On the Role of Pulmonary Haptoglobin (pHp) and its Receptor

(CD163) in the Human Lung

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Mahdi Abdullah

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1- Berichterstatter: Priv.-Doz. Dr. rer. nat. Torsten Goldmann

2- Berichterstatter: Prof. Dr. rer. nat. Heinz Fehrenbach

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List of Abbreviations

A549	Modified tumor alveolar epithelial cell line
ACTH	Adrenocorticotropic hormone
AEC II	Alveolar Epithelial cell type 2
AEC	3-Amino-9-Ethyl Carbazole
AM	Alveolar Macrophages
APPs	Acute Phase Proteins
APR	Acute Phase Reaction
APS	Ammonium Persulphate
BAL	Bronchoalveolar Lavage
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine Serum Albumin
CD163	Haptoglobin receptors
cDNA	complementary DNA
CHAPS	3-[(3-Cholamidopropyl) dimethyl-Ammonium]-1-propanesulfonate
CKII	Casein kinase II
COPD	Chronic Obstructive Pulmonary Disease
CRP	C-reactive protein
DAPI	4', 6-diamidino-2-phenylindole
DEPC	Diethyl Pyro Carbonate
DEX	Dexamethasone glucocorticoids
DMEM	Dulbecco/Vogt Modified Eagle's minimal essential
DMF	N.N.Dimethylformamide
DNase	Deoxyribonuclease
dNTPs	deoxy Nucleoside triphosphate
DTT	Dithiotreitol
FCS	Fetal Calf Serum
FITC	Fluoresceinisothiocyanat
GAPDH	Glyceraldehde-3-phosphate-dehydrogenase
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
Hb	Hemoglobin

HDL	High density lipoprotein
HEPS	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
Hgp	Hemoglobin haptoglobin binding protein gene
HIER	Heat Induced Epitope Retrieval
HOPE	Hepes-glutamic acid buffer mediated Organic solvent Protection Effect
Hp	Haptoglobin
Hpr	Haptoglobin-related protein
IGEPAL	Tert-Octylphenoxy poly (oxyethylene) ethanol
IHC	Immunohistochemistry
ISH	In Situ Hybridization
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemotracttant Protein-1
NaN3	Sodium azide
NBT	Nitro Blue Tetrazolium
NSCLC	Non-Small Cell Lung Cancer
PAM3	Lipopeptide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pHp	Pulmonary Haptoglobin
PKC	Protein Kinase C
PMSF	Phenylmethysulfonylfluriod
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT	Reverse Transcription
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAA	Serum Amyloid A
SCLC	Small Cell Lung Cancer
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SP-B	Surfactant protein B

SRCR	Scavenger Receptor Cysteine-Rich
SSC	Saline -Sodium Citrate buffer
STAT	Signal Transducers and Activators of Transcription
STST	Short-Term Stimulation of Tissues
TAE	Tris-acetate-EDTA
TB	Tuberculosis
TMAs	Tissue Microarray Arrays
TNF-α	Tumor necrosis factor alpha
T-TBS	Tween - Tris Buffer Saline

1. Introduction

This study faces towards enlightening the role of pulmonary Haptoglobin (pHp) and its receptor (CD163) in the human lung. Haptoglobin (Hp) is long time known as a common acute phase serum protein derived mainly from the liver. On the contrary, information about pHp available to date is very limited to some animal studies; nearly negligible information is available from the human system. Here, we demonstrate that pHp is abundantly expressed in the human lung and various lung diseases. Furthermore, it is shown that Hp is playing a major immuno-regulatory role in the defense against infections. The following chapters provide a brief overview of the current knowledge of Hp and its receptor CD163 in general and more specifically their involvement during human lung infection. Moreover, the role concerning the expression in cancer is described. Finally, the aims of this study are defined.

1.1 Haptoglobin (Hp).

1.1.1 The Hp genes and occurrence.

Hp is an acute phase plasma glycoprotein (with a concentration of 0.45–3 mg/ml in the blood plasma) composed of α1, α2, and ß polypeptide chains (size around 15, 20, and 40 kDa) in different combinations, the β-chain is generally more conserved between species. First reports on Hp date back to studies performed Jayle and Polonovski [64] describing Hp as a ''plasma substance'' interacting with hemoglobin (Hb). The Hp gene is localized on 16q22 [45, 89]. The primary function of Hp is binding free Hb for protection against oxidative stress or reactive oxygen species (ROS) and to facilitate the Hb-uptake by Hb scavenger receptors like CD163 [72]. After more than 60 years of research Hp is recognized as a multifunctional protein involved in the regulation of a variety of processes like immune responses, angiogenesis, prostaglandin synthesis and reverse cholesterol transport. Due to genetic polymorphism, Hp has been subject to considerable interest in the field of biochemical genetics [29, 78, 123]. The human Hp gene exists in two major allelic forms denoted Hp1 and Hp2 [20, 157]. The latter arose by an intragenic duplication of a 1.7 kb DNA fragment of the Hp1 gene after the divergence of human [25, 87, 135]. The Hp genotypes show differences in

geographical distribution. In the northwestern part of Europe, the Hp1-1 genotype is carried by approximately 16% of individuals, the Hp2-2 genotype by 36%, and the Hp2-1 genotype by 48% corresponding to allele frequencies of (0.4) Hp1 and (0.6) Hp2. The Hp1 allele frequency is lowest in South-East Asia (0.1) and highest in South America (0.8). Some studies indicate a possible association between specific Hp genotypes/phenotypes and particular disorders, including cardiovascular disease, autoimmune disorders and cancerous diseases [145, 150]. However, a possible advantage of one Hp allele over the other is not clear. In addition to the Hp gene, primates have a gene encoding the so-called haptoglobin-related protein (Hpr). The human Hp and Hpr genes are homologous and closely linked with the Hpr gene positioned 2.2 kb downstream of the Hp gene on chromosome 16 [20, 130].

1.2 Hp – an Acute Phase Protein

1.2.1 The Acute Phase Reaction and Acute Phase Proteins

Hp belongs to the group of Acute Phase Proteins (APPs) which appear during the Acute Phase Reaction (APR). The APR was defined for the first time in 1941 by Abernethy and Avery [3] and describes the organism's response to injury, infection or trauma of a tissue as well as to immunological disorders. It comprises a complex cascade of reactions to prevent further tissue damage, to eliminate any infective organisms and to enhance the healing process in order to restore homeostasis. It is initiated by macrophages of the affected tissue or by blood monocytes which release a wide range of mediators including cytokines. These cytokines act on fibroblasts and endothelial cells in the near vicinity causing a second release of cytokines. Only this second wave of cytokines triggers the actual cascade of complex reactions as part of the APR occurring locally and systemically. Locally, cytokines mediate leukocyte recruitment, in particular neutrophils and mononuclear cells, to the sites of inflammation. Systemically, they act on the immune system, bone marrow, brain and liver, and the reaction comprises the generation of a febrile response, an increase in adrenocorticotropic hormone (ACTH) secretion, leukocytosis and alteration of the hepatic APP gene expression. This change of hepatic APP expression leads to increases as well as decreases of APP plasma concentrations dividing them into positive and negative

APPs [52]. Since Hp is produced at elevated levels during the APR, it is categorized as a positive APP [131].

1.2.2 Cytokine control of APPs synthesis

The synthesis of APPs is controlled by cytokines and acts directly upon specific receptors on the surface of hepatocytes prompting APPs production [111]. APPs can be divided into two major categories according to their regulators: type 1 APPs production is induced by interleukin (IL-1) and tumor necrosis factor-alpha (TNF-α), whereas type 2 APP synthesis is elicited by IL-6 [17]. IL-6 is believed to be the primary stimulator of most APP genes; however, there is evidence that IL-1 and TNF-α can amplify the effects of IL-6 [52]. Hp is ranked as type 2 APPs in humans, but as type 1 in the rat [17] induced by IL-6, the actual Hp gene transcription within a cell is mediated by signal transducers and activators of transcription proteins STAT of which STAT3 has been described as the main signaling protein in mice hepatocytes *in vitro* [17, 68]. After binding of IL-6 to its receptor, STAT3 is activated at the cytoplasmic side of the IL-6 receptor by phosphorylation. Once activated it translocates to the nucleus. In mice, the three main regulatory elements of the Hp gene promoter are known to be recognition sites for the transcription factor CCAAT/enhancer binding protein beta (C/EBPβ), flanking a STAT interaction site, Binding of STAT3 to this interaction site has been identified as the key up regulator of murine Hp gene transcription induced by IL-6, whereas binding of other STAT proteins, e.g. STAT5, exerts inhibitory effects [68, 149]. Hp is mainly found in the plasma, but is also present in many other body fluids in mammals such as milk, urine, cerebrospinal fluid, amniotic fluid and saliva [56]. In addition, Hp expression has been reported in a variety of extra hepatic tissues. Hp mRNA could be detected in spleen, thymus, heart, lung, kidney and skin of the rat and human after stimulation with lipopolysaccharides [65, 82, 133, 158]. Hp mRNA was also found in murine adipocytes at a basal level and elevated levels after LPS challenge [44]. Moreover, murine lung epithelial cells express Hp mRNA [159]. There is also evidence of Hp mRNA in the reproductive tract, Hp mRNA expression was shown in rabbit oviductal tissue from 6h post-conception to day 3 and in the uterus on days 5 and 6 post-conception [55] and in human endometrium

[129]. In addition, macrophages, eosinophiles and epidermal keratinocytes express Hp in humans [2, 155, 159].

Besides their direct regulatory effect, cytokines can also act via the pituitary-adrenal axis on APP production. They mediate an increased release of glucocorticoids by causing a higher secretion of ACTH during the APR. Glucocorticoids, in turn, enhance the APP production in hepatocytes on the one hand, and reduce the release of cytokines from monocytes and macrophages on the other hand [53]. However, the effects on Hp expression in hepatocytes via this pathway appears low compared to the direct route [88]. The signaling pathway of ACTH on the Hp promoter has yet to be fully characterized; a direct glucocorticoid receptor binding site in the murine Hp promotor is suspected [110].

1.2.3 Species-specific APP response during APR

Several plasma proteins are known as APPs, but depending on the species the protein pattern of each single APP, during the APR is highly varying. In humans, the C-reactive protein (CRP) besides serum amyloid A (SAA) shows the highest increases during an APR, whereas, Hp increases only moderately [52], In cattle, Hp and SAA are considered as the most prominent APPs, whereas CRP is normally present in circulation while its concentration remains unchanged during an acute phase [35]. In contrast, CRP is recognized as a major reactant in pigs and same is true for Hp [76]. Similarly in the dog, CRP is classified as a major APP, whereas in the rat α_2-macroglobulin and α_1-acid glycoprotein are the APPs with the greatest increase of concentration during the APR [35, 52].

1.3 Hp: Structural aspects and phenotypes

Hp phenotypes are closely related to some of human diseases and also differentiation binding with receptor [78]. Molecular heterogeneity of human Hp, related to the two different alleles Hp1 and Hp2 was reported in 1955 by Smithies who distinguished the three major Hp phenotypes Hp1-1, Hp2-1, and Hp2-2 based on their electrophoretic migration patterns [134]. The Hp1-1 homozygote shows a single fast-migrating protein band, while Hp2-2 homozygote shows a series of slower migrating bands. The Hp2-1 heterozygote displays a different series of slowly migrating bands, and additionally a band of much fainter intensity than Hp1-1. Hp proteins are composed of α- and β-subunits, representing a complement control protein domain and a serine protease domain, respectively. The Hp β-chain (40 kDa) is invariant in humans and contains four glycosylation sites of the type Asn-X-Thr/Ser (see Figure 1). In contrast, the α-chain occurs in two major variants (Hp1 α-chain: 15 kDa, Hp2 α-chain: 20 kDa) encoded by the two allelic forms of the Hp gene, Hp1 and Hp2. Compared to the α-chain, the β-chain is generally more conserved between species [74]. The basic α and β unit of Hp is synthesized as a single chain precursor containing an 18 residue hydrophobic leader peptide (Figure 1). The latter direct secretion of the protein product and is cleaved of the mature Hp protein found in blood plasma [74]. In the endoplasmic reticulum, the single chain Hp precursor is cleaved carboxy-terminally to Arg102 Hp1 or Arg161 Hp2 by the complement C1r-like protein [153], resulting in α- and β-chains, corresponding to the amino-terminal and carboxy-terminal part of the precursor. They remain closely connected stabilized by a disulfide bond. After cleavage, the carboxy-terminal arginine of the α-chain is removed, presumably by carboxypeptidase resulting in different characteristic chains.

