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# Expression and release of chemokines associated with apoptotic cell death in human promonocytic U-937 cells and peripheral blood mononuclear cells

Assoziation von Expression und Sekretion von Chemokinen mit apoptotischem Zelltod in promonozytischen U-937 Zellen und mononukleären Zellen aus peripherem Blut

## Dissertation

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Gewidmet meinen Eltern

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# 1. Inflammation

For centuries humans have intuitively identified inflammation with fire as a result of the experience of redness, heat, and pain associated with its occurence. Interestingly, scientific investigation of inflammation has extended this analogy. At the microscopic level, inflammation is described as an accumulation of leukocytes that "spread" within tissues and then ultimately "burn out" and heal or lead to "smoldering" conditions. Similarly, at the molecular level, leukocytes use an oxidative mechanism, in essence a form of biologic fire, that destroys microorganisms and damages tissues. Despite the essential truth of our intuitive sense of inflammation, the objective understanding has come slowly.

While earlier advances were made with regard to manipulating humoral immunity as in the development of vaccines, in recent decades the focus of investigation has shifted towards revealing the "molecular language" that dictates the observed events and determines the quality, intensity, and duration of inflammation. The complexity of the immunoinflammatory system which utilises this language consisting of both humoral and cellular signals is a reflection of millions of years of environmental challenges.

Inflammation is a defense reaction of living tissue to injury and infection. As such, the primary objectives of any inflammatory process are localization (in order to contain the potentially injurious reaction, e.g. tuberculous granulomas) and elimination of the foreign agent. Consecutively, a period of repair of damaged tissue is promoted.

Traditionally, inflammation is classified in the terms of the clinical onset and duration of the response. Specifically, responses are *acute* when signs or symptoms occur within minutes to hours, *subacute* when the duration is days to weeks, and *chronic* if lasting weeks to months or even longer.

## 1.1. Acute responses

The earliest and simplest host response to irritation or infection is the transudate, a movement of fluid of low protein content from the intra- to the extravascular space. This reaction principally results from hemodynamic forces and is an attempt by the host to dilute the insulting agent. As vascular leakage increases with ongoing damage, the protein content of the fluid increases and will contain most plasma proteins as well as some cellular elements. At this point the fluid is an exsudate and contains "neutralizing factors", such as clotting factors, antibodies, and protease inhibitors.

Under most circumstances the fluid exsudative stage is naturally followed by the influx of polymorphonuclear neutrophilic leukocytes (PML). These cells begin to appear within hours and, depending on the nature of the inciting agent, can accumulate in massive numbers. These end-stage phagocytic cells have profound microbial killing capacity through oxidative mechanisms, which is subsequently discussed. In addition, their lysosomes harbour a variety of proteolytic enzymes that digest tissue components and microbes when released from activated or dying cells.

## 1.2. Chronic responses

### *1.2.1. Macrophage-rich responses*

The macrophage or mononuclear phagocyte is a highly sophisticated cell which participates in many aspects of inflammation (→ section 1.3.2.). It is capable of synthesizing and secreting a variety of cytokines depending on its state of differentiation. Histologically macrophages usually occur mixed with a variety of other leukocytes. Large numbers of them can be found in chronic pneumonitis, pleuritis, or peritonitis, as well as in lymph nodes draining sites of surgical implants or foreign bodies. Furthermore, they form foamy clusters in atherosclerotic plaques.

### *1.2.2. Eosinophil-rich responses*

Eosinophils occur as a minor cellular component in several inflammatory responses, but under some conditions they dominate. Specifically, their association with certain parasitic infestations (particularly helminths) and allergic conditions such as allergen-associated asthma is well known. The mechanisms of eosinophil recruitment and activation are just beginning to be elucidated. Some carcinomas, particularly of the urinary tract, produce cytokines that recruit eosinophils to the stroma of the tumor.

### *1.2.3. Plasma cell and lymphocyte-rich responses*

The lymphocyte and plasma cell-rich (lymphoplasmocytic) infiltrate is a commonly observed inflammatory pattern, especially at subepithelial sites. Lymphoid cells are major components of delayed-type hypersensitivity reactions and in tissue responses to viral infections. Interestingly, in some neoplasms, especially carcinomas and melanomas, an associated stromal lymphoplasmocytic response may indicate a better prognosis.

*1.2.4. Mixed-cell responses*

Chronic inflammatory responses most commonly involve a variety of leukocyte types as well as proliferation of local tissue elements. The mixed type of inflammation accompanies many conditions at many sites. Chronic ulcerative colitis is a notable example of this heterogenous pattern of inflammation in which there is ongoing acute cryptitis with neutrophil infiltration and an interstitial lymphoplasmocytic infiltrate admixed with varying numbers of mononuclear phagocytes and eosinophils. Ulcer sites often show repair and regenerative changes with proliferation of fibroblasts, vessel components, and epithelium. It is not difficult to imagine the complexity of events that must occur to coordinate the recruitment and function of so many cell types.

## 1.3. Humoral and cellular participants of inflammation

Understanding inflammation requires a knowledge of the various components of the process. However, a detailed discussion of all the participating components is beyond the scope of this introduction which shall focus on cytokines and chemokines. Table 1 gives a brief overview over the other components.

Table 1

| **Participants of Inflammation** | |
|---|---|
| Humoral elements | Coagulation and fibrinolytic factors, kinins, complement factors, immunoglobulins |
| Blood-borne cells | Neutrophils (→ below), monocytes and mononuclear phagocytes (→ below), eosinophils, basophils and mast cells, platelets |
| Stromal elements | Endothelial and epithelial cells, fibroblasts, smooth muscle cells, matrix proteins |

*1.3.1. Neutrophils*

PMLs are the primary phagocytic cells involved in host resistance to bacterial infection. They represent the largest population (40 – 60%) of blood leukocytes and circulate for about 10 hours after release from the bone marrow. Under steady state conditions about

55% of the released cells are marginated by loose attachment to the endothelium. During maturation they develop cytoplasmic granules that contain a variety of molecules with antimicrobial activities (→ sections 2. and 3.). Because of the activity of these degradative enzymes and oxygen metabolites, PML-rich responses are usually associated with significant bystander damage of host tissues.

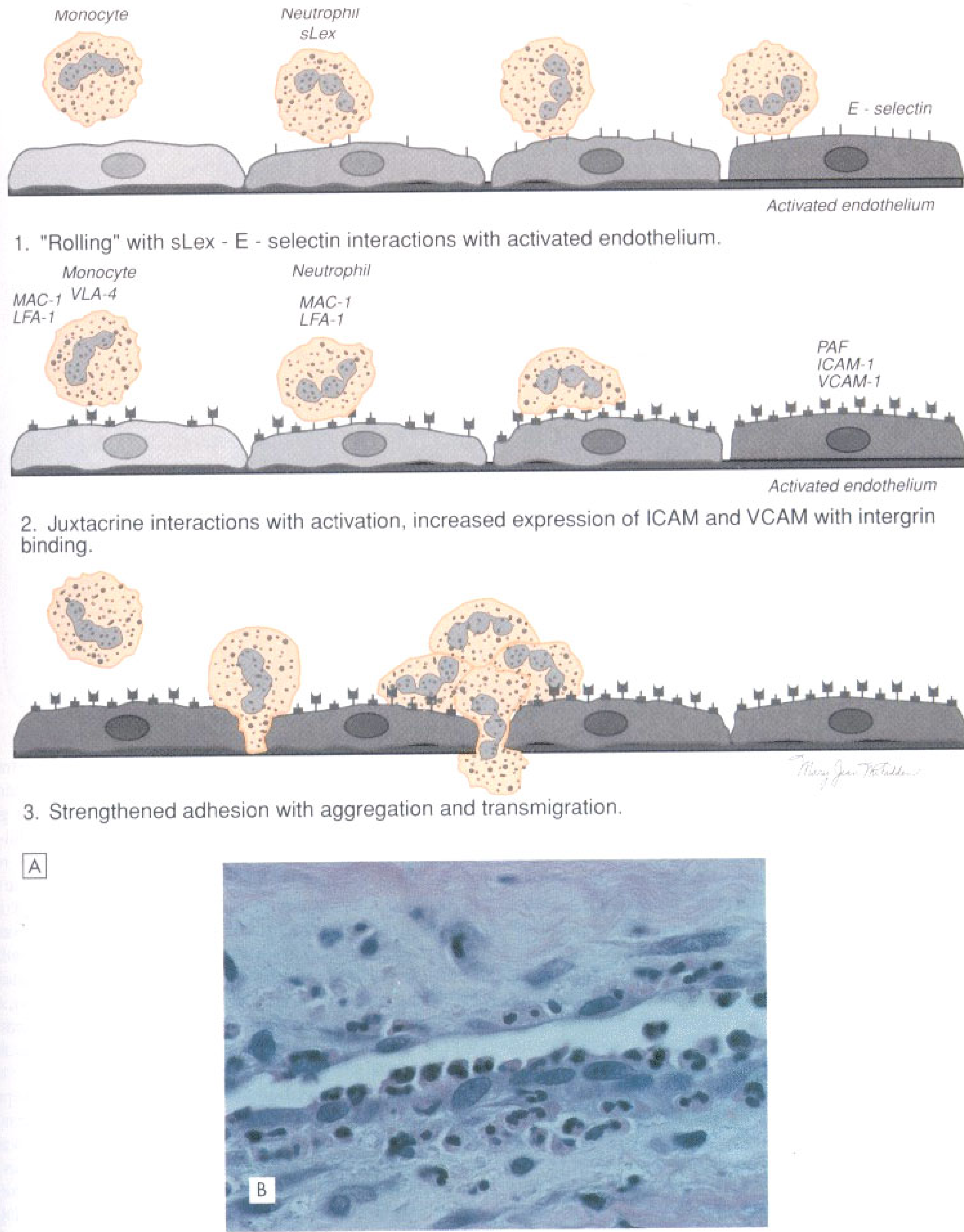



Fig. 1. Leukocyte-endothelial adherence during inflammatory responses. A: Stages of leukocyte adherence and aggregation. B: Photomicrograph showing leukocyte adherence and transmigration in an area of inflammation. [1]

Through a wide variety of membrane receptors, PML function can be regulated. E. g., receptors for complement fragment C3b and the immunoglobulin Fc region allow for more efficient phagocytosis of particles coated with these molecules. Receptors (integrins) for endothelial cell membrane proteins (addressins) and interstitial matrix proteins allow for local adherence of PMLs to sites of inflammation. Other receptors for chemotactic molecules (e.g. C5a and IL-8) and cytokines lead to PML activation and directed migration. Products of PMLs can also amplify inflammatory responses and regulate the activity of other cells. For example, granule plasminogen activator and membrane phospholipases promote vascular permeability and chemotaxis by respectively generating fibrin split products and metabolites of arachidonic acid. PML also actively synthesize and release cytokines with local and systemic effects.

*1.3.2. Monocytes and mononuclear phagocytes*

Monocytes and mononuclear phagocytes (MΦ), also known as macrophages or histiocytes, are distributed throughout virtually all tissues. They are released into the circulation as monocytes, where they remain for about 25 hours. As with the PML there is a

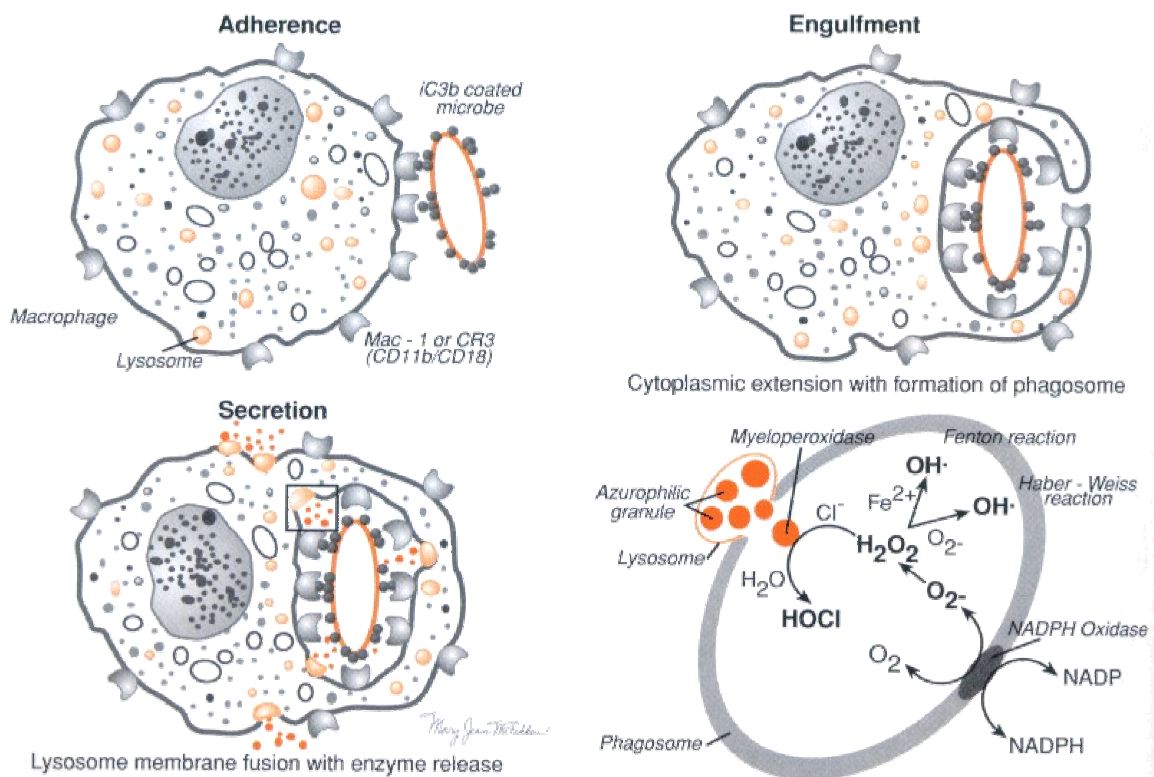



Fig. 2. Stages of phagocytosis of opsonized particle. Adherence is followed by engulfment and secretion. The biochemical reactions in the boxed area of the secretory cell are illustrated at lower right. [1]

large marginated pool. The monocytes then migrate to the tissues and become part of the tissue (fixed) macrophage population. These are found in high concentrations in the vasculature of the liver, spleen, and lungs where they remove effete cells and foreign bodies from the blood. Tissue macrophages also assist the regulation of immune responses and hematopoiesis.

Unlike PML, MΦ live longer and are capable of synthesizing a vast repertoire of bioactive products (➔ table 2). They also display a unique capacity to adapt to varied environmental challenges, a characteristic that is often enhanced and directed by signals provided by reactive lymphocytes as well as by humoral and bacterial products.

MΦ occur in varying numbers in nearly all forms of inflammation and likely participate in every stage of the inflammatory process. Local tissue macrophages can potentially initiate inflammation by producing cytokines in response to bacterial products such as endotoxin (lipopolysaccharides, LPS). Cytokines such as MΦ-derived IL-1 and TNF can induce expression of endothelial adhesion molecules, which begin local recruitment of circulating leukocytes. Other chemotactic molecules such as IL-8 and leukotriene $B_4$ can further attract inflammatory cells.

Another major function of the MΦ is phagocytosis, requiring the stages of attachment, engulfment, and destruction of the offending agent (➔ fig. 2). Although MΦ bear receptors for carbohydrates that allow for nonspecific attachment to target particles, the attachment and engulment process is greatly enhanced when the object is coated with activated complement fragments or immunoglobulins (opsonization). The destruction stage consists in fusion of the phagosome and lysosome, with release of attacking enzymes and reactive oxygen metabolites. This process is enhanced when the MΦ is activated. Activation is defined in terms of amplified functions, as compared to the initial baseline value, and occurs when the MΦ is exposed to molecular signals such as IFN-γ, LPS, and MCP-1. The activated state can vary, depending on the signal, but involves increased synthesis of enzymes, membrane receptors, and cytokines.

An important function of the activated MΦ is antigen processing and presentation. After phagocytosis, foreign proteins are degraded into peptides and transported to the membrane in association with MHC class II antigens. The membrane-exposed complex of foreign and MHC antigens serves to activate antigen-specific helper ($CD_4^+$) T-lymphocytes.

Table 2 [1]

| **Products of MΦ** | |
|---|---|
| Plasma regulated proteins | Coagulation factors, complement components, haptoglobin |
| Cytokines and growth factors | IL-1, IL-6, IL-1 receptor antagonist, TNF-$\alpha$, chemokines (IL-8, MCP-1, IP-10), growth- and colony stimulating factors (TGF-$\beta$, PDGF, G-CSF, GM-CSF, EPO, FGF) |
| Oxygen metabolites | $O_2^-$, $H_2O_2$, $OH^{\bullet}$ |
| Active lipid products | Prostaglandins, prostacyclin, leukotrienes, PAF |
| Matrix proteins | Fibronectin, proteoglycans |
| Enzymes | Proteases, collagenases, elastase, plasminogen activator, angiotensin convertase, phospholipase $A_2$, nucleases, lysozyme, hyaluronidase, amylase |
| Enzyme inhibitors | $A_2$-Macroglobulin, $A_1$-Antiprotease, fibrinolysis inhibitors |
| Other active molecules | Vitamin $D_3$, glutathione, nucleic acid derivates |

## 1.4. $Th_1$- and $Th_2$-Responses

As mentioned above, $CD_4^+$ T-helper (Th) cells become activated when they are exposed to a complex of antigen and MHC class II molecules. However, it has become apparent that there are differences in the way these cells respond once they have been activated.

If the activated Th-cell encounters IL-12, IL-15, and/or IL-18 that have been produced by other activated cells, the production and secretion of IL-2 and IFN-$\gamma$ is induced. This set of cytokines acts on neighboring MΦ and other phagocytic cells, triggering activation of intracellular enzyme systems that are effective in killing intracellular microorganisms such as Mycobacterium species or viruses. Such cells belong to the $CD_4^+$ subset known as $Th_1$ cells.

If, on the other hand, the activated T-cells produce and secrete a different set of cytokines, namely IL-4, IL-10, and IL-13, which inhibit MΦ activation and instead stimulate B-lymphocytes to produce antibodies, this type of cells is referred to as $Th_2$-cells. They mediate the release of eosinophils from the bone marrow and, among other functions, play an important role in infestations with extracellular parasites.

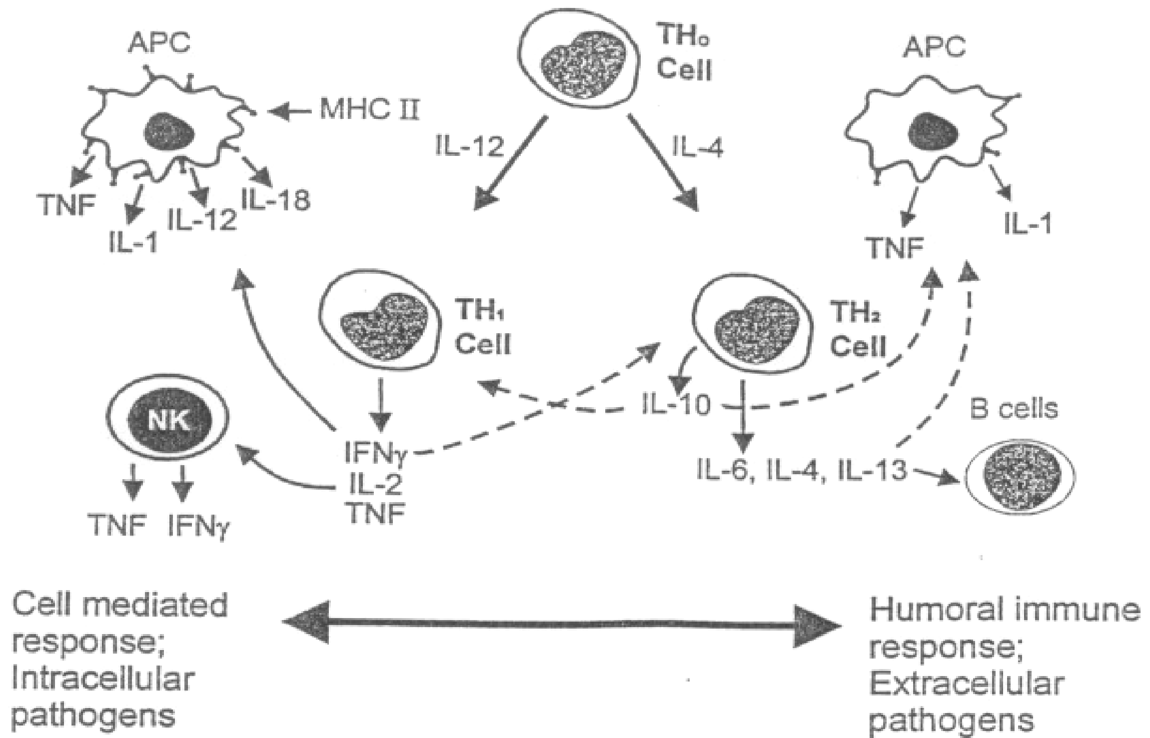


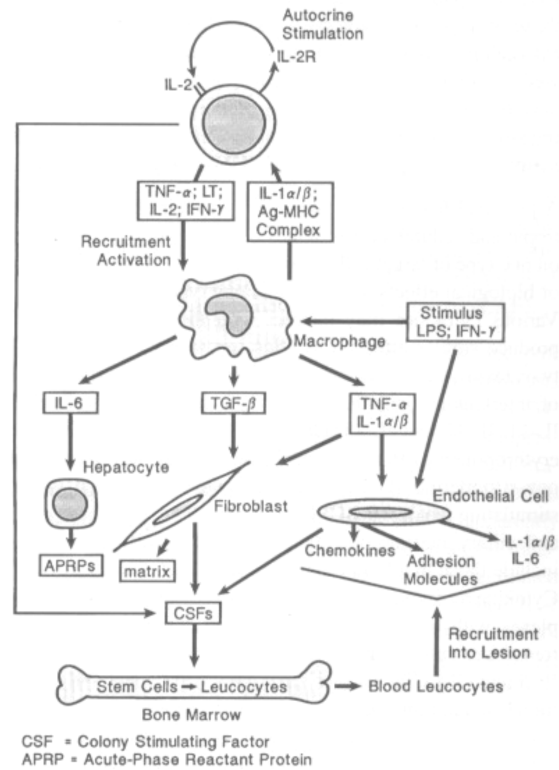

Fig. 3. The polarization of $Th_1$ and $Th_2$ responses by $CD_4^+$ Th cells and the role of the anti-inflammatory cytokines in T-cell differentiation. Solid lines indicate stimulatory pathways, dotted lines indicate inhibitory pathways. APC: antigen-presenting cell; Th0: uncommitted $CD_4^+$ Th cell precursor. [2]

# 2. Cytokines and Chemokines

As mentioned above, communication between cells is crucial, especially in the immune system where reactions to stimuli or insults like stress, infectious organisms, or dysplasia have to be intricately regulated. This is accomplished by the synthesis and release of a huge family of proteins, the cytokines, as well as by the expression and integration of their respective receptors into the plasma membrane of all kinds of body cells.

