Heme degradation pathway controls inflammation in model of mouse prostatitis

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Abstract

Acute or chronic inflammation in the prostate is implicated in pathogenesis of benign prostate hyperplasia (BPH) as well as development of prostatic intraepithelial neoplasia (PIN) and prostate cancer (PCa). Chronic prostatitis (inflammation in the prostate) is associated with high morbidity and negatively impacts life quality. Macrophages are critical regulators of inflammatory processes and are early immune cells responders. Among macrophage-associated genes, the stress-induced enzyme heme oxygenase-1 (HO-1), which degrades heme to carbon monoxide (CO), biliverdin and iron, has strong immunomodulatory effects in in vitro and in vivo disease models.

In this study, we investigated the specific role of HO-1 in macrophages on modulation of prostate inflammation. We established a mouse model of bacterial prostatitis in wild type mice, and evaluated the role of HO-1 in pathogen-induced prostate inflammation by using mice with conditional deletion of HO-1 in myeloid cells (Lys-M-Cre:Hmox1fl/fl). Immunohistological analysis showed increased infiltration of CD45 positive leukocytes and mitosis in the prostate glands as represented by phospho-Histone-H3 (p-HH3) staining in mice after bacterial challenge. Inflammation was accelerated in mice lacking myeloid-derived HO-1 with increased number of CD45 positive cells in the prostate glands. Further, we confirmed activation of the mitogen-activated protein kinase (MAPK) signaling pathway as observed by increased phosphorylation of extracellular signal-regulated kinase (Erk1/2) and induction of the inflammatory mediator interleukin-1β (IL-1β).

Since exogenous CO mimics the majority, if not all, of the HO-1 effects, we asked whether CO plays a role in the model of prostatitis in mice. CO suppressed proliferation as shown by decreased p-HH3 staining and p-Erk1/2 levels in the inflamed mice prostates. CO also suppressed bacteria-induced expression of lipid metabolic enzymes acyl-CoA synthetase-1 (ACSL1) and fatty acid synthase (FAS), which mediated macrophage activation in other inflammation models.

In summary, these results suggest that HO-1 plays a role in modulating pathogen-induced prostate inflammation via suppression of leukocyte influx and that exogenous CO may be used to block inflammation triggered proliferation and prevent PIN lesion formation.

Keywords: Prostate inflammation, myeloid derived HO-1, CO, lipid metabolism
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### Abbreviations

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<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>ACSL1</td>
<td>Long chain acyl-CoA synthetase</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow derived macrophage</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostate hyperplasia</td>
</tr>
<tr>
<td>CCL2</td>
<td>C-C motif ligand 2</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CRCP</td>
<td>Castration resistant prostate cancer</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger associated molecular pattern</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast growth factor-2</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of view</td>
</tr>
<tr>
<td>HH3</td>
<td>Histone H3</td>
</tr>
<tr>
<td>HO-1</td>
<td>Hemeoxygenase-1</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL (i.e. IL-1β)</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>LUTS</td>
<td>Lower urinary tract symptoms</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemoattractant Protein-1</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Monocyte-colony stimulating factor</td>
</tr>
<tr>
<td>NIH</td>
<td>National institutes of health</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide binding domain leucine rich repeat containing receptors</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIA</td>
<td>Proliferative inflammatory atrophy</td>
</tr>
<tr>
<td>PIN</td>
<td>Prostatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PCa</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TAMs</td>
<td>Tumor associated macrophages</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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1. Introduction

The majority of men will at some point during life experience prostate related problems. Benign prostate hyperplasia (BPH) and prostate cancer (PCa) are the most prevalent conditions of the urinary tract in males, despite progress in diagnosis and treatment in recent years (1). BPH affects 50% of men in their 50’s-60’s and over 90% in men over 80 years (2) and is caused by hyperproliferation of cells in the transition zone of the prostate gland (3). Enlargement of the prostate because of BPH may lead to obstruction of the urethra, pain, bladder dysfunction and urine retention (4, 5). PCa is one of the most common cancer types in the male population in Western countries (6, 7). In spite of improved screening methods and control in early stages, PCa still leads to a great number of cancer related deaths due to lack of effective treatment of advanced and metastatic disease (8). BPH and PCa are considered to be the result of several cooperating risk factors including heredity, age and hormones (9). In addition, a growing body of evidence based on human patient material samples as well as in vitro and in vivo studies have linked development and progression of proliferative disorders in the prostate to acute and chronic inflammation (10-13).

1.1. Inflammation

Inflammation is an essential component of the immune responses to pathogens or tissue injury and works to defend the host against harmful insults. It represents the initial response to any tissue damage and acts in a cause-independent manner to start the healing process of the affected area. The inflammatory processes are induced by the activation of tissue residing immune cells (leukocytes), primarily macrophages, in response to pathogens, stressed and damaged cells (14). Activation of macrophages occur via surface receptors (PRRs, pattern recognition receptors), such as toll-like receptors (TLRs) and nucleotide binding domain leucine rich repeat containing receptors (NLRs), recognizing signals from intruding microbes and stressed or damaged cells. Pathogen associated molecular patterns (PAMPs) including bacteria-derived lipopolysaccharides (LPS) or peptidoglycan bind TLRs, whereas nucleotides, such as extracellular ATP, mitochondrial DNA and other danger associated molecular patterns (DAMPs) related to cellular stress and necrosis, can activate multiple classes of receptors including NLRs (15). Upon activation, macrophages start to produce pro-inflammatory cytokines (i.e. TNF, IL-1β and IL-6) (14) and chemoattractants (i.e. monocyte chemoattracting protein-1, MCP-1). These molecules stimulate recruitment of bone-marrow derived myeloid cells (e.g. neutrophils and monocytes) to the site of inflammation. In response to PAMPs or DAMPs, recruited macrophages are polarized into a pro-inflammatory, cytotoxic phenotype (M1 type). Acting to eliminate bacteria or abnormal/tumor cells. By release of cytotoxic agents, including reactive oxygen species (ROS), as well as by activation of the adaptive immune system, the inflammatory cells work to clear tissues from pathogens and damaged cells (14). When the clearance of pathogens/cells is completed, macrophages phenotypically change grasping an anti-inflammatory profile (M2 type). M2 polarized macrophages orchestrate tissue repair and regeneration by production of anti-inflammatory cytokines transforming growth factor-beta (TGF-β) and interleukin-10 (IL-10) and stimulation of growth
factors, such as epithelial-, insulin-like- and vascular endothelial growth factor (EGF, IGF and VEGF) in order to accelerate healing processes and restore homeostasis in the injured tissue (16-18). Inflammation is absolutely necessary for host survival, but if not resolved it contributes to pathogenesis and development of various diseases. Chronic inflammation is a pathological condition that can be caused by autoimmune reactions or sustained infection. An unresolved inflammation is associated with presence of large numbers of immune cells, from which continuous release of ROS and pro-inflammatory mediators causes harm to the tissue by promoting cell death, inducing DNA damage and changes in the extracellular matrix (19). Examples of such syndromes are rheumatoid arthritis (20), diabetes mellitus (21, 22), atherosclerosis (23) and chronic inflammatory bowel disease (24). Hereditary factors have been identified as drivers in many immune mediated diseases, but only in combination with other risk factors they results in development of disease (25). Diabetes and cardiovascular disease are associated with dietary factors, smoking and the level of physical activity (26, 27). In addition, repeated contact with foreign substances or microbes in the colon, or infections in the urinary tract may trigger immune responses and activation of immune cells that are sustained over longer periods of time and influence function of these organs (28, 29). Chronic inflammation has also been strongly associated with development and progression of 20% of all cancer diseases (i.e. gastric cancer, colon cancer and prostate cancer) (16). Immune cell derived toxic molecule- and ROS incited DNA-damage in combination with defect repair and increased stimulation of proliferation have been shown to induce oncogene activation, loss of function of tumor suppressor genes, and malignant transformation of epithelial cells in sites of chronic inflammation (19, 30). Further, immune cells and inflammatory factors, such as cytokines and growth factors, including EGF, IGF and VEGF, are involved in growth and spread of established tumors by promoting cell migration, angiogenesis and remodeling of the extra cellular matrix (16).

