EICOSANOID AND CYTOKINE PRODUCTION BY HUMAN PERITONEAL MACROPHAGES

Characterization and Modulation

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EICOSANOIDEN EN CYTOKINEN PRODUCTIE VAN HUMANE PERITONEALE MACROFAGEN

Karakterisering en Modulatie

PROEFSCHRIFT

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"Bring me a fig from the banyan tree."
"Here is one, father."
"Open it."
"Here it is cut open."
"What do you see ?"
"Very tiny seeds, father."
"Cut open one of them."
"Here, it is cut open."
"What do you see ?"
"Nothing father."
"My dear boy ! This nothing which you do not perceive.
That huge tree has sprung up from it.
My dear boy, you must believe !
This very nothing is self to all that exists.
That is reality. That is self.
And that you are, my son !"

Chandogya Upanishad

Voor Govinda, Papa, Mama en Mark

Contents

Part I

General introduction

Chapter 1 Introduction

1.1 Macrophage functions

The human body has an extensive and specialized cellular network to defend against invading microorganisms, and to prevent the development and spread of neoplastic disease. The mononuclear phagocytic cells are important secretory cells that, through their receptors and secretory products, have a central and essential role in the initiation and maintenance of many immunologic and inflammatory processes. The secretory products are listed in Table 1.1 [1]. The mononuclear phagocyte system [monocytes and macrophages (Mϕ)] is complex, representing a heterogeneous group of cells found in organs and tissues throughout the body. All these mononuclear phagocytic cells are derived from a stem cell in the bone marrow [1,2].

Table 1.1 Secretory products of macrophages [1].

- Bioactive lipids
- Cytokines
- Complement components
- Coagulation factors
- Enzymes
  - neutral proteases
  - lipases
  - glucosaminidase
  - lysosomal acid hydrolases
  - deaminase
- Inhibitors of enzymes and cytokines
- Proteins of extracelluar matrix or cell adhesion
- Bioactive oligopeptides
- Sterol hormones
- Purine and pyrimidine products
- Reactive oxygen intermediates
- Reactive nitrogen intermediates

Inflammation is a multifunctional process, with both local and systemic features. The primary inflammatory response, the acute phase, is the migration of neutrophils, Mϕ and other leucocytes to the site of inflammation. Besides neutrophils, Mϕ also mediate the influx of leucocytes to the inflammatory site by generating chemotactic factors and providing a non-specific defence by phago- and pinocytosis combined with oxygen radical production and the release of lysosomal enzymes. In steady-state, Mϕ are also the key effector cells in the elimination of intracellular microorganisms, other pathogens, senescent cells and other waste products.
During the inflammatory reaction, besides the non-specific response, Mϕ are also involved in initiating and maintaining the specific response. They regulate the cellular immune response by the recognition, processing and presentation of the antigen to lymphocytes. By modulating the lymphocyte functions with their secretory mediators, they are positively involved in the humoral immune response. Further, Mϕ mediate tumor cytotoxity through direct cell-cell contact, inhibit the tumor cell division and

lyse antibody-coated tumor cells with their secretory products. During the inflammatory response, as well as in steady-state, Mϕ are involved in the regulation of the growth of committed haematopoietic progenitor cells by producing numerous homeopathic growth factors and providing a mechanical framework together with other stroma cells. Besides local effects, the inflammatory mediators also have systemical effects as they have access to the circulation and exert action at distant organs.
The most important mediators produced by Mϕ to regulate the specific response are eicosanoids and cytokines. Eicosanoids are biological active lipids and cytokines are polypeptides with a regulatory function [4-6].

1.2 Aim of the work

The aim of this series of investigations was to obtain more insight in the actions of inflammatory mediators synthesized by human macrophages (Mϕ). Information presently available on Mϕ and their inflammatory mediators have been obtained mainly from animals. The exact inflammatory mediator production by human Mϕ and their mediator interactions still have to be characterized.

1.2.1 Construction of the thesis

Chapter 2 provides background information on Mϕ and their inflammatory mediators for a better understanding of the series of experiments performed.
In these experiments the production and interactions of inflammatory mediators by human peritoneal Mϕ are characterized. Chapter 3 presents the investigation in which eicosanoid and cytokine production was studied *in vitro* using cells obtained from the rat peritoneal cavity during inflammatory response. All investigations following this experiment were performed using human Mϕ, which Mϕ were obtained from a sterile chronic inflammation site: the peritoneal cavity of patients with accumulation of ascitic fluid. In Chapter 4, the influence of cellular maturation and activation on the eicosanoid production of density-defined human peritoneal Mϕ is described.
Subsequently the profile, levels and interactions of eicosanoids and cytokines from human Mϕ are characterized and presented in Chapter 5, 6 and 7.
As Mϕ are predominantly present in ascites, the levels of eicosanoids, cytokines and ICAM-1, which plays a crucial role in the adhesion and migration of inflammatory cells, were measured in the ascitic fluid to correlate disease related patterns with these mediators for either diagnostic or prognostic purposes. These two studies are described in Chapter 8 and 9.

Mϕ have a central role in the initiation and maintenance of inflammatory diseases and, as shown in Chapters 5 and 7, the inflammatory mediator production of human Mϕ differ, in part, from Mϕ of other species. With the insight obtained on the production of inflammatory mediators by human peritoneal Mϕ and the interactions of these mediators with each other, human peritoneal Mϕ could be used as an *in vitro* model

to characterize the interactions of anti-inflammatory drugs with human Mϕ.
Non-selective drugs are very effective in inflammatory diseases; however, they may have a considerable risk of side effects and potential complications with prolonged systemic use. Selective inhibition and neutralization by therapeutics are currently being developed to overcome these risks. Their effects *in vivo* could be more predictable when also tested *in vitro* using human Mϕ. For this reason the role of Mϕ and their mediators in several inflammatory diseases are described and the differential effects of non-selective and selective acting therapeutics using an *in vitro* model of human peritoneal Mϕ, are discussed in Chapter 10.

1.3 References

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Chapter 2 The mononuclear phagocyte system and its inflammatory mediators

2.1 Mononuclear phagocyte heterogeneity

The mononuclear phagocytes are a heterogeneous type of cells with both phenotypical and functional properties. A distinction can be made between *inter-* and *intra*population heterogeneity. *Inter*population heterogeneity refers to differences between macrophage (Mϕ) populations located at different anatomical sites as summarized in Table 2.1 (Chapter 1), whereas *intra*population heterogeneity evidently refers to differences within the mononuclear phagocyte population from one particular morphological location [1-3].

Table 2.1 Cells of the mononuclear phagocytic system [1].

Macrophages	Location
- Committed stem cells	- Bone marrow
- Monoblasts	- Bone marrow
- Promonocytes	- Bone marrow
- Monocytes	- Bone marrow and blood
- Macrophages	
Kupffer cells	- Liver
Alveolar macrophages	- Lung
Free and fixed macrophages	- Spleen, lymph nodes, bone marrow, thymus, mucosa-associated lymphoid tissues, gastrointestinal tract, genitourinary tact, endocrine organs, colostrum
Histiocytes	- Connective tissue
Pleural and peritoneal macrophages	- Serous cavities
Microgliacytes	- Central nervous system
Osteoclasts	- Bone tissue
Synovial type A	- Joints
- Epithelioid cells	- Inflamed tissues
- Multinucleated giant cells	- Inflamed tissues

The development of the various mononuclear phagocytes can be considered to consist of multiple processes which all are under tight regulatory control. The steps that contribute to phagocyte development are proliferation, maturation, and differentiation of progenitor cells as well as functional activation, mostly of mature cells. These processes can be defined as follows. *Proliferation* is the replication of cells giving rise to daughter cells that express characteristics similar to those of their parental cells. *Maturation* is the process of cells acquiring features typical of cells in later stages of development and losing aspects of earlier stages. *Differentiation* is a specific type of maturation by which diversity is generated. Both maturation and differentiation are, in principal, irreversible. In contrast, *activation* is a reversible process by which cells are driven to express particular functions or by which the performance of existing

functions is enhanced [1-3]. Figure 2.1 presents a scheme of distinctive processes involved in mononuclear phagocyte development [1].

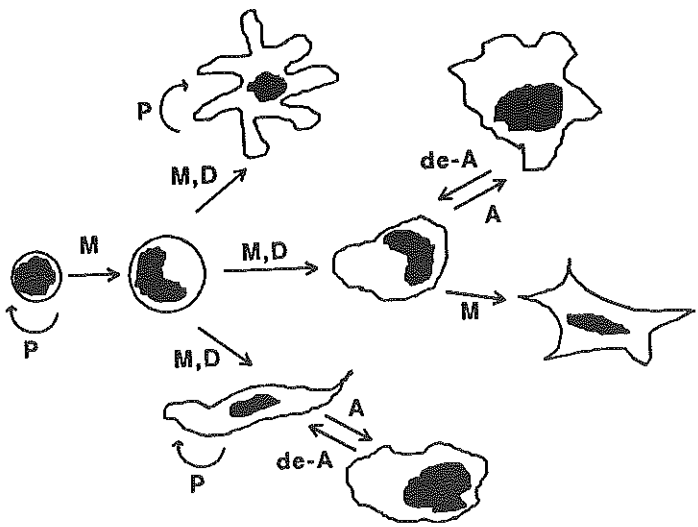

Figure 2.1 A scheme of distinctive processes involved in mononuclear phagocyte development . P = proliferation; M = maturation; D = differentiation, A = activation; de-A = deactivation [1].

Table 2.2 Schematic representation of peroxidase activity of human mononuclear phagocytes in culture, in co-expression with the differentiation antigen RFD7 [5].

Localisation of peroxidase activity	Monoblast	Pro-monocyte	Monocyte	Macrophage
Endoplasmatic reticulum	+	+	-	±
Nuclear envelope	+	+	-	±
Golgi apparatus	+	+	-	-
Lysosomal granules	+	+	+	-
RFD7	-	-	-	+

Variable phenotypic characteristics include size, morphology, intracellular enzymes and secreted substances (Table 1.1) as well as surface receptors. Many of these features can be used as markers to distinguish between development stages. Peroxidase activity patterns can, for example, be used to distinguish the developmental stages of mononuclear phagocytes. With electron microscopy the localisation of peroxidase activity can be detected. This peroxidase activity pattern has been important in

classifying the activation and developmental stages of mononuclear phagocytes. Besides the animal system, these studies have also been performed in human mononuclear cells [4,5]. The monoclonal antibodies against mononuclear phagocytes (mice and human) have facilitated the identification and functional investigation of the various cell surface molecules present, and have shown that the mononuclear phagocyte system is far more complex and heterogeneous than was previously thought [1-3].
Table 2.2 shows that during differentiation (6-day period *in vitro*) the peroxidase activity of human monocytes disappears, parallel with the expression of the differentiation antigen RFD7 on the cell surface [5].

2.2 Development of monocytes

The monocyte in the peripheral blood is derived from a stem cell in the bone marrow [6,7] by passing through different stages. The pluripotent haemopoietic stem cells (PHSC) self-generate and generate different progenitor cells [8]. One of them, the granulocyte/macrophage colony forming cell (GM-CFC) will form the macrophage and granulocyte lineage. In the macrophage lineage, GM-CFC will give rise to the restricted macrophage colony-forming cells (M-CFC) [9]. The M-CFC will generate the monoblast, which divides into two promonocytes [10,11]. The promonocyte will divide once, leading to two bone marrow monocytes [12]. These bone marrow monocytes, with a generation time of about 24 hours for mice will not divide further, and leave the bone marrow compartment and enter the blood stream [6].
The blood monocytes will distribute over a circulating and marginating pool [13] and stay there with a half-disappearance time of 17.4 hours in mice [14]; subsequently they migrate through the vascular wall into the tissues and do not return into the peripheral blood stream [6].
During an acute inflammatory reaction the number of monocytes increases at the site of the inflammation [6,14]. This is due to the decreased cell cycle time of the promonocytes, leading to an enhanced influx of monocytes into the peripheral blood compartment and a decreased half-disappearance time (10 hours in mice), leading to an increased efflux of monocytes out of the peripheral blood compartment [14].
Studies on the origin of mononuclear phagocytes and kinetics were mainly performed in mice. Kinetical studies and phenotypical studies on mononuclear phagocytes of other animal species gave similar results [rat (7, 15); rabbit (16); guinea pig (17,18)]. With bone marrow transplantation studies it was demonstrated that human Mϕ also originate from the bone marrow [19-21]. A few kinetical studies have been done on human mononuclear phagocytes. One study demonstrated that in healthy human the half-disappearance time of monocytes out of the peripheral blood compartment was 71 hours [22], whereas another study demonstrated that this half-disappearance time for healthy human was 8.4 hours. In patients with acute or chronic inflammation this monocyte turnover did not change [23].

2.3 Trans-endothelial migration of monocytes

Transendothelial migration is a multistep process in which three families of adhesion receptors are involved. The initial attachment of leucocytes to a vessel wall ("rolling") is mediated by selectins, present on endothelium and leucocytes. The rolling process is arrested by the activation of integrin and immunoglobin superfamily proteins and leads to firm attachment of the leucocytes, followed by shape changes and migration through the endothelium. Figure 2.2 shows the steps involved in the binding and trans-endothelial migration of leucocytes [24-26].

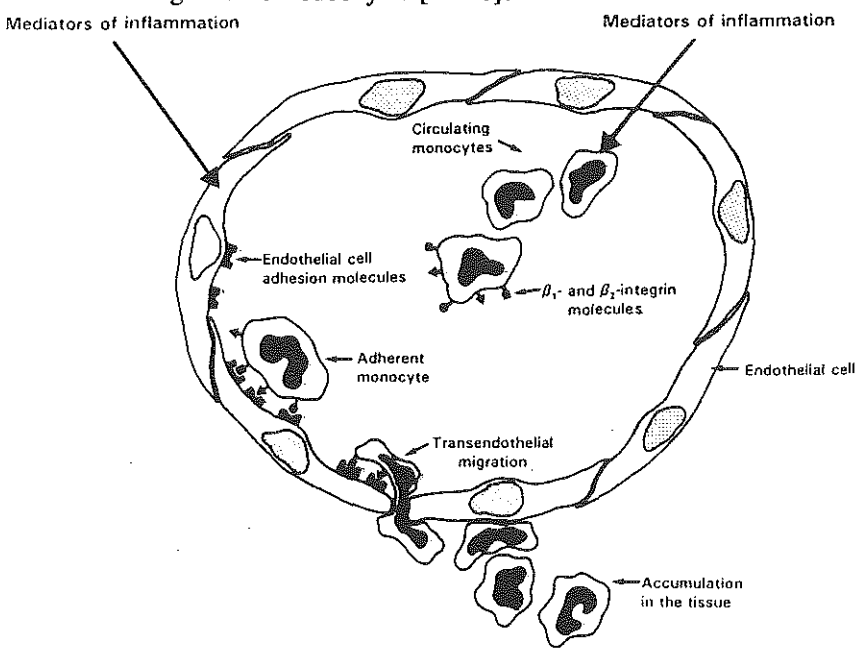

Figure 2.2 The subsequent phases of the migration of monocytes through vascular endothelium [27].

The selectins consist primarily of L-selectin, E-selectin and P-selectin. The immunoglobulin superfamily include the intercellular adhesion molecule 1 (ICAM-1) and the vascular cell adhesion molecule 1 (VCAM-1). The integrins include the specifically lymphocyte-function-associated molecule 1 (LFA-1) and the very-late-antigen 4 (VLA-4). The functions and specificities are shown in Table 2.3 [24-26]. ICAM-1 is one of the adhesion molecules which has a crucial role in transendothelial migration in inflammation. ICAM-1 is a single-chain membrane-bound glycoprotein that is encoded by genes of the super immunoglobulin family and consists of five extracellulair domains, a transmembrane portion and a cytoplasmatic tail. ICAM-1 is a ligand for LFA-1, which is only expressed on leucocytes [26,28]. ICAM-1 is expressed on endothelial cells, but also on a wide variety of other cells, including fibroblasts, epithelial cells, mucosal cells, human melanoma cell lines, lymphocytes

and monocytes [26,29,30]. Expression of ICAM-1 on different types of cells and the binding capacity of monocytes to macrovascular endothelial cells *in vitro*, is upregulated by cytokines such as interleukin 1 (IL-1), tumor necrosis factor α (TNF-α), interferon γ (IFN-γ) [29,31,32] and also by the chemoattractants like leukotriene B_4 (LTB_4) and complement factor 5a (C5a) [33]. These chemoattractants increase the speed of migration and control the orientation and movement of the monocytes in a specific direction.

Table 2.3 Adhesion molecules involved in leucocyte binding to endothelium [24-26].

Adhesion Molecule	Tissue distribution	Implicated in:
Selectins		
L-selectin	- All leucocytes, varying density	- Leucocyte rolling
P-selectin	- Megakaryocytes, platelets, endothelium	- Leucocyte rolling - Lymph node homing
E-selectin	- Cytokine-activated endothelium	
Integrins		
LFA-1	- Leucocytes	- Firm adhesion
VLA-4	- Leucocytes	- Homing to inflamed tissue
Immunoglobulin Superfamily		
ICAM-1	- Widespread constituve, cytokine inducible	- Transendothelial migration
VCAM-1	- Endothelium, epithelium, macrophage, dendritic cells, cytokine inducible	- Transendothelial migration

2.4 Development of macrophages

In normal, non-inflammatory situations, blood monocytes will differentiate through exudate Mϕ and exudate-resident Mϕ into resident tissue Mϕ [4,34] forming the various pools of local tissue Mϕ. In the steady state resident Mϕ will occur both freely as well as fixed in the tissue of most organs, each organ containing its own characteristic resident Mϕ populations [35,36].
Under steady-state conditions the monocytes replace the resident Mϕ. The lifespan of tissue Mϕ is not known exactly, but studies of bone marrow chimeras and repopulation kinetics following Mϕ depletion indicate a turnover time in the order of weeks in mice [37] and months in man [19,21]. Some studies suggest that the steady-state Mϕ populations are only maintained by local proliferation [38-40]. Others suggest that besides the replacement of resident Mϕ by monocytes, there is only a small number ($< 5\%$) of non-resident Mϕ which have not completed their cell division and they divide further locally [37,41].

During immunological stress conditions (acute and chronic) relatively larger numbers of monocytes enter the tissues and accumulate as responsive Mϕ. The local production of Mϕ does not play an important role in this increase [42-44].

2.5 Macrophage activation

The monocytes which migrate during immunological stress to the inflammatory area have a large and diverse potential for further development. It is not clear why these monocytes are more responsive to induction than resident tissue Mϕ, but probably this is because they interact with a larger variety of inflammatory mediators which are not present, or present in a lower concentration under conditions when there is no inflammation. The development state of the monocytes and the adherence at the inflammation site could also be of importance. These inflammatory monocytes are the young responsive Mϕ which probably have the potential for being activated in numerous different ways by inductive and suppressive signals, as put forward by the macrophage activation model presented in Figure 2.3 [45]. Each state represents the enhancement of one or more functions and the suppression of others [45,46].

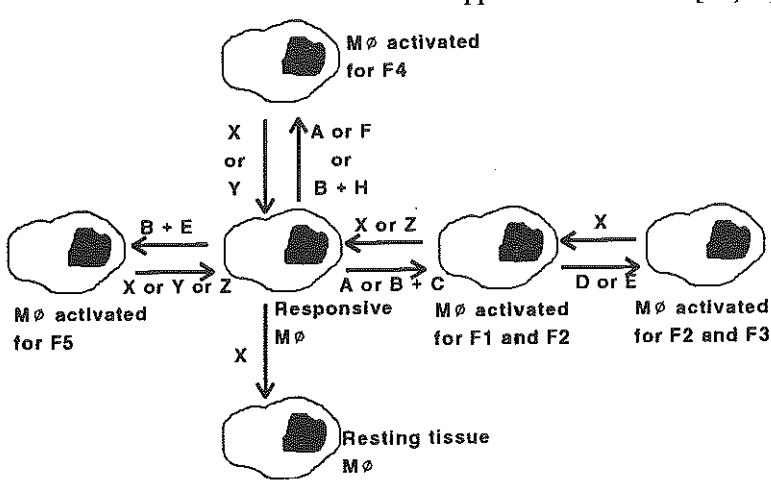

Figure 2.3 Model of macrophage activation. Responsive macrophages can develop in a large number of ways for complex functions (F1-F5), by receiving inductive signals (A-H) and/or suppressive signals (X-Z) [45].

This model of macrophage activation for multiple functions is based on the earlier developed model of Mϕ activation for tumor cytolysis, shown in Figure 2.4: Responsive Mϕ get "primed" by IFN-γ and "fully activated" by lipopolysaccharide (LPS). In each state certain markers appear or disappear and certain functions are turned off or on [47].

	Responsive Mϕ	Primed Mϕ	Activated Mϕ
Capacities	Ia - TFR ++++ LFA-1 - Secretion CP and TNF-α - Secretion ROI - Bind tumor cells -	Ia ++++ TFR - LFA-1 ++++ Secretion of CP and TNF-α - Secretion of ROI +++ Bind tumor cells ++++	Ia ++ TFR - LFA-1 ++++ Secretion of CP and TNF-α ++++ Secretion of ROI +++ Bind tumor cells ++++
Functions	Elevated chemotaxis and phagocytosis Proliferate	Elevated chemotaxis and phagocytis Do not proliferate Microbicidal Present antigen	Elevated Chemotaxis and phagocytosis Do not proliferate Microbicidal Kill tumor cells

Figure 2.4 Early developed macrophage activating model for tumor cytolysis. Interferon-γ (IFN-γ) primes the macrophage and lipopolysaccharide (LPS) converts it to the fully activated form. The capacities and functions of the macrophage varies with the stage of activation [47].

2.6 Modulators of macrophage activation

The extracellular signals which can regulate Mϕ activation are a wide range of cytokines (e.g. interferons, interleukins, colony stimulating factors and growth factors), eicosanoids and mediators which are not produced by cells such as LPS. Eicosanoids are biological active lipids, mainly produced by cells participating in the inflammatory responses. They interact with specific membrane receptors. Cytokines are soluble polypeptides which modulate cellular functions through interaction with specific membrane receptors. By initiating signal transduction, the eicosanoid- and cytokine-receptor complexes will alter the cell function. In section 2.8 the characteristics of the eicosanoids and cytokines will be described.

Lipopolysaccharide (LPS): LPS (or endotoxin) provides a potent and pleiotropic stimulus for mononuclear cells. It stimulates the release of pro-inflammatory cytokines like IL-1, IL-6, TNF-α, and also the release of IL-1 receptor antagonist (IL-1ra) and eicosanoids. It increases the adherence to endothelium and increases the respiratory burst activity [48,49].
LPS, a complex glycolipid, is the major component of the outermost membrane of Gram-negative bacteria. Structurally, LPS consists of a variable polysaccharide domain covalently bound to a diglucosamine based acylated phospholipid, lipid A. Lipid A domain is highly conserved and is responsible for most of the biological effects of LPS [50].
LPS binding protein (LBP) is a 60-kD acute phase protein present in sera [51] and synthesized in the liver [52]. It acts as an opsonin [53,54] as it binds to the lipid A moiety of LPS [55], the LPS-LBP complex then binds to the receptor CD14, a 55-kD glycoprotein, on mononuclear cells [56]. This binding leads to enhanced activation of

the mononuclear cells to produce mediators. Not known is whether the binding of the complex to CD14 alone is sufficient enough to induce production of mediators, or that CD14 does not issue signals but instead enables LPS to activate a second receptor. LPS may activate also certain receptors directly, without help from LBP or CD14 [57].

Interferon-γ (IFN-γ): IFN-γ is a cytokine [(20-25 kDa) 58] that is known as a factor that elicits the inflammatory responses of LPS on Mϕ [59]; however, this depends on the underlying activating state of the Mϕ [60]. IFN-γ is a product of T-lymphocytes and natural killer cells, that alters the function of Mϕ by interacting with a specific surface receptor on Mϕ [61].

Calcium: Calcium ionophore A23187 activates Mϕ by inducing a rapid calcium influx. [62].

2.7 Signal transduction mechanism

Signal transduction: Binding of agonists, the extracellular signal, to specific receptors on the outer surface of the lipid bilayer cell membrane usually causes activation of G-proteins on the inner surface of the cell membrane. G-proteins are a class of proteins that couple temporarily activation of receptors to either activation or inhibition of other effectors, e.g. activation of adenylyl cyclase, phospholipase C (PLC), cyclic 3',5'guanosine monophosphate (cGMP), phosphodiesterase and calcium and potassium channels, leading to the generation of second messengers, respectively cyclic 3',5' adenosine monophosphate (cAMP), 1,2 diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), cGMP and mobilization of Ca^{2+} [63].

These second messengers are able to activate a subgroup of protein kinases: the serine/threonine kinases. The other subgroup, the tyrosine kinases, are activated directly by their receptors. The protein kinases are enzymes which are part of the mechanism which regulate the cellular functions by phosphorylation. Phosphorylation alters the biological properties of the phosphorylated enzymes [64].
Protein phosphatases, dephosphorylating enzymes, are also involved in cellular regulation; however, the knowledge about their role *in vivo* is still premature [64,65]. Table 2.4 presents a summary of the protein kinases and protein phosphatases.

Phosphoinositide (PI) metabolism: The PI metabolism is an important source of second messengers. PLC phosphorylates phosphatidylinositol 4,5-biphosphate (PIP_2) into IP_3 and DAG. Both products are second messengers.
IP_3 will stimulate an intracellular Ca^{2+} influx from the endoplasmatic reticulum [66,67] and also elicits extracellular Ca^{2+} by activating Ca^{2+} release-activated channels and second-messenger-operated channels. There are also Ca^{2+} channels which are activated by other second messengers (e.g. Ca^{2+} and inositol 1,3,4,5-tetra phosphate (IP_4)), G-proteins or directly by a receptor [68].

The other second messenger released by the PI metabolism, DAG, in combination with Ca^{2+} will activate protein kinase C (PKC). PKC is a serine/threonine kinase that was first characterized by Nishizuki as a Ca^{2+} and DAG dependent enzyme [66,67]. PKC is now known to be a large family of proteins with at least 12 isotypes. Besides the PKCs which are Ca^{2+} and DAG dependent, there is a group of PKCs which does not depend on Ca^{2+}, and another PKC group which depends neither on DAG nor Ca^{2+}. Most probably, activation of individual PKC isotypes will lead to the phosphorylation of unique substrates [69].

Table 2.4 Protein kinases and protein phosphatases [64].

Enzymes	Regulates
Serine/Threonine Kinases	
Cyclic AMP-dependent protein kinase (type I and II)	cAMP
Cyclic GMP-dependent protein kinase	cGMP
Phosphorylase kinase	Ca^{2+}/calmodulin
Myosin light-chain kinase	Ca^{2+}/calmodulin
Ca^{2+}/calmodulin kinase I, III	Ca^{2+}/calmodulin
Ca^{2+}/calmodulin kinase II	Ca^{2+}/calmodulin
Protein kinase C	Ca^{2+}/phospholipid
Casein kinase II	Polyamine
Casein kinase I	Unknown
Glycogen synthase kinase 3	Unknown
Tyrosine Kinase	
Insulin receptor kinase	Insulin
PDGF receptor kinase	PDGF
EGF receptor kinase	EGF
Oncogen production	Unknown
Serine/Threonine Phosphatases	
Protein phosphatase-1	Inhibitor 1,2
ATP/Mg-dependent phosphatase	Glycogen synthase kinase 3; Inhibitor 1,2
Protein phosphatase-2A	Polyamines/G-substrate
Protein phosphatase-2B	Ca^{2+}/calmodulin
Protein phosphatase-2C	Mg^{2+}
Tyrosine Phosphatase	?

Phosphatidyl choline (PC) metabolism: PI is not the only source for DAG. In recent years it has become clear that PC is an important source for DAG at a relatively later phase. PC can be hydrolized by PLC directly into DAG or PC is hydrolized by phospholipase D (PLD) into phosphatic acid [(PA), 70], which is then converted by phosphatic acid phosphomonoesterase into DAG. This DAG then gives then probably sustained activation of PKC, which is essential for subsequent responses such as cell proliferation and differentiation [71]. PA and its metabolic product (lysoPA) may also be activators of PKC, PLC and PLD [72]. Phospholipase A_2 (PLA_2) hydrolizes PC into

free fatty acids and lysoPC. Cis unsaturated fatty acids and lysoPC enhance the DAG-dependent activation of PKC [71]. Figure 2.5 presents a schematic diagram of events following receptor activation by an external stimulus.

The Mϕ modulators: The transductional mechanism induced by IFN-γ and LPS have been intensively studied. IFN-γ triggers the signalling cascade by activating tyrosine kinases. This will induce a rise in intracellular levels of Ca^{2+}, raising the potential function of PKC, without initiating the phosphorylation [73] and they will also induce a set of immediate early genes [74].
LPS will initiate the rapid hydrolysis of PIP_2 by PLC, activating PKC [73]. Extracelluar calcium influx, induced by calcium ionophore, can directly activate calcium dependent enzymes [62].

Signal pathway after phosphorylation: The ionic changes (e.g. $[Ca^+]$ increase or Na^+/H^+ exchange) generated by the second messengers and the activated protein kinases and phosphatases will covalent modify regulatory proteins and enhance binding of nuclear regulatory factors, leading to the regulation at transcriptional and translation level of specific genes. Protein products of immediate-early genes in combination with ionic changes and modified proteins will lead to execution of rapid functions. The rapid response will lead to the initiation of chemotaxis, secretion of metabolites of arachidonic acid, neutral proteases, lysosomal hydrolases and radical oxygen intermediates (ROI). The protein products of the later appearing genes, also in combination with ionic changes and modificated proteins, can lead to enhanced potential for execution of complex functions. The long-term responses will include development changes [45].

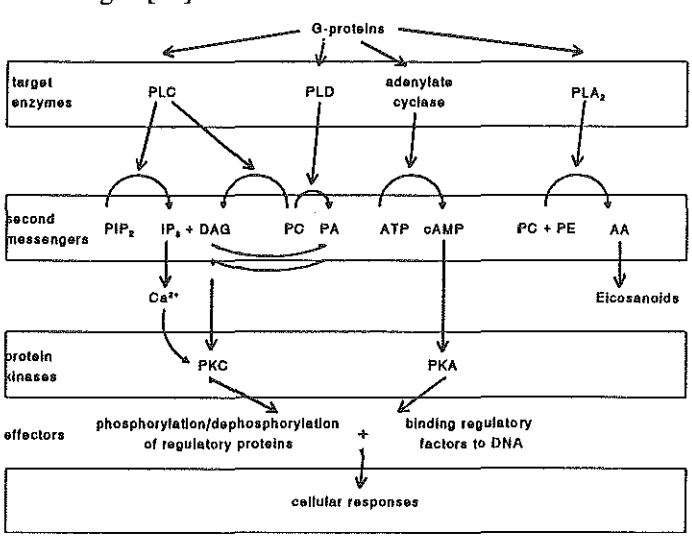

Figure 2.5 A schematic diagram of events following receptor activation by an external stimulus. See text for details and abbrevations used (PKA = protein kinase A).

2.8 Mediator production by macrophages

Important secretory products of the Mϕ, which are key mediators of immunity and inflammation, are the eicosanoids and cytokines.

2.8.1 Eicosanoids

Production: The eicosanoids are biological active lipids. They are not stored in cellular "pools", but are synthesized directly from free 20-carbon essential fatty acid-precursors (dihomo-γ-liolenic acid, arachidonic acid (AA), 5,8,11,14,17-eicosapentaenic acid). These free fatty acid-precursors become available when the cell membrane is disturbed and certain enzymes are activated. In man, AA is the most abundant precursor, and is in large amounts esterified to phospholipids [PC and phosphatidylethanolamine (PE)] and, to a smaller degree, in triacylglycerols in the cell membrane. AA is mainly ingested directly from the diet, but it can also be derived from dietary linoleic acid by anabolic desaturation and chain elongation. By hydrolysing the phospholipids containing the esterified arachidonate by PLA_2, free nonesterified AA will become available. This AA can also become available from the phospholipids by PLC or PLD and diacylglycerol lipase, or from the PI by the phosphatidylinositol-specific PLC [75,76]. The free nonesterified AA can then, besides efficient reesterfication into phospholipids, be further oxidized by two main enzyme systems: the fatty acid cyclooxygenase forming the enderoperoxyden (prostanoids) or the lipoxygenases forming the hydroperoxy fatty acids (leukotrienes).

Figure 2.6 shows the eicosanoids formed from AA through the lipoxy- and cyclo-oxygenase pathways. The initial product via the lipoxygenase pathway by 5-lipoxygenase is the relatively unstable 5-hydroperoxy eicosatetraenoic acid (5-HPETE). 5-HPETE can be reduced to 5-hydroxy eicosatetraenoate (5-HETE) or converted by dehydrase to the labile epoxide leukotriene A_4 (LTA_4). LTA_4 can be metabolized further into two pathways. Hydrolysing of LTA_4 by LTA_4 hydrolase yields LTB_4 and conjugation of LTA_4 with glutathione generates LTC_4. Stepwise cleavage of glutamate and glycine from LTC_4 by γ-gluamyltransferase and dipeptidase yields LTD_4 and LTE_4, respectively. Via 12- and 15-lipoxygenase the relatively unstable 5- and 12-HPETE are reduced to 12- and 15-HETE [76-78].
The initial product via the cyclooxygenase pathway by prostaglandin endoperoxide synthase (PGH), is the prostaglandin G_2 (PGG_2). PGG_2 is further oxidized by the same enzyme to the precursor of the prostanoids, PGH_2. PGH_2 is transformed to PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 (TXA_2). PGD_2 and PGE_2 by specific isomerases or nonenzymatic degradation, $PGF_{2\alpha}$ by reductase and TXA_2 by thromboxane synthase. TXA_2 is an unstable mediator which hydrolyses spontaneously to TXB_2. PGI_2 (unstable) by prostacycline synthase produces from PGH_2. PGI_2 is hydrolysed quickly to 6-keto-$PGF_{1\alpha}$ [6k$PGF_{1\alpha}$, 75-77].

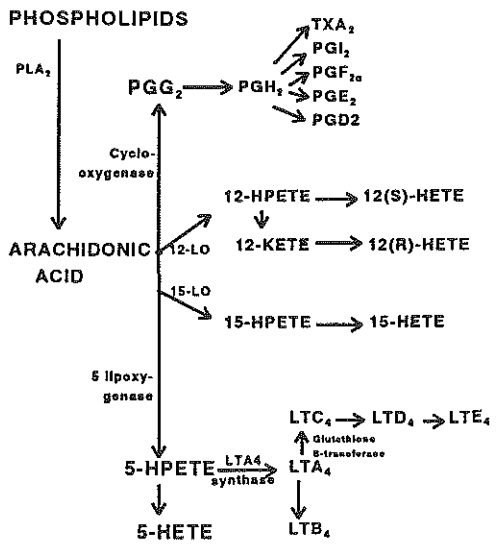

Figure 2.6 Eicosanoids formed from arachidonic acid by lipoxy- and cyclooxygenase enzymes [75,79].