Cytokines are proteins with the capacity to alter target cell functions in an autocrine, paracrine, or endocrine manner. They particularly modulate cellular parameters related to immune defense reactions associated with infection, inflammation, or cancer and can be distinguished from other cellular products which also fit this definition. These include lipids, vasoactive amines, neuropeptides, nucleotides, and even metabolites of oxygen and nitrogen, and are termed mediators. Refer to section 2.2. for a more detailed discussion of selected cytokines.

A large number of cytokines has been identified and characterized, and although new ones are being discovered at a breathtaking pace, we are still far from a comprehensive understanding of this incredibly complicated system. Nonetheless, Figs. 4 [2] → and 5 attempt a brief overview. Fig. 4 positions some important cytokines alongside their cells of origin and targets, Fig. 5 relates them to the stages of immunoinflammatory events. It must be mentioned that these stages are not necessarily sequential but usually represent concurrent events. Likewise, although certain cytokines dominate in some stages, overlap will always be observed because of cytokine pleiotropism. In general, inflammation likely depends upon a balance of proinflammatory (e.g. IL-1, IL-18, TNF-α, IL-8 etc.) and antiinflammatory (e.g. IL-18 binding protein, IL-1ra, IL-4, IL-10, IL-13 etc.) signals that constantly changes as the response evolves and resolves.



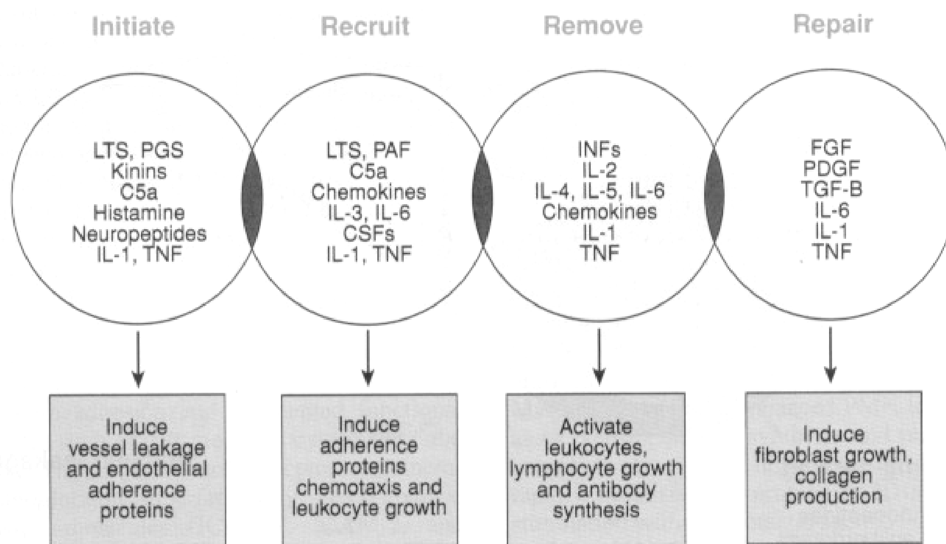




Fig. 5. Stages of immune and inflammatory responses. Cytokines can be associated with the stage of the response they most influence. Some cytokines can affect all stages, others are more restricted in their activity. [1]

## 2.1. Chemokines

The word chemokine is a shortening of *chemo*attractant cyto*kines*. This term indicates the main function of this family of small, secreted proteins, which is the induction of chemotaxis in a variety of leukocytes. To date, at least 40 distinct chemokines have been well characterized. In addition to their chemotactic capacity, they have a plethora of further, partly overlapping functions and are produced by a big variety of cells. Generally, chemokines act on more than one type of leukocyte, and in vitro responses include chemotaxis, enzyme release from intracellular stores, oxygen radical formation, shape change through cytoskeletal rearrangement, generation of lipid mediators and induction of adhesion to endothelium or extracellular matrix proteins. There is now considerable evidence to indicate that the coordinate expression of chemokines and adhesion molecules is responsible for selective trafficking of leukocyte populations from intra- to extravascular compartments. More recently, chemokines and their receptors have been shown to play a key role in the regulation of angiogenesis [3] and neuronal cell regulation [4].

As leukocyte recruitment appears to be an essential step in the development of inflammation, the topic of regulation of chemokine expression is of pivotal importance in the attempt to understand regulation, function, and pathophysiology of inflammatory processes. [5]

### *2.1.1. Structure and Function of Chemokines*

Chemokines are 6 to 14 kDa proteins with 20 to 70 percent homology in amino acid sequences. They have been subdivided into families on the basis of the relative position of their cysteine residues. There are at least four families of chemokines, but only two have been extensively characterized. The α- and β-chemokines which are encoded on chromosomes 4 and 17, respectively, appear to be the largest families. In the α-chemokines, one amino acid separates the first two cysteine residues (cysteine – any amino acid (X) – cysteine, or CXC), whereas in the β-chemokines, the first two cysteine residues are adjacent to each other (cysteine – cysteine, or CC).

The α-chemokines can be further subdivided into those that contain the sequence glutamic acid – leucine – arginine near the N-terminal, preceding the CXC sequence, and those that do not. The α-chemokines containing this sequence are chemotacic for neutrophils, whereas those not containing it act on lymphocytes.

The β-chemokines, in general, do not act on neutrophils, but attract monocytes, eosinophils, basophils, and lymphocytes with variable selectivity. As with the CXC family, the N-terminal amino acids preceding the CC residues are critical components of the biologic activity and leukocyte selectivity of these chemokines.

Several chemokines, e.g. IL-8, undergo N-terminal proteolytic processing after secretion, which alters their activity. This may reflect a general mechanism that allows local factors to regulate and amplify chemokine function.

*2.1.2. Chemokine Receptors*

Chemokines induce cell migration and activation by binding to specific G-protein coupled cell surface receptors, so-called sevenspanners, on target cells. Chemokine receptors are expressed on different types of leukocytes. On some cells they are constitutively expressed, while they are inducible on others: CCR1 and 2 are always found on monocytes but only after stimulation by IL-2 on lymphocytes. Some constitutive chemokine receptors can be downregulated.

Another interesting finding was that some chemokine receptors are expressed on T cells depending on their state of differentiation, e.g., CXCR3 being present on T helper cells in the cell-mediated $Th_1$-type immune responses, while an allergic $Th_2$ type reaction would favour CCR3. In this way, transient upregulation of chemokine receptors on leukocytes allows for specific amplification of either a $Th_1$- or $Th_2$-type response.

Some chemokine receptors are also expressed on nonhematopoietic cells, including neurons, astrocytes, epithelial cells, and endothelial cells. This suggests, in concordance with the findings mentioned at the beginning of this section, that the chemokine system has other roles in addition to leukocyte chemotaxis.

Chemokine receptors, like other members of the family of G protein coupled receptors, are functionally linked to phospholipases through $G_i$ or $G_q$ proteins. Many chemokine-induced signaling events are inhibited by Bordetella pertussis toxin, suggesting that chemokine receptors are linked to G proteins of the $G_i$ or $G_o$ class. Receptor activation leads to a number of cellular activation cascades, involving the second messengers $IP_3$, $Ca^{2+}$, and diacylglycerol which targets protein kinase C. Similar to other ligands of $G_i$ receptors, chemokines inhibit adenylyl cyclase and thus decrease cAMP levels. Interestingly, also Rho protein mediated processes (e.g. pseudopod formation) are induced, which take part in cell motility regulation. [6] Thus, chemokine receptors activate multiple

intracellular signaling pathways that regulate the intracellular machinery necessary to propel the cell in the required direction.

The production of proteins with cytokine-antagonizing functions is an important regulatory principle in cytokine biology. Examples are IL-1 receptor antagonist and IL-18 binding protein, which oppose IL-1 and IL-18 functions, respectively. Furthermore, the Duffy antigen receptor for chemokines (DARC), found on erythrocytes and endothelial cells, as well as soluble IL-1 receptors both function as sinks for their respective ligands. E.g., both CC and CXC type chemokines bind to the DARC, but do not induce calcium flux. Thus chemokines are cleared from the circulation, and local concentration gradients can be more easily upheld where needed.

## 2.2. Details about chemokines and cytokines relevant to my investigations

### *2.2.1. Interleukin 8*

In 1988, IL-8 was the first cytokine to be identified as an inducer of leukocyte migration. It is thus still considered a prototype of the chemokine family and many of the properties originally ascribed to IL-8 are still generally valid for the 50 or so human chemokines that have been described so far. IL-8 is produced and secreted by a wide variety of cells including MΦ, neutrophils, T-cells, mesangial cells, endothelial cells, fibroblasts, keratinocytes, hepatocytes, and chondrocytes. The encoding gene is located on chromosome 4 and some of the details of the intricate regulation of IL-8 expression have been elucidated. E.g., the 5´-promoter region of the human IL-8 gene is known to contain potential binding sites for a number of regulatory factors, including NF-κB and AP-1. On the transcriptional and translational level, members of the MAP-kinase stress pathway, particularly p38 MAP kinase, play a role in activation of IL-8 expression.

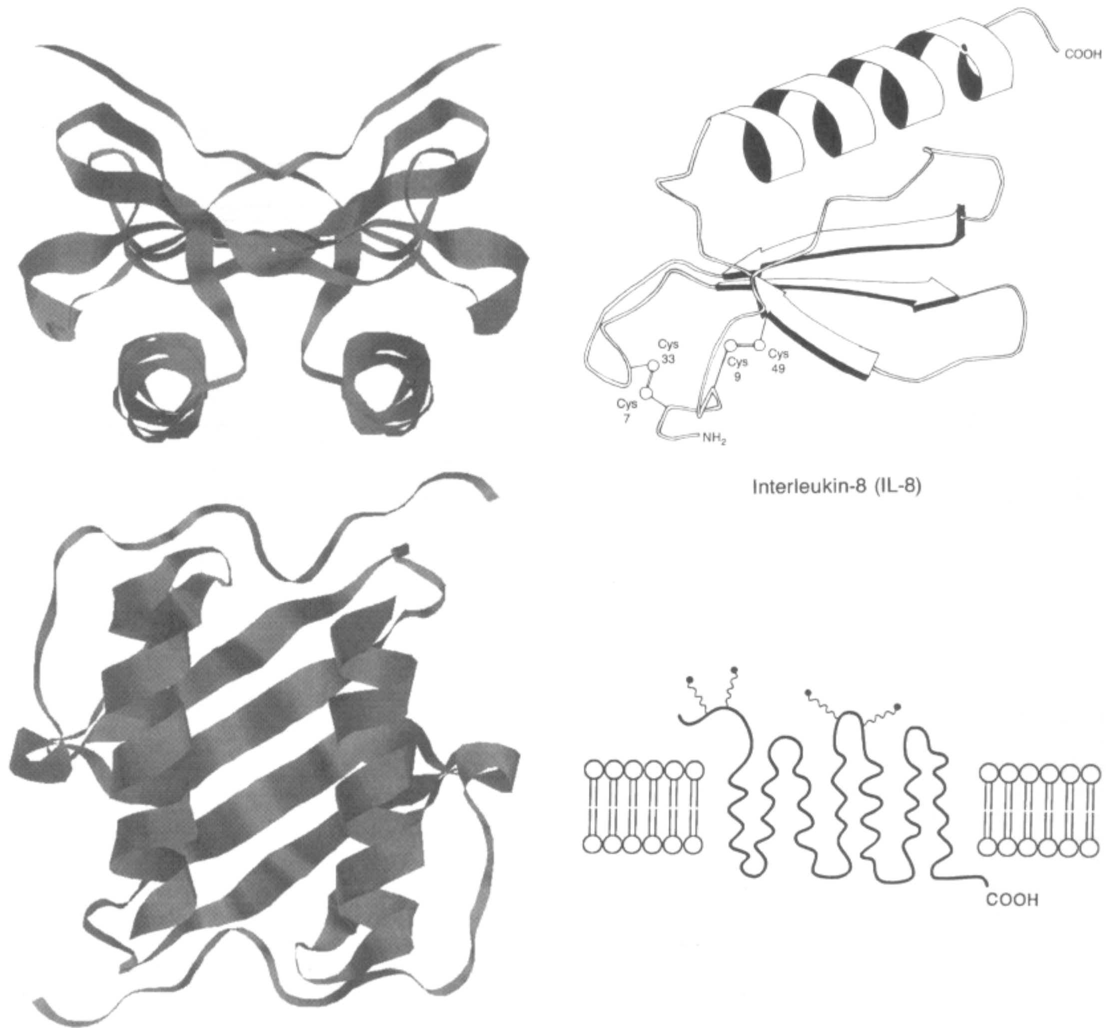



Fig. 6. Left: Human IL-8. Top right: A three dimensional minimized mean structure of the polypeptide backbone of human recombinant IL-8. Bottom right: Schematic representation of the seven transmembrane spanning IL-8 receptor which is G-protein coupled and belongs to the rhodopsin superfamily. [2]

The 8 kDa, 72 residue-protein IL-8 is a member of the α-subgroup of the chemokines (→ section 2.1.1.) and thus predominantly acts on neutrophils. It regulates their migration to inflammatory sites by enhancing their attachment to activated endothelial cells. Neutrophils encounter IL-8 bound to endothelial cells during selectin-mediated rolling. Since activated endothelial cells (and other immune cells) at inflammatory sites secrete IL-8, a chemokine concentration gradient forms along the vessel wall, with the highest IL-8 concentrations at the site of tissue damage. As neutrophils sample this gradient, they become activated and upregulate membrane expression of β-2-integrins. These adhesion molecules are rapidly integrated into the cell membrane and are then able to interact with their counterparts on the endothelial cells, ICAM-1 (intercellular adhesion molecule 1). It is

interesting to note that the surface expression of ICAM-1 on endothelial cells is greatly enhanced by IL-1, TNF-α, and IFN-γ.

In response to the localized, combinatorial effects of chemokines and other inflammatory mediators the gathering neutrophils rapidly undergo shape change, form pseudopods, and migrate across the microvessel wall to enter the infected or damaged tissue.

In addition to inducing chemotaxis and complementing processes, IL-8 has a plethora of further functions, and the list might become even longer as research continues. Refer to table 3 for a summary.

Table 3. Functions of IL-8

| **Cell Target** | **Effects** |
|---|---|
| Neutrophils | Chemotaxis; shape change; apoptosis ↓; respiratory burst ↑; degranulation ↑; lysosomal enzyme release ↑; cytosolic free $Ca^{2+}$ ↑; adherence to endothelial cell monolayers, fibrinogen, and subendothelial matrix proteins ↑; binding of complement factor C3b and LPS ↑; complement receptor type 1 ↑; surface expression of β-2-integrins ↑; phosphorylation of a 48 kDa cytosolic protein ↑; activation of 5-lipoxygenase and release of 5-HETE |
| T-cells | Chemotaxis |
| Basophils | Chemotaxis; histamine release ↑ or ↓; leukotriene release ↑ |
| Endothelial cells | Proliferation ↑; angiogenesis ↑ |
| Keratinocytes | Proliferation ↑ |
| Melanoma cells | Adhesiveness ↑ |

*2.2.2. Macrophage Inflammatory Protein 1α*

I chose MIP-1α as a representative of the family of CC- or β-chemokines (→ section 2.1.1.). As such it is encoded on chromosome 17. Like IL-8, MIP-1α is an 8 kDa protein. One of the stimuli for secretion of MIP-1α is LPS.

While the mode of action of MIP-1α is similar to IL-8 (→ section 2.2.1.), the cellular targets are not neutrophils, but preferably MΦ as well as T- and B-lymphocytes. Additional functions are listed in table 4.

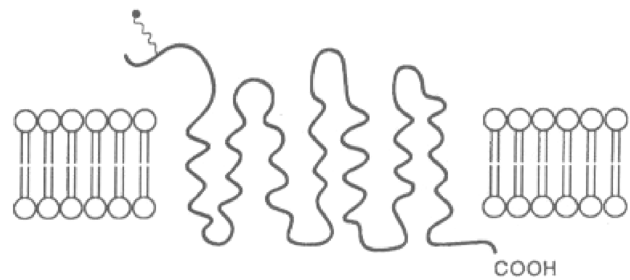


Fig. 7. [2] →

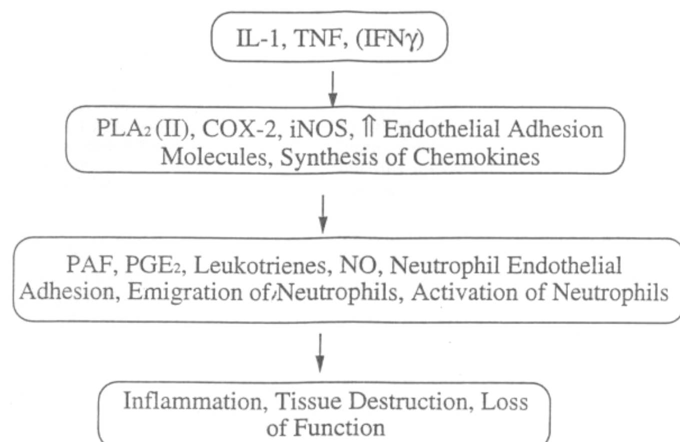
Table 4. Functions of MIP-1α

| **Cell Target** | **Effects** |
|---|---|
| MΦ | Chemotaxis; respiratory burst ↑; degranulation ↑; lysosomal enzyme release ↑; prostaglandin-independent endogenous pyrogen ↑; myelopoeitic enhancing activity |
| $CD_8^+$ T-cells | Chemotaxis; adherence to activated endothelial cells ↑ |
| B-cells | Chemotaxis |
| Stem cells | Growth inhibition |
| Basophils | Histamine release ↑ |
| --- | Binding of heparin |

*2.2.3. Interleukin 1*

Interleukins are a variety of polypeptide mediators that modulate leukocyte activity. The term interleukin was intended to bring a standard usage to a confusion of nomenclature that was based upon biologic activities.