1.2. Prostatitis

The prostate is an exocrine gland and a part the male reproductive system, which produces fluid nourishing and allowing for transport of sperm during and after ejaculation. The prostate is located below the urinary bladder, surrounding the upper part of the urethra (31). In such a proximity to the urine, prostate is constantly at risk of being exposed to potential urine reflux and infectious agents (i.e. bladder infection) (32). Prostate inflammation, called prostatitis, is a common urogenital tract condition in men over 50 years of age, which is frequently found in prostate specimen from biopsies or autopsies (33, 34). Prostatitis can be initiated by a variety of stimuli and has been associated with sexually transmitted organisms, such as Chlamydia trachomatis, Trichomonas vaginalis and Neisseria gonorrhea, viruses (e.g. human papilloma and herpes simplex), as well as gram negative bacteria (i.e. uropathogenic Escherichia coli) and the opportunistic pathogen Propionibacterium acnes (9, 35-40). P.acnes was able to establish chronic prostatitis in mice with persistent infiltrates of lymphocytes and macrophages 8 weeks after infection (41). When cocultured with prostate epithelial cells (RWPE1 cell line) P.acnes (isolated from human prostate tissue) activated inflammation related factors, such as nuclear factor kappa B (NFxB) and signal transducer and activator of transcription 3 (STAT3), and upregulated pro-inflammatory genes including IL-6, IL-8 and cyclooxygenase-2 (COX-2) (42). E.coli is one of the most frequent
uropathogens to be detected in prostate tissue samples and is also associated with increased infiltration of immune cells and expression of inflammatory mediators (43, 44). Of note, both *P. acnes* and *E. coli* have been shown to increase the production and release of IL-1β from macrophages via activation of the Nalp3-Caspase1 inflammasome (45, 46). In a rat model of sterile (non-bacterial) prostatitis, increased inflammasome activity was hypothesized to have significant impact on the pathology of chronic prostatic inflammation and related symptoms (47). Dietary factors, hormones and autoimmune responses contribute to the early steps of prostatitis. In addition, urine reflux and calcifications in the tubular tissue of the prostate increases risk of cytotoxicity from urine metabolites (e.g. uric acid crystals), physical trauma due to augmented pressure on the cells, and prostate infection, all contributing to trigger inflammatory responses (9). The National Institutes of Health (NIH) classifies prostatitis as one of four different categories: 1) Acute bacterial prostatitis, 2) Chronic bacterial prostatitis, 3) Non-bacterial chronic prostatitis and 4) Asymptomatic chronic prostatitis (48). An acute bacterial prostatic infection is usually successfully treated with antibiotics for a period of 2-4 weeks (49). Chronic prostatitis on the other hand is a poorly understood, but common condition in men and is often associated with recurrent urinary tract infections, severe lower urinary tract symptoms (LUTS) (bladder voiding and filling problems), urogenital pain and sexual dysfunction over months or years, even in men as young as 20 years old (50). Limited knowledge on the pathology, and symptoms overlapping with other prostate conditions have so far been hurdles in search for effective treatment. Chronic inflammation has in addition been strongly associated with hyperplasia and cancer in the prostate (13, 51, 52).

### 1.3. Prostate inflammation and the link to BPH and prostate cancer

Inflammation as a driver of hyperplasia and tumor development and progression in the prostate has been suggested in a great number of studies. As in many types of cancer, cytotoxic and oxidizing agents released from inflammatory macrophages are thought to promote accumulation of mutations and increase in genomic instability with subsequent cellular transformation that can initiate tumor formation in the prostate. Presence of lymphocyte and macrophage infiltrates (53-55), as well as expression of inflammatory cytokines (e.g. IFN-γ, IL-17, IL-15 and IL-8) (53, 54, 56-58) and growth factors (FGF-2, VEGF and TGF-β) (59) in tissue samples from BPH patients have been positively correlated with higher prostate volume and LUTS severity. Patients with documented BPH treated with non-steroid anti-inflammatory drugs showed beneficial effect on LUTS (60) and antibiotics significantly decreased prostatitis related elevated serum PSA (prostate specific antigen) (61). The regenerative potential within the inflamed prostate is thought rely on a complex interplay between immune cells, stromal components and epithelium, involving hormonal regulation by the male sex-hormones testosterone and dihydrotestosterone (62). The steroid hormone signaling controls the differentiation program of prostate epithelial cells by paracrine signaling in normal prostate and is known to be deregulated in the majority of advanced prostate cancers (63).

Moreover, patients biopsies with increased inflammation in the prostate, referred to as proliferative inflammatory atrophy (PIA) have been associated with pre-cancerous prostate intraepithelial neoplasia (PIN),
and potentially adenocarcinoma (64-66). Elkahwaji et al. showed that chronic bacterial infection of the prostate in a mouse model led to the development of PIN lesions within 3 months after induction of prostatitis by intraurethral injection of *E.coli*. After one week of infection the mice showed infiltration of leukocytes into the prostate stroma and after 26 weeks the prostate epithelium underwent transformation, shown by increased epithelial DNA-damage and proliferation, as well as by lower expression of the tumor suppressors PTEN and p27Kip1 (67). *P.acnes* isolated from human biopsies was also able to establish chronic prostatitis in a mouse model by transurethral inoculation, resulting in increased proliferation (represented by Ki67) and suppression of Nkx3 (prostate specific protein, often lost in hyperplasia and PCa) (41). The hypothesized association between bacteria induced prostatitis and tumor development was further supported by a study by Fassi Fheri and colleagues found *P.acnes* in over 80% of 71 biopsies from cancerous prostates, while bacteria were absent in healthy control tissues. In addition, long-term infection caused transformation with enhanced proliferation and anchorage-independent growth of prostate epithelial cells (42). Intracellular *P.acnes* infection in prostatic macrophages have also been found in significantly higher numbers in prostate cancer tissue samples than in normal tissue (68).