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MSALGAVIAL LLWGQLFAVD SGNDVTDIAD DGCPKPPEIA HGYVEHSVRY QCKNYYKLRT
    70         80         90        100        ↓ 110      120
EGDGVYTLND KKQWINKAVG DKLPECEADD GCPKPPEIAH GYVEHSVRYQ CKNYYKLRTE
   130        140        150        160       ↓ 170      180
GDGVYTLNNE KQWINKAVGD KLPECEAVCG KPKNPANPVQ RILGGHLDAK GSFPWQAKMV
   190        200        210        220        230        240
SHHNLTTGAT LINEQWLLTT AKNLFLNHSE NATAKDIAPT LTLYVGKKQL VEIEKVVLHP
   250        260        270        280        290        300
NYSQVDIGLI KLKQKVSVNE RVMPICLPSK DYAEVGRVGY VSGWGRNANF KFTDHLKYVM
   310        320        330        340        350        360
LPVADQDQCI RHYEGSTVPE KKTPKSPVGV QPILNEHTFC AGMSKYQEDT CYGDAGSAFA
             370        380        390        400
          VHDLEEDTWY ATGILSFDKS CAVAEYGVYV KVTSIQDWVQ KTIAEN
```

Figure 1: Amino acid sequence of human Hp. The signal peptide, the α-chain, and the β-chain are highlighted in green, grey, and blue respectively. Positions of cleavage to generate the mature protein are indicated by arrows. After cleavage between Arg102 and Ile103, Arg102 is removed by carboxypeptidase. The four carbohydrate attachment sites are indicated in brown, and cysteines are shown in red. The Hp2 sequence contains an identical duplicate of Hp1 residues α chain 19-160, and ß chain 162-406. Hp GenBank P00738-1.

By the use of electron microscopy, Wejman et al. have analyzed the overall shape of Hp, the Hp1-1 molecule was described as a barbell-like elongated structure with two spherical head groups of β-chains connected by a thin filament with a central knob [151] (see Figure 2). Besides its intriguing polymorphic nature, Hp is interesting from a protein evolutionary point of view due to a striking degree of sequence similarity between the Hp β-chain and members of the serine protease family. These are known to possess anti-inflammatory functions like trypsin, chymotrypsin, and elastase [11, 74]. Further studies are necessary to understand the relations between Hp phenotypes and correlating lung cancer types.
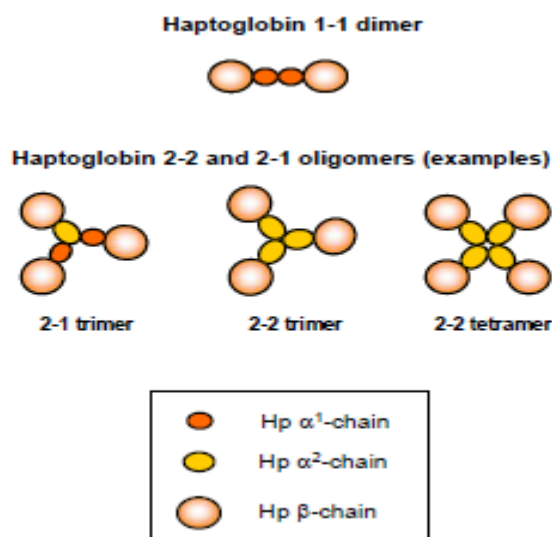
Figure 2: Different Hp phenotype shapes: Hp 1-1 molecule resembles a barbell. The dimer is connected by disulfide bonds. Hp2-1 and Hp 2-2 phenotypes display complicated structures of Hp due to variations in the chains. Examples of Hp 2-1 trimer, Hp 2-2 trimer and Hp2-2 tetramer are shown. Modified from [72].

1.4 Hp–Hb complex

To understand the complete function of Hp and its receptor, physiological issues of Hp binding to Hb are important. Hp–Hb binding is one of the strongest non-covalent interactions reported in biology [62]. A large number of studies on the Hp-Hb complex demonstrates that the nature of this essentially irreversible, but non-covalent binding has fascinated scientists for several decades [78]. The tetrameric structure of Hb, composed of two α and two β subunits, has been carefully studied by crystallographic methods [128]. All four globular subunits of Hb interact through non-covalent interactions, and each subunit binds a heme molecule. When released from red blood cells into plasma, Hb rapidly dissociates into αβ-dimers which bind to Hp in a 1:1 stoichiometry one Hb dimer per Hp αβ-unit (Figure 3) [4, 99].
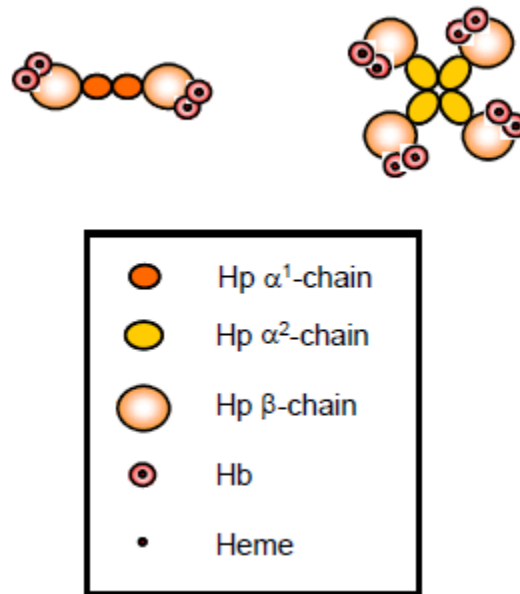
Figure 3: Hp-Hb complexes. The Hb dimer binds to Hp1-1 ß-chains as barbell axis in a trans-configuration. Also illustrated is the binding of Hb to the Hp 2-2 tetramer. Modified from [72].

1.5 Physiological functions of Hp in general

The main physiological tasks assigned to Hp are transport and immunomodulatory properties. The best recognized function of Hp is to bind free Hb and to transport it to the liver. More specifically, after the release of Hb into plasma, a physiological phenomenon associated with hemolysis or apoptosis of erythrocytes, Hp can minimize Hb-stimulated lipid peroxidation, and protect DNA as well as tissues against oxidative damage during hemolytic injury [50, 83]. Thus, physiologically, Hp has been considered to be an antioxidant [91]. The Hp-Hb complex cannot pass the glomerular filtration in the kidney due to its large molecular size, thereby preventing renal losses of the small Hb molecule. An overlapping role of Hp and hemopexin in resolution of hemolytic stress is indicated by the finding that Hp and hemopexin double- knockout mice are more susceptible to hemolytic damage than are wild type and single Hp / hemopexin- knockout mice [142]. Thus Hp and hemopexin constitute key players in the mechanisms protecting against undesirable effects of intravascular hemolysis. Some more important properties of Hp which are related by this study are explained in the following sections.

1.6 Hp and cancer

Hp normally originates from the liver. Elevated amounts of this protein in the blood plasma have been observed in infection, inflammation, and various malignant diseases, including lung and bladder cancer [18, 19], leukemia [93], breast cancer [10], esophageal squamous cell carcinoma [5], urogenital tumors [33], malignant lymphomas [39] and ovarian cancer [163]. To date, research over the past 60 years has revealed that Hp is a multifunctional protein involved in a variety of regulation processes including immune responses [102]. Previous reports have faced glycoprotein concentrations in the blood plasma of patients with primary or secondary carcinoma of the lung for statistical studies. Increased Hp levels have been found in the serum of patients suffering from lung cancer, therefore, those studies have revealed that Hp is a potential serum biomarker candidate for lung cancer [22, 40, 54]. The detection of a serum biomarker preferably indicating an early stage of the disease is helpful for immediate treatments and, therefore, for overall survival of patients.

1.7 Hp in angiogenesis and cell migration

Another important role of Hp in chronic diseases, serve as angiogenesis, processes which involves the formation of new blood vessels under normal (healthy) and pathological conditions. It plays an important role in a variety of physiological and pathological condition such as embryonic development, tumor growth, wound healing, and chronic inflammatory diseases [41]. The formation of new blood vessels involves dissolution of the underlying basement membrane, followed by cell migration, alignment, proliferation, differentiation into tubular structures, and finally reestablishment of a new basement membrane [85]. Stimulation of angiogenesis is a newly recognized biological function of Hp. The increased levels found in chronic inflammatory conditions may play an important role in tissue repair and be useful for the treatment of chronic inflammatory conditions and systemic vasculitis because of its ability to stimulate tissue repair and to compensate for ischemia by promoting the growth of collateral vessels [80]. Surprisingly, Hp2-2 is more angiogenic than the other Hp phenotypes [26]. In a variety of malignancies Hp concentration correlates with disease progression [98]. Angiogenesis is an important phenomenon in all of these conditions [42]. Further,

arterial expression of Hp by lipopolysaccharides in mice fibroblasts stimulated migration of wild-type fibroblasts but had no effect on migration of Hp knockout fibroblasts and it has been shown that flow induced arterial restructuring was delayed in Hp knockout mice. This new function of haptoglobin might be explained by facilitating cell migration through accumulation of a temporary gelatin matrix because cell culture showed that Hp is involved in the breakdown of gelatin [28].

1.8 Hp and its role in immunomodulation and inflammation

APPs are a class of proteins whose plasma concentrations increase positive APPs or decrease negative APPs in response to inflammation. This response is called the APR. In response to injury local inflammatory cells (neutrophils, granulocytes and macrophages) secrete a number of cytokines into the bloodstream like IL-1, IL-6 and IL-8, and TNF-α; the liver responds by producing a large number of acute phase reactants. At the same time, the production of a number of other proteins is reduced, therefore, referred to as negative acute phase reactants. No specific biological functions have yet been identified, a generally the function of APPs are considered to be anti-inflammatory in nature and include four key activities firstly inhibition of extracellular proteinases, secondly neutralization and clearance of harmful components such as released hemoglobin, heme, metal ions, reactive oxygen and nitrogen species, thirdly replenishment of the pool of proteins involved in blood coagulation and fibrinolysis and finally modulation of immune cell activity [13, 102, 103, 141, 149]. Among those, Hp has been reported to be a potent anti-inflammatory protein, inhibit the respiratory burst and to rise intracellular calcium in neutrophils following their stimulation with N-formylmethionyl-leucyl-phenylalanine (FMLP) [9, 102, 108, 122]. It suppresses lectin and LPS-induced proliferation of T lymphocytes, B cells and it modulates several macrophage functions [13, 101, 103, 124]. Hp is a powerful suppressor of lymphocyte function, as shown by the ability to inhibit the mitogenic response of lymphocytes to phytohemagglutinin and concanavalin A [13]. Different T helper (Th) lymphocyte subtypes known as Th1 and Th2 cells, are responsible for inducing and regulating the cellular and humoral immune response, respectively Th1 cells produce IL-2 and interferon gamma (IFN-γ) and induce strong IgG responses, thus favoring the cellular immune response, whereas Th2 cells produce IL-4, IL-5, IL-6, IL- 10 and

17

IL-13 and increase IgE production, thereby mediating a predominantly humoral and eosinophilic response. Many studies showed that Hp plays an important role in modulating the balance between Th1 and Th2 lymphocytes by promoting a predominantly Th1 cell response [1], [8] and [49]. Smark et al. demonstrated the immunomodulating effects of APPs and their relevance in cancer as a non-specific blocking factor protecting tumors against immunological attack [63, 124]. These authors subsequently reported that Hp selectively modulates the inflammatory response through its ability to suppress the synthesis of TNF-α, IL-10 and IL-12 by monocytes stimulated with LPS. More recently, it was further demonstrated that Hp is taken up and stored in monocytes and neutrophils within a cytoplasmic granular compartment and that it is subsequently secreted upon exposure to TNF-α [21] or *Candida albicans* [148]. However, the mechanism of Hp on the immune and inflammatory system remains largely unresolved. Some groups identified the promiscuous Mac-1 leucocyte integrin ß2 (CD11b/CD18) as a receptor for Hp on monocytes, macrophages, granulocytes, natural killer cells, and a small subset of B and CD8+ T lymphocytes [37]. Additionally, there is non-CD11b/CD18-mediated binding of Hp to mast cells and T lymphocytes through a yet unidentified receptor [38], and the Hp receptor is expressed on B cells. Hp blocks binding of CD22 to TNF-α activated endothelial cells thereby potentially reducing B-lymphocyte trafficking [51]. A model for the role of Hp in inflammation is depicted in figure 4.
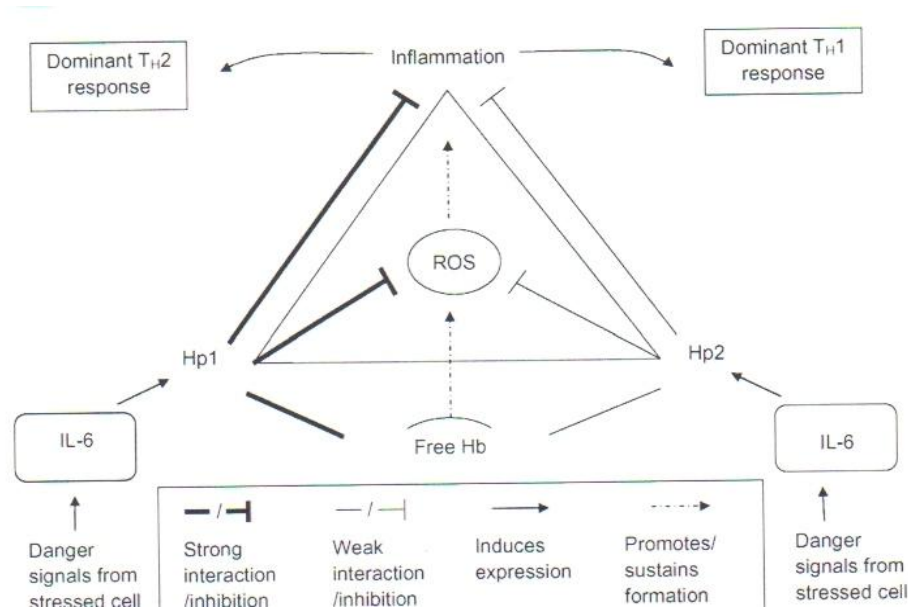
Figure 4: Model of the role of Hp in the infalmmatory response. Danger signals from stressed cells induce expression of IL-6 which in turn induces expression of Hp. The strong Hb binding, anti-oxidant and anti-inflammatory activity of Hp1 leads to a Th2 dominant response initiating healing and repair. In Hp2 shows weak ROS would allow the persistence of inflammatory stimuli, leading to a Th1 response and increased oxidative stress [115]

1.9 Hp and infection

Another important function of Hp is shown through increase during infection or inflammation [141, 149] in response to microorganisms such as *Haemophilus influenzae*. *Haemophilus influenzae* requires an exogenous heme source for aerobic growth in vitro. Hb or Hb-Hp satisfies this requirement. Heme acquisition from Hb-Hp is mediated by proteins encoded by hgp genes (hemoglobin haptoglobin binding protein gene A, B, C and D). Recently it was shown that deletion of the set of three hgp genes from a nontypeable strain of *H. influenzae* attenuated virulence in the chinchilla otitis media model of noninvasive disease [97]. Therefore, Hp affects bacterial proliferation. The combination of bacteria and blood in a wound can have lethal consequences, probably because hemoglobin iron supports prolific bacterial growth, when rats inoculated intraperitoneally with pathogenic *Escherichia coli* and small amounts of hemoglobin dye. Simultaneous administration of Hp, a naturally occurring hemoglobin-binding protein, fully protects against lethality. Therefore, Hp may not only accelerate the clearance of free hemoglobin, but also limit its utilization by adventitious bacteria and recorded as a natural bacteriostatic effect [34]. Also it has been shown that Hp can act as a

natural bacteriostatic by preventing the consumption of iron that is necessary for the growth of some pathogenic bacteria such as *Neisseria meningitides, Campylobacter jejuni and Bacteroides fragilis* [34, 107, 113].

1.10 Hp and chemoattractants

An attempt of this study was to characterize details about the functional effects of pHp in the human lung. Specifically, it has remained elusive so far as to how this protein might influence the inflammation in lung. Therefore, we analyzed for the first time potent chemo attractant cytokines from A549 cells stimulated to demonstrate the potential role of pHp in lung epithelial cells via enzyme-linked Immunoabsorbent assay (ELISA) technique. The alveolar surface area in the lung is lined with a thin layer of epithelial cells, consisting of squamous type I and cuboidal type II pneumocytes. These cells, together with alveolar macrophages (AM), serve a productive function in the lung against the outer environments. The majority of *in vitro* studies are performed on the immortalized cell line A549 and epithelium derived from the upper airway, there are relatively few studies describing the response of human alveolar epithelium to insults and no reports about Hp. Leukocyte recruitment into the alveoli is a multi-step process [120] and migration of leucocytes into tissue is facilitated by a number of factors such as cell adhesion molecules, cytokines, chemokine's and their corresponding signaling events. Soluble chemoattractants play an important role in leukocyte traffic into the lung [24]. Recent studies have suggested that the alveolar epithelium plays a role in modulating immune responses [136]. The cell line A549 can produce MCP-1, RANTES and IL-8 following stimulation by TNF-α and other pro-inflammatory cytokines, while rat type II pneumocytes produce MCP-1 in response to IL-1 and can be stimulated to produce a neutrophil chemoattractant [75, 109].

1.11 Hp and scavenger receptor

The monocyte or macrophage-specific protein CD163 also known as M130, RM3/1 antigen, or p155 functions as a receptor that specifically binds and internalizes the Hp-Hb complex [72, 86]. This scavenging system thus counteracts the accumulation of free Hb in the plasma. The binding of Hp-Hb to CD163 is

Ca2+-dependent and of high affinity. In contrast, Hb alone and Hp alone display low or no binding to CD163, respectively [72, 126]. The importance of Hp in efficient scavenging of Hb is thereby evident. Furthermore, receptor-binding of Hp-Hb is Hp phenotype dependent because CD163 exhibits a higher functional affinity for the complexes generated between Hb and Hp2-2 than it does for the smaller Hp1-1-Hb complex [72]. This observation is probably accounted for by the multiple receptor binding sites in oligomeric Hp-Hb complexes. Our understanding of the molecular pathways protecting against toxic heme was recently extended in a study by [61] which showed when the complex Hp-Hb with receptor in the surface of macrophage internalized and metabolized the Hb (see Figure 5). The toxic heme is converted to metabolites with overall anti-inflammatory activities by heme-oxygenase [67]. A recent study by Schaer et al. [125] reports the existence of an Hp-independent mechanism of CD163 mediated Hb clearance in plasma. According to this study, pHp and in parallel its receptor expressed upon stimulation with inflammatory in human lung which might mediate also Hb clearance and anti-inflammation.
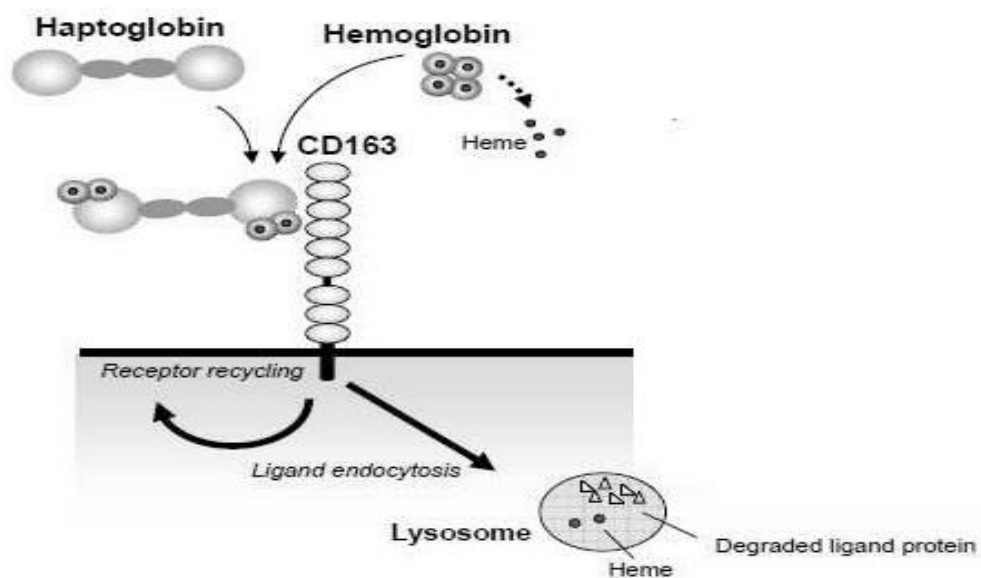
Figure 5:
Schematic presentation of the pathway for receptor-mediated endocytosis of free Hb and heme complex with Hp, Hb is cleared from the circulation or tissues in a complex with Hp by the macrophage scavenger receptor. Modified from [61]

1.11.1 CD163 expression and regulation