Before the baptism to its current name, IL-1 had various names, which reflects its highly pleiotropic characteristics. The molecule exists in two structurally related isoforms, IL-1α and IL-1β, which bind to the same receptor and exert similar functions. The



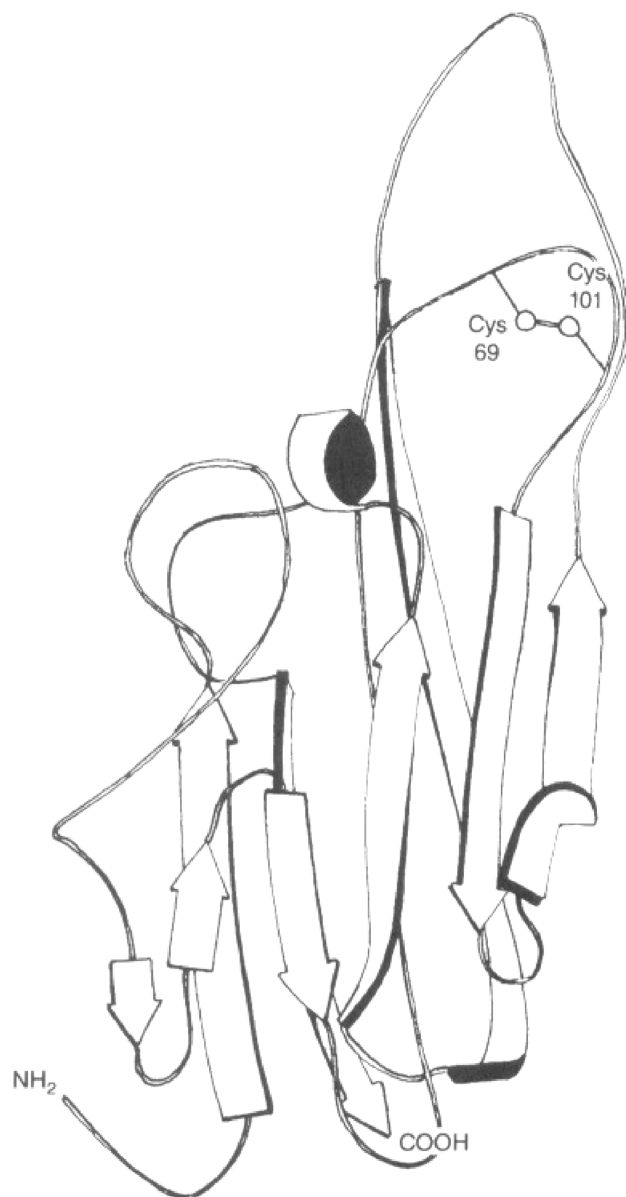

17.5 kD proteins are a product of a variety of cells, including MΦ, PML, lymphocytes, glial cells, endothelial and other cells. Microbial products (LPS, viruses) and other cytokines can

induce IL-1 synthesis. Its known in vitro proinflammatory biologic activities include stimulation of T-lymphocyte proliferation, chemokine synthesis, NO synthesis, prostaglandin synthesis, and expression of endothelial adhesion molecules. In vivo, IL-1 appears to contribute to neutrophilic leukocytosis, fever, and acute phase protein production. IL-1 also induces glucocorticoid release, which potentially provides an immunoregulatory feedback circuit. MΦ also produce IL-1 receptor antagonist, a natural inhibitor which tempers the biologic effects of IL-1.

### *2.2.4. Tumor Necrosis Factor α*

TNF-α was first discovered in the plasma of animals challenged with bacterial endotoxin and was found to cause necrosis in tumor cells in vivo and in vitro. TNF-α is a 17 kDa protein that usually exists as a trimer and is produced primarily by activated MΦ. Like IL-1 it is a highly pleiotropic cytokine and most of its functions overlap with those of IL-1. Parenteral administration of TNF causes fever, leukopenia, and hypotension. Animal studies indicate that, together with IL-1, TNF may be a critical mediator of septic shock and the cachexia of chronic disease. TNF has many inflammation-related actions, including the induction of multiple cytokines, adhesion molecule expression, bone and cartilage resorption, fibroblast proliferation, collagen synthesis, angiogenesis, and acute phase protein synthesis.

Fig. 9. Human TNF-α [2]

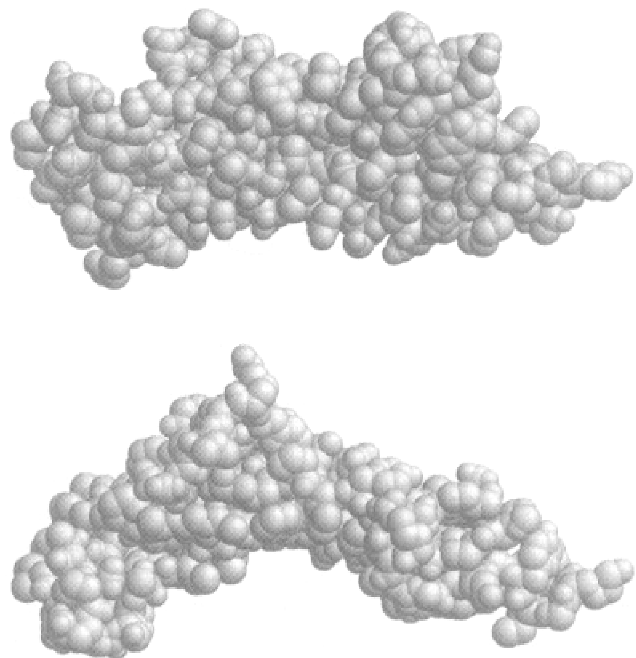


*2.2.5. Interleukin 10*

IL-10 is among the most important anti-inflammatory cytokines found within the human immune response. The 35 to 40 kDa protein is primarily synthesized by $CD_4^+$ $Th_2$ cells, MΦ, and B cells and circulates as a homodimer consisting of two tightly packed 160-amino acid molecules. IL-10 utilizes a cytokine receptor type II which activates the intracellular Jak (Janus tyrosine kinase) – Stat (signal transducer and activator of transcription) pathway [7]. In $Th_1$ cells, Stat3 DNA-binding results in suppression of cytokine production, including both IL-2 and IFN-γ. After engaging its 110 kDa receptor on monocytes, IL-10 suppresses their production of IL-1, TNF, IL-6, IL-12, and granulocyte colony-stimulating factor, as well as chemokine synthesis. Furthermore, it inhibits cell surface expression of major histocompatibility complex class II molecules on these cells and thereby impairs their capacity to present antigen to $Th_1$ cells. IL-10 also inhibits translocation of NF-κB after LPS stimulation and promotes degradation of mRNA for the pro-inflammatory cytokines. Moreover, IL-10 is a B cell stimulant, promoting proliferation, differentiation, and antibody production. It also synergizes with IL-3 and IL-4 to stimulate mast cell growth. The above characteristics underline the important antiinflammatory properties of IL-10 which are enhanced when it acts in concert with IL-4 and IL-13.

*2.2.6. Transforming Growth Factor β*

Fig. 10. Human TGF-$\beta_1$ [2]



TGF-β is the name of a family of cytokines which distinguishes itself from other ones by the ability of its members to influence almost every facet of the immune response, including the growth and differentation of precursors for multiple haematopoetic lineages, the proliferation and migration of mature immune cells (e.g. MΦ) and fibroblasts into sites of injury or response, and the suppression of such responses once they have been established. Furthermore, TGF-β plays a role in angiogenesis as well as cytokine regulation; in this regard especially its anti-

inflammatory actions (downregulation of IL-1 and TNF-α production in MΦ) play a role in the context of my experiments. The complimentary autocrine, paracrine, and even endocrine modes of TGF-β activity have been delineated through extensive *in vitro* and *in vivo* analyses of function [e.g. 8].

Today, three isoforms of TGF-β, called TGF-$\beta_{1,2,\text{ and }3}$, are known. They all bind to the same receptor. TGF-$\beta_1$, which is considered the prototype of the group, is the molecule I used as a modulating agent in my experiments. It is expressed by all immune cells and is the isoform most often acutely regulated in response to a variety of stress and disease signals.

The signals from TGF-β ligands are mediated by a receptor complex which is noteworthy for inducing an intrinsic serine-threonine kinase activity as opposed to receptors for other immunoregulatory cytokines that often signal through tyrosine kinase-mediated pathways. The downstream signalling cascade involves the SMAD proteins which carry the signals from the cell surface directly to the nucleus. [9]

# 3. Stress

Cells are complex units, the survival and function of which depends on the delicate balance between intra- and extracellular events. Understanding the causes and mechanisms of reversible and irreversible injury inflicted on cells, as well as their reaction to the respective form of stress or injury is a prerequisite for an appreciation of the biologic basis of disease processes. It is valuable for determining the therapeutic regimen, in anticipating the short- and long-term sequelae of a given pathologic process, and in implementing effective preventive measures. This section not only introduces some of the etiologic agents that can cause cellular stress, but also the mitogen-activated protein kinase (MAPK) family, an important signaling pathway in cellular responses to stress.

### 3.1. p38 Mitogen-Activated Protein Kinases and SB203580

Since the discovery of p38 MAPK in 1994, interest in the family of the MAPK has steadily been growing. In addition to their initial implication with inflammatory processes, a variety of functions has been reported, including mediation of signals triggered by

cytokines, growth factors, and environmental stress. More recently, MAPK have been associated with cellular development and differentiation, e.g. of cells of the immune system. This diversity not only underscores the central position of the MAPK in signal transduction, but also shows why clinical applications based on modulation of MAPK activity met with unexpected difficulties.

It is now known that there are four members of the MAP kinase family: the extracellular signal regulated kinases (ERK), p38, and c-Jun N-terminal kinases (JNK). They differ in their tissue distribution, regulation of kinase activation and subsequent phosphorylation of downstream substrates. The following section focuses on the p38 subgroup.

The MAPK families all consist of several members and in the case of p38 these are called p38α, β, γ, and δ. This is noteworthy, because despite amino acid sequence homologies of approximately 90 %, 63 %, and 61 % to p38α, respectively, only p38α and β are affected by the MAPK inhibitor SB203580.

This compound, which I used in my experiments, belongs to the pyridinylimidazoles. These substances were originally described as inflammatory cytokine synthesis inhibitors that subsequently were found to be selective inhibitors of p38 MAP kinases. SB203580 inhibits the catalytic activity of p38α and β MAP kinase by competitive binding to the ATP pocket.

While the α and the β isoforms are ubiquitously expressed, the expression of p38γ and δ is more restricted to skeletal muscle and glandular organs, respectively. All members of the p38 family possess a 12 amino acid activation loop containing a Thr-Gly-Tyr motif which is phosphorylated by upstream MAP kinase kinases (MKK).

As mentioned above, p38 MAPK are involved in a wide variety of signaling pathways. E.g., in cells exposed to pro-inflammatory cytokines or environmental stresses, p38 MAPK is activated which leads to phosphorylation of heat shock proteins (HSP) as well as strathmin which are involved in cytoskeletal reorganisation. HSP also have protective functions against oxdative stress. p38 MAPK also are involved in the regulation of phospholipase $A_2$, a key enzyme in the transduction of signals associated with inflammation and pain. In monocytes, p38 MAPK can upregulate c-Jun transcription and thereby influence host defense and inflammation. Moreover, through phosphorylation of p47, p38 kinases take part in enhancing the respiratory burst in activated neutrophils.

A view upstream in the signaling cascade reveals equally interesting details. For example, an isoenzyme of the above mentioned MKK, MKK6, appears to play a role in

cooperation with caspase 1 in signal transduction of apoptosis in T-cells. G-protein coupled receptors have also been found to be able to activate MAPK.

The greatest relevance of the MAPK to our studies, however, lies in their capacity to regulate cytokine biosynthesis in many different cell types. Transcript stability of IL-6 and IL-8 in monocytes is increased with enhanced activation of p38. Likewise, p38 MAPK-function is essential for TNF biosynthesis on a post-translational level in these cells and for IL-12 and IFN-γ expression in T cells. In B lymphocytes, p38 activates NF-kB and induces cell proliferation and expression of adhesion molecules. Nevertheless, p38 MAPKs also play their part in anti-inflammatory signaling: in dendritic cells they mediate IL-10-induced effects, such as downregulation of TNF-α production, suppression of endocytosis, and chemotactic migration. [10, 11]

With research showing ever new facets and details of MAPK-function, the enzymes and their specific inhibitors naturally evoke interest in possible clinical applications. Animal models have shown that SB203580 is capable to attenuate arthritis, decrease lethality of septic shock, reduce inflammatory activity in chronic obstructive lung disease (COPD) and

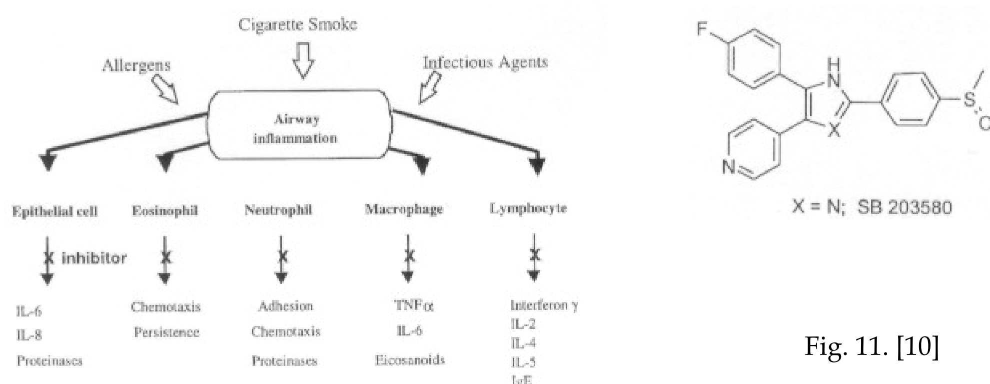



Fig. 11. [10]

asthma, and act as a protector of neurons as well as myocytes after ischaemic insults. In all cases, p38 activation in key cell types correlated with disease initiation and progression. Treatment with p38 MAP kinase inhibitors attenuated both p38 activation and disease severity. Structurally diverse substances have been tested extensively in preclinical studies; some newer generation compounds with fewer undesirable effects have even entered the clinical phases. [10]

## 3.2. Reactive Oxygen Species

Although oxygen is essential for the survival of cells it carries a definite risk of injury which occurs when cells are exposed to excess oxygen radicals. This occurs, among others, during immune responses or hyperoxic conditions as seen in treatments with high partial pressures of oxygen. The impact of these effects is particularly pronounced when the natural protectants of cells against the deleterious effects of activated oxygen species (i.e. antioxidants) are deficient in quantity.

PML and MΦ as well as certain local resident cells generate reactive oxygen species ($O_2^-$, $H_2O_2$, $OH^\bullet$) as their means of killing exogenous biologic materials or abnormal cells. For example, in bacterial sepsis (endotoxic shock) severe intravascular stasis of PML can occur within the lung. These neutrophils liberate oxygen radicals and damage capillary endothelium, causing diffuse alveolar damage, which progresses even further as storage pools of cellular antioxidants become depleted.

The potency and biologic toxicity of different reactive oxygen species varies considarably. $O_2^-$ is relatively weak and diffuses poorly across cell membranes. $H_2O_2$ is also a weak oxidant, but it can readily diffuse into the cytoplasm from organelles where it is produced. It also diffuses across plasma membranes. On the other hand, $OH^\bullet$ is an extremely reactive chemical that phagocytes use to attack microbes or necrotic cell debris, but it can also attack vital endogenous molecules within living cells and lead to cell injury or death. It is worthy of note that ionizing radiation produces $OH^\bullet$ along with a variety of other free radical species in a dose dependent manner. [12]

Reactive oxygen species cause cell injury by oxidation of critical cellular macromolecules such as proteins, DNA, and lipids. They can also oxidize thiols, leading to the formation of protein disulfides and protein mixed disulfides. They are also known to interact with cytoskeletal elements and interfere with mitochondrial oxidative phosphorylation and cause ATP depletion. Of considerable interest, however, is that $OH^\bullet$ and other reactive oxygen species interact with polyunsaturated fatty acids (PUFA) in a rapid-fire fashion to yield highly destructive PUFA radicals, lipid hydroperoxy radicals, and lipid hydroperoxides. This process is known as lipid peroxidation. The lipid peroxides are decomposed by transition metals, such as iron, to yield conjugated dienes, pentane, and ethane, which can be measured and serve as indicators of lipid peroxidation. Furthermore, toxic reactants are generated during lipid peroxidation. Lipid peroxidation propagates to cellular sites distant from the original injury, resulting in widespread membrane damage.

Finally these processes change the integrity of the membranes and result in influx of water and $Na^+$ and $Ca^{2+}$ ions. The cells can then be recognized by their enlarged size and their pale, cloudy, and granular cytoplasm. If the damage cannot be rapaired, death soon ensues.

### 3.3. Nitric Oxide

Nitric oxide (NO) was recognized in the 1980s as a free radical with important physiologic functions. First, it was identified as the endothelium-derived relaxing factor (EDRF) which plays a major role in the regulation of the vascular tone. Soon after, other functions were discovered: NO also is an effector molecule responsible for the cytostatic effect of activated MΦ on tumor cells and pathogens such as certain bacteria and viruses. The involvement of NO in the induction of apoptosis shall be discussed later in this section. NO can react with the superoxide radical ($O_2^-$) to form peroxynitrite ($ONOO^-$) or can induce or promote the formation of other reactive oxygen species. Radicals formed from NO are known to inhibit mitochondrial respiration. In addition, NO was found to act as a neuromediator implicated in long-term potentiation and it displays retrograde messenger activity.

NO is formed by the conversion of L-arginine to L-citrulline by a family of enzymes, the NO synthases (NOS), three of which have been identified to date. They are encoded by genes located on different chromosomes and differ in their properties. Type 1 or neuronal NOS (nNOS) and type 3 or endothelial NOS (eNOS) are expressed constitutively by various cell types, e.g. neuronal cells, vascular endothelium, skeletal myocytes, and others. In response to specific activators of these cells (e.g. neuromediators, bradykinin, etc.), the enzymes become activated by transient increases of the intracellular concentration of free $Ca^{2+}$. They then release brief `puffs´ of NO, creating small concentration gradients which are utilized in cellular signaling, e.g. via activation of soluble guanylate cyclase in neurotransmission or in relaxation of smooth muscle fibers of arterioles.

A third enzyme, the $Ca^{2+}$-independent type 2 or inducible NOS (iNOS) is transcriptionally regulated. iNOS is not expressed in resting cells in physiological situations, but can be induced following activation, mainly by cytokines, but also by other stimuli, such as hypoxia. Both cytokine release and hypoxia occur during the course of an inflammatory response and may act in synergy for the induction of iNOS. This implicates

that one major role of iNOS is to participate in the nonspecific defense of the host against invading pathogens and thus to contribute to innate immunity.

NO is often described as a double edged sword as it exerts opposite effects depending on its concentration and the target cell type. Although this is particularly true for its role in apoptosis, the focus of discussion in this dissertation shall be on the pro-apoptotic aspects since induction of apoptosis, through NO or other mediators, is an important means of the immune system in dealing with dysplastic or virus-infected cells.

NO interferes with enzymatic components of the mitochondria causing *reversible* inhibition of the respiration, but also irreversible inactivation of some mitochondrial enzymes containing Fe-S or heme moieties, to which it displays a high affinity. For a variety of proteins the binding of NO to heme leads to reversible inhibition of the functions of these proteins. One example is cytochrome oxidase: by competing with oxygen for this enzyme, low concentrations of NO inhibit electron transport. This in turn leads to impairment of respiration and ATP synthesis and finally to a release of mitochondrial $Ca^{2+}$ into the cytoplasm.

Other proteins are *irreversibly* inactivated by NO, such as enzymes of the respiratory chain (complexes I, II, and III) and of the citric acid cycle (aconitase) which contain Fe-S clusters, as well as GAPDH via S-nitrosylation. This results in iron release with consecutive formation of $OH^{\bullet}$, as well as an inhibition of glycolysis and further reduction of ATP production. These alterations of the energy metabolism of the cell will in turn affect $Ca^{2+}$ levels, this time by preventing an efficient activity of the $Ca^{2+}$-ATPases of the plasma membrane and the endoplasmatic reticulum, leading to dramatic increases of cytoplasmatic $Ca^{2+}$.

The above processes cause the inner mitochondrial membranes to swell, eliciting increased permeability for components of the mitochondrial matrix which are released into the cytoplasm. These include molecules involved in the control of apoptosis, such as apoptosis protease activating factor 1 (APAF-1) and cytochrome c. The latter, in conjunction with dATP, activates caspase 9 which stimulates the downstream caspase 3, leading to the proteolysis of inhibitor of caspase activated DNase (ICAD). The degradation of ICAD allows the CAD endonuclease to migrate to the nucleus and to exert its enzymatic activity, ultimately resulting in the internucleosomal cleavage of DNA.

In addition, NO is known to stimulate the process of pro-caspase activation in various cells as well as to inhibit ribonucleotide reductase, an enzyme involved in the production of deoxyribonucleotides, which leads to impairment of DNA synthesis and cell proliferation.

Finally, in some cells NO causes mutations and DNA strand breaks which leads to activation of the tumor suppressor protein p53. This in turn either induces a stable growth arrest or apoptosis. [13, 14]

### 3.4. Hyperosmolarity

Hyperosmolarity has recently gained clinical significance as research suggests an important role in the pathogenesis of cystic fibrosis as well as exercise-induced asthma. Recent investigations have shown that hyperosmotic stress induces the production and release of proinflammatory cytokines, such as IL-1 and IL-8, mediated by free oxygen radicals which activate p38 MAPK pathways. [15, 16]

### 3.5. Heat shock

Virtually all organisms respond to up-shifts in temperature (heat shock) by synthesizing a set of so-called heat shock proteins (HSP). The HSP are induced not only by heat shock but also by various other environmental stresses. Induction of HSP is regulated by the trans-acting heat shock factors (HSF) and the cis-acting heat shock element (HSE) present at the promoter region of each heat shock-sensitive gene. Usually, HSP are also expressed constitutively at normal growth temperatures and have basic and indispensable functions in the life cycle of proteins as molecular chaperones, as well as playing a role in protecting cells from the deleterious stresses. Molecular chaperones are able to inhibit the aggregation of partially denatured proteins and refold them using the energy of ATP. There are expectations for the use of molecular chaperones for the protection against and therapeutic treatment of inherited diseases caused by protein misfolding.