Sources: The inflammatory cells participating in the inflammatory response are the main source of a variety of eicosanoids. Tissues (e.g. skin, brain, intestine, kidney, heart, lung, spleen and blood vasculature) are also able to synthesize eicosanoids in response to a variety of stimuli [76,79]. The eicosanoid production is cell specific, (as summarized in Table 2.5) for human cells, but also depending on species origin and the type of stimuli which induces the eicosanoid production [80-91].

Table 2.5 Eicosanoid production by human cells (*=Table 2.6, *[n]=reference [80-91].

Cell type	LTB_4	LTC_4	5-HETE	PGE_2	PGD_2	6k-$PGF_{1\alpha}$	TXB_2
Macrophages	*	*	*	*	*	*	*
Neutrophils	*85/83	*83		*81		*81	*82
Eosinophils	*84	*83/84					
Mast cells	*80	*80	*80		*80		
Basophils	*91	*91					
Endothelial cells	*89			*86		*86/87/89	
Platelets				*81			*88
T-lymphocytes	*90						

Eicosanoid production by Mϕ: In Table 2.6 [92-125] an overview is given of eicosanoid production by resident and activated Mϕ of different species (human, rat and mice), different origin (alveolar, peritoneal, blood monocytes) and stimulated *in*

vitro by different exogenous stimuli [A23187, LPS, zymosan or (phorbol 12-myristate 13-acetate (TPA)]. The eicosanoid production by Mϕ is species dependent.
Besides species, tissue and exogenous stimuli differences, the experiments summarized in Table 2.6 also differ in the manner Mϕ were activated *in vivo*, the method used for the isolation of the cells, the incubation times of the cells *in vitro*, the concentrations of the stimuli, and the way eicosanoids were measured in the supernatant after the incubations were performed [high performance liquid chromatography (HPLC) and radio-immuno-assays (RIA)]. The concentrations of the eicosanoid production were expressed in ng/10^6 cells, ng/mg cell protein, % of total production, dpm/mg cell protein or only dpm/cpm. To compare the results the productions are expressed in Table 2.6 as: (1) not detectable: n.d; (2) just detectable but very low production: (+); (3) low production: +; (4) high production: ++; and (5) very high production: +++. A detailed overview of Table 2.6, is given below:
The 5-lipoxygenase production of human Mϕ and monocytes: In human peritoneal and alveolar Mϕ (resident and activated) the LTB_4 and 5-HETE production was very high when the cells were stimulated with A23187. Human alveolar Mϕ produce LTC_4; however, compared with the LTB_4 production of the peritoneal Mϕ as well as the alveolar Mϕ itself, LTC_4 production is much lower or not detectable.
The human blood monocytes also had a very high LTB_4 production and a high LTC_4 production compared with human Mϕ, but 5-HETE was not detectable when the monocytes were stimulated with A23187.
The 5-lipoxygenase production of rat Mϕ: In contrast to the human peritoneal and alveolar Mϕ, the LTB_4 production of resident peritoneal rat Mϕ were very low when stimulated with A23187 or LPS. In *in vivo* activated and *in vitro* A23187 stimulated peritoneal rat Mϕ, the LTB_4 production was not detectable. 5-HETE production was also low in resident peritoneal rat Mϕ, but higher in activated Mϕ (A23187 stimulated). LTC_4 production in resident peritoneal rat Mϕ (A23187) was only high when the rat was endotoxin tolerant.
The LTB_4 and 5-HETE production of resident alveolar rat Mϕ was higher than the production of resident rat peritoneal Mϕ (A23187 stimulation). Only activated rat Kupffer cells (A23187) had a very high LTB_4 and 5-HETE production level.
The 5-lipoxygenase production of murine Mϕ: Similar to the rat peritoneal Mϕ, the LTB_4 production of resident peritoneal murine Mϕ was also low (A23187 stimulated).
The cyclooxygenase production of human Mϕ: In human resident peritoneal Mϕ from continuous ambulatory peritoneal dialysis (CAPD) patients the PGE_2 and PGI_2 production was very high when these cells were stimulated with LPS. PGI_2, but not PGE_2 production, decreased when these Mϕ were activated *in vivo*.
With A23187 the PGE_2 production was lower and the PGI_2 production of the activated peritoneal Mϕ from CAPD patients was higher, in comparison to the same cells when they were stimulated *in vitro* with LPS. No data were available on the PGE_2 production of resident peritoneal Mϕ from CAPD patients stimulated with A23187, the PGI_2 production was low.
Of resident ascitic Mϕ only data on eicosanoid production were available of cells

stimulated with A23187; PGE_2 was not detectable and the PGI_2 production was low. Similar results as the ascitic Mϕ were observed with human resident alveolar Mϕ when they were stimulated with A23187 or LPS.
On the contrary, the PGE_2 production in cultured human blood monocytes was high when stimulated with A23187.
The cyclooxygenase production of rat Mϕ: In resident rat peritoneal Mϕ, PGI_2 was high when stimulated with A23187 and very high when stimulated with LPS. The PGE_2 production of these cells was low when stimulated with A23187 or LPS. While in active rat peritoneal Mϕ the PGE_2 was low to high (depending on the experiment) and PGI_2 production low, when stimulated with A23187.
In resident rat alveolar Mϕ the prostaglandin production is similar to resident human alveolar Mϕ when stimulated with A23187.
In resident rat Kupffer cells the PGE_2 and PGI_2 production was very low, and the PGD_2 was very high when stimulated with A23187. In *in vivo* activated rat Kupffer cells PGD_2 was also low after A23187 stimulation. In all the other Mϕ of different species or origin PGD_2 production was low, not detectable or not measured.
The cyclooxygenase production of murine Mϕ: In resident peritoneal murine Mϕ the PGE_2 and PGI_2 production was high when stimulated with LPS, PGI_2 was lower when the cells were stimulated with A23187.

Constitutive and inducible cyclooxygenase (COX): Prostaglandin endoperoxide synthase (PGH), the enzyme which exhibits both cyclooxygenase and peroxidase activity, also known as COX [126], has two isoforms: COX-1 and COX-2. COX-1 is encoded by a different gene than COX-2 [127,128]. COX-1 is constitutively expressed (stomach, intestine, kidney, platelets), while COX-2 expression is inducible (10-80 times in comparison to expression under normal physiological conditions) in a number of cells (e.g. monocytes, Mϕ, fibroblasts, synovium) by pro-inflammatory stimuli (growth factors, cytokines, endotoxin). Under normal conditions there is no expression in the gastrointestinal tract [129,130].
COX-1 and COX-2 are both localized in the endoplasmatic reticulum and nuclear membrane. [131].
The exact functions of the COX isozymes is not yet known, but recent studies have proposed that COX-1 is involved in the production of prostaglandins involved in cellular "housekeeping functions". These prostaglandins regulate the normal kidney and gastric functions and vascular homeostasis. Prostaglandins which are rapidly and transiently produced by COX-2 are most probably involved in inflammation and may also be involved in regulating cellular proliferation [129].

Table 2.6 Eicosanoid production by macrophages (Mϕ). nd=not detectable; (+)=very low production; +=low production; ++= high production; +++=very high production; CAPD = continuous ambulatory peritoneal dialysis; A23187 = calcium ionophore; LPS = lipopolysaccharide; TPA = phorbol 12-myristate 13-acetate; * = *in vitro* interferon-γ/LPS activated [92-125].

Mϕ origin	Stimulus	LTB_4	5-HETE	LTC_4	PGE_2	PGI_2	TXB_2	PGD_2
Human peritoneal Mϕ								
CAPD control	-				$+^{95/97}$	$+^{95/97}$	$+^{95/97}$	
CAPD peritonitis	-				$(+)^{95/97}$	$(+)^{95/97}$	$(+)^{95/97}$	
CAPD control	A23187				$+^{93}$	$+^{99}$	$+^{99}$	
CAPD peritonitis	A23187	$+++^{93}$	$+++^{93}$		$+++^{92}$	$++^{93}$	$++^{93}$	$(+)^{93}$
CAPD control	LPS				$+++^{95}$	$+++^{92}$		
CAPD peritonitis	LPS					$+^{92}$		
CAPD control	Zymosan	$+^{98}$					$+++^{96}$	
Ascites control	A23187	$+++^{96/98}$	$+++^{94/98}$		$nd^{94/98}$	$+^{98}$	$+^{94/98}$	$+^{98}$
Human alveolar Mϕ								
Normal	A23187	$++^{100}/+++^{101-105}$	$++^{100/103}/+++^{102}$	$nd^{101/102}/+^{103}$	$nd^{102}/+^{103}$	nd^{102}	$+^{100/103}/nd^{102}$	$nd^{102/103}$
,,	LPS	nd^{102}	nd^{102}	nd^{102}	$+^{102}$	nd^{102}	$+++^{102}$	nd^{102}
,,	TPA	$+^{100}$	$+^{100}$				$+^{100}$	
,,	A23/PMA	$+++^{100}$	$+++^{100}$				$++^{100}$	
,,	Zymosan	$+^{100/103/125}++^{101}$	$+^{100}$	$+^{103}$	$+^{103/125}$		$+^{100/103}++^{125}$	$++^{125}$
Chronic bronchitis	Zymosan	$++^{96}$					$++^{96}$	
Cigarette smoke	A23187	$+++^{104}$						
Asthmatic	A23187	$+++^{101}$		nd^{103}				
Human monocytes								
Normal	A23187	$+++^{105}$	nd^{105}	$++^{105}$				
Normal	Zymosan	$+^{105}$	nd^{105}	$+^{105}$				
Culture 1 day	A23187	$++^{103}$		$+^{103}$	$++^{103}$		$++^{103}$	
Culture 5 days	A23187	$++^{103}$		$+^{103}$	$++^{103}$		$++^{103}$	
Mϕ (alveolar)-1 day	A23187	$++^{103}$		$+^{103}$	$++^{103}$		$++^{103}$	
Rat Mϕ								
Peritoneal-resident	A23187	$+^{106/107/109/110}$	$+^{106/109}$	$nd^{106}+/++^{107}$	$+^{106/107/109/110}$	$+^{106/107/109}++^{110}$	$++^{106/107/109/110}$	$+^{106}++^{110}$
Peritoneal-resident	LPS	$+^{107}$		$(+)^{107}$	$+^{107}$	$(+)^{107}+++^{112}$	$++^{107}+++^{112}$	$(+)^{107}$
Peritoneal-resident	PMA	nd^{108}		nd^{108}	$++^{108}$	$+++^{108}$	$+^{108}++^{103}$	
Peritoneal-active	A23187	$nd^{111/113}$	$++^{111}$		$+^{111}++^{113}$	$+^{111}$	$+^{113}++^{111}$	$+^{111}$
Alveolar-resident	A23187	$++^{109}$	$++^{109}$	$+^{116}$	$+^{109/114}$	$+^{109/114}$	$+^{109}nd^{114}$	nd^{109}
Alveolar-resident	Zymosan				$+^{114}$	$+^{114}$	nd^{114}	
Interstitial-resident	A23187	$++^{109}$	$+^{109}$		$+^{109/115}$	$+^{109/115}$	$+^{109}nd^{115}$	$+^{109}$
Interstitial-resident	Zymosan				$+^{115}$	$+^{115}$	nd^{115}	
Kupffer-resident	A23187	$+^{117}$	$+^{117}(+)^{123}$		$nd^{117}(+)^{123}$	$(+)^{123}$	$+^{117/123}$	$++^{117/123}$
Kupffer-active	A23187	$+++^{117}$	$+++^{117}$		$+^{117}$		$+^{117}$	$+^{117}$
Kupffer-resident	Zymosan				$+^{124}$	nd^{124}	nd^{124}	$+++^{124}$
Kupffer-active*	Zymosan				$+++^{124}$	nd^{124}	nd^{124}	$+++^{124}$
Murine Mϕ								
Peritoneal-resident	A23187	$+^{122}$		$+++^{118}+^{119}$	$++^{118}+^{119}$	$+^{122}$	$+^{122}$	
,, ,,	LPS			nd^{119}	$++^{119/120}$	$++^{120}$		
,, ,,	Zymosan			$+^{119}++^{121}$	$++^{119/121}$	$+++^{121}$	$+^{121}$	
,, ,,	PMA			$nd^{118/119}$	$++^{118/119}$			
Peritoneal-activated	Zymosan			$+^{121}$	$+^{120/121}$	$+^{120/121}$	$+^{121}$	
Kupffer-resident	Zymosan				$+^{124}$	$+^{124}$	$++^{124}$	
Kupffer-activated*	Zymosan				$+++^{124}$	$+^{124}$	$++^{124}$	$+++^{124}$

Mechanism of action: Regulation of the eicosanoid production and action can be mediated at different levels as the eicosanoids are released extracelluar and their biological effects are receptor-mediated: (1) precursor-incorporation level; (2) phospholipid-release level; (3) cyclooxygenase and lipoxygenase enzyme level; and (4) receptor level.
The receptor classification has been based upon the amplication of classical pharmacological criteria including: (1) a comparison of the rank order of agonist potency; (2) the evaluation of the structure-activity relationships of agonists; (3) the use of selective antagonists; (4) radioligand binding analyses; and (5) characterizing of transduction processes. The leukotriene receptors are classified into LTB_4, LTC_4 and LTD_4 and the prostanoid receptors are classified into DP, EP1-3, FP, IP and TP receptors, as summarized in Table 2.7 [132]. The leukotrienes and prostanoids receptors are all G-protein coupled. These G-proteins stimulate or inhibit adenylate cyclase, stimulate PLC, PLA_2, stimulate cGMP-phosphodiesterase, modulate the opening of K^+ channels, stimulate Ca^{2+} mobilization and PKC activation [75,133,134].

Table 2.7 Eicosanoid receptors [132].

Nomenclature	Potency order	Predominant effectors
LTB_4	$LTB_4 > 12r\text{-}HETE$	IP_3/DAG
LTC_4	$LTC_4 \geq LTD_4 > > LTE_4$	-
LTD_4	$LTD_4 = LTC_4 \geq LTE_4$	IP_3/DAG
DP	$PGD_2 > PGE_2, PGI_2, PGF_{2\alpha}, TXA_2$	$cAMP\uparrow$
FP	$PGF_{2\alpha} > PGD_2 > PGE_2 > PGI_2, TXA_2$	IP_3/DAG
IP	$PGI_2 > > PGD_2, PGE_2, PGF_{2\alpha} > TXA_2$	$cAMP\uparrow$
TP	$TXA_2 = PGH_2 > > PGD_2, PGE_2, PGF_{2\alpha}, PGI_2$	IP_3/DAG
EP_1	$PGE_2 > PGF_{2\alpha}, PGI_2 > PGD_2, TXA_2$	IP_3/DAG
EP_2	$PGE_2 > PGF_{2\alpha}, PGI_2 > PGD_2, TXA_2$	$cAMP\uparrow$
EP_3	$PGE_2 > PGF_{2\alpha}, PGI_2 > PGD_2, TXA_2$	$cAMP\downarrow + IP_3/DAG$

Biological properties: The leukotrienes have potent biological actions in a number of areas. LTB_4 is regarded as an important inflammatory mediator *in vivo* as it primarily effects leucocytes (leucotactic), which results in chemotaxis, aggregation, increase of vascular permeability, superoxide release and degranulation. The cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4) primarily affect smooth muscle cells. These leukotrienes have an important role in bronchoconstriction, increase microvascular permeability in the airways and are able to decrease blood pressure by action on the heart [78,135].
Prostanoids exhibit distinct biological effects during inflammation: pro-inflammatory such as pain (PGE_2), vasodilation and edema formation and inhibition of platelet aggregation (PGD_2, PGE_2, PGI_2), platelet aggregation and vasoconstriction (TXB_2) [136]. PGE_2 and PGE analogues have probably also an anti-inflammatory role during inflammation via raising cAMP [137]. In 1973 it was already shown that PGE_1 suppresses acute carageenan-induced inflammation and chronic joint inflammation characteristic of adjuvant disease in the rat [138]. Different anti-inflammatory actions

of PGE_2 and its analogues are known, such as the inhibition of the TNF-α production [139], the inhibitory effect on IL-1 expression [140], reduction of granulomas [141] and mucosal protective effects [142].

Platelet activating factor (PAF): When in inflammatory cells, (e.g. Mϕ, neutrophils, eosinophils, platelets and endothelial cells) in response to different stimuli (e.g. A23187, TNF-α, zymosan) PLA_2 hydrolyses PC into AA. PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) can then be formed by acetyltransferase from lyso-PAF. There is substantial evidence that PAF has an important role in various physiological and pathophysiological episodes, e.g. platelet aggregation, stimulation of neutrophils and Mϕ, acute inflammation, asthma, shock, sepsis and multiple organ failure.
There are specific PAF receptors on inflammatory cells which are linked to G-proteins. When these receptors are activated, they activate PLA_2 and PLC, inducing the cascade of signal transduction mechanism and hereby also stimulating the eicosanoid production [143,144].

2.8.2 Cytokines

Cytokines are defined as follows: "Cytokines are regulatory proteins secreted by white blood cells and a variety of other cells in the body; the pleiotropic actions of cytokines include numerous effects on cells of the immune system and modulation of inflammatory responses" [145]. They are a heterogeneous group of proteins with a number of characteristics in common: (1) Cytokines are low molecular weight (<80 kDa) secreted proteins which are often glycolysed; (2) The constitutive production is usually low or absent: production is regulated by various inducing stimuli at the level of transcription and translation; (3) They are usually produced transiently and locally, the action radius is usually short (acting in a paracrine or autocrine, but not in an endocrine way; (4) Cytokines are extremely potent, generally acting at picomolar concentrations; (5) They interact with high affinity cell surface receptors specific for each cytokine or cytokine group; (6) Their cell surface binding ultimately leads to a change in the pattern of cellular RNA and protein synthesis, and to altered cell behaviour: increase/decrease cell proliferation, change in cell differentiation stage and/or change in differentiated functions; (7) Individual cytokines have multiple overlapping cell regulatory actions. The response of a cell to a given cytokine is dependent on the local concentration of the cytokine, the cell type and other cell regulators to which it is concomitantly exposed; (8) Cytokines interact in a network firstly by inducing each other, secondly by transmodulating cytokine cell surface receptors and thirdly by synergistic, additive or antagonistic interactions on cell function [145,146].
The cytokine receptors form a superfamily. They are all transmembrane glycoproteins made up of an extracelluar amino-terminal ligand binding domain, a short hydrophobic transmembrane region and a carboxy-terminal intracellular domain. Several soluble receptors have been found. They consist of the extracellular domain and are capable

Table 2.8 Characteristic properties of cytokines [Mϕ = macrophages; NK = natural killer cells, 148].

Cytokine	MW	Principal cell source	Primary type of activity	Preeminent effects
IL-1	17,500	Mϕ + others	Immunoaugmentation	Inflammatory, haematopoietic
IL-2	15,500	T-lymphocytes + LGL	T- and B-cell growth factor	Activates T-lymphocytes and NK cells
IL-3	14,000-28,000	T-lymphocytes	Haematopoietic growth factor	Promotes growth of early myeloid progenitor cells and enhance cytokine production of Mϕ [151]
IL-4	20,000	T_H-cells	T- and B-cell growth factor, promotes IgE reactions, suppresses cytotoxic and inflammatory function of Mϕ [152]	Promotes IgE switch and mast cell growth
IL-5	18,000	T_H-cells	Stimulates B-cells and eosinophils	Promotes IgA switch and eosinophilia
IL-6	21,000-26,000	Fibroblasts + others	Hybridoma growth factor; augments inflammation	Growth factor for B-cells and polyclonal immunoglobulin production
IL-7	25,000	Stromal cells	Lymphopoietin	Generates pre-B- and pre-T-cells and is lymphocyte growth factor
IL-8	8,800	Mϕ + others	Chemoattracts neutrophils and T-lymphocytes	Regulates lymphocyte homing and neutrophil infiltration
IL-10 [153]	35,000-40,000	T_H-cells, monocytes, Mϕ, B-cells	Immunosuppressant of Mϕ function	Inhibition T_H-cell responses directly and via Mϕ, immunostimulatory effects on B-cells
IL-11 [154]		Bone marrow stromal cells	Growth factor for IL-6 dependent plasmacytoma cells, functionally related to IL-6	
IL-13 [152]	10,000	T_H-cells	Suppresses cytotoxic and inflammatory function and stimulates antigen presenting function of Mϕ	Immunostimulatory effects on B-cells

G-CSF	18,000-22,000	Monocytes + others	Myeloid growth factor	Generates neutrophils
M-CSF	18,000-26,000	Monocytes + others	Mϕ growth factor	Generates Mϕ
GM-CSF	14,000-38,000	T-lymphocytes + others	Monomyelocytic growth factor	Myelopoiesis
IFN_{α} IFN_{β} IFN_{γ}	18,000-20,000 25,000 20,000-25,000	Leukocytes Fibroblasts T-lymphocytes + NK cells	Antiviral, antiproliferative, immunomodulating	Stimulates Mϕ and NK-cells, induce cell membrane antigens (eg MHC)
TNF-α	17,000	Mϕ + others	Inflammatory, immunoenhancing, tumoricidal	Generates vascular thromboses and tumor necrosis
TGF_{β}	25,000	Platelets, bone + others	Fibroplasia, immunosuppresion	Involved in wound healing and bone remodeling

of ligand-binding. Whether they are actively secreted or represent the degradation product of the receptor or cell turnover is uncertain [147].
The characteristic properties of the cytokines are presented in Table 2.8 [148]. IL-1α/ß, IL-6 and TNF-α are important pro-inflammatory mediators of the macrophage [149]. In Figure 2.7 their overlap is shown [150]. In the next sections the properties of these three cytokines will be discussed.

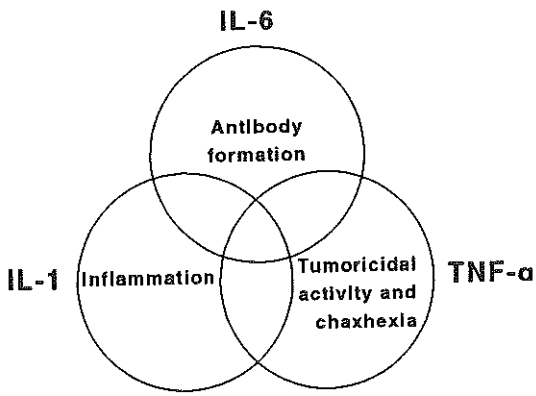

Figure 2.7 Redundancy and specifity in IL-1, IL-6 and TNF-α [150].

2.8.2.1 Interleukin 1

Interleukin 1 (IL-1) refers to two structurally related polypeptides [(17.5 kDa), 155,156], IL-1α and IL-1ß. IL-1 receptor antagonist (IL-1ra) is a third structurally related interleukin-1 which inhibits the activities of IL-1α/ß. All three forms are distinct gene products. IL-1ra is produced by the same cells that produce IL-1α and IL-ß [157,158].
A wide variety of agents (e.g. microbes, microbial products, inflammatory agents, plant lectins and antigens) can induce IL-1α and IL-1ß production [159]. The second messengers involved in the induction of IL-1 (α + ß) remains unclear. The leukotriene products are most probably involved [160]. IL-1α and IL-1ß appear to be under separate transcriptional control [161]. Transcription and translation of IL-1 are distinct and dissociated processes. Cells (human peripheral blood mononuclear cells) containing untranslated IL-1 mRNA are "primed", stimuli like LPS [the most common stimulus used for IL-1 (α + ß)] can rapidly trigger translation [162,163].
IL-1α and IL-1ß are synthesized by both leukocytic as well as nonleukocytic cells, but primarily they are a product of monocytes and Mϕ. Precursor IL-1α is fully active and remains cell associated. Biological inactive precursor IL-1ß is cleaved by IL-1ß converting enzyme into active IL-1ß and secreted by the cells [164,165]. IL-1 (α + ß) affects nearly every tissue [149,166]. The active forms of IL-1α and IL-1ß share the same biological properties by recognizing and activating the same receptor [167]: IL-1 receptor type I. So far, no response has been found via the type II IL-1 receptor

[168]. The exact signal transduction pathway following the binding of IL-1 to the cell surface receptor is not yet clear. It is hypothized that activation of a PLC, via coupling of IL-1 receptor to a G-protein, leads to hydrolyzing of phospholipids, liberating DAG. DAG then triggers protein kinases, which in turn phosphorylate multiple target proteins [169].
Proliferation of a variation of haematopoietic and other cells, regulation of pro-inflammatory events and mediation of tissue damage in inflammatory disease are considered as the main effects of IL-1α and IL-1ß.
IL-1ra binds to the same receptor as IL-1α and IL-1ß, but has no activating properties, it is a pure receptor antagonist [156]. IL-1ra is produced by human monocytes [158] and human Mϕ [170,171]. The regulation of the IL1ra production is differently regulated than the IL-1ß production in human monocytes [172]. During maturation of human monocytes into Mϕ *in vitro*, LPS stimulated IL-1ß production is downregulated [173], while IL-1ra production is greatly enhanced. Freshly isolated human monocytes do not produce IL-1ra unless stimulated with agents such as LPS [172], the enhanced IL-1ra production of these mature Mϕ is then constitutive [173].

2.8.2.2 Interleukin 6

Interleukin 6 (IL-6) is a glycoprotein with a molecular mass in the range 21 to 26 kDA, which has pleitropic activities that induces differentiation and regulates cell growth in a cell type dependent manner. It induces the differentiation of B-cells and promotes the growth of haematopoieic progenitor cells and differentiation and/or activation of T-cells and Mϕ. It is also the main regulator of acute phase responses in hepatocytes [174].
Even though IL-6 is produced by many different cell types including fibroblasts, endothelial cells, T and B cells, certain tumors, the monocytic cells are the major source [175,176]. Abnormal expression of the IL-6 gene is probably involved in the pathogenesis of diseases as IL-6 levels are increased in a variety of diseases, e.g. bacterial and parasite infection, viral infections, inflammatory or autoimmune diseases [(e.g. rheumatoid arthritis), 177].
The exact molecular events leading to the induced IL-6 production remains to be clarified [178]; however, it is known that a wide variety of agencies can induce the IL-6 production such as antigens/mitogens, LPS, IL-1, TNF-α, PAF, TPA, viruses, cAMP [179-181].
IL-6 binds to a specific cell-surface receptor, the ligand binding chain (gp80), which in turn associates with a signal-transducing chain (gp130). How this IL-6 receptor mediated signalling leads to the activation of various transcription factors is not yet known [182]. A recent paper shows that probably in the initial step the tyrosine kinase pathway is activated and not the protein kinases A (PKA)/PKC or Ca^{+}/calmodulin-dependent kinases [183].

2.8.2.3 Tumor necrosis factor α

Tumor necrosis factor α (TNF-α), a 17 kD polypeptide [184], was originally described as a factor which is induced by endotoxin and has anticancer activity [185,186]. It is clear now that TNF-α is involved in diverse pathological and physiological actions of the body. Various cell types are known to produce TNF-α [187], but just as for IL-1 and IL-6, monocytes and Mϕ are thought to be the responsible cells which contribute mostly to the local and systemic response to bacterial, viral and parasitic organism and their products by producing TNF-α [188,189]. An important biological effect of TNF-α is probably up-regulation of the immune response. Based on studies *in vitro* it is known that TNF-α directly affects a wide variety of cells, e.g. monocytes/Mϕ, T- and B-cells, eosinophils, neutrophils, fibroblasts and endothelial cells, hereby also inducing a wide range of other pro-inflammatory cytokines. In addition, TNF-α is also involved in cachexia, is a major mediator of endotoxin-mediated shock, has antibacterial effects, is probably involved in autoimmune diseases and is also involved in wound healing [187-189].
There are two distinct TNF-α receptors (TNF-R): TNF-R1 (P-75) and TNF-R2 (P-55) [190,191]. The specific functions of each receptor is still under debate; it is suggested that both receptors are active in signal transduction, TNF-R1 at high TNF-α concentrations, signalling a large number of TNF-α activities and TNF-R2 at low TNF-α concentrations, only signalling for the proliferation of primary thymocytes and T-cells [192]. It was shown with a human epithelial cell line that TNF-α up-regulates the expression of TNF-R1 but not TNF-R2, and that both receptors mediate, independently of each other, different functions [193]. In the signalling transduction pathway probably a G-protein and PLA_2 are involved [194,195].

2.9 Ascites

The peritoneal cavity is the endothelial one-cell-layer of the viscera. The peritoneal cavity normally contains less than 50 ml of fluid. Ascites is the accumulation of free fluid and salt in this peritoneal cavity. In sterile ascites peritoneal Mϕ are predominantly present. These cells have migrated from the capillary bed under the visceral peritoneum into the ascitic fluid.

2.9.1 Pathophysiology of ascites

Ascites results from exudation of fluid either from the surface of the liver or from the surfaces of the gut and its mesentry. The different causes are portal hypertension (75% cirrhosis, 5% cardiac failure), malignancies (10-12%), infections and others (8%-10%) as summarized in Table 2.9. Once formed, ascitic fluid can exchange with blood through an enormous capillary bed under the visceral peritoneum.

Table 2.9 Causes of ascites [197].

- Portal hypertension
Cirrhosis
Hepatic congestion
Congestive heart failure
Constrictive pericarditis
Budd-Chiari syndrome
Veno-occlusive disease
Portal vein thrombosis
- Infections
Secondary bacterial peritonitis
Tuberculous peritonitis
Fungal/parasitic peritonitis
- Malignancies
Peritoneal carcinomatosis
Mesothelioma
Metastatic liver disease
Hepatocellular carcinoma
Other intra-abdominal malignancies
- Other
Hypoalbuminaemia
Leakage from lymphatic tissue
Pancreas
Biliary tract
Renal failure
Myxoedema

Portal hypertension. The liver is literally the crossroads of the body. The portal and systemic circulations join here to drain through a common venous outflow. Liver cirrhosis is a diffuse process characterized by fibrosis and a conversion of normal architecture into structural abnormal nodules. Cirrhosis is a progressive disorder that has the potential of leading to portal hypertension and liver failure. Different disorders causing cirrhosis are classified as follow:
- Cirrhosis associated with alcohol abuse (60-70%)
- Postnecrotic cirrhosis (10-15%)
- Biliary cirrhosis: - primary + secondary (10%)
- Pigment cirrhosis (5%)
- Cirrhosis associated with Wilson's disease (rare)
- cryptogenic cirrhosis (10-15%)

The pathogenesis of ascites formation in patients with hepatic disorders has not been fully clarified. Several theories have explained the initiating event of renal sodium and water retention in cirrhosis. The peripheral arterial vasodilation hypothesis proposes that cirrhosis leads to peripheral arterial vasodilation as the initial event, resulting in a decreased effective plasma volume and the activation of compensating hormonal systems causing renal sodium retention.
Malignancy. Cancers have a propensity for invading blood vessels and may obstruct the hepatic vein, producing hepatic vein thrombosis; or they block the portal vein,

producing portal hypertension and, as mentioned earlier, portal hypertension leads to the formation of ascites.
Infections. Infection and malignancy, usually cause high protein ascites due to increased fluid and lymph production from the peritoneum and increased peritoneum permeability of the peritoneal capillaries, at the site of inflammation. Patients with ascites and cirrhosis may be complicated with a spontaneous bacterial peritonitis without an obvious primary source of infection. It probably reflects the deficient opsonic activity of ascitic fluid in these patients.

Others. Extravasation of pancreatic fluid, bile or lipid-rich lymph into the peritoneal cavity may also lead to chronic peritoneal fluid accumulation.

2.9.2 Treatment of ascites

When ascites develops in the setting of severe acute liver disease, resolution of ascites is likely to follow improvement in liver function. Ascites commonly develops in patients with stable or steadily worsening liver function. Therapeutic intervention is indicated both to prevent potential complications and to control progressive increase in ascites, which may become pronounced enough to cause physical discomfort. Bed rest is recommended and the uptake of water and especially salt is restricted. Diuretics are instituted when the above mentioned therapy fails to result in diuresis and weight loss.
Large volumes (10 liters in 1 hour) of ascites are removed by paracentesis in combination with concomitant intravenous infusion of albumin in amounts proportional to those removed. This is only done to speed the reduction of ascites in patients with tense ascites to make them more comfortable, it does not change the relapse or survival of the patient. A patient with cirrhosis and developing ascites has only a 40% change of being alive 2 years later [196]. Much depends on the major factor in the etiology of the fluid retention.
A minority of patients with advanced cirrhosis have "refractory ascites" and do not respond to the therapy. In some patients a side-to-side portacaval shunt may result in improvement in ascites or implantation of a plastic peritoneo-venous (Le Veen) shunt which has a pressure-sensitive, one-way valve allowing ascitic fluid to flow from the abdonimal cavity to the superior vena cava [196-199].

2.10 References

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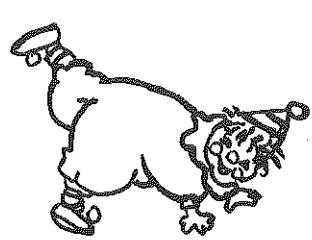
Part II

Cellular maturation and activation of macrophages



Chapter 3 Changes in eicosanoid and tumor necrosis factor α production by rat peritoneal macrophages during carrageenin-induced peritonitis

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3.1 Summary

Changes and correlations in cytokine and eicosanoid production by blood monocytes, non-purified and purified peritoneal cells during a carrageenin-induced peritonitis were investigated during ten days. The cells were isolated and stimulated *in vitro*. Cytokine and eicosanoid production of the non-purified fraction increased steadily during peritonitis.
During the whole episode of peritonitis the production capacity of granulocytes was very low and hardly any effect on the production capacity of macrophages (Mϕ) was observed. Cytokine and eicosanoid production of the non-purified fraction was mainly due to the presence of Mϕ. The production capacity of the peripheral blood monocytes was not similar to that of the peritoneal Mϕ.

