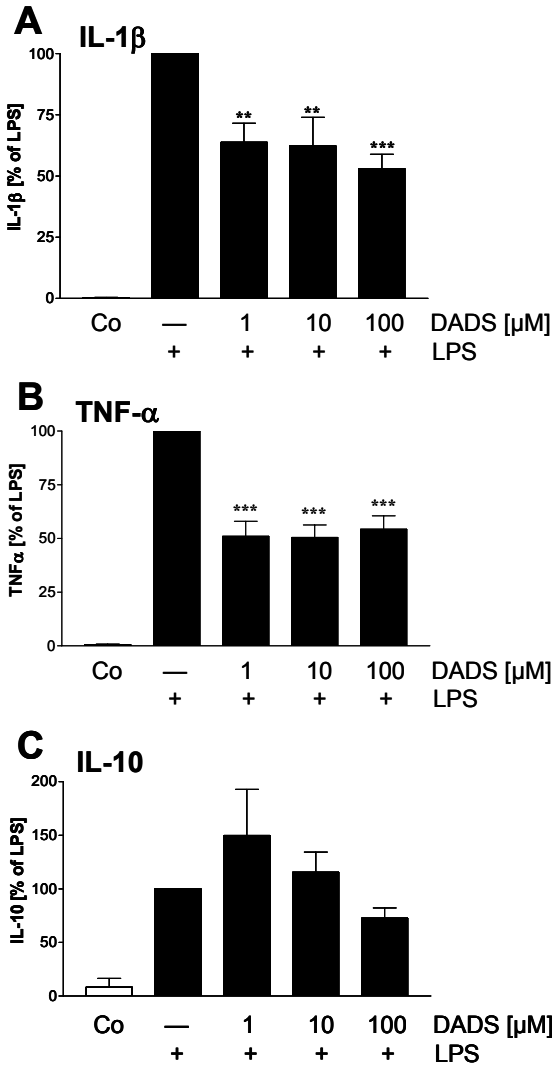


Figure 31: Effect of DADS on cytokine levels

Whole blood was incubated with various concentrations of DADS either alone or where indicated with 10 ng/ml LPS and subsequently incubated for 16 h. The levels of IL-1β , TNF-α and IL-10 were measured by ELISA as described under Methods*. Co represents cytokine levels of untreated cells. Bars represent*

*the mean ± SEM of values obtained from four healthy blood donors. **P<0.01, ***P<0.001 (ANOVA/Bonferroni).*

The major degradation product of alliin, allicin in concentrations up to 100 µM had no influence on IL-1β and TNF-α release and inhibited the liberation of IL-10 at a concentration of 100 µM (figure 32).

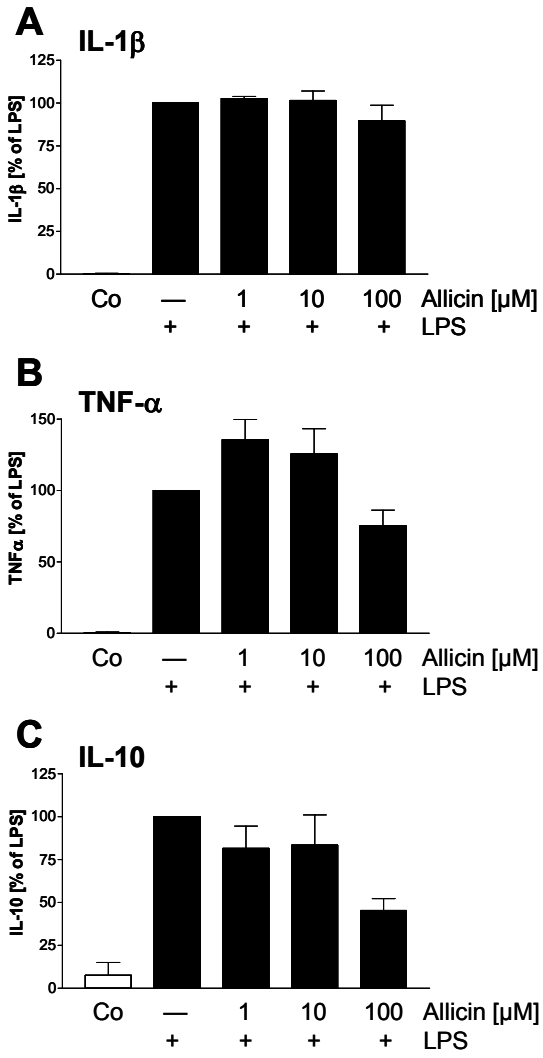


Figure 32: Effect of allicin on cytokine levels

Whole blood was incubated with various concentrations of allicin either alone or where indicated with 10 ng/ml LPS for 16 h. The levels of IL-1β, TNF-α and IL-10 were measured by ELISA as described under Methods. *Co represents cytokine levels of untreated cells. Bars represent the mean ± SEM of values obtained from four healthy blood donors. **P<0.01 (ANOVA/Bonferroni).*

$AMSO_2$ a major metabolite of DADS (Germain et al., 2002) did not reduce Il-1β (figure 33A), TNF-α (figure 33B) or IL-10 (figure 33C) liberation after LPS treatment. An other garlic constituent γ-glutamyl-cysteine (γ-GC) showed also no significant effect on the LPS-induced liberation of IL-1β (figure 33D), TNF-α (figure 33E) and IL-10 (figure 33F) in human whole blood.

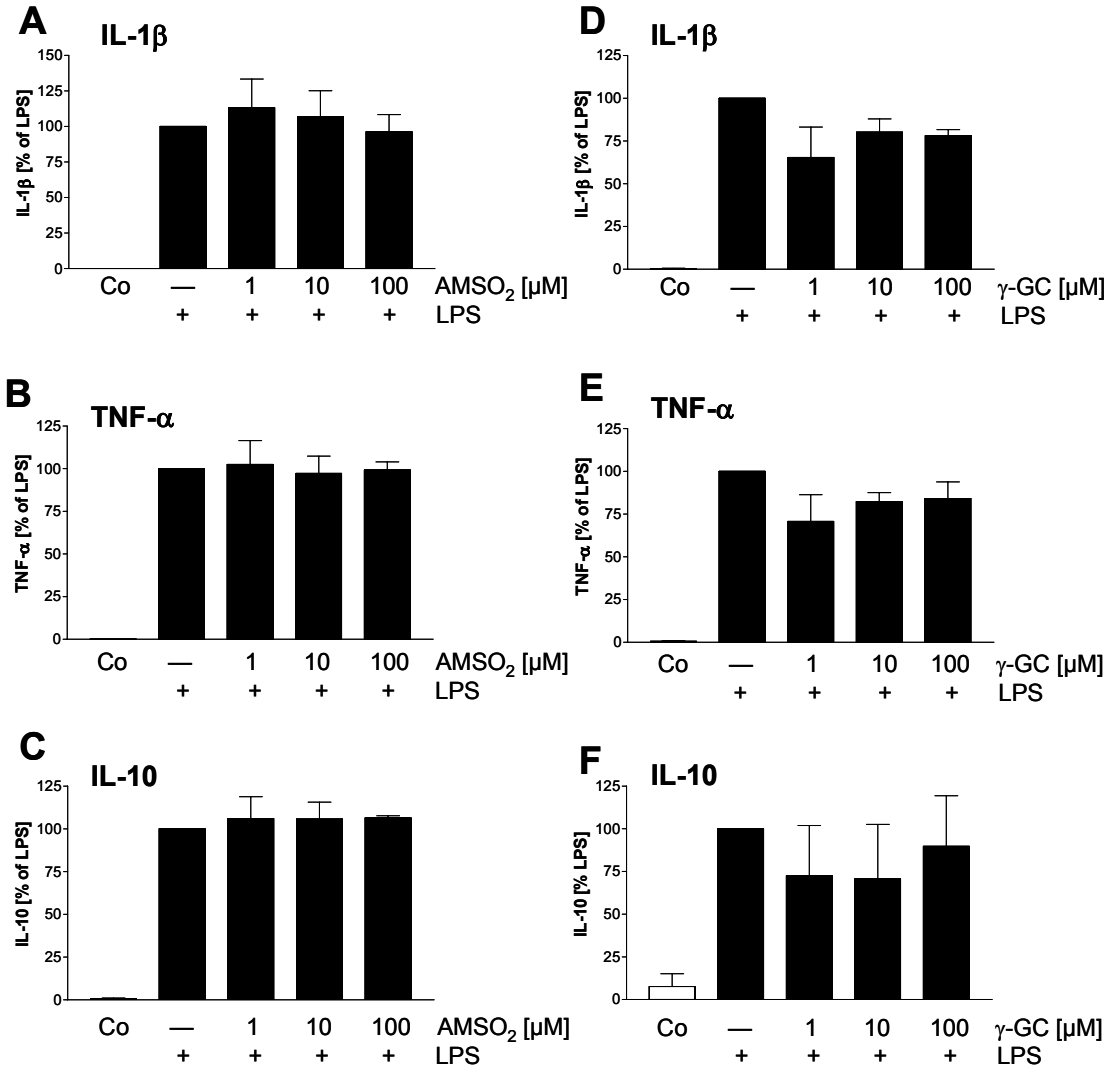


Figure 33: Effect of allyl-methyl-sulfone and γ-glutamyl-allyl-cysteine on cytokine levels

Whole blood was incubated with various concentrations of $AMSO_2$ or γ-GC either alone or where indicated with 10 ng/ml LPS for 16 h. The levels of IL-1β , TNF-α and IL-10 were measured by ELISA as described under Methods*. Co represents cytokine levels of untreated cells. Bars represent the mean ± SEM of values obtained from four healthy blood donors.*

1.6 Altered cytokine levels in human whole blood affect activation of NF-κB in HEK 293 cells.

The crucial question was: would a dGPE-mediated reduction of pro-inflammatory cytokines, such as TNF-α and IL-1β, and slightly increased amounts of anti-inflammatory IL-10 finally result in a reduced activation of NF-κB, and thus in a putative anti-inflammatory response. Incubation of transfected HEK 293 cells with cell-free blood sample supernatants of LPS-activated whole blood increased the luciferase activity about 8-fold compared to the supernatants of untreated blood (figure 34A). dGPE-treated supernatants led to a significant reduced NF-κB activity corresponding to their lowered pro-inflammatory cytokine levels. In agreement with the results obtained from measuring cytokine levels, blood sample supernatants treated with fertilized garlic showed a stronger inhibitory effect than samples treated with unfertilized garlic (figure 34A). To rule out that the observed result was due to a direct effect of dGPE (instead of modulated cytokine levels in blood samples) we pre-incubated transfected HEK 293 cells with dGPE (10 or 100 µg/ml) and activated cells with TNF-α (1 ng/ml). As shown in figure 34B, dGPE had no direct inhibitory effect on TNF-α-induced expression of the NF-κB-driven luciferase reporter gene.