1.4. **Macrophage phenotype regulates cancer development and growth**

Macrophages are excellent modulators of inflammatory responses and have crucial roles in destruction harmful pathogens and transformed cells, as well as in promoting healing processes and tissue regeneration. However, immune cells are active players in chronic inflammatory diseases and cancer. Tumor progression is shaped by residential and infiltrating innate immune cells. In general, tumors are characterized by a larger number of infiltrating myeloid cells and macrophages as compared to healthy tissues (69, 70). Tumor-associated macrophages (TAMs) are recruited to the tumor site by chemoattractants, especially CCL2, produced by cancer cells, and modulate angiogenesis, tumor growth and metastatic spread (71). TAMs are as well as normal macrophages classified into M1 or M2 subtype, induced by IFN-γ or IL-4/IL-13, respectively (72). M1 macrophages possess cytotoxic activity and are capable of eliminating cancer cells and reducing tumor growth (73). In contrast, the immuno-suppressive M2 like macrophages, associated with pro-angiogenic capacity in response to local hypoxia (74), have been linked to poor prognosis and chemotherapy resistance in cancer patients (75, 76). However, in early changes of the epithelium, called transformation, chronic pro-inflammatory responses are related to development of neoplasia (19). The macrophages present in chronically inflamed tissues share characteristics with M1 subtype, even though this polarization state generally is involved in anti-tumor immunity and infection defense. M1 macrophages have also been recognized to potently induce epithelial-mesenchymal-transition, a characteristic of tumor invasion and migration, in pancreatic ductal carcinoma (77). However, the paradigm of macrophage polarization into M1 and M2 subtype is a radical simplification of the reality. Mixed or hybrid populations of M1/M2 like macrophages with simultaneous production of pro-inflammatory (TNFα and IL-1β) and anti-inflammatory (IL-10) cytokines have been discovered in several studies, such as in early stages of PCa in transgenic mouse models (78) as well as in obesity related chronic inflammation in adipose tissue (79). Studies like these indicate a skewed inflammatory process with oxidative...
stress and regenerative potential creating a reactive microenvironment with increased concentrations of growth factors and damaged cells, favoring transformation and tumor development. The regulation and switching of the macrophage polarization in tumor microenvironments is poorly understood and illuminates the complexity of the crosstalk between cancer and the immune system. Deeper understanding of the underlying mechanisms of tumor development and the role of chronic inflammation may contribute to the finding of new treatment regimens and targets in the combat against cancer.

1.5. Heme degradation pathway

Heme is an organic, iron containing protoporphyrine IX molecule that when bound to hemo-proteins is involved in various essential biological processes, such as gas transport, electron transfer and drug metabolism. Free heme on the other hand have serious cytotoxic and pro-inflammatory properties, due to its highly oxidative potential (80). Degradation of heme and recycling of iron is performed by the hemeoxygenase enzymes (HO-1 and 2), which produce equimolar amounts of biliverdin, ferrous iron and carbon monoxide (CO) (81, 82). Biliverdin is immediately reduced by the biliverdin reductase (BVR) into the strong anti-oxidant bilirubin. HO-2 is constitutively active in various tissues sustaining homeostatic levels of heme in the body, while HO-1 is induced by increased intracellular concentrations of free heme, oxidative stress and bacterial endotoxin (83). HO-1 have been shown to have strong cytoprotective effects in in various cell types. By degrading excess free heme HO-1 directly prevents generation of ROS, oxidative stress and tissue damage. In addition, HO-1 and its produced metabolites have been shown to play important roles in immuno-modulation. Transgenic mice with HO-1 deficiency have been characterized with higher degree of inflammation and sensitivity to heme toxicity, indicating an anti-inflammatory function of HO-1 in these animals (84). Biliverdin and bilirubin are strong anti-oxidants countering free heme oxidative activity. The enzyme BVR has also been recognized as a transcription repressor of TLR4 and as an activator of Akt-IL-10 signaling, with strong influences on inflammation and cell death (85, 86).

Further, the HO-1 byproduct CO has been described a potent immunomodulator in various scenarios, such as sepsis, malaria (87, 88) and ischemia-reperfusion injury (8, 89) and have classically been associated with anti-inflammatory properties (90-92). Exogenously administrated CO prior to insult has been beneficial in several models, such as in case of promoting tolerance of transplanted allografts (93, 94). Also, anti-inflammatory effects of CO have been described by Otterbein and colleagues on immune cell activation, by blocking TNF release from endotoxin activated macrophages (91). Results from these studies and others suggest that CO has a therapeutic potential and is currently evaluated in several clinical trials in cases of for instance organ transplantation and pulmonary hypertension [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (2015-04-29).

Intriguingly, HO-1 and CO has recently also been implicated in immuno-stimulatory processes, for instance in driving to pathologic inflammation and insulin-resistance in in humans and mice with obesity and diabetes (87). In addition, CO treatment after infection acts to increase bacterial clearance (95) and even amplify pro-inflammatory responses in macrophages after bacterial challenge, by up-regulation of IL-1β activation and
release (96). In the later scenario CO protects from lethal sepsis through enhanced bacteria killing by macrophages, via a unique mechanism acting directly on bacteria via activation of the Nalp3-Caspase1 inflammasome. In the proposed mechanism HO-1 is induced by bacterial PAMP stimulation of TLR4 and produces CO. By diffusion, the CO reaches live bacteria in the intracellular space and provoke the pathogens to release extra cellular ATP, acting as a DAMP and co-stimulatory signal, binding to the macrophage P2X7 receptor (96). Nucleic acid stimulation of P2 receptors activates the internal NLR3 (Nalp3), which in association with Caspase 1 induces processing of IL-1β (97-100). We hypothesized that ATP released from necrotic cancer cells in the tumor microenvironment work in the same way to activate TAM. IL-1β has been shown to block proliferation and important signaling pathways of prostate cancer cells (101).