Keywords: eicosanoids . peritoneal macrophages . peritonitis . tumor necrosis factor-α

3.2 Introduction

Inflammation is the reaction to an injury such as an invasion by infectious agents. The blood supply to inflamed area increases, venular permeability increases and leucocytes migrate out of venules into the surrounding tissue.
At the site of an acute inflammation polymorphonuclear granulocytes (PMNs) produce large amounts of reactive oxygen intermediates, release a variety of hydrolytic enzymes and phagocytose pathogens [1]. By generating of chemotactic factors, the migration of mononuclear cells (monocytes/Mϕ and lymphocytes) to the site of inflammation is stimulated [2,3]. During the chronic phase of the inflammatory response, Mϕ, mononuclear phagocytes derived from blood monocytes [4], predominate over PMNs [3]. In addition to acting as a non-specific defence, activated Mϕ [5,6] will initiate and control the specific defence by presenting processed antigens to lymphocytes [7] and by cytotoxity through direct cell-cell contact with tumor cells or infected cells [8,9], during which they secrete cytokines and eicosanoids [10,11]. These inflammatory mediators also influence and regulate functions of other cells participating in the immune response [12,13].
Cytokines are proteins with regulatory functions. They can induce other cytokines with overlapping effects and form interactive networks with hormones and eicosanoids. Mϕ are a potent source of wide variety of cytokines [14], whereas granulocytes are reported to produce only a limited spectrum of cytokines [15-17].
Eicosanoids are bioactive lipids derived from arachidonic acid (AA) by cyclooxygenase and lipoxygenase pathways [18,19]. Eicosanoid production is species and tissue

dependent and often stimulus dependent [20,21].
The aim of this paper was to study cytokine (tumor necrosis factor α, TNF-α) and eicosanoid (leukotriene B_4, LTB_4; prostaglandin E_2, PGE_2; prostacycline (PGI_2, detected as 6-keto-prostaglandin $F_{1\alpha}$ ($6kPGF_{1\alpha}$) and thromboxane, TXB_2) production capacity of peritoneal PMNs, Mϕ and blood monocytes after the induction of a peritonitis with a carrageenin solution, a sulphated polygalactan which stimulates cell-mediated immunity [22]. It was of interest to determine whether, during an episode of peritonitis, changes occurred in (a) the differentiation of influxed cells; (b) the cytokine and eicosanoid production capacity of blood monocytes and peritoneal cells; (c) interactions between different types of peritoneal cells concerning their production capacity; and (d) if there was a correlation between the production of eicosanoids and TNF-α by blood monocytes and peritoneal Mϕ.

3.3 Materials and methods

Animals and treatment: Young male Wistar rats (13 weeks, approximately 200 gram, six rats per group) were injected intraperitoneally with 2 ml of a carrageenin solution (Marine Colloids Inc., USA, 1 mg/ml) on day 0. Six rats received an injection of saline (control group).

Cell isolation: On day 1 (that is 24 h after inducing the peritonitis with the carrageenin solution), three, seven, and ten peritoneal cells and monocytes from the blood were isolated. With each group, cells were also isolated from a control animal, which was considered as day 0.
Blood ($\pm$ 7 ml) was collected in tubes with 1ml EDTA (0.1 M) after decapitation. The blood was centrifuged (400xg, 4°C, 10 min) and from the leucocyte layer the monocytes were isolated into a 'monocyte' fraction by density gradient centrifugation using Percoll (d=1.064 g/ml, Kabi-Pharmacia, Sweden; 400xg, 4°C, 25 min). Monocytes were washed three times with phosphate buffer solution (PBS, 400xg, 4°C, 10 min) and suspended in Dulbecco's modification of Eagle's medium (DMEM + HEPES (GIBCO, UK) + penicillin/streptomycin ($5x10^4$ U/l / 50 mg/l, Flow Lab, UK) + foetal calf serum (10 % FCS, GIBCO, UK) + L-glutamine (600 mg/l Flow Lab, UK), 1.10^6 cells/ml).
The peritoneal cavities were washed twice with 20 ml PBS (pH 7.4, 4°C, Oxoid, UK). The washings per rat were pooled, centrifuged (400xg, 4°C, 10 min) and suspended in 5 ml DMEM. $5x10^6$ cells of this non-purified fraction were kept separate, the rest of this 'crude' fraction was separated on Percoll into a 'macrophage' and a 'granulocyte' fraction. These two purified fractions were washed three times with PBS (400xg, 4°C, 10 min) and suspended in DMEM (1.10^6 cells/ml).
A small sample of cells of each fraction was stained by Hemacolor (Merck, Germany) and the different cell types were counted using a microscope (Zeiss, standard 25, Germany). The viability of the cells was determined by trypan blue exclusion.

Cell incubation: One million leucocytes per ml DMEM were plated on plastic culture dishes (Costar, UK). The cells were triggered for 15 min by calcium ionophore A23187 (Calbiochem, USA), 1 μM final concentration in dimethylsulfoxide (0.1 % DMSO, Sigma, USA), 37°C ,7.5 % CO_2). Controls were incubated with DMSO (0.1 %).
The cells were also incubated for 24 h in the absence or presence of lipopolysaccharide (LPS, 10 μg/ml final concentration in PBS, LPS from E. coli 0111:B4 in PBS (Sigma, USA), 37°C, 7.5 % CO_2). As a blank PBS was added. At the end of the incubation the supernatant was centrifuged and kept at -80°C until required for analysis.

Cytokine production: TNF-α production in the samples was determined directly in the supernatant by bioassay. For this bioassay the TNF-α sensitive cell line WEHI-164 was used. The WEHI-164 cells were plated out in 96-wells plates ($2x10^4$ cells/50 μl/well, Costar, UK) and the samples (50 μl/well) or the human recombinant TNF-α (hr-TNF-α) standards (0.1 - 1000 u/ml hr-TNF-α, 50 μl/well) were added. After 24 h incubation (37°C, 7.5 % CO_2), MTT (tetrazolium salt, Sigma, USA) was added (0.125 mg/well) and after an incubation of 3 h the cells were lysated with buffer (20 % Sodium Dodecyl Sulphate (SDS) in 50 % N,N-dimethylformamide (DMF), pH 4.7, 100 μl/well) for 18 h. The absorbance was measured at 595 nm with an ELISA-reader (BIO RAD, model 3550, UK). The TNF-α production by the Mϕ was expressed as the cytotoxicity against WEHI-164 cells compared to the blank (DMEM complete) (23).

Eicosanoid production: Eicosanoid production (LTB_4, PGE_2, TXB_2 and $6kPGF_{1\alpha}$ from endogenous arachidonate of the samples was determined directly in the supernatant by radio immuno assays (RIA, Antibodies were obtained from Advanced Magnetics, USA; standards from Sigma, USA; and tritiated antigens from Amersham, UK). Cross-reactivities for individual antigens on antibodies were negligible.

Statistical analysis: Data are expressed as the mean ± standard error of the mean (SEM). Data were statistically analyzed with ANOVA followed by the Dunnett test or student's t-test. The correlations were determined by Pearson Correlation test. Data were considered significant when $p < 0.05$.

3.4 Results

Concentration and differentiation of cells obtained from the peritoneal cavity: Induction of peritonitis in rats caused an increase of cells in the peritoneal cavity on day 1.
The cell count dropped on the following days (Figure 3.1A). A pronounced influx of granulocytes was seen on the first day which was followed by an influx of Mϕ, resulting in a Mϕ / granulocyte ratio of 4:1 from day 3 to 10 (Figure 3.1B).

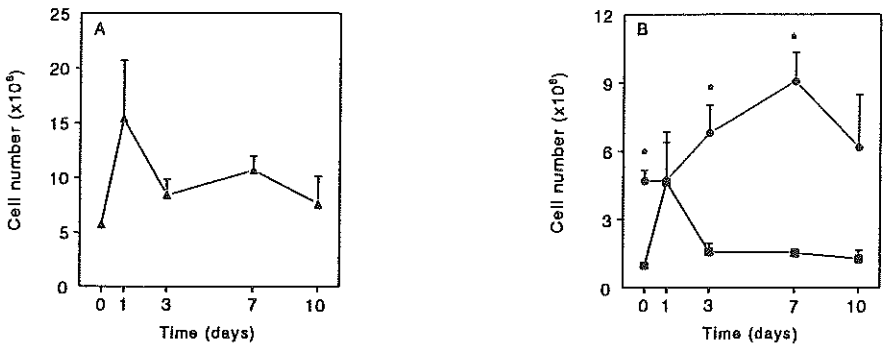

Figure 3.1 Time course (0-10 days) of cell number (A) and differentiation of the cells (B) during a peritonitis in rats; Mϕ(●), granulocytes(■); Mean ± SEM; n=6 per group; * = $p < 0.05$ macrophages vs. granulocytes.

Production capacity of cells to generate inflammatory mediators: The inflammatory mediator production per million cells stimulated *in vitro* (LTB_4; 15 min A23187; and PGE_2, TXB_2, $6kPGF_{1\alpha}$, TNF-α; 24 h LPS) by the peritoneal 'crude', 'macrophage' and 'granulocyte' fractions and the peripheral blood 'monocyte' fraction is shown in Figure 3.2A, 3.2B, 3.2C and 3.2D.

Cytokine and eicosanoid production by the crude fraction increased steadily during peritonitis. TNF-α and TXB_2 reached peak levels after 3 days, whereas PGE_2 and $6kPGF_{1\alpha}$ reached a plateau 7 days after induction of the peritonitis. In comparison with levels of other eicosanoids the LTB_4 production was low before and on the first day of the peritonitis and decreased significantly thereafter. By day 10 the production capacity returned to the initial level.

The production pattern of the cells from the macrophage fraction (Figure 3.2B) was similar to that of cells from the crude fraction (Figure 3.2A).

Inflammatory mediators were also produced by the granulocyte fraction (Figure 3.2C), although the production capacity of these cells was very low in comparison with cells from the crude and macrophage fraction. The pattern of inflammatory mediator production of the granulocyte fraction did not significantly change in time. On the tenth day after induction of the peritonitis, the concentration of the cells in the granulocyte fraction was too low to permit *in vitro* incubations.

The capacity of cells from the peripheral blood monocyte fraction to produce inflammatory mediators (Figure 3.2D) was at least four times lower in comparison with both the crude and macrophage fractions. The production capacity of the cells from the monocyte fraction was about the same with or without stimulus. The patterns of the TNF-α, PGE_2 and $6kPGF_{1\alpha}$ production of cells from the monocyte fraction were different from those of the macrophage and crude fraction. After induction of the peritonitis the TNF-α production of cells from the monocyte fraction increased significantly only on the tenth day, whereas PGE_2 and TXB_2 reached their highest level on the third day. The $6kPGF_{1\alpha}$ level did not change during the peritonitis. The LTB_4

production was negligible.

Correlations between inflammatory mediators produced in the same fraction: In the crude fraction (Figure 3.2A) there was a significant positive correlation between the production of PGE_2 and $6kPGF_{1\alpha}$ and PGE_2 and TXB_2 ($r = 0.6365$ and $r = 0.7145$). Similar significant correlations were found in the macrophage fraction (Figure 3.2B, $r = 0.5580$ and $r = 0.6106$).
In the monocyte fraction (Figure 3.2D) the only significant positive correlation between the inflammatory mediators was between PGE_2 and TXB_2 ($r = 0.8881$).

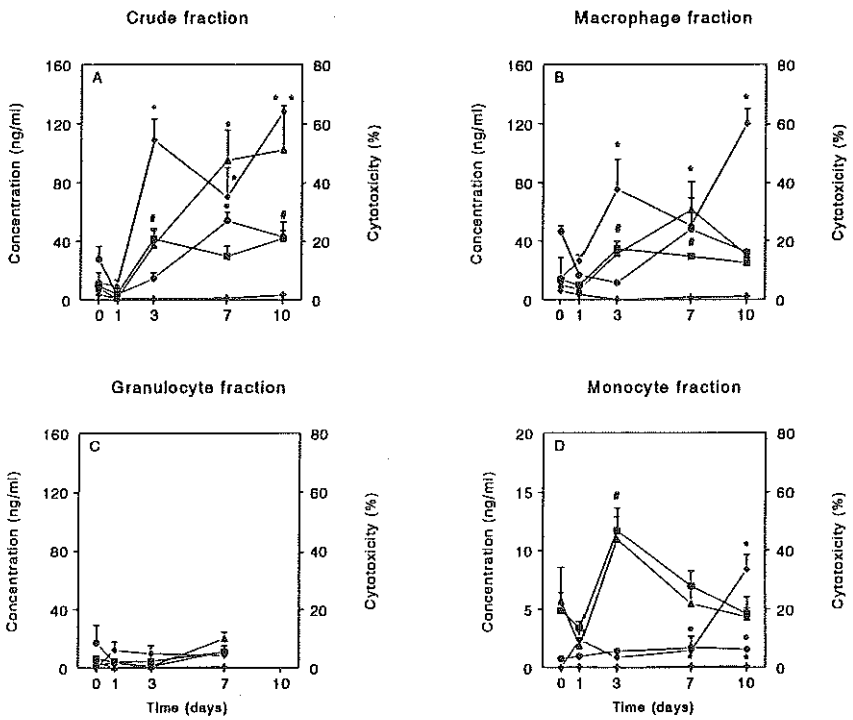

Figure 3.2 Production capacity of stimulated 'crude' (A), 'macrophage' (B), 'granulocyte' (C) and 'monocyte' (D) fraction to produce the eicosanoids LTB_4(+), PGE_2(▲), $6kPGF_{1\alpha}$(●), TXB_2(■) and the cytokine TNF-α(◆); LTB_4 production of 10^6 cells/ml determined in supernatant after 15 min 1 μM A23187 stimulation. PGE_2, TXB_2, $6kPGF_{1\alpha}$, TNF-α production of 10^6 cells/ml determined in supernatant after 24 h 10 μg/ml LPS stimulation; mean ± SEM; *, #, ● = $p < 0.05$ vs. day 0; n = 4-6.

Correlation between inflammatory mediators in different fractions: In Figure 3.2A and 3.2B the patterns of inflammatory mediators produced by the stimulated crude and macrophage cell fractions are presented. There was always a significant positive correlation between these two fractions. The correlation varied from $r = 0.5671$ for LTB_4 to $r = 0.8586$ for TNF-α. (PGE_2; $r = 0.6254$, $6kPGF_{1\alpha}$; $r = 0.7536$ and TXB_2

r = 0.7502). This correlation remained significant when we assumed that production of the cell inflammation mediators in the crude and macrophage fraction were only derived from the Mϕ (calculated to 100 % Mϕ; LTB_4 = 0.4649; PGE_2 = 0.5855, $6kPGF_{1\alpha}$ = 0.5506, TXB_2 = 0.7810; and TNF-α = 0.8674).

There were however some significant differences between the calculated (to 100 % Mϕ) production levels of the crude and macrophage fractions. When separate days were considered the production levels of the calculated crude fraction in comparison with the calculated macrophage fraction was on day 1 lower for PGE_2 and $6kPGF_{1\alpha}$, and on day 3 higher for LTB_4, TXB_2 and TNF-α (Figure 3.3 A-E).

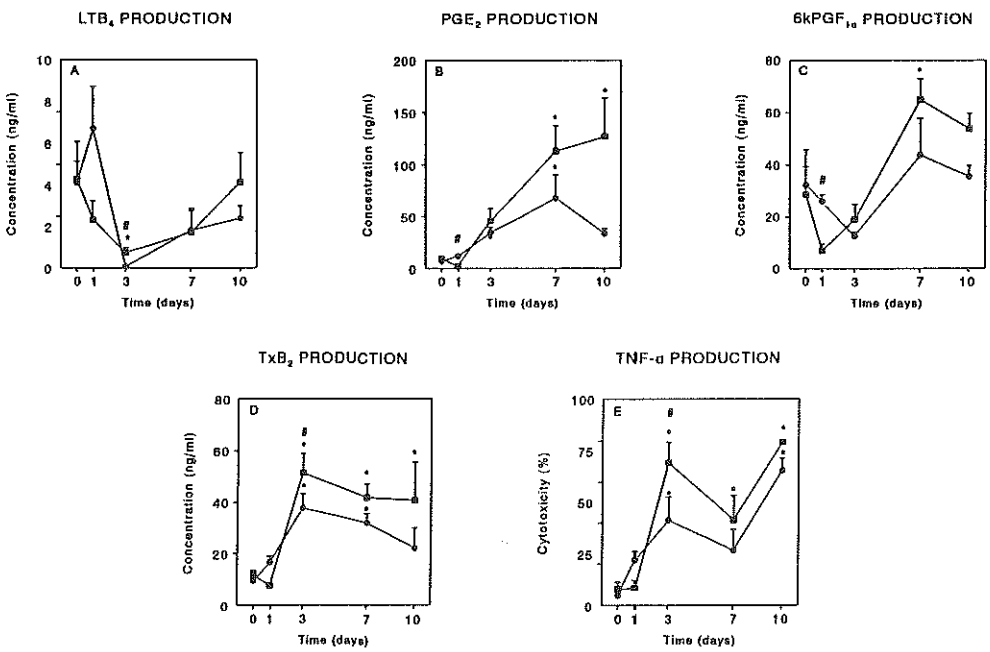

Figure 3.3 Production capacity of the stimulated 'crude' (■) and 'macrophage' fraction (●) (calculated to 100% Mϕ) to produce the eicosanoids LTB_4 (A), PGE_2 (B), $6kPGF_{1\alpha}$ (C), TXB_2 (D) and the cytokine TNF-α (E); LTB_4 production of 10^6 cells/ml determined in supernatant after 15 min 1 μM A23187 stimulation. PGE_2, TXB_2, $6kPGF_{1\alpha}$, TNF-α production of 10^6 cells/ml determined in supernatant after 24 h 10 μg/ml LPS stimulation; mean $\pm$ SEM ; * = $p < 0.05$ vs. day 0; # = $p < 0.05$ granulocytes vs. macrophages; n = 4-6.

Correlation coefficient between inflammatory mediators from monocyte and macrophage fraction: Only the basal TNF-α production of the cells from the monocyte and macrophage fractions, were clearly correlated (r = 0.5421).

3.5 Discussion

Characterization of inflammatory mediators produced by granulocytes and monocytes /Mϕ during induced inflammation in animal models, may be helpful to the understanding of inflammatory diseases and their treatment. The present study investigated the changes and correlations in cytokine and eicosanoid production capacity of peripheral blood monocytes, non-purified (crude fraction) and purified (macrophage and granulocyte fraction) peritoneal cells during a carrageenin-induced peritonitis in rats.

In the present study, where an influx of leucocytes into the peritoneum was achieved after carrageenin injection, the number of PMNs increased in the acute phase (day 1) and thereafter decreased quickly. On day 0, before inducing the peritonitis, there was a reasonable number of Mϕ present in the peritoneal cavity. The number of Mϕ increased from day 1 until seven days after induction of the peritonitis, resulting in a mainly a Mϕ cell population. Previous histological studies had similar results [24,25], although a quantitative and kinetic method showed that both monocytes and PMNs from rat migrate rapidly to the inflamed site, the migration of PMNs, however, also declined long before the migration of monocytes started to decrease [3]. The pattern of this migration can be explained with the function of the leucocytes in inflammation. In the initial phase both PMNs and Mϕ are involved in non-specific defence mechanisms [1,2]. In addition Mϕ will also initiate and control the specific defence [7-13]. The mechanism of this selective migration is not clear. Leucocyte adhesion to the blood vessel endothelium, which is followed by transendothelial migration, is a multistep process in which several adhesion molecules are involved [26,27].

Our experiment showed that the inflammatory mediator production capacity of the non-purified fraction, which was harvested from the inflamed peritoneal cavity, changed with time. The production of inflammatory mediators in the non-purified fraction was mainly caused by the presence of Mϕ during carrageenin-induced peritonitis. This is, first of all, based on the significant correlation between the eicosanoid and TNF-α production capacity of the macrophage fraction and the crude fraction, these correlations hardly changed when it was assumed that these fractions only contained Mϕ. Secondly, the production capacity of the granulocytes appeared to be very low. Moreover the production capacity of the granulocyte fraction did not correlate with the crude fraction.

When the production levels of the non-purified fraction was compared with the macrophage fraction, assuming that the inflammatory mediators were only produced by Mϕ, it was observed that the production of PGE_2 and $6kPGF_{1\alpha}$ had decreased on day 1 and the production of LTB_4, TXB_2 and TNF-α had increased on day 3. The results of these effects could be an amplification of the inflammation, due to the decrease of anti-inflammatory substances and the subsequent increase of pro-inflammatory substances.

The low production capacity of the crude fraction in the acute phase followed by an increasing production capacity in the chronic phase could also be explained by

presence of the Mϕ. Blood monocytes and resident Mϕ transform from primed into active Mϕ at the side of the inflammation, after which profound changes occur in morphology and function of these cells [4-6]. The Mϕ present at day 1 in the peritoneal cavity, probably were not primed *in vivo* at this time, which could be the reason that the eicosanoid and cytokine production capacity of these Mϕ were so low after stimulation *in vitro* with LPS. Once these Mϕ were primed *in vivo*, also the new recruded monocytes whom migrate into the peritoneal cavity, the production capacity of these monocytes/Mϕ steadily increased, showing *in vitro* a differentiated pattern for the eicosanoids and TNF-α. This pattern probably depends on the function of the metabolite.

As peripheral blood monocytes transform into peritoneal Mϕ [4], the correlation between these cells was investigated. The eicosanoid and TNF-α production capacity of blood monocytes was not similar to that of Mϕ during peritonitis. It was clearly shown that through influx and transformation of monocytes into Mϕ the synthesis of mediators completely changed.

In conclusion Mϕ are the main source of the inflammatory mediators whose production is dependent on the episode of the carrageenin-induced peritonitis. Characterisation of purified Mϕ *in vitro* in animal and human models will be helpful to the understanding of inflammatory diseases and their treatment. Peripheral blood monocytes do not reflect mediator production of Mϕ present at the inflammatory site.

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Chapter 4 Eicosanoid production by density-defined human peritoneal macrophages during inflammation

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4.1 Summary

Density-defined macrophages (Mϕ) isolated from fluids of patients with liver cirrhosis mainly generated the 5-lipoxygenase products leukotriene B_4 (LTB_4, 16 %) and 5-hydroxy-eicostetranoic acid (5-HETE, 24 %) and the cyclooxygenase products 12-hydroxy-heptadecatrieoic acid (HHT, 22 %) and thromboxane B_2 (TXB_2, 18 %). The synthesis of eicosanoids was linear with the maturity of the Mϕ subpopulations, suggesting that eicosanoid production is increased in *in-vivo* activated Mϕ.

Keywords: ascites . macrophages . subpopulations . eicosanoids

4.2 Introduction

During peritonitis a large number of inflammatory cells are present in ascites fluid. Ascites obtained from patients with alcoholic liver cirrhosis is a potential source of human Mϕ (approx. 86 %), which can successfully be used to study various aspects of their role in inflammation. It is likely that the mediators produced by these Mϕ contribute to the observed inflammatory changes.
In vitro activation of cells obtained from patients has shown that different eicosanoids are produced in higher concentrations during peritonitis [1].

Subpopulations of peritoneal Mϕ could reflect a continuum of functional changes induced by cellular maturation, exogenous stimuli or both. Maturation of monocytes into Mϕ takes place without cell division, but with an increase in cell volume [2]. The least mature Mϕ are expected to be found in the highest density layer of a density gradient and the more mature Mϕ in the lower densities [3]. The investigations described here were performed to obtain information about the ability of density-defined subpopulations of human peritoneal Mϕ and their ability to synthesize eicosanoids.

4.3 Materials and methods

Subjects: Peritoneal Mϕ were obtained from ascitic fluid of 3 patients with liver cirrhosis.

Cell isolation: Cells were isolated by centrifugation (1000xg) and washed with phosphate buffered saline (PBS, pH 7.4, Oxoid, UK). The concentrated cell suspension

was separated on a Percoll (Kabi-Pharmacia, Sweden) density gradient into six subpopulations (0 = non-separated cell suspension, 1 = 1.035 g/ml, 2 = 1.041 g/ml, 3 = 1.046 g/ml, 4 = 1.052 g/ml, 5 = 1.064 g/ml, 6 = 1.076 g/ml and 7 = pellet).

Cell incubation: One million density-defined cells in Dulbecco's minimal essential medium (Dulbecco's MEM, Gibco, UK) were labelled with ^{14}C-arachidonic acid (0.1 μCi, Amersham, UK) for 2 min and triggered by the calcium ionophore A23187 (2 μM) for 13 min. ^{3}H labelled standards (Amersham, UK) were added and the supernatants passed through Sep Pak C_{18} catridges (Waters Ass., USA).

Eicosanoid production: Exogenous eicosanoid formation was measured on RP-HPLC using a Nucleosil $5C_{18}$ column (3x200 mm,Chrompack, The Netherlands). A gradient of acetonitrile and 0.2 % triethylamine / 0.12 % trifluoroacetic acid / water (pH 3) was used. Cells were stained by Hemacolor (UK) to determine the cell differentiation. For each patient, per eicosanoid product, the production of the density defined leucocytes was compared with the density defined leucocytes with the highest production (= 100 %). The data are expressed as the mean $\pm$ SD (n = 3), with a correction for the number of Mϕ.

4.4 Results

Cell differentiation: When a comparison was made of the morphology of the macrophage subpopulations, we found the following: The Mϕ with the highest density (1.076 g/ml) were small round cells. With a declining density the nucleoplasmatic ratio also declined. The Mϕ in the lowest density layer (1.035 g/ml) were about the same size as the Mϕ in the second lightest density (1.041 g/ml), however their structure was much more irregular.
The cell distribution in percentages of total amount of recovered cells on the discontinuous gradient (with a cell recovery between 50 and 75 %) was 18 $\pm$ 2.0 % for fraction 1, 15 $\pm$ 2.1 % for fraction 2, 7 $\pm$ 2.4 % for fraction 3, 20 $\pm$ 5.6 % for fraction 4, 29 $\pm$ 2.8 % for fraction 5, 7 $\pm$ 4.4 % for fraction 6 and 1.7 $\pm$ 0.2 % for the pellet.
Each subpopulation contained mainly Mϕ; 95.5 $\pm$ 0.8 % in fraction 1, 94 $\pm$ 2.2 % in fraction 2, 95.8 $\pm$ 0.5 % in fraction 3, 91 $\pm$ 1.3 % in fraction 4, 81 $\pm$ 4.1 % in fraction 5, 77 $\pm$ 5.0 % in fraction 6 and no detectable amounts in the pellet. The non-separated cell suspension contained 86.3 $\pm$ 0.9 % Mϕ.

Eicosanoid production: The main lipoxygenase products of one million human peritoneal density-defined Mϕ from the 3 patients were 5-HETE (24 %), LTB_4 (16 %) and 15-HETE (7 %). The main cyclooxygenase products were HHT (22 %) and TXB_2 (18 %). In figure 4.1 the relationship between the eicosanoid production in percentages of the total formation per substance per patient and the density of the human peritoneal macrophage on the average for the 3 patients, is outlined.

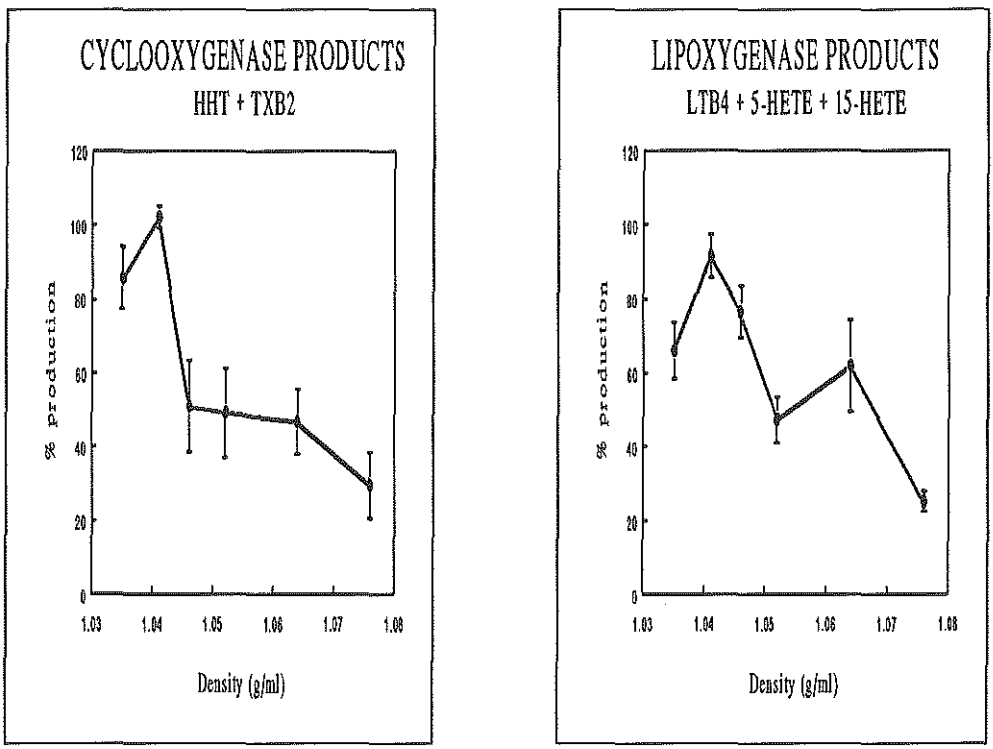

Figure 4.1 The average of the main eicosanoid production of one million human peritoneal density-defined Mϕ in percentages (highest production per eicosanoid and per patient is one hundred percent). A million human leucocytes were stimulated with A23187; the data are expressed as mean $\pm$ SD (n=3), with a correction for the number of Mϕ.

4.5 Discussion

Our preliminary results show that human peritoneal density-defined Mϕ also show morphological and biochemical heterogeneity, which might correlate with Mϕ maturation. Cell differentiation presumes that the least human mature Mϕ (the small round cells) are in the highest density gradient and the more mature Mϕ in the lower densities (cells with increasing cytoplasm). This has also been suggested for rat and human lung alveolar Mϕ [3-5].

The Mϕ subpopulations synthesize a variety of eicosanoids during inflammation, with the lipoxygenase products 5-HETE, LTB_4 and 15-HETE and the cyclooxygenase products HHT and TXB_2 as the main eicosanoid products.

The ability of human peritoneal Mϕ to synthesize eicosanoids seem to be density dependent. The Mϕ subpopulation of the highest density (1.076 g/ml) synthesize the least eicosanoids. The Mϕ subpopulations of lower densities have a higher level of eicosanoid production. The highest level of eicosanoid production is in the second

lowest dense macrophage subpopulation (1.041 g/ml).
Comparing the morphology and biochemical heterogeneity of the Mϕ subpopulations with each other, the following could be concluded: As the eicosanoid production is density dependent, we may assume it is depending on the maturity of the Mϕ. This is in agreement with previous findings with human Mϕ obtained from the peritoneal cavity during peritonitis. Freshly recruited Mϕ produced less eicosanoids than resident cells [5].
The Mϕ subpopulation of the lowest density has, compared to the subpopulation of the second lowest density a much more irregular shape and also produces less eicosanoids, these cells might be "dying".
The experiments described here confirm the idea that a relationship between the pattern and the abilities of human peritoneal density-defined Mϕ to produce eicosanoids exists.

4.6 References

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Part III

Human peritoneal macrophages: Production and interactions of their inflammatory mediators

Chapter 5 Production of inflammatory mediators by human macrophages obtained from ascites

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5.1 Summary

Ascites is a readily available source of human macrophages (Mϕ), which can be used to study Mϕ functions *in vitro*. We characterized the mediators of inflammation produced by human peritoneal Mϕ (hp-Mϕ) obtained from patients with portal hypertension and ascites.
The production of the cytokines interleukin 1ß (IL-1ß), interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) was found to be lipopolysaccharide (LPS) concentration dependent (0 - 10 μg/ml) with a maximal production at 10 μg/ml and also dependent on the time of exposure to the stimulus (0 - 36 h). IL-1ß, IL-6 and TNF-α production after LPS administration reached a plateau at 24 h.
In vitro stimulation for 24 h with LPS does not influence the eicosanoid production from endogenous arachidonate. Thirteen min of exposure of the cells to the calcium ionophore A23187 gives an significant increase in eicosanoid production from both exogenous and endogenous arachidonate. The main eicosanoids produced are the 5-lipoxygenase products leukotriene B_4 (LTB_4) and 5-hydroxy-eicosatetraenoic acid (5-HETE). The increase in production of the other eicosanoids are not significant.
The eicosanoid production depends on the stimulus concentration. The optimal A23187 concentration is 1 μM.
Oxygen radical production was measured in the Mϕ by a flowcytometric method. The fluorescence intensity of phorbol 12-myristate 13-acetate (TPA) stimulated and dihydro-rhodamine 123 (DHR 123) loaded hp-Mϕ increases significantly after 15 min.
We conclude that LPS stimulation of hp-Mϕ from liver disease results in similar production of IL-1ß, IL-6 and TNF-α, but that the profile of the eicosanoid production of these Mϕ stimulated with LPS and A23187 differs from Mϕ of other origin and species.

Key Words: human peritoneal macrophages . eicosanoids . interleukin 1ß . interleukin 6 . tumor necrosis factor α . respiratory burst

5.2 Introduction

Mϕ are of central importance in the initiation and regulation of non-specific and specific immune responses. They take part as active phagocytic cells, serve as antigen presenting cells (APC) for T-lymphocytes and are a major source of a wide range of inflammatory products including eicosanoids and cytokines in different types of immune responses (e.g. host defence against microorganism, antitumor and chronic inflammation).
When monocytes or tissue Mϕ are stimulated, both develop into active Mϕ. Profound changes then occur in morphology and function. Major changes are: increase in size, more complex and numerous surface folds and an increase in the numbers of vacuoles, lysosomes, phagosomes and endoplasmatic reticular elements. The adhesiveness and expression of Ia antigens increases. The Mϕ become more sensitive for agents which

trigger oxygen radicals and they secrete a large range of products including enzymes, coagulation factors, proteins of complement system, factors regulating cell activities and proliferation, bioactive lipids, nucleotide metabolites and reactive oxygen metabolites [1,2].
In vivo Mϕ activation appears to be mediated and/or modulated by microbial pathogens and their products, lipid mediators and cytokines [3,4]. *in vitro* activation can be initiated by a variety of agents, including A23187 [5], LPS [6,7] or cytokines [8,9]. The cytokines IL-1 [10], IL-6 [11] and TNF-α [12] are important Mϕ cytokine products when stimulated with LPS [7], which is one of the most potent stimuli of Mϕ [13].
Arachidonic acid (AA) can be metabolized to biologically active eicosanoids. The cyclooxygenase pathway generates the prostanoids and the 5-lipoxygenase pathway produces leukotriene LTB_4 and 5-HETE [14,15]. Eicosanoids exhibit distinct biological effects [16-19]. Like the cytokines they have an important function in controlling the immune reaction. A23187 is one of the most potent stimuli of eicosanoid production [5,20,21].
The respiratory burst is a characteristic feature of phagocytes. The production and release of radical oxygen intermediates (ROI) by Mϕ plays a major role in several of the effector functions of these cells. Oxygen radicals are of critical importance for antibacterial defence. They are also involved in tumoricidal activities, in suppression of lymphocyte proliferation [22,23] and have been implicated as important mediators of tissue damage in inflammation [24,25]. Oxygen radicals may be involved in a positive feedback mechanism of inflammation; superoxide anion (O_2^-) can induce IL-1 production in monocytes and polymorphonuclear leucocytes (PMNs) [26].
We have previously isolated human peritoneal Mϕ (hp-Mϕ) from ascitic fluid, obtained from patients with liver cirrhosis to investigate various aspects of their metabolism and the role of Mϕ in inflammation [27,28]. The aim of this study is to analyze the profile of eicosanoid production, the levels of cytokine production and the respiratory burst activity in this type of hp-Mϕ, regarding the potential influence of liver disease on these parameters, in comparison with other types of Mϕ.