CD163 is expressed exclusively in cells of the monocyte lineage [114]. Monocytes display low CD163 expression, but expression is highly induced during *in vitro* differentiation into macrophages [23, 112, 138]. High levels of CD163 are detected in a number of different mature tissue macrophages, including Kupffer cells (liver) and red pulp macrophages (spleen) [144, 164]. Human CD163 is synthesized as a 130 kDa glycoprotein single chain transmembrane molecule belonging to the scavenger receptor cysteine-rich superfamily (SRCR) consisting of nine extracellular cysteine-rich scavenger segments and a short cytoplasmic tail existing in at least three variants due to alternative splicing. It has close homology to a few other membrane proteins like CD5, CD6 and WC1 which all are characterized as extracellular domains composed exclusively of the cysteine rich scavenger receptors class B domains [79, 119]. In *vitro* studies on CD163 regulation in cultured monocytes and macrophages have revealed that CD163 is tightly regulated by pro- and anti-inflammatory mediators and the expression level is subject to regulation by a variety of factors. For instance, CD163 mRNA and protein is highly inducible by glucocorticoids [23, 58, 59,125, 138, 144]. CD163 is also up-regulated by the acute phase mediator IL-6 and the anti-inflammatory cytokine IL-10 and exhibited the strongest up-regulation [23, 138, 154]. In contrast, pro-inflammatory mediators like TNF-α, INF-γ, and LPS act as negative modulators of CD163 expression [23]. In addition to being present on the macrophage surface, continuous shedding of the extracellular CD163 domain leads to substantial amounts of soluble receptors in plasma which is belonging to inflammatory stimuli. High expression has been observed in macrophages of inflamed tissues during the healing phase of acute inflammatory reactions, atherosclerotic lesions, inflamed joints, chronic inflammatory diseases, wound healing process and in case of systemic inflammation [43, 112, 116, 165]. Furthermore, recent data indicate that soluble CD163 may be a valuable diagnostic parameter for monitoring macrophage activation in inflammatory conditions [94].

1.11.2 CD163: On the role in intracellular signaling and immunomodulation

The most well defined function of CD163 is removal of Hp-Hb complexes. In addition, a number of reports suggest a role for CD163 in intracellular signaling. Antibody-mediated cross linking of CD163 at the cell surface has been reported to trigger a tyrosine kinase-dependent signaling cascade resulting in intracellular Ca2+ mobilization, synthesis of inositol triphosphate, and secretion of IL-6 and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) [112, 144]. Similar studies indicate participation of casein kinase II (CKII) in the mechanism leading to secretion of IL-6 and furthermore, suggested that protein kinase C (PKC) may have a function in CD163 mediated signaling [112, 117]. Interestingly, IL-6 induces expression of both Hp, and CD163 [92]. Furthermore, IL-10 up-regulates CD163 and Hp [81]. The regulation of CD163 expression by pro- and anti-inflammatory mediators and the secretion of anti-inflammatory cytokines in response to ligand binding to CD163 strongly suggest a function of CD163 in immunomodulation and ant-inflammation (see Figures 6 and 7). Furthermore, the metabolites carbon monoxide, biliverdin, and free iron, resulting from intracellular degradation of the CD163 internalized heme, display overall anti-inflammatory effects [94, 164].
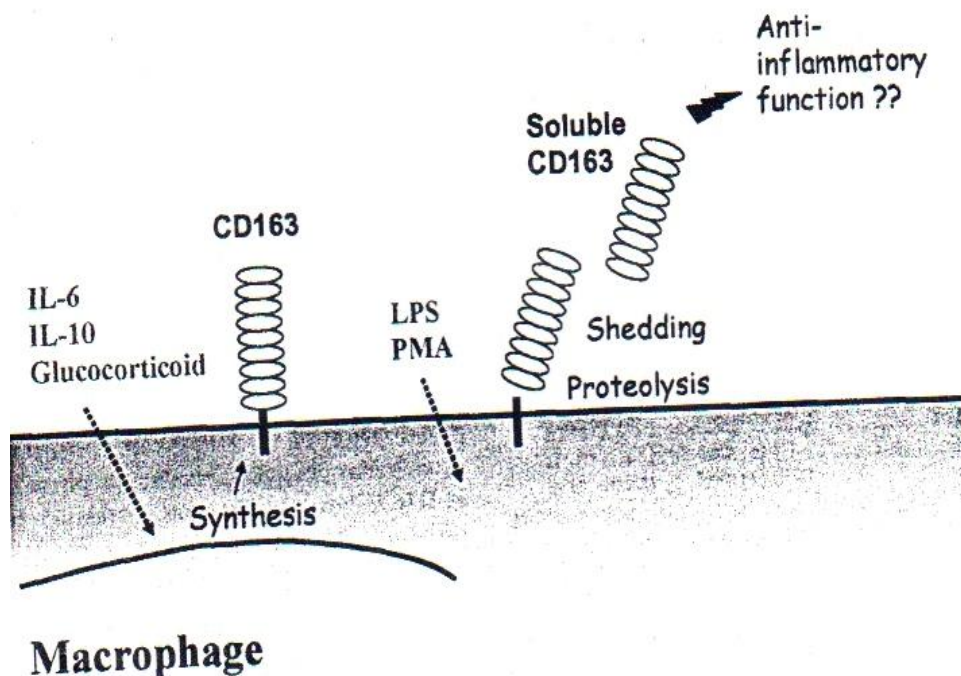
Figure 6:
Regulation of CD163 synthesis and shedding. Synthesis and expression of CD163 induce by anti-inflammatory cytokine and glucocorticoids with shedding of receptor via proteolysis as a result of stimulation either with LPS or Phorbol Merasate Esters (PMA). The punctuated arrows indicate the stimulatory effect of various substances on CD163 synthesis and shedding [94]
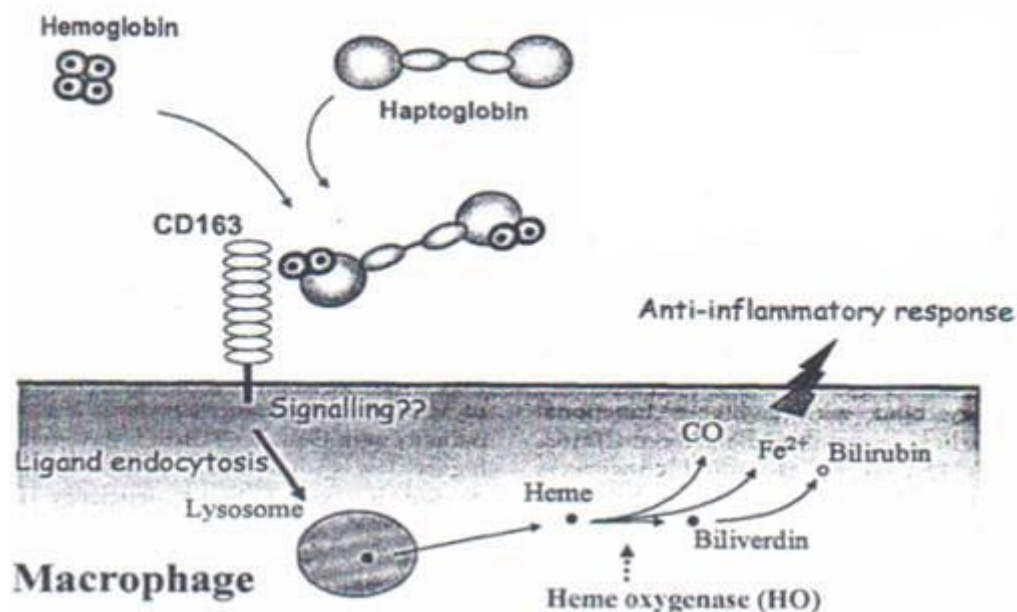
Figure 7:
Function of the Hp-Hb complex on the surface of a macrophage showing an anti-inflammatory response with an intracellular signaling cascade leading to secretion of anti-inflammatory cytokines [94].

1.12 Short Term Stimulation of Tissues

Tissue culture is the growth of tissues or cells separated from an organism. This is typically facilitated via use of a liquid, semi-solid, or solid growth medium. Cells or tissues are grown and maintained at an appropriate temperature and gas mixture (typically, 37°C % and 5% CO2) for mammalian cells in a cell incubator (Heraeus, Germany). Little is known about the complex interactions taking place within the human lung when stimulated with inflammatory agents. As part of a large scaled investigation aimed at improving the facilities available today to study of more detail about human lung function, an *ex vivo* tissue culture model with application of HOPE technique was used [77] (*H*epes–glutamic acid buffer mediated *O*rganic solvent *P*rotection *E*ffect) (DCS Innovative Diagnostik Systeme; Hamburg, Germany) [47, 105, 146]. This model was designated "Short Term Stimulation of Tissues" (STST). STST enables to gain insights into the cellular events taking place in response to inflammatory agents (LPS and Pam3), inflammatory cytokines (IL-6), glucocorticoids dexamethasone and different pathogen infection

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(*Haemophilus influenzae*, *Streptococcus pneumoniae* and *Chlamydia pneumoniae)*. Effects were studied for APP Hp and its receptor. The application of *ex vivo* models via cultivation using vital tissues has already been described for other functional studies in the human system and are long known [31, 48, 96, 121, 143]. The current study describes brief details about pHp and its receptor via analysis of the induction of expression, synthesis and the secretion with tools of molecular pathology in the human lung and for the first time in an *ex vivo* tissue culture system.

1.13 Aim of the study

The roles and regulation of Hp in extra-hepatic tissues are poorly understood. Based on data obtained by transcription microarray analyses, this study was conducted to investigate the expression and synthesis of pHp and its receptor in human lungs and in lung cancer. Additionally the expression of pHp and CD163 in chronic human lung diseases (sarcoidosis, COPD and tuberculosis) was examined.

Further, the regulation of pHp and CD163 was studied using *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Chlamydia pneumoniae,* different inflammatory agents, inflammatory cytokines, and glucocorticoids on the mRNA and protein levels.

Moreover, a possible secretion of pHp and CD163 by the human lung is analyzed, as well as a possible co-localization with Surfactant protein B (SP-B). Finally, the immunomodulatory effects of pHp onto cells of the human lung are investigated.

To obtain universally valid results, several different model systems are applied, all of them are human: STST-model, bronchoalveolar lavage (BAL), A549 cells and primary alveolar epithelial cell type two (AEC II).

Methodologically, the following techniques were used for read out: Transcription microarrays, *in situ hybridization*, conventional RT-PCR, Real-time-PCR, immunohistochemistry, immunocytochemistry, immunofluorescence, confocal laser microscopy, Western blot and slot blot analysis.

2. Material and Methods

2.1 Transcription microarray data

Two NSCLC specimens, five tumor-free lung tissues and one sample of tumor-free lung was not subjected to cultivation and were differently stimulated *in vitro* within the STST model [77]. After HOPE-fixation, total RNA was extracted (following RNeasy Mini kit instructions, Qiagen, Germany) from the tissues and proceeded to Imagenes (GmbH, Germany) for application to an Agilent 44k transcription microarray, including quality control, labeling, hybridization to Agilent 44k and subsequent read. Results of active and silent genes were received in regulation values concerning all different transcripts.

Sample 1: NSCLC 16 h medium without stimulation.
Sample 2: NSCLC 16 h stimulated with carboplatin and gemcitabine.
Sample 3: tumor-free lung 16 h medium without stimulation.
Sample 4: tumor-free lung 16 h infected with *Haemophilus influenzae*.
Sample 5: tumor-free lung without cultivation.
Sample 6: tumor-free lung 16 h medium without stimulation.
Sample 7: tumor-free lung 16 h stimulation with inactive grass pollen allergen.
Sample 8: tumor-free lung 16 h stimulation with active grass pollen allergen.

2.2 Samples origin and collection

Human lung tissues were obtained from patients of Großhansdorf Hospital and University of Lübeck after lobectomy / pneumonectomy. Specimens were the pathological characterized from trained pathologists at the Clinical and Experimental Pathology in Borstel using common immunohistochemistry markers (e.g. TTF-1, SPA, SPB, CK5/6, ZP2 and CD56)

As a fixative, either HOPE or formalin was used. 115 specimens were collected for investigation of expression of pHp in human lung cancer and tumor free lung. The patients had a mean age of 65 years (range from 45 to 85 years; 61 males, 54 females). Among these, 47 adenocarcinomas, 42 squamous cell carcinomas, 13 small cell lung cancer and 13 normal lungs were defined.

942 specimens from 82 human lungs for *ex vivo* tissue culture STST model were collected. See table 1 for exact numbers of specimens cultured via STST model.

This study was approved by the ethical committee of the University of Lübeck (reference number 03/158) and is in compliance with the Helsinki declaration.

Table 1: Total number of specimens cultured in STST model with different stimuli and medium (control)

Targets	LPS	Medium	DEX	Medium	IL-6	Medium	Pam3	Medium
Hp	80	80	63	63	14	14	57	57
CD163	101	101	67	67	14	14	75	75
Total	181	181	130	130	28	28	132	132

In addition to lung tissue samples, cells and precision cut lung slices were used. Among these, nine BAL samples from the Medical Clinic Borstel of patients who underwent bronchoalveolar lavage during routine intervention were used, as well as primary AEC II (from the University of Freiburg, Germany) and A549 cells (American Type Culture Collection). Furthermore, 10 single precision cut lung slices were stimulated with inflammatory agents and HOPE-fixed and 11 formalin-fixed probes from chronic diseases sarcoidosis, tuberculosis (TB) and chronic obstructive pulmonary disease (COPD) were included.

Additionally 30 stimulated specimens with pathogenic microorganisms (*Haemophilus influenzae*, *Streptococcus pneumoniae* and *Chlamydia pneumoniae* for 24h [31, 156]) were used.

2.3 STST model
In an *ex vivo* STST model, the responsiveness of 942 samples to different stimuli were examined. Vital specimen of tumor-free lung tissues, at least 5 cm away from the tumor and about 1cm x 1cm in size were used. Specimens were cultured in RPMI 1640 (Invitrogen, Germany) supplemented with 10% fetal calf serum (FCS) (PPA, Austria), 2% 1M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic

acid, Invitrogen, Germany), 1% of 10mM sodium pyruvate (Biochrom, Germany), and 1% of Penicillin-Streptomycin (Invitrogen, Germany).

In general tissues culture was conducted at 37°C with 5% CO_2 (Heraeus, Germany) and different times of incubation and concentration of inflammatory agents (10 min, 30 min, 2h, and 24h). For each plate, two specimens were incubated in each well for different fixation, one for HOPE and the other for formalin fixation. Specimens were cultured with different inflammatory agents, inflammatory cytokines and the glucocorticoid dexamethasone. The current study used lipopolysaccharides (LPS), Pam3 (represents the Gram-positive compound peptidoglycan), glucocorticoids dexamethasone (DEX) and IL-6. Additionally, 96 specimens were cultured in medium without stimulation 2h for washing from blood and to reduced minimal level of pre-existing Hp. For stimulation the following compounds with according concentrations were used: LPS: 200ng/ml, 50ng/ml and 10ng/ml (EMC micro collections GmbH, Germany), PAM3 200ng/ml (EMC micro collections GmbH, Germany), human Interleukin-6 50ng/ml (Sigma-Aldrich Chemie GmbH); dexamethasone 50 ng/ml and 10ng/ml (Merck, Germany). The general procedure of the STST model is shown below in figure 8.

Short-Term Stimulation of Tissues
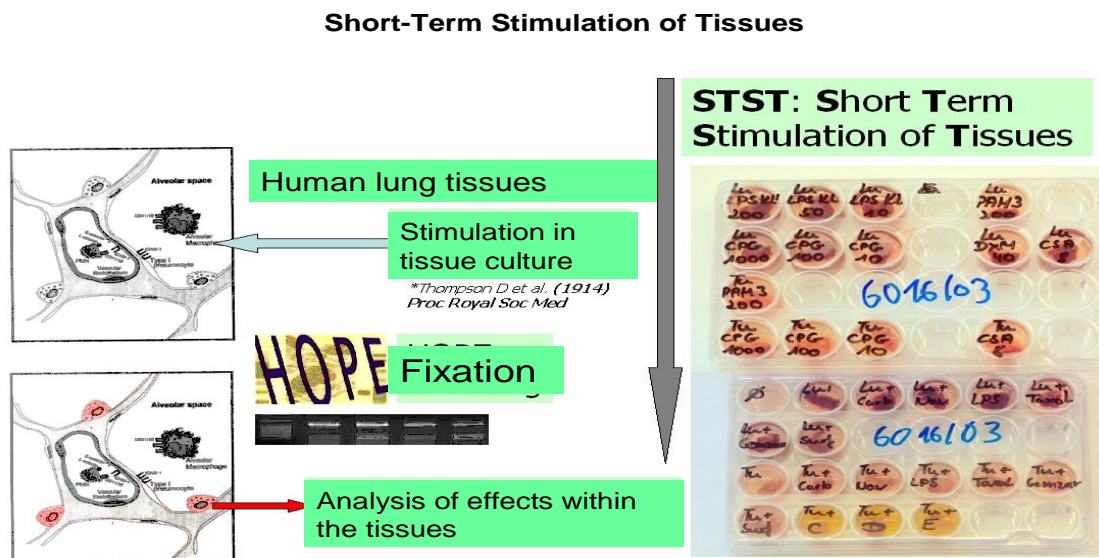
Figure 8: Presentation of short term tissue culture and eventual events of tissues during stimulation, fixation and analysis the effects with molecular tools [77].

2.4 Fixation and collection of supernatants

After the end of incubation time, specimens were either fixed with HOPE or 4% formalin. Supernatants of the STST model, BAL, AEC II and A549 cells were collected and centrifuged at 6000 rpm for 5 min and kept at -80 for further analysis.

2.4.1 HOPE fixation and preparation of paraffin blocks

Tissues were processed for HOPE fixation as previously described [105] and as shown in figure 9. All specimens which were used, were freshly cut from human lung after lobectomy
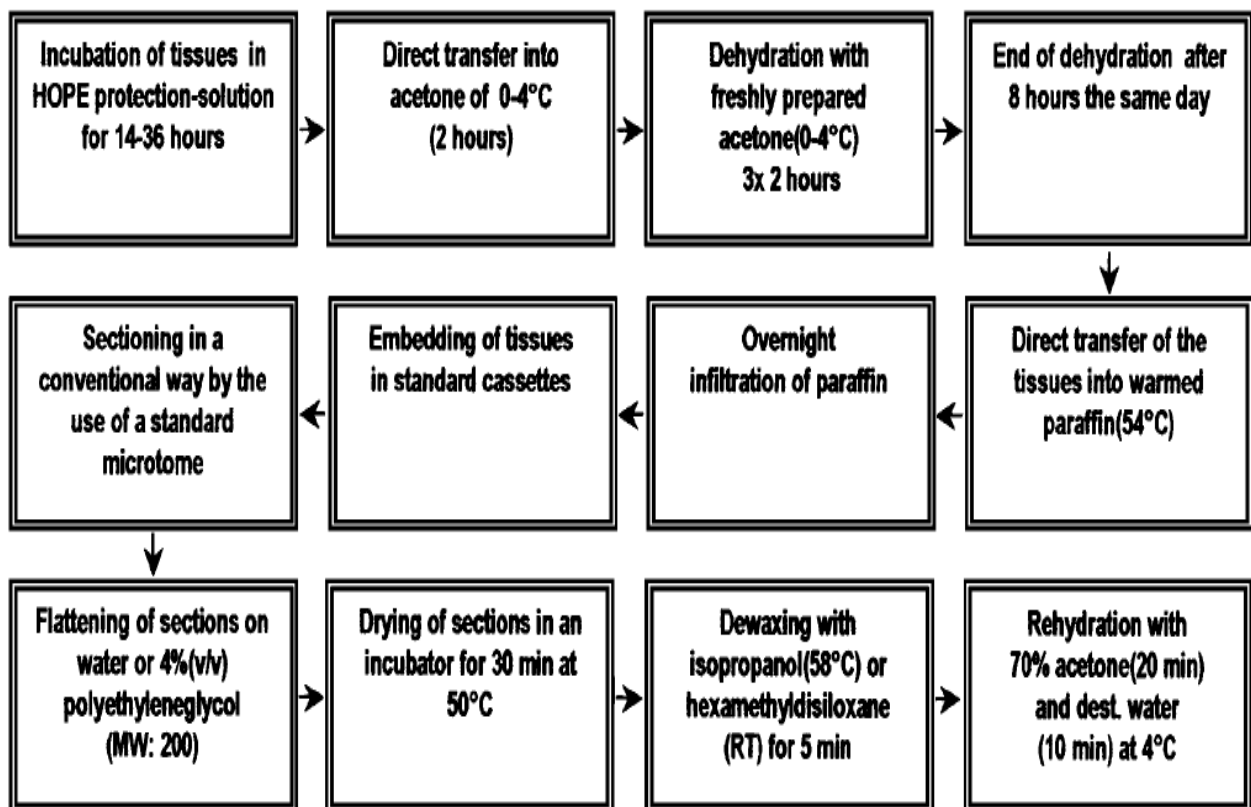
Figure 9: Tissue-processing with the HOPE-fixation technique, schematic representation of the standard protocol [105]

2.5 Tissue microarrays

For IHC and ISH all specimens were arranged in tissue microarrays (TMAs) as described in figure 10 (MTA-Booster, Beecher Instruments, ALPHELYS, France). A hollow needle was used to remove tissue cores as small as 0.6 mm in diameter from regions of interest in paraffin-embedded tissues. These tissue cores were inserted in a recipient paraffin block in a precisely spaced array pattern. Sections from the block were cut using a microtome (Leica SM, 2000R, Germany), and mounted on super frost slides (Menzel-Gläser, Germany). Six different TMAs were produced, containing 115 formalin-fixed tissue specimens for investigation of pHp in tumor and tumor free lung and one TMA for ISH containing 20 HOPE-fixed lung specimens and 20 lung cancer tissues. Furthermore, 21 TMAs including formalin-fixed samples from the STST model were used. Additionally, two TMAs containing 30 Hope-fixed specimens from an *ex vivo* STST model stimulated with different types of pathogenic microorganisms (*Haemophilus influenzae*, *Chlamydia pneumoniae* and *Streptococcus* pneumoniae) were used [31, 156] .
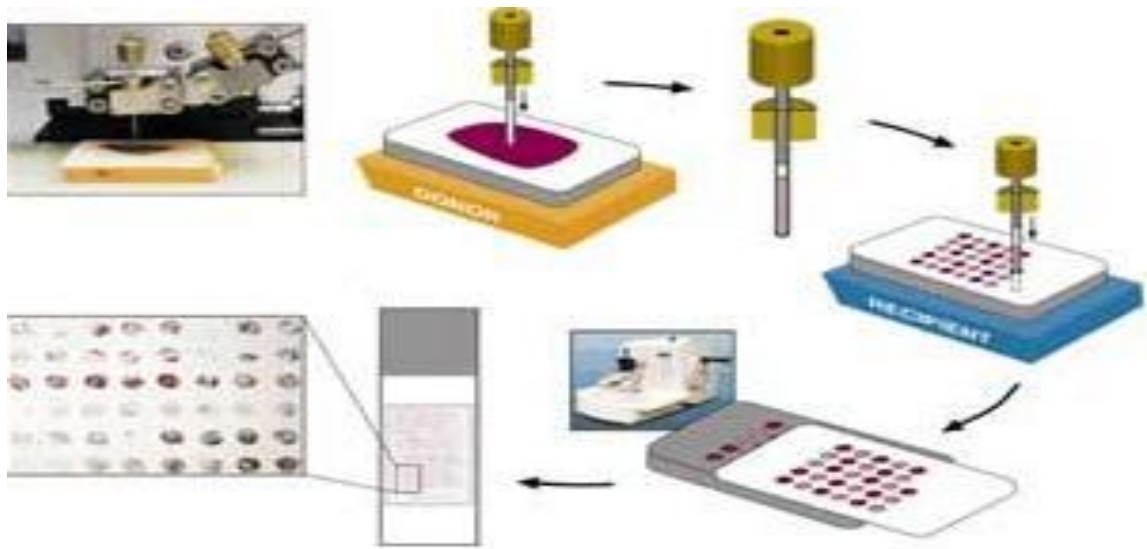
Figure 10: Schematic view of procedure in tissue micro array techniques. www.microarraystation.com

2.6 Sectioning of HOPE-fixed paraffin-embedded tissue

Paraffin-embedded tissues were cut on a microtome. Two different water baths were used, one at room temperature and the second at 35-39°C. Sections were transferred onto the surface of the room temperature water bath first, then to the second water bath in order to allow them to stretch out at 35-39°C. After this, the tissue sections were placed carefully on a slide, drip-dried and placed on drying rack. All sections were allowed to dry in an incubator at 45°C for about 30 min or preferably at 37°C overnight. Dried sections were stored at 4°C.

2.7 Immunohistochemistry on HOPE-fixed tissue sections

Immunohistochemistry (IHC) from HOPE-fixed sections was cut, deparaffinized with incubation for 10 min in pure isopropanol at 60°C and subsequent washing in fresh isopropanol. After air-drying, the deparaffinized slides were transferred in 70% acetone for 10 minutes at 4°C for rehydration and washed with DEPC-treated water for 10 min at 4°C. To block endogenous peroxidases, deparaffinized and rehydrated tissue sections were placed in a cuvette with 3 % H_2O_2 for 10 minutes. After blocking, slides were washed in a cuvette with aqua dest. and transferred immediately into a new cuvette with T-TBS pH 7.6 with 0.05% Tween 20 (Tween-Tris buffered saline, sigma-Aldrich, Germany).

Immunodetection with either anti-haptoglobin antibody (mouse anti-human; monoclonal anti-haptoglobin antibody; clone HG-36, dilution 1:100; Abcam, U.K.) or anti-CD163 antibody (mouse anti-human; monoclonal anti-CD163 antibody; clone 10D6, dilution 1:100; Diagnostic BioSystems, Netherlands) diluted in antibody diluent (Zytomed, Berlin, Germany) was done for 55 minutes at room temperature. Slides were washed with T-TBS pH 7.6 (twice 3min). The detection system (ZytochemPlus HRP polymer kit, Zytomed System, Berlin) was applied for 30 min and color reaction was performed using 3-Amino-9-Ethylcarbazole/ H_2O_2 as substrate (AEC 160mg in 10ml of N-N-Dimethylformamid Sigma Chemie GmbH, Germany). Color reagent was prepared as one drop of AEC in 2 ml acetate buffer pH 5.2 (0.1M glacial acetic acid and 0.1M sodium acetate trihydrate, Merck, Germany) and one drop of 0.3% H_2O_2 mixed and filtered with a syringe filter (Spectrum, USA). Sections were incubated with 200 µl of prepared solution

for 3-15min. Slides were counterstained using Mayer's hemalum (Merck, Germany) and covered with either Kayser's glycerin gelatin (Merck, Germany) or with synthetic aqueous mounting medium "Aquatex" (Merck, Germany).

Negative controls were included in every staining series as well as positive sections from human liver to ensure even results. Slides were analyzed on a microscope and photomicrographs taken with a CCD camera (Leica Microsystems, Germany).

2.8 IHC on formalin fixed paraffin embedded tissue sections