HO-1 has also been associated with cancer (89). Conflicting results from multiple studies indicate that HO-1 effects differ depending of cancer type and stage of progression (102). An elevated epithelial HO-1 expression has been detected in renal cancer, colon cancer and pancreatic cancer (103-106). On the other hand, HO-1 expression was proven repressed in macrophages present in non-small lung carcinoma (107). In prostate cancer, HO-1 has been suggested to interfere with several different processes and pathways, linked to both tumor progression as well as anti-tumor activity. HO-1 overexpressing PC3 cell xenografts in immunocompromised mice showed significant reduction in tumor growth compared to control mice implanted with wild type PC3 cells. In vitro, HO-1 induction resulted in reduced proliferation and migration in PC3 cells (108) as well as blockage of NFκB signaling and angiogenic factors, such as VEGF-receptor (109). CO treatment mimics HO-1 effects and suppressed the growth of LNCaP and PC3 cells by inhibiting phosphorylation of the cell cycle guardian RB, (110) and to cause growth arrest in moderately differentiated prostate tumors in mice by inducing mitotic catastrophe (111). During normal conditions HO-1 operates in the cytoplasm, but nuclear localization of a truncated and enzymatically restricted form of the protein (112) has been associated with prostate cancer (105). Nuclear HO-1 has been recognized to modulate Androgen Receptor activity through interference with STAT3, a transcriptional activator that is often deregulated in cancer (113). In addition, high epithelial expression of HO-1 has been positively correlated with castration resistant prostate cancer (CRPC) and tumor suppressor PTEN deletion (114). Inhibition of HO-1 in CRPC reduced invasion and metastasis (106). Since inflammation has been assigned a “hallmark of cancer” (115) and estimated to be a direct or indirect cause of tumor development in 20 % of all cancers, a huge research is currently conducted trying to identify new targets for treatment, as well as diagnostic and prognostic factors. The polarization status of macrophages has been proven to be determinants of function in chronic inflammatory settings and in cancer development and progression. Previous data from our group showed strong induction of HO-1 in macrophages after infection with E.coli or Escherichia faecalis, strains often involved in urinary tract infections and were associated with M1 like phenotype (96). At present, the function of macrophage derived HO-1 in bacteria induced prostate inflammation has not been investigated.
1.6. Lipid metabolism in inflammation and prostate cancer

Lipids are used for many essential processes by the cells, including storage of energy, generation of cellular membranes, and synthesis of lipid derived hormones. In normal cells, fatty acids are generally originating from food, while de novo synthesis of fats is almost absent, with exception of liver, breast and adipose tissue (116). Cancer cells were early described to have a deregulated metabolism, characterized by increased glycolysis, in spite of adequate oxygen levels (117). Highly proliferative tumor cells are also dependent on increased lipid production to cover the need of energy and to be able to generate new cells. Several lipid metabolic enzymes have been shown to be upregulated in various types of cancer, including prostate cancer (118). Fatty acid synthase (FAS) is active in the de novo synthesis of fatty acids and has been associated with tumor progression, malignant transformation and poor prognosis (116). Overexpression of FAS in prostate cancer cells lead to augmented proliferation and inhibition of cell death in vitro (119). Mechanistically, FAS has been associated with activation of PI3K-Akt signaling (implicated in many cases of PCa) (120) and androgen signaling in the prostate (121). Long chain acyl-CoA synthetase 1 (ACSL1) is one of 5 iso-forms of an enzyme that works to activate fatty acids for further metabolic processes by coupling them to Coenzyme A, generating acyl-CoA molecules. Except for being upregulated in tumors (116), ACSL1 is activated by bacterial infection via a TLR4 dependent mechanism and mediates the phospholipid turn over in LPS stimulated macrophages (122). In addition, conditional deletion of ACSL1 in macrophages prevented acceleration of atherosclerosis in diabetic mice. This indicates a role of ACSL1 in promoting the polarization of macrophages into pro-inflammatory phenotype, mediating chronic inflammation in this disease model (123). Since lipid metabolic enzymes, such as FAS and ACSL1 have been shown to regulate immunological reactions and/or malignant transformation and heme degradation pathway is linked to metabolic stress, we hypothesized that ACSL1/FAS are regulated in our model of prostatitis by the presence of HO-1/CO.

1.7. Aim

In this study, we investigated the role of HO-1 in macrophages and its effects on bacteria induced inflammation in the prostate. To do this, we established a mouse model of bacterial prostatitis, induced by intraurethral inoculation of E.coli (43, 65, 67), and examined how selective deletion of HO-1 in myeloid cells and application of CO influence the development of inflammation. We focused on the following questions:

1. How does selective deletion of HO-1 in macrophages influence development of inflammation that leads to tumorigenesis in the prostate?
2. Will application of CO regulate inflammation in the prostate to prevent development of cancer?

In addition, we used in vitro based methods to look at the influence of macrophage derived factors and CO on malignant phenotype in prostate derived cell lines PNT1A (transformed epithelium) and PC3 (adenocarcinoma). As a sub-aim, we investigated whether lipid metabolic enzyme expression in inflamed prostate tissue is regulated by CO treatment.
The goal of the study is to obtain valuable knowledge about the inflammatory process and its regulation in bacterial prostatitis, focusing on the heme degradation pathway enzyme HO-1. Targeting inflammation and inflammation regulated factors could possibly represent an alternative in anti-cancer therapy. Since CO have shown suppressive effects on prostate tumor cells \textit{in vitro} and in xenograft experiments, there is a hope for future clinical applications of this gaseous molecule as therapy in prostate cancer/prostatitis patients.

2. Material and Methods

2.1. Bacteria

\textit{Escherichia coli} MG1655, originally acquired from the Keio library, was provided by Bernard Strauss (Massachusetts Institute of Technology [MIT], Cambridge, Massachusetts, USA). This strain of bacteria was used in bacterial infection experiments \textit{in vitro and in vivo}. Bacteria were inoculated in Luria Broth (LB) medium (Invitrogen™, Life Technologies, Carlsbad, CA, USA) from a frozen aliquot obtained from a single colony, and incubated overnight (o/n) at 37 °C on orbital shaker. Fresh LB was inoculated with 1 ml aliquot of the o/n culture and sub-cultured for 1.5h at 37 °C. Cells were pelleted at 3220 x g (5 min) and resuspended in sterile Phosphate Buffered Saline (DPBS -Cl -Mg) pH 7.4 (Gibco® Life Technologies). Optical density of bacteria suspension was measured at 600 nm (OD$_{600}$) and adjusted with PBS to 0.45. Serial dilutions of stock suspension (1:1000-1:10$^9$) were plated in duplicates on LB-agar and incubated at 37 °C o/n. Colony counts were averaged and used to estimate the concentration of bacteria (CFU/ml) in the prepared stock with OD$_{600}$=0.45. Appropriate volumes of the bacteria dilutions were used to prepare injections for infection \textit{in vivo} or \textit{in vitro} treatments.