5.3 Materials and methods

Subjects: The hp-Mϕ were obtained from ascitic fluid of 12 patients with liver cirrhosis, 2 patients with congestive heart failure, 1 patient with primary sclerosing cholangitis, 1 patient with malignant mesothelioma and 1 patient with the Budd-Chiari syndrome (Table 5.1). None of the patients were on drugs which have been reported to influence the lipoxygenase and cyclooxygenase enzymes.

Cell isolation: The hp-cells were isolated by centrifugation of the ascitic fluid at 400xg (4°C) and washed with phosphate buffered saline (PBS, pH 7.4, 4°C, Oxoid, UK). The concentrated hp-cell suspension was separated on Percoll (d=1.064 g/ml,

Table 5.1 List of patients with ascites (n = 17).

Number	m/f	- age	Diagnosis
1.	male	- 68	biliary liver cirrhosis
2.	male	- 65	congestive heart failure
3.	female	- 50	alcoholic hepatitis and liver cirrhosis
4.	female	- 72	alcoholic liver cirrhosis
5.	male	- 69	alcoholic liver cirrhosis
6.	female	- 41	alcoholic liver cirrhosis
7.	female	- 65	alcoholic liver cirrhosis
8.	male	- 69	congestive heart failure
9.	female	- 38	Budd- Chiari syndrome
10.	male	- 46	alcoholic liver cirrhosis
11.	male	- 65	cryptogenic liver cirrhosis
12.	male	- 59	alcoholic liver cirrhosis
13.	male	- 60	alcoholic liver cirrhosis
14.	male	- 39	primary sclerosing cholangitis
15.	female	- 42	alcoholic liver cirrhosis
16.	male	- 39	alcoholic liver cirrhosis
17.	male	- 82	malignant mesothelioma

Kabi-Pharmacia, Sweden). A small sample of cells was stained by Hemacolor (Merck,Germany) and the different cell types were counted under a microscope (Zeiss, standard 25, Germany). The viability of the cells was tested by trypan blue exclusion.

Cytokine production: For the measurement of the cytokine production one million leucocytes per ml RPMI complete [RPMI-1640 + HEPES (GIBCO, UK) + penicillin /streptomycin ($5x10^4$ u/l / 50 mg/l, Flow Lab, UK) + foetal calf serum (10 % FCS, GIBCO, UK) + L-glutamine (600 mg/l Flow Lab, UK)] were plated on plastic culture dishes (Costar, UK). The cells were stimulated with the addition of increasing concentrations of lipopolysaccharide (0 - 10 μg/ml, 36 h, LPS from E. coli 0111:B4 in PBS, Sigma, USA) or incubated in the absence or presence of LPS (10 μg/ml) for an increasing time period (0 - 36 h, 37°C, 7.5 % CO_2). As blanc PBS was added. The net cytokine production is the production with LPS minus blanc of cells incubated for the same period of time. At the end of the incubation the supernatant was filtered (0.22 μm, Millipore, France) and kept at -70°C till analyzing. IL-1ß and IL-6 production in the supernatant of the samples were measured by ELISA (IL-1ß: Medgenix, Belgium, IL-6: Hycult, Holland). TNF-α production of the samples was determined with a bioassay and partly by ELISA (Cistron, USA). For this bioassay the TNF-α sensitive cell line WEHI-164 was used. The WEHI-164 cells were plated out in 96-wells plates ($2x10^4$ cells/50 μl/well, Costar, UK) and the samples (50 μl/well) or the human recombinant TNF-α (hr-TNF-α) standards (0.1 - 1000 u/ml hr-TNF-α, 50 μl/well) were added. After 24 h of incubation (37°C, 7.5 % CO_2), MTT (Tetrazolium salt, Sigma, USA) was added (0.125 mg/well) and after an incubation of 3 h the cells were lysed with buffer (20 % Sodium Dodecyl Sulphate (SDS) in 50 % N,N-

dimethylformamide (DMF), pH 4.7, 100 μl/well) during 18 h The absorbance was measured at 595 nm with an ELISA-reader (BIO RAD, model 3550, UK). The TNF-α production by hp-Mϕ is expressed as the cytotoxicity against WEHI-164 cells compared to blanc (RPMI complete) (29).

Eicosanoid production: For the measurement of eicosanoid production from exogenous arachidonate, $1x10^6$ leucocytes per ml PBS (+ Ca^{2+}, pH 7.4, Oxoid, UK) in polypropylene tubes (Greiner, The Netherlands) were labelled with ^{14}C-AA (0.125 μCi, 55.7 mCi/mmol, Amersham, UK) for two min and triggered by A23187 (0 - 5 μM final concentration in dimethylsulfoxide (DMSO, Sigma, USA) for 13 min at 37°C. Controls were incubated with DMSO. ^{3}H-labelled standards consisting of 6-keto-prostaglandin $F_{1\alpha}$ ($6kPGF_{1\alpha}$), PGE_2, LTB_4, 12-HETE and 15-HETE (approximately 0.01 μCi of each eicosanoid, Amersham, UK) were added for recovery calculations and as retention time markers. The supernatants were then passed through Sep Pak C_{18} cartridges (Waters Ass., USA) and eluted with methanol, dried and dissolved in 200 μl methanol. Eicosanoid formation was measured on RP-HPLC using a Nucleosil $5C_{18}$ column (3x200 mm, Chrompak, The Netherlands). 100 μl sample was injected onto the column. As eluens a gradient of acetonitrile and 0.2 % triethylamine / 0.132 % trifluoracetic acid / water (pH >> 3) was used. The eicosanoid formation is expressed as the mean percentage of total formation of the most common metabolites per patient.
In these samples, both A23187 stimulated and non-stimulated, eicosanoid production from endogenous arachidonate was measured (LTB_4, PGE_2, thromboxane (TXB_2) and $6kPGF_{1\alpha}$). In the cytokine samples (24 h, 10 μg/ml LPS in RPMI) also the eicosanoid production from endogenous arachidonate was measured by radio immuno assays (RIA; Antibodies were obtained from Advanced Magnetics, USA and standards from Sigma, USA). The exogenous eicosanoid production is given as net production. This is the production of the cells with A23187 minus control values.

Respiratory burst activity: ROI production was measured with DHR 123 (Molecular probes, USA). The stock solution was 1 mg/ml in N,N-dimethylformamide (Sigma, USA); further dilution was performed with PBS. DHR 123 is oxidized intracellularly to fluorescent rhodamine 123 (R123) by hydrogen peroxide.
Leucocytes ($0.25x10^6$/ml) were incubated in RPMI in polypropylene tubes with increasing concentrations of TPA (0 - 1000 ng/ml, TPA diluted in DMSO:PBS = 1:10, Sigma, USA, 10 min, 37°C, blanc is 10 % DMSO) directly followed by incubations with increasing concentrations of DHR 123 (0 - 100 ng/ml, 15 min, 37°C). In the time experiments TPA and DHR 123 were added to the cells at the same time and incubated for an increasing time period (0 - 1 hour, 100 ng/ml TPA, 333 ng/ml DHR 123, 37°C). Samples were put on ice straight after the incubations. The fluorescence intensity [mean channel number (MCN)] was measured with a fluorescence activating cell scanner (FACS, Becton Dickinson Immunocytometry Systems, USA). R123 is detected as Fluorescence 1 (green; 500-530 nm) (30,31).

5000 cells per sample were measured. The data were evaluated with the FACS SCAN program system (Becton Dickinson Immunocytometry Systems, USA). The net ROI production is the fluorescence intensity (in MCN) of the TPA stimulated and DHR 123 loaded cells, minus the fluorescence intensity (in MCN) of the non stimulated cells which were also loaded with DHR 123 and incubated for the same period of time.

Statistical analysis: The data are expressed as the mean ± standard error of the mean (SEM). Data were statistically analyzed with Anova followed by the Student T-test or the student T-test only; data were considered significant when $p < 0.05$.

5.4 Results

Cell differentiation: The Percoll separated cell suspension was microscopically examined and the following composition of cells was found: 94.6 ± 1.2 % Mϕ, 4.0 ± 1.1 % lymphocytes and 1.2 ± 0.4 % granulocytes (n=17). The viability of the cells was 86 ± 1.3 % (range: 94.2 - 75.8).

Cytokine production: To determine LPS concentration for optimal IL-1ß, IL-6 and TNF-α production, hp-Mϕ were stimulated with increasing concentrations of LPS (0-10 μg/ml) for 36 h as shown in Figure 5.1. The highest production was with the highest LPS concentration used. All subsequent experiments were performed with 10 μg/ml LPS.
The time dependent net IL-1ß, IL-6 and TNF-α production from 0 to 36 h with LPS is shown in Figure 5.2. The maximal net cytokine production for all three cytokines was reached at 24.
The IL-1ß (n=16), IL-6 (n=10) and TNF-α (n=10/17) production without or after stimulation with 10 μg/ml LPS for 24 h is shown in Table 5.2. LPS stimulation resulted in a significant increase in the production of each cytokine.
Mϕ from 6 out of 16 patients had no IL-1ß production without LPS, all patients had IL-6 production without LPS stimulation and 6 out of 10 patients had no TNF-α production without LPS.

Table 5.2 Cytokine production by hp-Mϕ. IL-1ß, IL-6 and TNF-α generation by 10^6 hp-Mϕ/ml stimulated for 24 h with 10 μg/ml LPS. Data are expressed as mean ± SEM and statistically analyzed by Student T-test.

PRODUCT	CONTROL	LPS	n
IL-1ß (ng/ml)	3.0 ± 1.2	8.7 ± 1.5 *	16
IL-6 (ng/ml)	6.1 ± 1.4	32.6 ± 7.1 *	10
TNF-α (ng/ml)	0.1 ± 0.1	2.7 ± 1.1 *	10
TNF-α y(% killing)	21.6 ± 5.1	47.7 ± 3.9 *	17

* $p < 0.05$ (increase vs controls). n = number of patients.

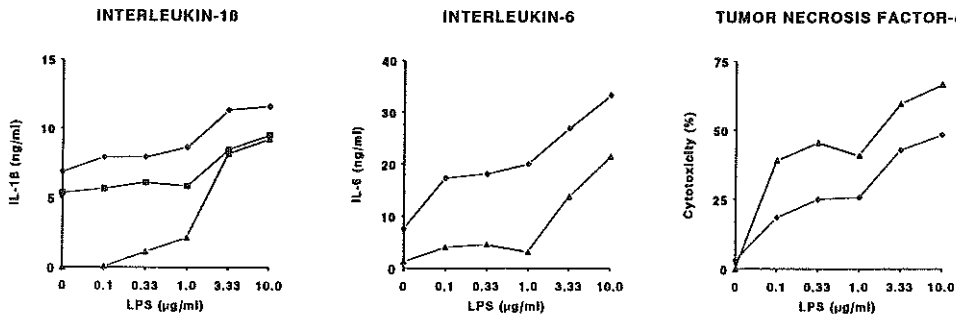

Figure 5.1 Interleukin 1ß (n=3, ng/ml, left figure), interleukin 6 (n=2, ng/ml, middle figure) and tumor necrosis factor α (n=2, % killing, right figure) production by hp-Mϕ (10^6/ml) stimulated with an increasing concentration LPS; 0 - 10 µg/ml. The results are shown separately per patient (■, ◆, ▲).

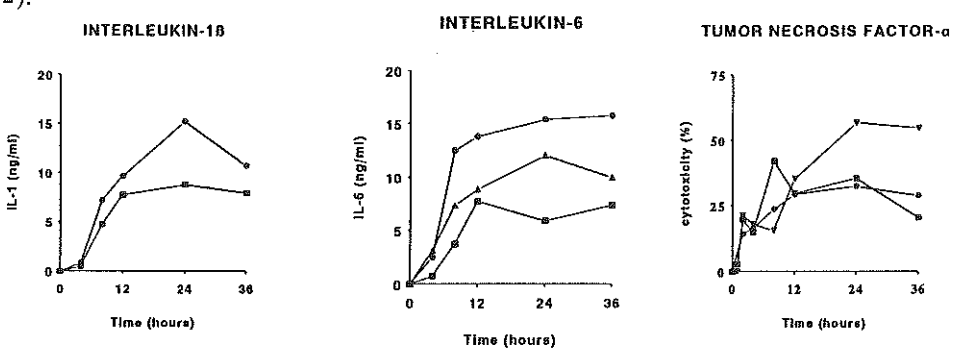

Figure 5.2 The time dependency of net interleukin 1ß (n=2, ng/ml, left figure), interleukin 6 (n=3, ng/ml, middle figure) and tumor necrosis factor α (% killing, n=3, right figure) production of hp-Mϕ (10^6/ml) stimulated with 10 µg/ml LPS. The cells were stimulated for 0 - 36 h. The results are shown separately per patient (■, ●, ▲).

Eicosanoid production: First the A23187 concentration was determined (0 - 5 µM) which yielded the most eicosanoid production. Figure 5.3 (top and middle) shows the LTB_4 and 5-HETE production from added ^{14}C-AA (exogenous production) and Figure 5.3 (bottom) the endogenous LTB_4 production as measured by RIA of 3 patients. The optimal concentration is 1 - 2 µM. Consequently all following experiments were performed with 1 µM A23187.

All eicosanoids formed from exogenous arachidonate by hp-Mϕ after A23187 stimulation are listed in Table 5.3A. The main net eicosanoids were the 5-lipoxygenase products LTB_4 and 5-HETE, the minor net eicosanoids were the cyclooxygenase products hydroxy-heptadecatrieoic acid (HHT) and TXB_2. Other eicosanoids were less then 1.5 % of ^{14}C-AA which was converted per product.

To investigate if eicosanoid production from exogenous and endogenous arachidonate were related to each other, four eicosanoids were measured in the same samples by RIA, as in which ^{14}C-AA conversion was determined by HPLC (Table 5.3B).

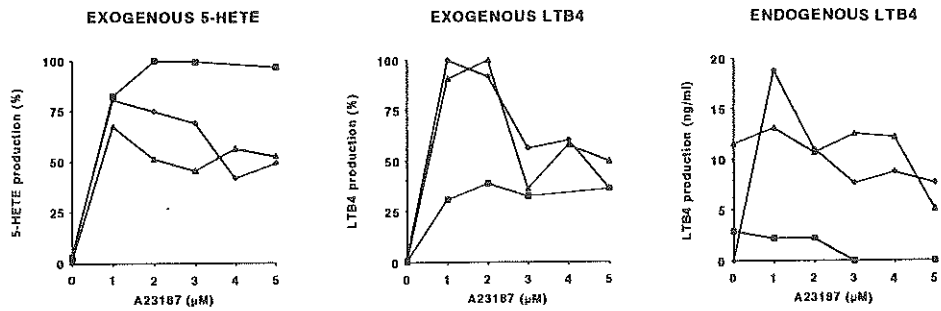

Figure 5.3 Eicosanoid formation from exogenous (left figure; 5-HETE, middle figure; LTB_4, % of total formation per patient) and endogenous arachidonate (right figure; LTB_4, ng/ml) production of hp-Mϕ (10^6/ml). Cells were prelabeled with ^{14}C-AA (2 min), triggered with 1 μM A23187 for 13 min and ^{3}H labelled standards were added afterwards. The results of three patients are shown separately; (■, ◆, ▲).

Table 5.3A Eicosanoid production from exogenous arachidonate by hp-Mϕ. Profile of eicosanoid production from exogenous ^{14}C-arachidonic acid in 10^6 hp-Mϕ/ml after A23187 stimulation (1 μM, 13 min, expressed as percentages of total formation per patient).

NET EXOGENOUS LIPOXYGENASE PRODUCTS A23187 (%)		NET EXOGENOUS CYCLOOXYGENASE PRODUCTS A23187 (%)		n
LTB_4	32.1 ± 3.9 *	$6kPGF_{1\alpha}$	0.5 ± 0.3	5
5-HETE	57.2 ± 3.9 *	TXB_2	2.6 ± 1.3	5
di-HETE	1.4 ± 0.4	HHT	4.3 ± 1.6	5
12-HETE	0.3 ± 0.2	$PGF_{2\alpha}$	0.9 ± 0.5	5
15-HETE	0.7 ± 0.3	PGE_2	0.1 ± 0.04	5
		PGD_2	0.1 ± 0.1	5

* $p < 0.05$ (increase vs controls).

The main eicosanoid was also a 5-lipoxygenase product (LTB_4). Cyclooxygenase production did not increase significantly with A23187.
Furthermore eicosanoid production from endogenous arachidonate was measured in the same samples as in which the cytokine production was measured (24 h LPS). LPS did not influence significantly eicosanoid production from endogenous arachidonate over 24 h as measured by RIA (Table 5.4).

Table 5.3B Eicosanoid production from endogenous eicosanoid by hp-Mϕ. Eicosanoid production from endogenous arachidonate in 10^6 hp-Mϕ/ml with and without A23187 stimulation (1 μM, 13 min, expressed as ng/ml).

	CONTROL (ng/ml)	A23187 (ng/ml)	n
LIPOXYGENASE PRODUCT LTB_4	1.0 ± 0.3	7.6 ± 1.7 *	11
CYCLO-OXYGENASE PRODUCTS			
PGE_2	0.1 ± 0.1	0.24 ± 0.1	11
TXB_2	1.8 ± 0.2	1.8 ± 0.3	5
$6kPGF_{1\alpha}$	0.4 ± 0.4	0.4 ± 0.3	5

* $p < 0.05$ (increase vs controls).

Table 5.4 Eicosanoid production from endogenous arachidonate by hp-Mϕ. Eicosanoid production from endogenous arachidonate in 10^6 hp-Mϕ/ml with LPS (10 μl/ml, 24 h, expressed as ng/ml). LPS stimulation does not significantly ($p < 0.05$) stimulate the eicosanoid production (vs. controls).

	CONTROL (ng/ml)	LPS (ng/ml)	n
LIPOXYGENASE PRODUCTS LTB_4	1.0 ± 0.2	1.2 ± 0.3	16
CYCLO-OXYGENASE PRODUCTS			
PGE_2	1.7 ± 0.3	2.1 ± 0.2	16
TXB_2	3.4 ± 0.3	3.2 ± 0.2	5
$6kPGF_{1\alpha}$	2.7 ± 0.3	3.1 ± 0.3	5

Respiratory burst: To measure the respiratory burst activity of the hp-Mϕ, the method published by Emmendorffer et al. using DHR 123 for human neutrophils (31) was modified for peritoneal Mϕ. The optimal net formation of ROI loaded with DHR 123 (0 - 1000 ng/ml, 10 min) and stimulated with TPA (0 - 1000 ng/ml, 15 min) is given in Figure 5.4 (top left and top right). All subsequent experiments were performed with 100 ng/ml TPA and 333 ng/ml DHR 123.

To check whether this method depended on the cell activity, the ROI production wasalso measured in cells (n=4) which were kept on ice during the stimulation and loading (10 + 15 min). In Figure 5.4 (left bottom) the temperature dependency for the ROI production at 4°C (n=4) and 37°C (n=10) is shown. There was no significant ROI production increase at 4°C, but TPA increased the net ROI production significantly at 37°C. The respiratory burst activity dependence on the time of stimulus is shown in Figure 5.4 (bottom right). The ROI production was significantly different after 15 min of stimulation. DHR 123 and TPA were given at the same time now.

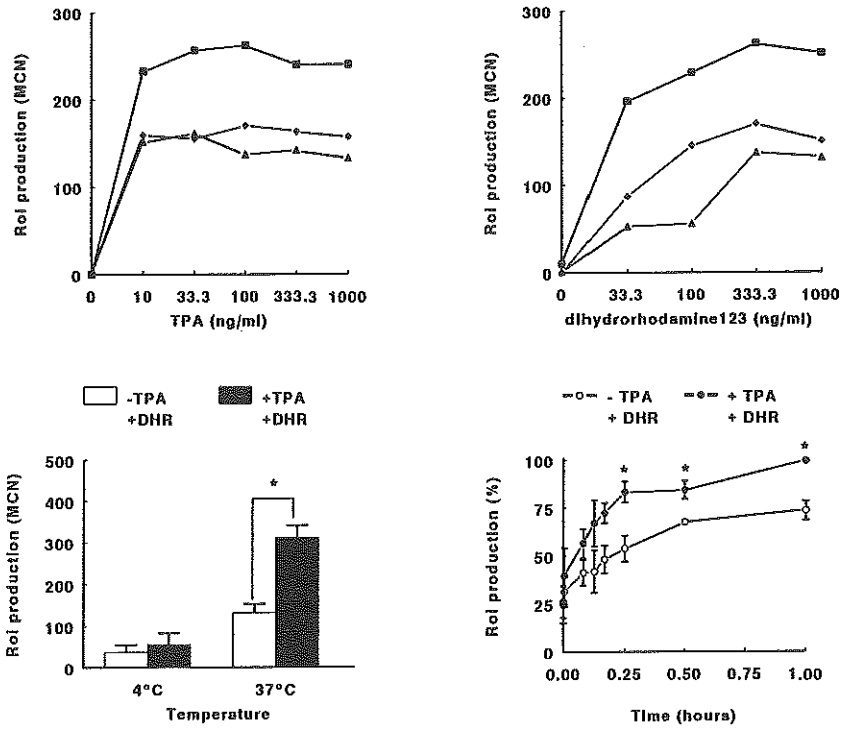

Figure 5.4 The radical oxygen intermediates (ROI) of hp-Mϕ ($0.25x10^6$/ml) was measured with dihydrorhodamine 123 (DHR 123). Left top figure shows the dependency of net ROI production on phorbol 12-myristate 13-acetate (TPA) concentration (0 - 1000 ng/ml, 10 min TPA + 15 min 333 ng/ml DHR 123, 37°C, n=3). Right top figure shows the dependency of net ROI production on DHR 123 concentration (0 - 1000 ng/ml, 10 min 100 ng/ml TPA + 15 min DHR 123, 37°C, n=3). The results of three patients are shown separately; (■, ◆, ▲). Left bottom figure shows the spontaneous (open bar) and TPA-stimulated (closed bar) respiratory burst activity at 4°C (n=4) versus 37°C (n=10) (10 min blanc or 100 ng/ml TPA + 15 min 333 ng/ml DHR 123). In these 3 graphs the ROI production is given in mean channel number (MCN). Data are expressed as mean ± SEM and statistically significant (*) when $p < 0.05$ vs. controls (spontaneous respiratory burst). Right bottom figure shows the stimulus time dependency (n=3). Blanc (○) or TPA (●) and DHR 123 were given at the same time (t= 0 - 1 hr, 100 ng/ml TPA, 333 ng/ml DHR 123, 37°C, n=3). The ROI production is expressed as % increase of maximal increase (= 100 %) per patient with TPA. Data are expressed as mean ± SEM and statistically significant (*) when $p < 0.05$ vs. controls (t = 0 h).

5.5 Discussion

Aim: In previous experiments we have shown that ascites is a good source of hp-Mϕ which can be used to study eicosanoid production by human Mϕ [27,28,32]. These Mϕ can be used as a model for testing the effects of anti-inflammatory drugs on the eicosanoid production *in vitro*, as we have shown previously with drugs such as Malotilate [33] and E6080 [34].

By gaining more information on the inter-relationships between the inflammatory

mediators, such as eicosanoids, cytokines and oxygen radicals of the hp-Mϕ we expect to expand our *ex vivo* anti-inflammatory drug testing model with human inflammatory cells.
The aim of this study was to characterize in addition to the eicosanoid production other cell functions of hp-Mϕ including cytokine production and respiratory burst activity.

Results: We investigated the cytokine production of hp-Mϕ stimulated with LPS as this is an important stimulus for Mϕ *in vivo* [7,13].
The eicosanoid production was determined both with LPS and A23187 as this stimulus is known to be a potent trigger for lipoxygenase products derived from Mϕ [5,20,21].
The experiments showed that the production of the cytokines IL-1ß, IL-6 and TNF-α was LPS concentration dependent and also dependent on the time of exposure to the stimulus.
LPS stimulation influenced the net IL-1ß, IL-6 and TNF-α production significantly, but did not have an appreciable effect on the eicosanoid production from endogenous arachidonate.
A short stimulation with A23187 gave a significant increase in lipoxygenase products from exogenous and endogenous arachidonate. The eicosanoid production from exogenous and endogenous arachidonate depended on the A23187 concentration.
Production of measurable amounts of ROI *in vitro* of the hp-Mϕ can be triggered by TPA. With the lowest concentration of the stimulating agent the ROI production was maximal, higher concentrations of TPA did not change the level of production. The measurement of ROI production was DHR 123 concentration dependent. Beyond 333 ng/ml the ROI production slightly decreased, the DHR 123 concentration was no longer a limiting factor.
With optimal concentrations of TPA and DHR 123 the net respiratory burst activity increased significantly. When stimulus and DHR 123 were given at same time, the increase was significant after 15 min.

Comparison of cytokine and eicosanoid production in Mϕ from different species and/or origin: Similar kinetics as we had found for LPS induced cytokine production have been previously reported for human alveolar Mϕ [35-37] hp-Mϕ obtained from continuous ambulatory peritoneal dialysis (CAPD) patients [38,39], a murine macrophage cell line [40] and murine peritoneal Mϕ [41-43].
In all these observations the cytokine production increased with increasing LPS concentration with a maximum in the range 1 - 10 μg/ml LPS. Human alveolar Mϕ had with LPS a maximal IL-1ß (0 - 42 h and IL-6 (0 - 72 h) production by 24 h [a slight increase at 72 h for IL-6), 35,36].
The IL-1 production of the murine macrophage cell line P388D1 (0 - 18 h) and murine peritoneal Mϕ (Alderey Park, 0 - 24 h) increased in time with LPS [40,42]. Peritoneal Mϕ from C3H/HEN and C3H/HEJ mice had with LPS (0 - 72 h) a maximal Il-1 production at 24 h [41]. The IL-6 production was maximal between 8 - 12 h for the murine macrophage cell line [40].

TNF-α production of Mϕ of other origin or species usually reached a maximal level at an earlier time then our hp-Mϕ. TNF-α (0 - 42 h) production of human alveolar Mϕ peaked between 4 - 8 h [35]. The murine macrophage cell line stimulated with LPS reached a plateau at 2 h [40]. Rat peritoneal Mϕ reached a maximum at 6 h [44] and guinea pig peritoneal Mϕ around 8 h [45].

In our experiments LTB_4 and 5-HETE were the main eicosanoids formed (89 %), when the Mϕ were stimulated with A23187. In normal bovine alveolar Mϕ A23187 stimulated the release of both the cyclooxygenase and lipoxygenase products [21], also in resident mouse peritoneal Mϕ [46,47]. Rat peritoneal Mϕ and rat Kupffer cells mainly synthesized cyclooxygenase products after A23187 stimulation [32].
In alveolar Mϕ from healthy volunteers, exogenous arachidonic acid was predominantly metabolized via the 5-lipoxygenase pathway [20,48,49], but also cyclooxygenase products were formed, when stimulated with A23187 [20,49].
Leukotrienes are known to mediate inflammatory reactions. LTB_4 is an important chemotactic agent for leucocytes [16], generates superoxide anion in neutrophils [17], whereas the sulfidopeptide leukotrienes (LTC_4, LTD_4 and LTE_4) primarily affect smooth muscle contraction, increase vascular permeability [18] and promotes lysosomal enzymes [19].
Specific inhibition of the enzyme 5-lipoxygenase has therapeutical potential in a variety of inflammatory conditions shown by different animal models and clinical trials [50-52] and as mentioned before we have tested the drugs Malotilate [33] and E6080 [34] on the eicosanoid production in our *in vitro* model.

The pattern of eicosanoid metabolism depends on the stimulus applied [20,21,48], as well as the maturity of the cell [49], site of origin of macrophage and the species [32]. In relation to this, *in vivo* priming might be another important point for influencing the eicosanoid and cytokine profile when the cells are stimulated *in vitro*. Cytokines and eicosanoids regulate also their own and each other's release *in vivo* and *in vitro* [53-55]. The exact interaction of these mediators involved in human macrophage inflammation still has to be investigated.

Comparison of cytokine and eicosanoid production in hp-Mϕ from liver cirrhosis and CAPD patients: The high cytokine production of our hp-Mϕ with LPS was probably due to priming of the cells *in vivo*. The patients, where the cells were isolated from, have a chronic inflammation. Peritoneal Mϕ obtained from CAPD patients during peritonitis and compared to Mϕ obtained from the same type of patients during a infection free period, stimulated with LPS *in vitro*, released significantly more IL-1ß and TNF-α [39,56] whereas their prostacyclin release (measured as $6kPGF_{1\alpha}$, without and with LPS) had declined sharply [38].
The TNF-α bioactivity of the liver cirrhosis Mϕ seems lower then Mϕ of CAPD patients with peritonitis, however TNF-α was measured by different bioassays. Although the methods are comparable the % killing is higher with the ^{3}H-thymidine

Table 5.5 Comparison of cytokine and Prostaglandin synthesis by human peritoneal macrophages from liver cirrhosis and continuous ambulatory peritoneal dialysis (CAPD) patients without and with peritonitis. Compared are the eicosanoid and cytokine synthesis without stimulus (24 h PBS = blancs) and with stimulus (24 h LPS = LPS), LPS = 5 μg/ml CAPD hp-Mϕ (10^6 cells/ml) and 10 μg/ml with liver cirrhosis hp-Mϕ (10^6 cells/ml), n = number of patients.

Liver Cirrhosis (this study)			n	CAPD [38,39,56]			
				Control	n	Peritonitis	n
IL-1ß (ng/ml)	blancs	3.0 ± 1.2	16	0.3 ± 0.1	9	0.2 ± 0.1	8
	LPS	8.7 ± 1.5		1.0 ± 0.2		6.6 ± 2.8	
TNF-α (% killing)	blancs	21.6 ± 5.1	17	4.3 ± 5.1	21	12.9 ± 4.1	12
	LPS	47.7 ± 3.9		15.8 ± 6.5		61.9 ± 7.7	
TNF-α (ng/ml)	blancs	0.1 ± 0.1	10	1.0 ± 0.3	12	1.0 ± 0.3	12
	LPS	2.7 ± 1.1		2.6 ± 0.4		9.3 ± 4.3	
$6kPGF_{1\alpha}$ (ng/ml)	blancs	2.7 ± 0.3	5	19.1 ± 3.6	10	3.4 ± 1.4	10
	LPS	3.1 ± 0.3		21.4 ± 4.3		4.0 ± 1.2	
PGE_2 (ng/ml)	blancs	1.7 ± 0.3	5	15.1 ± 2.7	10	9.4 ± 2.6	10
	LPS	2.1 ± 0.2		27.4 ± 4.5		31.0 ± 4.4	

incorporation cytostatic assay compared to the MTT tetrazolium cytotoxic assay [29]. Therefore the TNF-α bioactivity of hp-Mϕ from liver cirrhosis patients compared to Mϕ from CAPD patients with peritonitis could be in the same range. The concentrations of TNF-α measured by ELISA in supernatants of liver cirrhosis patients however showed a TNF-α production comparable to Mϕ from CAPD patients without peritonitis.

A pronounced difference between the Mϕ from liver cirrhosis and the Mϕ of CAPD patients with peritonitis was observed in the PGE_2 production. The PGE_2 release of human peritonitis Mϕ without LPS was initially much higher then the hp-Mϕ in our observations. Furthermore a significant increase in PGE_2 production in response to LPS was seen (no difference compared to infection-free Mϕ of CAPD patients).
In conclusion the Mϕ of liver cirrhosis patients have striking features with Mϕ from CAPD patients with peritonitis (see Table 5.5).

Oxygen radical production: In response to TPA the ability to generate oxygen radicals was enhanced. TPA stimulates the intracellularly and extracellularly respiratory burst of phagocytes [57,58]. The flow cytometric method we have used here reflects the total production of H_2O_2 inside single, stimulated, cells. DHR 123 is oxidized by a hydrogen peroxide and peroxidase dependent system. This method with DHR 123 seemed to be a highly sensitive indicator for the respiratory burst [59-61]. In comparison with human monocytes and neutrophils the respiratory burst of monocyte-derived Mϕ was very low when measured with the luminol-amplified chemiluminescence assay [61], which measures intra- and extracellular H_2O_2 and O^{2-} [58]. We were able to measure with DHR 123 a clear increase in the respiratory burst activity, when the Mϕ were stimulated with TPA. Our results were similar to Banati [30] whom measured the respiratory burst of rat peritoneal Mϕ with DHR 123. The Mϕ exhibit spontaneous respiratory burst activity already before stimulation and after stimulation the fluorescence intensity had increased about a factor 3, our ROI production (in MCN) increased a factor 2.4.

Conclusion: Ascitic fluid is an easily available source of large quantities of hp-Mϕ. In this study we have characterized the eicosanoid and cytokine production and respiratory burst activity of human Mϕ isolated from peritoneal ascitic fluid. The IL-1ß, IL-6 and TNF-α kinetics of hp-Mϕ stimulated with LPS is similar to that of human lung Mϕ, peritoneal Mϕ of CAPD patients and peritoneal Mϕ of other species. The cytokine production is LPS concentration dependent and dependent on the time of exposure to LPS. The profile of eicosanoid production depends on both the species origin, location and the stimulus applied. LPS did not effect the eicosanoid production from endogenous arachidonate of the hp-Mϕ, while a stimulation with A23187 both increased the lipoxygenase production from exogenous and endogenous arachidonate. The Mϕ of liver cirrhosis patients have striking features with Mϕ from CAPD patients with peritonitis, probably due to priming of the Mϕ *in vivo*.
With optimal concentrations of TPA and DHR 123 the net respiratory burst activity in the hp-Mϕ increased significantly. Whether a relation exists between formation of cytokines and eicosanoids and the respiratory burst activity of the hp-Mϕ still has to be examined.
Characterization of hp-Mϕ function show that ascites may be a promising source of Mϕ to set up a model for testing the effects of anti-inflammatory drugs *in vitro* on different types of inflammatory mediators produced by human Mϕ.