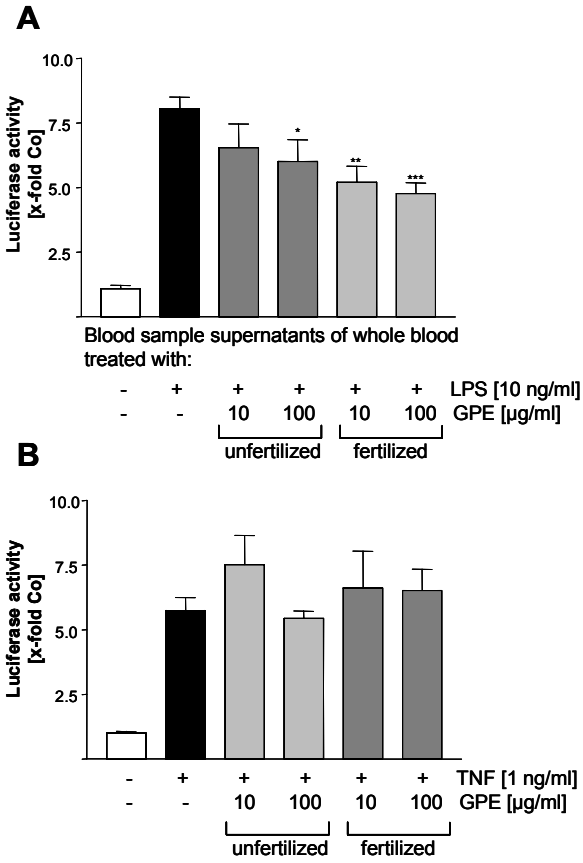
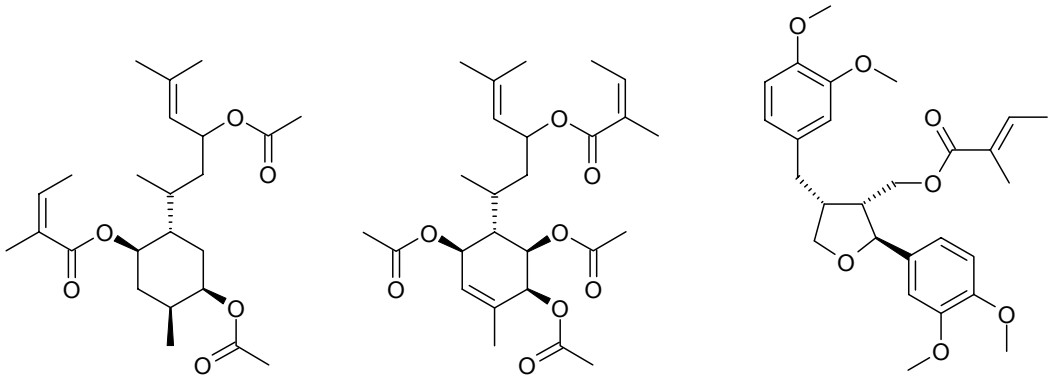


Figure 34: Garlic powder extract (dGPE)-treated and LPS activated blood samples induce lower NF-κB activity than blood samples activated with LPS alone.

*(A) HEK293 cells were incubated for 6 h with 5 µl of cell-free supernatant from whole blood samples treated as indicated. (B) Cells were pre-incubated with GPE for 2 h and subsequently stimulated with 1 ng/ml TNF-α for additional 6 h. Subsequently, a luciferase reporter-gene assay was performed. Co represents luciferase activity of cells treated with supernatant of untreated blood samples (A), or untreated cells (B) respectively. Bars represent the mean ± SEM, of at least three independent experiments performed in triplicate. *P<0.05, **P<0.01, ***P<0.001 (ANOVA/Bonferroni).*

2. Edelweiss (*Leontopodium alpinum* Cass.)

Edelweiss has been used by inhabitants of the Alps in traditional medicine for the treatment of bronchitis, fever and to cure inflammatory diseases of the gastrointestinal tract in man and animals (Bitschnau T., 1991; Kiene K., 1992b). We, therefore, focused on the possibly underlying molecular mechanisms of single constituents isolated from *L.alpinum*. We received bisabolane A, bisabolane CD and MAB F7 from Prof. Dr. H. Stuppner (University of Innsbruck, Austria). The constituents were isolated from roots of *Leontopodium alpinum* Cass., the purity was found to be >98 % as judged by HPLC. All structures are depicted in figure 35.



Bisabolane A Bisabolane CD MAB F7

Figure 35: Structure of constituents of Leontopodium alpinum Cass.

2.1 Constituents of *L. alpinum* inhibit TNF-α-induced NF-κB transactivation

2.1.1 Bisabolane A and bisabolane CD inhibit NF-κB transactivation.

We started to examine whether Bisabolane A, CD or MAB F7 were able to inhibit TNF-α-mediated NF-κB transactivation. HEK 293 cells were transfected with pNF-κBluc and pRL-TK as described in *Materials and Methods*. Transfected cells were pre-incubated with bisabolane A and bisabolane CD (1-100 μM) for 2 h. Subsequently cells were activated with 1 ng/ml TNF-α for 6 h. Bisabolane A as well as bisabolane CD

significantly inhibited TNF-α induced NF-κB transactivation in concentrations of 100 µM (figure 36). Unfortunately, the observed effect was not dose-dependent and therefore we did not conduct further experiments with bisabolane A and bisabolane CD.

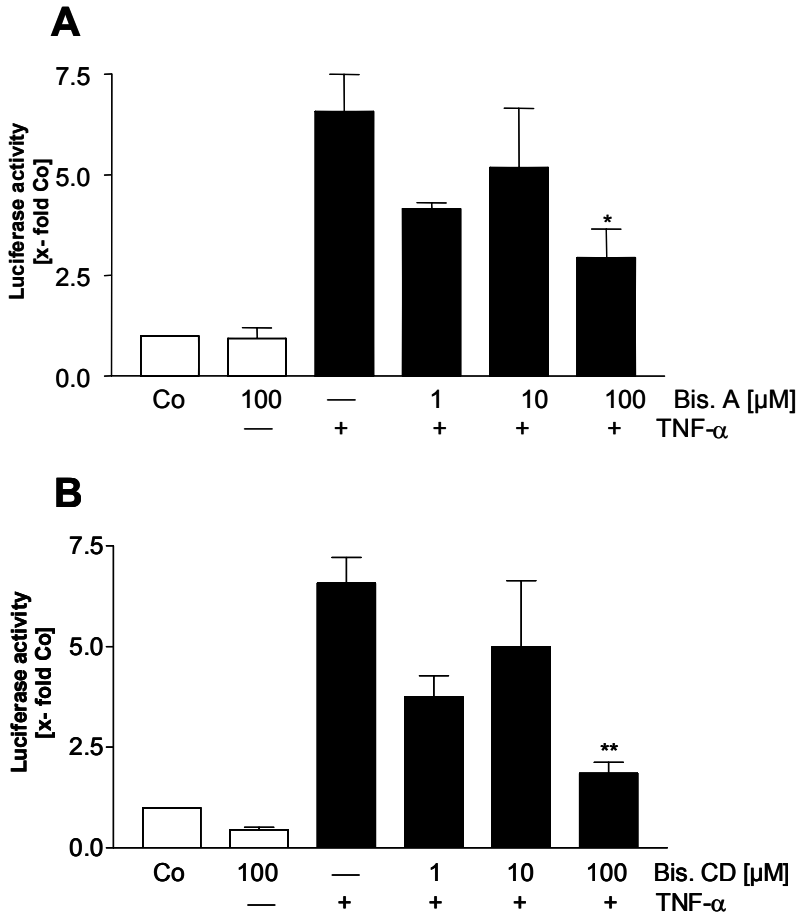


Figure 36: Bisabolane A and bisabolane CD inhibit NF-κB-driven luciferase activity

*HEK 293 cells were pre-incubated with various concentrations of bisabolane A (A) and bisabolane CD (B) for 2 h. Afterwards cells were activated with 1 ng/ml TNF-α for 6 h. Co represents luciferase activity of untreated cells. Bars represent mean ± SEM of three independent experiments performed in triplicate. *p<0.05, **p<0.01 (ANOVA/Bonferroni).*

2.1.2 MAB F7 inhibits TNF-α-mediated NF-κB transactivation but not nuclear translocation and DNA-binding of NF-κB.

Stimulation with 1 ng/ml TNF-α resulted in a strong increase of NF-κB activity. Pre-treatment of transfected HEK 293 cells with MAB F7 for 2 h inhibited dose-dependent TNF-α activated NF-κB transactivation with a maximal inhibition at 50 µM. 50 µM of MAB F7 alone had no influence on NF-κB activity (figure 37). Since MAB F7 exhibited a more effective and dose-dependent inhibition of NF-κB than Bisabolane A & CD, we focused on the mechanisms how MAB F7 mediates its effects.