## 3.6. Cytotoxic Substances

Antineoplastic drugs have effected cures in some forms of cancer, and can produce remissions and alleviation of symptoms in other forms. However, these agents are cytotoxic also to normal cells and can provoke severe adverse reactions, particularly in cells that are rapidly replicating, such as cells of the gastrointestinal mucosa, the germinal epithelium, and the hair folliculi, as well as stem cells in the bone marrow. A variety of chemotherapeutic agents is used in clinical practice. Among others, these include alkylating agents (e.g. cyclophosphamide, busulfane, etc.) and antibiotics (e.g. bleomycin, mitomycin, etc.) which suppress cell growth by interacting with DNA replication, antimetabolites (e.g. methotrexate, 5-fluorouracil, azathioprin, etc.) which enter into and disrupt cellular metabolic functions, and alkaloids (e.g. vincristin, paclitaxel, etc.) that interfere with mitotic spindle integrity. However, the ultimate outcome commonly produced by many of the cytotoxic substances is the induction of apoptosis in target cells.

### *3.6.1. VP-16*

VP-16 or etoposide is a naturally occurring substance derived from the mayapple plant and, together with teniposide, belongs to the family of the epipodophyllotoxins. It is an inhibitor of topoisomerase II, an essential enzyme that plays a role in virtually every cellular DNA process. This enzyme interconverts different tertiary and quaternary structures of DNA by passing one nucleic acid segment through a transient double-stranded break which has been generated in a second segment. By virtue of its capability to rearrange double-stranded DNA, topoisomerase II is able to regulate DNA over- and underwinding, and can resolve knots and tangles in the genetic material. Beyond these important physiological functions, topoisomerase II is the target for successful anticancer drugs, such as VP-16 and teniposide, used to treat human malignancies which include various neoplasms of the lung, Hodgkin´s disease, non-Hodgkin´s lymphomas, and acute myeloid leukaemia (AML), as well as refractory testicular cancer. These agents are referred to as topoisomerase II poisons, because they transform the enzyme into a potent cellular toxin. Topoisomerase II poisons act by increasing the concentration of enzyme-cleaved DNA complexes that

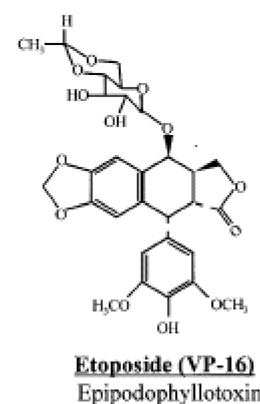



Fig. 12 [17]

normally are fleeting intermediates in the catalytic cycle of topoisomerase II. This results in an accumulation of enzyme-mediated breaks in the genetic material of treated cells and ultimately triggers apoptotic cell death.

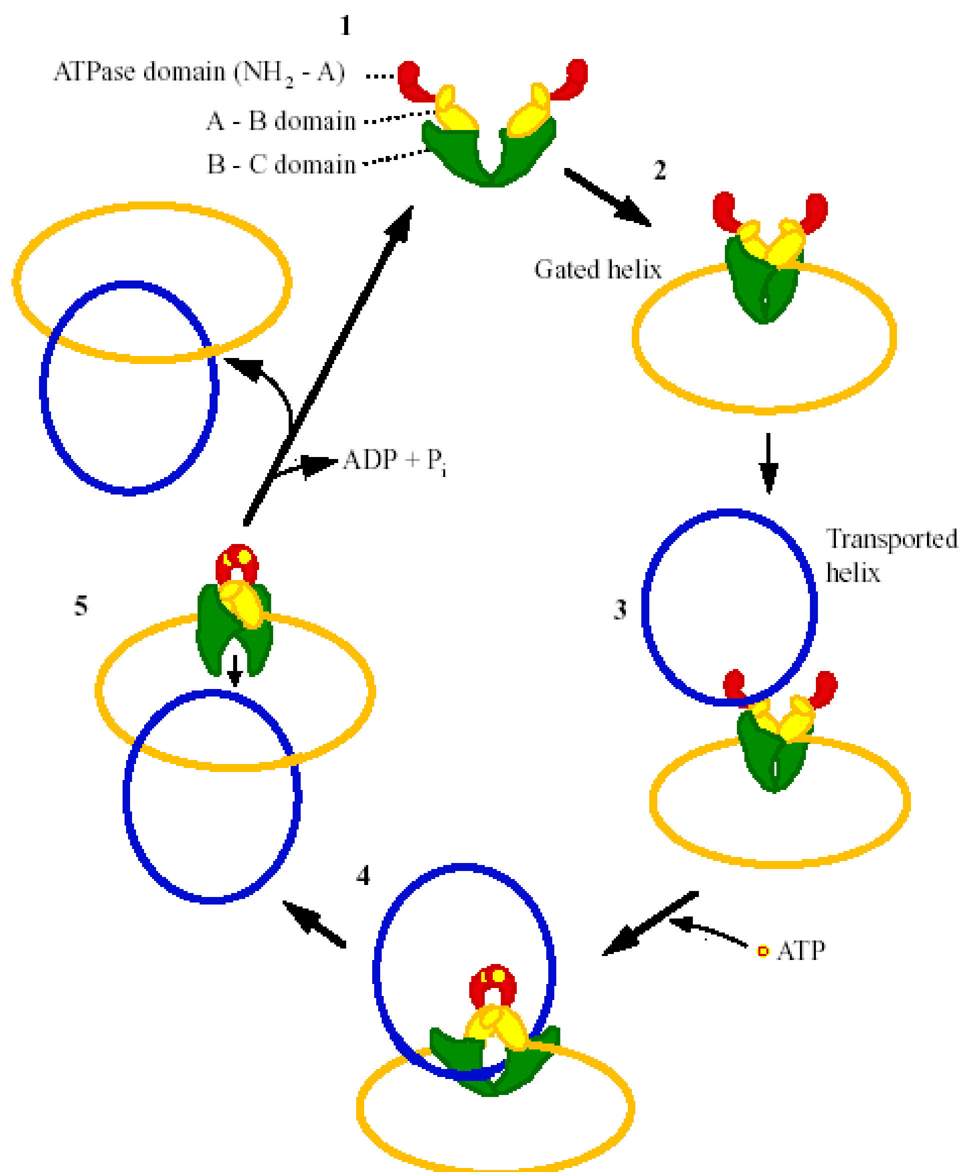



Fig 13. A model for DNA topoisomerase II action. The enzyme is composed of discrete structural domains bordered by protease sensitive sites (A, B, and C). The free topoisomerase II dimer depicted in **1** is shown bound to the gated DNA helix (orange) in stage **2**. Stage **3** shows the prestrand passage cleavable complex in which the gated helix is in equilibrium between cleavage and religation. The transported DNA helix (blue) is entering the complex between the ATPase domains. ATP then binds to the complex and induces a conformational change, whereby the transported helix is passed through the break in the gated helix. Stage **4** represents the circular clamp intermediate, which is trapped by the nonhydrolysable ATP analog AMPPNP. In the presence of ATP, this intermediate is transient and is likely to rapidly proceed to stage **5**, where the transported helix is released through the C-terminal gate. ATP is then hydrolysed to allow the enzyme dimer to return to its original conformation. [17]

*3.6.2. 2-Chlorodeoxyadenosine*

2-CdA belongs to the family of the antimetabolites and to the subgroup of the purine analogs. The action of these drugs evolves from their ability to disrupt the synthesis of nucleic acid. However, 2-CdA is unique compared with traditional antimetabolite drugs in that it is equally active against dividing *and* resting lymphocytes because of its interactions with adenosine deaminase. By that means 2-CdA decreases not only DNA synthesis, but also purine metabolism. Hence, in clinical practice, 2-CdA is of special importance in diseases in which most cells are in the resting phase, such as chronic lymphocytic leukemia (CLL). Especially in the rare hairy-cell form of CLL extraordinary therapeutic successes have been achieved. Furthermore, 2-CdA is effective against several forms of non-Hodgkin´s lymphoma. As for VP-16 and U-937 cells, it has been demonstrated that 2-CdA is a potent inducer of apoptosis in PBMC. [18]

Sections 3.2. to 3.5. have presented some examples for forms of cellular stress. One aspect common to all these stressors is the induction of a set of proteins with different functions, some of which are protective such as HSPs and hemeoxygenase 1 (HO-1), others of which have signaling properties like IL-8. One of the new hypotheses of this work is that stress inflicted on cells by cytotoxic substances is dealt with in a way similar to the previously observed reactions after exposure to oxygen radicals, NO, etc, namely, among others, by the induction of chemokine synthesis and secretion.

# 4. Apoptosis and Necrosis

## 4.1. Necrosis

When early pathologists started looking at the first slides with stained tissues over 100 years ago they looked mostly at sections of organs in which injury was extreme and sudden: ischemia after occlusion of a major artery, physical or chemical trauma, or overwhelming infection. Necrosis was the term they applied to what they saw.

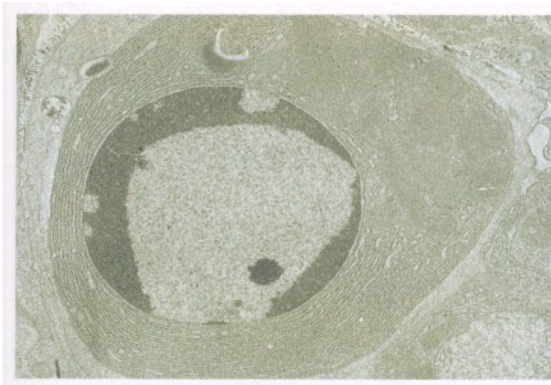
In necrosis the critical organelle is the mitochondrion, which begins to swell early on. High-amplitude swelling crystals, probably of calcium phosphate, begin to precipitate in the mitochondrial lumen. If the process is not reversed, the mitochondrion is no longer able to maintain its ionic gradients or oxidative phosphorylation, and the cell runs out of energy.

Starving for ATP, the plasma membrane's ion pumps fail, water floods in, and the cell swells and bursts. Lysis releases the cell's intracellular contents into the extracellular milieu, where they have no business being; these internal lipids, proteases and small molecules are intensely proinflammatory. They attract white cells, primarily macrophages, from around the body. Given the extent of damage, this is usually desirable because the local facilities for dealing with damage can be overwhelmed. The net effect of the inflammatory process is the removal of debris, resolution of injury, and, if the stroma has been damaged, scar formation.

## 4.2. Apoptosis

A different morphology of cell death was described in 1972 by Kerr, Wyllie, and Currie [19]. It was seen in cells for which death was normal and predictable, i.e., cells scheduled to live a shorter time than the body as a whole. It was also seen in cells dying of relatively minor injury compared to that which leads to necrosis, e.g., around the periphery of ischemic lesions. Because this death seemed to be more physiologic than necrosis, it was named apoptosis, from a word in the Hippocratic corpus (apo- away; ptosis falling), meaning the loss of leaves from trees or petals from flowers.

The defining morphologic feature of apoptosis is a collapse of the nucleus. Chromatin, which is normally composed of mixed open and condensed regions, heterochromatin and euchromatin, becomes supercondensed, appearing as crescents around the nuclear envelope



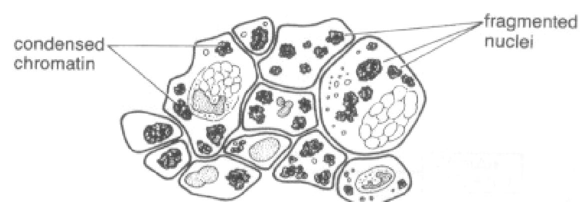



Fig. 14 [1] ←A , B↑

and eventually as spherical featureless beads. The structural correlate of this morphologic change is the fragmentation of DNA, initially into quite large pieces of about 50 to 70 kbp and then, in some but not all cells, into units of one or several nucleosomes in length. (A nucleosome consists of a core of histone proteins wrapped by about 180 bp of DNA and is the first stage of compaction of DNA.) This degradation reflects the action of an endonuclease on the DNA in the linkers between nucleosomes, where the strand is

accessible to the enzyme because it is not protected by histones. The identity of the endonuclease is still unresolved, although caspase-activated DNAse, a new candidate, has attracted considerable attention. Because cells can only repair a few simultaneous double stranded brakes in their DNA, the extensive DNA damage in apoptosis, up to 300.000 breaks per chromosome, means that even if there were no other changes, the cell would certainly never successfully divide again.

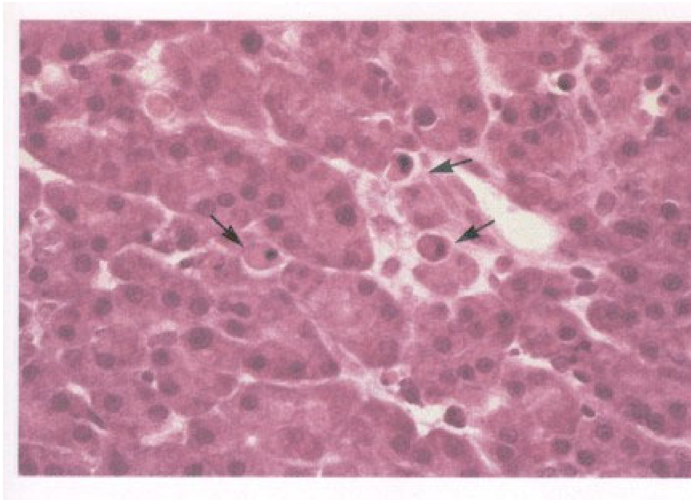


Fig 15 [1]

However, other changes do take place. Early in apoptosis, cells shrink remarkably, losing about a third of their volume in a few minutes. The mechanism for this process is not yet understood, but it must involve shifts in ions, as well as water, perhaps through the unusual activation of plasma membrane associated ion channels or pumps. This shrinkage is quite apparent in cell culture and also in vivo, where apoptotic cells in tissue sections often pull away from their neighbours. As might be expected, there are also cytoskeletal changes that accompany shrinkage, and the result is a peculiar, vigorous "boiling" action of the plasma membrane, which has been called zeiosis or blebbing. By this action, the apoptotic cell usually tears itself apart into apoptotic bodies, some of which contain chromatin. It is not known how, or even if, these changes lead to cell death. This is because early in apoptosis, while the cell is still fully viable (i.e., able to exclude vital dyes like trypan blue), it is recognized by another cell and phagocytosed; it dies within the phagocyte. Therefore the real goal of all the morphologic changes may be to ensure that the cell gets taken up by another before it has had a chance to spill its dangerous contents. For further details, see [20]

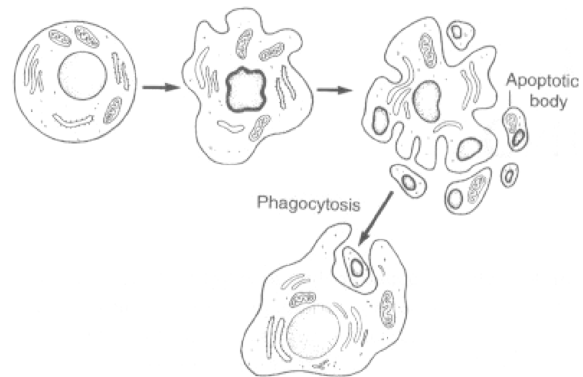



Fig. 16 [1]

### 4.3. Induction of Apoptosis, Fas and Fas-Ligand

Thymocytes die when exposed in culture to a concentration of glucocorticoid equal to that physiologically achieved each day at the peak of the circadian cycle. Wyllie [21] showed in 1980 that this death process met the criteria of apoptosis. Steroids work almost exclusively by inducing changes in gene expression, usually by transcriptional activation. But could transcriptional activation lead to death? It seemed impossible, but when thymocytes were treated with a lethal concentration of dexamethasone, in the presence of blockers of transcription, the cells did not die. The steroid did not kill the cells; rather, it induced them to kill themselves. That is, when they were prevented from expressing what came to be called death genes at either the transcriptional (mRNA) or translational (protein) levels, they could not die. The important point that these experiments made was this: If one cell type has death genes, then all cells in the body do because they share the same genome. Therefore any cell in the body could be made to undergo apoptosis if we could understand how to get it to turn on these genes, and any cell that intended to die might be prevented from doing so by a reverse strategy. The results also suggested that in addition to programmed cell death there was a cell death program.

Recent investigations have provided a more detailed insight into the induction of apoptosis via this death program. Besides NO or TNF-α, Fas and Fas-Ligand have been identified as important mediators of that process. Fas is a transmembrane receptor protein, also known as $CD_{95}$ and APO-1, capable of inducing DNA-damage and consequent apoptosis in cells on the surface of which it is expressed. Its cytoplasmic domain is referred to as the above mentioned death domain; it is required to transmit the apoptotic signal. The intracellular transduction is carried out by the proteolytic caspase cascade, starting with caspase 8. It is triggered by the binding of FasL, the ligand of Fas, to its receptor.

While the receptor Fas is a common protein found in many cell types, FasL is much more restricted in its expression. FasL is expressed on cytotoxic T lymphocytes, where it contributes to their cytotoxic function and mediates the elimination of peripheral T cells following an immune response. Constitutive expression has also been detected in immunoprivileged sites such as the eye and testis. [22] The Fas – FasL system as a whole has essential roles in immunological self-tolerance and the homeostasis of the lymphocyte population.

Long before the discovery of apoptosis, cytotoxic agents have been used to treat malignant diseases on the basis of empirical data – knowledge about their mode of action

has caught up with some delay. Today the approach is reversed and new ideas about potential therapeutic strategies can be based on sound fundaments layed by research; a good example is NO which has been found to induce programmed cell death in various cells, as mentioned above. Now the possibility for a reintroduction of a normal apoptotic process in leukaemic cells by manipulating their susceptibility to NO or other proapoptotic stimuli can be envisaged.

# 5. Cells used in our investigations

## 5.1. U-937

U-937 is a monocytoid cell line. It was derived by Sundstrom and Nilsson in 1974 from malignant cells obtained from the pleural effusion of a 37 year old caucasian patient with diffuse histiocytic lymphoma. Studies since 1979 have shown that U-937 cells can be induced to terminal monocytic differentiation by incubation with several substances, including IFN-γ and TNF. They express the Fas antigen and are sensitive to TNF and anti-Fas antibody-induced apoptotic cells death. They are capable of synthesizing IL-8, lysozyme, β-2-microglobulin, TNF-α, and other important regulators of cell physiology.

## 5.2. Peripheral Blood Mononuclear Cells

PBMC were obtained from a pool of 12 healthy donors (→ Materials and Methods). By centrifugation in Ficoll Hypaque solution PBMC were separated from red blood cells, platelets, and the polymorphonuclear fraction of white blood cells. This provided us with a mixture of approximately 40 – 60 % MΦ and a strongly varying ratio of natural killer (NK) cells and T- and B-lymphocytes, depending on the particular donor. A further 24 h incubation period on polystyrene plates caused the MΦ to become adhesive. Washing away the supernatant thus yielded an approximately 95 % pure culture of MΦ.

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# 6. Reagents

TNF-α was kindly provided by Knoll AG (Ludwigshafen). N-tosyl-L-phenylalanine-chloromethyl ketone (TPCK), SB203580, and VP-16 were from Calbiochem-Novabiochem GmbH (Bad Soden, Germany). CdA, Polymyxin B (PmxB), Histopaque-1077, and human AB serum were purchased from Sigma (Deisenhofen, Germany). (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)-amino] diazen-1-ium-1,2-diolate (DETA-NO) was from Alexis GmbH (Grünberg, Germany). TGF-$\beta_1$ was kindly provided by Dr. N. Cerletti (Novartis, Basel). IL-10 was purchased from R&D Systems (Wiesbaden, Germany). All other chemicals were either from Merck (Darmstadt, Germany), Sigma, Roth GmbH (Karlsruhe, Germany), or from Calbiochem-Novabiochem GmbH.

# 7. Cells

### 7.1. Cultivation and Treatment of Human Promonocytic U-937 Cells

The human monocytic cell line U-937 was obtained from the American Type Tissue Collection (Rockville, MD). The cells were maintained in RPMI 1640 supplemented with 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) heat-inactivated FCS (GIBCO BRL, Eggenstein, Germany) using 175 $cm^2$ polystyrene flasks (Greiner, Frickenhausen, Germany). For the experiments, 3 ml of cell suspensions were seeded into 6-well polystyrene plates (Greiner) at $10^6$ cells/ml. Modulators of IL-8 release were given 30 min before exposure to VP-16. After the indicated time periods, cells were centrifuged and cell-free supernatants were assayed for cytokine concentrations. Cells were lysed either for determination of apoptotic DNA fragmentation or for analysis of expression of specific mRNA species. VP-16, TPCK, and SB203580 were dissolved in DMSO. DMSO concentrations in the cultures were 0.026% (v/v) or below. DMSO did not influence release of IL-8 from U-937 cells (711 ± 150 pg/ml versus 714 ± 147 pg/ml for control and DMSO (0.026%), respectively; n = 4). In addition, controls were corrected for vehicle. All incubations of either U-937 cells or PBMC were performed at 37 $^{o}C$ and 5% $CO_2$ for the indicated time periods.