5.6 References

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Chapter 6 Effect of a novel 5-lipoxygenase inhibitor, E6080 on the eicosanoid production of human peritoneal macrophages

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6.1 Summary

This study was performed to determine the selectivity of the 5-lipoxygenase inhibitor 6-Hydroxy-2-(4-sulfamoylbenzylamino)-4,5,7-trimethylbenzothiazole hydrochloride (E6080) on the *in vitro* eicosanoid metabolism in human peritoneal cells of patients with ascites. The IC_{50} for E6080 on the formation of the 5-lipoxygenase products leukotriene B_4 (LTB_4) and 5-hydroxy-eicosatetraenoic acid (5-HETE) was respectively 3.7 μM and 1.7 μM. The production of the other lipxygenase (8-, 12-, 15-HETE) and cyclooxygenase products [hydroxy-heptadecatrieoic acid (HHT), 6-keto-prostaglandin $F_{1\alpha}$ ($6kPGF_{1\alpha}$), thromboxane B_2 (TXB_2), $PGF_{2\alpha}$, PGE_2 and PGD_2) were not significantly inhibited in the dose range we have studied (0.3 μM - 30 μM). E6080 is specific and equipotent to most of the known potent 5-lipoxygenase inhibitors.

Keywords: E6080 . 5-lipoxygenase inhibitor . arachidonic acid metabolism . human peritoneal cells

6.2 Introduction

One potential approach for the treatment of chronic inflammatory diseases is to suppress the release of inflammatory mediators.
Eicosanoids are among the most important inflammatory mediators and modulators of both the initial and late phase of the inflammatory reaction.
Leukotrienes, a group of 5-lipoxygenase products belonging to the eicosanoids, have marked effects on the neutrophil migration, neutrophil and monocyte aggregation, release of lysosomal enzymes, capillary permeability, induction of pain and smooth muscle contraction [1,2]. Different animal models and clinical trials have shown that drugs which block leukotriene synthesis by specific inhibition of the enzyme 5-lipoxygenase, have therapeutic potential in a variety of inflammatory conditions such as inflammatory bowel disease [3], rheumatoid arthritis [4], anaphylaxis [5,6,7], asthma [8,9,10], endotoxic shock [11] and edema and pleurism [12,13].
Several 5-lipoxygenase blockers have been described, of varying specificity. Recently Tsunoda et. al. reported that the new compound E6080 suppressed anaphylaxis and bronchospasm in animal models, probably by blocking leukotriene synthesis [5,8]. To determine whether this compound is indeed a specific and potent 5-lipoxygenase blocker and also effective in human cells, we examined its effects on the pattern of eicosanoid production by human peritoneal cells. We compared our findings on specificity and potency with those reported for other 5-lipoxygenase blockers.

6.3 Materials and methods

Subjects: Peritoneal cells were obtained from ascites of five patients with portal hypertension (5 patients). None of the patients were on drugs which have been reported to influence the lipoxygenase and cyclooxygenase enzymes.

Cell isolation: The human peritoneal cells were isolated by centrifugation of the ascitic fluid at 400xg (4°C) and washed with phosphate buffered saline (PBS, pH 7.4, 4°C, Oxoid, UK). A small sample of the cells were stained by Hemacolor (UK) and the different cell types were counted under a microscope (Zeiss, standard 25, Germany).

Incubation: One million peritoneal cells suspended in Dulbecco's minimal essential medium (Dulbecco's MEM, Gibco, UK) were incubated for two min with 6-Hydroxy-2-(4-sulfamoylbenzylamino)-4,5,7-trimethyl-benzo thiazole hydrochloride (E6080, Eisai Co., Japan; dose range: 0.3 μM - 30 μM), labelled with ^{14}C-arachidonic acid (0.125 μCi, 55.7 mCi/mmol, Amersham, UK) for two min and triggered by the calcium ionophore A23187 (2 μM, final concentration) for 13 min at 37°C. ^{3}H labelled standards consisting of 6kPGF$_{1\alpha}$, PGE$_2$, LTB$_4$, 12-HETE and 15-HETE (approx. 0.01 μCi of each eicosanoid, Amersham, UK) were added for recovery calculations and as retention time markers. The supernatants were then passed through Sep Pak C$_{18}$ cartridges (Waters Ass., USA) and eluated with methanol, dried and dissolved in 200 μl methanol.

Eicosanoid production: Exogenous eicosanoid formation was measured on RP-HPLC using a Nucleosil 5C$_{18}$ column (3x200 mm, Chrompack, The Netherlands). 100 μl sample was injected onto the column. As eluens a gradient of acetonitrile and 0.2% triethylamine / 0.12% trifluoracetic acid / water (> > pH 3) was used.

Statistical analysis: The data are expressed as the mean $\pm$ standard error of the mean (SEM, n=5). The IC$_{50}$ values were calculated by BMDP software module for non-linear curve-fitting [14]. The curve was fitted to a four parameter logistic function [15].

6.4 Results

Cell differentiation: Microscopically the following cells were found: 77 $\pm$ 10.4 % macrophages (MÝ), 16 $\pm$ 11.3% Neutrophils, 7 $\pm$ 5.0 % lymphocytes and 0.1 $\pm$ 0.1 % eosinophils.

Eicosanoid production: Figure 6.1 shows the main lipoxygenase products formed by human peritoneal cells consisting of LTB$_4$ (16.7 %), 5-HETE (15.6 %) and 15-HETE (4.5 %). The minor lipoxygenase products were 8-HETE (1.3 %) and 12-HETE (1.0 %). The main cyclooxygenase products were HHT (20.0 %), 6kPGF$_{1\alpha}$ (19.6 %) and

TXB_2 (6.2 %) and as minor cyclooxygenase products $PGF_{2\alpha}$ (3.1 %), PGE_2 (2.4 %) and PGD_2 (1.8 %) were formed.

Eicosanoid inhibition: In figure 6.2 the influence of E6080 on the production of the most common eicosanoid products of the human peritoneal cells, is presented. E6080 inhibited, in a concentration range of 0.1 μM -30.0 μM, the 5-lipoxygenase products LTB_4 and 5-HETE in a selective manner. The IC_{50} values for LTB_4 and 5-HETE were respectively 3.7 and 1.7 μM. The production of the other lipoxygenase and cyclooxygenase products was not significantly inhibited in the dose range studied.

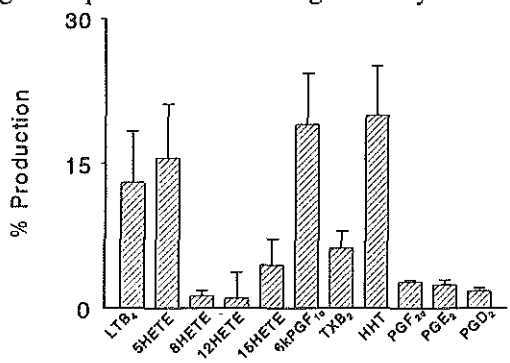

Figure 6.1 Arachidonic acid metabolites formed by peritoneal cells of patients with ascites, expressed as the mean percentage of total formation of the most common metabolites $\pm$ SEM (n = 5).

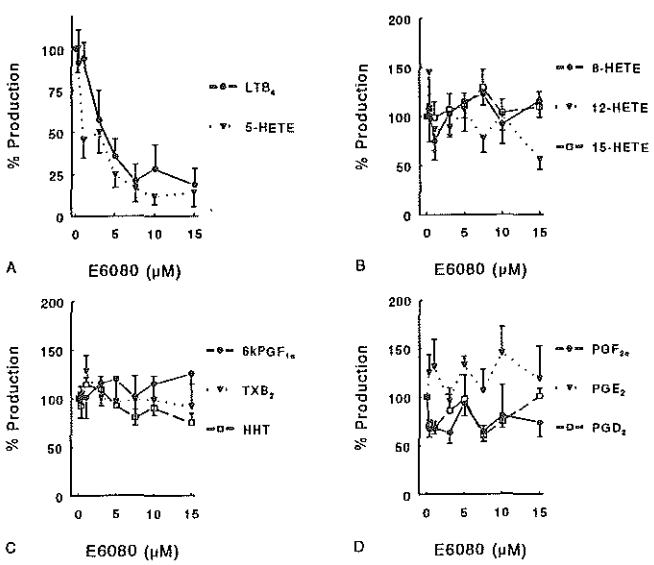

Figure 6.2 Effect of E6080 on the formation of the 5-lipoxygenase products LTB_4 and 5-HETE (1A), the other lipoxygenase products, 8-HETE, 12-HETE and 15-HETE (1B) and cyclooxygenase products, $6kPGF_{1\alpha}$, TXB_2 and HHT (1C), $PGF_{2\alpha}$, PGE_2 and PGD_2 (1D) in human peritoneal cells. Each point represents the mean $\pm$ SEM (n = 5). Control = 100 % of metabolite formation.

The effects of E6080 on 5-, 12- and 15-lipoxygenase and cyclooxygenase are summarized in table 6.1 and compared with reported IC_{50} values for the following drugs: A64077, AA861, MK861, BWA137C and CGS 8515.

Table 6.1 Comparison of the potencies and selectivities of some novel 5-lipoxygenase inhibitors and E6080 *in vitro* (REF = reference; > > = higher then; n.d.= not detected).

DRUG	REF	IC_{50} (μM)			
		LTB_4	5-HETE	12/15-LO	CO
E6080 *in vitro* *in vivo*	this paper 8	3.7 approx 1	1.7 n.d.	> > 30 n.d.	> > 30 n.d.
A64077	4,16	0.16 - 2	0.53	> > 100	4.8 - 25
AA861	12,16,23	0.08-0.8	0.01-0.8	100 (12-LO)	15
MK-886	9,19	0.0025-1.1	0.003-0.013	> > 1.1	> > 1.1
BW A137C	24	0.1 - 0.8	n.d.	2.2 - 15	22 - 32.4
CGS 8515	13	0.1-3.8	0.02-1.0	> > 30 12-LO > > 500 15-LO	60 - 340

6.5 Discussion

Human peritoneal Mϕ (hp-Mϕ) are an easily available source of inflammatory cells, which can be used to test new drugs for the effects on inflammatory mediators and may be a first choice model for anti-inflammatory drugs tested *in vitro* before clinical studies are started.
E6080 proved to be a potent 5-lipoxygenase inhibitor in human peritoneal cells (Mϕ, neutrophile granulocytes, eosinophils and lymphocytes) with an IC_{50} between 1.7 - 3.7 μM.
According to Tsunoda et. al. the IC_{50} of E6080 on LTB_4 production in an antigen inhalation sensitized guinea pig model, measured in broncho alveolar lavage fluid, was approximately 1 μM [8]. This suggest that the IC_{50} values of E6080 found *in vitro* for human peritoneal cells for 5-lipoxynase, are similar to those found *in vivo* in the anaphylactic shock animal model.
E6080 is an orally active 5-lipoxygenase inhibitor [8] which we have shown now by examining overall eicosanoid production, to be specific. E6080 inhibits, in a concentration range from 0.1 to 30 μM, the 5-lipoxygenase products LTB_4 and 5-HETE in a selective manner without effecting the other lipoxygenase and

cyclooxygenase products.
Potent specific 5-lipoxygenase inhibitors for clinical use are not available as yet, although clinical trials with some novel 5-lipoxygenase inhibitors are at present performed.
A-64077 (Zileuton) seems to be a useful oral 5-lipoxygenase inhibitor. *In vitro* in different models the IC_{50} value is quite low [4,16]. In clinical trials in patients with ulcerative colitis A-64077 caused a significant fall in the median LTB_4 levels [3], and in other clinical trials it ameliorated the asthmatic response to cold, dry air [17] and attenuated allergen-induced nasal congestion and the release of 5-lipoxygenase products in nasal-rinse [7]. However, results in atopic asthma have been disappointing [10].
MK-886 seems to be a very potent and specific 5-lipoxygenase inhibitor (So far as known the only 5-lipoxygenase inhibitor through a new mechanism: 5-lipoxygenase activating protein (FLAP) [18]) for human PMNLs *in vitro* [9,19], orally active in different animal models [9,20,21], which is now undergoing clinical trials [22].
AA861, as shown in table I, has marked lower IC_{50} values for LTB_4 and 5-HETE then those obtained for E6080 [12,16,23]. However, AA861 is orally less effective when compared with E6080 on bronchospasm in actively sensitized conscious guinea pigs [8] and protection of PAF-induced death in mice [5]. The difference between *in vitro* and *in vivo* results might be due to poor bioavailability [8].
Other more or less equipotent 5-lipoxygenase inhibitors are the following substances: **BW A4C and BW A137C** [24], given orally, been found to be effective in a bronchial anaphylaxis anaesthezided guinea pig model [6], and **CGS 8515** inhibits inflammatory response in carrageen-induced pleurisy, sponge inflammation in rats [13] and endotoxic shock in rats [11].
E6080 is equipotent to most of the known 5-lipoxygenase inhibitors. We have shown that it is effective in human peritoneal leucocytes. E6080 is a promising 5-lipoxygenase blocker for clinical use as it is very selective in the wide dose range we have used.

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Chapter 7 Interactions between cytokines and eicosanoids: a study using human peritoneal macrophages

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7.1 Summary

To examine the interactions between the main pro-inflammatory cytokines and eicosanoids produced by human inflammatory cells, human peritoneal macrophages (hp-Mϕ) were isolated from ascitic fluid of patients with portal hypertension. Interactions between interleukin 1ß (IL-1ß), interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) were studied by addition or inhibition of several cytokines and eicosanoids: Human recombinant IL-1ß (hrIL-1ß) addition, LTB_4 addition and 5-lipoxygenase inhibition (6-Hydroxy-2-(4-sulfamoylbenzylamino)-4,5,7-trimethylbenzothiazole hydrochloride; E6080), PGE_2 addition and cyclooxygenase inhibition (indomethacin).
In hp-Mϕ hrIL-1ß stimulated the LTB_4 production, while the PGE_2 production was inhibited. HrIL-1ß had no significant effect on IL-6 production in hp-Mϕ. LTB_4 did not regulate IL-1ß and IL-6 production. Increasing PGE_2 down regulated the TNF-α production, but did not effect the IL-1ß and IL-6 production.

Key words: human peritoneal macrophages . eicosanoids . cytokines . interactions

7.2 Introduction

It is known that Mϕ are a principal source of inflammatory mediators such as cytokines and eicosanoids, and that many differences exist both in their production of and interactions between mediators depending on the species origin, tissue origin, stimulus and cell maturity. These differences must be considered when *in vitro* systems are used as models for human inflammatory diseases.
In previous studies we have characterized the production of the cytokines IL-1ß, IL-6 and TNF-α and the lipoxygenase and cyclooxygenase products by hp-Mϕ isolated from ascitic fluid of patients with portal hypertension [1]. The cytokines IL-1 [2], IL-6 [3], and TNF-α [4] are important pro-inflammatory cytokine products of Mϕ upon stimulation with lipopolysaccharide [(LPS), 5]. The cytokine release of hp-Mϕ was time and dose LPS dependent. The IL-1ß, IL-6 and TNF-α had similar kinetic plots in hp-Mϕ [1] human lung Mϕ [6], peritoneal MÝ (p-Mϕ) of continuous ambulatory peritoneal dialysis (CAPD) patients [7] and p-Mϕ of other species [8]. In contrast, the eicosanoid profile in Mϕ seems to be more dependent on the species origin, on the tissue from which the Mϕ were derived [9] and the stimulus applied [10]. Thus rat p-Mϕ synthesize mainly cyclooxygenase products [9] and mice p-Mϕ synthesize both cyclo- and lipoxygenase products when the cells are stimulated by calcium ionophore [11]. In hp-Mϕ a stimulation with calcium ionophore increased the lipoxygenase products [1]. Usually peripheral blood monocytes of healthy donors or resident p-Mϕ

from rats or mice are used to study interactions between cytokines and eicosanoids. However peripheral blood monocytes differ from mature Mϕ and have different profiles of inflammatory mediators [12]. Resident Mϕ also differ from activated Mϕ, as *in vivo* priming also influences the profile of inflammatory mediators produced by the hp-Mϕ [7]. IL-1 is known to stimulate 5-lipoxygenase [13] and PGE_2 production [14,15], leukotrienes are known to be involved in the regulation of IL-1 production [16,17] and interactions between IL-1 and IL-6 have been reported [18,19]. All these experiments have been performed with cells derived from different species and/or tissues or different cell types.
As differences exist in the production of inflammatory mediators, it is expected that the interactions between eicosanoids and cytokines also depends on the species origin, tissue origin, stimulus applied and cell maturity. Following the above concept, we have attempted to characterize *in vitro* the interactions between IL-1ß, IL-6, TNF-α, LTB_4 and PGE_2, the major pro-inflammatory cytokines and eicosanoids of hp-Mϕ.

7.3 Materials and methods

Subjects: The hp-Mϕ were obtained from ascitic fluid of 6 patients (= n, 5 males and 1 female, age 39 - 82 years) with ascites, due to portal hypertension, undergoing therapeutic paracentesis. Patients were mainly on drugs such as diuretics which have not been reported to affect cytokine and eicosanoid production.

Cell isolation: The hp-cells were isolated from the ascitic fluid by centrifugation at 400xg (4°C) and washed with phosphate buffered saline (PBS, pH 7.4, 4°C, Oxoid, UK). After that the concentrated hp-cell suspension was separated on Percoll (d = 1.064 g/ml, Kabi-Pharmacia, Sweden). To establish the hp-cell purity a small sample of cells was stained by Hemacolor (Merck, Germany) and the different cell types were counted under a light microscope. The following composition of cells was found: 92.5 ± 2.4% Mϕ, 6.1 ± 2.2 % lymphocytes and 1.5 ± 0.7 % granulocytes (number of patients (n) = 6). The viability of the hp-cells was tested by trypan blue exclusion method. The viability of the cells was 84.1 ± 1.9 % (range: 78.5 - 90.6).

Cell incubation: Cells were plated on plastic culture dishes (Costar, UK) at a density of 10^6 cell/ml in RPMI medium [RPMI-1640 supplied with HEPES (25 mM, GIBCO, UK), penicillin/streptomycin ($5x10^4$ U/l / 50 mg/l, Flow Lab, UK), foetal calf serum (10 % FCS, GIBCO, UK) and L-glutamine (600 mg/l Flow Lab, UK)]. The cells were first incubated for 10 min with different mediators and inhibitors of inflammatory mediators (37°C, 7.5 % CO_2): LTB_4 (Sigma, USA), a 5-lipoxygenase inhibitor E6080 (Eisai Co., 43 Japan), PGE_2 (Sigma, USA), Indomethacin (Merck Sharp and Dohme, NL), hrIL-1ß (British Bio-technology, UK).
Thereafter cells were incubated in the absence or presence of a final concentration of 10 µg/ml LPS in PBS (PBS; 10 % final concentration, Sigma, USA, 37°C, 7.5 % CO_2) for 24 h or stimulated by a final concentration of 1 µM calcium ionophore

A23187 in dimethyl sulfoxide (DMSO; 0.1 % final concentration, Sigma, USA, 37°C, 7.5 % CO_2) for 15 min. At the end of the incubation the supernatant was filtered (0.22 μm, Millipore, France) and kept at -80°C till analyzing. Cell viability was not influenced by increasing concentrations of hrIL-1ß, LTB_4, E6080, PGE_2 and indomethacin in comparison to controls (24 h incubations).

Cytokine analysis: The cytokine production was measured in the supernatant of the samples incubated for 24 h without or with 10 μg/ml LPS. IL-1ß, IL-6 and TNF-α products were measured by ELISA (IL-1ß: Medgenix, Belgium, sensitivity = 2 pg/ml. IL-6: Hycyte, The Netherlands, sensitivity = 2 pg/ml; TNF-α: Cistron Biotechnology, USA, sensitivity = 10 pg/ml).

Eicosanoid production: LTB_4 and PGE_2 production from endogenous arachidonate was measured in supernatant of the samples stimulated with A23187 or LPS by radio immuno assays (RIA's; antibodies were obtained from Advanced Magnetics, USA, tritiated antigens from Amersham, UK and standards from Sigma, USA). Cross reactivities for individual antigens on antibodies were negligible.

Statistical analysis: The data are expressed as the mean $\pm$ standard error of the mean (SEM). Data were statistically analyzed with ANOVA followed by Dunnett's test, or only the Student's T-test or Wilcoxon-test. The non-parametric Wilcoxon-test was used for statistical analysis as the range of the production between the patients was fairly wide. This lack of homogeneity was nonpermissive for the student's T-test. The correlations were determined by Pearson's Correlation test. Data were considered significant when $p < 0.05$.

7.4 Results

Cytokine and eicosanoid production of hp-Mϕ: To determine the basal cytokine and eicosanoid production of the hp-Mϕ, the cells were incubated *in vitro* without and with 1 μM A23187 for 15 min (Table 7.1A) and without and with 10 μg/ml LPS for 24 h (Table 7.1B). The LTB_4 and PGE_2 production of the hp-Mϕ were low after 15 min of incubation without stimulus. Only the LTB_4 production increased with A23187 stimulation. 24 h of incubation without or with LPS did not influence the LTB_4 production. The PGE_2 production was 10 fold higher when the cells were incubated without stimulus for 24 h and further increased twofold when the cells were stimulated with LPS. Hp-Mϕ produced the cytokines IL-1ß, IL-6 and TNF-α when they were incubated without stimulus for 24 h. The production of these cytokines increased then significantly when the hp-Mϕ were stimulated *in vitro* with LPS.

The interactions between cytokines and eicosanoids were studied by addition or inhibition of several mediators: hrIL-1ß addition, LTB_4 addition and 5-lipoxygenase inhibition (E6080), PGE_2 addition and cyclooxygenase inhibition (indomethacin). The

results are presented in percentages, control is set as 100 % (Tabel 7.1 gives the production of controls in pg or ng per ml).

Table 7.1 (A) PGE_2 and LTB_4 production (pg/ml) by 10^6 hp-Mϕ/ml stimulated for 15 min with 1 μM A23187 (Control is 15 min incubation with DMSO). (B) LTB_4, PGE_2, IL-1ß, TNF-α (pg/ml) and IL-6 production (ng/ml) by 10^6 hp-Mϕ/ml stimulated for 24 h with 10 μg/ml LPS (Control is 24 h incubation with PBS). Data are expressed as mean ± SEM and statistically analyzed by Wilcoxon-test (W), * = $p < 0.05$ (increase vs control), n = number of patients, ns = not significant.

Table 7.1A

PRODUCT	CONTROL	A23187	n	W
LTB_4 (pg/ml)	652 ± 416	4944 ± 1662	6	*
PGE_2 (pg/ml)	52 ± 26.3	66 ± 18.4	3	ns

Table 7.1B

PRODUCT	CONTROL	LPS	n	W
LTB_4 (pg/ml)	611 ± 125	629 ± 141	3	ns
PGE_2 (pg/ml)	692 ± 167	1345 ± 168	6	*
IL-1ß (pg/ml)	825 ± 417	2979 ± 1146	5	*
TNF-α (pg/ml)	195 ± 81	3292 ± 1803	6	*
IL-6 (ng/ml)	8 ± 2.0	37 ± 11.3	6	*

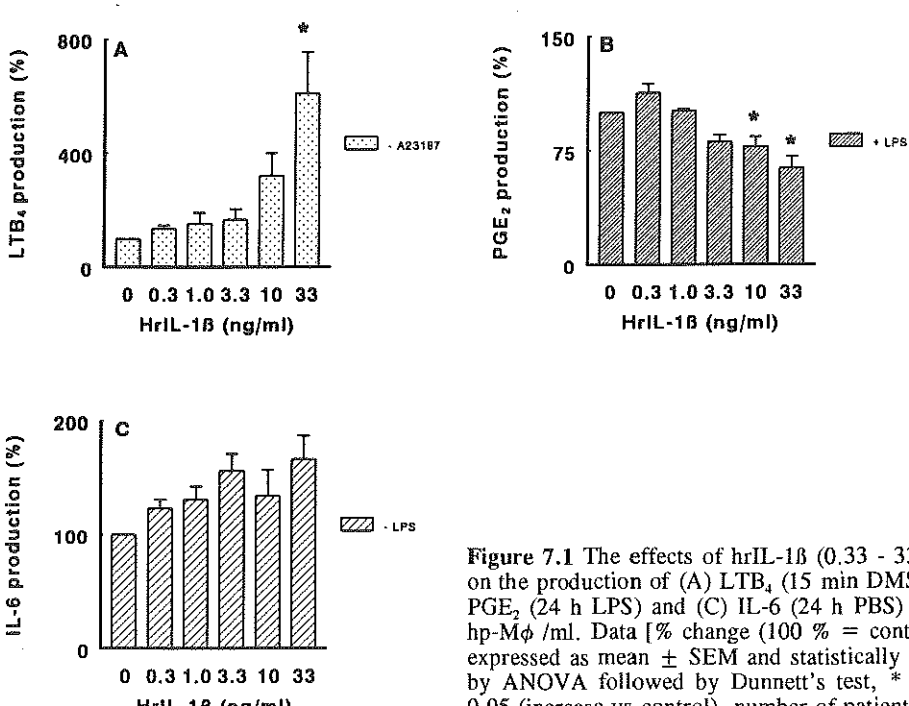

Figure 7.1 The effects of hrIL-1ß (0.33 - 33 ng/ml) on the production of (A) LTB_4 (15 min DMSO), (B) PGE_2 (24 h LPS) and (C) IL-6 (24 h PBS) of 1.10^6 hp-Mϕ /ml. Data [% change (100 % = control)] are expressed as mean ± SEM and statistically analyzed by ANOVA followed by Dunnett's test, * = $p <$ 0.05 (increase vs control), number of patients = 5.

Addition of hrIL-1ß: As the 24 h incubation without or with stimulus did not influence the LTB_4 production of the hp-Mϕ (Table 7.1B), we only studied the effect of hrIL-1ß on the LTB_4 production when the cells were incubated for 15 min without or with A23187. LTB_4 production was dose dependently increased when the cells were incubated for 15 min without A23187 and with hrIL-1ß (Figure 7.1A). PGE_2 production was stimulated by LPS (Table 7.1B). This production was dose dependently decreased by hrIL-1ß (Figure 7.1B). HrIL-1ß (24 h without LPS) did not significantly affect IL-6 production in hp-Mϕ (Figure 7.1C).

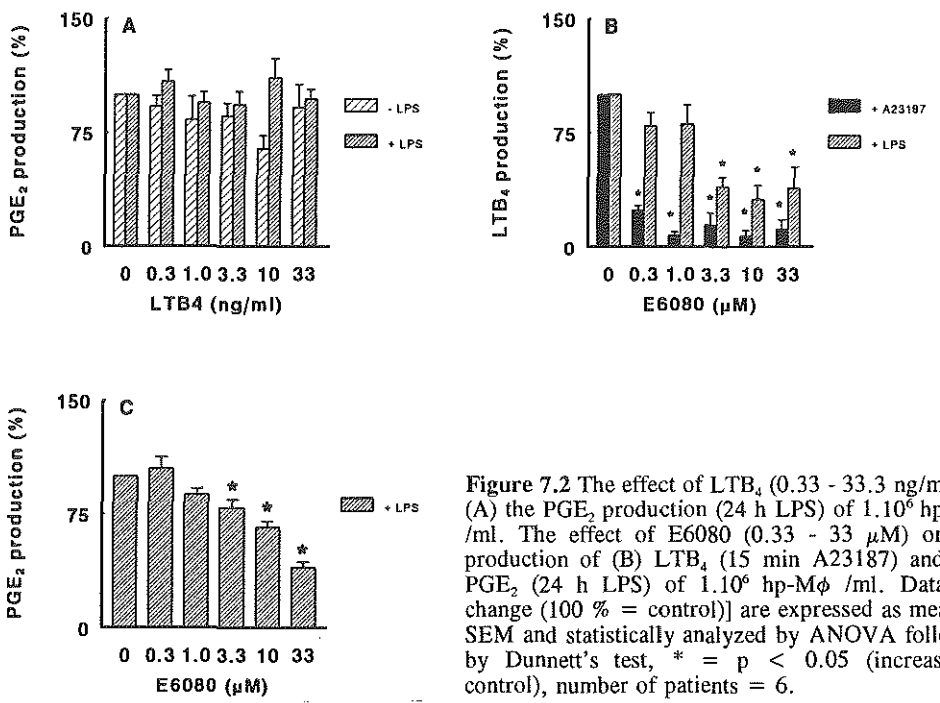

Figure 7.2 The effect of LTB_4 (0.33 - 33.3 ng/ml) on (A) the PGE_2 production (24 h LPS) of 1.10^6 hp-Mϕ /ml. The effect of E6080 (0.33 - 33 μM) on the production of (B) LTB_4 (15 min A23187) and (C) PGE_2 (24 h LPS) of 1.10^6 hp-Mϕ /ml. Data [% change (100 % = control)] are expressed as mean ± SEM and statistically analyzed by ANOVA followed by Dunnett's test, * = $p < 0.05$ (increase vs control), number of patients = 6.

LTB_4 addition and 5-lipoxygenase inhibition: PGE_2 production increased significantly when the hp-Mϕ were incubated for 24 h with LPS (Table 7.1B). Adding LTB_4 to hp-Mϕ followed by incubations of the cells for 24 h without (0.33 - 33.3 ng/ml LTB_4: 92 ± 7.0 %; 86 ± 15.1 %; 86 ± 8.3 %; 64 ± 8.7 %; 91 ± 15.2 %, n = 6) or with LPS (Figure 7.2A) did not have significant effects on PGE_2 production. As expected the 5-lipoxygenase inhibitor E6080 significantly reduced A23187 stimulated LTB_4 production (IC_{50} value of approx. 0.2 μM, Figure 7.2B). Although LPS (24 h) did not stimulate the LTB_4 production, E6080 also inhibited without or with LPS (24 h) the LTB_4 production dose dependently (0.33 - 33.3 μM E6080: 79 ± 9.0 %; 80 ± 12.6 %; 39 ± 6.1 % ($p<0.05$); 31 ± 9.1 % ($p<0.05$); 38 ± 14.3 % ($p<0.05$), IC_{50} value of approx. 2.5 μM, n = 3). E6080 also inhibited the LPS stimulated PGE_2 production dose dependently (IC_{50} value of approx. 25 μM, Figure 7.2C), although

E6080 did not significantly affect the LPS stimulated IL-1ß (0.33 - 33.3 μM E6080: 89 ± 6.3 %; 77 ± 8.7 %; 79 ± 7.7 %; 67 ± 13.0 %; 56 ± 14.8 %, n = 6) and IL-6 production (0.33 - 33.3 μM E6080: 109 ± 10 %; 109 ± 11 %; 118 ± 11 %; 110 ± 9 %; 122 ± 16 %, n = 6) at concentrations which significantly inhibited the LTB_4 production.

PGE_2 addition and cyclooxygenase inhibition: Exogenous PGE_2 dose dependently reduced LPS stimulated TNF-α production by hp-Mϕ (Figure 7.3), although exogenous PGE_2 did not significantly affect LPS stimulated IL-1ß (0.33 - 33.3 ng/ml PGE_2: 99 ± 7.6 %, 111 ± 9 %; 99 ± 7.5 %; 95 ± 5.1 %; 94 ± 5.4 %, n = 6) or IL-6 production (0.33 - 33.3 ng/ml PGE_2: 101 ± 11 %; 111 ± 13 %; 105 ± 11 %; 114 ± 8 %; 105 ± 10 %, n = 6). Indomethacin in combination with LPS stimulation (24 h) almost completely inhibited the PGE_2 production at the lowest dose (0.33 - 33.3 μM indomethacin: 5 ± 1.3 %; 5 ± 1.3%; 5 ± 11.3 %; 11 ± 4.9 %; 5 ± 1.1 %, n = 6). Indomethacin did not significantly affect LPS stimulated TNF-α production, although there was an increase (3.3 - 33.3 μM indomethacin: 206 ± 80 %; 246 ± 51 %, n = 6), neither directly the LPS stimulated (24 h) IL-1ß (0.33 - 33.3 μM indomethacin: 103 ± 8 %; 129 ± 28 %; 104 ± 9 %; 118 ± 11 %; 122 ± 16 %, n = 6) and IL-6 production (0.33 - 33.3 μM indomethacin: 86 ± 7.5; 87 ± 6.8; 95 ± 5.9; 91 ± 8.8; 96 ± 10.7, n = 6).

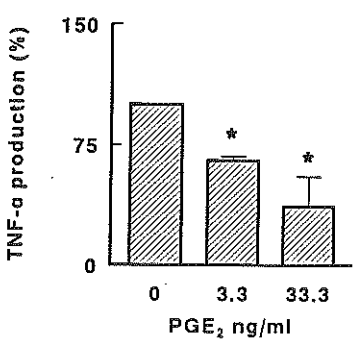

Figure 7.3 The effect of PGE_2 (0.33 - 33.3 ng/ml) on TNF-α production (24 h LPS) of 1.10^6 hp-Mϕ /ml. Data [% change (100 % = control)] are expressed as mean ± SEM and statistically analyzed by ANOVA followed by Dunnett's test, * = $p < 0.05$ (increase vs control), number of patients = 6.

7.5 Discussion

It is well established that the profile of eicosanoid production of Mϕ depends on the species origin, tissue origin and the stimulus applied. We expected that the profile of interactions between inflammatory mediators such as the cytokines and eicosanoids would also vary. We observed the following interactions:

Interaction between hrIL-1ß and LTB_4 and PGE_2: Two forms of IL-1 exist: IL-1α and IL-1ß. Precursor IL-1α is fully active and remains cell associated. Biological inactive precursor IL-1ß is cleaved by IL-1ß converting enzyme into active IL-1ß and secreted by the cells [20]. To study the interactions of IL-1 with the eicosanoids and other cytokines, hrIL-1ß was used because IL-1ß is mainly found outside the cells. Our

results indicate that exogenous hrIL-1ß had reverse effects on the production of several eicosanoids by the hp-Mϕ: An increase of LTB_4 and a decrease of PGE_2 production. This is in contrast with cells of other species origin and or tissue origin (e.g. murine lymphoma cells, human neutrophils, monocytes from healthy donors, human dermal fibroblasts), in which LTB_4 and PGE_2 production were stimulated [21,13-15]. In murine resident peritoneal Mϕ PGE_2 production was only stimulated at a low dose of IL-1ß [22].

Influence of lipoxygenase inhibition on IL-1ß and PGE_2: It has been reported that LTs are involved in the regulation of IL-1 production by activated murine peritoneal macrophages and human blood monocytes, based on studies with dual inhibitors of 5-lipoxygenase and cyclo-oxygenase [16,17]. A study with specific 5-lipoxygenase inhibitors showed that at concentrations which completely inhibited LT synthesis the IL-1 production of activated murine peritoneal macrophages [23] and human peripheral blood monocytes of healthy donors [(IL-1ß), 24] was not reduced. Our study confirms this finding using the 5-lipoxygenase inhibitor E6080 in human peritoneal inflammatory cells. This 5-lipoxygenase inhibition had no significant effect on IL-1ß and IL-6 production at concentrations which significantly inhibited the LTB_4 production after LPS stimulation. We conclude that LTB_4 does not regulate IL-1ß and IL-6 production in hp-Mϕ. In our study the 5-lipoxygenase inhibitor had no effect on LPS stimulated PGE_2 production at low concentrations ($< 3.3\ \mu M$) but at higher concentrations PGE_2 production was significantly inhibited. This could be explained by the fact that this was achieved directly with lower LTB_4 concentrations (In 24 h. incubations LTB_4 was also inhibited by E6080 ($\geq 3.3\ \mu M$)), another possibility is that this effect was through a non selective inhibition of the cyclooxygenase enzyme, although in earlier findings it was shown that E6080 was a specific 5-lipoxygenase inhibitor when macrophages were stimulated with A23187 and the exogenous eicosanoid production was measured by HPLC [25].