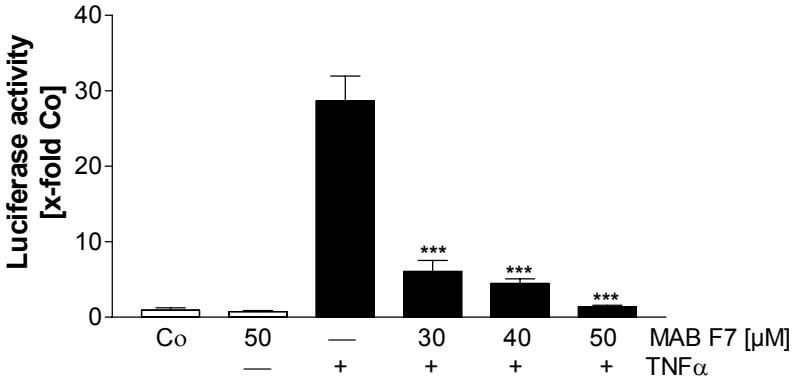


Figure 37: MAB F7 inhibits TNF-α-mediated NF-κB-driven luciferase activity in HEK 293

*Transfected HEK 293 cells were pre-incubated with various concentrations of MAB F7 for 2 h. Then cells were activated with 1 ng/ml TNF-α for 6 h. Co represents luciferase activity of untreated cells. Bars represent mean ± SEM of three independent experiments performed in triplicate. ***p<0.001 (ANOVA/Bonferroni).*

Next we tested whether this effect is due to a reduced DNA binding respectively an impaired nuclear translocation of NF-κB. For that reason we pre-treated HUVECS and Jurkat cells with MAB F7 for 2 h before stimulation with 5 ng/ml TNF-α for 30 min. Nuclear extracts were prepared and assayed for NF-κB-binding activity by EMSA. As it can be seen in figure 38 TNF-α led to a strong induction of NF-κB DNA binding, however MAB F7 did not inhibit DNA binding in neither HUVECS nor Jurkat cells.

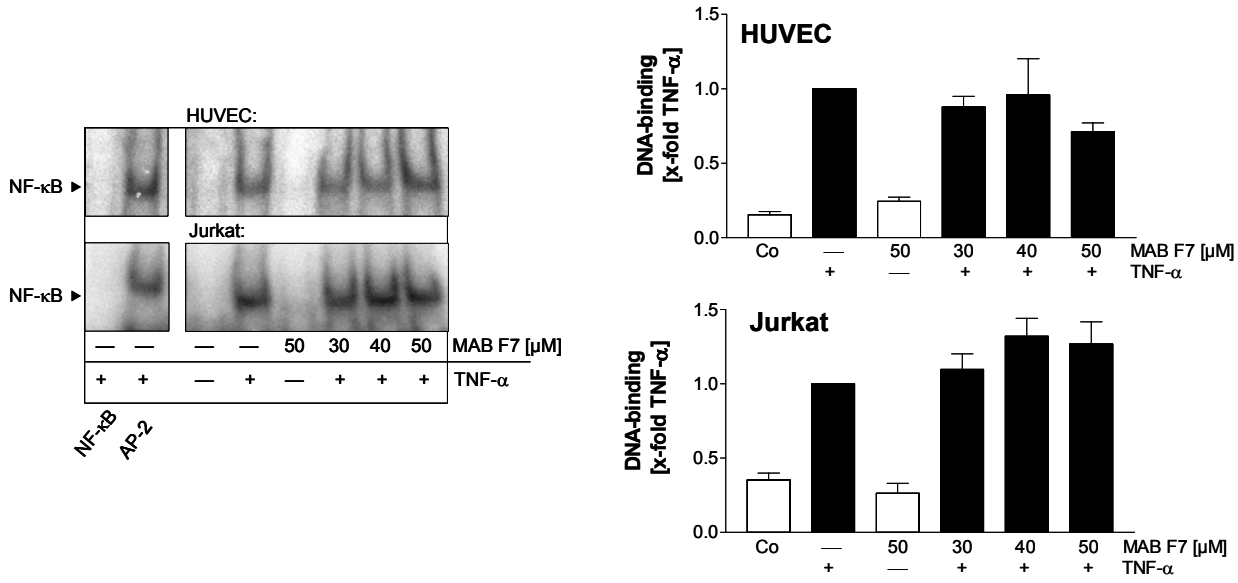


Figure 38: MAB F7 does not impair TNF-α-mediated NF-κB DNA binding
Left panel: HUVECSs or Jurkat were either left untreated or were treated with MAB F7 as indicated. After 2 h TNF-α (5 ng/ml) was added for additional 30 min. Equal amounts of nuclear extracts were employed to EMSA as described under Methods*. The first two lanes of each gel show DNA binding of nuclear extracts of TNF-α-activated cells incubated with a 100-fold excess of unlabeled NF-κB (unlabeled competitor, line 1) or AP-2 (unlabeled noncompetitor, line 2) consensus oligonucleotides. All experiments were performed at least three times. Representative gel shift experiments are shown.*
Right panel: Quantitative evaluation of densitrometric data of all experiments. Co represent NF-κB DNA-binding activity of untreated cells. Bars represent the mean ± SEM of at least three independent experiments.

We examined further the required incubation period to block NF-κB activation by MAB F7. Cells were incubated with MAB F7 for 2 h, 1 h, 30 min and 15 min before the addition of TNF-α, simultaneously with TNF-α, or 15 min, 30 min, 1 h, 2 h and 4 h after the addition of TNF-α. We found that MAB F7 inhibited NF-κB transactivation effectively until 2 h after the addition of TNF-α. However, when MAB F7 was added 4 h after TNF-α, no effect on NF-κB activity was seen (figure 39).

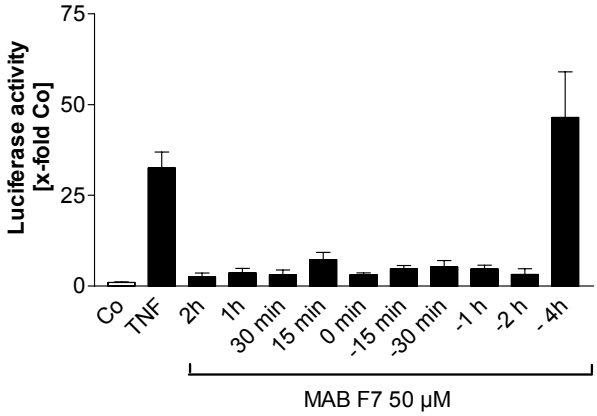


Figure 39: MAB F7 still inhibits NF-κB-driven luciferase activity 2 h after the addition of TNF-α

Transfected HEK 293 cells were incubated with 50 µM MAB F7 for 2 h, 1 h, 30 min and 15 min before, at the same time (0 min), or 15 min, 30 min, 1 h, 2 h and 4 h after the addition of TNF-α (-15 min, -30 min,...-4 h) . Cells were activated with 1 ng/ml TNF-α for 6 h at time point 0 min. Co represents luciferase activity of untreated cells. Bars represent mean ± SEM of three independent experiments performed in duplicate.

2.2 MAB F7 does not mediate cytotoxic effects in HUVECS

In order to rule out that the effects seen are caused by cytotoxic side-effects, we incubated HUVECSs with 50 µM MAB F7 in the absence or presence of TNF-α for 24 h. Cell viability was determined by propidium-iodide staining as described in *Methods*. Treatment of cells with MAB F7 had no significant effect on cell viability. Likewise, cells co-treated with TNF-α and MAB F7 were as viable as cells incubated with TNF-α alone (figure 40)

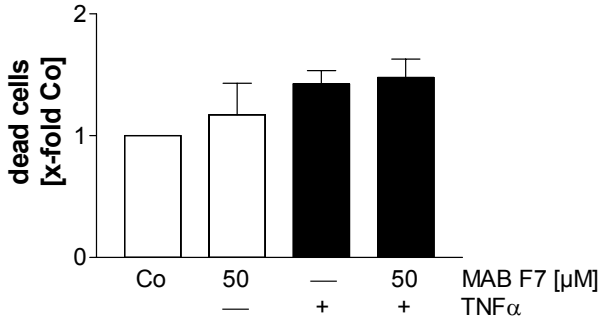


Figure 40: MAB F7 does not show cytotoxic effects after 24 h

HUVECS were treated with 50 µM MAB F7 for 24 h in the absence or presence of TNF-α. After 24 h the amount of dead cells was determined by propidium-iodide staining as described under Methods. Co

represents the relative amount of dead untreated cells. Bars represent the mean ± SEM of three independent experiments performed in duplicate.

2.3 MAB F7 inhibits the expression of ICAM-1 and E-selectin.

In order to prove that MAB F7 affects NF-κB-regulated genes we examined whether the TNF-α-induced expression of endothelial adhesion molecules ICAM-1 and E-selectin is inhibited by MAB F7. Stimulation of HUVECS with TNF-α increased ICAM-1 and E-selectin expression on the cell surface. Surface expression of ICAM-1 and E-selectin was reduced to nearly basal levels by pre-treatment with 50 µM MAB F7 (figure 41).