### 7.2. Isolation and Cultivation of PBMC

The study protocol and consent documents were approved by the *Ethik Kommission* of the Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main. Healthy volunteers abstained from using any drugs during two weeks before the study. Adherent PBMC, which constitute a monocyte-enriched fraction, were isolated as follows: Blood was drawn in heparinized syringes. PBMC were isolated by centrifugation through Ficoll Hypaque (Sigma) at 1000 g for 13 min. PBMC were washed twice in isotonic NaCl solution and cultivated in RPMI 1640 supplemented with 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% (v/v) heat-inactivated human AB serum. For evaluation of cytokine expression PBMC were resuspended at 3 x $10^6$ cells/ml and were seeded in polystyrene 24-well plates or 6-well plates at 1 ml or 2 ml per well, respectively. Nonadherent cells were removed by aspiration after a 2.5 h incubation period. CdA was dissolved in DMSO (final concentration of DMSO: 0.1% or 0.025%). In all experiments performed either with PBMC or with whole blood cultures, polymyxin B (PmxB) was added to the different concentrations of CdA (final concentration of PmxB: 1 μg/ml) in order to ensure that the results were not due to endotoxin contamination either from the CdA or from the cell culture medium. Unstimulated controls were incubated with vehicle (PmxB and DMSO).

### 7.3. Human Whole Blood Cultures

Whole blood cultures were performed as follows: Vehicle or CdA in the respective concentrations were added to culture medium (RPMI 1640 supplemented with 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin). As mentioned earlier, CdA was routinely treated with PmxB. Heparinized blood was mixed with an equal volume of culture medium and 1 ml aliquots were transferred into round-bottom polypropylene tubes (Greiner). The sealed tubes were incubated upright at 37 $^{o}$C and 5% $CO_2$ for the indicated time periods. Controls were incubated with vehicle only (PmxB and DMSO). Thereafter, Triton X-100 was added to a final concentration of 1%. The tubes were mixed until the blood became transparent. The lysed blood was stored at -70 $^{o}$C until IL-8 concentrations were determined by ELISA.

# 8. Methods

## 8.1. Enzyme-Linked Immunosorbent Assay

*8.1.1. Principles*

The ELISA is a highly versatile, sensitive, and quantitative technique. The protocol can be varied not only to detect specific antibodies or cell surface or soluble antigens, but also for epitope mapping and other purposes. In my experiments, I applied the antibody-sandwich method to quantify IL-8, MIP-1α, TNF-α (➔ below), and oligonucleosomes (➔ section 8.1.3.).

In this protocol, a capture antibody is absorbed onto a microtiter plate. After a blocking step in which the non-specific binding sites are saturated with incubation buffer, which contains a certain mix of proteins (e.g. 10 % fetal calf serum), the test solution containing the respective antigen is added. The plate is left at room temperature for two hours, then the wells are thoroughly washed. Another one hour period follows, in which the immunocomplexes are incubated with secondary reactants covalently coupled to an enzyme. Unbound conjugates are washed out and chromogenic substrate is added. As the substrate is hydrolyzed by the bound enzyme conjugate, a coloured product is generated. Finally, this product is detected with a microtiter plate reader. The amount of product generated is proportional to the amount of analysate in the test mixture (➔ fig. 1).

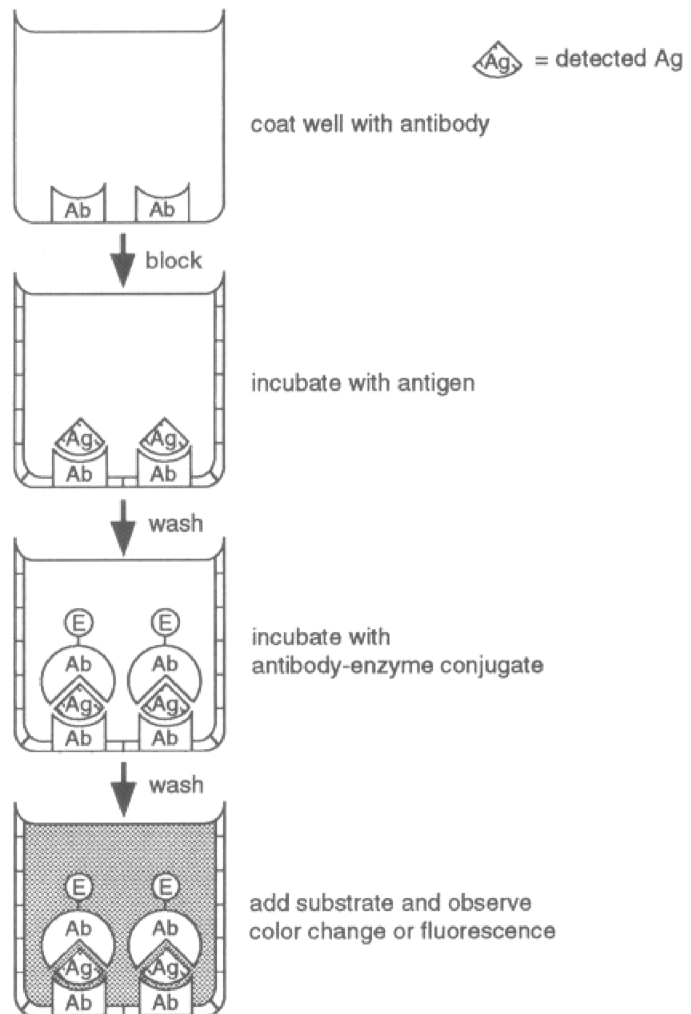


Fig. 1. [22a]

*8.1.2. Details*

Cell culture supernatants were analyzed for concentrations of IL-8 and TNF-α (Pharmingen) by ELISA according to the manufacturer´s instructions. For human MIP-1α, I used a sandwich ELISA which was established recently [23]. Briefly, a 96-well plate (Greiner) was coated overnight with 100 ng/well of anti-human MIP-1α monoclonal antibody (Pepro Tech Inc., Frankfurt, Germany) in 100 µl/well of coating buffer (0.1 M carbonate, pH 9.5). All incubations were carried out at room temperature. After blocking with 400 µl of 10% (v/v) FCS in phospate-buffered saline (PBS) for 2 h and a single wash with 0.05% (v/v) Tween-20 in PBS (washing-buffer), 100 µl/well of MIP-1α standards (R&D Systems, Wiesbaden-Nordenstadt, Germany) or samples were added in the respective dilution made in PBS/10% (v/v) FCS. Plates were incubated for 2 h. After six washing steps in washing buffer, 33 ng/well of the polyclonal anti-MIP-1α antibody (Pepro Tech Inc., in 100 µl/well TBST (Tris-buffered saline, pH 7.5, containing 0.5% (v/v) Tween-20)/0.3% (v/v) of FCS) was added and incubated for 1 h. Thereafter, plates were washed six times with washing buffer, followed by a 0.5 h incubation with 100 µl/well of anti-rabbit IgG-horseradish peroxidase conjugate (Biorad, München, Germany) as the detecting antibody at a 1:15000 dilution in TBST. After nine washes and addition of substrate (Sigma), color development was determined using $OD_{450/595\ nm}$ in an ELISA reader (BioRad).

*8.1.3. Cell Death Detection ELISA and DNA Fragmentation Assay*

8.1.3.1. Principles

During apoptosis, $Ca^{2+}$ and $Mg^{2+}$ dependent endogenous DNA endonucleases are activated as described in section 4.2. One of these enzymes cleaves double stranded DNA at the most accessible internucleosomal linker region, generating mono- and oligonucleosomes. In contrast, the DNA of the nucleosomes is tightly complexed with the core histones and is therefore protected from cleavage by the endonuclease. The DNA fragments yielded are discrete multiples of a 180 bp subunit which can be made visible as a "DNA ladder" by agarose gel electrophoresis after extraction and separation of the fragmented DNA. This was performed in the experiment described in section 11.1. The enrichment of mono- and oligonucleosomes in the cytoplasm of the apoptotic cell is due to the fact that DNA degradation occurs several hours before plasma membrane breakdown.

8.1.3.2. DNA Fragmentation Assay

U-937 cells (20 x $10^6$ cells/85 mm polystyrene plate) were incubated for 6 h in the presence or absence of VP-16 (3.1 μg/ml). Thereafter, cells were lysed in lysis-buffer (50 mM Tris/HCl pH 7.5, 20 mM EDTA, 1% NP-40) and cytosolic DNA fragments characteristic of apoptotic cell death were isolated as follows: The lysates were incubated at 56 °C in 1% SDS containing 30 μl RNase A. After 2 h, proteinase K at a final concentration of 1 mg/ml was added and the mix was incubated at 37 °C over night. The lysates were then precipitated over night at -20 °C by addition of 0.5 volumes $NH_4$ and 2.5 volumes 100% ice-cold ethanol. The precipitates were washed twice in ice-cold 70% ethanol, dried, and resuspended in 40 μl DEPC-$H_2O$. This solution was incubated at 37 °C over the weekend. Finally, electrophoresis was performed using a 1.5% agarose gel. DNA was stained using ethidium bromide at 0.5 μg/ml.

8.1.3.3. Cell Death Detection ELISA

The cell death detection ELISA by Boehringer Mannheim is based on the quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. This permits a specific determination of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates.

In the first incubation step, anti-histone antibody is fixed absorptively onto the wall of the well of the microtiter plate. Subsequently, non-specific binding sites on the wall are saturated by treatment with incubation buffer (blocking solution). During the second incubation step, the nucleosomes contained in the sample bind via their histone components to the immobilized anti-histone antibody. In the third step, anti-DNA-peroxidase (POD) reacts with the DNA part of the nucleosome. After removal of unbound POD conjugate by a washing step, the amount of POD retained in the immunocomplex is determined photometrically with ABTS® (2,2′-azino-di-[3-ethylbenzthiazoline sulfonate (6)] as substrate. For further details see manufacturer´s instructions.

## 8.2. Reverse Transcription Polymerase Chain Reaction

*8.2.1. Principles*

The theoretical basis of the PCR was probably first described in a paper by Kleppe et al. in 1971 [24]. However, this technique did not excite general interest until the mid-1980s, when Kary Mullis and co-workers developed it into a method that could be used to

generate large amounts single-copy genes from genomic DNA [25]. In 1988 Saiki et al. introduced the thermostable DNA polymerase from *Thermophilus aquaticus* which not only greatly facilitated the process, but also improved the specifity and increased the yield of the desired products [26].

Like ELISA, PCR has a wide range of applications, including, among others, the measurement of somatic rearrangements and transcriptional switches of selected genes, studies of gene polymorphisms and the variability of gene expression. In addition to studying DNA, PCR can be extended by reverse transcription (RT) in order to detect specific strands of mRNA. This combination, which was first performed by Rappolee et al. in 1989 [27], has greatly widened the spectrum of applications for PCR and is particularly useful when the clinical specimen are small or the number of cells is limited.

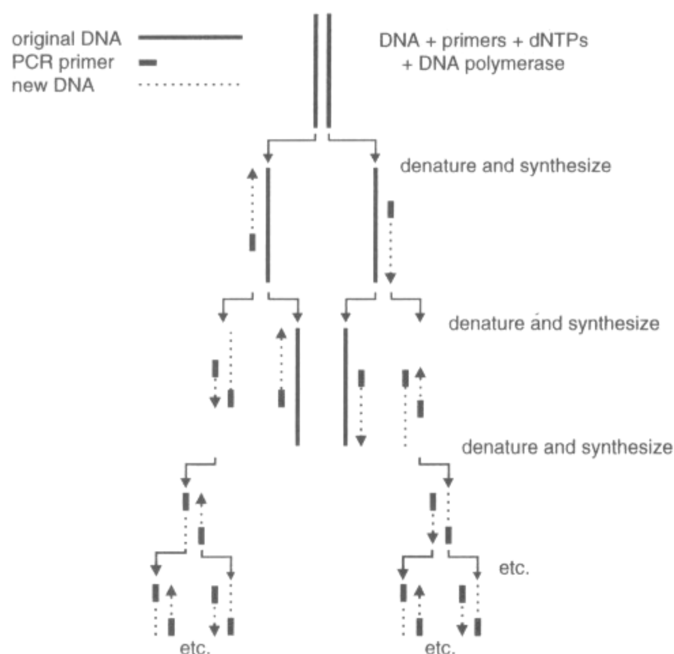


Fig. 2. [27a]

RT makes use of reverse transcriptase, an enzyme found in RNA viruses which are capable to synthesize cDNA from mRNA. The resulting cDNA mixture is then treated according to the PCR protocol described below using primers that are specific for the RNA to be detected.

During PCR, DNA is amplified enzymatically. The first step simply entails mixing template DNA, two appropriate oligonucleotide primers, *Taq* DNA polymerase, deoxyribonucleoside triphosphates (dNTPs), and a buffer. Once assembled, the mixture is cycled a varying number of times (→ section 8.2.2.) through temperatures that permit denaturation, annealing, and synthesis to exponentially amplify a product of specific size and sequence (→ fig. 2). The PCR products are then displayed on an ethidiumbromide-stained agarose gel to visualize the bands.

*8.2.2. Details*

Total RNA was isolated from U-937 using Trizol-Reagent (GIBCO-BRL) as suggested by the manufacturer. After extraction by chloroform, precipitation by isopropanol, and a wash in 70% ethanol, RNA was dissolved in DEPC-treated water and stored at -70 $^0$C. Total RNA concentrations were determined using a Gene Quant (Pharmacia Biotech., Freiburg, Germany) photometric device. 500 ng of RNA were used for RT-PCR (GeneAmp RNA PCR kit, Perkin-Elmer Corp., Weiterstadt, Germany) according to the manufacturer´s instructions. Reverse transcription and PCR were performed on a thermocycler (Perkin Elmer Corp.). The protocols were as follows: RT: 42 $^0$C for 30 min, 94 $^0$C for 5 min; PCR: 94 $^0$C for 1 min (1 cycle), followed immediately by 94 $^0$C for 1 min, 60 $^0$C for 1 min, and 72 $^0$C for 2 min (with variable number of cycles) and a final extension phase at 72 $^0$C for 7 min. To investigate IL-8 mRNA expression the number of cycles was 27. For MIP-1α 30 cycles and for glyceraldehydphosphate dehydrogenase (GAPDH) 25 cycles were used. The sequences of the primers were as follows: IL-8 (F): 5´-ATg AcT Tcc AAg cTg gcc gTg gcT-3´; IL-8 (R): 5´-TcT cAg ccc TcT TcA AAA cTT cTc-3´ [28]; MIP-1α (F): 5´-cgc cTg cTg cTT cAg cTA cAc-3´; MIP-1α (R): 5´-TgT ggA ggT cAc Acg cAT gTT-3´ [23]; GAPDH (F): 5´-Acc AcA gTc cAT gcc ATc Ac-3´; GAPDH (R): 5´-Tcc Acc Acc cTg TTg cTg TA-3´ [28]. PCR products were run on a 2% agarose gel containing 0.5 µg/ml ethidium bromide. Length of the amplicons were 452 bp, 289 bp, and 281 bp for GAPDH, IL-8, and MIP-1α, respectively.

*8.2.3. Establishing an RT-PCR to detect Fas-Ligand*

One prominent signaling pathway associated with programmed cell death is the Fas / Fas-Ligand system. Studies have shown that the expression of FasL is upregulated during the process of apoptosis induced by TNF-α and cAMP in PBMC. In order to detect FasL mRNA, I established an RT-PCR, using $MgCl_2$ concentrations, the duration of the cycle stages (holds, denaturation, annealing, and synthesis), as well as the number of cycles as variables. Primers were self-designed following these guidelines: gc-content >40% for strong annealing, melting temperatures ($T_m$) >59 $^0$C and not more than 3 $^0$C apart, no base repetitions of more than three, no internal loops, no complementary sequences longer than two bp, at 3´-ends last two bases only g or c (for stronger annealing at the site of initiation of synthesis), and finally no other good match in the genome as determined by BLAST search. Note that not all of these criteria can always be met. Primers were synthesized by Gibco BRL. While RT was performed as described in the previous section, I used the

improved hot start *TaqGold* DNA polymerase (Perkin Elmer Corp.) to avoid unspecific results. As opposed to the *AmpliTaq* enzyme I previously used, hot start *TaqGold* polymerase is activated only by a 10 min incubation at 94 $^0$C, thus preventing premature DNA synthesis. The protocol listed below yielded a specific band (→ fig. 3) with the length of the FasL mRNA (XX350 bp). This band was cut out of the gel, sequenced (→ section 8.4.) and identified as the correct fragment using BLAST search.

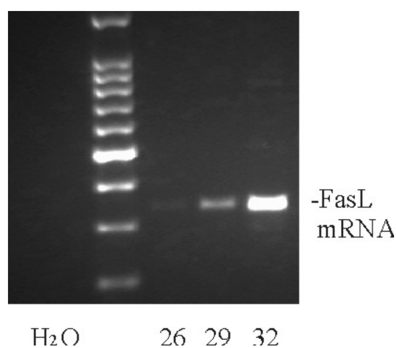


Fig. 3. Establishment of the RT-PCR to detect FasL mRNA. PBMC were incubated for 16 h with 250 µM cAMP. The number of PCR cycles is indicated.

The following protocol was performed using a thermocycler (Perkin Elmer Corp.): 94 $^0$C for 10 mins (1 cycle), followed immediately by 94 $^0$C for 1 min, 56 $^0$C for 1 min, and 72 $^0$C for 2 min (30 cycles) and a final extension phase at 72 $^0$C for 7 min. A $MgCl_2$ concentration of 1.3 mM proved optimal. The sequences of the primers were as follows: FasL (F): 5´-ggA TTg ggc cTg ggg ATg TTT cA-3´; FasL (R): 5´-TTg Tgg cTc Agg ggc Agg TTg TTg-3´. Visualization was performed as described in section 8.2.1.

## 8.3. Sequencing

DNA sequencing was performed using the ABI-Prism 310 Genetic Analyser (PE Biosystems) based on the dideoxynucleotide chain termination method. In the termination labeling mix, the four dideoxy terminators (ddNTPs) are tagged with fluorescent dyes. This technique permits the simultaneous sequencing of all four reactions (A, C, G, and T) in one reaction tube. The probes are separated electrophoretically using a micro capillary. As each dye terminator emits light at a different wavelength when excited by laser light, all four colours corresponding to the four nucleotides can be detected and distinguished within a single run. Raw data were evaluated by the ABI-Prism sequencing analysis software. The sequencing reaction, as listed in the table, was performed in a thermocycler (GeneAmp 2400, PE Biosystems) with 25 cycles of the following temperature steps: 96 °C for 10 s, 55 °C for 5 s, and 60 °C for 2 mins. For the detection process, probes were prepared according

to manufacturer´s instructions. The mix was constructed as follows: 1 µl of DNA (50 ng PCR derived DNA), 2 µl of sequencing premix, 1 µl of primer solution, and 6 µl $H_2O$.

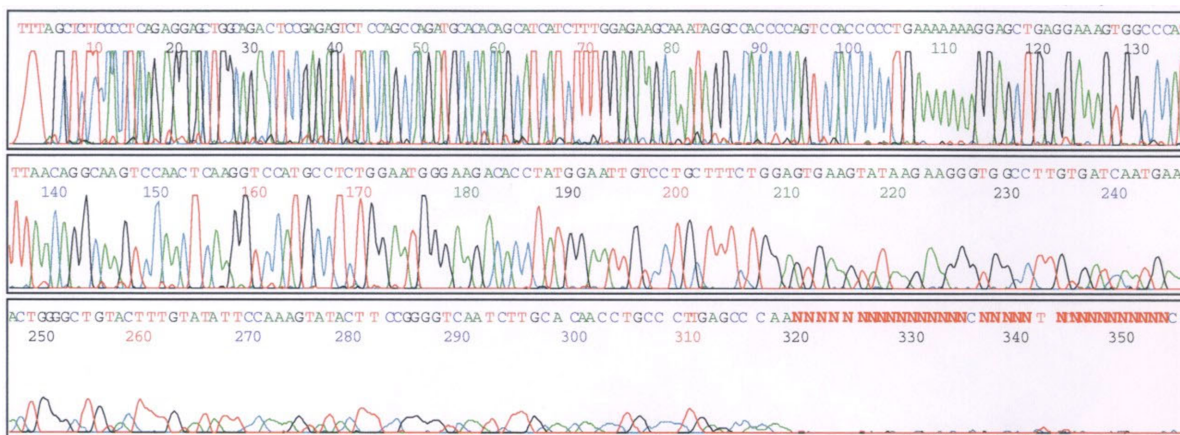

Fig. 4. Result of the sequencing procedure of the PCR amplicon of the FasL RT-PCR.

## 8.4. Determination of Cell Viability

Viable cells were quantified by measuring the amount of soluble formazan produced by cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) according to the manufacturer´s instructions. After the indicated time periods under various experimental conditions, cells were resuspended and a 20 µl sample was removed and diluted in 200 µl. The 220 µl samples were put onto a 96-well plate. 20 µl of CellTiter 96$^{@}$AQ$_{ueus}$ One solution-MTS Reagent (Promega, Mannheim, Germany) were added to each well. After 1h at 37 $^{0}$C, absorbance was measured at 490 nm. Various cell numbers were used to ascertain the linearity of the assay.