For Immunohistochemical staining of formalin fixed paraffin embedded tissue sections TMA sections were applied. The sections were deparaffinized with xylene (two times 10 min). Rehydration of the tissues was performed in graded ethanol series for two minutes each step (2 times 100%, 96%, 90%, 80%, 70%; and 2 times aqua dest.). Endogenous peroxidases were blocked as mentioned above. For antigen retrieval, Heat Induced Epitope Retrieval (HIER) in citric buffer pH 6 solution (0.1M citric acid monodihydrate and sodium citrate dihydrate Merck, Germany) was applied for 35 min. Immunostaining and detection were the same as in the previous section (2.7).

2.9 Cell culture and cytospin preparation

A549 cells and primary AEC II were cultured in modified high glucose DMEM (4.5 g/l) medium with L-glutamine (PPA, Austria) supplemented with 1% penicillin/streptomycin solution (Invitrogen; Karlsruhe, Germany) and 10% heat-inactivated FCS (PPA, Austria). BAL cells were cultured in RPMI 1640 with L-glutamine (PPA, Austria). Cells were generally incubated at 37°C in 5% CO_2 for 48h. After 48h medium was discarded, cells were washed with PBS and incubated with Trypsin-EDTA (PPA, Austria) for 10 min. Then, cells were transferred to a fresh falcon tube with 10 ml medium and centrifuged with 1500 rpm at 4°C for 5 min (Rotixa 50 RS Hettich, Germany).

The supernatants were discarded and sedimented cells adjusted in equal amount of medium. To obtain a total cell count and viability of cells, an aliquot of the cell suspension was diluted 1:10 with 0.4% Trypan Blue (Sigma-Aldrich, Germany)

and counted on a standard hemocytometer. Generally, 1×10^6 cells /ml medium were used.

For stimulation, 1×10^6 cells were allowed to adhere to the wells overnight, before the medium was discarded and replaced by medium containing the stimuli (inflammatory agents, cytokine and glucocorticoids dexamethasone) for 24h or 35h. For each stimulus, medium controls without stimuli were included. After end of incubation, supernatants were collected for proteomic analysis. For cytospin preparation, washed cells were centrifuged for 10 min with 1200 rpm at 4°C. Adhered cells were removed with 5ml of Trypsin-EDTA and centrifuged for 10 min with 1200 at 4°C. The supernatant was discarded and the pellet resuspended in PBS with 25µl of Trypan blue for cell counting and viability assessment. For cytocentrifugation, 0.5×10^6 of viable cells were centrifuged with 750 rpm on a high accelerating cytospin 2 centrifuge (Shandon; Frankfurt, Germany) for 5 minutes. Slides were examined under a microscope to ensure homogenous separation and dried in a desiccation chamber overnight.

2.9.1 Fixation of cytospin slides
After complete drying of slides, half of the cytospins were fixed with HOPE overnight at 4°C or with 4% formalin for 10min at 4°C. Dehydration was performed via incubation with 70% acetone in DEPC treated water for 1h, then a series of incubations with acetone 70% (6 times, 10 min each). After dehydration, sample slides were immersed in liquid paraffin and stored until further analysis.

2.9.2 Immunostaining of cytospin slides
For immunocytochemistry (ICC), the cytospins were deparaffinized, rehydrated and treated like described for IHC (2.7).

2.10 Slot blot analysis
Supernatants of STST model, BAL, primary AEC II and A549 cells were analyzed by slot-blot and subsequent immunodetection (PR 60 and PR 648 slot blot filtration device with vacuum pump, Amersham Biosciences, UK). Total amount of protein

was quantified via Bradford assay (Protein Assay Kit, PIERCE, Rockford, USA) and a Helios ß spectrophotometer (Unicam, UK). For analysis 100-500µg protein were loaded each well.

Nitrocellulose transfer membrane (Whatman GmbH, Germany) and filter paper was soaked with buffer T-TBS pH 7.6 and membranes placed into in the device. By using the vacuum pump for 2-3 min, samples were transferred from every well onto the membrane. After loading the samples, the membrane was three times washed with 50µl of T-TBS pH 7.6 for 2-3 min by the help of the vacuum pump.

Immunostaining started by incubating the membrane with 5% Roche blocking solution (Boehringer Mannheim, Germany) for 1h and subsequent washing. The blocked membrane was incubated with either anti-haptoglobin antibody (mouse anti-human; monoclonal anti-haptoglobin antibody; clone HG-36, dilution 1:100; Abcam, U.K.) or anti-CD163 antibody (mouse anti-human; monoclonal anti-CD163 antibody; clone 10D6, dilution 1:100) for 50 min at room temperature. After incubation with primary antibody, the membrane was washed again with T-TBS pH 7.6 (3 times at 5 min). Secondary antibody (goat-anti-mouse; AP-conjugated; absorbed against human; Dianova, Hamburg, Germany) was applied for 1 h in a dilution 1:10 000 at room temperature and washed (twice, 5min) with T-TBS pH 7.6 and finally with T-TBS pH 9.5 for 5min. Color reaction with NBT/BCIP system (Nitro Blue Tetrazolium chloride/5-Bromo-4-Chloro-3-Indolyl Phosphate; Boehringer Mannheim, Germany) was done according to manufacturer's instructions. Medium controls were used in parallel to stimulated samples. For analyzing slot-blot results, Corel photo–paint and Band Leader software (Band Leader, UK) were applied.

2.11 Bronchoalveolar lavage

2.11.1 Preparation of bronchoalveolar lavage

Bronchoalveolar lavage (BAL) fluids from nine patients (three cases of sarcoidosis, one case of chronic eosinophilic pneumonitis, two cases of neutrophilic alveolitis, one case of bronchiectasis and two healthy donors) who underwent BAL as a standard diagnostic procedure at Medical Clinic in Borstel were collected. Immediately after lavage, the aspirated fluids were pooled and placed in 50ml plastic tube and centrifuged at 1200 rpm for 10 min at 4°C. The sedimented cells

were resuspended with 30 ml of PBS by using a vortex device and processed as described in section (2.9).

An aliquot of each cell suspension was placed on a standard hemocytometer to obtain total cell counts. The stimulated samples were used for cytospins preparation as described in (2.9). Before further analysis, clinical information was obtained from Medical Clinic in Borstel.

2.12 Immunofluorescence staining

HOPE and formalin fixed cytospins from primary AEC II, A549 cells, and BAL were used for immunofluorescence analysis. The general procedure is the same as described for IHC (2.7).

Instead of enzyme-conjugated secondary antibody, goat anti-mouse labeled with fluorescence isothiocyanate FITC was used (Alexa Fluor 488, Invitrogen, Germany) in a dilution 1:200 for 40 min in dark environment. Counter staining and mounting medium DAPI was used (4'-6-diamidino-2-phenylindole, Vector Laboratories, Burlingame, USA) and slides were covered with cover class. The samples were examined with a fluorescence microscope (Nikon Eclipse 80*i*) and slides were kept at 4°C in dark environment.

2.13 Principal molecular biology methods used

2.13.1 General laboratory practice

Particular care was taken in the work with nucleic acids. Generally, working benches were cleaned with 80% ethanol. Prior to molecular biology analysis and isolation of RNA all benches and pipettes were additionally wiped with 0.1 M HCl. The microtome was cleaned with chloroform before cutting. Glass and metal ware was baked at 180°C for at least 6 h. Thermostable plastic ware was autoclaved at 121°C for 20 min and sterile gloves were always used during the working-procedures. Chemicals, composition of buffers and reagents, instruments used in this study are listed in the appendix.

2.14 Ribonucleic acid preparation

2.14.1. Total ribonucleic acid extraction

Total ribonucleic acid (RNA) was extracted from different specimens: 13 human, HOPE-fixed lung cancer tissues, tumor-free lung tissues and liver samples. RNA was also extracted from 11 *ex vivo* cultured tissues with inflammatory agents, inflammatory cytokines, dexamethasone and pathogenic microorganisms (*Haemophilus influenzae, Chlamydia pneumoniae* and *Streptococcus pneumoniae*). Furthermore, from 11 samples of chronic diseases (sarcoidosis, tuberculosis, and COPD) in parallel with tumor-free lung RNA was extracted according to manufacturer's recommendations (RNeasy Mini kit, Qiagen, Germany). After destroying residual DNA by DNase I treatment (Invitrogen, Germany), cDNA was synthesized by reverse transcription (Superscript II, Invitrogen, Germany). The schematic procedure for isolation of RNA is shown in figure 11.
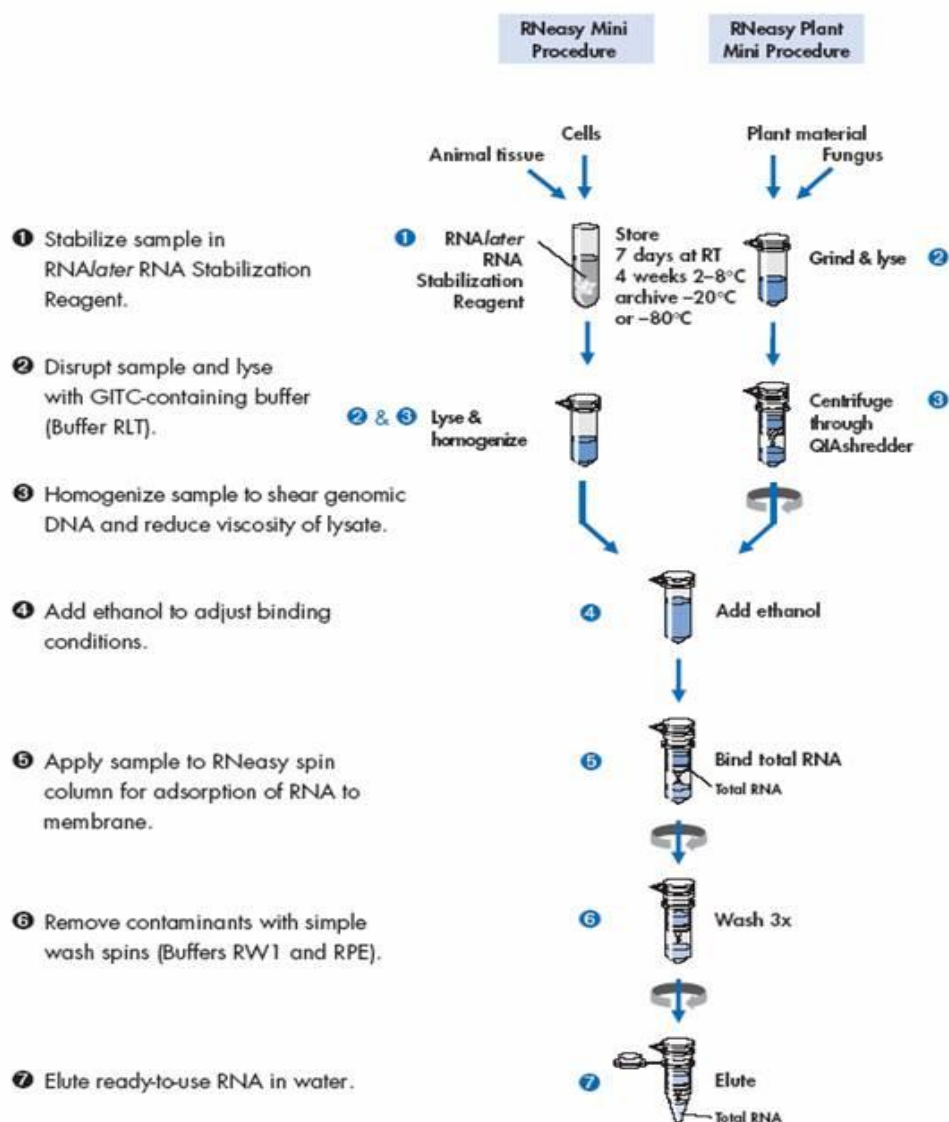
Figure 11: Schematic view of the procedure for the isolation of RNA (RNeasy Mini kit, Qiagen, Germany)

2.14. 2 Quantification of RNA by optical density

The total RNA dissolved in the DEPC-treated water was quantified by measuring the absorption of nucleic acids at 260 nm in a spectrophotometer (Gene Quant pro RNA/DNA Calculator, Amersham Pharmacia Biotech, and Cambridge, England). For this, RNA was diluted 1:100 (2 µl RNA+98 µl DEPC-treated water). For assessing protein contamination, the ratio of 260/280 nm was determined. Only samples with a ratio > 1.6 were kept at -80 °C and used for further analysis.

2.14.3 Deoxyribonuclease digest

All RNA samples underwent a Deoxyribonuclease (DNase) digest of DNA to prevent false positive results produced by any residual genomic DNA in the subsequent DNA amplification reactions. For each assay, 1 µl of DNase I was added to 1 µg of RNA with the appropriate volume of buffer (10x DNase I reaction buffer, Invitrogen, Germany) and incubated at room temperature for 15 min.

2.14.4 Reverse transcription PCR (conventional)

The reverse transcription PCR (RT-PCR) serves to synthesize a strand of complementary DNA (cDNA) from an RNA template. The enzyme reverse transcriptase (RT) catalyzes this reaction in the presence of primers. For this work, primers which anneal to the poly-A-tail of mRNAs (Oligo-dT15 500µg/ml, Invitrogen, Germany) were chosen. The protocol was used for the transcription of 1-2 µg total RNA in a reaction volume of 20 µl. The RNA was denatured at 65°C for 10 min and immediately cooled down to 4°C to preserve its linearized structure. The primers annealed to this denatured RNA during 10 min at 70°C, followed by the reverse transcriptase activity for 50 min at 42°C via (SuperScript II Reverse Transcriptase 200U/µl Invitrogen, Germany). Finally, the preparation cooled to 4°C. See Table 2 and 3 for reagents used and their final amounts in 20µl reaction volume as well as program cycle.

Table 2 -Reagents and amounts for reverse transcription (final volume 20μl)

RT reagents	Final amount in 20 µl
RNA	1 -2µg (8µl)
DNase 10x PCR running buffer (200 mM Tris-HCL, 500 mM KCL)	1 µl
DNase I	1 µl
ETDA 25 mM	1 µl
Oligo-dT15 500 µg/ml	1 µl
First Strand buffer	4 µl
DTT 0.1M	2 µl
dNTPs10mM (dATP, dCTP, dGTP and dTTP)	1 µl
Superscript II 200Units/µl	1 µl

Table 3- Program cycle for RT-PCR

Program cycle for RT-PCR		
Cycle	Temperature	Time
1	65°C	10 min
2	70°C	10 min
3	25°C	10 min
4	25°C	10 min
5	42°C	50 min
6	70°C	15 min
7	4 °C	44h

2.14.5 Polymerase Chain Reaction (PCR)

PCR is a technique which amplifies specific DNA segments. Similar to the RT, the key reagents which were used are the primers and an enzyme, the DNA polymerase. However, contrary to the RT, the PCR primers for this study were chosen to act not at random, but specifically for a DNA region and, hence, for the genes of interest. The principle of the PCR is the 20 to 40-fold cycling using a thermo cycler System (Biometra, Germany).

2.14.5.1 Selection of primers for the genes of interest

The main genes of interest were Hp, CD163 and GAPDH as a house keeping gene. Species specific primers were applied to samples of human haptoglobin origin, and human CD163. PCR products were sequenced (at National Reference Center for Mycobacteria, Research Center Borstel) and sequences verified by applying the Blast-algorithm. Primers were designed using the DNAsis program. Table 4 provides details of the primer sequences, the PCR fragment size and the primer sequences used in this study.

Tab. 4: Details of PCR primer, the PCR fragment size and fragment sequences the forward and reverse primer sequences for the genes Hp, CD163 and GAPDH.

Type of primer	Fragment size	Fragment sequences
Hp	341	Forward 5'-AGGCATTATGAAGGCAGCAC-3 Reverse 3'-CTTCCAGGCTGAAATCTTGC-5'
CD163	431	Forward 5'-AGACCTGGACTATTAAATGC-3 Reverse 3'-CGTTGTGAGTCATGCCTTAT-5'
GAPDH	257	Forward 5'-AGAACGGGAAGCTTGTCATC-3 Reverse 3'-TGCTGATGATCTTGAGGCTG-5'

2.14.5.2 Implementation of the PCR

Generally, a Master-Mix was prepared containing 10x PCR Running buffer, $MgCl_2$, dNTPs, Taq DNA polymerase (Invitrogen, Germany) and the forward and reverse primers as in Table 4. 42 µl of this mixture were added to 8 µl of cDNA to achieve a total PCR reaction volume of 50 µl. The PCR reagents, temperature and time condition for the complete PCR is shown in Table 5 and 6.

Tab. 5: PCR reagents used and their final concentration in 50 µl

PCR reagents	Final concentration in 50 µl
Ultra-pure H_2O	Add to (50 µl)
10x PCR buffer 200 mM Tris-HCL,500 mM KCL	5 µl
$MgCl_2$ 50 mM	2 µl
dNTPs 10 mM (dATP, dCTP, dGTP and dTTP)	1 µl
Primer forward 20 µM	2 µl
Primer reverse 20 µM	2 µl
Taq DNA polymerase 5 Units /µ 500 U	0.3 µl

Tab. 6: Temperature and time condition of the PCR steps

	Fragment		
	HP	CD163	GAPDH
Hot start	99°C	99°C	99°C
Denaturation	94°C 1min	94°C 1 min	94°C 1min
Primer annealing	58°C 1min	55°C 1 min	57°C 1min
Extension	72°C 1min30s	72°C1 min 30s	72°C1m30s
Number of cycles	39	39	35
Final extension	72°C15 min	72°C15 min	72°C15 min
Cooling	4°C 22h	4°C 22h	4°C 22h

2.14.5.3 Visualization of PCR products by DNA agarose gel electrophoresis

PCR products were checked by DNA gel electrophoresis with 5x DNA loading buffer containing Xylene-cyanol or Bromophenol-blue (Sigma, Germany). PCR products were separated on 2.4-2.8% agarose gels (Invitrogen, Germany) containing 10 µl Ethidium bromide (Invitrogen, Germany) in 1xTAE (Tris-Acetate-EDTA) buffer at 80-90 volts (Biometra, Germany) for 20-25 min. After the electrophoresis, DNA bands were visualized by a UV-transilluminator (Gel documentation system, INTAS, Germany) and the fragment size was assessed by comparison to a DNA molecular weight marker (pBr322-*Msp*1, NewEnglandBiolabs, NJ, USA).

2.15 Real-time-PCR

RNA isolation and cDNA synthesis was conducted as mentioned above. PCR amplification was performed using Light Cycler® Carousel-Based System (Roche Molecular Biochemicals, Penzberg, Germany) with light cycler software 4 LL.2.0 for analysis of data and specific primer of Hs-Hp-1-SG QuantiTect Primer Assay (200) from (Qiagen, Germany). Relative quantification of mRNA from a standard curve included in each assay and expression level of Hp was normalized for GAPDH (forward primer 5'-AGAACGGGAAGCTTGTCATC-3 and reverse primer 3'-TGCTGATGATCTTGAGGCTG-5'). Each experimental sample was finally expressed as the relative mRNA expression compared with the expression level of infected lung, stimulated lung on *ex vivo* tissue culture and chronic diseases.

2.16 In situ hybridization

The localization of pHp mRNA at the cellular level can be achieved by in *situ hybridization* (ISH) applied to tissue sections. Isolation of RNA and cDNA synthesis was done as described above. PCR products of Hp and CD163 were used for generating Hp mRNA complementary probe of DIG–labeled DNA with its specific primers.

First, the double-stranded PCR products were denaturized by boiling 1 µl of PCR product with 15 µl of RNase free water. Probes were labeled with digoxigenin by random primed labeling using DIG high-prime Kit (Boehringer, Mannheim,

Germany) overnight according to the manufactures recommendation [48]. Concentration of the labeled probes were estimated by comparison with Dig-labeled control DNA of a given concentration and direct detection of probes spotted onto positively charged nylon membranes [105]. The yield of the probe was quantified by a direct detection method (dot blot arrays).

A series of dilutions of Dig-labeled DNA probe was applied to a positively charged nylon membrane. In parallel, the nylon membrane was loaded with a series of Dig-labeled DNA of known concentration, ranging from 10 ng/µl to 30 pg/µl, serving as standard control. The nylon membrane was subjected to immunological detection of Dig-labeled DNA by anti-DIG antibody conjugated with alkaline phosphatase (Roche) and NBT/BCIP as color reagent. The probe concentration determined by eye assessment of the staining intensities of the probe compared to the standard control.

Tissue sections of 1 micrometer thickness were cut as a previously described in material & methods, deposited on room temperature SuperFrost® Plus microscope slides (Menzel-Gläser; Braunschweig, Germany) and dried at 40°C. Deparaffinization and rehydration was performed according to the same protocol used for IHC. Hybridization solution was composed of 2 ng/ml freshly denatured probe, 250 µg/ml yeast tRNA, 0.1%SDS and 50%formamide (Boehringer, Mannheim, Germany) in PBS.