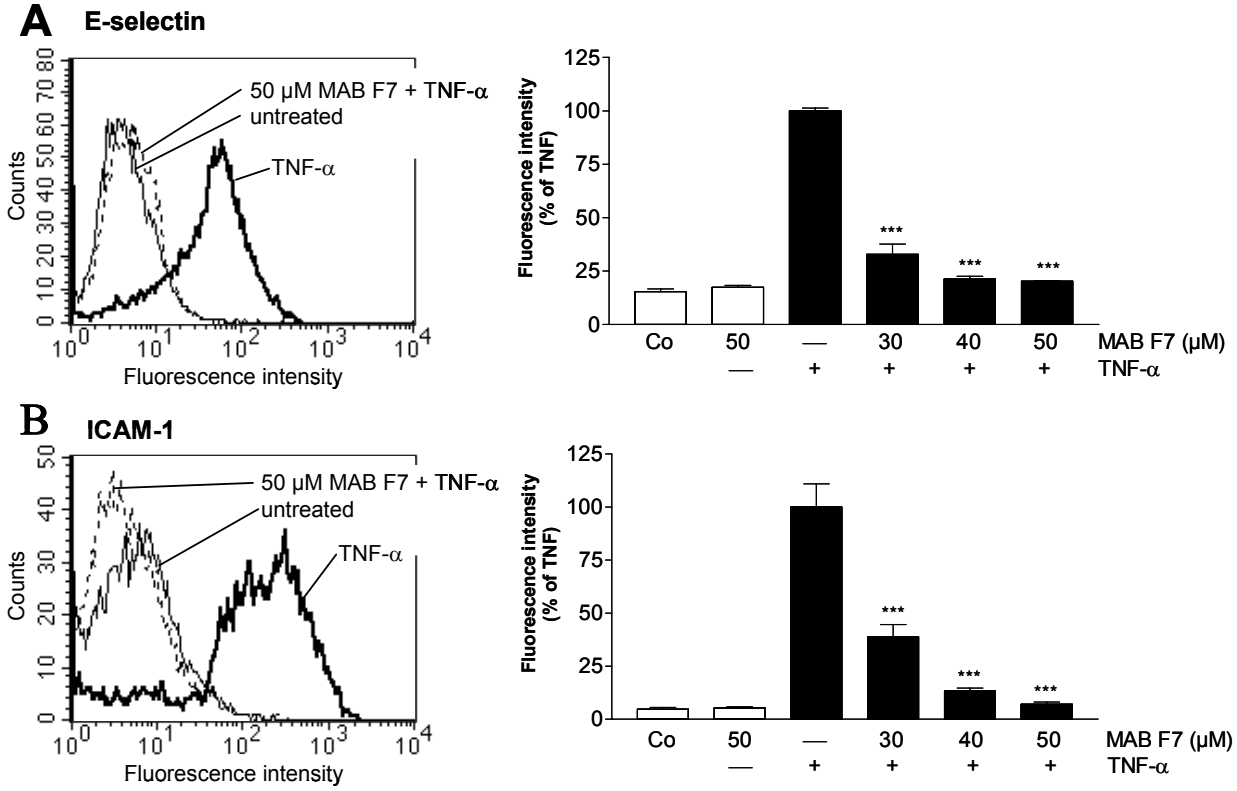


Figure 41: MAB F7 inhibits the expression of ICAM-1 and E-selectin on the cell surface.

HUVECSs were either left untreated or were pre-treated with MAB F7 as indicated. After 2 h TNF-α (5 ng/ml) was added for additional 4 h (E-selectin) or 16 h (ICAM-1), respectively. Expression of E-selectin and ICAM-1 was quantified by flow cytometry as described under Methods*. Co represents adhesion molecules expressed on the surface of untreated cells. Data in the left panels show representative histograms demonstrating numbers of cells* vs. *fluorescence intensity at 520 nm. Bars represent the mean ± SEM of at least three independent experiments. ***P<0.01 (ANOVA/Bonferroni).*

Additionally, we examined whether MAB F7 inhibits the TNF-α-induced expression of total ICAM-1 in HUVECSs. Western blot analysis of whole cell lysates for ICAM-1 revealed that co-treatment with TNF-α and 30 µM MAB F7 significantly increased ICAM-1 levels. However co-treatment with 50 µM MAB F7 led to a significant decrease of total ICAM-1 (figure 42).

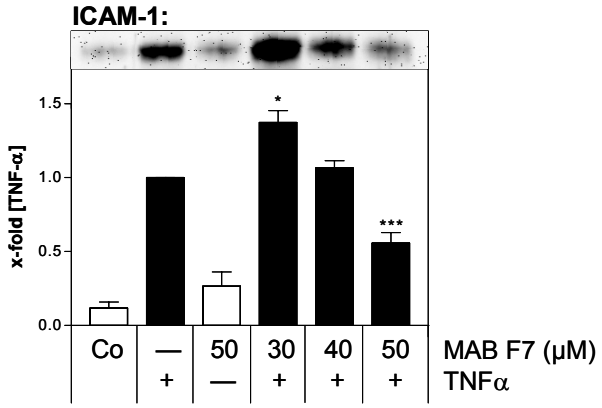


Figure 42: MAB F7 inhibits the TNF-α-induced expression of ICAM-1

*HUVECSs were either left untreated or were pre-treated with MAB F7 as indicated. After 2 h TNF-α (5 ng/ml) was added for additional 16 h. Co represents ICAM-1 expression of untreated cells. The top figure shows one representative western-blot of four independent experiments. The lower diagram shows the densitrometric evaluation of all four experiments as mean ± SEM. *P<0.05, ***P<0.001 (ANOVA/Bonferroni).*

2.4 MAB F7 does not interfere with TNF-α-induced expression of ICAM-1 mRNA.

In order to proof, that MAB F7 interacts with the NF-κB pathway and thereby reduces transcription of ICAM-1, we examined whether MAB F7 inhibits the TNF-α-induced expression of ICAM-1 mRNA by real-time RT-PCR. Surprisingly, MAB F7 did not inhibit TNF-α-mediated expression of ICAM-1 mRNA. 10 µM MG-132, a well established NF-κB inhibitor, reduced the amount of ICAM-1 mRNA to 20 % (figure 43).

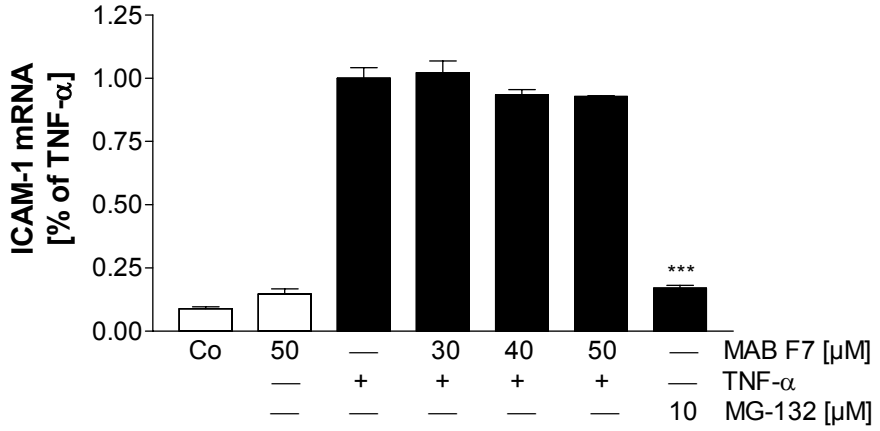


Figure 43: MAB F7 does not interfere with TNF-α induced expression of ICAM-1 mRNA

*HUVECSs were pre-treated with MAB F7 or MG-132 as indicated and subsequently incubated for 4 h with 5 ng/ml TNF-α. ICAM-1 mRNA levels were determined by real-time RT-PCR. Normalization of ICAM-1 mRNA was performed by evaluating against 18s RNA levels. Bars represent the mean ± SEM of three independent experiments performed in triplicate. ***P<0.001 (ANOVA/Bonferroni).*

2.5 MAB F7 inhibits protein synthesis in HUVECS and HEK 293 cells.

Since we now had the evidence that MAB F7 does not inhibit the TNF-α-mediated transcription of ICAM-1, we examined other mechanisms how MAB F7 could inhibit the TNF-α-induced expression of adhesion molecules. Since MAB F7 reduced E-selectin and ICAM-1 levels as well as the activity of the firefly luciferase we decided to check whether MAB F7 inhibits protein synthesis in general.

2.5.1 MAB F7 inhibits the expression of P_{cmv}-driven green fluorescent protein in HEK 293 cells.

In order to demonstrate that the MAB F7-mediated inhibition of luciferase activity as demonstrated before (see III.2.1.2) was not due to a specific inhibition of NF-κB activity but rather due to an interference with protein synthesis, we transfected HEK 293 with a plasmid containing a $P_{CMV\ IE}$ – driven green fluorescent protein. Right after the transfection procedure cells were incubated for 16 h with various concentrations of MAB F7. As positive control we used the known protein-synthesis inhibitor

cycloheximide (CHX) (Obrig et al., 1971). Expression of GFP-protein was determined by fluorescent microscopy. A representative field was chosen under normal light conditions (figure 44, left panel) and subsequently observed under UV-light (excitation wave-length: λ_{ex} = 450 – 490 nm; emission wave-length: λ_{em} = 520 nm) (figure 44, middle panel). Treatment with MAB F7 led to a decreased expression of green fluorescent protein (figure 44 C&D), compared to untreated cells (figure 44 B). Cells treated with cycloheximide also showed impaired levels of GFP (figure 44E)

Quantitative analysis of GFP-expression was carried out by flow cytometry. Dot-plot analysis of untransfected cells, as depicted in figure 46 (lane A, right panel), shows a unique population of cells, expressing a direct proportional increase in green fluorescence (FL-1H, λ_{em} = 530 nm) and red fluorescence (FL-2H, λ_{em} = 585 nm) after excitation with an argon-laser (λ_{ex} = 488 nm). Since cells expressing GFP emit more green fluorescent light (FL-1H) than red fluorescent light (FL-2H), they can easily be detected and quantified as a second population as depicted in figure 46, lane B, right panel. Events lying in region R1 (figure 44, lane A, right panel) were considered as GFP-expressing cells. Quantitative analysis showed that MAB F7 dose-dependently reduces GFP expression. After treatment with 50 µM MAB F7 only 35 % of the GFP-transfected cells expressed green fluorescent protein compared to untreated cells. CHX treatment decreased the expression of GFP to 30 % compared to untreated cells (figure 45).