Effect of PGE_2 on TNF-α: In our studies PGE_2 ($\geq$ 3.3 ng/ml) down-regulated the TNF-α production of hp-Mϕ. This was reported before only in different animal models: Activated murine peritoneal Mϕ [26], resident peritoneal rat Mϕ and murine cell lines [27]. The decreased PGE_2 concentration [5 % of the LPS stimulated control level (1345 ng/ml)] did not significantly effect the TNF-α production by hp-Mϕ, however the trend was an increase in TNF-α production. It seems that PGE_2 only significantly inhibits TNF-α production of hp-Mϕ when it has reached a certain level. Probably this level was not reached when the hp-Mϕ were only incubated with LPS for 24 h, otherwise indomethacin would have given a significant increase in the LPS stimulated TNF-α production.

Influence of IL-1ß on IL-6: In our studies hrIL-1ß did not significantly influence IL-6 production by hp-Mϕ, although in human monocytes of healthy volunteers hrIL-1ß induced IL-6 production was seen [19]. Cell maturity is probably responsible for this

difference between monocytes and macrophages, as in two other studies hrIL-1ß was shown to stimulate IL-6 synthesis in human blood monocytes, but not in monocyte-derived macrophages and human alveolar macrophages [12,28]. Also species origin and tissue origin of the cells probably effects the interaction between IL-1ß and IL-6 production. RIL-1 was able to induce IL-6 *in vivo* in mice [29] and hrIL-1ß induced IL-6 production in human endothelial and smooth muscle cells [20].

Conclusion: There are differences between the interactions of cytokine and eicosanoid production by hp-Mϕ and Mϕ of other species origin, but also similarities. In inflammatory processes the regulation of the sequential release of pro- and anti-inflammatory mediators could be important for the outcome of the disease. For our understanding of inflammatory diseases and their treatment, *in vitro* investigations with inflammatory cells are very important, although results obtained from incubations with cells from other species, tissue origin or maturity should be interpreted with caution, as this study shows these cells differ from human cells in their profile of interactions between and their production of cytokines and eicosanoids. When human inflammatory diseases are to be studied, hp-Mϕ isolated from ascitic fluid are therefore suitable cells to examine the interactions between cytokines and lipid inflammatory mediators.

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Part IV

Levels of inflammatory mediators in ascitic fluid

Chapter 8 Levels of soluble intercellular adhesion molecule 1, eicosanoids and cytokines in ascites of patients with liver cirrhosis, peritoneal cancer and spontaneous bacterial peritonitis

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8.1 Summary

The levels of the eicosanoids leukotriene B_4 (LTB_4), prostaglandin E_2 (PGE_2), prostacycline (PGI_2) and thromboxane B_2 (TXB_2), the cytokines interleukin 1ß (IL-1ß), interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) and soluble intercellular adhesion molecule 1 (sICAM-1) were measured in ascites and plasma samples of patients with liver cirrhosis (53), peritoneal cancer (26) and spontaneous bacterial peritonitis (SBP; 10) to assess their value as a possible diagnostic and prognostic parameter in the course of the disease. Soluble ICAM-1, of the eicosanoids PGE_2 and LTB_4 and the protein concentration in ascites were all significantly elevated in ascites of patients with peritoneal cancer in comparison to ascites of patients with liver cirrhosis. In ascites of patients with SBP, IL-6 concentration was significantly elevated and the protein concentration was significantly lower in comparison to the other two groups. None of these parameters however seems to be of practical use as a diagnostic parameter, as there is an overlap between all the levels of these mediators in ascites of liver cirrhosis, peritoneal cancer and spontaneous bacterial peritonitis group. Soluble ICAM-1 levels were much higher in plasma compared to ascites, contrary to IL-6 levels which were much higher in ascites than in plasma. Soluble ICAM-1 in ascites correlated with sICAM-1 in plasma ($r = 0.6926$, $p = 0.0001$). Soluble ICAM-1, IL-6 and the number of polymorphonuclear cells (PMNs) in peritoneal fluid correlated during episodes of infection in patients with a peritonitis. For this reason sICAM-1 and IL-6 could be of prognostic value for patients with a peritonitis.

Keywords: Soluble ICAM-1 . interleukin 6 . ascites . liver cirrhosis . peritoneal cancer . spontaneous bacterial peritonitis

8.2 Introduction

Ascites is the accumulation of fluid in the peritoneal cavity. The main causes of ascites are liver cirrhosis, malignancies involving the peritoneum and inflammatory processes. Early diagnosis of the probable cause of the ascites is useful for guiding further investigation and treatment [1]. Ascites may be complicated by bacterial infection, a condition known as spontaneous bacterial peritonitis (SBP). SBP has a high mortality unless diagnosed and treated at an early stage [2]. Ascites of all causes contains inflammatory cells, mainly macrophages (Mϕ), which produce inflammatory mediators. As the pattern of mediators produced by inflammatory cells depends in part on the stimulus and on the stage of inflammation, we decided to measure a wide variety of inflammatory mediators or products of inflammation to determine whether the different causes of ascites were associated with specific patterns of inflammatory mediators in ascites. Based on prior work in which we characterized production of

inflammatory mediators by human peritoneal Mϕ [(hp-Mϕ), 3] we measured arachidonic acid products, several pro-inflammatory cytokines, and an adhesion molecule. The lipoxygenase product LTB_4 [4] and the cyclooxygenase products PGE_2, TXA_2 and PGI_2 [detected as 6-keto-prostaglandin $F_{1\alpha}$ ($6kPGF_{1\alpha}$), 5] were measured as these are products of hp-Mϕ [3]. We also measured the pro-inflammatory cytokines IL-1ß, IL-6 and TNF-α [6,7], which are also produced by hp-Mϕ [3].
In addition we measured the sICAM-1. ICAM-1 plays a crucial role in the adhesion and migration of inflammatory cells. ICAM-1 is a single-chain membrane-bound glycoprotein [8] which is expressed on endothelial and other cells [8-10]. Expression of ICAM-1 is upregulated by the cytokines IL-1, TNF-α, interferon γ (IFN-γ) ([9-12] and also by the chemoattractants LTB_4 and C5a [13]. Expression of ICAM-1 on tissue leads to the release of a soluble form into the circulation. The biological function of this soluble form of ICAM-1 is not yet clear and little is known about the relation between expression of ICAM-1 on tissue and its soluble form. Soluble isoforms of adhesion molecules can be found in the circulation of normal humans [14].
Although there are publications on IL-6 and TNF-α in ascites, especially during SBP [15-18], we are not aware of reports on ascitic eicosanoid and ascitic sICAM-1 levels. Soluble ICAM-1 in plasma or serum [14,19-24] and the cytokines IL-1, IL-6 and TNF-α in plasma, serum or ascites [15,16,22,25-29] have been found to be elevated in patients with different diseases including malignancies and liver cirrhosis. Therefore we measured the levels of sICAM-1, the representative eicosanoids LTB_4, PGE_2, PGI_2 and TXB_2 and the pro-inflammatory cytokines IL-1ß, IL-6 and TNF-α in ascites of patients with liver cirrhosis, peritoneal cancer and SBP to increase our understanding of inflammatory mediators involved in peritoneal inflammation, to determine if these mediators showed a specific disease related pattern and whether this pattern could be of diagnostic and prognostic value.
Simultaneously we measured the same parameters in blood samples of these patients to determine if there was a close correlation between ascites and plasma values.

8.3 Patients and methods

Patients: Eighty nine ascites samples were obtained. Some patients were drained several times in the period we collected the samples. Usually there was a time period of several weeks in between (totally 63 patients were drained). These samples were divided into 3 groups: The liver cirrhosis group [number of samples = 53, sex (male/female (m/f)) = 33/20, age (range) = 58 (27-82)], the peritoneal cancer group [number of samples = 26, sex (m/f) = 18/8, age (range) = 61 (37-81)] and the SBP group [number of samples = 10, sex (m/f) = 7/3, age (range) = 48 (22-59)].
A patient with ascites was classified as having SBP if the number of PMNs was higher then $0.25x10^9$ cells/l in the ascites and the bacterial culture of the ascitic fluid was positive or in case of the culture was negative, the PMNs count in the ascites should be higher then $0.5x10^9$ cells/l (Bac *et al.*, 1993a). A patient with renal failure on continuous ambulatory peritoneal dialysis (CAPD) was classified as having a peritonitis

if the number of PMNs is higher then $0.10x10^9$ cells/l in the dialysates and the bacterial culture is positive (Vas, 1989).
In the liver cirrhosis group there were 29 samples obtained from patients with alcoholic liver cirrhosis, 13 samples from patients with hepatitis B or C virus induced cirrhosis, 3 samples from partients with primary biliary cirrhosis or sclerosing cholangitis, 5 samples from patients with Budd Chiari and 3 samples from patients with other diseases.
Of the malignancy related ascites group all patients had metastized disease. Eight samples were obtained from patients with colon carcinoma, 7 samples from patients with pancreatic carcinoma, 3 samples from patients with liver carcinoma, 2 samples from patients with peritoneal mesothelioma, 2 samples from patients with gastric carcinoma, 1 sample from a patient with lung carcinoma, 1 sample from a patient with a sarcoma and 2 samples from patients with an unknown primary source.
In the SBP group 4 samples were obtained from a patient with hepatitis B virus induced cirrhosis and this patient had a *Staphylococcus epidermidis* infection in the ascites (PMNs number varied between $0.5x10^9$ and $4.2x10^9$ cells/l), 3 samples were obtained from a patient with a pancreas carcinoma and a portal vein thrombosis and this patient had a *Klebsiella* infection in the ascites (PMNs number varied between $0.6x10^9$ and $15.6x10^9$ cells/l), 2 samples were obtained from patients with liver cirrhosis, one of these patients had an *Escherichia coli* infection in the ascites ($4.3x10^9$ PMNs/l), the other patient had a *Streptococcus mitis* infection in the blood, the ascitic culture of this patient was negative ($7.8x10^9$ PMNs/l in ascites). One sample was obtained from a patient with an acute liver failure and an *Escherichia coli* infection in the ascites ($10.5x10^9$PMNs/l).
In 34 of these patients with ascites, blood (24 samples from the liver cirrhosis group, 6 samples from the cancer group and 4 samples from the SBP group) was collected in EDTA-tubes on the same day or the day after ascites drainage. Blood was also obtained from 17 healthy volunteers [sex (m/f) = 12/5, age (range) = 29 (23-40)].
Additionaly from two CAPD patients [sex (m/f) = 1/1, age (range) = 49 (41 - 57)] the drained dialysates were collected during and after an episode of peritoneal bacterial infection. The first CAPD patient had a *Klebsiella* infection. The number of PMNs varied between $0.1x10^9$ PMNs/l and $3.8\ x10^9$ PMNs/l. The second CAPD patient had *Enterococcus faecalis* infection. The number of PMNs varied between $0.2x10^9$ PMNs/l and $1.4\ x10^9$ PMNs/l.

Assays: The ascites, dialysates and blood were centrifuged at 1500xg directly after withdrawal. The centrifuged ascites, dialysates and plasma were stored at - 80°C until they were analysed. Soluble ICAM-1, TNF-α, IL-1ß and IL-6 were determined in the centrifuged ascites and plasma using commercially available ELISA kits (sICAM-1: British Biotechnology products, UK, sensitivity = 2.5 ng/ml. TNF-α: Eurogenetics, USA, sensitivity = 10 pg/ml. IL-1ß: Eurogenetics, USA, sensitivity = 2 pg/ml. IL-6: Hycult, The Netherlands, sensitivity = 2 pg/ml).
LTB_4, PGE_2, $6kPGF_{1\alpha}$ and TXB_2 were determined by radio immuno assays

(Antibodies: Advanced Magnetics - USA, ^{3}H-Antigen: Amersham - UK and standards: Sigma - USA) in centrifuged ascites and plasma which were passed through Sep Pak C_{18} cartridges (Waters Ass., USA).
In the dialysates only sICAM-1 and IL-6 were determined.
Protein concentrations in the ascites and plasma were determined with the pyrogallol-red-molybdate(IV) method (Instruchemie, The Netherlands).

Statistical analysis: Data are expressed as the mean ± standard error of the mean (SEM). Data were statistically analyzed with ANOVA followed by Student's T-test. The correlations were determined by Pearson Correlation test. Data were considered significant when $p < 0.05$.

8.4 Results

Soluble ICAM-1, cytokine and eicosanoid concentrations in ascites: Soluble ICAM-1 concentration was significantly elevated in ascites of patients with peritoneal cancer (385 ± 66 ng/ml, n = 26) compared to the concentration of sICAM-1 in ascitic fluid of patients with liver cirrhosis (151 ± 16 ng/ml, n = 53, p = 0.0000) and SBP (215 ± 27 ng/ml, n = 10, p = 0.0284). There was no significant difference between the concentrations in ascites from patients with liver cirrhosis and SBP (Figure 8.1A).

IL-6 concentration was significantly elevated in ascites of patients with SBP (103 ± 24 ng/ml, n = 10) compared to the concentration of IL-6 in ascites of patients with liver cirrhosis (12 ± 1.8 ng/ml, n = 53, p = 0.0000) and peritoneal cancer (24 ± 5.7 ng/ml, n = 26, p = 0.0000; Figure 8.1B).

Ascites protein concentration was significantly lower in SBP (10 ± 1.6 mg/ml, n = 7, p = 0.0104) compared to the liver cirrhosis group (26 ± 2.4 mg/ml, n = 46) and peritoneal cancer group (44 ± 2.6 mg/ml, n = 25, p = 0.0000). Ascites protein levels were significantly higher (p = 0.0000) in peritoneal cancer compared to the other two groups (Figure 8.1C).

Of the eicosanoids the LTB_4 and PGE_2 concentrations were significantly higher in ascites of patients with peritoneal cancer (n = 26) compared to the liver cirrhosis group (n = 47), and ascites LTB_4 and $6kPGF_{1\alpha}$ concentrations of peritoneal cancer were significantly increased in comparison to SBP (n = 10, Table 8.1). LTB_4 concentration in ascites of patients with SBP and PGE_2 concentration in ascites of patients with liver cirrhosis was significantly lower in comparison to the other two groups.

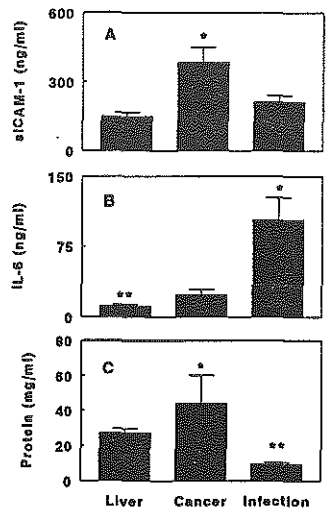

Figure 8.1 (A) Soluble ICAM-1 concentration (ng/ml) in ascites of patients with ascites due to liver cirrhosis (liver, n=53), peritoneal cancer (cancer, n=26) and SBP (infection, n=10). (B) Interleukin 6 concentration (ng/ml) in ascites of patients with ascites due to liver cirrhosis (liver, n=53), peritoneal cancer (cancer, n=26) and SBP (infection, n=10). (C) Protein concentration (mg/ml) in ascites of patients with ascites due to liver cirrhosis (liver, n=46), peritoneal cancer (cancer, n=25) and SBP (infection, n=7). * = significantly higher compared to the other two groups ($p < 0.05$), ** = significantly lower compared to the other two groups.

Table 8.1 Eicosanoid concentration in ascites of patients with liver cirrhosis, peritoneal cancer and SBP (Data is mean ± SEM, nd = not detectable, n = number of samples).

Eicosanoids	Liver cirrhosis (n)	Peritoneal Cancer (n)	SBP (n)
LTB_4 (pg/ml)	21 ± 5.4 (47)	193 ± 67 ** (26)	nd * (10)
PGE_2 (pg/ml)	27 ± 3.2 * (47)	122 ± 27 *** (26)	79 ± 20.7 (10)
$6kPGF_{1\alpha}$ (pg/ml)	145 ± 62 (47)	359 ± 110 **** (26)	113 ± 38 (10)
TXB_2 (pg/ml)	57 ± 8.1 (46)	70 ± 9.4 (26)	53 ± 3.2 (8)

* = significantly lower compared to other two groups ($p < 0.05$).
** = significantly higher compared to other two groups ($p < 0.05$).
*** = significantly higher compared to liver cirrhosis group ($p < 0.05$).
**** = significantly higher compared to SBP group ($p < 0.05$).

Soluble ICAM-1, cytokine and eicosanoid concentrations in plasma: In Table 8.2 the plasma levels of sICAM-1, cytokine and eicosanoid concentrations per patient group and controls were compared. No significant differences were seen between the three patient groups. Soluble ICAM-1 and IL-6 concentrations in plasma of the patients were significantly elevated in comparison to the controls. IL-1ß and TNF-α were not detectable in plasma of patients, IL-1ß, IL-6, TNF-α and LTB_4 were not detectable in control plasma.

Table 8.2 Soluble ICAM-1, cytokine and eicosanoid concentration in plasma of patients with liver cirrhosis, peritoneal cancer and SBP and in plasma of controls (Data is mean ± SEM, n = number of samples, nd = not detectable).

Mediators	Liver cirrhosis (n)	Peritoneal Cancer (n)	SBP (n)	Plasma control (n)
sICAM-1 ng/ml	781 ± 141 (24)	781 ± 367 (5)	1204 ± 271 (3)	228 ± 24 * (17)
IL-6 pg/ml	1191 ± 358 (24)	420 ± 215 (6)	1736 ± 394 (4)	nd * (17)
IL-1ß pg/ml	nd (17)	nd (6)	nd (4)	nd (17)
TNF-α pg/ml	nd (17)	nd (6)	nd (4)	nd (17)
LTB_4 pg/ml	22 ± 16.3 (23)	14 ± 14.1 (6)	54 ± 54.6 (4)	nd (17)
PGE_2 pg/ml	67 ± 21.1 (23)	68 ± 32.1 (6)	52 ± 48.0 (4)	70 ± 13.0 (16)
$6kPGF_{1\alpha}$ pg/ml	104 ± 67.4 (23)	14 ± 14.3 (6)	6.7 ± 4.7 (4)	8.8 ± 1.4 (16)
TXB_2 pg/ml	256 ± 126 (17)	141 ± 51 (6)	127 ± 32 (2)	153 ± 67 (14)

* significantly lower compared to the 3 patients groups ($p < 0.05$).

Table 8.3 Soluble ICAM-1, cytokine and eicosanoid concentration in ascites and plasma of patients (grouped all together) with liver cirrhosis, peritoneal cancer and SBP (Data is mean ± SEM, n = number of samples, nd = not detectable).

Mediators metabolites	Ascites (n)	Plasma (n)
sICAM-1 ng/ml	227 ± 24 (89)	820 ± 122 (32) *
IL-6 ng/ml	26 ± 4.4 (89) *	1 ± 0.3 (34)
IL-1ß pg/ml	10 ± 2.7 (59)	nd (25)
TNF-α pg/ml	65 ± 24.4 (78) *	nd (24)
LTB_4 pg/ml	72 ± 22.7 (84)	25 ± 13.0 (33)
PGE_2 pg/ml	63 ± 10.0 (84)	65 ± 16.4 (33)
$6kPGF_{1\alpha}$ pg/ml	206 ± 50 (84)	76 ± 47.3 (33)
TXB_2 pg/ml	61 ± 5.5 (81)	218 ± 86.2 (25)

* = significantly higher ($p < 0.05$).

Comparison between the sICAM-1, cytokine and eicosanoid concentrations in ascites and plasma: As we had only a small number of plasma samples of the peritoneal cancer group and the SBP group and there were no significant differences between the plasma levels of the three patient groups, all the plasma samples of patients were compared with the ascites samples, without subdivision, as calculated and given in Table 8.3. There were differences between plasma and ascites values: sICAM-1 concentration was much higher in plasma, while IL-6 and TNF-α concentrations were higher in ascites (Table 8.3).
There was a good relation between ascites sICAM-1 and plasma sICAM-1 ($r = 0.6926$ and $p = 0.0001$, $n = 27$, Figure 8.2); for the other metabolites there was no correlation.

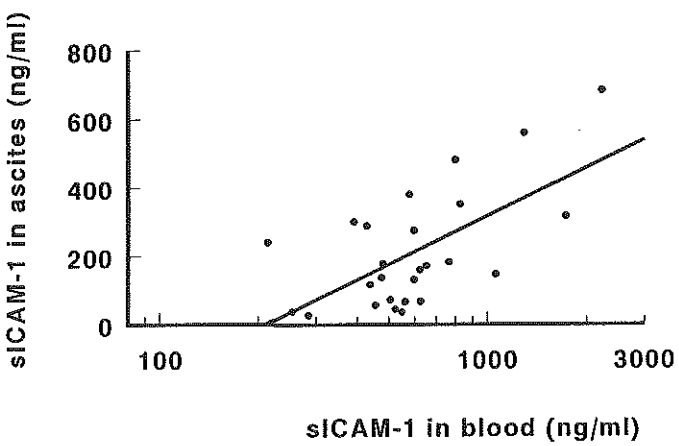

Figure 8.2 Correlation between sICAM-1 in ascites and plasma of patients with ascites due to liver cirrhosis, peritoneal cancer and SBP ($r = 0.6926$, $p = 0.0001$ and $n = 27$).

Soluble ICAM-1 and IL-6 concentration during peritonitis: In 2 patients with SBP, ascites was drained several times [patient 1: 5x (day 0, 25, 28, 32, 38) and patient 2: 4x (day 0, 6, 18, 24)] during 5 to 6 weeks. For both these patients sICAM-1 and IL-6 concentrations in ascites changed during this period: Patient 1 had no SBP on the first day (day 0) of drainage (negative culture, 0.1×10^9 PMNs/l). The sICAM-1 and IL-6 concentrations in the ascites were low (77 ng/ml sICAM-1 and 1 ng/ml IL-6). On day 25 the patient had a subtotal hepatic resection. After this operation the number of PMNs (1.7×10^9 PMNs/l), sICAM-1 (190 ng/ml) and IL-6 (72 ng/ml) had increased in the ascites. A *Staphylococcus epidermidis* infection was diagnosed on day 28 in the ascites (0.5×10^9 PMNs/l, 242 ng/ml sICAM-1, 56 ng/ml IL-6). During the peritonitis sICAM-1 and IL-6 concentrations peaked at day 32 (316 ng/ml sICAM-1, 119 ng/ml IL-6, 4.2×10^9 PMNs/l). Antibiotics were given after day 32, after which the sICAM-1 and IL-6 concentrations decreased (195 ng/ml sICAM-1, 79 ng/ml IL-6, 2.2×10^9 PMNs/l, Figure 8.3A).
Patient 2 had a *Klebsiella* peritonitis on the first day of drainage. In the ascites of this

patient the sICAM-1 and IL-6 concentrations and the PMNs number were high (351 ng/ml sICAM-1, 48 ng/ml IL-6, $15.6x10^9$ PMNs/l). Antibiotics were given for several weeks and the sICAM-1 concentration decreased to a low level (56 ng/ml). IL-6 concentration slightly increased (day 6, 60 ng/ml), but then also decreased to a very low level (0.3 ng/ml). At the same time also the number of PMNs decreased ($3.4x10^9$ PMNs/l on day 6, $0.6x10^9$ PMNs/l on day 18). At the last drainage the bacterial culture of the ascites was negative and the number of PMNs had further decreased ($0.5x10^9$ PMNs/l; Figure 8.3B).

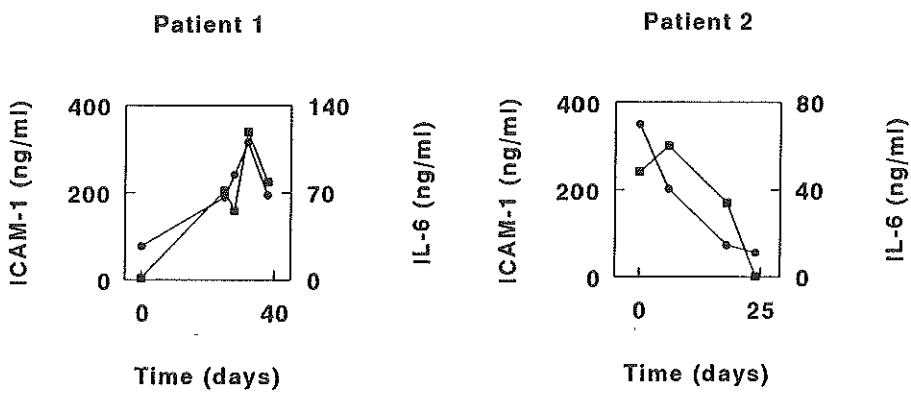

Figure 8.3 Soluble ICAM-1 (●) and IL-6 (■) concentrations (ng/ml) in ascites of 2 patients with SBP. (A) Patient 1 had no SBP on day 0 of drainage. The patient was operated on day 25, an *Staphylococcus epidermidis* infection started after this operation. Antibiotics were given after day 32. (B) Patient 2 had a SBP on the first day of drainage (*Klebsiella* infection). During several weeks antibiotics were given. At day 24 the patient was infection free.

The ICAM-1 and IL-6 concentrations were also measured in drained dialysates of 2 CAPD patients during an episode of peritoneal infection (patient 3: 4x (day 0, 1, 8, 13) and patient 4: 6x (day 0, 3, 6, 7, 10, 12). Patient 3 had on day 0 an untreated bacterial infection (*Klebsiella*, $1.4x10^9$ PMNs/l), the sICAM-1 concentration was 3 ng/ml and the IL-6 concentration 80 ng/ml. On day 1 antibiotics were given and the IL-6 concentration decreased (66 ng/ml), the sICAM-1 concentration (6 ng/ml) and the number of PMNs ($3.8x10^9$ PMNs/l) increased. On day 8 the sICAM-1 concentration had only decreased slightly, but the IL-6 concentration (3 ng/ml) and the number of PMNs ($0.1x10^9$ PMNs/l) had decreased. On day 13 the patient was free of infection (negative culture, $< 0.1x10^9$ PMNs/l), sICAM-1 was not detectable and the IL-6 concentration stayed low (3 ng/ml; Figure 8.4A).
Patient 4 had on day 0 a bacterial infection (*Enterococcus faecalis*, $1.4x10^9$ PMNs/l) and before antibiotics had been given, the sICAM-1 concentration was 21 ng/ml and the IL-6 concentration 12 ng/ml. On day 3 after antibiotics had been given, IL-6 concentration increased slightly (13 ng/ml), but sICAM-1 concentration (9 ng/ml) and also the number of PMNs in the dialysates ($0.8x10^9$ PMNs/l) decreased. Between day 6 and 10 the infection was still present, but the concentration of sICAM-1 and IL-6

and the number of PMNs decreased (day 6: 0.9 ng/ml sICAM-1, 0.8 ng/ml IL-6, $0.5x10^9$ PMNs/l; day 7: 0 ng/ml sICAM-1, 2 ng/ml IL-6, $0.2x10^9$ PMNs/l; day 10: 0 ng/ml sICAM-1, 0.9 ng/ml IL-6, $0.1x10^9$ PMNs/l). On day 12 the patient was free of infection (negative culture, PMNs < $0.1x10^9$), sICAM-1 was not detectable and the IL-6 concentration (0.8 ng/ml) was also low (Figure 8.4B).
The correlation between sICAM-1, IL-6 and the number of PMNs in the ascites and the drained dialysates in those patients with a peritonitis was for sICAM-1 and PMNs 0.7201 (p = 0.0002), for IL-6 and PMNs 0.4454 (p = 0.0378) and for sICAM-1 and IL-6 0.4800 (p = 0.0238).

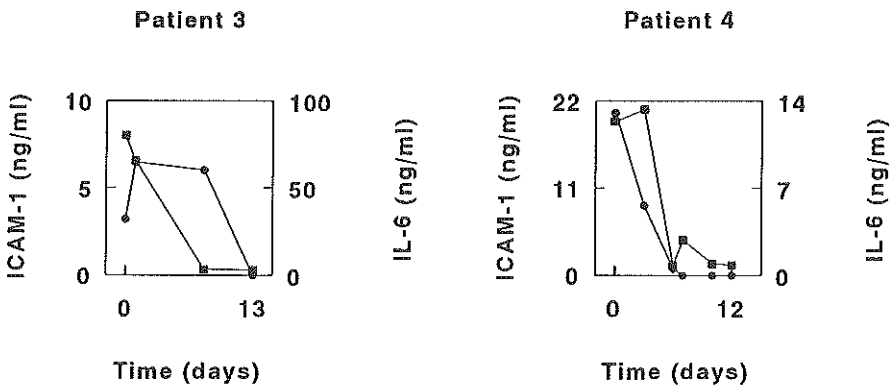

Figure 8.4 Soluble ICAM-1 (●) and IL-6 (■) concentrations (ng/ml) in drained dialysates of 2 CAPD patients during an episode of peritoneal infection. (A) Patient 3 had on day 0 a peritonitis (*Klebsiella*) and no antibiotics was given yet. On day 1 antibiotics had been given. On day 13 the patient was infection free. (B) Patient 4 had also on day 0 a peritonitis (*Enteroccus faecalis*) and no antibiotics was given yet. On day 12 the patient was infection free.

8.5 Discussion

The present study shows that there are significant differences in the concentrations and patterns of inflammatory products in ascites due to different disorders. Soluble ICAM-1 and the eicosanoids PGE_2 and LTB_4 all clearly differentiated between peritoneal cancer and liver cirrhosis: these metabolites were all higher in ascites of patients with peritoneal cancer.
Ascitic sICAM-1 and IL-6 also increased during episodes of peritoneal infection in two patients with SBP as well as in infected dialysates of two CAPD patients. Soluble ICAM-1 levels were much higher in plasma than in ascites, in contrast to IL-6 levels which were much higher in ascites than in plasma. Soluble ICAM-1 in ascites of all the patients correlated with sICAM-1 in plasma of these patients. The sICAM-1 levels in plasma of patients were significantly higher than the plasma levels of the controls. The cytokines IL-1ß and TNF-α were low or not detectable in ascites or plasma of patients with liver cirrhosis, peritoneal cancer or SBP.

Soluble ICAM-1 concentration in ascites and plasma: Although there were significant differences in concentrations of sICAM-1 in ascites, in the different disorders plasma levels of sICAM-1 were not significantly different, probably due to the small number of plasma samples in the peritoneal cancer group and the SBP group. Other studies showed that sICAM-1 levels were elevated in sera of patients with different type of diseases in comparison to controls [14,21,22] including acute liver disease [(chronic hepatic C virus infection), 24], chronic liver diseases [(Primary biliar cirrhosis and primary sclerosing cholangitis), 19] and patients with different forms of cancer [20,23].
Soluble ICAM-1 is probably released more to the blood stream circulation than locally within the peritoneal cavity as the sICAM-1 levels in plasma of all the patients was significantly higher than levels in ascites of all the patients. Adhesion to and penetration through the vascular endothelium is a mandatory step for leucocyte migration and accumulation at sites of inflammation. De novo or up-regulated expression of adhesion molecules on sinusoidal lining cells in inflamed liver biopsy specimens indicates that these endothelial cells actively modulate their phenotype in response to environmental factors, thus playing a key role in the recruitment of leucocytes in acute and chronic liver inflammation [30] and probably leading to the release of ICAM-1 locally.
Malignant melanomas are usually surrounded by an heavy inflammatory infiltrate, the inflammatory cells often express activation associated markers [31] and the malignant melanomas express ICAM-1 [32]. An explanation for the significant differences of sICAM-1 concentration in ascites of the different groups in our study could be that cancer cells are also a source of sICAM-1. A study showed that an unstimulated human melanoma cell line expressed ICAM-1 on the cell surface but did not release significant levels of sICAM-1. Stimulation with TNF-α, IL-1 and IFN-γ, but not IL-6, then caused the release of sICAM-1. The same group showed in the same publication that in nude mouse models bearing s.c. human melanoma tumours, the serum contained human sICAM-1 [33]. These two studies show clearly that only the cancer cells in these models could have been the source of the soluble form of ICAM-1. In humans, cancer cells will most probably be an important source for he release of sICAM-1 locally into the peritoneal cavity. Assuming that cancer cells are a source of sICAM-1, this may provide cancer cells additional escape mechanisms eg. ineffective recognition of cell surface ICAM-1 by cytotoxic lymphocytes or blocking immune recognition by the blocking the ligand for ICAM-1, lymphocyte-function-associated antigen 1 (LFA-1), on the leucocytes [8,34].

All our patients with peritoneal cancer had high levels of sICAM-1 in the ascites. All these patients have late stage cancer, therefore it was not possible to correlate the disease progression of these patients with the sICAM-1 level in the ascites or plasma of these patients. Other studies did show that the levels of sICAM-1 in humans correlated with liver metastasis in a variety of tumours, and with disease progression in melanoma cells [20,23].

IL-1ß, IL-6 and TNF-α concentrations in ascites and plasma: In previous studies we have isolated Mϕ from ascitic fluid of patients with liver cirrhosis. These Mϕ had a high IL-6 production capacity [3]. The Il-6 levels in the ascites of the patients used for this study were always much higher in the peritoneal cavity than in their plasma, indicating that IL-6 was produced locally in the peritoneal cavity by Mϕ. Endothelial cells are also able to produce IL-6 [35,36]. IL-1ß and TNF-α were hardly detectable in ascites or plasma of patients with liver cirrhosis, peritoneal cancer and PBS. Other reports however show besides IL-6 levels, also elevated IL-1 and TNF-α plasma levels in patients with ascites caused by liver diseases in comparison to controls were found [25,27]. In SBP patients, in comparison to non infected patients, elevated TNF-α levels in ascites [18] and plasma [26] were also found. Similar to our results, one other report also describes significant elevated IL-6, but not IL-1 or TNF-α levels in ascites and serum of SBP patients [17].
Besides Mϕ and endothelium, tumor cells should also be considered as potential sources for IL-6. Some tumor cells have been shown to produce IL-6 [37,38]. High IL-6 levels may account in different ways for the alterations which are associated with malignancy [39]. IL-6 is a potent growth factor for myeloma/plasmacytoma cells and its deregulated expression may be involved in the oncogenesis of human multiple myelomas [40].
The liver is an important site of synthesis and the main clearance organ of circulating cytokines. The rapid hepatic clearance of circulating cytokines may normally constitute an important mechanism limiting the systemic action of cytokines. Lack of hepatic clearance may be one reason for disturbances of the immune system in patients with hepatic cirrhosis [41] and probably also patients with liver metastasis. IL-6 is important in liver diseases, it induces acute-phase protein synthesis, in particular fibrinogen production, in hepatocytes [40]. The high IL-6 production during the bacterial infection is probably a direct effect of the bacteria on the macrophages in the peritoneum:lipopolysaccharide (LPS), a bacterial membrane component, is known to be one of the most potent stimuli of Mϕ [42]. Increasing LPS concentrations increases the IL-6 production capacity of human peritoneal Mϕ *in vitro* [3].