\textbf{Optimizing of an \textit{in vivo} dose of bacteria}: Intraurethral injection of a single dose of bacteria at 2x10$^5$, 2x10$^7$ and 2x10$^8$ CFU/animal (n=2/group) was performed. Mice injected with 50 µl PBS and naïve mice, respectively, were used as negative controls. Inflammation as tested by increased number of CD45+ cells in tissue was evaluated in the prostates harvested 2 weeks after infection. A higher degree of inflammation was observed in mice injected with the highest dose of bacteria, 2x10$^8$ CFU/ml, which therefore was used in all following infections \textit{in vivo}. Further since the PBS-injected mice also showed signs of inflammation in the prostate they were hereafter used as a model of sterile inflammation, while mice catheterized but not injected with PBS or bacteria (‘tubing’ group) were used as negative controls in addition to naïve animals.

2.2. Mice

C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) 7-10 weeks of age weighing 20-25 g were used (after at least three days of acclimatization) to establish models of pathogen induced and sterile prostatitis through intraurethral (i.u.) instillation of \textit{Escherichia coli} MG1655 (2x10$^8$ CFU/ animal) and PBS, respectively (n=3-6/group). Injections were done using a 30G syringe connected to lubricated polyvinyl tubing (outer
diameter 1.2 mm) inserted into the urinary bladder via the urethra. The procedures were performed under 3 % isoflurane induced anesthesia. After procedure the mice were monitored for changes in behavior associated with pain or distress and body weight records were kept. The animals were kept at all times in ventilated cages (up to 5 individuals per cage) in a 12 hours light-dark cycle provided water and food ad libitum. The procedures were approved by Institutional Animal Care and Use Committees at Beth Israel Deaconess Medical Center, Harvard Medical School, MA, USA. Prostates and blood samples were harvested from the different mice groups 2h, 6h, 24h and 3 weeks after injection and analyzed by immunohistochemistry (CD45 and pHH3), real time PCR (rtPCR) with primers against IL-1β and IL-10 and western blot. The same time points as indicated above were chosen for investigation of the role of carbon monoxide (CO) in this model. Animals injected with E.coli or PBS as well as negative controls inhaled CO (250 ppm) for 1h, starting 1h after injection. The role of myeloid derived HO-1 in bacteria-induced or sterile prostatitis was investigated by using conditional knockout mice deficient in HO-1 in myeloid cells. These animals were generated by crossing mice having the Hmox1 gene flanked with LoxP sites (Hmox10/0), provided by the RIKEN BRC through the National Bio-Resource Project, Japan, with transgenic mice expressing Cre recombinase under control of the myeloid specific LysM-promoter (LysM-Cre) (Jackson Laboratories). LysM-Cre:Hmox10/0 homozygotes were verified by PCR based genotyping (124).

2.3. Bone marrow isolation and bone marrow derived macrophages (BMDMs)

Intact femurs were isolated from WT mice euthanized by overdose of xylazine and cleaned from soft tissues. Bones were crushed in a mortar and cells were washed in RPMI (Life Technologies) containing 1% Anti-microbial & anti-fungal solution (A/A) (Life Technologies) (wash medium). Cells were passed through 40 µm strainers and spun at 453 x g for 5 min and resuspended in fresh wash medium. Red blood cells were lysed using Buffer EL (Qiagen, Hilden, Germany) for 5 min. The reaction was blocked by RPMI containing 15 % Fetal Bovine Serum (FBS) (Atlanta Biologicals, Norcross, GA, USA). Cells were washed two times with the wash medium before resuspended and diluted in macrophage differentiation media (RPMI 15 % FBS 1 % A/A containing M-CSF, 20 ng/ml (Peprotech, Rocky Hill, NJ, USA). Cells were plated in cell culture treated 100-mm dishes (1 plate per femur isolated) and differentiated at 37 °C, 5 % CO₂, 95 % humidity for 5 days. Media was changed on day 3 after isolation and the macrophages were used for experiments on day 5.

2.4. Bacteria stimulation and CO treatment of BMDMs in vitro

BMDMs differentiated with M-CSF (20 ng/ml) for 5 days were scraped off the culture dishes and suspended in fresh RPMI, 10 % FBS, 1 % Penicillin-Streptomycin medium (Life-Technologies). The cells were counted and replated in 100-mm dishes (3x10^6 cells per plate). Bacteria (E.coli MG1655) were prepared as described above (see section ‘Bacteria’). Macrophages were washed twice in PBS to remove residual antibiotics and were then stimulated with bacteria (10 MOI) for 1h in antibiotic free culture media. Sterile PBS pH 7.4 was added to control plates. Macrophages from stimulated and control groups were treated with CO (250 ppm) or air for 1h. Bacteria were removed after 2 hours stimulation and medium containing antibiotics (1 ml medium per 170 000
cells) was added to the plates. Supernatants from the BMDMs were harvested 24h after stimulation, filtered and stored at -20 °C until use.

2.5. Cell culture and soft agar colony assay

One preliminary colony assay was performed in triplicates to investigate anchorage independent growth in prostate epithelial PNT1A (transformed cell line) and PC3 (prostate adenocarcinoma cell line) cells using a three dimensional culture system in soft agar. A modified version of a protocol (Wallert and Provost Lab, Minnesota State University Moorhead, MN, USA)

http://web.mnstate.edu/provost/Soft%20Agar%20Assay%20Protocol%202012.pdf (2014-11-04) was followed using 6 well plates with 5000 cells per well. A base layer with 0.5 % sterile agar RPMI 10 % FBS 1% penicillin/streptomycin (Life Technologies) was casted into 6 well plates, 1 ml per well and solidified. Cells grown to 60-80 % confluence in RPMI 10 % FBS were deattached by trypsinization and diluted to the final concentration of 1x10^4 cells/ml in RPMI 20 % FBS 2 % P/S. Each suspension was mixed 1:1 with sterile soft agar 0.7 % (37-39 °C) (final concentration 0.35 % agar 10 % FBS) and applied to each well on top of the base layer and let incubate o/n).

The cells in the soft agar were treated for 2-3 weeks with supernatants from differentiated BMDMs treated with bacteria at 10 MOI and CO (see section ‘Bacteria stimulation and CO treatment of BMDMs’). Medium was changed every third day (0.5 ml per well per time). RPMI 10 % FBS was used as negative control.