## 8.5. Electrophoresis Mobility Shift Assay

### *8.5.1. Principles*

The mobility shift DNA-binding assay using gel electrophoresis (EMSA) was designed to either investigate the presence of transcription factors bound to nuclear DNA, or to find regulating sequences (promoters) of genes. A knowledge of the cellular content of these regulatory structures is valuable, since important processes, e.g. the development of an adult organism from a fertilized ovum or the maintenance of biochemical characterisitcs of the developed tissue, are known to be dependent on their presence.

Promoters are usually located upstream from the genes the expression of which they influence, and can be identified by their capability to bind transcription factors. If a protein binds to a DNA fragment, the molar mass of the newly formed complex is considerably higher than that of the DNA molecule alone. The presence or absence of the regulator can then be visualized by gel electrophoresis, as migration of the larger complex is retarded (→ fig. 5).

The transcription factors are located in the nucleus of a cell, thus the first step of the EMSA protocol is to create unfractioned protein-extracts from the nuclei. Before these extracts can be mixed with DNA containing the regulating sequence, the DNA has to be incubated with a restriction endo-nuclease which cleaves the long molecule into smaller fragments. With the appropriate experimental design it is now possible to detect the presence of a certain trans-cription factor, e.g., as in our case, NF-κB. If, however, the position of the regulating sequence is to be determined, this can be accomp-lished with a restriction map.

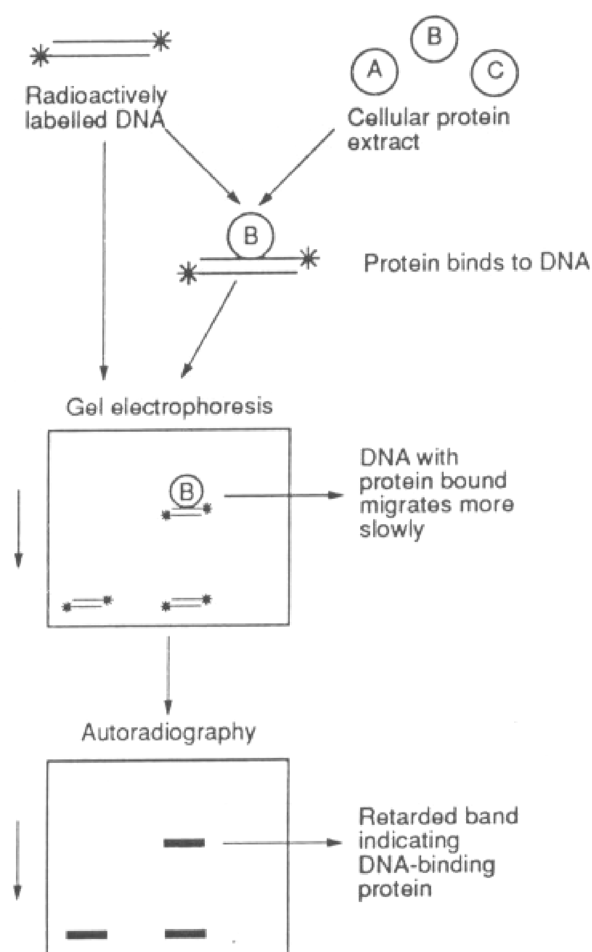


Fig. 5. [28a]

*8.5.2. Details*

Nuclear extraction was performed as follows: After washing the culture plate first with PBS, then with 0.1 mM EDTA in PBS the cells were transferred to Eppendorff cups and pelleted by centrifugation (2 min at 6000 rpm). The supernatant was completely removed and the pellet resuspended in 300 µl cold hypotonic homogenisation buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM ethylene diamine tetraacetic acid (EDTA), 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF)). After homogenisation, the resulting solution was allowed to swell on ice for 1 or 2 h, after

which 20 µl of 10% Nonidet P-40 (NP40) was added, resulting in a final concentration of 0.6%. This mix was vortexed, centrifuged for 1 min at 14 000 rpm, and resuspended in 70 µl ice-cold nuclear extract buffer (25% glycerol, 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). This solution was put on a shaker at full speed in the cold room for 20 min and again centrifuged for 5 min at 4 °C and 13 000 rpm. 1 µl of the resulting supernatant containing the desired nuclear extract was used to determine the protein content (Bio Rad). The rest was either used directly for the EMSA reaction or was stored at -70 °C.

The EMSA procedure was begun with radioactive labeling of the complementary DNA strands with T4 polynucleotide kinase using [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol). DNA-protein binding reactions were performed for 30 min on ice in a mixture (20 µl) of 4% Ficoll, 20 mM HEPES pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 1mM PMSF, 0.25 mg/ml bovine serum albumin (BSA), 2 µg of poly [dI-dC], and 20 000 dpm of $^{32}$P-labeled oligonucleotides. Sequences of oligonucleotides were as follows: NF-κB: 5´-ccc tac tgg gga ctc tcc ctt t-3´. DNA-protein complexes were separated from free DNA probe by electrophoresis through native 4.0% polyacrylamide gels at 40 mA in 0.5x Tris borate-EDTA. Gels were fixed in 10% acetic acid and 10% isopropanol. After vacuum drying, the gels were exposed to Kodak X-Omat XAR film at -80 °C for 12-48 h.

# 9. Statistics

For experiments using U-937 cells and PBMC/whole blood cultures, data are shown as mean ± SD or mean ± SEM, respectively. Data are presented either as $OD^{405/495\ nm}$, pg/ml, ng/ml, or as percent change compared to unstimulated control (100%) and were analyzed by unpaired Student´s t test (U-937 cells) or paired Student´s t test (PBMC, whole blood cultures) on raw data using Sigma Plot (Jandel Scientific).

# III. Results

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10.2.1. Induction of IL-8 and MIP-1α Expression by TNF-α, IL-1, and LPS

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# 10. Testing the Systems: Induction of IL-8 and MIP-1α Expression in U-937 Cells and PBMC by TNF-α, IL-1, and LPS and its Modulation by IL-10 and cAMP

In order to verify my results, the experimental systems, procedures, and substances had to be tested for validity; e.g. reproducibility of procedures, stable dose-response relations of cellular responses, sensitivity of cells to modulating agents, and others. To accomplish this, I selected well-documented stimuli and modulators of IL-8 and/or MIP-1α expression and reproduced previous findings.

## 10.1. U-937

### *10.1.1. Dose-Response Relation of IL-8 and MIP-1α mRNA Synthesis and Protein Release after Stimulation with TNF-α*

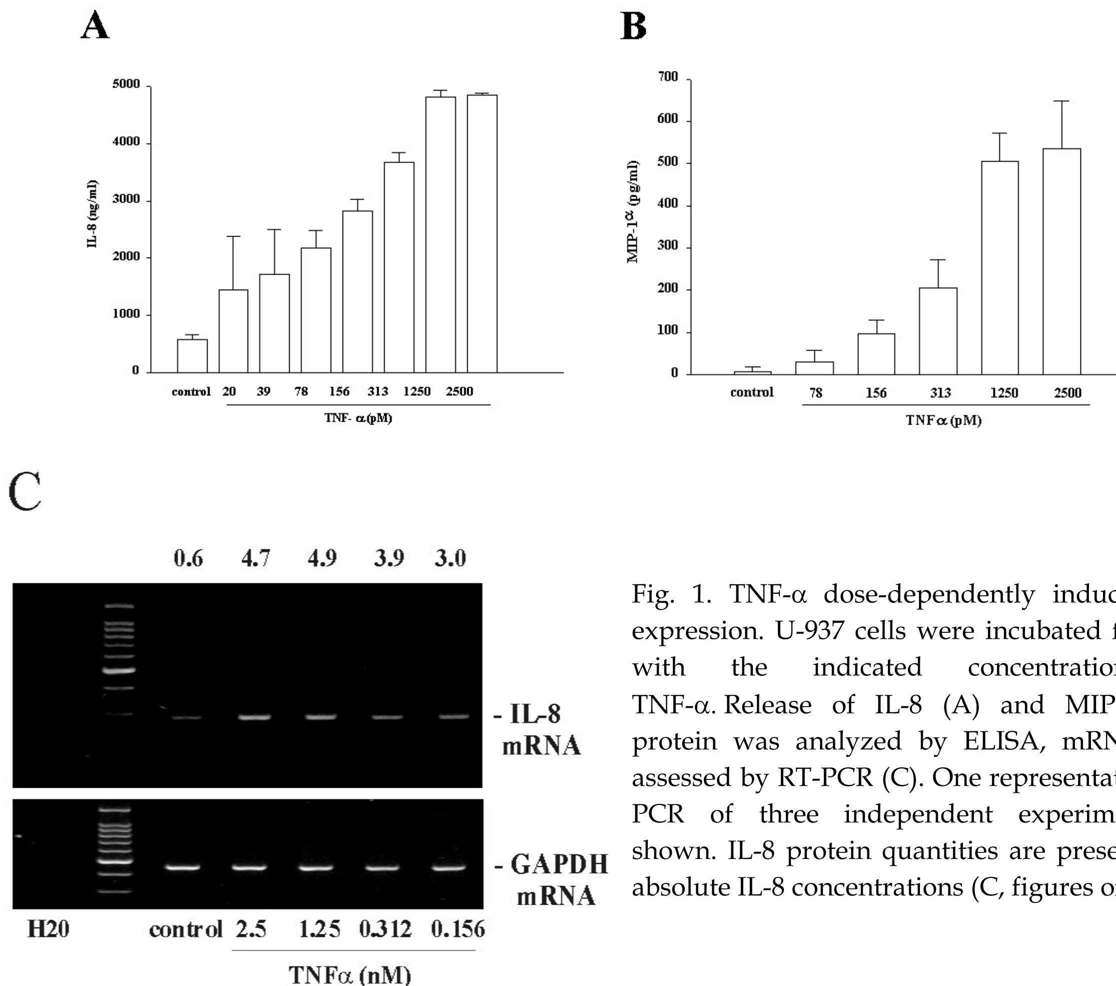



Fig. 1. TNF-α dose-dependently induces IL-8 expression. U-937 cells were incubated for 19 h with the indicated concentrations of TNF-α. Release of IL-8 (A) and MIP-1α (B) protein was analyzed by ELISA, mRNA was assessed by RT-PCR (C). One representative RT-PCR of three independent experiments is shown. IL-8 protein quantities are presented as absolute IL-8 concentrations (C, figures on top).

TNF-α is a prominent pro-inflammatory cytokine. It was among the earliest proteins of this family to be investigated thoroughly for structure and functions. Today it is known to exert a wide range of actions, among others the induction of multiple cytokines, including IL-8 and MIP-1α. Therefore, TNF-α is an ideal molecule to serve as stimulus when the validity of a system is to be tested. As expected, coincubation with TNF-α dose-dependently upregulated IL-8 gene expression and protein release (fig. 1).

## 10.2. PBMC

### *10.2.1. Induction of IL-8 and MIP-1α Expression by TNF-α and LPS*

As in U-937 cells, TNF-α is a potent stimulator of pro-inflammatory actions also in PBMC. Lipopolysaccharides (LPS or endotoxin) are the surface molecules of gram-negative bacteria which cause the responses of the immune system to these microorganisms. If present at high concentrations, LPS eventually overwhelms host defense mechanisms and septic (endotoxic) shock ensues. Thus, endotoxin constitutes another inducer of the pro-inflammatory chemokines IL-8 and MIP-1α as demonstrated in fig. 2.

### *10.2.2. Modulation of inflammatory cytokine mRNA and Protein Levels by IL-10 and cAMP*

The prototype role which IL-1 and TNF-α play in the family of the pro-inflammatory cytokines is taken by IL-10 in the anti-inflammatory field. By potently suppressing inflammatory cytokine production, IL-10 attenuates the potentially harmful fire of inflammation. That is what makes this molecule one of the most important regulators in the delicate equilibrium of the cytokine concert.

Although reports on regulatory roles of the cyclic AMP signaling system on cytokine production are not entirely uniform, potent inhibition of TNF-α production in human MΦ, as well as upregulation of IL-10 and TGF-$\beta_2$ expression suggest that this pathway may represent a principle of MΦ-deactivation. Therefore, I chose to verify cAMP as an inhibitor of the LPS-induced cytokine response. As shown in fig. 2, dibutyryl-cAMP (a synthetic analog to cAMP with increased stability and slow-release properties) potently suppresses TNF-α as well as MIP-1α production in PBMC. Release of IL-8 was partially reduced by dBcAMP.

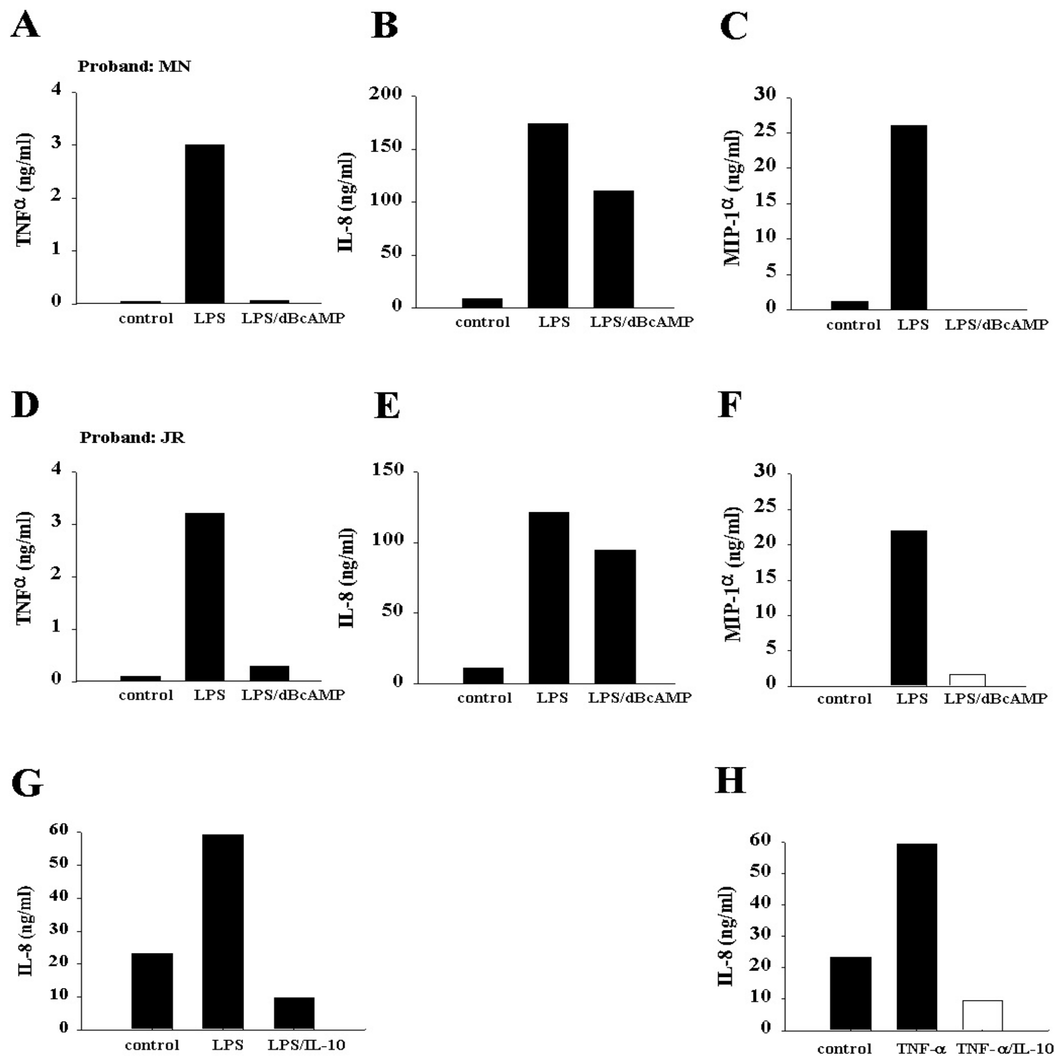



Fig. 2. (A-G) LPS induces release of IL-8, MIP-1α, and TNF-α; the responses can be reduced by coincubation with cAMP. (H) TNF-α stimulates IL-8 secretion which can be inhibited by IL-10. PBMC from two donors (JR, MN) were incubated for 19 h (A-G) with 10 ng/ml LPS in the presence or absence of (A-F) 250 µM cAMP or (G) with 10 ng/ml IL-10. (H) PBMC were stimulated with 5 nM TNF-α either toghether with or without 10 ng/ml IL-10. Release of IL-8 and TNF-α protein were analyzed by ELISA. Data are expressed as absolute cytokine concentrations.

### 10.3. Whole Blood Culture

*10.3.1. IL-8 Expression is induced by IL-1β and can be inhibited by IL-10*

IL-1β shares a variety of properties with TNF-α and was used to induce IL-8 release in WBC, our experimental model representing the microenvironment of the blood compartment. The anti-inflammatory actions of IL-10 are the subject of advanced studies with clinical relevance, e.g. in the fields of inflammatory bowel disease and rheumatoid arthritis [30, 31]. It has extensively been show that, in its role as a $Th_1$ inhibitor, IL-10 potently suppresses chemokine release from immunocompetent cells. Also in my experiments IL-10 strongly reduced IL-1-stimulated IL-8 release from the blood cells of all donors investigated (fig.3).

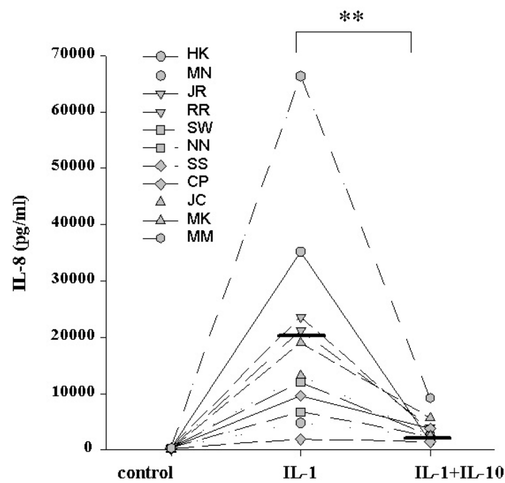



Fig. 3. IL-1 induces IL-8 release which can be inhibited by IL-10. WBC from 11 different donors were incubated for 24 h with 2nM IL-1β in the presence or absence of 10 ng/ml IL-10. Release of IL-8 was determined by ELISA. Data are expressed as absolute IL-8 concentrations.

# 11. Expression of Chemokines is induced by VP-16 and is associated with apoptotic Cell Death in U-937 Cells.

Exposure to oxygen radicals, NO, and other stressors causes chemokine secretion in U-937 cells. One of the hypotheses presented in this work is that this reaction can also be induced when stress is inflicted on cells by cytotoxic substances. Section 11.1. presents data showing that VP-16 is a potent inducer of apoptosis in my U-937 system; section 11.2. demonstrates the accompanying expression of chemokines.

### 11.1. Incubation with VP-16 causes apoptotic Cell Death in U-937 Cells

I chose the DNA topoisomerase II inhibitor VP-16 as cytotoxic agent in the U-937 system, because the compound is a well characterized inducer of apoptosis in these cells [32-34]. Induction of apoptosis with accompanying DNA fragmentation was observed by gel-electrophoresis after 6 h of exposure to VP-16 (3.1 µg/ml) (fig. 4). Fig. 5 shows that ongoing apoptotic cell death was evident 6 h and 8 h (A) as well as 19 h (B) after onset of incubation with VP-16. A stringent dose-response curve was observed (B). At the late 19 h time point viability, as determined by the MTS-assay, was 36.7% ± 7.5%, 42% ± 8.1%, and 28.1% ± 6.9% for VP-16 at 3.1 µg/ml, 6.3 µg/ml, and 12.5 µg/ml, respectively (control set at 100%, n = 4). After 19 h of exposure to these concentrations of VP-16 and consistent with previous data [34], the majority of the remaining cells showed morphological signs of apoptosis as examined by light microscopy (data not shown).

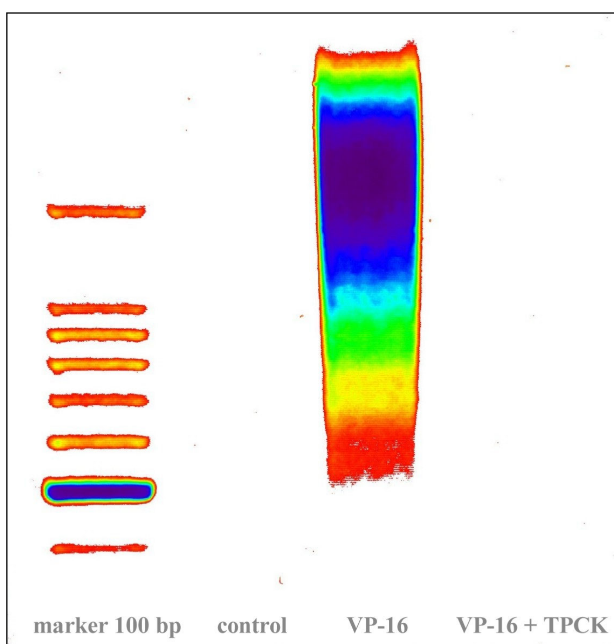



Fig. 4. VP-16 induces apoptotic fragmentation of DNA, TPCK inhibits this process. U-937 cells were incubated for 6 h with 3.1 µg/ml VP-16 in the presence or absence of 50 µM TPCK. DNA fragmentation was visulized by gel-electrophoresis.