Hybridization was carried out overnight at 46°C in moist chambers. Slides were washed by the following steps: 2x SSC (Saline- Sodium Citrate) twice for 10 min at ambient temperature, then 0.2x SSC twice for 30 min at 50°C. For detection of signals, specimens were then incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody as previously described [105, 48]. Slides were washed in DIG washing buffer for 15 min at room temperature. 1% blocking reagent in maleic acid buffer (Boehringer, Mannheim, Germany) was then applied for 30 min at room temperature. Immunological detection of labeled probes was achieved by incubating with anti-digoxigenin antibody conjugated with alkaline phosphatase (Anti-DIG AP-Roche Germany) at dilution of 1/ 7500 in 1% blocking reagent for 30 min at room temperature. Slides were washed in washing buffer twice for 15 min, and afterwards incubated in detection buffer (Roche) at pH 9.5 for 5 min at room temperature. Color reaction with NBT/BCIP (Boehringer Mannheim, Germany)

was performed at room temperature and the staining process was observed under a microscope & stopped after 10 min by incubation in de-mineralized water. Slides were then counterstained with hematoxylin (Merck, Germany) and examined under a microscope.

2.17 Sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) from paraffin-embedded samples was performed as follows: Specimens were deparaffinized by 2 times of xylene treatment (1 ml; 10 min) and two steps of ethanol treatment (1ml; 10 min) with respective removal of the supernatants before drying in a vacuum centrifuge (1 h; SpeedVac 110; Savant, Germany). Proteins were extracted at 4°C over night using a lysis buffer containing (7 M urea; 2 M thiourea; 4% CHAPS; 2% IGEPAL; 1% Triton-X; 100 mM Dithiotreitol [DTT]; 5 mM PMSF; and 0.5 mM Tris).

Lysates were denatured at 100°C for 5 min and protein pellets were resuspended in 5 µl of sample buffer (LDS sample buffer, Invitrogen, Germany). For quantification of total protein, a commercial Bradford-Assay (Pierce Kit, Pierce, CA, USA) was used via a spectrophotometer (Helios ß photometer, Unicam, UK).

DTT was used to ensure reducing conditions (linearization and chain-separation), and this solution was applied to the SDS-PAGE consist of (4% stacking gel 0.5 M Tris/HCl pH 6.8 and 12 % resolving gel 1.5 M Tris/HCl pH 8.8 Novex minicell, Invitrogen, Germany) with a standard protein marker (Novex Sharp Protein Standard, Invitrogen, Germany). 5-10 µg/ml of total protein was loaded onto SDS-PAGE.

Separation conditions were set as follows: 50 V for 10 min, 200 V for another 60 to 70 min (Biometra, Germany).

After separation, separated proteins were transferred onto a nitrocellulose membrane using the iblot gel transfer system (Invitrogen, Germany) according to the instructions of the manufacturer. Membrane was blocked for 1h using 5% DIG wash and block buffer (Boehringer Mannheim, Germany). Primary antibody either anti-haptoglobin antibody (mouse anti-human; monoclonal anti-haptoglobin antibody; clone HG-36, dilution 1:100; Abcam, U.K.) or anti-CD163 antibody

(mouse anti-human; monoclonal anti-CD163 antibody; clone 10D6, dilution 1:100; Diagnostic BioSystems, Netherlands) was incubated for 60 min, and membrane was washed 3 times in Tris-buffer pH 7.6. Secondary antibody (goat-anti-mouse; AP-labeled; absorbed against human; Dianova, Hamburg, Germany) was applied for 1 h, dilution 1:10 000 and 1:5000 for CD163 and washed 2 times with buffer from above. Finally, Tris-buffer at pH 9.5 was used to prepare membrane for color reaction using the NBT/BCIP system (Boehringer Mannheim, Germany) according to the manufacturer's protocol. Supernatants from tissue culture, A549 cells and BAL were carried out with same procedure as mentioned above.

2.18 Measurement of Monocyte Chemotactic Protein-1 and Interleukin-8 concentration

To investigate the role of the pHp during the initial local response to inflammatory stimuli in human lung, chemokine production by A549 cells after stimulation with LPS (200ng/ml) was measured. A549 cells were cultured up to 30h in the presence or absence of LPS and haptoglobin protein (150ng/ml, Abcam, U.K) and supernatants were collected at end of incubation time. The chemokine-concentrations within the supernatants were measured by ELISA Kits for Monocyte Chemotactic Protein-1 (MCP-1) (R & D Systems GmbH, Germany) and for interleukin-8 (IL-8) (BioSourceCytoSet, USA). Optical density readings were performed utilizing a Microplate Reader at 450nm wavelength and analyzed with a microplate manager/PC data analysis software (Bio-Rad, laboratories, Hercules, USA).

2.19 Indirect immunofluorescence staining and confocal laser microscopy

For microscopic analysis of cellular localization or co-localization of pHp (rabbit anti-human; polyclonal anti-haptoglobin antibody; dilution 5 µg/ml; Abcam, U.K), and SP-B(SP-B) (mouse anti-human; monoclonal anti-SP-B antibody; clone, SPM158 dilution 1: 50; Zytomed, Berlin, Germany), an indirect immunofluorescence and confocal laser microscopy was used.

As secondary antibodies, either goat anti-mouse (conjugated with FITC green) or goat anti-rabbit (conjugated with Texas Red) were applied.

Cytospin slides fixed either with formalin or HOPE were used for comparison BAL, primary AEC II and A549 cells as well as specimens stimulated with inflammatory cytokine IL-6 were performed.

The procedure for immunoreactions is started by incubation of cytospins and specimens with two primary and secondary antibodies separately as previously described in material and methods IHC section. Mounting medium containing DAPI for DNA-counterstain was used for covering and specimens, which were then analyzed using a Leica TCS SP5 confocal laser scanning microscope equipped with a custom-optical tunable filter and a 63x numerical aperture 1.32 plan-apochromatic oil-immersion objective (Leica Microsystems, Bensheim, Germany). Images were acquired with the Leica LA SAF software and further processed and assembled using conventional imaging software (FixFoto).

2.20 Statistical analyses
We have included standard errors of the means and p-values from Chi-square-tests in figures 20 A and B, 33A and B and 38A.

3. Results

3.1 Transcription microarray
Figure 12 shows the results of analysis of transcription microarrays in comparison to GAPDH. Hp transcription can be clearly observed in all specimens, without consideration of being NSCLC or tumor free. The level of transcription was comparably high in all tissues analyzed, even exceeding the GAPDH level in the case of the non-cultivated sample 5.
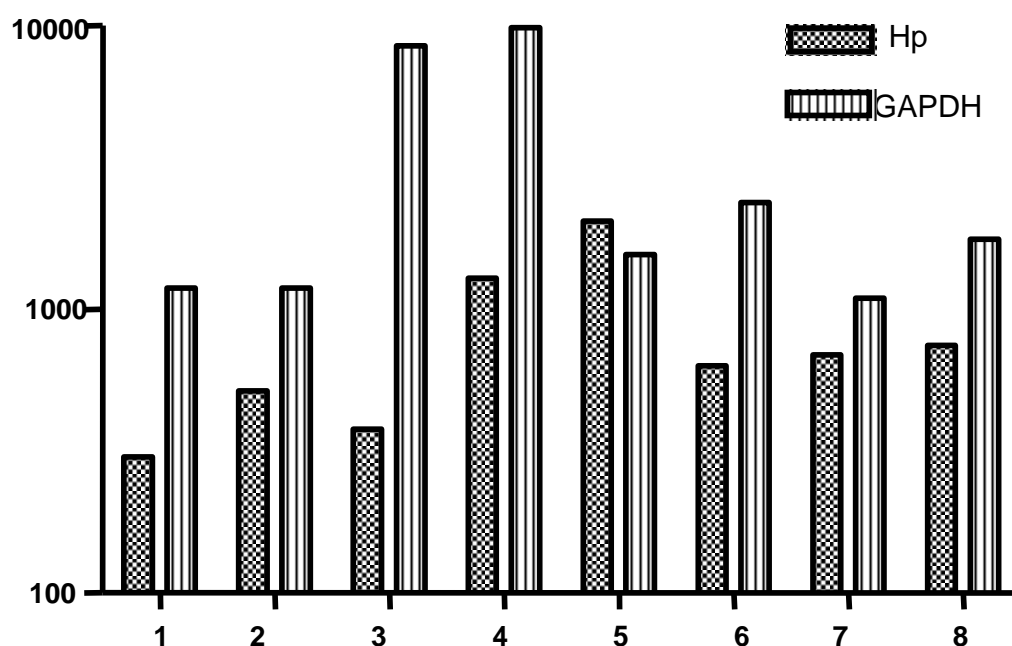
Figure 12:
Transcription microarray data of Hp in different tissue samples in respective comparison to GAPDH. Y-axis represent relative units (Log) and X-axis represent: samples tumor+ medium (1), tumor+ chemotherapeutics+ medium (2), lung+ medium (3), lung+ *Haemophilus influenzae*+ medium (4), lung HOPE-fixed uncultured (5), lung+ medium uncultured (6), lung+ inactive allergen PHLP1 (7), lung+ active allergen PHLP1 (8)

3.2 IHC

3.2.1 Expression of pHp in lung cancer tissue and tumor free lung

Figure 13 shows the amount of positive cases in relation to the total number of cases and the type of tissue investigated. The results of IHC are displayed in figure14 and 15. Among lung cancers, pHp signals appeared mostly in adenocarcinomas (19/47 cases pHp-positive, 40.4%), showing perinuclear and granular staining of tumor cells (Figure 14A). While in squamous cell carcinoma tissues, only 2 of 42 specimens were pHp-positive in tumor cells and few cells showed signal (4.8%) as shown in figure 14B. However, 8 cases (19%) showed stained cells of AEC II surrounding the tumor cells (Figure 14C). Small cell carcinoma staining revealed that 2 of 13 tumors were positive for pHp protein (15.4%), (Figure 14D). Concerning tumor-free lungs, all thirteen cases were positive for pHp and showed signals in alveolar macrophage (AM), while in AEC II cells were found to be positive signals in four cases and in four bronchi were stained (Figure 15 A, B and C). Negative controls showed no signals and in parallel to all IHC staining, liver sections were used as positive controls in which Hp appears as a granular staining in the cytoplasm (Figure 15D).

47
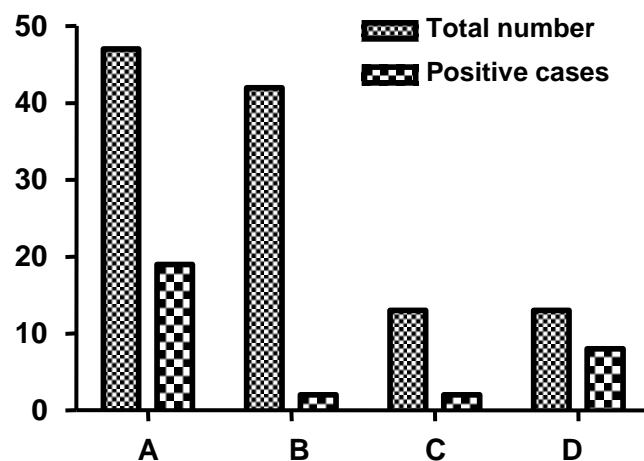
Figure 13:
Expression of pHp in different tumor entities and tumor free lung tissues determined by immunohistochemistry, X axis represented total number of cases analyzed in left bar and positive cases in right bar for adenocarcinomas (A), squamous cell carcinomas (B), small cell carcinomas (C), and tumor free lung tissues (D).
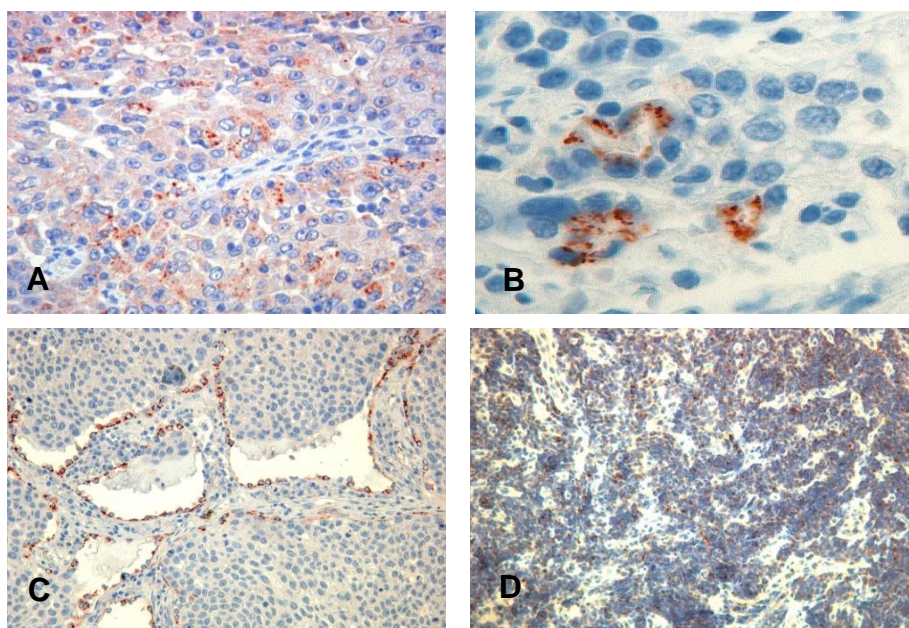
Figure 14:
Immunohistochemical detection of pHp in different subtypes of human lung cancers including adenocarcinomas (A), squamous cell carcinomas (B), AEC II surrounding squamous carcinomas (C) and small cell carcinomas (D), (magnification: upper panel 800x, lower panel 400x).
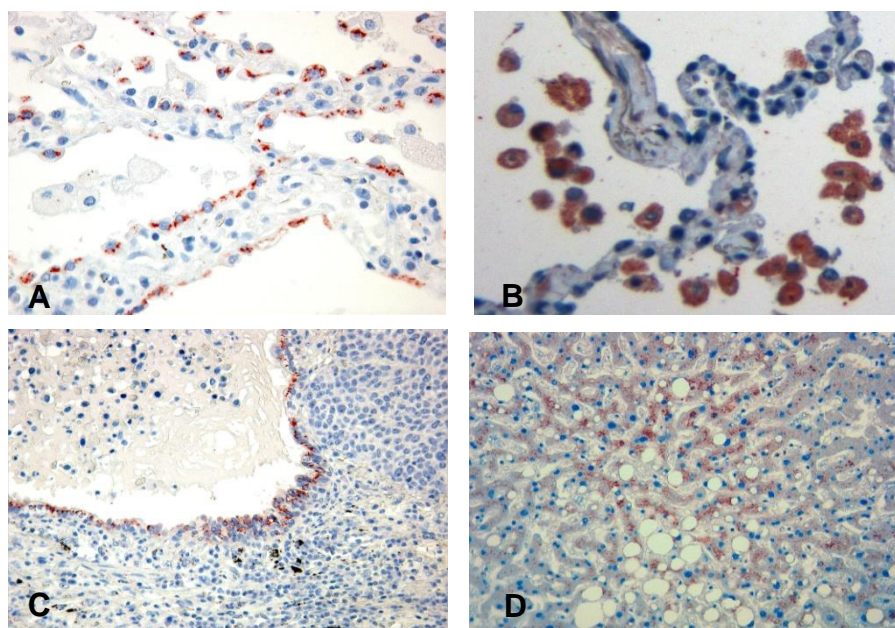
Figure 15:
Immunohistochemical detection of pHp in different non-malignant cell types of human lung tissues and liver sections as positive control: AEC II cells (A), AM (B), bronchi (C) and liver (D) (Magnification: Upper and lower panel 400x).

3.2.2 Transcription of pHp mRNA in human lung cancer and tumor free lung

RT-PCR was performed to additionally address transcripts of pHp. 13 NSCLC tissues, 2 corresponding tumor-free lung tissues and one liver sample were analyzed by RT-PCR. pHp mRNA was detected in all NSCLC and tumor-free lung and, as expected, in the liver. This confirms the IHC results and addresses the transcription of the pHp gene in human lung tissues and lung cancer for the first time (Figure 16, 17)
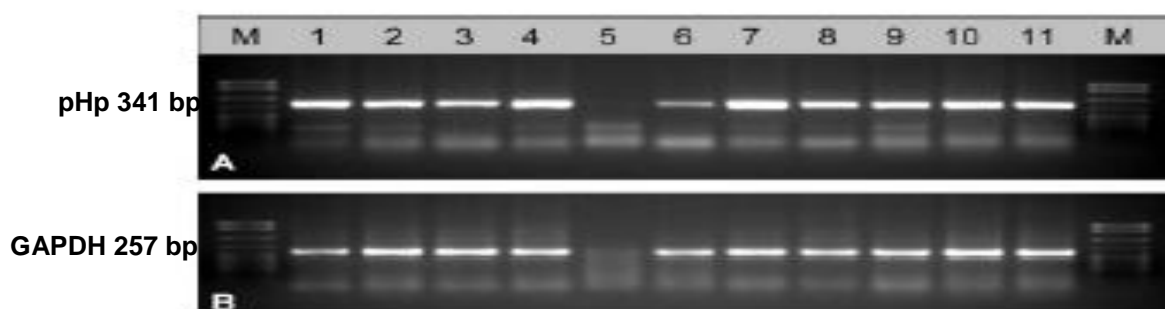
Figure 16:
RT-PCR targeting pHp-transcripts in ten specimens of NSCLC. Products were separated on agarose gels and stained with Ethidium bromide. Panel (A) shows the 341bp fragment of pHp; Panel (B) the 257 bp fragment of GAPDH. Molecular weight marker: M=pBr322/*Msp1*
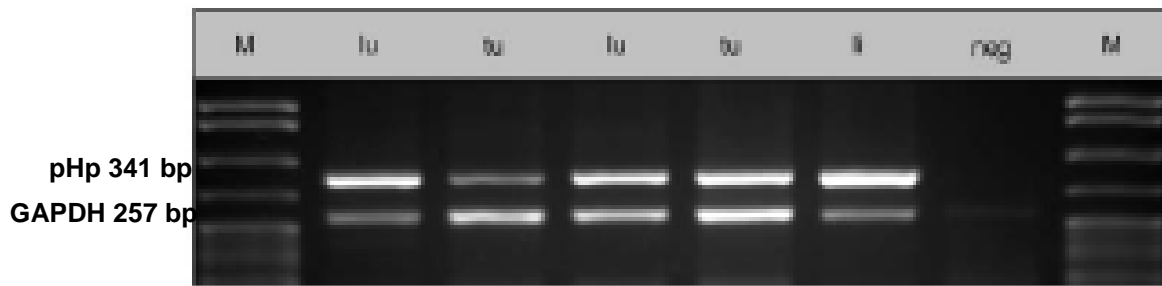
Figure 17:
RT-PCR targeting pHp (upper bands, 341 bp) and GAPDH (lower bands, 257 bp) for tumor free lung (Lu), lung tumor (tu), liver (li) and negative control. M= pBr322/*Msp1*

3.2.3 Cellular localization of pHp in human lung cancer and tumor free lung

For cellular localization of pHp-transcripts, 17 cases of carcinomas were additionally analyzed by application of *in situ* hybridization. In all investigated eleven cases of adenocarcinomas and in four of six cases of squamous cell carcinomas, signals can only be detected in tumor cells as shown in figure 18 A and B. While in tumor free lung, AM, AEC II cells, and epithelial cells of airways such as bronchi displayed signals of pHp transcripts as shown in figure 18 C and D. Negative controls did not show any signals.
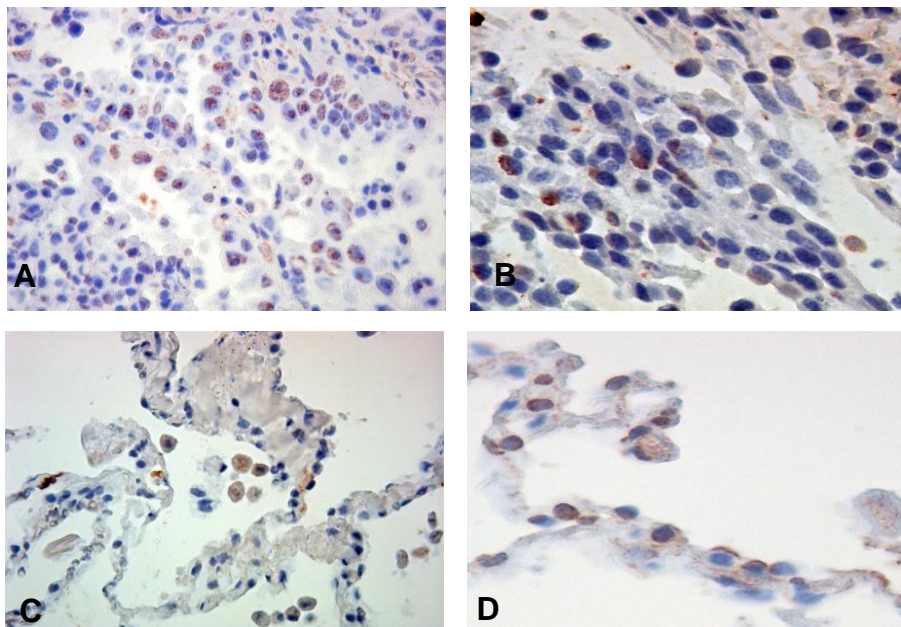
Figure 18:
In situ hybridization targeting pHp transcripts using a 341 bp digoxigenated DNA-probe on human lung tissues: adenocarcinoma (A, 200x), squamous cell carcinoma (B, 200x). In tumor free lung expression of pHp mRNA in AM (C, 200x), and AEC II (D, 400x) cells

3.2.4 Level of pHp protein in tumor and tumor free lung

Western-blot analysis was performed to compare the amounts of pHp-protein from NSCLC, tumor-free lungs and liver. Comparable amounts of pHp in NSCLC compared to the liver tissues and tumor-free lung are portrayed in figure19. Tumor free lung showed comparably weaker signals. All signals appeared at a molecular weight 40 kDa which represents the size of a β-chain of pHp.
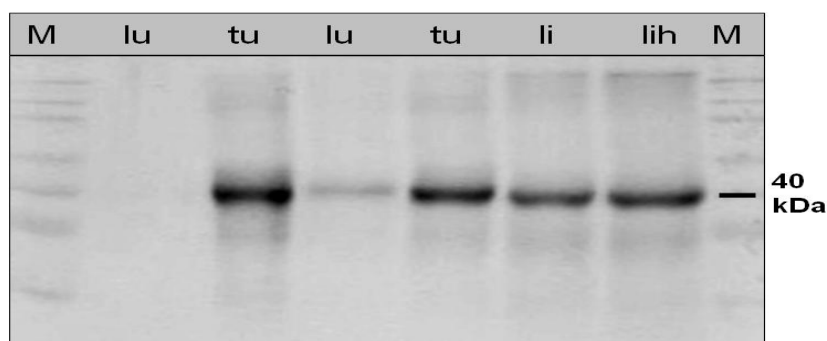
Figure 19:
Western immunoblot showing pHp in tumor-free lung (Lu), tumor (tu) and liver (li, lih) (lih for liver high protein concentration) and marker (M): M=Novex Sharp Prestained Molecular Weight Marker

3.3 IHC in human lung ex vivo tissue culture

3.3.1 Effects of IL-6, LPS, Pam3 and DEX on the production of pHp protein and its receptor

An overview upon the investigated cases and related numbers of cases showing pHp expression in STST is shown in figure 20A. pHp appears to be modulated by inflammatory stimuli. In 67 out of 80 specimens (83.7%), expression of pHp could be induced by LPS. The lipopolysaccharides stimulate pHp-expression, compared to medium control, in a dose and time dependent manner and the effects were recorded in AEC II and AM (Figure 21A and B). Strong staining was induced with LPS 200 ng/ml for 24h compared to lower concentration (10 and 50 ng/ml). As for LPS, DEX stimulates pHp production within AEC II and AM, in 54 out of 63 specimens (85.7%) in a time and dose dependent manner as displayed in figure 22A and B. Pam3 shows similar effects on pHp expression in AM and AEC II in a time-dependent manner (study did not assess dose-dependent manner) in 41 specimens from 57 specimens (71.9%). Finally, in 12 out of 14 cases (85.7%),

induced expression of pHp was observed due to IL-6 as shown in figure 23B and D.

Additionally, alveolar epithelial cells lining bronchi and bronchioles appeared to show positive signals in all specimens concerning IHC. In general, pHp appears as granular staining inside the cytoplasm of positive cells. Together with these results, IHC showed that infiltrating inflammatory cells are positive for Hp as a reaction of inflammation, tumor or due to other unknown pathological condition. There is an evidence for expression of Hp in granulocytes, monocytes and lymphocytes as well as AM but not in eosinophiles (Figure 24A-C). Furthermore, this study showed that the expression of pHp and CD163 is correlated to each other and hemorrhagic specimens showed high intensity of signals for both molecules (Figure 24D and F).

The induction of CD163 by analysis in STST model as shown in figure 20B was observed as follows: Up-regulation by IL-6 in 11 specimens from 14 (78.5%), by DEX in 52 specimens from 67 (77.6%) and due to Pam3 in 35 from 75 (46.6%) specimens. Interestingly, downregulation of CD163 by LPS can be observed in 48 from 101 specimens (47.5%) as shown in figure 25, 26, 27. Expression of CD163 could be addressed in a dose and time dependent-manner by induction with inflammatory stimuli and especially by DEX. Conversely, stimulation with LPS results in a downregulation in time and dose-dependent manner. In general, the staining of CD163 was localized in the cell membranes of AM.
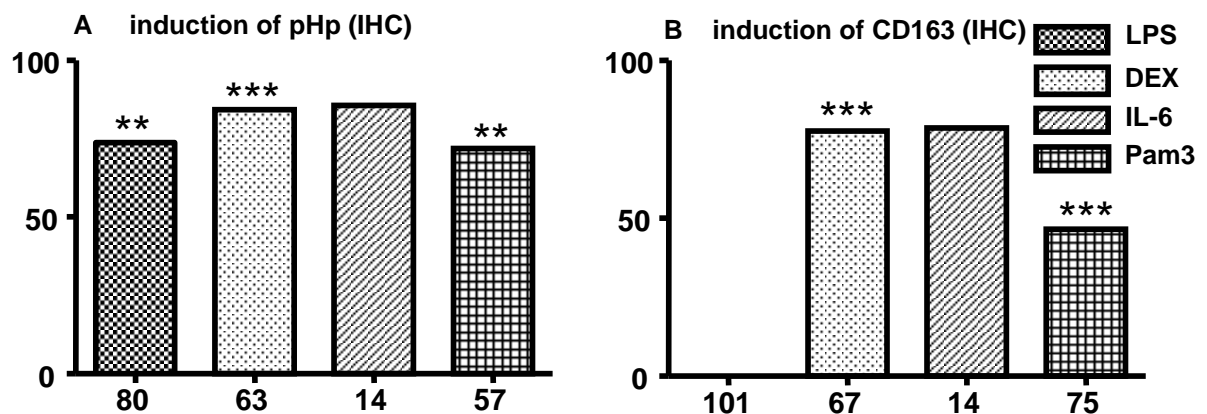
Figure 20 (A, B): Induction of pHp (A) and CD163 (B) by different inflammatory agents in the STST model. On the X-axis the total numbers of investigated stimulated tissues are given. On the Y-axis percentages of cases showing regulated expression are given (**p<0.01, ***p<0.001).

52
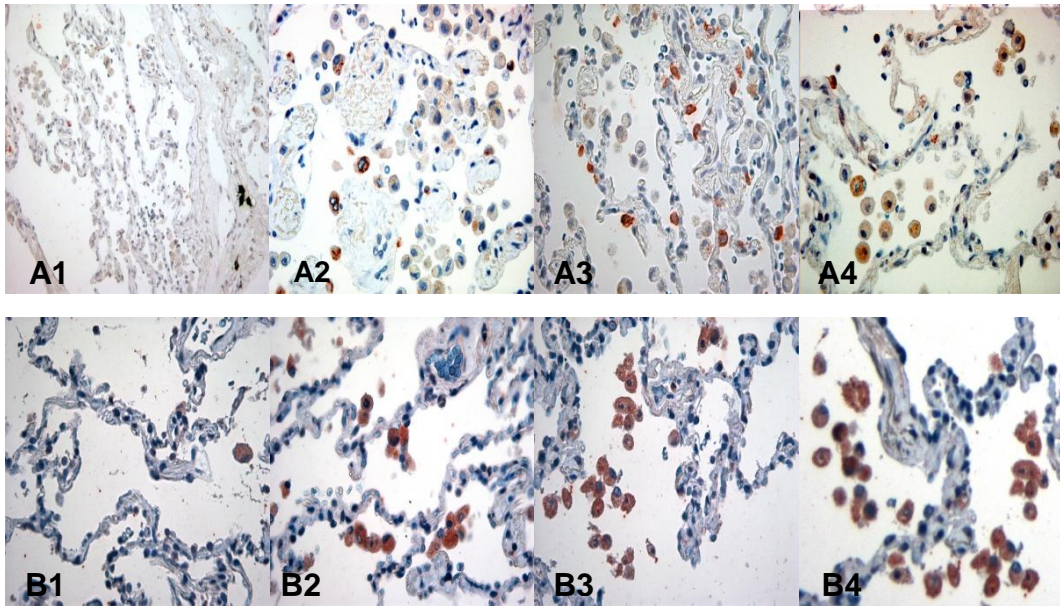
Figure 21:
Immunohistochemical detection of pHp after stimulation with LPS at different concentrations (A) and incubation times (B). A1: Medium control, A2: LPS (10ng/ml), A3: LPS (50ng/ml), A4: LPS (200ng/ml). B1: Medium control, B2: LPS (10 min), B3: LPS (3h), B4: LPS (24h). B2 – B4: 200 ng/ml LPS (Magnification: Upper and lower panel 200x, except A1: 100x).
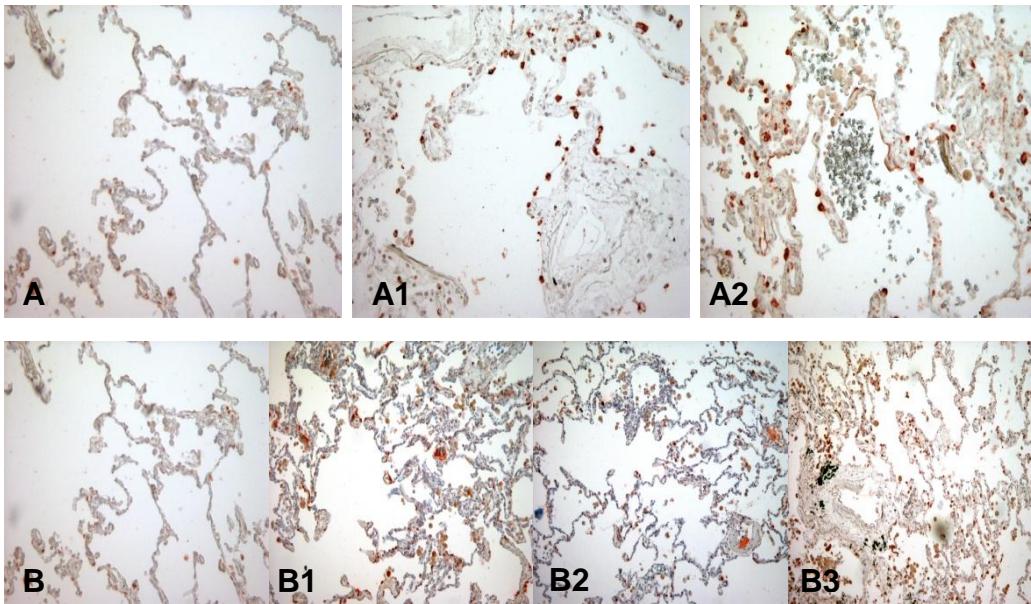
Figure 22:
Immunohistochemical detection of pHp after stimulation with DEX at different concentrations (A) and incubation times (B) of DEX. A: Medium control, A1: DEX (10ng/ml), A2: DEX (50ng/ml), B: Medium control, B1: DEX (10 min), B2: DEX (3h), B3: DEX (24h), B1-B3: 50ng/ml DEX (Magnification: Upper and lower panel 100x)
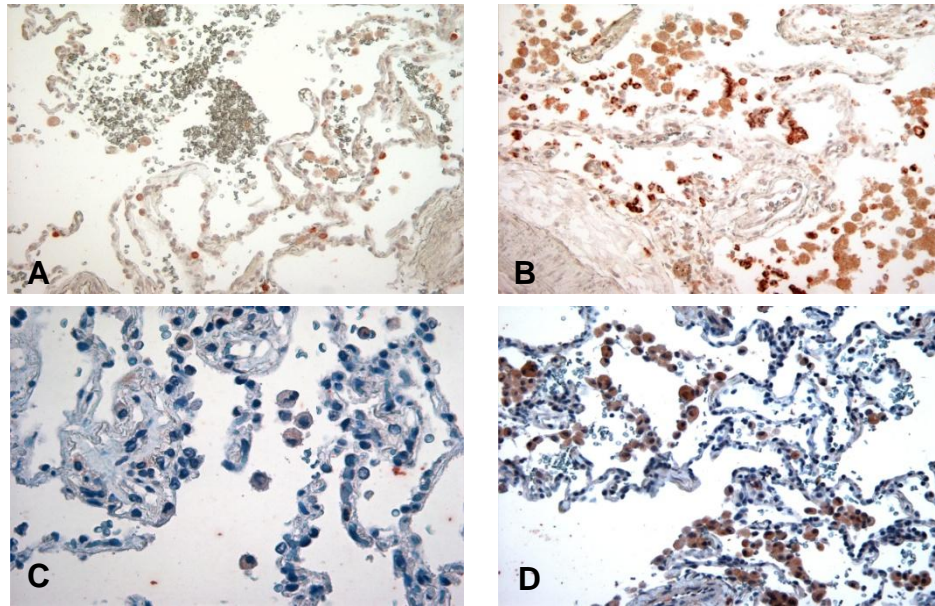
Figure 23:
Immunohistochemical detection of pHp after stimulation with 200ng/ml Pam3 (B), 50ng/ml IL-6 (D) and medium controls (A and C) (Magnification: Upper and lower panel 200x.
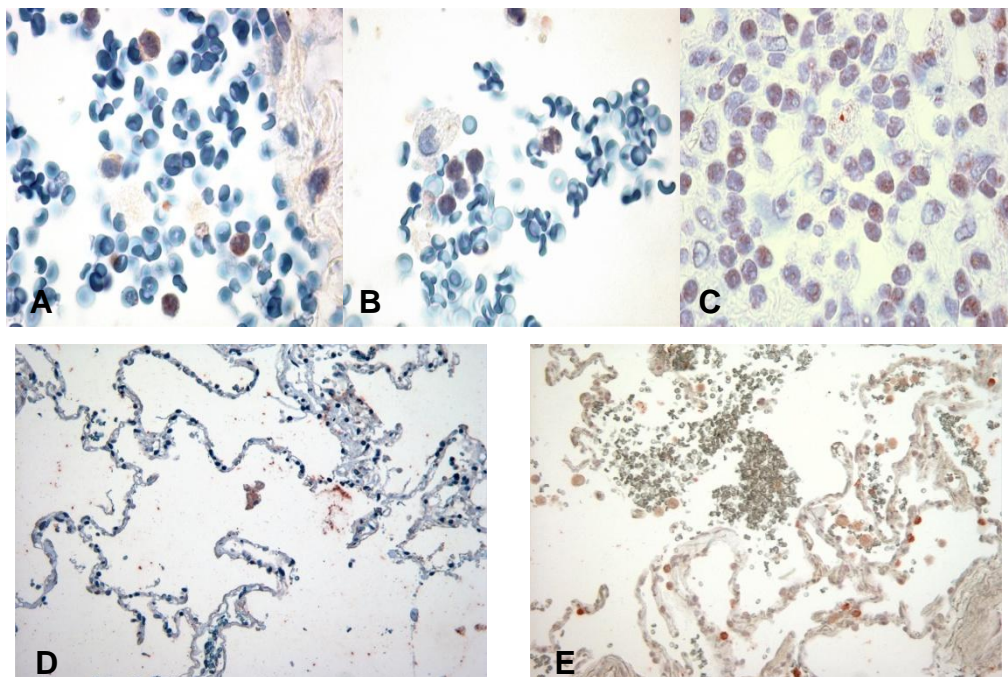
Figure 24:
Immunohistochemical detection of Hp in inflammatory cells: (A) monocytes, (B) neutrophiles, (c) lymphocytes as well as in hemorrhagic lung (E) compared to tumor free lung (D). (Magnifcation: Upper panel 400x and lower panel 200x).
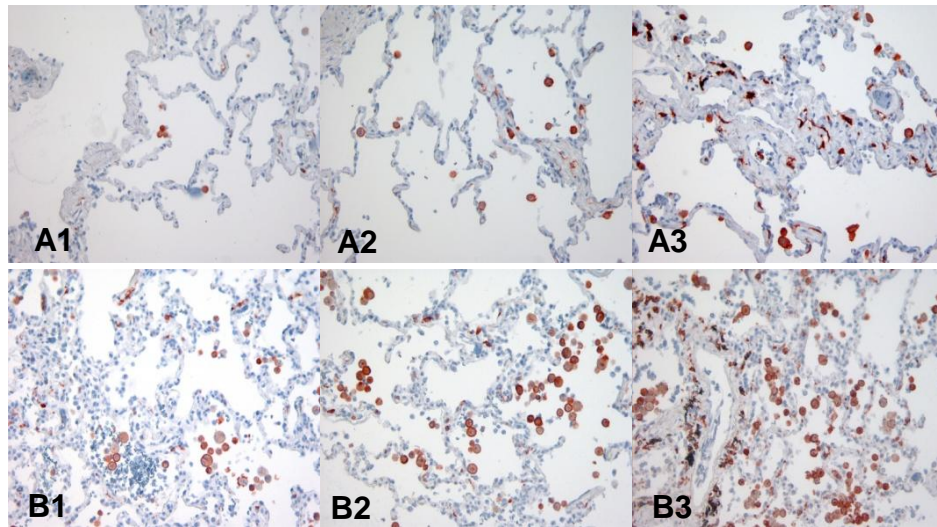
Figure 25:
Immunohistochemical detection of CD163 after stimulation with DEX at different incubation times (A), and different concentrations (B). A1: Medium control, A2: DEX (2h), A3: DEX (24h), A2-A3 50ng/ml DEX. B1: Medium control, B2: DEX (10ng/ml), B3: (50ng/ml). (Magnification: Upper and lower panel 200x)
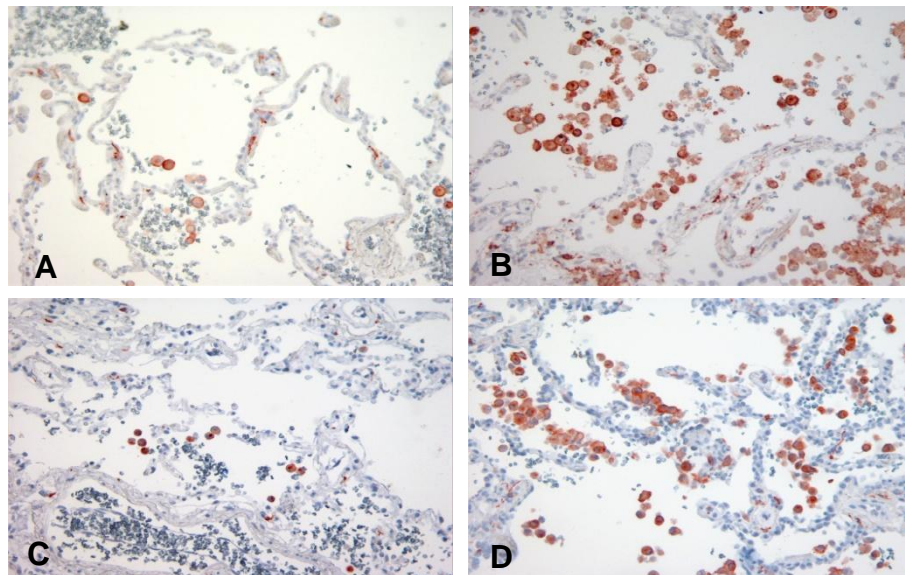
Figure 26:
Immunohistochemical detection of CD163 after stimulation with 50ng/ml IL-6 (B) and 200ng/ml Pam3 (D), (A and C medium controls), (Magnifcation: Upper and lower panel 400x)
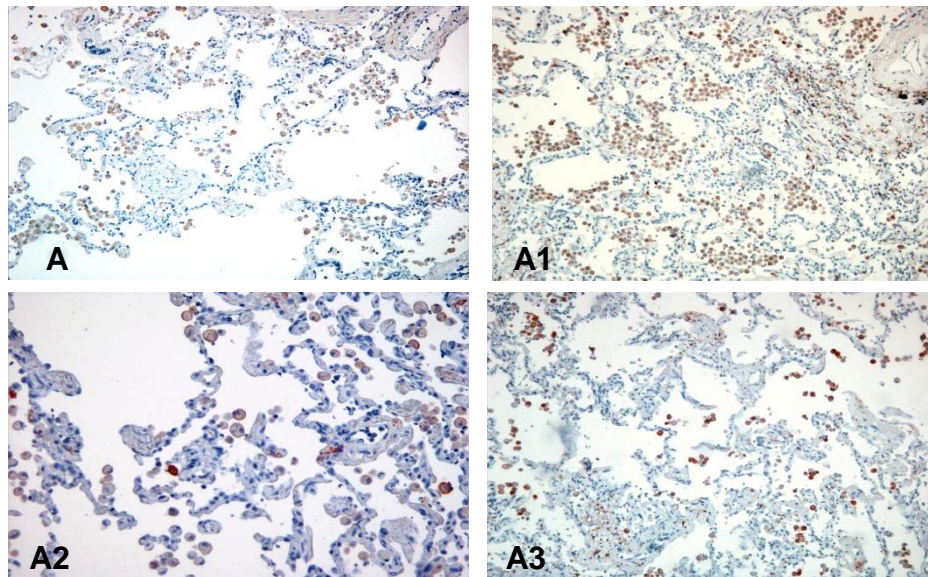
Figure 27:
Immunohistochemical detection of CD163 after stimulation with different incubation times of 200ng/ml LPS. A: Medium control, A1: (10 min), A2: (2h), A3: (24h) (Magnifcation: Upper and lower panel 200x)
.

3.3.2 ICC of pHp and CD163 with BAL cells, primary AEC II cells and A549 cells.

To further confirm expression and induction of pHp and its receptor in human lung, cytospins from BAL, primary AEC II and A549 cells were investigated. From nine different patients BAL cells were stimulated and the expression of pHp and CD163 was analyzed. The diagnoses were as follows: Three cases of sarcoidosis, two cases of neutrophilic alveolitis, two samples from healthy lung and one case of chronic eosinophilic pneumonitis and bronchiectasis, each.