2.6. Western blot

Prostate tissue was sonicated in lysis buffer (50 mM Tris-Cl pH 7.0, 150 mM NaCl, 0.5 % NP-40, 2 mM EDTA, 25 mM NaF, 0.1% SDS, 1 tablet/10 ml buffer Complete Mini Protease Inhibitor Cocktail) (Roche, Indianapolis, IN, USA). The lysates were centrifuged (Microfuge 18 Centrifuge, Beckman Coulter, Bromma, Sweden) for 20 min at 18 000 x g and supernatants were transferred to clean Eppendorf tubes. The centrifugation was repeated once more and clear supernatants were transferred to new Eppendorf tubes. The protein concentrations in the samples were measured using Pierce BCA Protein Assay (Thermo Scientific, Waltham, MA, USA) according to manufacturer’s instructions. The samples were adjusted with dH2O to equal concentration. Samples were prepared for electrophoresis by adding 4x sample buffer (Life Technologies) containing 10 % β-Mercaptoethanol and boiled for 5 min. Samples were loaded (20-25 µg/well) in NuPAGE® Novex® 4-12% Bis-Tris Protein Gels (Life Technologies) using Precision Plus Protein™ Kaleidoscope™ (Bio Rad, Hercules, CA, USA) as size marker. Electrophoresis was performed in MES SDS running buffer (Life Technologies) at 90-120V. Separated proteins were transferred to PVDF membranes (Amersham, Piscataway, NJ, USA) in transfer buffer (33 mM Tris, 0.19 M Glycine, 20 % Methanol) at 80V for 1.5h. Membranes were blocked in 5 % fat-free milk in TBS (50 mM Tris-HCl, pH 7.0, 150 mM NaCl) for 1h followed by incubation with primary antibodies: HO-1 1:1000 (ab13248, Abcam, Cambridge, MA, USA), ACSL1 1:1000 (D2H5) (#9189 Cell Signaling, Beverly, MA, USA), Fatty Acid Synthase 1:1000 (C20G5) #3180 Cell Signaling), IL-1β 1:500
(ab1413, Millipore (Billerica, MA, USA), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) 1:1000 (#9101, Cell Signaling, Beverly, MA, USA) Cleaved Caspase-1 p10 (M-20) 1:1000 (sc-514, Santa Cruz Biotech, Dallas, TX, USA), TLR4 1:1000 (ab13556, Abcam, Cambridge, MA, USA), β-Actin (A5316 Sigma Aldrich, St. Louis, MO, USA) o/n at +4°C. After overnight incubation, membranes were washed in 1x Tris-Buffered saline (TBS) for 2*10 min and then incubated with Horse Radish Peroxidase (HRP)-conjugated secondary antibodies (Anti-mouse IgG, HRP-linked Antibody (#7076, Cell Signaling) or Anti-rabbit IgG, HRP-linked Antibody (#7074, Cell Signaling) at 1:5000 dilution for 1h at RT on orbital shaker, followed by washing twice for 15 min in TBS. Proteins were detected on radiofilm after signal development with SuperSignal™ West Pico Chemiluminescent Substrate and SuperSignal™ West Femto Chemiluminescent Substrate (Thermo Scientific). The results were correlated with a loading control represented by the house-keeping protein β-Actin. Before re-probing with different antibodies, membranes were stripped in ReBlot Plus Strong Antibody Stripping Solution (Millipore, Billerica, MA, USA) for 10 min. Membranes were washed in TBS for 20 min and stored until re-probed.

2.7. RNA isolation and Real-time PCR

Total RNA was isolated from prostate tissues according the manufacturer’s instructions using RNeasy Mini RNA Isolation Kit (Qiagen, Hilden, Germany). cDNA was synthesized with iScript™ cDNA synthesis kit (BioRad) using 1 µg isolated RNA following the manufacturer’s protocol: 25 °C, 10 min; 37 °C, 2h; 85 °C 5 min. mRNA expressions were measured by real-time PCR using SYBR-Green PCR Master Mix (Applied Biosystems, Life Technologies) and the following primers: IL-1β, forward: 5′-TGGGCCTCAAAGGAAGAGA-3′, reverse: 5′-GGTGCTGATGTACCAGTTTT-3′; IL-10, forward: 5′-CCAAGCCTTATCGGAAATGAC-3′, reverse: 5′-TTTTCACAGGGGAGAATCG-3′; β-actin, forward: 5′-TAGACTTGCAGCAGGAGATGCC-3′, reverse: 5′-CCACAGGATTTCCATACCAAGA-3′. The following program was applied: 95 °C, 10 min; 94 °C, 30 sec; 58 °C, 55 sec; 72 °C, 1 min; 95 °C, 1 min; 55 °C, 30 sec; 95 °C, 30 sec (step 2-4 times 40 cycles). Standard software of Stratagen MxPro 3005P (Agilent Technologies, Santa Clara, CA, USA) was used to measure relative changes in mRNA levels.

2.8. Immunohistochemistry and immunostaining

Immunohistochemical stainings were performed for detection of markers of interest in the prostate tissue. Immunofluorescence and enzyme activity (Avidin-Biotin Complex) were used as detection systems. Sections were evaluated using light microscopy (Nikon, Tokyo Japan) or fluorescence microscopy (Zeiss Apotome Axiovert Fluorescence Microscope, Oberkochen, Germany) and pictures were taken at 10x and 40x magnification.

Tissue fixation and preparation of sections: Prostate tissues were fixed in Zinc fixative (0.1M Tris-HCl, 0.5g/L Calcium Acetate, 5g/L Zinc Chloride, 5g/L Zinc Acetatedihydrate) for 48h, dehydrated in a series of alcohols with increasing percentages and treated with xylene, and then embedded in paraffin. The paraffin blocks were cut in 5 µm sections using microtome and mounted on microscope glass slides (Thermo Scientific, Waltham,
MA, USA). Deparaffinated and rehydrated sections were washed in dH₂O three times for 2.5 min and placed in PBS pH 7.4 for 5 min.

**Immunostaining:** Fixation of sections to slide and antigen retrieval were performed following the optimized protocol for each antibody as specified below (see CD45, p-HH3 and HO-1, respectively). After antigen retrieval sections were washed twice for 5 min in PBS and then incubated with 7% Horse Serum diluted in PBS for 30 min to block unspecific binding. Primary antibodies were applied on each section for overnight incubation at +4 °C. The following day slides were washed 3 times in PBS followed by 10 min blocking in 0.5 % hydrogen peroxide solution. After an additional wash in PBS, biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) were diluted 1:300 in PBS and applied on each section and incubated at room temperature (RT) for 1h. Slides were washed twice for 5 min in 1x PBS. Avidin-Biotin peroxidase complexes were prepared from ABC-kit (VECTASTAIN®, Vector Labs) (2 drops of A, 2 drops of B in 10 ml PBS) and was let to stabilize at RT for 30 min and then applied on the slides and incubated at room temperature for 30 min. The slides were washed twice for 5 min in 1xPBS before addition of 3, 3’-diaminobenzidine (ImmPACT™ DAB Peroxidase (HRP) Substrate, Vector Labs) to develop the staining for 1-2 min. The color reaction was blocked in dH₂O for 5 min twice. Nuclei were counter-stained with hematoxyline and the sections were dehydrated in alcohol and xylene, covered in mounting medium and cover slips. The slides were evaluated microscopically (Nikon) and pictures were taken at 10x, and 40x magnification.