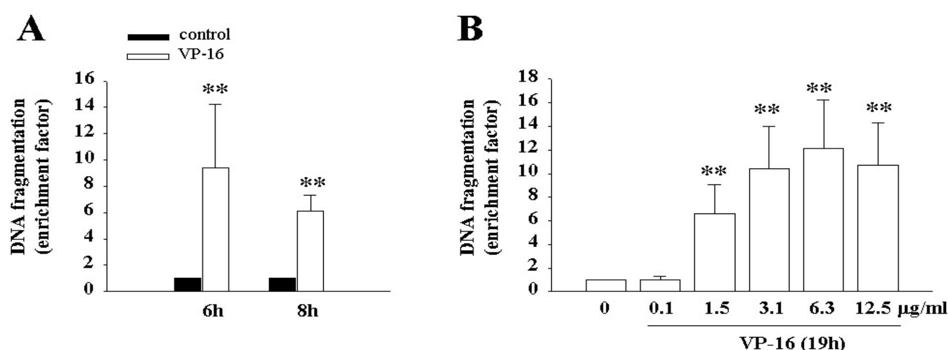



Fig. 5. VP-16 induces apoptosis. U-937 cells were incubated for 6 h and 8 h with 6.3 µg/ml VP-16 (A) or for 19 h with the indicated concentrations of VP-16 (B). Apoptosis was analyzed by ELISA. Mean DNA fragmentation (enrichment factor) ± SD (n=3) are shown. **: $p < 0.001$ compared to control.

### 11.2. VP-16 promotes Synthesis and Release of IL-8 and MIP-1α in U-937 Cells

In parallel with induction of apoptosis, VP-16 significantly increased protein release of IL-8 and MIP-1α (fig. 6A & B). The threshold concentration for VP-16-induced apoptosis as well as chemokine release was 1.56 µg/ml for both responses (fig. 6). The lower concentration tested (0.16 µg/ml) did neither induce apoptosis nor chemokine secretion. As expected, VP-16 also upregulated expression of IL-8 and MIP-1α mRNA which could be detected in the viable cell fraction after 19 h of exposure to VP-16 (fig. 6C).

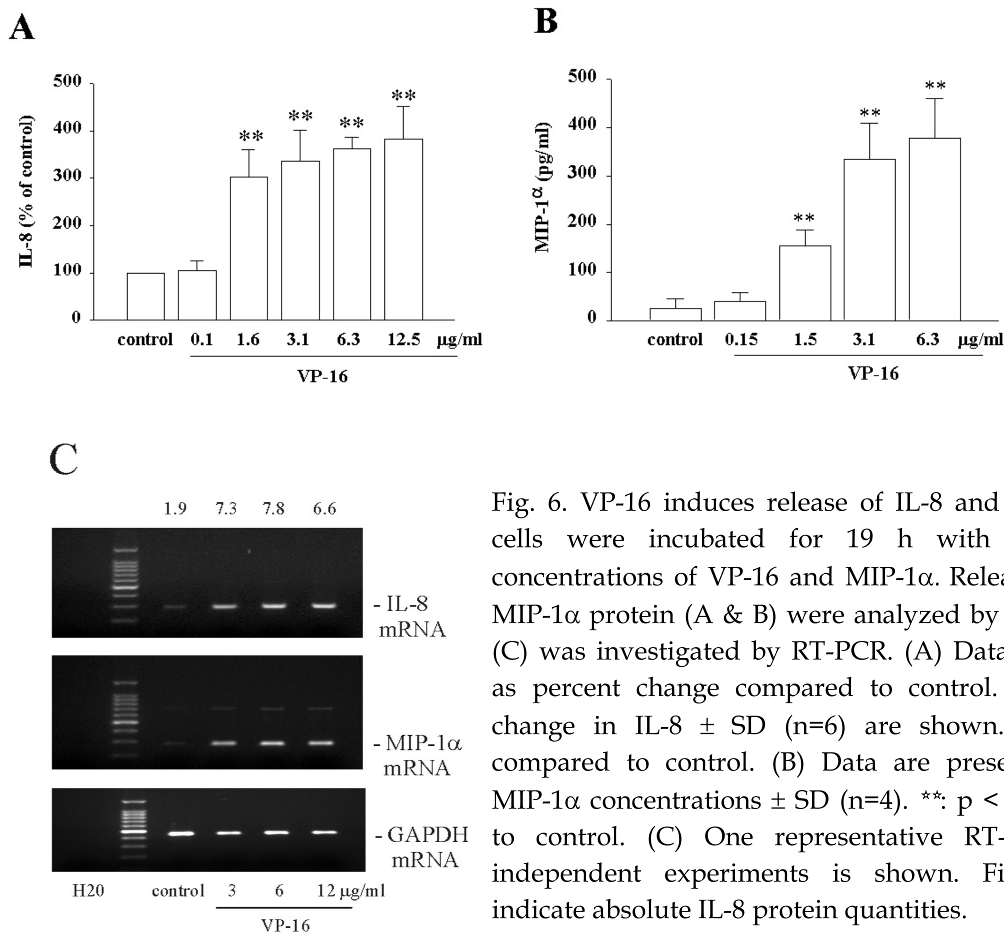



Fig. 6. VP-16 induces release of IL-8 and MIP-1α. U-937 cells were incubated for 19 h with the indicated concentrations of VP-16 and MIP-1α. Release of IL-8 and MIP-1α protein (A & B) were analyzed by ELISA, mRNA (C) was investigated by RT-PCR. (A) Data are expressed as percent change compared to control. Mean percent change in IL-8 ± SD (n=6) are shown. **: $p < 0.01$ compared to control. (B) Data are presented as mean MIP-1α concentrations ± SD (n=4). **: $p < 0.01$ compared to control. (C) One representative RT-PCR of three independent experiments is shown. Figures on top indicate absolute IL-8 protein quantities.

The kinetics of VP-16 (6.3 µg/ml)-induced chemokine mRNA expression and protein release revealed induction of mRNA for IL-8 and MIP-1α after 4 h of incubation. At this 4 h time point, no reduction of cell viability was detectable (96.3% ± 9.8% versus control set at 100%, n = 3, MTS assay). First significant release of IL-8 protein induced by VP-16 was measurable after 6 h of treatment with VP-16 (226.5 ± 53.8% compared to control, $p < 0.05$, n = 3, fig. 7A). Induction of apoptosis corresponded to release of IL-8, regarding both the kinetics and the dose-response relationship. VP-16-induced MIP-1α protein release exhibited

a similar dose-response relationship as observed for IL-8 release and induction of apoptosis (fig. 7C & D). However, the amount of protein was considerably lower compared to IL-8 and the kinetics of MIP-1α release appeared to be delayed, which is in accordance with previous studies with LPS-stimulated adherent PBMC [23].

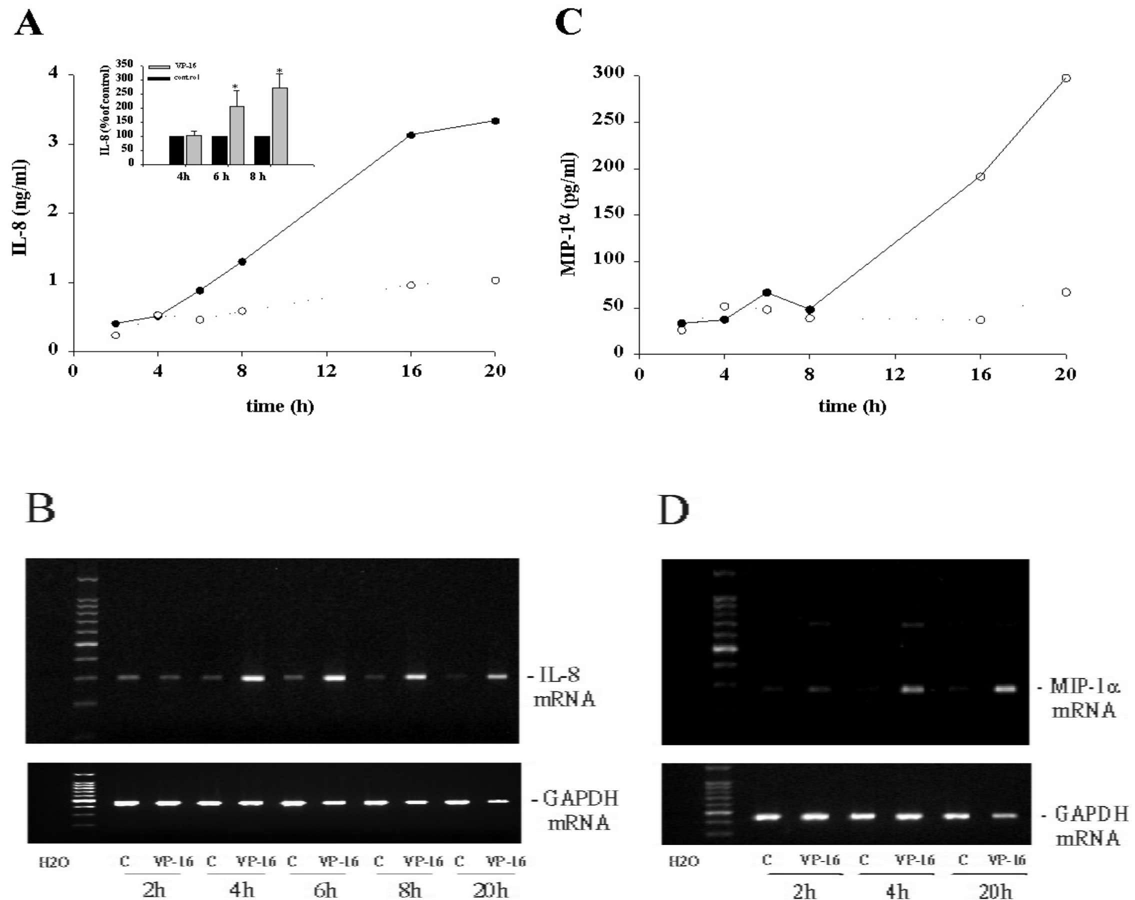



Fig. 7. Kinetics of VP-16-induced IL-8 and MIP-1α expression. (A & C) U-937 cells as unstimulated control (o) or exposed to 6.25 µg/ml VP-16 (•) were incubated for the indicated time periods. Supernatants were assayed for IL-8 (A, insert) or MIP-1α (C) by ELISA. The results of one representative kinetics experiment out of three independently performed are shown. Data are expressed as absolute IL-8 or MIP-1α concentrations (A & C) or as mean percent change in IL-8 ± SD (n=3). *: $p < 0.05$ compared to control (insert). (B & D) Kinetics of VP-16-induced IL-8 and MIP-1α mRNA expression of the same experiment were evaluated by RT-PCR.

After a 19 h incubation period I was still able to detect cells which stained positive for MIP-1α antigen. In unstimulated U-937 cells no cells positive for MIP-1α could be identified (fig. 8). Compared to VP-16 as a stimulus, incubation of U-937 cells with TNF-α (45 ng/ml, 19 h incubation) resulted in release of MIP-1α in a similar concentration range (597 pg/ml ±

Fig. 8. VP-16 induces expression of MIP-1α. U-937 cells were incubated for 19 h with vehicle or 6.25 μg/ml VP-16. Expression of MIP-1α protein was evaluated by immunohistochemistry. This experiment was performed by Dr. Stefan Frank and Dr. Heiko Kämpfer and is displayed with their friendly permission.

160 pg/ml versus 5 pg/ml ± 9 pg/ml for TNF-α stimulation versus control, n = 6, $p < 0.01$). Release of TNF-α remained rarely detectable at all time points investigated (6 h, 8 h, 19 h) in U-937 cells exposed to 6.25 μg/ml VP-16 and was below 10 pg/ml in all culture supernatants examined. Chemokine production by U-937 cells in our studies was not likely the result of release of preformed chemokines for two reasons: First, induction of apoptosis and release of chemokines was accompanied by early induction of expression of chemokine mRNA (fig. 7). Second, apoptosis of U-937 cells achieved by high concentrations of DMSO (4%) [35] was not associated with enhanced release of IL-8 (data not shown).

Both IL-8 and MIP-1α are NF-κB-dependent genes. Therefore, I investigated the degree of NF-κB activation in U-937 cells exposed to VP-16. Fig. 9 shows an upregulated binding activity of the transcription factor.

Fig. 9. Transcription factor NF-κB is activated when U-937 cells are exposed to VP-16. U-937 cells were incubated for the indicated times with 3.1 μg/ml VP-16. Binding of NF-κB to its consensus DNA-binding element was evaluated by EMSA.

# 12. Modulation of VP-16-induced Chemokine Expression and Apoptosis in U-937 Cells

To further investigate signal transduction pathways by which VP-16-induced apoptosis and chemokine expression are mediated, I examined the effects of the following modulators of cellular responses:

## 12.1. Serine Protease Inhibitor $N^{\alpha}$-tosyl-L-phenylalanine-chloromethyl ketone (TPCK)

Previous studies have identified TPCK as a potent inhibitor of VP-16-induced apoptosis in U-937 cells [32]. Figs. 4 and 10B demonstrate that TPCK protected the cells from induction of apoptosis (no detectable DNA fragmentation in fig. 4, strong reduction of the quantitiy of free histones, fig. 10B). Interestingly, also release of IL-8 protein was prevented when TPCK was added (fig. 10A).

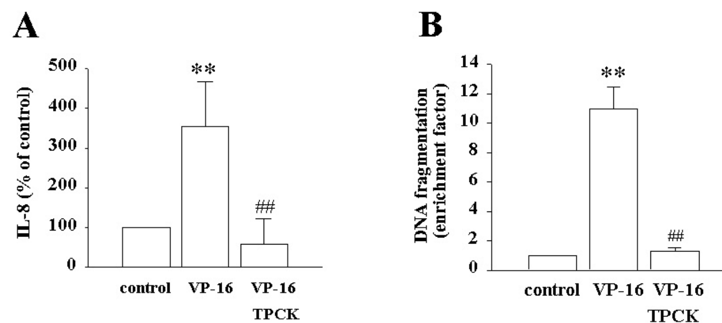



Fig. 10. Modulation of VP-16-induced IL-8 release or apoptosis by TPCK. U-937 cells were incubated for 19 h with 3.1 µg/ml VP-16 with or without 50 µM TPCK. Release of IL-8 (A) and apoptosis (B) were determined by ELISA. IL-8 release is expressed as percent change compared to control. Mean percent changes in IL-8 ± SD (n=4) are shown. **: $p < 0.01$ compared to control; ##: $p < 0.01$ compared to VP-16 alone and not significantly different from control. Apoptosis is quantified as mean DNA fragmentation (enrichment factor) ± SD (n=3), **: $p < 0.01$ compared to control; ##: $p < 0.01$ compared to VP-16 alone and not significantly different from control.

## 12.2. p38 Mitogen Activated Protein Kinase (MAP-Kinase) Inhibitor SB203580

Activation of p38 MAP-kinases holds a central position in signal transduction pathways which regulate cytokine production in response to inflammatory stimuli or stress (➔ section 3.1.). In accordance with this concept, SB203580 inhibited release of IL-8 after exposure to VP-16 (fig. 11A). Interestingly, the apoptotic process remained unaffected (fig. 11B).

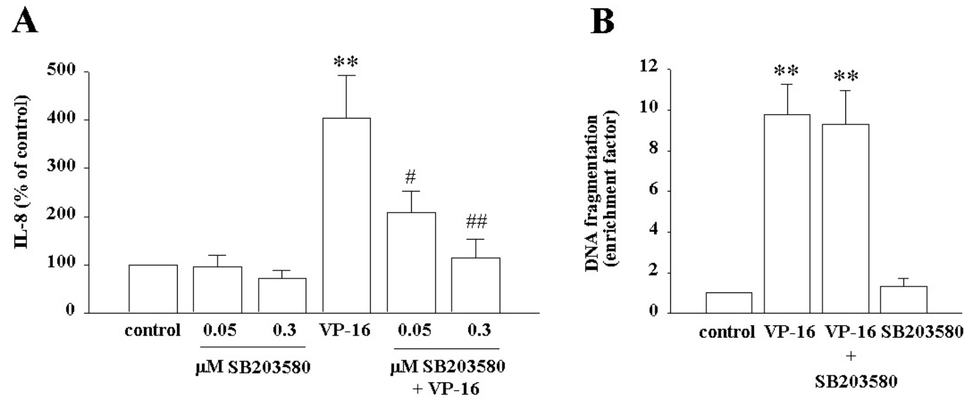



Fig. 11. Modulation of VP-16-induced IL-8 release or apoptosis by SB203580. U-937 cells were incubated for 19 h with 3.1 µg/ml VP-16 in the presence or absence of the indicated concentrations of SB203580 (A) or with 0.3 µM SB203580 (B). Release of IL-8 (A) and apoptosis (B) were determined by ELISA. IL-8 release is expressed as percent change compared to control. Mean percent changes in IL-8 ± SD (n=6) are shown. **: $p < 0.01$ compared to control; ##: $p < 0.01$ compared to VP-16 alone and not significantly different from control; #: $p < 0.05$ compared to VP-16 alone. Apoptosis is quantified as mean DNA fragmentation (enrichment factor) ± SD (n=3), **: $p < 0.01$ compared to control; ##: $p < 0.01$ compared to VP-16 alone and not significantly different from control.

## 12.3. Transforming Growth Factor $\beta_1$

TGF-β is a versatile molecule which is often acutely modulated in response to a variety of stress and disease signals. It influences many aspects of the immune response. TGF-β mediates antiinflammatory responses via suppression of inflammatory cytokine synthesis. In our experiments, TGF-$\beta_1$ had effects similar to SB203580, as it inhibited VP-16-induced IL-8 release (fig. 12), but did not interfere with apoptosis (data not shown).

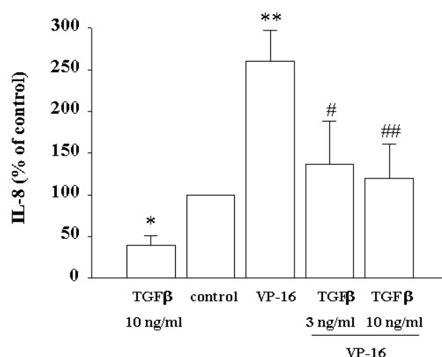



Fig. 12. Modulation of VP-16-induced IL-8 release by TGF-$\beta_1$. U-937 cells were incubated for 19 h with the indicated concentrations of VP-16 and/or TGF-$\beta_1$. Release of IL-8 was determined by ELISA. IL-8 release is expressed as percent change compared to control. Mean percent changes in IL-8 ± SD (n=4) are shown. **: $p < 0.01$ compared to control; *: $p < 0.05$ compared to control; ##: $p < 0.01$ compared to VP-16 alone; #: $p < 0.05$ compared to VP-16 alone.

# 13. Determination of IL-8 mRNA Half-Life

Along with increased synthesis and release of proteins, modulation of the half-lives of their respective mRNAs is another concept in cytokine biology that is utilized to promote the efficacy of immune responses. Therefore, I determined the half-life of VP-16-induced IL-8 mRNA.

As shown in fig. 13, VP-16-induced IL-8 mRNA is degraded with a half-life of 4.6 h. Background expression of IL-8 was too low in these cells to be determined by RT-PCR with the necessary accuracy. Thus I could not determine the half-life of background IL-8 mRNA in these experiments. However, in a previous study this background half-life was determined to be 0.6 h [36]. Although additional experiments are necessary to verify these findings, it appears that, besides NF-κB activation, mRNA stabilisation may contribute to VP-16-induced increase of IL-8 protein levels in U-937 cells.

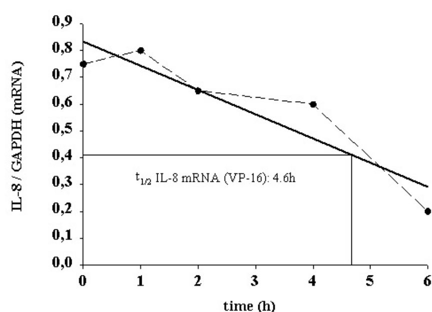



Fig. 13. Determination of IL-8 mRNA half-life in U-937 cells coincubated with 3.1 μg/ml VP-16. Cells were treated with VP-16 for 6.5 h. Thereafter, transcription of additional IL-8 mRNA was blocked using actinomycin D which was added at time point 0 h.

# 14. 2-Chlorodeoxyadenosine (CdA) enhances IL-8 and TNF-α Release from PBMC in parallel to strong Induction of Apoptotic Cell Death.