After 24 h of incubation with inflammatory stimuli, only weak expression of pHp was observed. After 35h of stimulation, up-regulation of pHp expression was observed in cells from healthy subjects. Furthermore, strong staining of pHp was recorded with DEX, IL-6, LPS and Pam3, respectively, after 35h of incubation compared to medium control (Figure 28). The samples from non-healthy subjects showed weak up-regulation between stimulated and non-stimulated BAL cells.

In primary AEC II, stronger signals appeared upon stimulation DEX, LPS and Pam3 for 35h compared to 24h as shown in figure 29 A1-A4. This study did not assess expression of IL-6. Expression of pHp in A549 cells showed a slight up-regulation after 35h compared to 24h especially with IL-6, but other stimuli also

displayed distinct expression (Figure 29 B1 and B2). Generally, pHp showed stronger signals after 35h than after 24h of stimulation.

CD163 is exclusively expressed in AM. The results in BAL cells showed up-regulation with IL-6, DEX and Pam3 but downregulation was recorded with LPS (Figure 30F). CD163 is not expressed by A549 cells and primary AEC II cells. Although both fixation-techniques were used, clearer signals were observed in HOPE-fixed material.
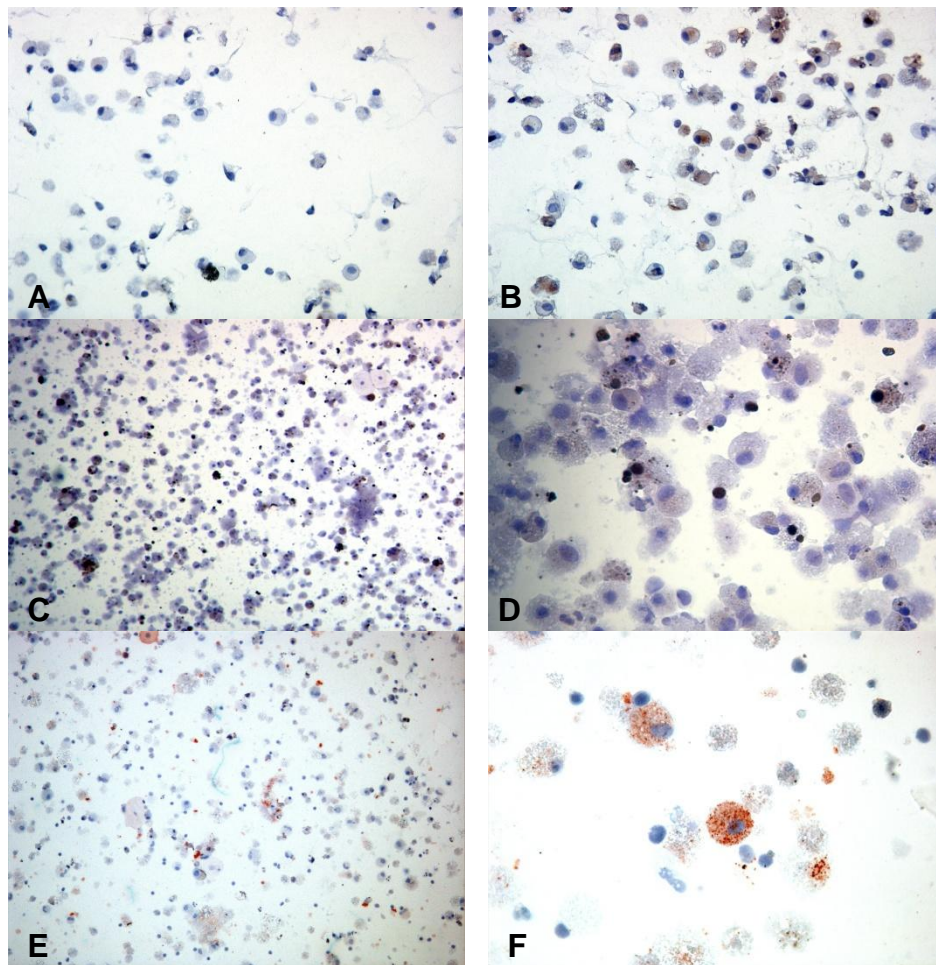
Figure 28:
Immunocytochemical detection of pHp in BAL cells after stimulation with inflammatory stimuli. A and C: Medium control 24h and E: Medium control 35h. B: 200ng/ml LPS for 24h, D: 50ng/ml DEX for 24h and F: 50ng/ml DEX for 35h. (Magnification: All figures at 100x except D and F at 400x).
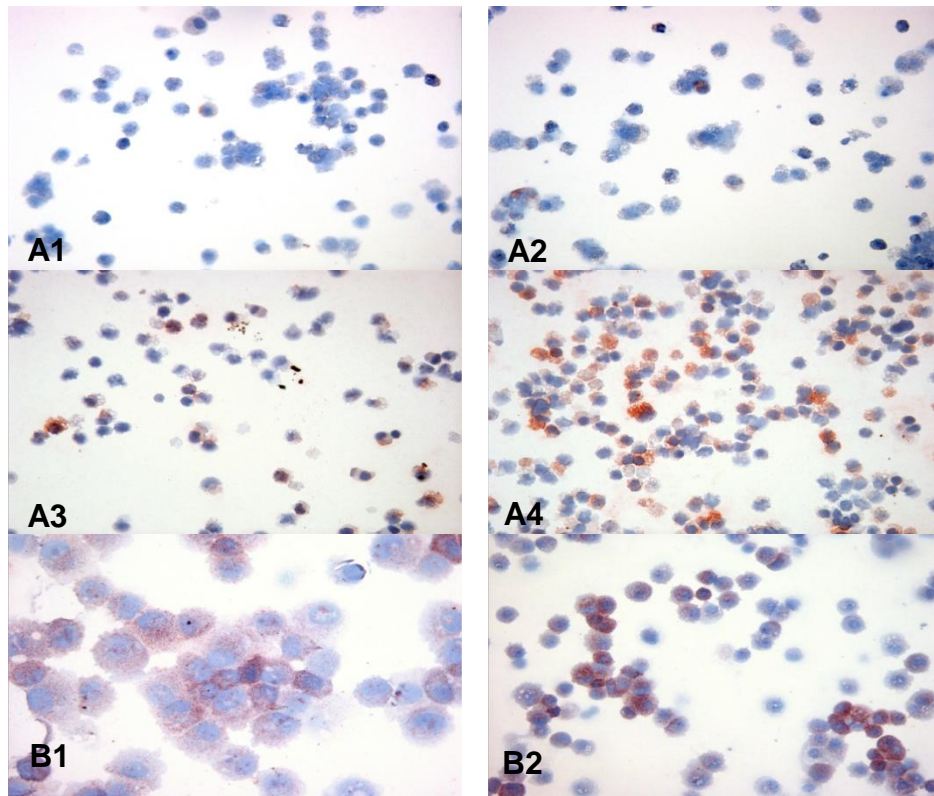
Figure 29:
Immunocytochemical detection of pHp in primary AEC II after stimulation with 200ng/ml Pam3 for 24h (A2) and for 35h (A4): Medium control (A1-A3) and in A549 cells after stimulation with 50 ng/ml IL-6 for 35h (B2): compared to medium control B1. (Magnification: All images at 200x and B1 at 400x).
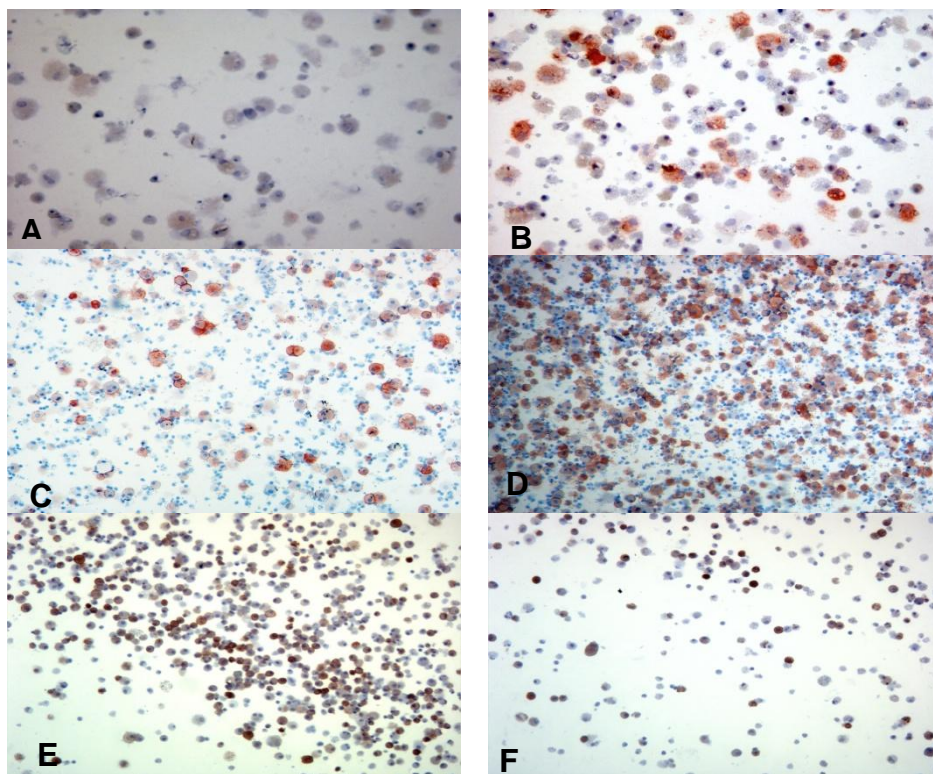
Figure 30:
Immunocytochemical detection of CD163 in BAL after stimulation with 50 ng/ml IL-6 for 24h (B). 50ng/ ml DEX for 24h (D) and 200 ng/ml of LPS for 24h (F). A, C and E are medium controls. (Magnification: All images at 400x except E and F at 200x).

3.3.3 Induction of pHp and CD163 in STST model with human pathogens (*Haemophilus influenzae, Streptococcus pneumoniae* and *Chlamydia pneumoniae*)

IHC results revealed expression of pHp and downregulation of its receptor in 10 specimens stimulated in STST model with each *Haemophilus influenzae* and *Streptococcus pneumoniae* after 24h but not necessarily its receptor with the latter. pHp and CD163 expression were demonstrated in AEC II and AM. Even stronger regulation of pHp and its receptor was observed upon *Chlamydia pneumoniae* infection after 24h of stimulation. Again, signals were located in AEC II and AM (Figure 31).
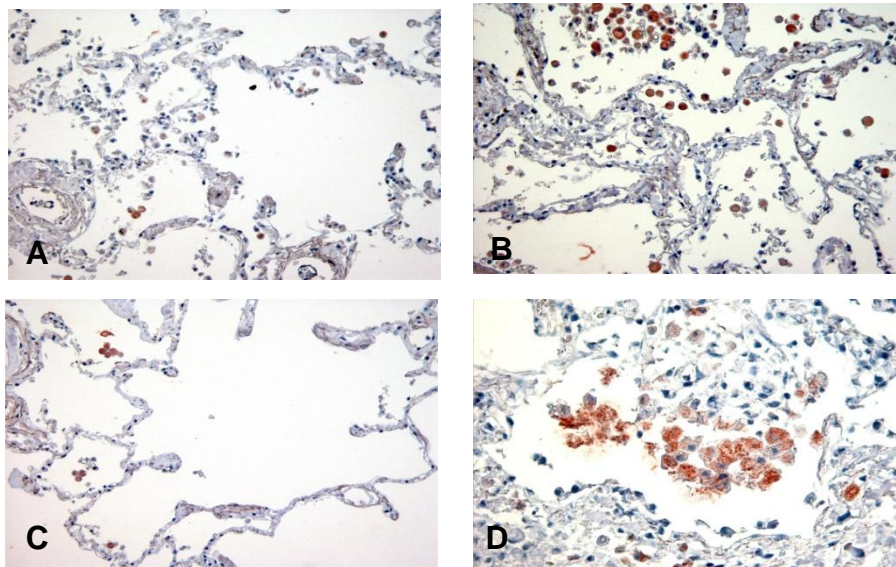
Figure 31:
Immunohistochemical detection of pHp in *Haemophilus influenza* (B) and *Chlamydia pneumoniae* (D) stimulated human lung tissues. (A and C) shows medium control. (Magnfication: Upper and lower panel show magnification of 200x).

3.3.4 Expression of pHp and CD163 in chronic diseases

To compare expression of pHp and its receptor within chronic diseases, eleven cases of each (COPD, sarcoidosis and TB) were analyzed as shown in figure 32. In case of COPD, the invading inflammatory cells, as well as the obstructed bronchi show strong pHp staining in A AEC II and AM. Additionally, CD163 is only expressed by the AM surrounding the obstruction and in no other cells or in the inflamed bronchi. Expression of pHp in sarcoidosis reveals weak expression in granuloma-forming cells, while CD163 is strongly expressed in these cells and in all macrophages surrounding the granulomatous lesion. In cases of tuberculosis, data showed strong expression of pHp and CD163 from AEC II and AM surrounding the granulomatous lesion.
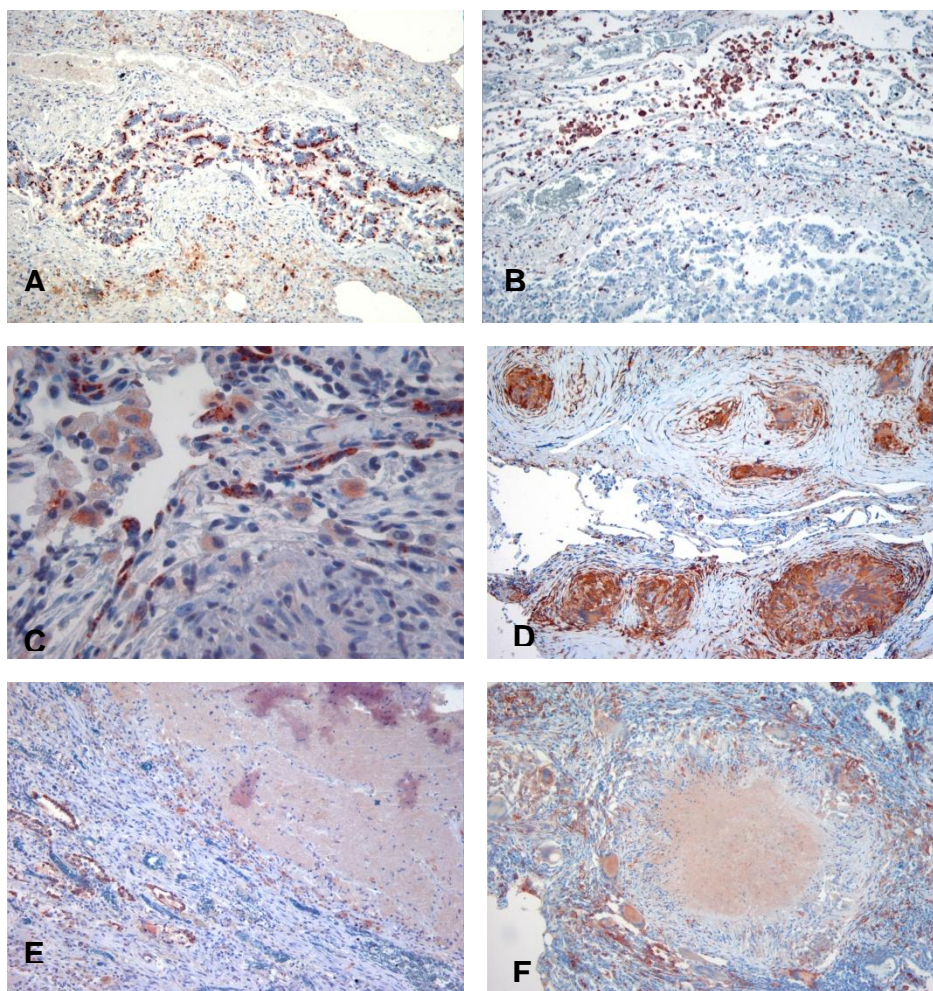
Figure 32:
Immunohistochimcal detection of pHp (A, C, E) and CD163 (B, D, F) in COPD (A and B), sarcoidosis (C and D) and tuberculosis (E and F). All three panels show a magnification of 200x and 400x (C), respectively.

3.3.5 Immediate secretion of pHp and CD163 from tissues and cells upon stimulation

For further analysis, the secretion of pHp and CD163 by tissues and cells into culture-supernatant was analyzed by immuno slot-blot. The total number of investigated cases and percentage of observed secretion in medium of tissue culture are displayed in figure 33 A. Just 10 min after incubation with LPS, IL-6, Pam3 and DEX, a slight increase of pHp protein in culture supernatant compared to medium control can be observed and this is dramatically increased after 24h. The results also show, that intensity of pHp protein in specimens which are not incubated in medium before culture is more than in specimens after culturing in medium for 3h. Therefore, pre-existing Hp needs to be washed away by incubation for 3h in medium before stimulation as shown in figure 34 and 35.

As for stimulation of cells, nine BAL samples were equally treated with inflammatory agents. After 24h of stimulation, only very weak signals can be observed, while stimulation for 35h showed stronger secretion of pHp as shown in figure 36.

Secretion of CD163 was induced by application of LPS, DEX, Pam3 and IL-6 tissue and BAL cell culture. Total numbers of investigated cases and observed secretions in percent are shown in figure 33 B. No secretion was observed in primary AEC II and A549 culture supernatants.

A   induction of pHp secretion

B   induction of CD163 secretion
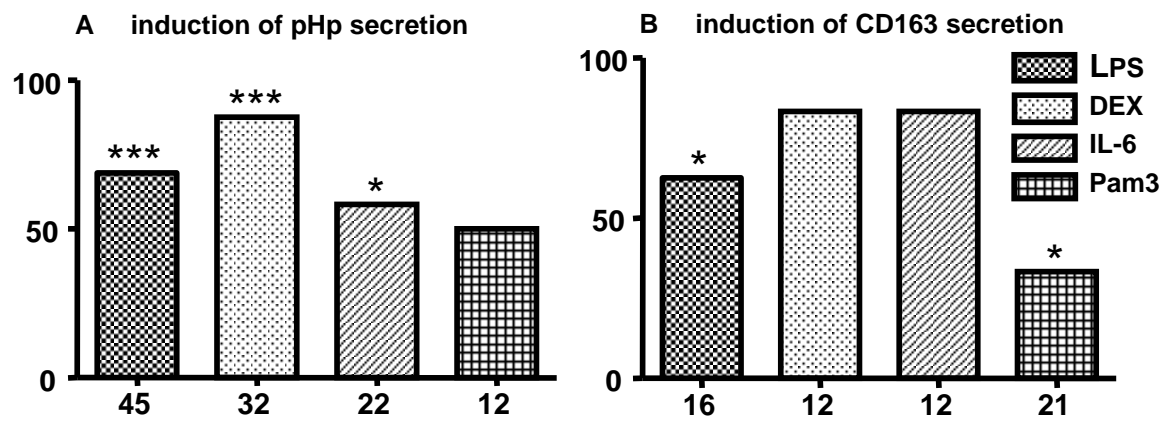
Figure 33:
Slot-blot analysis of supernatants for pHp (A) and CD163 (B) with different stimulated lung specimens by STST model. Y-axis: percentages of cases showing secretion. X-axis: total number of supernatants analysed with different types of stimuli (*p<0.05, **p<0.01, ***p<0.001).
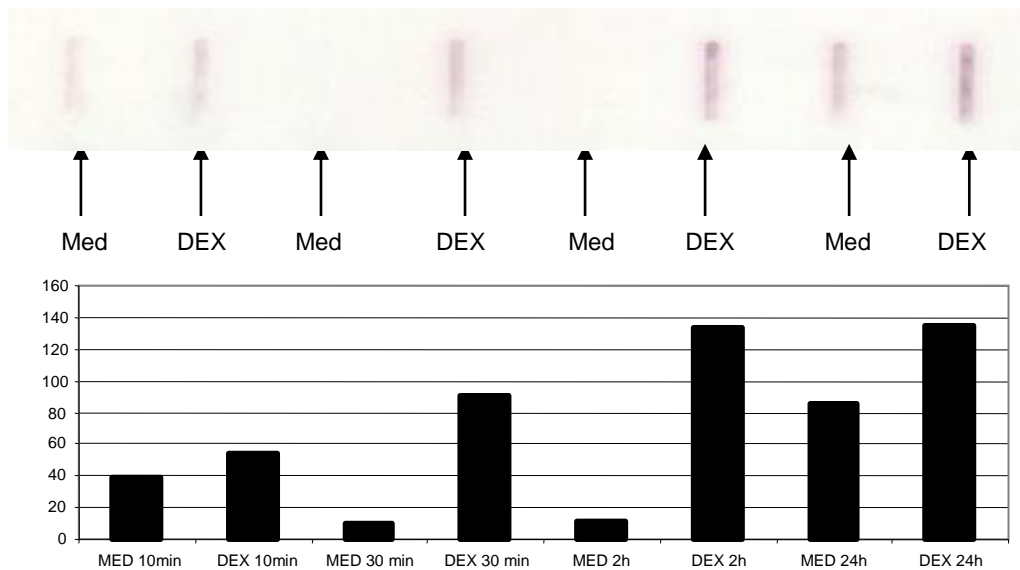
Figure 34:
Secretion of pHp with 50ng/ml DEX stimulated lung by STST model determined by slot-blot analysis. Upper panel: different bands of pHp upon DEX stimulation parallel to medium control at different incubation times. Lower panel represent Band Leader results. Specimens were incubated in medium for 3h before culture.
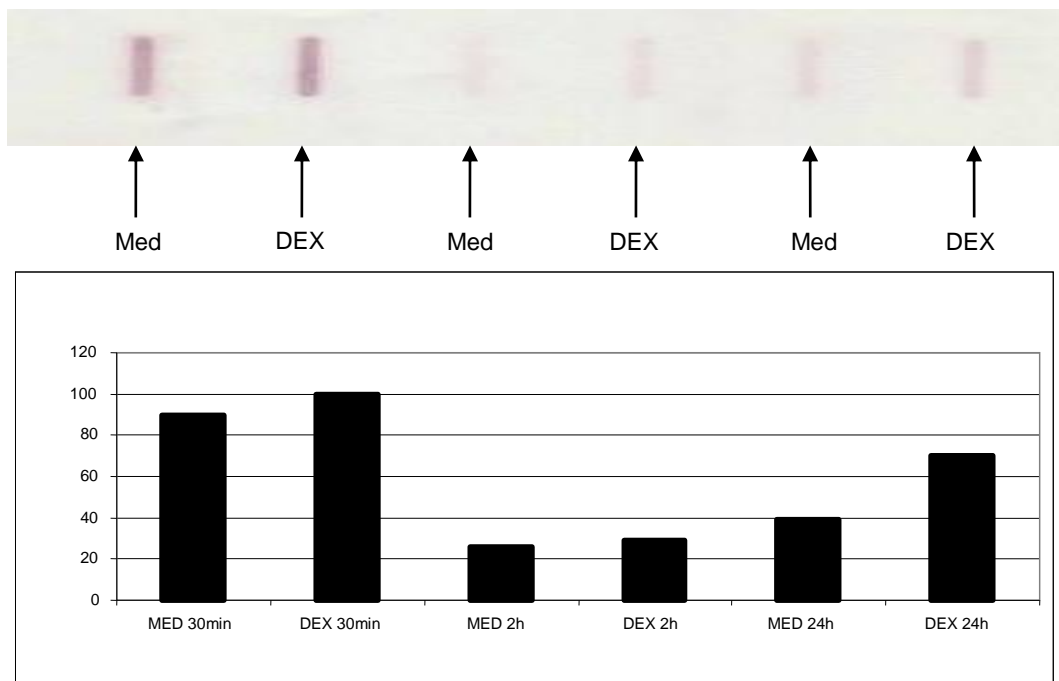
Figure 35:
Secretion of pHp with 50ng/ml DEX stimulated via STST model determined by slot blot analysis. Upper panel: different bands of pHp upon DEX stimulation parallel to medium control with different incubation times. Lower panel represent Band Leader results. Specimens not incubated before culture.
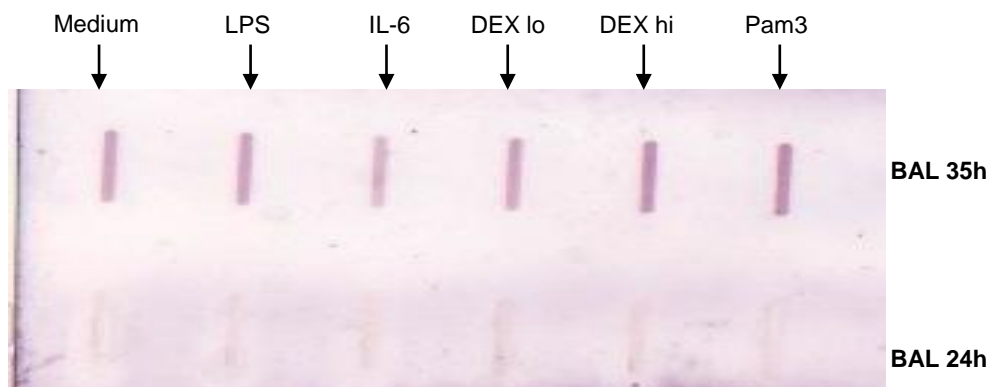
Figure 36:
pHp detection in supernatants of BAL via induction with different inflammatory stimuli. Upper band shows secretion after 35h of incubation and lower band for 24h of incubation.

3.3.6 Immunofluorescence microscopy shows secretion of pHp upon stimulation with LPS, Pam3, DEX and IL-6

Immunofluorescence microscopy of ICC-positive cytospin prepared from BAL cells and primary AEC II, reveals that pHp appears to be modulated by the inflammatory mediators LPS, Pam3, DEX, and IL-6. Vesicular signals close to the cell membranes can be observed as shown in figure 37. Expression and secretion in vesicles was clearer observed in HOPE-fixed material compared to formalin and signals were stronger after 35h compared to 24h of stimulation. Weak signals could also be detected in primary AEC II and A549 cells (data not shown).
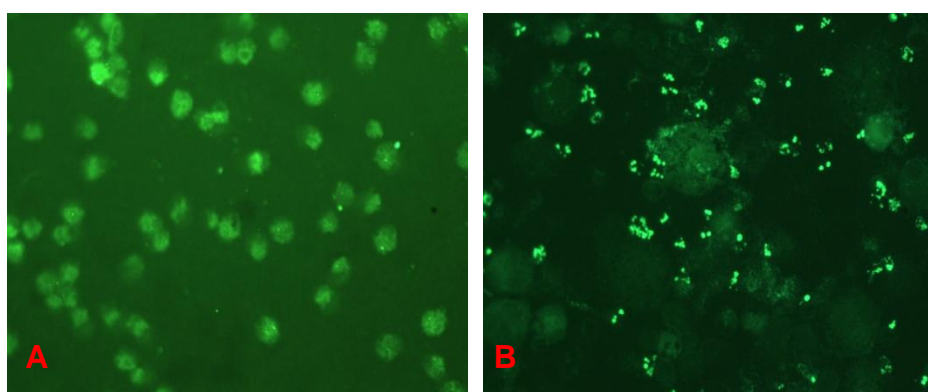
Figure 37:
Immunofluorescence detection of pHp in stimulated BAL cells (35h) showed secretion of pHp as vesicles near AM. A: Medium control and B: 50ng/ml DEX. Both figures show magnification of 400x.

3.3.7 Transcriptional regulation of pHp and CD163 in response to different stimuli.

To further verify the mRNA data of pHp and CD163, STST stimulated tissues were subjected to RT-PCR. The data showed constant amount of pHp mRNA levels in inflammatory response with different concentrations and incubation times. pHp mRNA can be detected after stimulation with DEX, LPS, IL-6 and Pam3 (data not shown). In Real-time PCR up-regulation by DEX, LPS, IL-6 and Pam3 was determined; hereby only DEX affected tissues in a dose- and time dependent manner (Figure 38A). Concerning LPS, transcription of pHp could only be observed with high concentrated LPS (200 ng/ml) stimulation after 24h. Furthermore, CD163 mRNA amounts vary dose-dependently according to RT-PCR data in DEX and IL-6-stimulated lung (IL-6 not shown) (Figure 38B). No

variation could be observed due to Pam3, but LPS caused a downregulation of CD163, which verifies the results of IHC (data not shown).
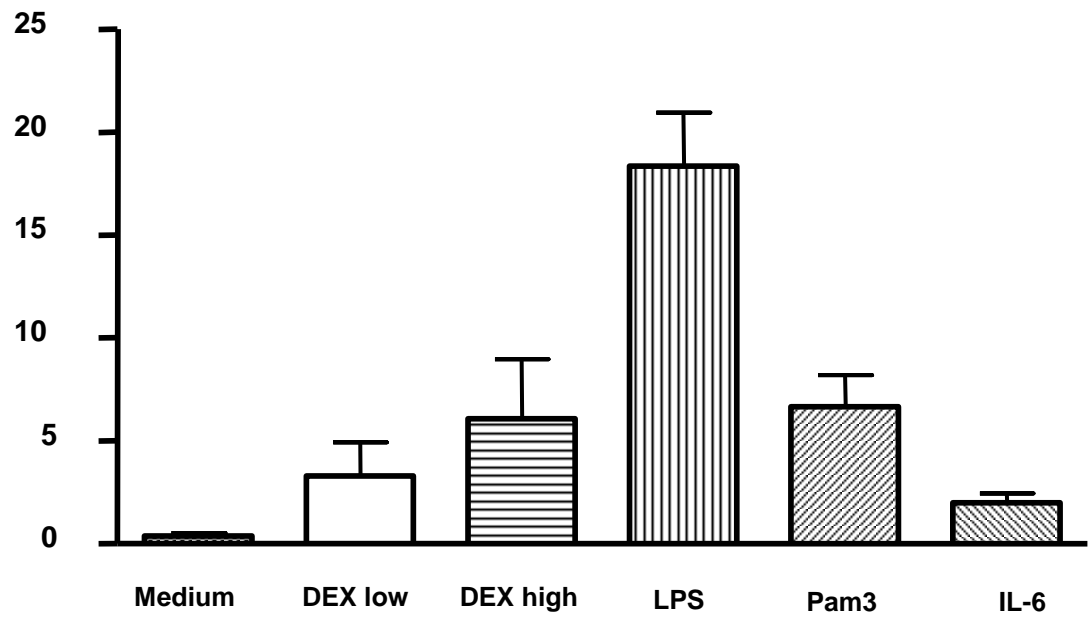
Figure 38 A:
pHp mRNA levels in STST model compared to medium control. The results are displayed as normalized ratio to intern calibrators and house-keeping gene GAPDH. The effects of inflammatory simuli are displayed on x-axis. All results are duplicates (N=2).
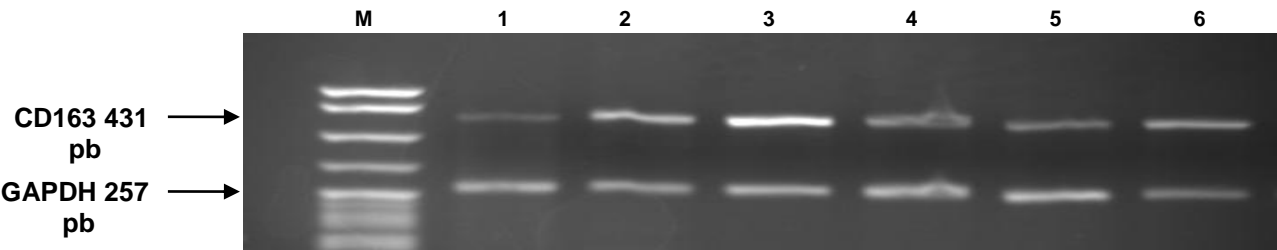
Figure 38 B:
RT-PCR with CD163 primer and cDNA from medium control tissue & stimulated tissues. Upper band: lane 1 and 4: Tumor free lung. Lane 2 and 5: 10ng/ml DEX. Lane 3 and 6: 50ng/ml. Lower band shows GAPDH. M= pBr322Msp1.

3.3.8 Transcriptional regulation of pHp and its receptor in response to infection and in chronic diseases.

Haemophilus influenzae and *Chlamydia pneumoniae*-infected tissues were analyzed for transcription of pHp. The results show constant amounts of pHp mRNA from infected and non-infected lungs by conventional RT-PCR (Figure 39 A, B). In conventional RT-PCR data shows downregulation of CD163 mRNA with *Haemophilus influenzae* compared to medium as shown in figure 40. Only the effects upon *Haemophilus influenzae* are displayed.
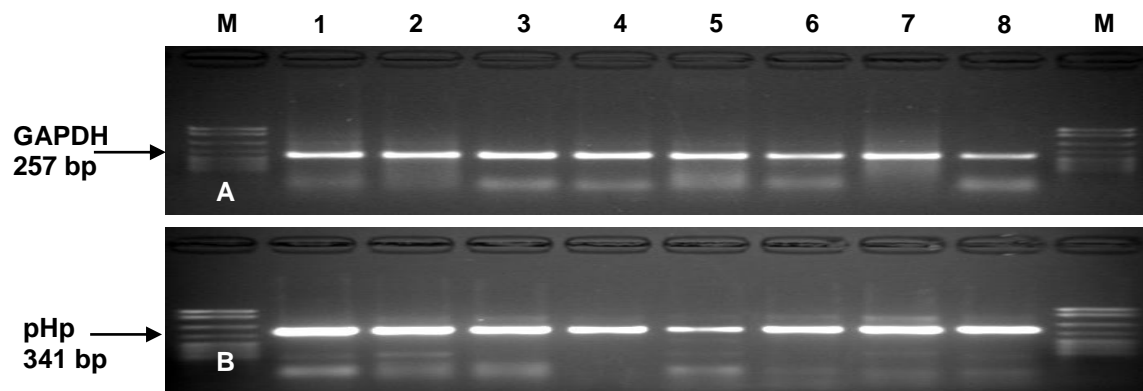
Figure 39:
RT-PCR with pHp (B) and GAPDH (A) primer with cDNA from medium control & stimulated lungs (*Heamophilus influenzae*). Lane 2, 4, 6, and 8: Infected lung. Lane 1, 3, 5, and 7: Medium control. M= pBr322Msp1
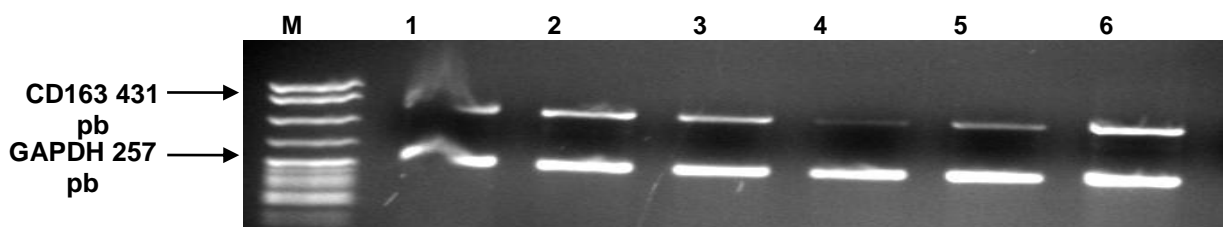
Figure 40:
RT-PCR with CD163 (Upper band) primer with cDNA from medium controls & stimulated lungs *(Haemophilus influenzae),* Lane 1, 3 and 6: medium control. Lane 2, 4 and 5: *(Haemophilus influenzae)*. Lower band GAPDH, M= pBr322Msp1

In Real-time PCR, pHp mRNA shows upregulation in chronic diseases in COPD and sarcodiosis compared to tumor free lung as shown in figure 41.

66
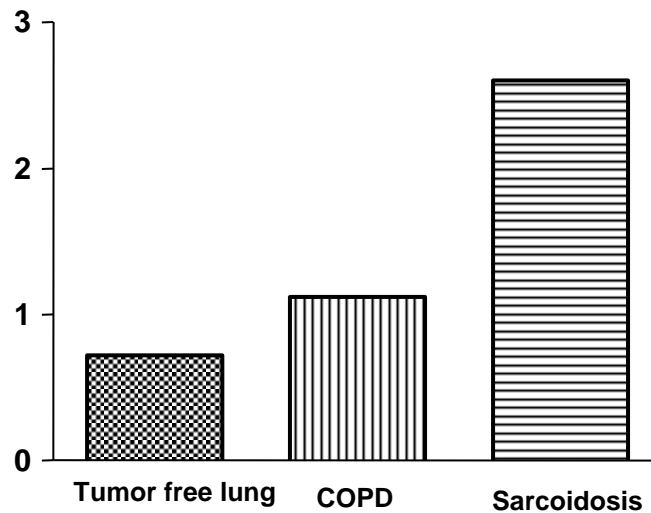
Figure 41:
pHp mRNA levels in chronic diseases compared to tumor free lung. The results are displayed as normalized ratio to intern calibrators and house-keeping gene GAPDH. X-axis represents COPD, sarcoidosis and tumor free lung (single experiments).

3.3.9 Localization of pHp mRNA in stimulated tissues

For further confirmation and localization of pHp mRNA in human lung, we additionally analyzed eleven single specimens in STST model stimulated with IL-6. In all specimens stimulated with IL-6, signals were generated within the AM and AEC II cells. Negative controls did not show any signals (Figure 42).
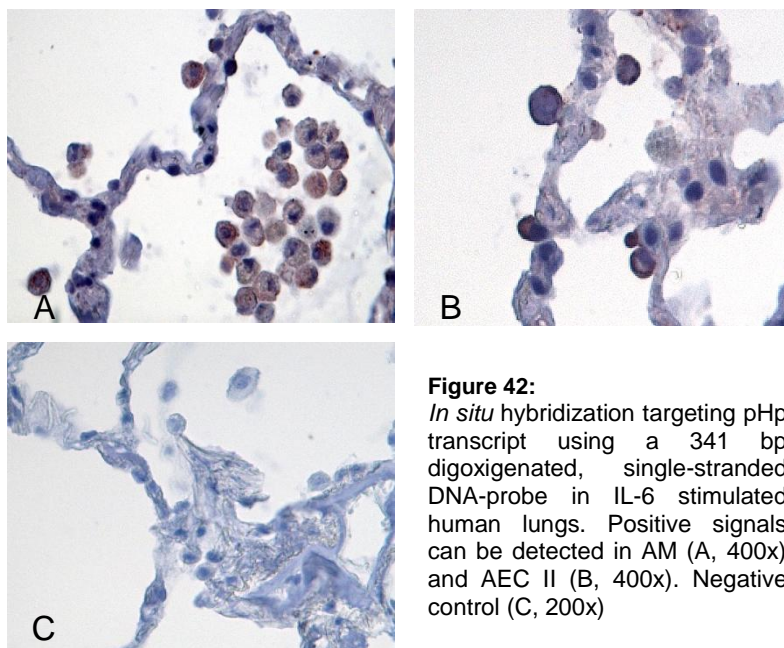
Figure 42:
In situ hybridization targeting pHp transcript using a 341 bp digoxigenated, single-stranded DNA-probe in IL-6 stimulated human lungs. Positive signals can be detected in AM (A, 400x) and AEC II (B, 400x). Negative control (C, 200x)

3.3.10 Effects of IL-6, LPS, Pam3 and DEX on the level of pHp protein and its receptor

Western blot analysis was performed to compare the levels of pHp-protein and CD163 from lysates and supernatants of stimulated tissues and cells. Constant levels of pHp protein can be found in lysates and supernatants of STST model. The molecular weight is around 160 kDa which represents the size of Hp 2-2 depending on non-reducing conditions. In cases of BAL signals in the supernatants have been observed after 35h of stimulation. Depending on the stimulus, constant variations of pHp can be seen (Figure 43). No positive signals can be displayed in supernatants of primary AEC II cells and A549 cells, respectively. Western blotting targeting CD163 in supernatants of BAL culture and cell lysates revealed upregulation due to DEX and IL-6 stimulation and downregulation with LPS (data not shown).

Figure 43:
Western blot analysis from supernatants of stimulated BAL cells with different agents. pHp signal represent 2-2 according to molecular weight. Lane 1: medium control. Lane 2: 10ng/ml LPS. Lane 3: 200ng/ml LPS. Lane 4: 10ng/ml DEX. Lane 5: 50ng/ml DEX. Lane 6: IL-6 50ng/ml and lane 7: 200ng/ml Pam3. M=Novex Sharp Prestained Molecular Weight Marker

3.3.11 Chemokine production by A549 cells in response to LPS

After cultivation of A549 cells and stimulation with LPS and pure Hp protein, the concentration of two important chemoattractant cytokines, MCP-1 and IL-8 was measured in the supernatants. When A549 cells were cultured in medium alone, concentration of IL-8 and MCP-1 was 1272 pg/ml and 418 pg/ml. Addition of 200 ng/ml LPS increased both concentrations to 3713 pg/ml and 2219 pg/ml. Addition of 150 ng/ml pure haptoglobin protein elevated the concentrations of IL-8 and MCP-1 finally around 10-fold, reaching 35275 pg/ml and 84604 pg/ml as shown in figure 44A and B.
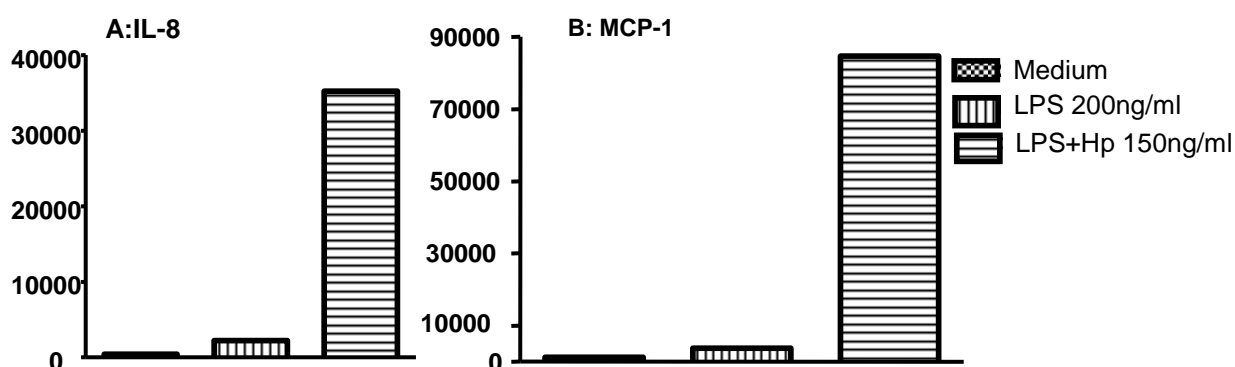
Figure 44:
Concentration of IL-8 (A) and MCP-1 (B) in supernatants of A549 cells stimulated with 200 ng/ml LPS and 150ng/ml of Hp protein.Y-axis represents concentration of cytokines (pg/ml). X-axis displays stimulation.

3.3.12 Co-localization of pHp with SP-B in AEC II and A549 cells

Simultaneously addressed sub-cellular localization of pHp protein and SP-B by indirect immunofluorescence staining and confocal laser microscopy showed partial co-localization pHp and SP-B in A549 cells. The signals appear as distinct points (yellow signals) within the cytoplasm of IL-6 stimulated A549 as shown in figure 45 A. Furthermore, we demonstrate a complete co-localization of pHp in AEC II of tissue sections stimulated with IL-6 as shown in figure 45B.

Figure 45 A:
Partial co-localisation of pHp protein and SP-B. Red channel refers to pHp (A) and green channel to SP-B (B). Overlay of A and B shows yellow signals within cytoplasmic apartments of IL-6 stimulated A549 cells (C).

Figure 45 B:
Co-localisation of pHp protein and SP-B in AEC II. Red channel refers to pHp expression (A) and green channel to SP-B expression (B). Overlay shows yellow signals within cytoplasmic apartments of AEC II (C).

4. Discussion

4.1 pHp in lung cancer

Immunosuppression can be a major problem in cancer, impairing immune defenses against the tumor, vitiating attempts at immunotherapy, and predisposing the patients to infection. The nature and mechanism of immunosuppression are not fully understood. Specific unresponsiveness to tumor-associated antigens has been reported in a number of experimental systems [100]. In addition, non-specific suppression of a broad spectrum of immune responses may occur. Correlated with the latter phenomenon is the presence of nonspecific immunosuppressive factors in sera and ascites fluids of cancer patients [14, 98, 104].

Apffel and Peters [7] who first proposed that carbohydrate-rich plasma proteins produced by the liver, called "symbodies", localize on the surface of cancer cells and enable them to escape the immunologic attack of the host; these carbohydrates-rich plasma proteins which modulate immune function, are now identified as APRs. It has been unclear, whether immunosuppressive factors associated with cancer are a unique product of tumor cells, or whether they resemble substances found in other conditions and perhaps represent a normal feedback mechanism that has been deranged during the development of tumors. To address these questions, we conducted the analysis of the expression of the APP pHp in human lung cancer. This study showed that pHp is strongly expressed and synthesized in lung cancer, with expression levels that reach the expression levels found in the liver. Considering the immunoactive properties of pHp, it is conceivable that tumors produce Hp either innately or by inducing other parts of the body systems, such as the liver [15]. The expression of pHp in human lung tissues is observed for the first time and cellular localization observable by cytoplasmic granular staining in adenocarcinomas while in squamous cell carcinomas negligible expression of pHp was observed in the tumor cells. Therefore, pHp could be used as a diagnostic marker for the sub-differentiation of lung cancers. Among healthy cells, AEC II as well as epithelial cells of airways (bronchi and bronchioles) were frequently stained with high intensities. According to this, it is possible that Hp as an APP is involved in a variety of immunoreactive processes. The variation of staining between adenocarcinomas and squamous cell carcinomas could be due to the different cell types from which the carcinomas derive.