Marker specific procedures for immunostaining:

**CD45:** Deparaffinated, rehydrated and washed sections were cooled down in refrigerated PBS (4 °C) for 3 min. Antigen retrieval and fixation of tissue was performed putting the sections in cold acetone with 7 % formalin for 2.5 min. The slides were washed in cold PBS for 4-5 min and then transferred to 1x PBS at RT. Primary antibody: Rat Anti-mouse CD45 antibody (#553076 BD Pharmingen, San Diego, CA) 1:50 dilution in PBS. Secondary antibody: Biotinylated Rabbit Anti-Rat IgG (BA-4000, Vector Laboratories, Burlingame, CA, USA), 1:300 dilution in PBS.

**pHH3:** Deparaffinated, rehydrated and washed sections were fixed in 2% paraformaldehyde for 3 min and washed twice for 5 min in PBS. Sections were acidified in 0.1 M sodium citrate pH 6.0 for 5 min at RT and then transferred to warm solution of 0.1 M sodium citrate pH 6.0 (+93 °C) for 25 min. The container with hot citrate buffer and the slides were put in ice-water bath for 20 min to cool down. After antigen retrieval the slides were placed into RT PBS. Primary antibody: Rabbit anti-pHH3 (Ser10) #9701 (Cell Signaling, Beverly, MA, USA) 1:100. Secondary antibody: Biotinylated Goat Anti-Rabbit IgG (BA-1000, Vector Laboratories, Burlingame, CA, USA)

**HO-1:** Fixation and antigen retrieval were performed as above for pHH3 with exception of warm sodium citrate being applied for 15 min. Primary antibody: Rabbit anti-HO-1 (ab13248, Abcam, Cambridge, MA, USA) 1:100.
Secondary antibody: Biotinylated Goat Anti-Rabbit IgG (BA-1000, Vector Laboratories, Burlingame, CA, USA)

**Immunofluorescence staining:** Sections were fixed with 2% paraformaldehyde for 10 min following washing with PBS 3 times and permeabilization with 0.05 % Triton X-100 in PBS for 5 min and then washed twice for 5 min in 1xPBS. 7 % Horse Serum diluted in PBS was used for blocking for 30 min. Primary antibodies applied on each section: ACSL1 (D2H5) Rabbit mAb (#9189 Cell Signaling, Beverly, MA, USA) 1:300 and CD45: Rat Anti-mouse CD45 antibody (#553076 BD Pharmingen, San Diego, CA) 1:50. Sections were incubated with primary antibodies overnight at +4 °C. The following day the slides were placed in PBS twice for 5 min and incubated at RT in darkness for 1h with secondary antibodies diluted 1:300 (Anti-Rat Alexa Fluor® 488, anti-Rabbit Alexa Fluor® 594, Invitrogen/Life Technologies, Molecular Probes. After careful washing 2x5 min in PBS the nuclei were stained with Hoechst-33258 (Molecular Probes, Invitrogen) in dilution 1:10000. Slides were left for 5 min in PBS and then 5 min in MQ-H₂O before they were left to air-dry in darkness. The slides were covered with Gelvatol, (Sigma) and glass coverslips. Zeiss Apotome Axiovert Fluorescence Microscope was used to evaluate the fluorescence staining.

2.9. Statistical analyses

All values are expressed as the average ± SD. All experiments were performed in duplicates or triplicates. Mice number: n=3-6/group. Samples were analyzed using 1-way ANOVA followed by Tukey’s multiple comparison test or Student’s t test (2 tailed). A result was considered significant if the p value was lower than 0.05. Analyses were performed using SPPS13.0 and/or Microsoft Office Excel 2007.

3. Results

3.1. Establishment of in vivo model of pathogen-induced prostatitis

To investigate the role of myeloid-derived HO-1 and CO in pathogen-induced inflammation in the prostate in vivo, we established a mouse model of prostatitis by injecting live *E.coli* into the urinary bladder via a urethral catheter as previously described (65). We have first optimized a dose of bacteria and found that a dose of 2x10⁸ CFU *E.coli* suspended in 50 µl sterile PBS was sufficient to induce prostate inflammation. Experimental mice were injected intraurethrally (i.u.) with *E.coli* while control groups consisted of mice injected with the same volume of PBS or non-treated mice (naïve animals) (**Figure 1A**). Prostates from each group were harvested after 2h, 6h, 24h and 3 weeks after single dose of bacteria and were analyzed by immunoblotting, real time PCR and/or immunohistochemistry. There were no significant differences in the body weights between PBS versus *E.coli* groups 20 days after infection (**Figure 1B**).

Immunohistological staining revealed increased infiltration of CD45+ leukocytes into the prostate tissue (**Figure 1C-D**), primarily to the stroma, as early as after 6h in both PBS or *E.coli* treated groups in comparison
to naïve controls. The infiltration of CD45+ cells was further increased in prostates from *E.coli* injected mice at the 24h time point and was significantly higher than PBS group, with a sustained effect on leukocyte infiltration up to 3 weeks after a single injection of bacteria.

Further, we performed analyses of proliferation by immunostaining with antibody against a mitosis marker, phospho-Histone H3. Expression of P-HH3 showed was strongly induced after *E.coli* injection (Figure 1E-F). The number of P-HH3+ cells per FOV was elevated between 6h to 3 weeks in bacteria injected mice as compare to naïve animals. PBS injected mice also showed a significant increase in p-HH3 staining only after 24h as compare to normal tissue, but no difference was detected in earlier or later time points.

Real time PCR performed on the prostate tissues from mice injected with bacteria (Figure 1E) showed a marked increase in pro-inflammatory cytokine pro-IL-1β mRNA. In contrast, IL-10 expression was suppressed by *E.coli* as well as PBS groups as compare to normal prostate from naïve mice.