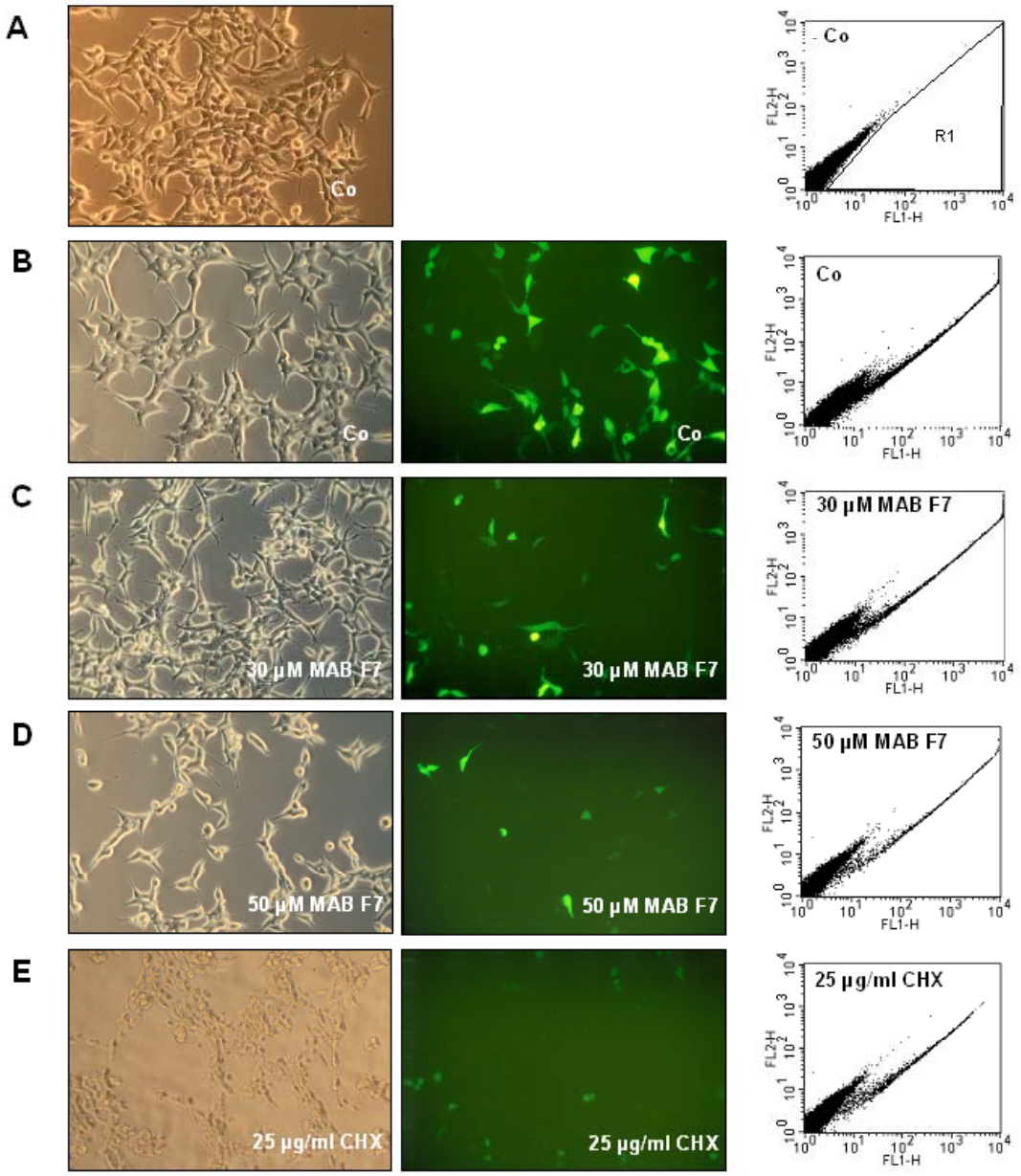


Figure 44: MAB F7 inhibits the expression of green fluorescent protein

Left panel shows a representative field of untransfected or transfected HEK 293 cells observed by normal light. Middle panel shows the same field of transfected cells under UV-light (λ_{ex} = 450 – 490 nm; λ_{em} = 520 nm). Right panel shows dot-plots of HEK cells demonstrating red fluorescence (FL-2H: λ_{em} 585 nm) vs. *green fluorescence (FL-1H: λ_{em} 530 nm). All events within region (R1) were considered as GFP-expressing cells. Lane A represents cells not transfected with pEGFP-N1. Lane B: untreated transfected cells. Lane C: transfected cells treated with 30 µM MAB F7 for 16 h. Lane D: transfected cells treated with 50 µM MAB F7 for 16 h. Lane E: transfected cells treated with 25 µg/ml cycloheximide (CHX) for 16 h. Representative pictures and dot-plots of three independent experiments performed in triplicate are shown.*

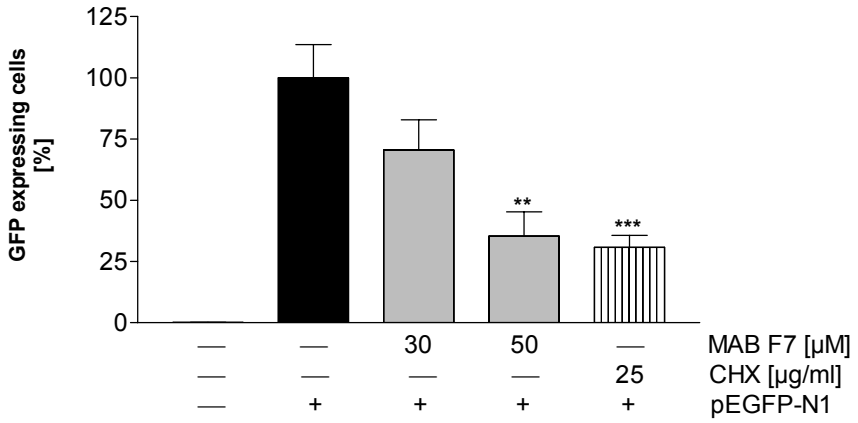


Figure 45: MAB F7 inhibits the expression of green fluorescent protein

*HEK 293 cells were either transfected with pEGFP-N1 or were left untreated. Transfected cells were incubated for 16 h with various amounts of MAB F7 or CHX as indicated. Bars represent the percentage of GFP-expressing cells. The number of untreated, transfected cells expressing GFP was set as 100 %. Values are represented as mean ± SEM of three independent experiments performed in triplicate. **p<0.01, ***p<0.001 (ANOVA/Bonferroni).*

2.5.2 MAB F7 inhibits [^{3}H]-leucine incorporation in HEK 293 cells and HUVECS

Since we could show that MAB F7 and CHX were able to decrease the expression of a CMV-driven GFP-reporter gene, we examined whether MAB F7 would also be able to reduce [^{3}H]-leucine incorporation in HEK 293 cells. Therefore we co-incubated HEK 293 cells with MAB F7 or CHX and [^{3}H]-leucine (1µCi) for 24 h. As it can be seen in figure 46 treatment with MAB F7 reduced dose-dependently [^{3}H]-leucine incorporation. Leucine uptake was reduced to 10 % by 50 µM MAB F7. CHX treated cells incorporated only 4 % leucine compared to untreated cells.

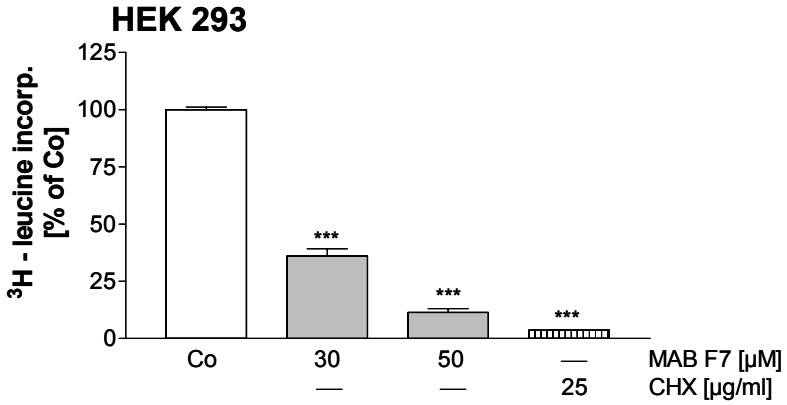


Figure 46: MAB F7 inhibits [^{3}H]-leucine incorporation in HEK 293 cells

*HEK 293 cells were co-incubated for 24 h with MAB F7 and 1µCi [^{3}H]-leucine as indicated. The incorporated radioactivity was determined with a liquid scintillation counter. Co represents leucine uptake in 24 h of untreated cells, set as 100 %. Bars represent the mean ± SEM of three independent experiments performed in triplicate. ***P<0.001 (ANOVA/Bonferroni).*

Furthermore we examined whether HUVECS incorporate less leucine after MAB F7 treatment and whether this effect was reversible. We co-incubated HUVECS with MAB F7 and [^{3}H]-leucine for 24 h. To investigate the reversibility of this effect we incubated the cells with MAB F7 for 24 h. After that period we removed the old medium washed the cells and added fresh medium with 1µCi [^{3}H]-leucine and incubated them for additional 24 h. MAB F7 inhibited dose-dependently leucine integration. HUVECSs treated with 50 µM MAB F7 incorporated only 2 % leucine compared to control cells. This effect seems to be reversible: cells treated with 30 µM MAB F7 integrated 4 times more leucine when MAB F7 was removed than cells co-treated with MAB F7. Cells treated with 50 µM MAB F7 incorporated 8 times more leucine after removal of MAB F7. However, the overall uptake is still significantly lower than that of control cells. CHX showed similar effects as MAB F7 (figure 47).