Correlation between IL-6 and sICAM-1: The high sICAM-1 and IL-6 levels and low IL-1ß and TNF-α levels in the patients with ascites could indicate that there is a connection between IL-6 and sICAM-1: IL-6 could induce ICAM-1 expression and also the release of the soluble form. In an auto-immune mouse model ICAM-1 was newly induced in the hepatic sinusoids of controls by intravenous injection of recombinant mouse IL-6 and elevated production of IL-6 by hepatic mononuclear cells also correlated with this ICAM-1 expression [43]. On the other hand incubation of cultured monolayers of endothelial cells with IL-6 did not effect their adhesiveness for monocytes [12] and IL-1 α/ß, TNF-α and IFN-γ but not IL-6 activate increase the permeability of human endothelial cells *in vitro* [44].

It has not been shown previously that both sICAM-1 and IL-6 levels in ascites of

patients with SBP and in the dialysis fluid of CAPD patients with SBP correlated with episodes of infection. Soluble ICAM-1 and IL-6 levels were high when the patients had an infection, decreased when antibiotics were given and were hardly detectable when the patients were free of infection.

Eicosanoids in ascites and plasma: It is not quite clear why the LTB_4 production is much higher in ascites of the peritoneal cancer group than in the liver cirrhosis group. Probably cancer cells in the peritoneal cancer group activate the inflammatory cells to produce LTB_4. This elevation may in turn stimulate ICAM-1 expression [45]. The release of the vasodilating agents PGE_2 and PGI_2 [5] could also act as mediators involved in the induction of ascites [46]. In patients with a liver disease endothelial cells, macrophages and hepatocytes or the disability of the liver to clear these vasodilators [47] could be responsible for the PGE_2 and PGI_2 production. In patients with cancer, the cancer cells itself could also be responsible for the high concentration of the PG's in ascites. A number of experimentally induced tumours in animals as well as those occurring naturally in humans have been found to produce high amounts of PG's and PGE_2 in particularly [48,49].

Conclusion: In conclusion the levels of sICAM-1, the eicosanoids LTB_4 and PGE_2 and the cytokine IL-6 and the protein concentration in ascites are parameters which showed a specific disease related pattern, however for diagnostic use they may not be suitable as all the parameters of the 3 different groups, in which we had divided our patients, overlapped. However as sICAM-1 levels in ascites correlated with sICAM-1 levels in plasma and both sICAM-1 and IL-6 correlated with episodes of infection (in ascites of patients with SBP and drained dialysates of CAPD patients with SBP), sICAM-1 together with IL-6 seem to be parameters which could be of use to monitor for patients with SBP.

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Chapter 9 High interleukin 6 production within the peritoneal cavity in decompensated cirrhosis and malignancy-related ascites

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9.1 Summary

To assess the diagnostic and prognostic value of interleukin 6 (IL-6), interleukin 1β (IL-1β), and tumor necrosis factor α (TNF-α) assays in plasma and ascites, we measured these cytokines in 8 patients with malignancy-related ascites and 32 patients with decompensated cirrhosis. Five patients had an episode of bacterial peritonitis (BP) during which period one or more ascitic fluid samples were analyzed. IL-6 and TNF-α were not significantly different between the cirrhotic and malignant two groups: ascitic IL-6 13816 $\pm$ 15314 vs 28138 $\pm$ 23403 pg/ml, plasma IL-6 542 $\pm$ 719 vs 559 $\pm$ 604 pg/ml; ascitic TNF-α 19 $\pm$ 50 vs 12 $\pm$ 31 pg/ml, plasma TNF-α 3,4 $\pm$ 8,2 vs 6,1 $\pm$ 13,8 pg/ml. During an episode of BP there was a significant increase only in ascitic IL-6, (133268 $\pm$ 99743 pg/ml) which declined after antibiotic treatment. None of the parameters was associated with the 6 months survival (11 of the 40 patients died within 6 months). There was a correlation ($r = 0.675$; $p = 0.002$) between plasma IL-6 levels and the Child-Pugh score in patients with liver cirrhosis, but not with the etiology of the liver disorder. Plasma IL-6 levels correlated with immunoglobuline A (Ig A) levels ($r = 0.649$; $p = 0.004$) but not with CRP, ESR, fibrinogen, Ig M or Ig G.
Il-6 is produced within the peritoneal cavity in hepatic and malignant ascites. There is a sharp increase in the local production of IL-6 during an episode of bacterial peritonitis.
This increase was not detectable for TNF-α. It remains to be assessed what the physiological meaning is for this over-production of locally generated cytokines and if this relates to the poor prognosis of patients with cirrhosis and infectious disorders.

Key words: interleukin 6 . tumor necrosis factor α . ascites . cirrhosis . malignancy

9.2 Introduction

IL-6, also called interferon β_2, or hepatocyte-stimulating growth factor, is produced by several cell types such as monocytes, fibroblasts and endothelial cells. Monocytes seem to be the most important in this process [1,2]. IL-6 acts in concert with other cytokines such as IL-1 and TNF-α and leads to a variety of different actions including maturation of B cells for immunoglobulin synthesis, and induction of the synthesis of acute-phase proteins by hepatocytes [3]. IL-6 appears to be a key member of the IL family, however many of the interactions with other lymphokines are poorly understood. Elevated serum levels of different cytokines in chronic liver disease have been reported [4,5]. The pattern of cytokine elevation is stage dependant and is only moderately affected by the type of liver disease. There are some preliminary reports, suggesting high IL-6 production in ascitic fluid during spontaneous bacterial peritonitis [6-8], and without peritonitis [9]. A correlation between survival and elevated TNF-α [10] and high serum IL-6 levels [11,12] has been established for patients with alcoholic hepatitis. A profound increase of IL-6 and TNF-α after the onset of septicaemia which

lasted much longer in patients with cirrhosis compared to controls may be related to the high mortality of infectious complications in cirrhotic patients [13,14].
In order to assess the diagnostic and prognostic value of these cytokines, we measured plasma as well as ascitic fluid IL-6, TNF-α and IL-1β in patients with ascites of different etiology, including carcinomatous peritonitis and different causes of decompensated cirrhosis and portal hypertension. In some patients with an episode of SBP cytokines were measured serially in ascitic fluid. Plasma and ascitic fluid IL-6, TNF-α and IL-1β levels were compared to clinical data, biochemical measurements, diagnosis and prognosis.

9.3 Material and methods

Patients: Thirty-two patients with hepatic ascites and 8 patients with malignancy-related ascites were prospectively studied from June to December 1993. Sixteen patients had alcoholic cirrhosis, 7 patients had hepatitis B or C virus induced cirrhosis, 5 patients had primary biliary cirrhosis (PBC) or sclerosing cholangitis (PSC) and 4 patients had other another liver disease (auto-immune disease 2x, cryptogenic 1x and M Wilson 1x). Of the malignancy-related ascites group all patients had metastasized disease (2 patients had pancreatic adenocarcinoma, 2 peritoneal adenocarcinomatosis with unknown primary tumor, 1 gastric carcinoma, 1 peritoneal mesothelioma, 1 coloncarcinoma and 1 leiomyosarcoma). Five patients developed spontaneous BP (SBP) during the course of the disease, four patients with cirrhosis and one patient with a peritoneal adenocarcinomatosis. For further analysis patients are divided in three groups; cirrhosis (n = 28), malignancy-related ascites (n = 7) and bacterial peritonitis (n = 5). In 23 out of the 40 patients, plasma samples were available, taken within 24 h after paracentesis. In those 40 patients a total of 56 ascitic fluid samples were available for biochemical analysis, cultures and cytokines assays. If more than one sample was analyzed in a patient, the patient's average was used in the statistical analysis. Patient characteristics are given in Table 9.1. Survival was assessed during a period of six months.

Cytokine assay: Ascites and blood were obtained under sterile conditions and immediately cooled to 0^0C and centrifuged. Aliquots of cell-free samples were stored at -70 ^{0}C till assay. Il-6 was determined in ascites and plasma by an ELISA (Hycult, Uden, The Netherlands) and TNF-α (Eurogenetics, Tessenderlo, Belgium) and IL-1β (Eurogenetics, Tessenderlo, Belgium). The determination threshold for IL-6 was 2 pg/ml, for TNF-α 10 pg/ml and for IL-1β 2 pg/ml.

Blood and ascitic fluid sampling: Blood samples were taken within 24 h after the paracentesis was performed. In addition to routine biochemical assessment, plasma C reactive protein, IgG, IgM, IgA, and fibrinogen levels were measured. All ascitic fluid samples were cultured with bedside inoculation in blood culture bottles. Total protein, lactate dehydrogenase, cholesterol, triglycerides and leucocyte count with differential

counting was measured in each ascitic fluid sample. In all patients with cirrhosis the severity was graded by the Child-Pugh score. Spontaneous bacterial peritonitis was defined as a positive ascitic fluid culture with more than $0.25x10^9$ polymorphonuclear cells/l.

Table 9.1 Clinical and laboratory characteristics of the patients (Mean ± SD).

	Hepatic ascites group 1	Malignant ascites group 2	Bacterial peritonitis group 3
Number of patients	28	7	5
Age yrs (mean ± SD)	54 ± 15	58 ± 14	42 ± 17
Gender M/F	17/11	6/1	2/3
Child-Pugh B/C	13/15		2/3
serum bilirubine µmol/l	84 ± 101	47 ± 87	122 ± 156[a]
Ascites leucocytes 10^9 cells/l	0.1 ± 0.1	0.7 ± 0.7[b]	8.8 ± 6.5
Ascites LDH U/l	80 ± 61[c]	295 ± 217	274 ± 253
Ascites total protein g/l	10 ± 7	36 ± 9[d]	11 ± 6
Ascites cholesterol mmol/l	0.5 ± 0.4	1.9 ± 0.9[e]	0.4 ± 0.4
Ascites triglyceride g/l	0.4 ± 0.4	0.8 ± 0.3	1.1 ±1.8
Plasma IL-6 pg/ml	542 ± 719	559 ± 604	1637 ± 907
Ascites IL-6 pg/ml	13816 ± 15314	28138 ± 23403	133268 ± 99743[f]
Ascites/plasma IL-6 ratio	29.2	30.3	56.7

[a] : p=0.01 group 3 versus group 1 and 2
[b] : p=0.002 group 2 versus group 1 and 3
[c] : p=0.002 group 1 versus group 2 and 3
[d] : p=0.004 group 2 versus group 1 and 3
[e] : p=0.004 group 2 versus group 1 and 3
[f] : p=0.002 group 3 versus group 1 and 2

Statistical analysis: Data are summarized as the mean ± standard deviation (SD). The Mann-Whitney U-test was used to compare data between two groups. The Kruskal-Wallis test was used to compare data between more than 2 groups. Correlations were estimated by the Spearman rank correlation coëfficient. p-Values (two-tailed) of less than 0.05 were considered statistically significant.

9.4 Results

Ascitic fluid IL-6 levels were higher in patients with malignancy-related ascites compared to hepatic ascites (28138 ± 23403 pg/ml vs 13816 ± 15314 pg/ml), but due to the wide scatter of values this did not reach statistical significancy (p = 0.21). However during an episode of SBP there was a marked increase in ascitic fluid IL-6 levels (133268 ± 99743 pg/ml, p=0.002 when compared to hepatic and malignant ascites) while plasma values (1637 ± 907 pg/ml vs 542 ± 719 pg/ml, p = 0.07) were barely influenced by this sharp rise in ascitic fluid levels (Figure 9.1).

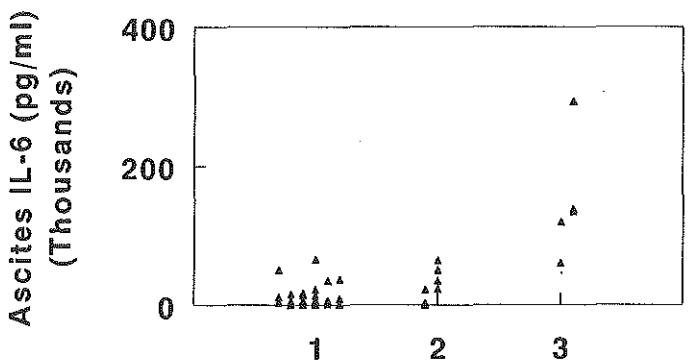

Figure 9.1 Ascitic fluid IL-6 levels in hepatic ascites (1), malignant ascites (2) and bacterial peritonitis (3).

In 2 patients ascitic fluid levels were assessed after antibiotic treatment and declining values were obtained, finally similar to other patients with hepatic ascites without infection. The type of micro-organism causing the ascitic fluid infection (*Escherichia coli* 2x, *Klebsiella pneumoniae* 1x, *Staph. Epidermidis* 1x, and culture-negative 1x) did not seem to influence the IL-6 levels. TNF-α levels were not statisticaly different between the patients with hepatic ascites and those with malignant ascites. Ascitic TNF-α 19 ± 50 pg/ml vs 12 ± 31 pg/ml and plasma TNF-α 3.4 ± 8.2 vs 6.1 ± 13.8 pg/ml. As TNF-α was only detectable in plasma in 7 patients and in ascites in 8 patients, no reliable statistical analysis was possible. Even in the patients with bacterial peritonitis and in some terminally ill patients with malignant ascites TNF-α could not be detected in ascites and plasma. IL-1β was not detected in any ascites or plasma samples.

None of the parameters could differentiate between patients who survived more than six months and the eleven patients who died during this period. There was a correlation between plasma IL-6 and the Child-Pugh score (r = 0.675; p = 0.002, Figure 9.2).

No relation could be established between the diagnosis of the underlying liver disorder (alcoholic, viral or PBC/PSC) and the cytokine profile (Figure 9.3). Plasma IL-6 levels

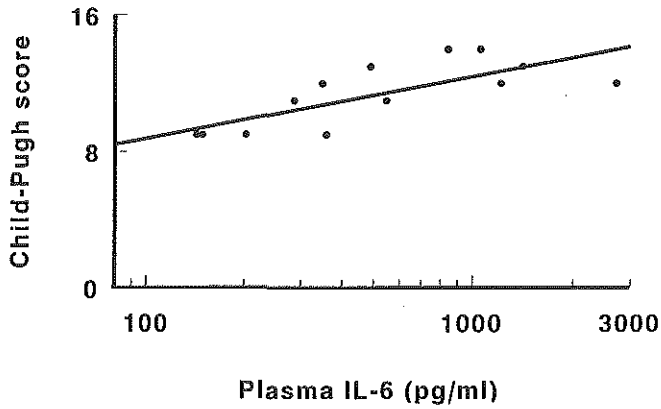

Figure 9.2 Correlation between the Child-Pugh score and plasma IL-6 levels in patients with cirrhosis.

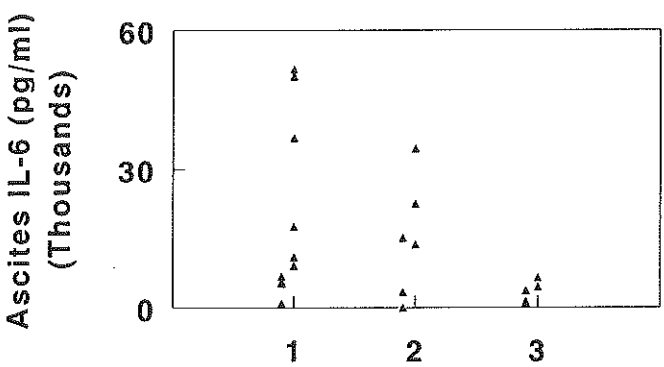

Figure 9.3 Ascitic fluid IL-6 levels in patients with alcoholic (1), viral (2), or PBC/PSC liver disease (3).

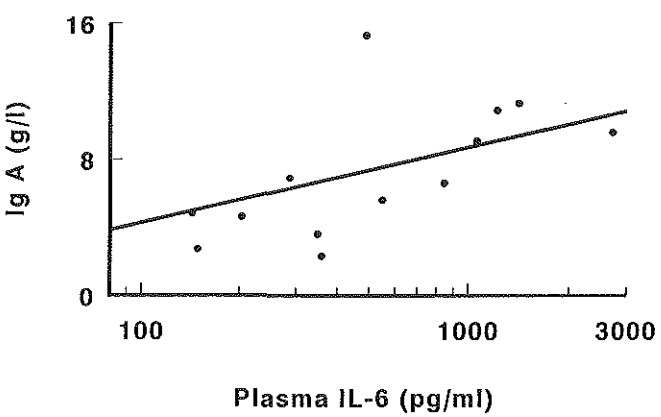

Figure 9.4 Correlation between plasma IL-6 levels and IgA concentration.

correlated with serum Ig A levels ($r = 0.649$; $p = 0.004$, Figure 9.4) and slightly with C reactive protein ($r = 0.49$; $p = 0.04$) but not with Ig M ($r = 0.30$; $p = 0.22$), Ig G ($r = 0.34$; $p = 0.15$), and fibrinogen ($r = -0.17$; $p = 0.53$).

9.5 Discussion

In this study we found evidence for a high production of IL-6 locally produced within the peritoneal cavity. The ascitic fluid levels were 30 times higher than plasma levels for hepatic as well as for malignant disease. Apparently, even the presence of sterile ascites generates enough inflammation of the peritoneum with an increase of locally active monocytes and macrophages to cause these high levels of cytokines. Hepatic clearances of locally generated cytokines may be diminished in patients with cirrhosis, however this mechanism does not seem to be of much importance, as malignant ascites with an intact liver function and normal hepatic clearance generates the same high ascites/plasma ratio's. More evidence for the local production within the peritoneal cavity comes also from the fact that during bacterial inflammation of the ascitic fluid ascites levels are about ten times higher, with an ascites/plasma ratio of about 60, compared to ascitic fluid levels without infection. Ascites dynamics in patients with cirrhosis suggests that there is a continual shift between ascitic fluid and the circulation with peritoneal free-water clearances of 4-5 l/hour [15] and a high lymph flow within the thoracic duct rich in protein and albumin content [16,17]. Therefore the IL-6 is quickly cleared from the circulation as plasma levels are low and not very much influenced by the presence of bacterial peritonitis. Indeed the half-life of IL-6 has been determined to be only a few minutes [3].
High IL-6 synovial fluid levels of patients with rheumatoid arthritis with little activity in serum have been demonstrated [18] as well as high cerebrospinal fluid levels in patients with acute meningitis, but not in those with chronic or non-infectious neural disease [3]. Local production of IL-6 in acute and chronic liver disease has also been demonstrated by endothelial, Kuppfer, and infiltrating mononuclear cells in liver tissue, expression being more pronounced in areas with most inflammation [19,20]. Many of the activities of TNF-α, IL-1, and IL-6 indicate a local rather than a systemic function for these cytokines. IL-6 shares many of its biological activities with TNF-α and IL-1, and probably all three cytokines act in concert directing inflammatory and immunological reaction. For example, IL-1 and TNF-α can stimulate IL-6 production, but IL-6 suppresses TNF-α and IL-1β production [21]. The fact that we did not find elevated TNF-α levels in plasma and ascites of most patients does not seem to concur with other studies. Most studies describe an increase in ascitic fluid levels during bacterial inflammation of both IL-6 and TNF-α [6-8]. Several explanations could be given for this discrepancy. First of all there might be a difference in the methodology measuring TNF-α [22]. For example Propst et al. [6] used an enzyme amplified immunoassay (EASIA) which might detect lower levels of TNF-α than the assay we used. Zeni et al. [7] used a competitive inhibition radioimmunoassay with the use of coated monoclonal antibodies to TNF-α, fixed to the inner plastic surface of the tubes.

Also Devière et al. [8] made use of an immunoradiometric assay to measure TNF-α. Another explanation for the undetectable levels of TNF-α levels might be sought in the presence of high concentration of soluble TNF receptors in ascites, which might be a potential source of interference for immunoassays [23,24]. Van Zee et al. [25] found a reduction of TNF-α immunoactivity, as determined by ELISA, when soluble type I and type II TNF-α receptors were added to plasma. The same might be applicable for IL-1 receptor antagonists. The low concentration of IL-1 in ascites, even during periods of acute bacterial inflammation, was also confirmed in other studies [6,7].
Plasma IL-6 levels correlated with the severity of the underlying liver disease, indicated by the Child-Pugh score, but not with the etiology of the cirrhosis. This stage dependency suggests that porto-systemic shunting or impaired hepatic clearance may also contribute to the sustained elevation of endogenous cytokines. Bacterial lipopolysaccharide-induced macrophage stimulation due to prolonged endotoxaemia in patients with chronic liver disease could be responsible for activation of the cytokine cascade. A correlation between serum Ig A levels and IL-6 concentration has been described before [26] and seems to represent increased B cell maturation directly caused by IL-6. Why this correlation has repeatedly been found only for Ig A levels and not for Ig M and Ig G levels is not entirely clear. It might be that B-cells producing immunoglobulin Ig A are simply more responsive to the stimulatory effect of IL-6. In our findings there is no evidence that this effect was restricted to patients with alcoholic liver disease.
What is the potential pathogenetic role of the cytokine imbalance in patients with cirrhosis?
Although locally produced IL-6 might provide stimulatory signals for the hepatocytes, resulting in liver regeneration, excess of cytokines could lead to tissue damage and fibrosis [27].
Prolonged increased levels of IL-6 and TNF-α after septicaemia have been found in cirrhotics and there is some evidence suggesting that increased cytokine levels makes the host more susceptable to endotoxaemia [13]. This may be related to the poor prognosis of infectious complications during cirrhosis [14]. Furthermore, it does seem possible that the very high concentrations of IL-6 locally produced within the peritoneal cavity during bacterial peritonitis, is contributing to the decompensation of liver disease often found in patients with SBP.
Finally, we found evidence for high IL-6 production in the ascites in patients with and without cirrhosis. Plasma IL-6 levels in patients with cirrhosis are stage-dependant but do not correlate with the etiology. Due to the broad scatter of values in plasma as well in ascitic fluid, IL-6 can not be used to differentiate between different etiologies of ascites in the individual patient. The exact effects of IL-6, whether beneficial or harmful to the patient are at present not well understood, therefore it is too early to speculate on the therapeutic use of IL-6 antagonists, for example in patients with recurrent bacterial infection of the ascitic fluid.

9.6 References

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Part V

General discussion and summary

Chapter 10 General discussion

Inflammatory mediators are involved in allergy and acute and chronic inflammatory diseases. To treat these type of diseases, the specific role of the inflammatory cells and their mediators should be understood. Human peritoneal macrophages (Mϕ) obtained from ascitic fluid could be used as a human *in vitro* Mϕ model. Our human *in vitro* Mϕ model could contribute to understanding the mechanism of human inflammatory diseases and the search for therapeutic agents which act as specific inhibitors or neutralizers of inflammatory mediators, and thus finally diminish the whole inflammatory process.
To understand the mechanism of human inflammatory diseases care should be taken with when using only animal models. When *in vitro* studies are done, preferably human *in vitro* models should be used. As our studies show, the profile of the eicosanoid production of human peritoneal Mϕ was not similar to the eicosanoid profiles of Mϕ from other species. The interactions between the eicosanoids and cytokines of human peritoneal Mϕ were also different compared with to the interactions of eicosanoids and cytokines of Mϕ from other species.

Eicosanoid and cytokine production of human peritoneal Mϕ: We measured the exogenous eicosanoid profile of the human peritoneal Mϕ by high performance liquid chromatography (HPLC). These Mϕ mainly produced the 5-lipoxygenase products leukotriene B_4 (LTB_4) and 5-hydroxy eicosatetraenoic acid (5-HETE) when stimulated with calcium ionophore A23187. The levels of the cyclooxygenase products were low and did not significantly increase with A23187. Lipopolysaccharide (LPS) did not influence the endogenous eicosanoid production at all.
Our study with rat peritoneal Mϕ shows that Mϕ produce only cyclooxygenase products (Chapter 3). It is not easy to compare the results of human peritoneal Mϕ with Mϕ of different origin and/or species characterized by other groups, as only a few eicosanoids are measured per experiment. An overview is given in Table 2.6 in the Introduction (Chapter 2). This table shows clearly that human peritoneal Mϕ, isolated from a chronic inflammation site (ascites), differs from resident and activated rat and murine Mϕ, isolated from different locations and stimulated with different stimuli. Peritoneal rat Mϕ mainly produce cyclooxygenase products. Murine peritoneal Mϕ produce, besides cyclooxygenase products, also LTC_4 when stimulated with A23187.
The eicosanoid profile of normal alveolar Mϕ and peritoneal Mϕ isolated from continuous ambulatory peritoneal dyalisis (CAPD) patients with peritonitis stimulated with A23187, was very similar to our human peritoneal Mϕ isolated from ascitic fluid. There were not enough data available on the eicosanoid production of human alveolar Mϕ activated *in vivo* and of resident peritoneal Mϕ isolated from CAPD patients, to make a good comparison with our human peritoneal Mϕ.
The production of the pro-inflammatory cytokines interleukin 1ß (IL-1ß), IL-6 and tumor necrosis factor α (TNF-α) from human peritoneal Mϕ is similar to the profile of Mϕ of other origin and species stimulated with LPS [1-9]. The cytokine production increased with increasing LPS concentration and the production level of IL-1ß and IL-6

reached a plateau around 24 hours. TNF-α always peaked before IL-1ß and IL-6. Only the TNF-α production from Mϕ of other species always peaked at an earlier time than the TNF-α production from the human peritoneal Mϕ [1,6,10,11].

Eicosanoid and cytokine interaction of human peritoneal Mϕ: To understand the mechanism of human inflammatory diseases, it should be known how inflammatory cells regulate their own and each other's eicosanoid and cytokine production *in vivo*. As a contribution to unravel this complexity, we characterized *in vitro* the interactions between the eicosanoids LTB_4 and prostaglandin E_2 (PGE_2) and the cytokines IL-1ß, IL-6 and TNF-α of human peritoneal Mϕ, by the addition and inhibition of several eicosanoids and cytokines.
Human peritoneal Mϕ have a positive interaction between IL-1ß and LTB_4, a negative interaction between IL-1ß and PGE_2 and no significant interaction between IL-1ß and IL-6. These last two results are in contrast with cells of other species origin and/or tissue origin in which IL-1ß stimulated the LTB_4, PGE_2 and IL-6 production [12-16]. In human peritoneal Mϕ the leukotrienes are not involved in the regulation of IL-1ß and IL-6. With dual inhibitors it was reported that leukotrienes are involved in IL-1 production [17,18]; however, with specific inhibitors similar results as ours were obtained for IL-1 with activated murine peritoneal Mϕ and human peripheral blood monocytes of healthy donors were found [19,20]. In our study, when LTB_4 was selectively inhibited PGE_2 also decreased. With increasing LTB_4, the PGE_2 levels did not change.
Increasing PGE_2 down-regulated the TNF-α production. This has been reported before, but only in different animal models. In contrast to Mϕ of other species with decreasing PGE_2 no significants effect on the TNF-α production of human peritoneal Mϕ were seen [21,22].
This study shows that the cytokines and eicosanoids influence each other's production differently than in Mϕ of other species. This could have consequences when therapeutics are being developed, which specifically inhibit or neutralize inflammatory mediators in disease. They could have different effects in *in vitro* and *in vivo* studies using inflammatory cells of other species.

10.1 The human peritoneal macrophage *in vitro* model

Decreasing the activity of the pro-inflammatory mediators in disease and achieving improvement of these diseases could be done by using different strategies. Inflammatory diseases are now treated by drugs which inhibit the mediators non-selectively: The most important and effective drugs are the glucocorticoids and the non-steroidal anti-inflammatory drugs (NSAID). More specific inhibitors and neutralizers are in the development stage.
As Mϕ have a central and essential role in the initiation and maintenance of many immunologic and inflammatory processes, the effects of specific inhibitors or neutralizers can be first tested on Mϕ, following our human *in vitro* Mϕ model.

Our experiments in which the eicosanoid and cytokine pattern of the human peritoneal Mϕ was characterized and the experiments in which the interactions of human peritoneal Mϕ with several eicosanoids and cytokines was studied, have shown that the eicosanoid and cytokine production and the interactions of these mediators of human peritoneal Mϕ differ from Mϕ of other species. Therefore, to study the effects of selective inhibitors and neutralizers *in vitro* on human cells, the usefulness of these compounds *in vivo* will be more predictable and probably lead to a better understanding of their mechanisms.

There are several options for selective inhibition or neutralization of the mediators involved in inflammatory diseases:

(1) Selective inhibition of the leukotrienes with specific 5-lipoxygenase inhibitors,
(2) Increasement of the levels of anti-inflammatory cytokines which inhibit the pro-inflammatory mediators of the Mϕ,
(3) Neutralization of the pro-inflammatory mediators with specific antibodies or soluble cytokine receptors against these mediators,
(4) Receptor blockade of the inflammatory mediators with receptor antibodies or receptor antagonists against the receptors of these inflammatory mediators,
(5) Inhibition of the influx of leucocytes through blocking the adhesion molecules on the inflammatory cells and endothelial cells with antibodies. Consequently Mϕ and other inflammatory cells will not migrate through the vessel wall to the site of inflammation.
(6) A combination of different therapies as mentioned above, may lead to a synergistic effect.

The search for specific inhibitors or neutralizers of mediators involved in the inflammatory process is still in the elementary stage. Testing has started in several animal models and a few clinical trials have been done. Several of these options as mentioned above (1-4,6) can be tested in our *in vitro* model.

The following section firstly presents a description of several inflammatory diseases (asthma, inflammatory bowel disease (IBD) and septic shock) in which Mϕ and their inflammatory mediators have a significant contribution. Then the mechanism of action of several drugs which are available for clinical use will be described. Thereafter, a description is given of selective inhibitors and neutralizers of inflammatory mediators which are currently under investigation, together with a discussion on the use of peritoneal Mϕ.

10.2 Mϕ and their inflammatory mediators in diseases

Asthma: Asthma is a disorder characterized by varying degrees of reversible airway obstruction in association with exaggerated airway responsiveness to a wide variety of physical and pharmacological agents. Until recently, asthma has been thought of as

solely an immunological disorder involving the release of broncho-active mediators from mast cells that are triggered by antigen via immunoglobulin E-dependent mechanisms. However, allergen mediated bronchospasm is not the fundamental feature present in all asthmatics, and is not the basic pathophysiological alteration present in all asthmatic diseases.
It is now widely accepted that asthma is a chronic inflammatory disease. Superimposed on this chronic inflammatory state are acute inflammatory episodes, that correspond to exacerbations of asthma [23-25].

Many different inflammatory cells and their mediators are involved in asthma. Mast cells have an important role in initiating the acute responses to allergen and other stimuli (exercise, hyperventilation, fog); eosinophils and T-lymphocytes are more important in the chronic inflammatory response. Mϕ are probably important in both phases. The Mϕ are the major cells in the airway lumen, and in broncho-alveolar lavages and in biopsies it is found that their number has markedly increased in asthmatic patients. Following antigen challenge *in vivo* they become activated (morphological changes) and *in vitro* it has been shown that they are able to respond to antigen by releasing chemo-attractants and pro-inflammatory mediators.
Mediators such as histamine (derived mast cells), prostaglandins and leukotrienes (released from Mϕ and mast cells) are important in the acute and subacute inflammatory response. They contract airway smooth muscle, increase airway hyperresponsiveness, increase microvascular leakage, increase airway mucus secretion and attraction of other inflammatory cells. Cytokines play a dominant role in chronic inflammation. IL-3, IL-4 and IL-5 secreted by T-lymphocytes are important for mast cells (survival), B-cells (production of Ig E) and eosinophils (differentiation, survival and priming), respectively, respectively. Cytokines as IL-1, IL-6 and TNF-α produced by the Mϕ, in combination with the cellular cooperation of the Mϕ with the other inflammatory cells (e.g. T-lymphocytes, eosinophils) and endothelial cells, are important in the amplification of the inflammatory response [23-31].

Inflammatory bowel disease (IBD): This disease encompasses at least two forms of intestinal inflammation: ulcerative colitis (UC) and Crohn's disease (CD). The causes of these bowel diseases are unknown. They are defined empirically by their typical clinical, pathological, radiological, endoscopic and laboratory features.
The pathogenesis of IBD can be divided into two stages. The first is the initiating event which triggers the inflammatory response, but this event remains unknown. The second is the amplification of the inflammatory response which involves the inflammatory cells, including Mϕ, neutrophils, lymphocytes and mast cells [32].
It is known that in IBD, the production of pro-inflammatory cytokines as IL-1, IL-8 and TNF-α, which can be produced by Mϕ, are increased [33-35]. Also several eicosanoid products (15-HETE, hydroxy-heptadecatrieoic acid (HHT), thromboxane (TXB_2), PGE_2 and LTB_4), which can be produced by Mϕ, are present in increased amounts in association with IBD [36-38]. Oxygen free radicals generated by

neutrophils and Mϕ may also have a role in tissue injury [32].

The incidence of UC is inversely associated with smoking, and clinical relapses have been associated with cessation of smoking. Conversely, CD has been directly associated with smoking [39,40]. The correlation between smoking and CD could be a direct effect of nicotine on the Mϕ. In CD predominantly Mϕ and lymphocytes are infiltrated, while in UC the lamina propria is densely infiltrated by neutrophils [32]. In our human *in vitro* Mϕ model we observed that nicotine enhances the IL-6 production of *in vitro* LPS stimulated Mϕ (personal observation). Smoking could increase locally the IL-6 levels in patients with CD by affecting the Mϕ present in the lamina propria; however, further investigations have to be performed to clarify the mechanism of action.

Septic shock. Circulatory shock from gram negative bacterial sepsis, produces a spectrum of pathophysiological alterations including cardiopulmonary, renal, haematologic and metabolic dysfunction leading to vascular collapse.
Many mediators of the pathogenesis of sepsis have been described, e.g. TNF-α, IL-1, IL-6, and lipoxygenase products [41,42]. All these mediators could be products derived from Mϕ, because LPS, the major component of the outermost membrane of gram-negative bacteria, is a potent stimulus for Mϕ (see section 2.6).

A central mediator of sepsis is not known yet, although IL-1 and TNF-α have been commonly proposed for this role; however, these levels are not in all patients high. This is most probably due to differences in the release of endotoxin, local mediator production by Mϕ and the immunological status of the patient. Different methods of measurement may also influence the results [43].
Usually, the mediators are measured in the blood plasma of the patients. In our study, patients with SBP who have a high risk to develop a septic shock had very high levels of IL-6 in their ascitic fluid, although the IL-6 levels were much lower in the blood (Chapter 8).