Recently, it has been reported that CdA efficiently induces apoptosis after a 24 h incubation period in resting PBMC [37]. I confirmed these findings in adherent PBMC after exposure to CdA (fig. 14A). In parallel, I observed augmented release of IL-8 from the same set of cells (fig. 14B), as well as in whole blood cultures (WBC) (fig. 14E). The amounts of IL-8 released from adherent PBMC were always higher than those secreted from WBC; this is most likely due to inevitable activation of monocytes during isolation and cultivation of adherent PBMC. In six out of the eight PBMC donors, I detected induction of TNF-α release (fig. 14C). Fig. 14D shows a correlation analysis comparing TNF-α and IL-8 protein levels; IL-8 release correlated significantly with release of TNF-α ($r = 0.86$). Induction of IL-8 was evident in the presence of polymyxin B (PmxB; final concentration: 1 μg/ml), which was routinely added to CdA and vehicle. At this concentration PmxB completely suppressed LPS (10 ng/ml)-induced release of IL-8 (24.1 ± 7.9 ng/ml, 172.9 ± 6.9 ng/ml, 29.1 ± 9.4 ng/ml for PmxB alone, LPS, and LPS/PmxB, respectively, $n = 3$). In four out of the eight donors tested, the experiments were performed in the presence and absence of PmxB in controls and CdA stimulations. I did not observe a more dramatic enhancing effect of CdA on IL-8 release in the absence of PmxB (22.7 ± 10.8 ng/ml versus 48.4 ± 9.3 ng/ml for control and CdA in the absence of PmxB, $n = 4$, $p < 0.01$; 15.6 ± 7.2 ng/ml versus 40.4 ± 7.8 ng/ml for control and CdA in the presence of PmxB, $n = 4$, $p < 0.01$). From this I conclude that CdA-induced IL-8 as well as TNF-α release is not a result of a latent endotoxin contamination in the CdA preparation. When unstimulated PBMC were lysed after a 20 h incubation period by three cycles of freezing and thawing in culture supernatant, no significant difference in IL-8 concentrations was observed between these whole cell lysates and the respective, equally treated, cell-free culture supernatant alone (112.4% ± 16% compared with cell-free supernatant only, set at 100%, $n = 3$). Thus, I conclude that CdA-induced IL-8 release from adherent PBMC is not a consequence of release of preformed IL-8. As shown in Fig. 14D, CdA was also active in inducing IL-8 release in whole blood cultures.

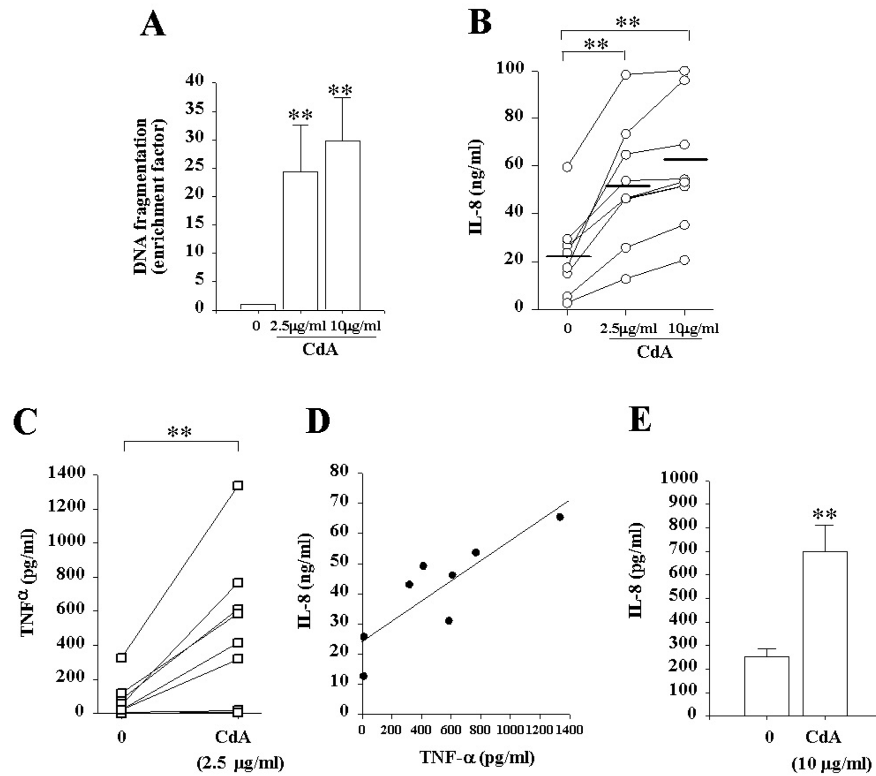



Fig. 14. CdA induces apoptosis as well as IL-8 and TNF-α release from adherent PBMC and augments release of IL-8 from WBC. PBMC (A, B, and C) or WBC (E) were stimulated for 24 h with the indicated concentrations of CdA. Apoptotic cell death (A), release of IL-8 (B, E), and TNF-α (C) were determined by ELISA. (A) Apoptosis is quantified as mean DNA fragmentation (enrichment factor) ± SEM (n=3), **: $p < 0.01$ compared to control. (B, C) Data are expressed as absolute cytokine concentrations. **: $p < 0.01$ compared to control. (D) Production of TNF-α is plotted against IL-8. The correlation is significant at $r = 0.86$. (E) Data are expressed as mean IL-8 concentrations ± SEM (n=9), **: $p < 0.01$ compared to control.

# IV. Discussion

Apoptosis is an active, organized strategy for safe removal of unwanted cells in multicellular organisms. This type of cell death is supposed to avoid the initiation of an inflammatory response before the dying cell disintegrates, which inevitably would lead to release of potentially hazardous cell constituents. The prevention of this spillage is accomplished by early phagocytosis [38].

Since rapid phagocytosis plays an essential role in the pathophysiology of apoptosis as described above, efficient attraction of potent phagocytes is likely to be a critical parameter of the process. This is what renders chemokine expression during apoptotic cell death an interesting topic of investigation. I have shown that initiation of programmed cell death in the promonocytic cell line U-937 by the cytotoxic drug VP-16 is associated with expression and release of the chemokines IL-8 and MIP-1$\alpha$. Induction of mRNA for MIP-1$\alpha$ and IL-8 was evident as early as 4 h after onset of stimulation with VP-16. IL-8 protein release was significantly augmented after 6 h of exposure to VP-16.

In the present study, I do not address the molecular basis of VP-16-induced chemokine expression in further detail. However, in accordance with previous observations [34] I was able to detect activation of the transcription factor nuclear factor-κB (NF-κB) in U-937 cells through VP-16. As NF-κB is an important mediator in the signaling pathway of IL-8 induction, these results provide a link between VP-16-induced cellular stress and expression of IL-8 and other NF-κB-dependent genes.

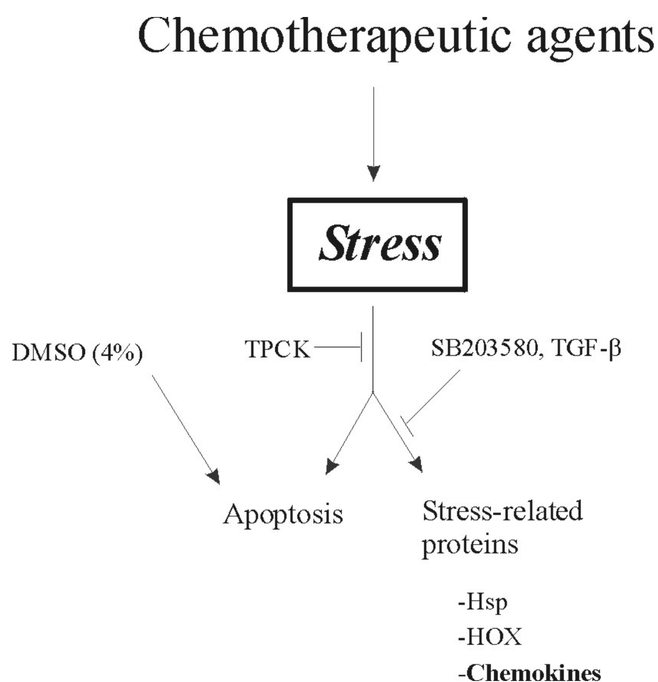
I determined the half life of VP-16-induced IL-8 mRNA to be 4.6 hours, which is relatively high compared to the previously reported background half life of 40 mins [36]. Although additional studies are necessary, these results suggest that VP-16 may enhance the stability of IL-8 mRNA. Such an effect should contribute to upregulation of IL-8 protein by VP-16. In this context it is worthwhile to note that IL-8 mRNA contains several AU-rich sequences in its 3´ untranslated region that have previously been shown to render mRNA transcripts more susceptible to degradation. In turn, these AU-rich sequences and proteins binding to them may be targeted by strategies aiming at an increase of IL-8 mRNA stability.

It is necessary to exclude the possibility that release of chemokines occurs due to secondary necrosis. When I performed MTS viability assays after a 4 h incubation period with 6.3 μg/ml VP-16, I was not able to detect a significant decrease in staining and hence cell number, despite induction of IL-8 under these conditions. I can thus conclude that initiation of chemokine expression in our experiments is a rapid process and not likely due to secondary necrosis. I furthermore infer that induction of chemokine expression is compatible with apoptotic death in these cell cultures. Parallel experiments with the two NO-donors (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)-amino]diazen-1-ium-1,2-diolate (DETA-NO) and sodium nitroprusside revealed that the respective efficacy to induce IL-8 in U-937 cells is linked to the capacity of each compound to induce apoptosis in these cells [11]. This observation provides further evidence for the close association of chemokine induction and apoptotic cell death in this experimental system.

Cellular responses to various forms of stress are frequently mediated by activation of p38 MAP-kinases. These responses include release of cytokines, such as IL-1, TNF-α [39], or IL-8 [28]. In agreement with this concept, inhibition of p38 MAP-kinases using the specific inhibitor SB203580 prevented IL-8 release in response to stress induced by the cytotoxic drug VP-16. However, SB203580 did not modulate VP-16-induced apoptosis. This observation corresponds with a recent study which reports that p38 MAP-kinases are not involved in apoptosis induced by UV irradiation in U-937 cells [40].

TGF-β is an important endogenous modulator of immune responses. [8] In our experiments, coincubation with TGF-$\beta_1$ inhibited VP-16-induced IL-8 secretion, but, similar to SB203580, the apoptotic process remained unaffected.

Fig. 1 →




In contrast to SB203580 and TGF-$\beta_1$, the serine protease inhibitor TPCK prevented both induction of apoptosis as well as IL-8 secretion. I assume that VP-16-induced IL-8 secretion and apoptosis share a proximal signaling pathway which is

dependent on the activity of a TPCK-sensitive serine protease. More downstream the pathways of IL-8 induction and apoptosis split. As a result, SB203580 and TGF-$\beta_1$ inhibit IL-8 release only (➔ fig. 1). I suggest that the pathways leading to chemokine upregulation and to apoptotic death evolve partially common and/or parallel and that both represent cellular reponses to stress. This concept is supported by recently published data which demonstrate that caspase inhibitors completely block apoptotic death induced by Fas-associated pro-apoptotic stimuli in human smooth muscle cells, but only modestly decreased MCP-1 transcription. The authors conclude that the terminal events of the apoptotic signaling cascade initiated by Fas/FADD are not necessary for chemokine release [41].

The experiments with the promonocytic cell line U-937 were complemented by studies using human PBMC and whole blood cultures. It has recently been demonstrated that incubation with CdA induces a strong apoptotic response in resting PBMC [37]. In addition to being able to confirm this response when I exposed adherent PBMC to CdA, I observed an accompanying amplification of baseline IL-8 production. Interestingly, I also detected TNF-$\alpha$ protein release in these experiments.

CdA significantly augmented release of IL-8 not only from PBMC, but also from human whole blood cultures. Although the molecular mechanisms underlying CdA-induced IL-8 induction cannot be addressed thoroughly in this system, it is interesting to note that CdA is able to induce IL-8 in the physiological microenvironment of the blood compartment. This fact acquires further significance when one recalls that CdA is a chemotherapeutic drug which is administered intravenously for the treatment of different kinds of haematologic neoplastic diseases, such as hairy cell leukaemia or chronic lymphatic leukaemia, and a number of other malignancies.

The next logical step in our series of experiments was to try an endogenous inducer of apoptosis to further generalize our statements. Our choice fell on the Fas – FasL system, for it does not only constitute an important mediator of programmed cell death, but also has special relevance for our experimental settings as it plays an essential role in immunological self-tolerance and homeostasis of the lymphocyte population. To elaborate whether FasL was expressed by U-937 cells and/or PBMC exposed to the cytotoxic agents VP-16 or CdA, respectively, and was then active as an inducer of apoptosis in an auto- and

paracrine manner, I established an RT-PCR to detect FasL mRNA. Furthermore, I stimulated U-937 cells with an activating monoclonal anti-Fas antibody and assayed the supernatants for chemokines. Unfortunately, these studies could not be completed as planned, as I detected a strong LPS contamination in the anti-Fas antibody.

When apoptosis was defined, it was claimed that this form of cell death was antiinflammatory *per se* or at least occured strictly without inducing inflammation, since it was theorized to be a physiologic way of cells to die. This naturally implies a process that causes the smallest possible amount of disturbance to the surrounding cells, tissues, and organ systems. The present study is in disharmony with this concept: In addition to the fact that chemokines alone are capable of inducing local inflammatory responses, I observed release of TNF-α, a prototype of the pro-inflammatory cytokines, from adherent PBMC after incubation with quantities of CdA that also induced apoptosis. Moreover, protein levels of TNF-α in the supernatants of these experiments correlated well with the respective IL-8 contents. These results imply that TNF-α might play a role as mediator during the induction of IL-8 expression through CdA.

In recent times the number of studies which support opposition to the anti-inflammatory concept of apoptosis has steadily been growing. For instance, FasL, a major endogenous activator of apoptotic cell death, has recently been implicated as a mediator of induction of inflammatory genes [42]. Corresponding to the present data, FasL is indeed able to trigger release of IL-8 in rheumatoid arthritis synoviocytes [43] as well as in bronchiolar epithelial cells [44]. It is also capable to induce inflammatory angiogenesis with leucocyte recruitment *in vivo* [45]. These reports provide evidence for a possible role of FasL as a link between apoptosis and chemokine release.

Another prominent pro-inflammatory cytokine that has recently been associated with the execution of the apoptotic program is IL-1. It has been found that induction of apoptosis goes along with release of IL-1 in murine peritoneal exsudate cells [46]. Likewise, another study shows a close connection of the kinetics of apoptosis, phagocytosis, and the synthesis of IL-1β and IL-8. The authors found cytokine upregulation in primary macrophages and THP-1 cells cocultured with murine apoptotic CTLL-2 cells [47]. Furthermore, gene expression of the murine IL-8 homologue MIP-2 as well as of both IL-1α and IL-1β in murine peritoneal exudate cells was inducible by contact with apoptotic T-cells [45]. The coculture supernatants from these experiments were able to initiate accumulation of

neutrophils *in vivo*, thus proving that chemokines released in association with apoptotic cell death are biologically active.

The following three studies provide further *in vivo* data, underscoring the physiological relevance of the pro-inflammatory aspects of apoptosis. Uchimura et al induced apoptosis of lymphoid organs in mice by whole-body X-ray irradiation. Apoptotic cell death was accompanied by transient upregulation of MIP-2 mRNA, as well as accumulation of neutrophils. Both processes peaked at 9 h and were significantly reduced when the experiment was performed in $p53^{-/-}$ mutants which are resistant to radiation-induced apoptosis [48]. Another study reports similar effects in murine ischemia/reperfusion-injured kidneys. In addition to upregulation of MIP-2 and neutrophil influx, the authors also found functional impairment of the kidney. Interestingly, these effects could be abrogated by i.p. injection of a pan-caspase inhibitor, hinting at an important role of these enzymes in cytokine induction accompanying apoptosis [49]. Schaub et al [41] performed their experiments in injured vessel walls of carotid arteries of live rats. Besides recruitment of macrophages into the wounds, this report reveals further details about the mechanisms of chemokine release paralleling apoptosis: The upregulation of chemokines induced by Fas-associated pro-apoptotic stimuli in human smooth muscle cells was significantly, but not completely, reduced by blockage of IL-1α activity. This emphasizes the role of IL-1α as a mediator, but also provides evidence for an additional, IL-1-independent pathway for Fas induction of chemokine expression.

The latter studies [41, 48, 49] obviously stand in contrast with reports showing deactivation of LPS-stimulated human macrophages after contact with apoptotic cells [50, 8].

When critically assessing these results, one should keep in mind that our study investigates the consequence of stress-induced apoptosis in the cytokine producer itself. This situation is definitely different from the experimental setting of the other studies presented above [45, 50, 8].

Recently published reports furthermore supply data regarding cellular stress inflicted by chemotherapeutic drugs. In agreement with our results, Lee et al [51] state that the chemotherapeutic agent taxol can induce IL-8 in human ovarian cancer cells. Taxol is a microtubule-stabilizing agent which is antiproliferative and pro-apoptotic. IL-8 induction correlated with apoptosis in this system. Similarly, platinum compounds were shown to upregulate the protein levels of IL-8 and IL-1 in human umbilical vein endothelial cells [52]. Moreover, augmentation of IL-8 release was detected in human $CD_4^+$ T-cells during

ongoing apoptotic cell death induced by crosslinking of $CD_2$ and/or $CD_{28}$ after antigen exposure and propagation with IL-2 [53].

Based on the published data and the results of the present study, I conclude that the presence of cellular stressors such as chemotherapeutic drugs initiates apoptosis in U-937 cells, adherent PBMC, and WBC. This event is associated with expression of chemokines. I suggest that the resulting chemokine gradients serve to "tag" cells which have been irreparably damaged by the respective insult and which are about to undergo apoptosis, as targets for invading phagocytes. These processes may represent an advantageous strategy which contributes to efficient and safe removal of apoptotic cells.

# V. Summary

Removal of apoptotic cells by macrophages or resident semi-professional phagocytes is a prominent principle with important implications for the pathophysiology of chronic inflammatory diseases, viral infections, or cancer. To characterize mechanisms which may determine the fate of apoptotic cells, I investigated chemokine expression in apoptotic promonocytic U-937 cells or PBMC. Exposure of U-937 cells to the anti-cancer drug etoposide (VP-16), an inducer of apoptosis in these cells, was associated with increased expression of the chemokines IL-8 and macrophage inflammatory protein 1α (MIP-1α). Upregulation of IL-8 mRNA expression by VP-16 was observed as early as 4 h after onset of treatment and was still detectable after 19h of exposure. A serine protease inhibitor prevented both VP-16-induced apoptosis and release of IL-8, whereas inhibition of p38 MAP-kinases reduced IL-8 secretion only. Moreover, I observed that incubation with 2-chlorodeoxyadenosine (CdA) upregulated release of IL-8 from adherent PBMC in parallel to induction of apoptosis. In these cells a modest but significant induction of TNF-α release by CdA was also detected. In addition, CdA augmented release of IL-8 from whole blood cultures. By facilitating adequate recruitment of phagocytes to sites of cell death, stress-induced upregulation of chemokines associated with apoptosis may contribute to mechanisms aiming at efficient removal of apoptotic cells.

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# VII. Zusammenfassung

Das Abräumen apoptotischer Zellen durch Makrophagen oder ortsgebundene Gewebsphagozyten ist ein wichtiger Prozeß, dessen Ablauf die Pathophysiologie von verschiedenen Krankheitsbildern entscheidend beeinflussen kann. Dazu zählen unter anderem chronisch entzündliche Erkrankungen, Virusinfektionen und Neoplasien. Zur Charakterisierung dieser Vorgänge untersuchte ich die Chemokinfreisetzung in apoptotischen promonozytischen U-937 Zellen und mononukleären Zellen aus peripherem Venenblut (PBMC). In der Zellinie konnte ich zeigen, daß die Expression der Chemokine Interleukin 8 (IL-8) und Macrophage Inflammatory Protein 1α (MIP-1α) durch das in diesen Zellen Apoptose induzierende antineoplastische Medikament Etoposid (VP-16) hochreguliert wird. Diese Steigerung der mRNA-Expression war bereits 4 Stunden nach Beginn des Experiments nachweisbar und blieb bis zum 19 Stunden-Zeitpunkt erhalten. Durch Zugabe eines Serin Protease Inhibitors waren sowohl die VP-16-induzierte Apoptose als auch die IL-8 Freisetzung hemmbar, während ein p38 MAP-Kinase Inhibitor nur die IL-8 Sekretion beeinträchtigte.

Desweiteren zeigten meine Studien, daß in adhärenten PBMC, in denen ich mit 2-Chlorodeoxyadenosin (CdA) Apoptose induzierte, die IL-8 mRNA Expression und Proteinproduktion mit dem Zelltod einhergingen. Unter diesen Bedingungen konnte ich außerdem eine signifikante Hochregulation der TNF-α Produktion feststellen. Auch in der Vollblutkultur induzierte CdA IL-8.

Die durch Zellstreß hervorgerufene Hochregulation von Chemokin-mRNA und Protein ist demnach eng mit dem parallel ablaufenden programmierten Zelltod assoziiert. Dies impliziert, daß die situationsgerechte Rekrutierung von Phagozyten an Orte, an denen Apoptose stattfindet, einen wichtigen Mechanismus zur Beseitigung sterbender Zellen darstellt.

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Mit großer Freude
Diese Arbeit zu gutem Ende gebracht zu haben
Vielen Dank Euch allen,

Frankfurt am Main, am 1. August 2002