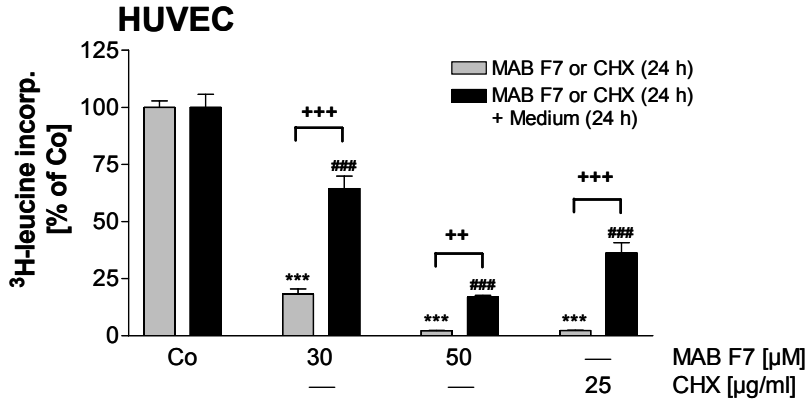


Figure 47: MAB F7 reversibly inhibits [^{3}H]-leucine incorporation in HUVECSs

*Light grey bars represent HUVECSs co-incubated for 24 h with MAB F7 or CHX and 1µCi [^{3}H]-leucine. Black bars represent HUVECS incubated for 24 h with MAB F7 or CHX followed by medium change and 1µCi [^{3}H]-leucine incubation for additional 24 h. Co represents leucine uptake in 24 h of untreated cells, set as 100 %. The incorporated radioactivity was determined with a liquid scintillation counter. Bars represent the mean ± SEM of three independent experiments performed in triplicate., ***p<0.001 (light grey bars compared to Co); ###p<0.001 (dark grey bars compared to Co) (ANOVA/Bonferroni); ++p<0.01, +++p<0.001 (paired t-test)*

V. Discussion

Since the early 1990s' it is widely accepted, that atherosclerosis is not just a simple occlusion problem caused by a growing deposit of blood-lipids that eventually closes an affected blood vessel (Libby, 2002). More and more evidence have demonstrated that atherosclerosis is a progressive inflammatory disease. It has been demonstrated that active nuclear factor-kappa B (NF-κB) is present in atherosclerotic lesions (Brand et al., 1996). NF-κB plays an important role in the development of atherosclerosis by inducing the expression of cellular adhesion molecules (CAM), such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin (Chen et al., 1995), chemokines like monocyte chemoattractant protein-1 (MCP-1) and IL-8, or cytokines TNF-α and IL-1β (Collins and Cybulsky, 2001). High amounts of adhesion molecules are expressed in areas of the atherosclerotic lesions, where an increased accumulation of leukocytes can be detected (O'Brien et al., 1996). The cytokine tumor necrosis factor alpha (TNF-α) is a strong enhancer for both NF-κB activation and CAM expression (Haraldsen et al., 1996). Since garlic has a long tradition as a medical plant with assumed anti-inflammatory properties we examined in the first part of the present work the influence of garlic on TNF-α-induced activation of NF-κB and CAM expression.

1. Anti-inflammatory properties of garlic (*Allium sativum* L.)

1.1 Garlic does not affect TNF-α-induced NF-κB activity and expression of ICAM-1 and E-selectin.

We found that none of the different garlic extracts and the various single garlic compounds (i.e. DADS, AM, γ-GC, allicin, SAC or $AMSO_2$), showed significant inhibitory effects on TNF-α induced NF-κB activity in a human embryonic kidney cell line (HEK 293) or in human endothelial cells (HUVECS). However, it has been described recently that pre-treatment with SAC is able to inhibit TNF-α-induced, and hydrogen peroxide-induced NF-κB activation in endothelial cells and T-cells (Geng et al., 1997; Ide and Lau, 2001; Ho et al., 2001). Yet, these results were achieved after a pre-incubation period of 24 h with concentrations of SAC in a milli-molar range. Whether these conditions can be reached in human plasma is questionable (Koch, 1996;

Rosen et al., 2001). Further, it is probable that these effects are not mediated by a specific inhibition of NF-κB by SAC. It seems more likely that cysteine as metabolite of SAC mediates this effect. As it has been described previously, cysteine and N-acetyl-cysteine are capable to inhibit NF-κB activation by modulating the levels of intracellular glutathione and glutathione disulfide (Mihm et al., 1995; Oka et al., 2000).
The TNF-α-mediated expression of ICAM-1 and E-selectin in HUVECS was not affected by DADS and AM treatment. This is supported by an *in vivo* study where it was shown that aged garlic extract (AGE) does not influence the expression of endothelial adhesion molecules or the adherence of leukocytes to the endothelium in rabbits on a cholesterol-enriched diet (Campbell et al., 2001). However, recently another group reported that garlic extracts are able to reduce leukocyte migration through endothelial monolayers *in vitro* (Hofbauer et al., 2001). These contradictory results can be due to the different garlic preparations and the different test systems used by each individual group. However, it can not be excluded that other transcription factors, important for the activation of ICAM-1, like AP-1, Ets-1, STAT or Sp1 are targeted by garlic. Likewise garlic may prevent the adhesion of leukocytes after stimulation with different agents, e.g. oxLDL, INF-γ (Roebuck and Finnegan, 1999; Dwivedi et al., 2001). Especially for aged garlic extract (AGE) it has been demonstrated, that it is capable to reduce LDL oxidation *in vitro* and *in vivo* and may contribute to decrease the expression of adhesion molecules by anti-oxidative effects (Munday et al., 1999; Lau, 2001; Borek, 2001).

1.2 Garlic inhibits NF-κB activity *via* a modulation of cytokine release in human whole blood

1.2.1 Garlic extracts modulate LPS-induced liberation of inflammatory cytokines in human whole blood.

The liberation of pro- and anti-inflammatory cytokines of blood cells, i.e. monocytes, T-cells and other lymphocytes is a further important factor in the process of inflammation (Cotran et al., 1999). Up to now very little is known about the interrelationship of garlic and cells of the immune system.
We could show that DMSO extracts of dried garlic powders inhibit the LPS-induced liberation of the pro-inflammatory cytokines IL-1β and TNF-α. In parallel, the levels of the anti-inflammatory cytokine IL-10 remained unchanged or even tended to be increased. These findings are basically in line with a recent publication using fresh garlic juice (Ghazanfari et al., 2000; Hodge et al., 2002). However, in contrast to this

study, we were unable to detect an influence on the cytokines INF-γ and IL-6. This may be due to the different garlic preparations used (fresh garlic juice *versus* garlic powder extract) and different experimental set-ups. LPS is a strong inducer of various transcription factors, like NF-κB or AP-1 in monocytes (Guha and Mackman, 2001). Recent studies revealed that garlic extracts and SAC are capable to inhibit NF-κB activation after stimulation with LPS and INF-γ in mouse macrophages (Kim et al., 2001). Interestingly the inhibitory effect of SAC after LPS and INF-γ stimulation was evident at 250-fold lower concentrations than described for TNF-α-stimulated cells (Geng et al., 1997). This indicates that garlic-mediated effects are dependent on the used stimulus and cell type.
On the other hand, we could show that aqueous garlic extracts can act as strong inducers of IL-1β, TNF-α and IL-10 in human whole blood. This finding is supported by recent studies using the garlic constituent alliin or aged garlic extract. These studies show an increase of pro-inflammatory cytokine IL-1β, IL-2, IL-12, INF-γ and TNF-α in blood monocytes and spleen cells (Salman et al., 1999a; Kyo et al., 2001). Additionally, it was demonstrated that treatment with aqueous garlic extracts increased the concanavalin A-induced (Con A) proliferation of T-lymphocytes stronger than ethanolic garlic extracts. This finding is correlated with increased IL-2 and IL-4 secretion (Colic et al., 2002; Kyo et al., 2001). These effects may be provoked by immunological active lectins within the different garlic preparations (Dam et al., 1998; Morioka et al., 1993).

An attempt to identify the effective constituents of GPE was made by employing allicin and DADS, two major conversion products of alliin after its interaction with alliinase. Allicin had no inhibitory effect on the production of IL-1β and TNF-α, but rather decreased IL-10 levels. DADS decreased the LPS-induced release of TNF-α and IL-1β already at low concentrations (1 μM) and may thus contribute at least to the observed effect for GPE. $AMSO_2$ and γ-GC had no effect on LPS-induced cytokine release.

1.2.2 Modulation of cytokine release inhibits NF-κB activity in HEK 293 cells.

Most interestingly, we showed that stimulation of HEK 293 cells with blood supernatants treated with garlic powder extracts and LPS resulted in a significantly lowered NF-κB activity compared to cells incubated with LPS-treated blood supernatants. The inhibition of NF-κB correlated very well with the reduced levels of IL-1β and TNF-α measured in the blood supernatants. TNF-α and IL-1β are two major cytokines that mediate inflammation and are involved in the progression of atherosclerotic lesions. Especially the activation of the endothelium, *via* NF-κB, is

induced by these two cytokines (McGill et al., 1998). In recent years the possibility that infections, combined with other factors, can be responsible for the genesis of the atherosclerotic lesions has been rediscovered (O'Connor et al., 2001). By suppressing the LPS-induced liberation of IL-1β and TNF-α garlic may inhibit the genesis of atherosclerosis.

In summary, we could show that possible anti-atherogenic effects mediated by single garlic constituents or garlic extracts are not mediated *via* direct inhibition of NF-κB or NF-κB regulated genes. Moreover, it seems that modulation of cytokine release in blood cells may be the key of garlic-induced NF-κB inhibition.

1.3 Modulation of cytokine-release and subsequent NF-κB inhibition by garlic is sulfur dependent.

Further we could demonstrate that sulfur-fertilization of garlic plants leads to an increase in total sulfur and an increase in alliin, allicin, allyl-cysteine and γ-glutamyl-cysteines in garlic dry powder. Extracts from sulfur-fertilized garlic inhibited IL-1β and TNF-α release more efficiently than unfertilized garlic. Additionally, stimulation of HEK cells with supernatants treated with sulfur-fertilized dGPE resulted in lower NF-κB activity than dGPE of unfertilized garlic. In earlier studies the quality of the garlic powders used was often neglected, which could be one reason of the achieved contradictory results concerning a beneficial effect of garlic (Isaacsohn et al., 1998; Reuter et al., 1996). Moreover, the presented data point to sulfur-compounds as active principle of garlic with respect to the modulation of LPS-induced cytokine levels. This is an important finding as until now not much attention has been paid to sulfur-fertilization during the cultivation of garlic as medicinal product. These findings are in line with recently published results of the sulfur-dependent anti-platelet activity of onions (Goldman et al., 1996).

For the first time we could show, that inhibition of LPS-induced liberation of pro-inflammatory cytokines IL-1β and TNF-α is dependent on the sulfur content of garlic powders used.