Epithelial damage in sepsis probably results from persistent and repetitive inflammatory insults, probably locally initiated and controlled by the Mϕ. These insults produce sufficient damage that, down regulation (e.g. cytokine inhibition by PGE_2, T-cell inhibition by Mϕ) of the inflammatory reaction can no longer occur; this may lead to a state of metabolic anarchy in which the body can no longer control its own inflammatory response [41,44].

10.3 Therapy: strategies and application in diseases

10.3.1.1 Glucocorticosteroids

Glucocorticosteroids [GCs, e.g. hydrocortisone (cortisol) and cortisone] are hormones

which are synthesized and released by the adrenal cortex when they are needed. Synthetic compounds are also available, e.g. Prednisolone (prednisone), Methylprednisolone, betamethasone and dexamethasone.
When given therapeutically, GCs have powerful anti-inflammatory and immunosuppressive effects. They inhibit both the early and late manifestations of inflammation by reduction of the blood supply, the accumulation of leucocytes at the site of inflammation, and by directly effecting Mϕ and other inflammatory cells [45].

GCs directly affect Mϕ by decreasing the function and production of their inflammatory mediators [26]. The production of pro-inflammatory cytokines as IL-1, IL-6, IL-8 and TNF-α [46], and also IL-4, a cytokine which has inhibitory effects on Mϕ [(e.g. IL-1α, TNF-α and PGE_2 production), 47], are inhibited [48]. GCs also inhibit eicosanoid production. It was first thought that GCs inhibit eicosanoids through the upregulation of lipocortin 1 - a calcium and phospholipid binding protein which inhibits the activity and synthesis of PLA_2 [49]. Recently it has been suggested that lipocortin 1 has a key function in the GCs regulation of inflammatory and immune responses, not only by inhibiting the eicosanoid production, but also through other mechanisms. It seems that though lipocortin 1 has no signal sequence for secretion, the protein can be secreted by cells and there are receptors for lipocortin 1 on phagocytes. These receptors influence the inflammatory action of the cells by mechanisms independent of inhibition of PLA_2 activity. Lipocortin 1 has also, for example, inhibitory effects on cytokine induced cell migration and release of reactive induced oxygen species. Besides anti-inflammatory effects, lipocortin 1 also protects the brain against damage and has selective effects on the release and actions of several neuro-endocrine factors. It is possible that, besides lipocortin 1, there are other mechanisms through which the GCs control the inflammatory and immune response, that will be revealed in the future [50,51].

In clinical use GCs are used for anti-inflammatory therapy or immunosuppression, for example:

- In asthma GCs are the most effective treatment to decrease the number of inflammatory cells and the release of inflammatory mediators; they also reduce the microvascular permeability and mucus secretion [26,52].
- In IBD GCs are effective for the treatment of mild to severe UC or CD [32].
- In patients with septic shock GCs did not reduce the mortality [53,54], while in animal models they were effective [55,56]. This could be due to the time of administration, in animals GCs were administered before LPS, and the balance between the toxic effects of the inflammatory mediators and the protective actions of these mediators against the infection [46].

GCs are usually very effective in the therapy of cytokine and eicosanoid mediated pathologies; however, their use is limited, as GCs probably inhibit the synthesis of all cytokines and eicosanoids. This leads to a considerable risk of side effects and

potential complications when prolonged systemic use is needed [45].

10.3.2 Non-steroidal-anti-inflammatory drugs

Non-steroidal-anti-inflammatory drugs (NSAIDs) are divided into different chemical classes: salicylic acids (e.g. aspirin), propionic acids (e.g. ibuprofen), acetic acids (e.g. indometacin), fenamates, oxicams and pyrazolones. They are anti-inflammatory, have anti-analgesic effects and anti-pyretic effects by primarily effecting cyclo-oxygenase in the eicosanoid pathway, thus inhibiting the prostanoids [45].
By decreasing the generation of PGE_2 and PGI_2, NSAIDs will reduce in particular the vasodilation and erythema. However, not all the anti-inflammatory power appears to stem from prostaglandin inhibition, NSAIDs also have the ability to disrupt interactions within the cell membranes, in particular signals which are transduced through the G-proteins e.g. cell-cell adhesion of neutrophils to the blood vessel walls and de-activation of neutrophils [(e.g. superoxide anion production), 57].

Clinical side effects of NSAIDs when used in the long term are stomach irritation, gastrointestinal bleeding and ulceration [(prostaglandins are needed to regulate acid over production and to synthesize the mucus barrier that prevents its self-digestion), 58,59].

The two isoforms of cyclooxygenase (COX), COX-1 and COX-2, are both sensitive for NSAIDs, but there are clear pharmacological differences. Recent studies on cyclooxygenase (COX-1 and COX-2) support the hypothesis that the side effects of NSAIDs correlate with their ability to inhibit COX-1 (the regulator of normal kidney and gastric functions and vascular homeostasis), while the anti-inflammatory (therapeutic) effects of these agents are due to their ability to inhibit COX-2 [(pro-inflammatory functions), 60-63].
For example, aspirin and indomethacin are strong inhibitors of COX-1 (at anti-inflammatory dose IC_{50} COX-1:IC_{50} COX-2 = 166 and 60 respectively). They are also the two NSAIDs that cause the most gastric damage. The NSAIDs diclofenac and naproxen inhibit COX-1 and COX-2 almost equally (ratio IC_{50} COX-1:IC_{50} COX-2 = 0.7 and 0.6 respevtively). At anti-inflammatory doses they are less ulcerogenic than aspirin or indomethacin. BF 389 (ratio IC_{50} COX-1:IC_{50} COX-2 = 0.2) and NS 398 (IC_{50} COX-2 $<$ 30 nM, no IC_{50} for COX-1) two experimental drugs, are potent and selective COX-2 inhibitors and have little or no gastric ulcerogenicity.
The development of selective COX-2 inhibitors will probably lead to anti-inflammatory drugs without side effects which are associated with the inhibition of COX-1 [60-62]. The GCs block the synthesis of COX-2 but not of COX-1 [63].

Blocking of cyclooxygenase diverts arachidonic from PGE_2 to the lipoxygenase products LTB_4 in the gastric mucosal, this may also contribute to mucosal injury [64]. However, BW755C, a dual COX (ratio IC_{50} COX-1:IC_{50} COX-1 = $\pm$ 1) and 5-

lipoxygenase inhibitor, is an experimental anti-inflammatory agent with little or no ulcerogenic activity. This is probably due to less inhibition of COX-1 at anti-inflammatory concentrations (as hypothized above) and less production of LTB_4 [60]. Possibly *in vivo* with decreasing PGE_2, TNF-α increases. In our experiments (Chapter 7) we have shown that *in vitro* exogenous PGE_2 can down-regulate the TNF-α production of human peritoneal Mϕ and there was a trend of increasing TNF-α with decreasing PGE_2. This increasing TNF-α could also be a source of the side effects caused by NSAIDs.

10.3.3 Neutralization of the effects of pro-inflammatory mediators

By direct interference with the effects of the key cytokines which sustain the inflammation, therapeutic intervention can probably be achieved. The interaction between Mϕ and the other inflammatory cells will be disturbed (less activation) and less pro-inflammatory mediators will then be produced, leading to a decreased influx of Mϕ and less activation of the Mϕ present locally at the inflammation site. This will then lead to a decrease of physiological responses. Cytokines and other inflammatory mediators which are anti-inflammatory or/and have a positive effect on the homeostasis will then probably not recover or be disturbed.

10.3.3.1 5-lipoxygenase inhibitors

Drugs which only block the 5-lipoxygenase production, also have therapeutic potential. The leukotrienes have marked effects on neutrophils and other inflammatory cells. By blocking the local 5-lipoxygenase production, less neutrophils and monocytes/Mϕ will migrate to the site of inflammation and the inflammatory cells will also be less activated at the site of inflammation, releasing less inflammatory products. This has been shown in different animal models and clinical trials. For example:

- Asthma: We have shown *in vitro* that E6080 inhibits specific LTB_4 production (Chapter 6). Tsunoda et al. have shown that in antigen sensitized guinea pigs, E6080 dose dependently inhibited bronchospasm, inhibition of leucocyte infiltration and decreased bronchoepithelial damage [65]. In clinical trials, specific 5-lipoxygenase inhibitors tended to reduce the symptoms [66,67].
- Ulcerative colitis: Zileuton, a 5-lipoxygenase inhibitor, has proven effective in an experimental model of colitis, showing that leukotrienes play an important role in the pathogenesis of IBD [68].
  Selective 5-lipoxygenase inhibitors have a potential effectiveness in the management of UC [28] by decreasing LTB_4 in rectal dialysis of patients with active UC [36].
- Septic shock: Selective 5-lipoxygenase inhibitors are able to protect against shock. In rat studies it was demonstrated that the initial hypotension induced by endotoxin was attenuated by a 5-lipoxygenase inhibitor, probably by directly effecting the LTs. Sulfopeptide LTs are potent vasoconstrictors, they probably

affect systemic pressure by directly increasing vasculator permeability. Plasma immunoreactive 6kPGF$_{1\alpha}$ which was increased in the later phase of endotoxic shock was reduced with a 5-lipoxygenase inhibitor, probably by blocking LTC_4 and LTD_4 which stimulate prostacyclin synthesis and release from endothelial cells [42].

The effectiveness of new and existing 5-lipoxygenase inhibitors can be tested in our *in vitro* model, as we have shown with E6080. The usefulness of these specific 5-lipoxygenase inhibitors *in vivo* will then be more predictable.

10.3.3.2 Anti-inflammatory cytokines

The effects of the pro-inflammatory cytokines can be inhibited by application of the proper anti-inflammatory cytokine(s), the inflammatory reaction may then down-regulated. IL-4, IL-10 and IL-13 suppresses cytotoxic and inflammatory functions of Mϕ.

- IL-4 has so far only been tried in patients with cancer. Recombinant human IL-4 had anticancer effects in animal models; however, when tried in patients with renal cancer or melanoma, it did not produce meaningful activity [69].

Besides Mϕ, intestinal epithelial cells are also a source of IL-8. In inflammatory IBD the source of IL-8 is not yet clear. From studies on epithelial cell lines it became clear that IL-4 and IL-10 could not down-regulate the IL-8 production. This difference in regulation should be taken into account when down-regulation of the IL-8 production with 'suppressive' cytokines is warranted [70].

Our *in vitro* model will be very useful to test the effects of anti-inflammatory cytokines on the eicosanoid and cytokine production. As we have shown in Chapter 7, there are similarities but also differences between eicosanoids and cytokines in comparison to Mϕ of other species. By studying the effects of natural occurring cytokine inhibitors on human Mϕ, their effect *in vivo* will be more predictable. Not only will pro-inflammatory cytokines be influenced, but most probably also several eicosanoids, as the cytokines influence the eicosanoid production.

10.3.3.3 Cytokine antibodies and soluble cytokine receptors

Inflammatory mediators can be neutralized with specific antibodies or with soluble cytokine receptors.

- For example, IL-6 has also pro-inflammatory effects in rheumatoid arthritis. Anti-IL-6 monoclonal antibodies resulted in clinical and biological improvement of patients with severe rheumatoid arthritis. Further studies are required to evaluate the real benefit and the mode of action of this monoclonal antibody [71].

With the measurement of Mϕ products in the ascitic fluid and plasma of patients with ascites due to different etiology, we have shown that IL-6 is very high in patients with spontaneous bacterial peritonitis (SBP; Chapters 8 and 9). IL-6 could be used to monitor disease activity in patients with SBP. Local treatment with antibodies against IL-6, may reduce the risk for the development of septic shock.

Naturally occurring soluble receptors have been found for a variety of cytokines. These soluble cytokine receptors bind to their respective cytokines. The *in vivo* function of soluble receptors is not yet known. When there are soluble cytokines presents, the cytokines may bind to the soluble receptors and possibly not to their specific receptors on the target cells. *In vitro* it has been shown that soluble receptors can block cell activation [72,73].
However, it is also possible that the soluble cytokine receptors (and also the autoantibodies) transport the cytokine through the circulation and present it to the cell surface receptors and the target cells. The activity of the cytokine is then enhanced as it is protected from proteolysis and these soluble receptors act then as a reservoir of active or potentially active factors. Enhancing activities have been demonstrated for the soluble IL-6 receptor [74].

Our *in vitro* model will be useful to test the effect of inhibiting the effect of IL-1 with IL-1 receptor antagonist (IL-1ra). As we have shown in Chapter 7, IL-1 increases LTB_4 production and decreases PGE_2 production.

10.3.3.4 Receptor antibodies and receptor antagonists

Blockade of the receptors to which the inflammatory mediators bind with receptor antibodies or receptor antagonists is another possibility. *In vivo* in a mouse model, it was shown that IL-1 induced catabolic host changes, associated with inflammation, could be blocked with monoclonal antibodies directed against the IL-1 receptor [75]. IL-1ra is a naturally occurring antagonist, so far the only one known, blocking the binding of IL-1α and IL-1ß to the IL-1 receptor [76].

- In a rabbit model with immune-mediated bowel inflammation IL-1-ra was a potent anti-inflammatory cytokine, preventing acute colitis and the appearance of chronic inflammation [77,78].
- Administration of IL-1ra, after inducing a bacterial sepsis in animals (rats and rabbits), also showed significant improvement and survival [79,80].
- In an open-label trial in patients with sepsis syndrome or shock, IL-1ra (continuous infusion) seemed in the initial evaluation to be safe and to provide a dose-related survival. Plasma IL-6 was also dose-dependently decreased [81].

Our *in vitro* model will be useful to test the effect of inhibiting the effect of IL-1 with IL-1ra. As we have shown in Chapter 7, IL-1 increases LTB_4 production and decreases PGE_2 production.

10.3.3.5 Cellular adhesion molecules antibodies

Cellular adhesion molecules have an important role in the migration of leucocytes to the site of inflammation (see paragraph 2.3). Inhibiting the infiltration of leucocytes with specific monoclonal antibodies will have therapeutical potential in acute inflammatory events, chronic inflammation and malignancy. This has been shown in experimental models of human diseases e.g. Colitis [82] and allergic asthma [83].

10.3.3.6 Combinations

A combination of different therapies mentioned above may have a synergistic effect, as the production of inflammatory mediators is a result of a variety of cells which are activated by interacting with each other and their mediators. Understanding which inflammatory cells and mediators are crucial for the induction of the allergic response or inflammation is of prime importance, followed by knowledge on which cells and mediators enhance the inflammation. Subsequently a variety of combinations may be possible.

It was shown, for example, that LTD_4 and PAF receptor antagonists, synergistically decrease the antigen-induced airway microvascular leakage in an animal model [84]. In asthma and in IBD the possibilities are the blocking of the cell infiltration (antibodies to cellular adhesion molecules or inhibition of the chemoattractants, e.g. anti-IL-8 monclonal antibody or specific inhibition of LTB_4) combined with antibodies or soluble receptors or anti-inflammatory cytokines, decreasing the pro-inflammatory cytokines.
As suggested by Fischer et al., in patients with septic shock anti-cytokine therapy in combination with standard anti-microbial agents in patients with bacterial sepsis may be an effective strategy [85].

A variety of combinations are possible for inflammatory diseases. Our *in vitro* Mϕ model will be of use to study which combination of specific inhibitor(s) and/or neutralizers will have a synergistic effect in reducing the inflammatory production by Mϕ. This could be done in combination with other human cells with which the Mϕ and their mediators interact during the inflammatory response. This work could then contribute to the understanding of the mechanism of human inflammatory diseases and help in finding therapeutic agents which act as specific inhibitors and/or neutralizers of inflammatory mediators, and will finally abolish the whole inflammatory process.

10.4 References

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Chapter 11 Summary

Ascitic fluid is a readily available source of human peritoneal macrophages (hp-Mϕ). Characterization of the production profile of purified human Mϕ *in vitro* will be helpful for our understanding of inflammatory diseases and their treatment. These Mϕ can then be used as a human *in vitro* model to characterize the interactions of anti-inflammatory drugs with Mϕ. Mϕ are very important in inflammatory diseases, as through their receptors and secretory products, they have a central and essential role in the initiation and maintenance of immunological and inflammatory processes.

Chapter 3: Using the carrageenin-induced peritonitis rat model, we have shown that *in vitro* cytokine and eicosanoid production during an episode of inflammation is mainly due to the presence of Mϕ. The inflammatory mediator production capacity of Mϕ was dependent on the phase of the peritonitis. The eicosanoid and cytokine production *in vitro* was very low on the first day after inducing the peritonitis (the acute phase), then it steadily increased, showing a differentiated pattern in the chronic phase. The synthesis pattern of the inflammatory mediators of peripheral blood monocytes had completely changed in comparison to monocytes which had migrated into the inflamed peritoneum and transformed into peritoneal Mϕ. *In vivo* priming of the Mϕ leads to a change and a higher eicosanoid and cytokine production capacity of the Mϕ *in vitro*.

Chapter 4: Using hp-Mϕ, we have shown that the eicosanoid production depended on the maturity of the Mϕ. Density-defined hp-Mϕ showed morphological and biochemical heterogeneity. The least mature Mϕ, the small round Mϕ, synthesized the least eicosanoids. The more mature Mϕ, the Mϕ with more cytoplasm, had a higher level of eicosanoid production. Mϕ which are more mature, are probably more activated *in vivo* and this activation leads to a higher eicosanoid production capacity *in vitro*.

Chapter 5: The hp-Mϕ were obtained from ascites of patients of varying etiology, mainly liver cirrhosis. This is a sterile chronic inflammation site. Lipopolysaccharide (LPS) stimulation *in vitro* resulted in similar production pattern of the pro-inflammatory cytokines as the LPS stimulated Mϕ of other origin and species. However, the profile of the eicosanoid production of hp-Mϕ stimulated *in vitro* with calcium ionophore A23187 and LPS differed from A23187 and LPS stimulated Mϕ of other origin and species. The main eicosanoids produced by the hp-Mϕ when stimulated *in vitro* with A23187, were the 5-lipoxygenase products LTB_4 and 5-HETE. The increase of the cyclooxygenase products was not significant. LPS did not influence the eicosanoid production. The production of the pro-inflammatory cytokines interleukin 1ß (IL-1ß), IL-6 and tumor necrosis factor α (TNF-α) was LPS concentration dependent and also depended on the time of exposure to the stimulus. The oxygen radical production in these hp-Mϕ was measured by a flow cytometric method. The production increased significantly, when the hp-Mϕ were stimulated with phorbol 12-myristate 13-acetate (TPA).

Chapters 6 and 7: The interactions between the eicosanoids LTB_4 and PGE_2 and the cytokines IL-1ß, IL-6 and TNF-α of hp-Mϕ, were characterized by the addition and inhibition of several of these mediators. The cytokines and eicosanoids influenced each other's production. There was a positive interaction between IL-1ß and LTB_4, a negative interaction between IL-1ß and prostaglandin (PGE_2) and no significant interaction between IL-1ß and IL-6. When the LTB_4 production was inhibited with the specific 5-lipoxygenase inhibitor E6080, the PGE_2 production decreased. Increasing LTB_4 had no effect on PGE_2. Increasing PGE_2 down-regulated the TNF-α production. As we expected, because the profile of eicosanoid production of hp-Mϕ was not similar to the profiles of Mϕ from other origin or species, the profile of interactions between the eicosanoids and cytokines of hp-Mϕ was partly different compared with the profile of eicosanoid and cytokine interactions of Mϕ from other origin and species. These differences in eicosanoid and cytokine production and interactions profiles establish that when mediators in human inflammatory diseases are to be studied, care should be taken with animal models. When *in vitro* studies are done, preferably human *in vitro* models should be used.

Chapters 8 and 9: Mϕ are usually the main inflammatory cells present in ascites of patients due to all causes. Corresponding to the pattern of mediators produced by Mϕ, which depends on the stage of activation of the Mϕ and the stimulus applied, the pattern of the soluble intercellular adhesion molecule 1 (sICAM-1) concentration and the eicosanoid LTB_4 and PGE_2 concentration, measured directly in ascitic fluid of patients, also showed a specific disease related-pattern. The disease-related pattern of the different parameters may not be suitable for diagnostic use as the parameters of the three different groups (liver cirrhosis, peritoneal cancer, spontaneous bacterial peritonitis) overlapped. However, as sICAM-1 levels in ascites correlated with sICAM-1 levels in plasma and both sICAM-1 and IL-6 correlated with episodes of infection, sICAM-1 together with IL-6 seem to be parameters which could be of prognostic value for patients with spontaneous bacterial peritonitis (SBP).

Chapter 10: An outline is given of common diseases; asthma, inflammatory bowel disease (IBD) and septic shock, in which Mϕ and their inflammatory mediators (e.g. eicosanoids and pro-inflammatory cytokines) through a co-ordinated cellular co-operation with other inflammatory cells are involved in the acute and chronic inflammation. Besides non-selective working drugs, these diseases may be treated with selective acting therapeutics e.g. by selective inhibition of the eicosanoid production, by increasing levels of anti-inflammatory cytokines, by neutralizing pro-inflammatory cytokines, through receptor blockade of inflammatory mediators, or a combination of specific inhibitors and/or neutralizers.
As human Mϕ differ from Mϕ of other species, the effects of selective acting therapeutics *in vivo* will be more predictable when tested *in vitro* using human inflammatory cells. Most of these therapeutics can be widely tested to study their effect on a variety of mediators involved in the defence mechanism.

Samenvatting

Ascites vocht is een makkelijk verkrijgbare bron voor humane peritoneale macrofagen (hp-Mϕ). Het *in vitro* karakteriseren van het productie profiel van humane peritoneale Mϕ zal zeer nuttig zijn voor het begrijpen van ontstekings ziekten en hun behandelingen. Deze Mϕ kunnen dan gebruikt worden als een humaan *in vitro* model om de interacties tussen Mϕ en ontstekingsremmende geneesmiddelen te bestuderen. Mϕ zijn zeer belangrijk in ontstekings ziekten, omdat ze door middel van hun receptoren en hun uitscheidings producten een centrale en essentiële rol innemen in de ontwikkeling en handhaving van immunologische en ontstekingsprocessen.

Hoofdstuk 3: Gebruikmakend van een carrageenine-geïnduceerde peritonitis rat model, hebben we laten zien dat de *in vitro* cytokinen en eicosanoïden productie tijdens het ontstekings proces voornamelijk afkomstig waren van aanwezige Mϕ. De productiecapaciteit van ontstekingsmediatoren door Mϕ was afhankelijk van de fase van de peritonitis. De eicosanoïden en cytokinen productie was *in vitro* zeer laag op de eerste dag na het induceren van de peritonitis (de acute fase). Daarna nam de productiecapaciteit van de ontstekingsmediatoren geleidelijk toe, waarbij tijdens de chronische fase een gedifferentieerd patroon tot stand kwam.
Het productieprofiel van ontstekingsmediatoren van perifere bloedmonocyten was volledig veranderd in vergelijking met monocyten die naar het ontstoken peritoneum waren gemigreerd en tot Mϕ waren getransformeerd. *In vivo* 'priming' van Mϕ leidt tot verandering en een hogere productie van eicosanoïden en cytokinen door Mϕ *in vitro*.

Hoofdstuk 4: Met hp-Mϕ hebben we laten zien dat de eicosanoïden productie afhankelijk is van de rijpheid van de Mϕ. Dichtheids-gedefineerde humane peritoneale Mϕ vertonen een morfologische en biochemische heterogeniteit. De minst rijpe Mϕ, de smalle ronde Mϕ, produceerden de minste hoeveelheid eicosanoïden. De meer rijpere Mϕ, de Mϕ met meer cytoplasma, hadden een hogere eicosanoïden productie. Mϕ die rijper zijn, zijn waarschijnlijk *in vivo* meer geactiveerd en dit leidt tot een hogere eicosanoïden productie capaciteit *in vitro*.

Hoofdstuk 5: De hp-Mϕ werden verkregen uit ascites van patiënten met verschillende oorzaken voor deze aandoening, voornamelijk levercirrose. Het ontstoken peritoneum is een steriele chronische ontstekingsplaats. Lipopolysaccharide (LPS) stimulatie *in vitro* van de hp-Mϕ, resulteerde in een vergelijkbaar productiepatroon van ontstekings-stimulerende cytokinen als de door LPS gestimuleerde Mϕ die van andere oorsprong en species waren. Echter, het eicosanoïden productie profiel van hp-Mϕ die *in vitro* met calcium ionofoor A23187 of LPS werden gestimuleerd, verschilden van met A23187 of LPS gestimuleerde Mϕ die van andere oorsprong en species waren.
De voornaamste eicosanoïden, geproduceerd door humane Mϕ gestimuleerd *in vitro* met A23187, waren de 5-lipoxygenase producten LTB_4 en 5-HETE. De netto productie van de cyclooxygenase producten was verwaarloosbaar klein. LPS beïnvloedde de eicosanoïden productie niet. De productie van de ontstekings-stimulerende cytokinen

interleukine 1ß (IL-1ß), IL-6 en tumor necrose factor α (TNF-α) was afhankelijk van de concentratie van LPS en ook afhankelijk van de incubatietijd met de stimulus.
De zuurstof radicaal productie in deze humane peritoneale Mϕ werd gemeten met behulp van een 'flow'cytometrische methode. Wanneer de cellen met 'phorbol 12-myristate 13 acetate' (TPA) werden gestimuleerd, nam de zuurstof radicaal productie van de humane peritoneale Mϕ significant toe.

Hoofdstukken 6 en 7: De interacties tussen de eicosanoïden LTB_4 en PGE_2 en de cytokinen IL-1ß, IL-6 en TNF-α van humane peritoneale Mϕ werden gekarakteriseerd door de toevoeging en remming van verschillende mediatoren. De cytokinen en eicosanoïden beïnvloedde elkaars productie. Er was een positieve interactie tussen IL-1ß en LTB_4, een negatieve interactie tussen IL-1ß en PGE_2 en geen interactie tussen IL-1ß en IL-6. Toen de LTB_4 productie werd geremd met behulp van de specifieke 5-lipoxygenase remmer E6080, nam de PGE_2 productie ook echter af. Toenemende toegevoegde LTB_4 had geen effect op PGE_2 synthese. Toenemende PGE_2 verlaagde de TNF-α productie.
Zoals we verwachtten, omdat het productie profiel van humane peritoneale Mϕ gevormde eicosanoïden anders was dan dat van Mϕ van andere oorsprong en species, verschilden de interacties tussen eicosanoïden en cytokinen van humane peritoneale Mϕ gedeeltelijk van interacties tussen eicosanoïden en cytokinen bij Mϕ van andere origines.
Deze verschillen in eicosanoïden en cytokinen productie en onderlinge interacties bevestigen dat wanneer mediatoren in humane ontstekings ziekten worden bestudeerd, zeer voorzichtig met diermodellen moet worden omgegaan. Wanneer *in vitro* studies worden uitgevoerd, verdienen humane *in vitro* modellen uiteraard de voorkeur.

Hoofdstukken 8 en 9: In ascites van patiënten, veroorzaakt door allerlei aandoeningen, zijn Mϕ meestal de voornaamste aanwezige ontstekingscellen. Overeenkomstig het profiel van mediatoren geproduceerd door Mϕ, dat afhankelijk is van het activatie stadium van de Mϕ en de toegediende stimulus, vertonen de in ascites gemeten concentraties van 'soluble intercellular adhesion molecule 1' (sICAM-1) en de eicosanoïden LTB_4 en PGE_2, specifiek aan ziekten gerelateerde patronen.
De aan ziekten gerelateerde patronen van de verschillende parameters is waarschijnlijk niet geschikt voor diagnostisch gebruik, omdat alle parameters van de drie verschillende groepen, waarin de patiënten waren ingedeeld (lever cirrose, peritoneale kanker, spontane bacteriële peritonitis (SBP)) overlap vertoonden. Echter, omdat de sICAM-1 niveaus in ascites correleerde met sICAM-1 niveaus in plasma en zowel sICAM-1 als IL-6 correleerde met episoden van infectie, zijn sICAM-1 en IL-6 parameters die samen mogelijk prognostische waarde kunnen hebben voor patiënten met SBP.

Hoofdstuk 10: In de afsluitende discussie worden als hoofdlijn een aantal veel voorkomende ziekten besproken zoals, asthma, darmontstekingen en septische shock,

waarin de Mϕ en hun ontstekingsmediatoren (eicosanoïden en ontstekingsstimulerende cytokinen) door middel van een gecoördineerde cellulaire coöperatie met andere ontstekingscellen betrokken zijn bij acute en chronische ontstekings processen. Deze ziekten kunnen met behalve niet-selectieve ontstekingsremmende geneesmiddelen, waarschijnlijk ook behandeld worden met selectief werkzame therapeutica, bijvoorbeeld door middel van selectieve remming van de eicosanoïden productie, het toe laten nemen van ontstekingsremmende cytokinen, het neutraliseren van ontstekingsstimulerende cytokinen, receptorblockade van de ontstekingsmediatoren of een combinatie van genoemde stoffen.
Omdat humane Mϕ verschillen van Mϕ van andere species, zal het effect van selectief werkzame therapeutica *in vivo* beter voorspelbaar zijn als ze *in vitro* getest worden met gebruikmaking van humane ontstekingscellen. De meeste van deze therapeutica kunnen uitgebreid worden getest om hun effect te bestuderen op een verscheidenheid van ontstekingsmediatoren, betrokken bij het verdedigingsmechanisme.

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Curriculum Vitae

Wanda Murel Pruimboom was born on February the 20th, 1965, in Leerdam. She attended primary school in Muiden and Morphett Vale (South-Australia), and secondary school in Naarden (HAVO), Greenwood, Western Australia and finished it in Bussum (VWO) in 1984.
In 1990 she got her university degree (Masters) in Molecular Scientifics at the Agriculture University in Wageningen. During this study she was involved in three research projects in the following departments at the Agriculture University in Wageningen: Molecular Genetics Division, Department of Heredity (Prof. dr. H. van de Broek); Department Toxicology (Prof. dr. J.H. Koeman); Cellbiology Division, Department of Experimental Animal Morphology and Cellbiology (Prof. dr. W.B. van Muiswinkel). For her practical work she spent 5 months at the Department of Biochemistry, Flinders University in South Australia (Prof. A.W. Murray).
From 1990 till 1994 she was doing a PhD research program in the Department of Pharmacology of the Erasmus University Rotterdam (head: Prof. dr. P.R. Saxena).
She is married to Nallappa Govinda Rajan, a software engineer.

List of publications

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Detection of eicosanoids (LTB_4, PGE_2, $6kPGF_2$, TXB_2), cytokines (IL-1, IL-6, TNF-α) and soluble ICAM-1 in ascitic fluid from patients with portal hypertension, cancer and bacterial peritonitis.

9. IAIS Symposium on novel molecular approaches to anti-inflammatory therapy, 1994, Toronto, Canada.
Detection of soluble ICAM-1, cytokines (IL-1, IL-6, TNF-α) and eicosanoids (LTB_4, PGE_2, TXB_2, $6kPGF_{1\alpha}$) in ascites of patients with portal hypertension, peritoneal cancer and spontaneous bacterial peritonitis.

10. XIIth International Congress of Pharmacology, 1994, Montreal, Canada.
Detection of soluble ICAM-1, cytokines (IL-1, IL-6, TNF-α) and eicosanoids (LTB_4, PGE_2, TXB_2, $6kPGF_{1\alpha}$) in ascites of patients with portal hypertension, peritoneal cancer and spontaneous bacterial peritonitis.

List of abbreviations

AA	arachidonic acid
APC	antigen presenting cell
ATP	adenosine triphosphate
A23187	calcium ionophore A23187
BP	bacterial peritonitis
cAMP	cyclic 3',5' adenosine monophosphate
CAPD	continuous ambulatory peritoneal dialysis
CD	Crohn's disease
COX	cyclooxygenase
cGMP	cyclic 3',5' guanosine monophosphate
C5a	complement factor 5a
DAG	1,2-diacylglycerol
DHR123	dihydrorhodamine
DMEM	dulbecco's modification of eagle's medium
DMF	N, N-dimethylformamide
DMSO	dimethyl sulfoxide
ELISA	enzyme linked immunosorbent assay
E6080	6-hydroxy-2-(4-sulfamoylbenzylamino)-4,5,7-trimethylbenzothiazole hydrochloride
FACS	fluorescence activating cell scanner
FCS	foetal calf serum
GCs	glucocorticosteroids
GM-CFC	granulocyte/macrophage colony stimulating forming cell
h	hour
HETE	hydroxy eicosatetraenoic acid
HHT	hydroxy-heptadecatrieoic acid
HPETE	hydroxyperoxy eicosatetraenoic acid
HPLC	high performance liquid chromatography
hp-Mϕ	human peritoneal Mϕ
hr	human recombinant
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
IFN	Interferon
Ig	immunoglobine
IL	interleukin
IL-1ra	interleukin 1 receptor antagonist
IP_3	inositol 1,4,5-triphosphate
IP_4	inositol 1,3,4,5-tetra phosphate
$6kPGF_{1\alpha}$	6-keto-$PGF_{1\alpha}$
l	litre
LBP	LPS binding protein
LFA	lymphocyte-associated-molecule
LPS	lipopolysaccharide
LT	leukotriene
Mϕ	macrophage(s)
M-CFC	macrophage colony forming ells
MCN	mean channel number
m/f	male/female
min	minutes
μg	microgram
mg	milligram
ml	milliliter
MTT	tetrazolium salt
mRNA	messenger RNA
n	number
ng	nanogram

NK	natural killer cells
ns	not significant
NSAID	non-steroidal- anti-inflammatory drugs
PA	phosphatic acid
PAF	platelet activating factor
PBC	primary biliary cirrhosis
PBS	phosphate buffer saline
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PG	prostaglandin
PGH	prostaglandin endoperoxide synthase
PGI_2	prostacycline
PHSC	pluripotent haemopoietic stem cells
PI	phosphatidyl inositol (phosphoinositide)
PIP_2	phosphatidyl inositol 4,5-biphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholiase D
p-Mϕ	peritoneal Mϕ
PMNs	polymorphonuclear cells
PSC	primary sclerosing cholangitis
r	correlation
RIA	radio immuno assay
RNA	ribonucleic acid
ROI	radical oxygen intermediates
RP-HPLC	reversed-phase HPLC
R123	rhodamine 123
SBP	spontaneous bacterial peritonitis
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
sICAM	soluble ICAM
T_H-cell	T-Helper cell
TNF	tumor necrosis factor
TNF-R	TNF-α receptor
TPA	phorbol 12-myristate 13-acetate
TX	thromboxane
u	units
UC	ulcerative colitis
VCAM	vascular cell adhesion molecule
VLA	very-late-antigen