Different haptoglobin phenotypes or Hp polymorphisms could be associated with different tumor types. Numerous studies showed that Hp1-1 and Hp2-1 are more common than Hp2-2 [10, 12, 18] and that the geographical distribution is important due to differences in gene sequence concerning Hp, such as the absence of Hp in Caucasian people or the low Hp1 allele frequency in Southeast Asia [78]. This leads to the speculation that most cases of adenocarcinomas might belong to Hp 1-1 or 2-1, while squamous or small cell carcinomas might belong to a patient carrying Hp 2-2. Beckman et al [18] reported that a group of patients with squamous cell carcinomas and small cell carcinomas showed no significant difference in Hp compared to a control group, which is in agreement with this study. In patients with pulmonary adenocarcinomas, the frequency of Hp 2-2 is lower compared to the control group, with a corresponding increase in the Hp 1-1 and Hp 2-1 phenotype [18, 29]. Further studies are necessary to understand the relations between Hp phenotypes production of tissues and correlating cancer types. Another recent study showed that Hp serves as a natural inhibitor of collagen degradation, is locally expressed by fibroblasts in the arterial wall [28] and is involved in cell migration processes and arterial restructuring such as angiogenesis. Moreover, collagen turnover is an important phenomenon in many of pathological conditions, like tumor cell invasion, wound healing, cell growth, metastasis, and enhancement of collagen degradation. This is related to a severe tissue destruction and malfunction, which is often encountered in pathological processes, such as arthritis and metastasis.

Thus, the high expression of pHp in adenocarcinomas may be correlated with their comparably high potential to metastasize. This reveals a possible role of pHp within functions of the extracellular matrix, during cell migration and suggests another role for this protein in cancer [70, 132, 152]. In general, our results and above mentioned studies which showed that increased levels of Hp synthesis appear during lung cancer genesis, and that higher amounts of Hp can be observed in the blood plasma of those patients, suggested that Hp could be a potential serum tumor biomarker for lung cancers. We are in accordance with this presumption due to our finding of Hp in the tumor itself. The increase in Hp expression in tissues and serum may enable to invasion and surveillance mechanisms of tumor cells [14, 54, 71, 127]. It has been shown that Hp possess a wide distribution of receptors in the surface of immune and inflammatory cells and

interference with functions of these cells [13, 102, 103, 104]. Therefore, via these receptors Hp may act as a natural antagonist for receptor ligand activation of the immune system.

Arredouani et al [8] further described the role of Hp affecting the immune system, showing that Hp directly affects T-cells and suppresses T-helper-cells cytokine production and associated mechanisms. However, it is not clear whether this is an indirect phenomenon caused by modulation of antigen-presenting cell function or whether Hp directly acts on T cells [8]. Furthermore, Seder et al [127] showed that passive removal of Hp out of the plasma of cancer patients restores the immune response at least temporarily. Another important finding related to extra hepatic Hp, is that this APP differs from normal liver Hp which is more glycosylated. Numerous studies have demonstrated an increase in the serum level of Hp, alteration in the degree and pattern of glycan adducts [6, 57, 73]. It is likely that these changes may encompass host defense as well as tumor promoting mechanisms. Evidence favoring a host defense mechanism is provided by a study demonstrating pro-apoptotic activity of Hp toward hepatocarcinoma cells in culture [69]. The importance of the additional group determinant in cell adhesion suggests that abnormal Hp glycosylation could interfere with tumor invasion through binding endothelial selectins, thus supporting metastasis [66, 71, 140], while an increase of siasylation group protects tumor cells from host response, thus conferring a degree of immunity [57]. More studies are needed to confirm these findings in lung cancer tissues.

4.2 Regulation of pHp and CD163 upon inflammatory agents and glucocorticoids

A generally strong anti-inflammatory environment is necessary in the lung, as the lung is the organ with the highest exposure to the atmosphere and a large number of potential injuries from chemical agents, microorganisms, organic, and inorganic dusts. It is not surprising that a variety of intra-and extra-cellular antioxidant defenses are advantageous for the lung, so if antioxidants were not synthesized in high and significant quantity in the lung, it would not doubt undergo a marked inflammatory response.

However, it could be too slow for the lung to wait for expression of Hp from other organs; therefore, a fast mechanism for removal of harmful pro-inflammatory effect of Hb is needed. It has been generally considered that the extracellular antioxidant

activity identified with the serum proteins such as ceruloplasmin, transferrin, and Hp is produced primarily in the liver and the presence of these proteins in Bronchoalveolar lavage fluids has been assumed to be likely derived from the serum, although contributions may be made by the serum [158].

The current study performed the induction and regulation of pHp with its receptor in a human *ex vivo* tissue culture model, AEC II, A549 cells and BAL cells with inflammatory agents, inflammatory cytokine and glucocorticoids.

This study leaves no doubt that pHp is synthesized and regulated as a pulmonary source pHp from primary AEC II, AM and bronchi. This is in agreement with Yang et al [158], [159] who found that the airway epithelium is a major site of Hp gene expression in baboons and mice during inflammation. In humans Hp was not expressed in normal healthy lung (in a very small n of 6), but got activated in AM during allergy and eosinophily. The roles of Hp in healthy lung and lung defense have not been elucidated. Results from this study indicate that a major pathway for the removal of Hb in the lung involves the formation of Hp-Hb complexes, because both of Hp and its receptor are expressed in majority of human lung specimens associated with hemorrhage. Numerous pathologic conditions are associated with lung injuries either due to infection or lung cancer; therefore, red blood cells can be present in the lower respiratory tract, resulting in the release of Hb and reactive iron. These products potentially participate in oxidant generation and contribute to injury [60, 161]. Ghio et al. [46] have shown that intratracheal instillation of whole blood in the rat can induce a neutrophilic lung injury with a marked increase in both TNF-α and Macrophage Inflammatory Protein -2 (MIP-2).

As a major APP, Hp has been shown to have several biologic functions including a role in Hb catabolism [30, 50, 160], so an elevated level of Hp and its receptor observed as local expression at site of inflammation or due to induction via inflammatory agents or infection might be a pathway for elimination pro-inflammatory oxidative agents either from lung cells itself as we have shown in our present and other studies [139]. It was demonstrated that exogenous Hp is taken up and stored in monocytes, neutrophils and macrophages within a cytoplasmic granular compartment and is secreted by these cells during the phagocytosis of *candida albicans* [21, 139, 148]. This might be indicating that Hp protein levels are enhanced locally at the sites of inflammation or due to induction by stimuli to modulate the activity of granulocytes.

Our results show that some of the inflammatory cells like neutrophils, monocytes and lymphocytes have signals for Hp with IHC staining as well as macrophages, but not eosinophiles. This is in disagreement with Yang et al. who have shown that Hp is been transcribed in eosinophiles of patients suffering from allergy [159]. In addition, it has been reported that Hp suppresses macrophage function such as LPS induced production of TNF-α, proliferation, cytokine production by T cells, and proliferation of B cells. Hp has further been shown to bind to mono- and poly-nuclear cells through CD11/CD18, CD22 and other yet undefined surface receptors. Therefore, via wide distribution of receptors Hp can modulate function of these cells and cause endocytosis of Hp, with exerting its immunomodulatory and anti-inflammatory effects through receptors mediated signaling [8, 9, 13, 15, 37, 51].

pHp and its receptor showed strong regulation in human lung upon stimulation in the STST model which was confirmed in BAL, AEC II and A549 cells. The synthesis of Hp is affected by many factors and the regulation of Hp is varying upon species and cells. For example, IL-6, IL-1, TNF-α and glucocorticoids can up-regulate the synthesis of Hp in bovine hepatic cell lines [162]. Whereas in rats the Hp gene was regulated by IL-6, IL-1, dexamethasone and TNF-α, while in humans Hp was regulated by IL-6 and TNF-α, which is in agreement with our results. IL-6 however, is the most common inflammatory cytokine mediator for Hp gene regulation in all species studied [44, 141, 155].

pHp is significantly regulated in AM and AEC II with inflammatory stimuli in IHC, Hp expression increases approximately three to six fold in the plasma of mice injected with LPS and regulation of Hp response to inflammation has been carefully studied in vitro hepatoma cells [88, 106]. This depends on an interaction between specific DNA sequences and the nuclear transcription factors, C/EBP ß (formally known as the IL-6 binding protein). There is a sequence in the 5 region of the regulatory region in the human Hp gene, that can direct synthesis of a heterologous gene Hp in the lung, as reported by D Amiento et al [27].

There was slight expression of pHp in non-stimulated BAL (sarcoidosis, chronic eosinophilic pneumonia, neutrophilic alveolitis and bronchiectasis). But, upon stimulation, even the healthy cases showed remarkable upregulation of pHp in BAL after inflammatory induction.

A mechanism, which is responsible for the transport of the high molecular weight Hp-Hb complexes from the respiratory tract to circulation, is yet to be established.

In this study, we have shown that CD163, which was recently identified as an Hp-Hb scavenging receptor [72] is mainly expressed in AM from tissue sections associated with hemorrhage and strongly regulated in the STST model. The abundance of pHp and CD163 expression in lung specimens associated with hemorrhage is higher compared to normal lungs. CD163 has a high affinity for Hp-Hb complexes but does not bind to Hp or Hb alone. Endocytosis of Hp-Hb complexes by AM could be the major pathway responsible for the removal of toxic and pro-inflammatory Hb from the circulation and from sites of local tissue destruction after trauma, infection or during inflammation. Increased production of pHp and CD163 in AM induced by the same agents could significantly contribute to the clearance of Hb and thus protect the lower respiratory tract against Hb-mediated oxidative damage. This is in agreement with [119] who found the presence of multiple glucocorticoid response elements in the proximal promoter region of the human CD163 gene as well as several consensus binding sites in for the STAT signaling pathway. In contrast to anti-inflammatory stimuli, pro-inflammatory mediators like LPS decrease CD163 mRNA levels due to the absence of consensus binding sites for the Rel protein (relish protein, discovered in Drosophila), located in the proximal promoter sequence of CD163 [118]. There is evidence that a metabolic pathway as interaction between Hp and CD163 regulates inflammation at least by two ways. First, CD163 is reported to directly induce intracellular signaling leading to the secretion of anti-inflammatory cytokines. Secondly, and perhaps even more important, the CD163-mediated delivery of hemoglobin to the macrophage may fuel an anti-inflammatory response, because heme metabolites have potent anti-inflammatory effects[23, 94]. Besides its role in scavenging Hp-Hb complexes, several studies of CD163 on regulation and function have shown that CD163 is an anti-inflammatory element. In vitro studies on CD163 regulation in cultured monocytes and macrophages have shown that CD163 is tightly regulated by pro-and anti-inflammatory mediators [23, 94,125], which is in good agreements with this study.

Here, for the first time, immunohistochemical techniques were used to detect pHp and CD163 in human lungs. High expression of CD163 have already been

observed in macrophages of inflamed tissues such as atherosclerotic lesions, inflamed joints and systemic inflammation [43, 112, 116]. It is likely that the elevated levels of pHp and its receptor especially in the vicinity of AM, promote a direct role as anti-inflammatory elements.

4.3 pHp is a secretory protein

Hp is known as a secretory protein [16] which is in agreement with this study as expectable. pHp is secreted from vesicles by AM under inflammatory conditions shown in cytospins of BAL. pHp was also detected in supernatants of BAL cultures and in ex vivo tissue culture. This secretion happened shortly in a time- and dose-dependent manner, which strongly indicates a fast innate immune response of pHp against inflammatory stimuli.

Transcription of pHp mRNA and IHC results differ slightly for low-dose of LPS (10, and 50 ng/ml) stimulation, because time- and dose-dependence has only been observed in IHC. LPS and DEX both possess specific responsive elements in promoter region of human gene [44, 125]. High amounts of transcription of pHp mRNA were recorded upon LPS 200 ng/ml at 24h, which is in good agreement with IHC and slot blots that also showed fast secretion in dose- and time-dependent manner. This can be explained by secretion of pre-existing Hp protein [21, 148]. pHp secretion starts after 10 min of stimulation and reaches its maximum after 24h, when transcription starts to be also induced.

However, the levels of CD163 mRNA show a downregulation upon LPS, but high protein levels in the supernatants. This can be explained by LPS causing a shedding of CD163 from the surface of the macrophages, which known as a soluble form of hemoglobin scavenger receptor (sHbSR) [32]. Increased levels of this receptor are recorded in patients with myelo-monocytic leukemia and infection [95].

4.4 pHp and its receptor in infection and chronic diseases

This study, and above mentioned studies demonstrate that pHp and its receptor contribute to the reestablishment of homeostasis after local or systemic infection by propagating various anti-inflammatory activities and participate important roles in chronic diseases via high expression in protein and mRNA level.

We can speculate that they also might be responsible to maintain chronic diseases in chronic condition, which could be the case in COPD, tuberculosis and sarcoidosis.

Both pHp and CD163 are known to serve as immunomodulators during inflammation with elevated levels in the healing phase of acute inflammatory reactions, in chronic inflammatory diseases, during wound healing processes and systemic vasculitis because of their ability to stimulate tissue repair and to compensate for ischemia by promoting the growth of collateral vessels [80]. This suggests a functional role for those glycoproteins in the downregulation of inflammatory processes [125, 165].

pHp and CD163 deliver anti-bacterial effects through depleting Hb, which is the main source for bacteria requirement. In a recent study it was shown that the deletion of a set of three hemoglobin haptoglobin binding protein (hgp) genes from a nontypeable strain of *H. influenzae* attenuated virulence in the chinchilla otitis media model of noninvasive disease [97].

4.5 pHp possesses chemoattractant effects

The enhanced production of the APP pHp in response to inflammatory stimuli suggests that it might augment the effector limb of the acute inflammatory response by alteration in the level of chemokines.

The significant role of pHp in the secretion of potent chemoattractant chemokines in A549 cells stimulated with pro-inflammatory cytokines like LPS strongly indicates a role of pHp as a chemoattractant. This is in agreement with studies which observed that pro-inflammatory cytokines such as TNF-α induce AEC II to produce chemoattractants that could provoke the trans endothelial migration of CD3+ lymphocytes and CD14+ monocytes [36, 84]. Furthermore, numerous studies showed effective role of chemoattractants on AEC II, A549-cells, and in rat and human lungs [75, 109, 137]. Our results prove for the first time that the effect of pHp in human alveolar epithelial cells contribute to development of the local inflammatory response by increased production of major chemoattractant cytokines (MCP-1 and IL-8), which are responsible for attracting monocytes, lymphocytes and polymorphonuclear cells.