2. Anti-inflammatory properties of the edelweiss constituent MAB F7

Edelweiss preparations were used as cure against inflammatory maladies of the gastro-intestinal tract and bronchitis (Pickl-Kerk, 1995; Bitschnau, 1991). Since the transcription factor NF-κB and the adhesion molecules ICAM-1 and E-selectin represent important factors in the process of inflammation, we supposed that constituents of edelweiss mediate their anti-inflammatory properties by inhibition of NF-κB activity and thereby reduce the expression of adhesion molecules.

2.1 Effects of MAB F7 on mediators of inflammation

At first our attention was turned towards possible effects of edelweiss constituents on TNF-α-induced NF-κB transactivation activity. In a reporter-gene assay we could demonstrate the dose-dependent reduction of NF-κB transactivation by MAB F7, bisabolane A and bisabolane CD. Yet, we showed by electrophoretic mobility shift assay (EMSA) that MAB F7 was not able to inhibit TNF-α-mediated NF-κB DNA-binding activity in HUVECS and Jurkat T-cells. The contradictory results can be explained by the fact that the activity of NF-κB is not only dependent on the DNA binding of NF-κB dimers, but also on direct modifications of NF-κB proteins (Zhong et al., 2002; Chen et al., 2001; Wang and Baldwin, 1998; Sakurai et al., 1999). It has been demonstrated that protein kinase A (PKA) can increase NF-κB activity by phosphorylation of p65. This increase is associated with an enhanced recruitment of the transcriptional co-activator CBP/p300 (Zhong et al., 1998). Further it was shown by Chen et al, that CBP/p300 acetylates p65. Acetylation of p65 prevents the binding to IκB and thus the IκB-dependent export of NF-κB (Chen et al., 2001).

Secondly, we revealed by FACS analysis and western blot, that MAB F7 inhibited dose-dependently the TNF-α-mediated expression of ICAM-1 and E-selectin in HUVECS. Ahn and colleagues showed recently that lignans from *Magnoliae fargesii* inhibited the TNF-α-induced expression of ICAM-1 and VCAM-1 in the human leukemic monocyte cell-line THP-1 (Ahn et al., 2001).
In order to close the gap between NF-κB activation and the expression of adhesion molecules we performed real-time RT-PCR experiments. We could show that pre-treatment of HUVECS with MAB F7 does not inhibit the TNF-α-induced expression of ICAM-1 mRNA.

The inhibitory effect of plant lignans on NF-κB seems to be strongly dependent on the chemical structure of the lignan, the cell-line and on the stimulus used. For example, it has been reported, that arctigenin a dibenzylbutyrolactone type lignan, inhibits LPS-induced NF-κB activation in RAW macrophages (Cho et al., 2002). Further it was demonstrated by Baumann et al., that 1H-cyclopenta[b]benzofuran lignans (rocaglamides) from *Aglia* species inhibit NF-κB activity after TNF-α and PMF stimulation in T lymphocytes but not in HeLa and PC12 cells (Baumann et al., 2002). Both groups showed that IκBα levels were increased after treatment with the various lignans. Interestingly another transcription factor, i.e. SP-1 was not affected (Baumann et al., 2002). This stands in contrast to a recent study showing that the lignan Malachi 4 inhibits the replication of human immunodeficiency virus type 1 (HIV-1). This effect is not mediated by inhibition of NF-κB but of SP-1 (Gnabre et al., 1995).

Taken together, our results suggest that MAB F7 inhibits the TNF-α mediated expression of ICAM-1 and E-selectin not *via* the NF-κB pathway.

2.2 Effects of MAB F7 on protein synthesis

The fact that MAB F7 did inhibit the expression of firefly-luciferase protein, ICAM-1 and E-selectin led us to the assumption that this effect might be linked with inhibition of protein synthesis. This hypothesis was confirmed by the finding that MAB F7 inhibited dose-dependently the incorporation of [^{3}H]-leucine in HEK 293 cells and HUVECs. For HUVECs we could show that this effect seems to be reversible. However, leucine uptake was still significantly reduced 24 h after removal of MAB F7, pointing to a long persisting interference with protein synthesis. Secondly, we could show, that MAB F7 blocked the expression of CMV-driven GFP-protein in HEK 293 cells. This supports the results we obtained with the leucine uptake and indicates that MAB F7 inhibits the protein synthesis of reporter genes, regardless of the used promoter. Additionally, this result explains how MAB F7 reduced the activity of the firefly luciferase reporter, even 2 h after TNF-α stimulation. Previously, it has been shown that proliferation of endothelial cells occurs mainly at branch orifices, where atherosclerosis is initiated (Wright, 1972; Kunz et al., 1978). Akimoto and colleges demonstrated that laminar shear stress induces a reversible cell cycle arrest in G_1-phase by the up-regulation of p21 (Akimoto et al., 2000). Besides, the authors suggest that increased cell proliferation might destabilize the endothelium and may cause atherosclerosis. Therefore, it is tempting to speculate that the MAB F7-induced reduction of cell proliferation may help to stabilize the endothelium.

These results are conclusive with other studies demonstrating anti-proliferative effects of various plant lignans on macrophage cell lines and breast cancer cell lines (Filleur et al., 2001; Cho et al., 2001a; Bohnenstengel et al., 1999). Other effects of lignans reported by various groups, such as the inhibition of NO-synthase expression (Cho et al., 2002) and NO production (Cho et al., 2001a), the release of TNF-α, IL-1α and IL-1β (Yesilada et al., 2001; Cho et al., 2001a; Cho et al., 2001b) and inhibition of platelet aggregation (Chen et al., 2000) may be a result of the anti-proliferative effect.

In summary we could show that MAB F7 blocks the TNF-α-induced expression of E-selectin and ICAM-1 in human endothelial cells. This effect is not mediated by a specific inhibition of NF-κB activation as demonstrated by EMSA and real-time RT-PCR. We showed that MAB F7 mediates a reversible block of protein synthesis in endothelial cells without altering cell viability.

VI. Summary

The present work examined molecular pathways involved in the proposed anti-inflammatory properties of garlic (*Allium sativum* L.) and edelweiss (*Leontopodium alpinum* Cass.).

Single garlic constituents and metabolites, i.e. diallyl disulfide (DADS), allyl mercaptane (AM), allyl methylsulfone ($AMSO_2$), γ-glutamylcysteine (γGC) and S-allylcysteine (SAC) failed to inhibit TNF-α-mediated activation of nuclear factor-κB (NF-κB) in HUVECs and HEK 293 cells. Hydrophilic and lipophilic extracts of various dried garlic powders had also no effect on NF-κB activation.
As a consequence of this, DADS and AM also failed to inhibit the TNF-α-induced expression of adhesion molecules ICAM-1 and E-selectin.

DMSO garlic extracts (dGPE) inhibited the LPS-induced liberation of pro-inflammatory cytokines IL-1β and TNF-α in a human *ex vivo* whole blood model, whereas the liberation of the anti-inflammatory cytokine IL-10 remained unchanged. The observed effect proved to be sulfur-dependent, since garlic powders fertilized with 200 U of sulphate (~ 320 kg $CaSO_4$/ha) significantly reduced IL-1β and TNF-α release after LPS stimulation. From all single garlic constituents tested only DADS reduced IL-1β and TNF-α release. Allicin diminished IL-10 release, and $AMSO_2$ and γGC where completely ineffective.

Treatment of HEK 293 cells with dGPE and LPS-activated blood supernatants lowered NF-κB activity compared to cells treated with LPS-activated supernatants. The blood supernatants treated with sulfur-fertilized garlic showed again lower NF-κB activity as their unfertilized counterparts. This may be a possible mechanism, contributing to the anti-inflammatory effects of garlic.

We showed for the first time that sulfur-fertilization of garlic augments the anti-inflammatory actions of garlic powder extracts. This increase is correlated with enhanced levels of sulfur constituents in garlic dry powder.

MAB F7 a lignan from *L. alpinum* inhibited the TNF-α-induced expression of the adhesion molecules ICAM-1 and E-selectin *via* a general inhibition of protein synthesis. It could be excluded that MAB F7 interferes directly with the NF-κB pathway.

In summary, this thesis work supplies new insights in the proposed anti-inflammatory actions of garlic extracts and constituents. It reveals that sulfur-fertilization of garlic plants may contribute to the improvement of garlic as medical plant. In addition, it provides first informations on the molecular actions of MAB F7, a lignan from edelweiss. To what extend these effects contribute to the postulated anti-inflammatory properties of *L. alpinum* remains unclear and is subject of further investigation.

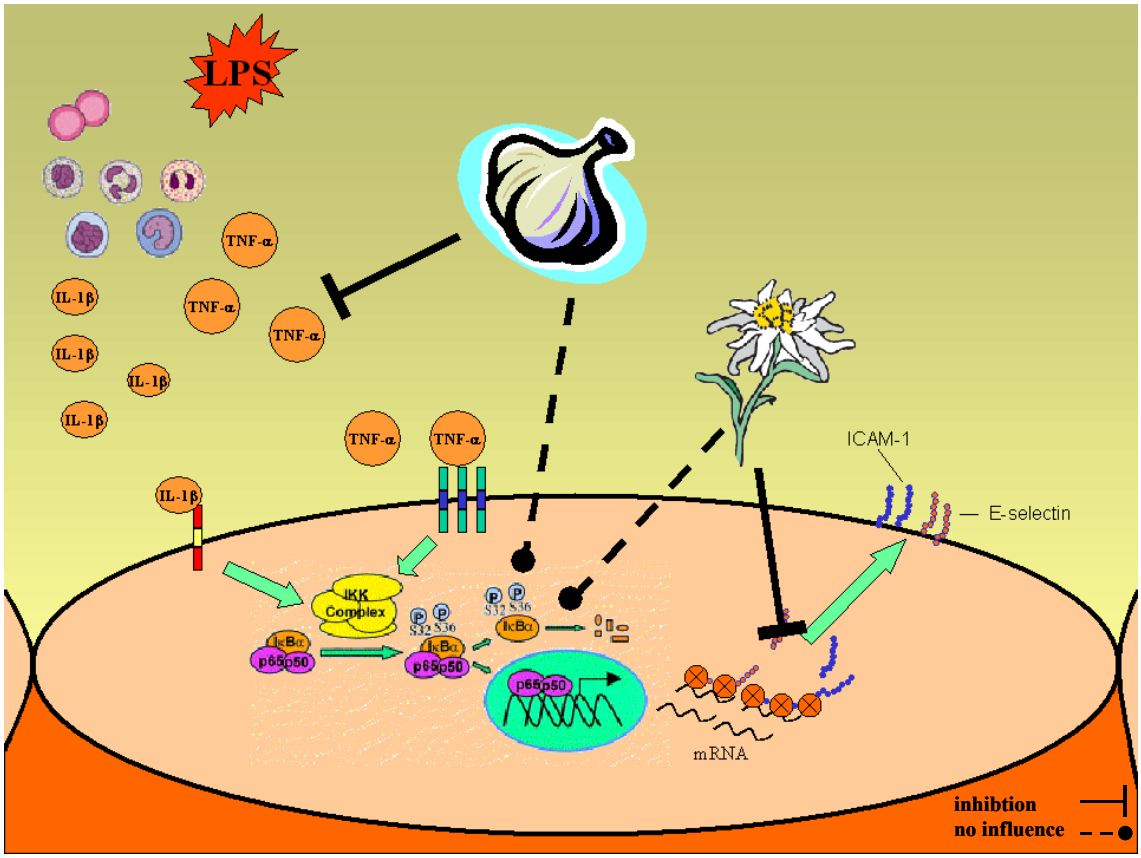


Figure 48: Summary of the postulated effects.
Garlic (Allium sativum L.) inhibits LPS-induced liberation of IL-β and TNF-α in human whole blood leading to an overall impaired NF-κB activation in adjacent tissue. Edelweiss (Leontopodium alpinum Cass.) inhibited TNF-α-induced E-selectin and ICAM-1 expression by interfering with protein synthesis.

The present studies about garlic were part of the EU Garlic & Health project supported by the European Community.
(QLK1-CT-1999-00498; www.plant.wag-ur.nl/projects/garlicandhealth).

VII. Appendix

1. Abbreviations

AGE	Aged garlic extract
aGPE	Aqueous garlic powder extract
AM	Allylmercaptane
AMS	Allyl methylsulfide
$AMSO_2$	Allyl methylsulfone
AP-1/2	Activator protein 1/2
ApoB	Apolipoprotein B
APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphate
Bq	Bequerel
BSA	Bovine serum albumine
CAM	Cell adhesion molecule
CBP	CREB binding protein
cDNA	complementary DNA
CHX	Cycloheximide
cpm	Counts per minute
CRE	cAMP-responsive enhancer-binding protein
cRNA	complementary RNA
Ci	Curie (1 Ci = $3.7x10^{10}$ Bequerel)
Co	Control
Da	Dalton
DADS	Diallyl disulfide
dGPE	DMSO garlic powder extract
DLR	Dual luciferase reporter gene assay
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dNTP	Desoxynucleosidtriphosphate
DTT	Dithio threitol
ECGM	Endothelial cell growth medium
ECL	Enhanced chemoluminescence reagent
EDTA	Ethylene diamine-N,N,N´,N´ tetra acid
EGTA	Bis(aminoethyl)glycolether-N,N,N´,N´-tetra acid
EMSA	Electrophoretic mobility shift assay
END	Enterodiol

ENL	Enterolactone
FITC	Fluoresceinisothiocyanate
FCS	Fetal calf serum
G-CSF	Granulocyte-colony stimulating factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
γ-GC	γ-glutamylcysteine
H	Hour
HBS	HEPES buffered saline solution
HDAC	Histone deacetylase
HDL	High density lipoprotein
HEK 293	Human embryonic kidney cell line 293
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HIV-1	Human immunodeficiency virus type-1
HUVECs	Human umbilical vein endothelial cells
ICAM	Intracellular cell adhesion molecule
IκB (-α/-ε)	Inhibitory protein (-α/-ε)
IKK	IκB-kinase
IL	Interleukin
INF	Interferone
JNK	c-Jun N-terminal kinase
L	Liter
LB	Lennox Broth
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
m	Milli (10^{-3})
M	Molar
MAB F7	2-methyl-but-2-enoic acid 4-(3,4-dimethoxy-benzyl)-2-(3,4-dimethoxy-phenyl)-tetrahydro-furan-3-ylmethylester
MAT	Matairesinol
MCP	Monocyte chemoattractant protein
M-CSF	Macrophage-colony stimulating factor
MG-132	Carbobenzoxyl-leucinyl-leucinyl-leucynal
µ	Micro (10^{-6})
min	Minute
MOPS	3-[N-Morpholino]propansulfonic acid
mRNA	messenger Ribonucleinic acid
NEMO	NF-κB essential modulator
NES	Nuclear export signal

% (m/v)	Mass per volume per cent
n	Nano (10^{-9})
NF-κB	Nuclear factor kappa B
OD	Optical density
oxLDL	Oxidised low density lipoprotein
PAA	Polyacrylamide
PAGE	Polyacrylamide-gel electrophoresis
PBC	Peripheral blood cells
PBS	Phosphate buffered saline solution
PCR	Polymerase chain reaction
PI	Propidium iodide
PMSF	Phenylmethylsulfonylfluoride
Poly[dIdC]	Polydesoxyinosine-desoxycytosine
ppm	Parts per million
RNA	Ribonucleinic acid
RNAse	Ribonuclease
rpm	Rotations per minute
RT	Room temperature
SAC	S-allylcysteine
sec	Second
SECO	Secoisolariciresinol
SEM	Standard error of the mean value
SDS	Sodium dodecyl sulfate
SMC	Smooth muscle cells
SRE	Serum response element
STAT	Tignal transducers and activators of transcription
STE	Sodium chloride, Tris, EDTA buffer
TAE	Tris, acetate, EDTA buffer
TBE	Tris, borate, EDTA buffer with Triton X-100
TBS-T	Tris buffered saline solution with Tween
TGF	Transforming growth factor
TE	Tris-EDTA buffer
TEMED	N,N,N´,N´-tetramethylethylendiamine
TNF-α	Tumor necrosis factor alpha
tot RNA	total RNA
Tris	Tris-hydroxymethyl-aminomethan
U	Unit
V	Volt
VCAM	Vascular Cell Adhesion Molecule
% (v/v)	Volume per volume

W	Watt

2. Alphabetical order of companies

Acros Organics	(Schwerte, Germany)
Amersham	(Braunschweig, Germany)
Beckmann Instruments	(Munich, Germany)
Becton Dickinson	(San Jose, CA, USA)
Biochrom	(Berlin, Germany)
BioRad Laboratories	(Munich, Germany)
Biosource	(Nivelles, Belgium)
Bio Whittaker Bioproducts	(Heidelberg, Germany)
Berthold	(Bad Wildbad, Germany)
Calbiochem	(Schwalbach, Germany)
Carl Roth	(Karlsruhe, Germany)
Clontech	(Palo Alto, CA, USA)
Dianova	(Hamburg, Germany)
Endogen	(Bonn, Gemany)
Gibco/BRL	(Eggenstein, Germany)
GraphPad Software	(San Diego, CA, USA)
Greiner	(Nürtingen, Germany)
ICN Biomedicals	(Eschwege, Germany)
Jackson Immunolab	(Hamburg, Germany)
MBI Fermentas	(St.Leon Roth, Germany)
NEN	(Cologne, Germany)
PAA Laboratories	(Linz, Austria)
PAN	(Aidenbach, Germany)
Packard	(Rodgau - Jügesheim, Germany)
PE Applied Biosystems	(Foster City, CA, USA)
PharMingen	(San Diego, CA, USA)
Promega	(Heidelberg, Germany)
Promocell	(Heidelberg, Germany)
Qiagen	(Hilden, Germany)
R & D Systems GmbH	(Wiesbaden, Germany)
Riedel-de-Haen	(Seelze, Germany)
Roche	(Mannheim, Germany)
Sarstedt	(Nürnbrecht, Gemany)
Santa Cruz	(Heidelberg, Germany)
Serotec LTD	(Wiesbaden, Germany)

Sigma-Aldrich	(Taufkirchen, Germany)
Stratagene	(Heidelberg, Germany)
TPP AG	(Trasadingen, Switzerland)
USB	(Cleveland, OH, USA)

3. Publications

3.1 Abstracts

H-P Keiss, V.M. Dirsch, T. Hartung, A.M. Vollmar

Extracts from Garlic dry powders modulate LPS-induced liberation of cytokines.

Naunyn-Schmiedeberg's Archieves of Pharmacology, Vol. 365, Suppl. 1

H-P Keiss, S. Schwaiger, M. Dobner, H. Stuppner, A.M. Vollmar, V.M. Dirsch

A lignan from *Leontopodium alpinum* inhibits the TNF-α-induced expression of ICAM-1 and E-Selectin in a NF-κB independent manner.

Naunyn-Schmiedeberg's Archives of Pharmacology, Vol. 367, Suppl. 1

3.2 Original Publications

V.M. Dirsch, H-P Keiss, A.M. Vollmar

Garlic metabolites fail to inhibit the activation of the transcription factor NF-kappaB and subsequent expression of the adhesion molecule E-selectin in human endothelial cells

Eur.J.Nutr., in rebuttal

H-P Keiss, V.M. Dirsch, T. Hartung, T. Haffner, L. Trueman, J. Auger, R. Kahane, A.M. Vollmar

Garlic (*Allium sativum* L.) modulates cytokine expression in LPS-activated human blood leading to an overall inhibitory effect on NF-κB activity.

J.Nutr., in rebuttal

H-P Keiss, S. Schwaiger, M. Dobner, H. Stuppner, A.M. Vollmar, V.M. Dirsch

A lignan from *Leontopodium alpinum* inhibits the TNF-α-induced expression of ICAM-1 and E-Selectin in a NF-κB-independent manner.

Manuscript in preparation

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