Furthermore, we co-localized pHp with SP-B which is known as a protein expressed from in AEC II as part of the surfactant-system. SP-B is stored in the

cells and secreted into the alveolar space [147]. Recently, it has been shown that SP-B contributes to the anti-inflammatory properties of surfactant [90].

In conclusion, our data show for the first time the expression of pHp in lung cancer tissues, especially in adenocarcinomas. The ability of pHp to affect immune responses could play an important role in lung carcinogenesis, including tumor escape from immunological attack. We have identified pHp and its receptor, synthesized secreted and regulated in the human *ex vivo* tissue culture model STST. The results were confirmed in AEC II, A549 cells and BAL cells. Moreover, the study showed that A549 cells secrete the chemoattractant cytokines MCP-1 and IL-8 in response to pure Hp protein.

Taken together, we show that pHp and its receptor are broadly expressed in the human lung. Both molecules are highly inducible on the protein- and mRNA-level, they are secreted, and pHp was co-localized with SP-B. These results strongly support a role of these molecules within the host defense of the human lung.

5. Abstract

This work focuses on the analysis of pulmonary haptoglobin and its receptor CD163 in humans. The knowledge about human pulmonary haptoglobin available to date is very limited.

First, we show that pulmonary haptoglobin exists and that this molecule gets constantly synthesized in the human lung by alveolar epithelial cells type II, alveolar macrophages, and bronchial epithelia with expression levels comparable to the liver. In lung cancer, we show that pulmonary haptoglobin is frequently expressed by adenocarcinomas-cells, but it is nearly absent in squamous cell carcinomas and small cell lung cancer. Therefore, pulmonary haptoglobin can be used as a diagnostic marker for sub-differentiation of lung cancer. In chronic infections of the human lungs (chronic obstructive pulmonary disease, tuberculosis, sarcoidosis), we found high levels of pulmonary haptoglobin and CD163 suggesting a functional role for these molecules in the course of these diseases.

Secondly, the regulation of pulmonary haptoglobin and its receptor CD163 was broadly analyzed using different model systems. We found that pulmonary

haptoglobin is constantly upregulated upon stimulation with lipopolysaccharide, lipopeptide, dexamethasone, interleukin-6, *Haemophilus influenzae, Streptococcus pneumoniae* and *Chlamydia pneumoniae* in human lung tissues, A549 cells and bronchoalveolar-lavages. The regulation of CD163 behaves like pulmonary haptoglobin, except that CD163 is downregulated by lipopolysaccharide due to shedding of this receptor from the cell surface. These regulation experiments were performed on the protein- and the mRNA-level with consistent data in a large scaled study (n=942).

Third, it is demonstrated that pulmonary haptoglobin is secreted by human lung tissues and cells. This secretion is regulated by lipopolysaccharide, lipopeptide, dexamethasone, and interleukin-6. Moreover, the secretion of pulmonary haptoglobin is a comparably fast mechanism, which is a strong hint that pulmonary haptoglobin is an independent local immunomodulator.

Fourth, to understand the function of pulmonary haptoglobin as an immunomodulator, we used haptoglobin-protein as a stimulus in A549 cells and show that this leads to elevated release of interleukin-8 and monocyte chemoattractant protein-1.

Finally we show a cellular co-localization of pulmonary haptoglobin and surfactant protein b under application of confocal microscopy.

These results for the first time prove a role of pulmonary haptoglobin and CD 163 in the immunodefense of the human lungs. This knowledge might lead to the development of new therapeutic regimens, especially for chronic disorders, in the future.

6. References

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7. Appendix (Reagents and buffers)

7.1 Reagents for IHC

Antibody diluent   (Zytomed, Berlin, Germany)

Acetate buffer pH 5.2   A-(Glacial acetic acid 0.1 M) B-(sodium acetate, trihydrate 0.1M Merck, Germany), 21 ml from solution (A) +79 ml solution (B) pH 5.2 + 900 ml aqua dest

Citric buffer PH 6.8   A-(Citric acid monohydrate 0.1M) B-Tris sodium citrate dihydrate 0.1M Merck, Germany) 18 ml from (A) +82 ml from (B) pH 6.8+ 900 ml aqua dest

0.05% Tween 20   (Sigma, Chemie, GmbH, Germany)

HRP polymer kit plus   (Zytomed System, Berlin, Germany)

Tris buffer PH 7.6 1M Trishydroxyaminomethyle (Merck, Germany)

Xylene   (J.T Baker, Netherlands)

Isopropanol   (Merck, Germany)

Acetone   (J.T Baker, Netherlands)

Ethanol   (Merck, Germany)

Mayer's hemalum   (Merck, Germany)

Kaysers glycerin gelatin   (Merck, Germany)

0.3% H2O2   (Merck, Germany)

Aqueous mount   (Zytomed, Berlin, Germany)

3-Amino9-Ethyl-Carbazole (AEC) 160mg/10ml DMF (sigma, Chemie, GmbH, Germany)

Dimethylformamid   (Merck, Germany)

Primary antibody: (Mouse anti-human; monoclonal anti-CD163 antibody; clone 10D6, dilution 1:100; Diagnostic BioSystems, Netherlands)

Primary antibody: (Mouse anti-human; monoclonal anti-haptoglobin antibody; clone HG-36, dilution 1:100; Abcam, U.K)

7.2 Reagents for western blot and slot blot

Running buffer (Laemmli) (50 mM Tris; 384 mM Glycine; 0.1 % SDS)

SDS-PAGE 4 % stacking gel (0.5 M Tris/HCl pH 6.8); 12 % resolving gel (1.5 M Tris/HCl pH 8. 8)

Reducing buffer (200 mM Tris/HCl pH 8.0; 20 mM EDTA; 25 % Glycerin; 2 % SDS ;1 % DTT; 0. 02 % Bromphenolblue

Ammoniumpersulphate 10%   (Bio-Rad, Hercuber, Germany)

Acrylamide   (Carl Roth, GmbH, Kalsruhe, Germany)

Sodium-Dodecyl-Sulphate (SDS) 10 % (Carl Roth, GmbH, Karlsruhe, Germany)

NBT/BCIP (NitroBlueTetrazolium chloride/

5-Bromo-4-chloro-3-indolyl phosphate)   (Boehringer, Mannheim, Germany)

Temed   (Carl Roth GmbH, Karlsruhe, Germany)

Tris-buffer saline pH 7.4

Tris-buffer saline pH 9.5

Roche blocking buffer 5%   (Boehringer Mannheim, Germany)

lysis buffer (7 M urea; 2 M thiourea; 4 % CHAPS; 2 % IGEPAL; 1 % Triton-X; 100 mM DTT; 5 mM PMSF; 0.5 mM Tris)

Urea   (Carl Roth GmbH, Kalsruhe, Germany)

LDS   (Invitrogen, Germany)

IGEPAL   (Sigma, St. Louis, USA)

Triton x   (Merck, Germany)

Dithiotreitol DTT   (Carl Roth GmbH, Kalsruhe, Germany)

Thiourea   (Carl Roth GmbH, Kalsruhe, Germany)

PMSF   (Carl Roth GmbH, Kalsruhe, Germany)

Bromphenolblue   (Merck, Germany)

Xylene   (J.T Baker, Netherlands)

Ethanol   (Merck, Germany)

Standard protein marker   (Novex Sharp, Invitrogen, Germany)

Coomassie (Bradford) Protein Assay Kit.   (PIERCE, Rockford, USA)

Secondary antibody (goat-anti-mouse, AP-labeled, absorbed against human, Dianova, Hamburg, Germany)

Secondary antibody (goat-anti-rabbit, AP-labeled, absorbed against human, Dianova, Hamburg, Germany)

7.3 Reagents for RT-PCR

RNeasy Mini kit	(Qiagen, Germany).
DNase I	(Invitrogen, Germany)
DNase running buffer 10x	(Invitrogen, Germany)
SuperScript TM II Reverse Transcriptase	(Invitrogen, Germany)
Tag DNA polymerase	(Invitrogen, Germany)
Oligo- dT^{15} primers	(Invitrogen, Germany)
First strand buffer 5x	(Invitrogen, Germany)
DTT	(Invitrogen, Germany)
Human haptoglobin primers	
Glyceraldehde-3-phosphate-dehydrogenase GAPDH primer	
Human CD163 primers	
pBr322-*Msp*1	(NewEnglandBiolabs, NJ, USA)
Magnesium chloride MgCL2	(Invitrogen, Germany)
dNTPs (10mM dATP, dGTP, dCTP, dTTP)	(Invitrogen, Germany)
EDTA25mM	(Invitrogen, Germany)
PCR running buffer 10x	(Invitrogen, Germany)
2.4 – 2.8% agarose gel	(Invitrogen, Germany)
Ethidium bromide solution	(Invitrogen, Germany

Running buffer TAE1x (Tris–base 40mM; 0. 5 M EDTA pH 8; glacial acetic acid pH 7. 5).

DNA loading buffer Bromophenol blue A-(stock solution) 20ml Glycerol 99% 25 ml 1xTAE buffer and 0. 4 gram bromophenol blue)

B-(Work solution)   (15ml 5xTAE buffer + 1ml stock solution)

DNA loading buffer xylene cyanol A-(stock solution) 0.025 g xylene cyanol+ 3ml Glycerol 99% add to 10 ml with 1xTAE buffer,

B-(Work solution) 100 µl stock+ 630 µl 1xTAE buffer +270 µl Glycerol

Marker (pBr322-*Msp*1) 10 µl conc. +15 µl bromophenol blue+75 µl 5x TAE buffer

DEPC treated water (Sigma, Germany) 1ml/ 1litter aqua dest. and autoclaved for 2h

Xylene cyanol	(Sigma, GmbH, Germany)
Glycerol 99%	(Sigma, USA)

7.4 Reagents for in situ hybridization

DIG-High Prime (Boehringer, Mannheim, Germany)

2ng/ml freshly denatured probe DNA Hp

250µg/ml yeast tRNA (Boehringer Mannheim, Germany)

0.1 %SDS (Boehringer Mannheim, Germany)

50%formamide (Boehringer Mannheim, Germany)

EDTA 0.2 M (Boehringer Mannheim, Germany)

Nylon membrane (Boehringer Mannheim, Germany)

Anti –DIG AP (Boehringer Mannheim, Germany)

DIG wash and block buffer (Boehringer Mannheim, Germany)

Tris-buffer saline pH 7.4

Tris-buffer saline pH 9.5

NBT/BCIP (Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyphosphate)

(Boehringer Mannheim, Germany)

Maleic acid buffer (Boehringer Mannheim, Germany)

20x SSC buffer NaCl 3M, 300mM Tris sodium citrate dehydrate with1.8 ml 1N HCl

pH 7 (Merck, Germany)

Xylene (J.T Baker, Netherlands)

Ethanol Merck, Germany)

Hematoxylin (Merck, Germany)

Acetone (J.T Baker, Netherlands)

ß-Mercaptoethanol (Sigma, GmbH, Germany)

7.5 Reagents for preparation of cytospin, cell and tissue culture

RPMI 1640 (PPA, Austria)

RPMI 1640 with L- Glutamine (PPA, Austria)

2% 1M HEPES (Invitrogen, Germany)

1% 10mM sodium pyruvate (Biochrom, Germany)

Trypsin EDTA (PPA, Austria)

Trypan blue 0.4% (Sigma, Aldrich, Germany)

Bovine serum BSA-PBS (PPA, Austria)

DMEM high glucose 4,5g/L with L-Glutamine medium (PPA, Austria)

1% Penicillin and Streptomycin (Invitrogen; Karlsruhe, Germany)

7.6 Reagents for preparation immunofluorescence and indirect laser confocal microscopy

DAPI   (Vector Laboratories, Burlingame USA)

Alexa Fluor 488   (Invitrogen, Germany)

Taxes red   (Invitrogen, Germany)

Primary antibody: (rapid anti-human; polyclonal anti-haptoglobin antibody; dilution 5 µg/ml; Abcam, U.K)

Primary antibody: (mouse anti-human; monoclonal anti- SP-Bantibody; clone, SPM158 dilution 1:50; Zytomed, Berlin, Germany)

7.7 Main instruments

Autoclave   (GLA 40, Germany)

Digital camera   (Leica Microsystems, Germany)

Centrifuge   (Hettich, EBA 12, Germany)

Centrifuge   (Hettich, Mikro 12-24, Germany)

Centrifuge   (Shandon; Frankfurt, Germany)

Centrifuge   (Rotixa 50 RS Hettich, Germany)

Microscope   (Leica TCS SP5 confocal microscope)

Microscope   (Leica Microsystems, Germany)

Fluorescence microscope   (Nikon Eclipse 80i)

Microtome   (Leica, SM 2000 R, Germany)

pH Meter   (Digital pH-/mV/ thermometer, Germany)

Shaker   (IKA Labortechnik, USA)

Rotar   (TYP VF 2, Jancke and Kunkel, Germany)

Vortex   (Heto RK-20 VS, Denmark)

TMAs   (MTA-Booster, Beecher Instruments, ALPHELYS, France)

Homogenizer   (Janke & Kunkel, IKA-Werk, GmbH& Co KG, Germany)

Vacuum centrifuge (Speed Vacuum SC 110, Savant, Germany)

Spectrophotometer (Amersham Pharmacia Biotech, Gene Quant pro RNA/DNA Calculator, Cambridge, England)

Spectrophotometer   (Helios ß, Unicam, UK)

CO2 cell culture incubators (Heraeus, Germany)

Pressure cooker   (Germany)

Microwave oven   (Germany)

Water purification system Milli-Q Advantage A10 system, Germany)

iblot gel transfer system with nitrocellulose membrane (Novex minicell, Invitrogen, Germany

UV-transilluminator   (Gel documentation system, INTAS, Germany)

Light Cycler$^®$ Carousel-Based System (Roche Molecular Biochemicals, Penzberg, Germany)

SDS-PAGE power supply   (Bimetal, Germany)

DNA gel electrophoresis tank   (Biometra, Germany)

Thermo cycler   (Biometra, Germany)

Hybridizer   (Hybaid, Omnislde, MWG-Biotech, Germany)

Slot blot machine (PR 60 and PR 648 slot blot Filtration Manfolds with vacuum pump)   (Amersham Biosciences, UK)

7.8 Software
BLAST

DNAsis.

BandLeader with Corel Photopaint

Light cycler software 4 LL.2.0

Leica Application suite LAS AF

Microplate Manager/PC Data Analysis software (Bio-Rad, laboratories, Hercules, USA)

FixFoto

Acknowledgements

Curriculum vitae

Full Name   : Mahdi Ali Abdullah

Nationality   : Iraqi

Date and place of birth: 10.04.1974, / Nineveh (Mosul), IRAQ

Marital status: Married

Languages   : Kurdish, Arabic, German and English

Education

1980-1986: Bazkertan Primary School, Nineveh (Mosul), IRAQ

1986-1992: Brtelaa Secondary School for Boys, Nineveh (Mosul), IRAQ

1994-1999: Collage of Veterinary Medicine, University of Mosul, IRAQ

Qualifications

Bachelor degree in Veterinary Medicine and Surgery (1999) University of Mosul, IRAQ

Master degree in Veterinary Pathology (2002) University of Mosul, IRAQ

Employment and responsibilities

Since December 2005: Teaching Assistant Instructor at the Department of Veterinary Pathology, College of Veterinary Medicine, University of Dohuk, IRAQ

Since August 2007: PhD student, Research Center Borstel, Germany at the Department Clinical and Experimental Pathology

Workshops and courses attended

-April 2008: Workshop on "Molecular Diagnostic Tools" organized by the Dohuk Research Center, College of Veterinary Medicine and the Research Center Borstel, Germany

- June 2008: "Technical Workshop on Bioinformatics " organized by EU-FB6 INCO-DEV INCOME EU-FP6 PRIORITY ConFluTech, Research Center Borstel, Germany

-September 2008: "Borsteler Herbestseminar Für Pathologie und Biomedizin " organized by Clinical and Experimental Pathology, Research Center Borstel, Germany

-May 2008: Oral presentation "Expression of haptoglobin in human infected lung "2rd Kurdistan Conference on Biological Sciences, University of Dohuk, IRAQ

-April 2009: Poster presentation "On the role of acute phase protein haptoglobin and its receptor in the human lung", visit of the advisory board, Research Center Borstel, Germany

-May 2009: Workshop "ConFlu", tech workshop on AIV, Sequencing, Phylogeny and Bioinformatics" organized by ConFluTech, Uppsala, Sweden, and the Research Center Borstel, Germany.

-June 2009 seminar "On the role of haptoglobin and its receptor in human lung" Research Center Borstel, Germany.

-June 2009: Poster presentation (3th Lübecker Graduate Student's Day) "Haptoglobin and its role human lung", University of Lübeck.

-November 2009: Oral presentation "On the expression of haptoglobin in human lung" Herbsttagung der Sektion Zellbiologie. Research Center Borstel, Germany.

-November 2009: Poster presentation "32nd Symposium of the North-German Immunologists" On the role of the acute phase protein haptoglobin in the human lung. Research Center Borstel, Germany.

-Particcipated in the mentoring program in the Research Center Borstel during the dissertation.

Publications

Original articles

1- Abdullah M, Schultz H, Kähler D, Branscheid D, Dalhoff K, Zabel P, Vollmer E, Goldmann T. Expression of the acute phase protein haptoglobin in human lung cancer and tumor-free lung tissues. Pathol. Res. Pract. 2009; 205, 639-647.

2-Abdullah M, Marwitz S, Kähler D, Schultz H, Kugler C, Zabel P Vollmer E Goldmann T. Pulmonary haptoglobin (pHp), a new marker for adenocarcinomas of the lung. Pathology. *in press*

3- Goldmann T, Kähler D, Schultz H, Abdullah M, Lang Ds, Stelmllmacher F, Vollmer E. On the significance of Surfactant protein-A within the human lungs; Diagnostic pathology 2009, 4:8

4. Marwitz S, Zeiser T, Schultz H, Kähler D, Abdullah M, Hauber HP, Zabel P, Vollmer E, Goldmann T. The human placenta releases substances that drive lung cancer into apoptosis. Diagnostic Pathology 2009, 4:27

5- Kähler D, Alexander C, Schultz H, Abdullah M, Branscheid D, Lindner B, Zabel P, Vollmer E, GoldmannT. Proteomics out of the archive: Two-Dimensional electrophoresis and mass spectrometry using HOPE-fixed, paraffin-embedded tissues. J. Histochem Cytochem. 2010 Mar, 58(3): 221-8.

6- Drömann D, Rupp J, Rohmann K, Osbahr S, Ulmer AJ, Marwitz S, Röschmann K, Abdullah M, Schultz H, Vollmer E, Zabel P, Dalhoff K, Goldmann T. The TGF-beta- Pseudoreceptor BAMBI is strongly expressed in COPD lungs and regulated by nontypeable *Haemophilus influenzae*. Respiratory Research 2010, 11: 67.

Published abstracts

1. Schultz H, Kähler D, Branscheid D, Abdullah M, Vollmer M, Zabel P, Goldmann T. Analysis of the expression of Transketolase-like enzyme 1 (TKTL1) in Non Small Cell Lung Cancer tissues and tumor free lung specimens.

2. Kähler D, Branscheid D, Abdullah M, Vollmer E, Alexander C, Lindner B, Zabel P, Goldmann T. 2-D-electrophoresis and molecular investigations using HOPE-fixed tissues from Non Small Cell Lung Cancer.

3. Schultz H, Abdullah M, Kähler D, Nakashima M, Dalhoft K, Zabel P, Vollmer E, Goldmann T. Expression of haptoglobin in different benign and malignant cell types of the human lung.

4. Kähler D, Abdullah M, Branscheid D, Schultz H, Vollmer E, Alexander C, Linder B, Zabel P, Goldmann T. 2-D-electrophoresis and peptide mass fingerprinting using HOPE fixed non small cell cancer specimens.

5. Kähler D, Schultz H, Abdullah M, Vollmer E, Goldmann T. Molecular high throughput analyses and automated detection systems.

Declaration

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