To evaluate signaling pathways that are implicated in the inflammatory processes in the prostate, we tested for MAPK and cytokine expression. We observed an induction of phospho-Erk1/2 and cMyc at 6h and 24h after injections of PBS or *E.coli* (Figure 1H), indicating activation of the MAPK-Erk1/2 signaling pathway, which is involved in proliferation and tissue regeneration in prostates of these mice. However, the result from the 3 weeks’ time-point was inconsistent and not taken into consideration, for example the naïve animal showed strong in induction of p-Erk1/2, indicating faulty biopsy harvesting. The established model was used to investigate the role myeloid derived of HO-1 in the settings of prostate inflammation.
Figure 1: Model of pathogen-induced prostatitis by transurethral instillation of *E. coli* in male wild type mice. (A). Illustration of the model established and used throughout the study. In the first step male WT mice (C57BL/6) were inoculated intraurethrally (i.u.) with *E. coli* M1655 (2x10^8 CFU/animal) or injected with PBS i.u. Controls used were catheterized but not injected (tubing) or naïve (untreated) mice. After procedure mice were kept at standard conditions until sacrifice; 2h, 6h, 24h and 3 weeks after injection, respectively. Prostates were harvested and analyzed for inflammation and proliferation by Immunohistochemistry (IHC) Immunoblotting and/or real-time (rt) PCR. In later experiments Mice were treated with carbon monoxide (CO), 250 ppm for 1h, starting 1h after injection, and were compared to mice kept in air. After establishment the model was also applied in conditional HO-1 knockout mice (*LysM-Cre:HOx1^fl/fl*) (All data shown in this figure represent wild type mice kept in standard conditions in air after procedure until time of euthanazation). (B). Left: Body weight records (grams) of mice from day 0 through day 20 after injection of PBS or *E. coli* (2x10^8 CFU/animal), each curve representing one individual. Right: Change in body weight (grams) in mice inoculated i.u. with *E. coli* versus PBS injected animals from day 0 to day 20 after procedure. (C-D) Quantification and representative pictures of CD45+ cells (leukocytes) in prostate 6h, 24h and 3 weeks after i.u. injection of PBS or *E. coli* (2x10^8 CFU/animal). (E-F) Number of CD45+ cells per field of view (FOV) in prostates from mice injected i.u. with PBS or *E. coli* (2x10^8 CFU/animal) versus Naïve controls at time points 6h, 24h and 3 weeks, respectively (n=2-3 individuals/group). Data shown as average ± SD. * P<0.05, ** P<0.01 *** P<0.001. (G). Expression of cytokines IL-1β (left) and IL-10 (right) presented as mRNA fold change in PBS or *E. coli* injected mice (2x10^8 CFU/animal) versus naïve controls (value normalized to 1) 2h after injection (n=3-4 animals/group). Data shown as average ± SD *P<0.05. (H). Immunoblot of phospho-Erk 1/2 and cMyc in prostate lysates from mice 6h, 24h and 3 weeks after i.u. injection of PBS or *E. coli* (2x10^8 CFU/animal). N=Naïve, β-Actin=loading control.
3.2. **HO-1 is induced in prostatitis and influences the infiltration of leukocytes to the prostate after intraurethral E.coli infection**

We found that HO-1 was induced in mice prostates early on (2h) after i.u. injection of PBS or *E.coli* in comparison to naïve individuals (Figure 2A). Cells positive for HO-1 were localized by IHC primarily to the stroma after 3 weeks (Figure 2B). Since our hypothesis suggest that HO-1 is present in immune cells in the prostate, we further investigated the role of myeloid-derived HO-1. We used conditional knockout mice lacking HO-1 specifically in innate immune cells (*LysM-Cre:Hmox1fl/fl*) and compared the number of CD45+ and p-HH3+ cells per FOV (Figure 2C-F) in these animals with wild type mice after challenge with bacteria or PBS i.u. as above. Quantification of IHC staining from mice 3 weeks after injection showed that the infiltration of CD45+ cells was augmented in *LysM-Cre: Hmox1fl/fl* mice as compare to *Hmox1fl/fl* mice in both PBS and *E.coli* exposed group (Figure 2C-D). In contrast, the rate of mitosis, represented by p-HH3+ cells, was independent on the HO-1 levels in the myeloid cells (Figure 2E-F).
Figure 2: Role of myeloid-derived HO-1 in prostate inflammation. (A-B) Immunoblotting IHC staining of HO-1 in inflamed prostate from wild type mice, 2h and 3 weeks after intraurethral (i.u.) injection of PBS or bacteria, respectively. (A). Relative quantity of HO-1 measured by immunoblotting of prostate lysates from wild type mice sacrificed 2h after i.u. instillation of *E. coli* (2x10⁸ CFU) or injection of PBS, compared to naïve controls. β-Actin=Loading control (B). Immunohistochemical staining of HO-1 in prostate tissue from naïve mouse versus animals harvested 3 weeks after induction of prostatitis via i.u. injection of PBS and *E. coli*, respectively, performed as in A. Examples of stained cells are indicated by arrows. (C-D) Quantification and representative pictures of CD45+ cells in prostates from conditional myeloid-specific HO-1 knock-out mice (*LysM-Cre:Hmox¹⁺⁻¹⁻*) compared to *Hmox¹⁺⁻¹⁻* (WT) mice, 3 weeks after PBS or *E. coli* injection i.u. (2x10⁸ CFU/animal). (C). Number of CD45+ cells per FOV in prostate, 3 weeks after i.u. injection of PBS or *E. coli* in *Hmox¹⁺⁻¹⁻* versus *LysM-Cre:Hmox¹⁺⁻¹⁻* mice (n=2-3 animals/group). Results shown as average ± SD, *P<0.05, *P<0.001. (D). Representative pictures of CD45 staining in mouse prostate as described in C, positive cells are indicated by arrows. (E-F) Quantification and representative pictures of p-HH3+ cells in wild type (*Hmox¹⁺⁻¹⁻*) versus conditional HO-1 knock out mice (*LysM-Cre:Hmox¹⁺⁻¹⁻*), treated as in C. Results shown as average ± SD, **P<0.01, ***P<0.001. (E). Number of p-HH3+ cells per FOV in mice prostates as indicated in C. (F). Representative pictures of p-HH3 staining in mice described in D. Arrows indicate stained cells.
3.3. CO suppresses proliferation in the prostate

In this study we investigated the potential of CO to regulate inflammation in the prostate. We applied CO at 250 ppm for 1h, starting 1h after injection of bacteria i.u. Immunohistochemical staining with antibody against p-HH3 indicated a decreased number of mitotic cells in the prostates from CO treated mice as compare to control mice at 3 weeks after injection of PBS or E.coli (Figure 3A-B). This effect correlated with lower activation of p-Erk1/2 in the prostate lysates of CO treated mice after injection of bacteria as visualized with western blot (Figure 3C). Staining of CD45 in prostate did not show any significant difference in numbers of infiltrating leukocytes between CO treated groups and Air controls after 3 weeks (Figure 3D-E). However, we noticed changes in levels of pro-IL-1β in the prostate lysates of Air versus CO treated animals analyzed by immunoblotting. Induction of pro-IL-1β was observed in prostates of CO treated individuals after 6h. After 24h, the pro-IL-1β had disappeared in the group of mice exposed to CO, while the Air treated animals showed induction of named pro-cytokine. The cleaved, active form of IL-1β did not seem to follow the changing concentrations of its precursor at the time-points tested. However, this might be due to detection problems of too low amounts of the target protein in the lysates. (Figure 3F). Intriguingly, these data show that CO in this context suppresses proliferation and tissue regeneration in the inflamed prostate. In addition, a faster induction and following suppression of the pro-inflammatory cytokine IL-1β precursor in response to CO treatment indicates an effect on immune cell phenotype and/or activation, possibly leading to more effective resolution.
3.4. Inflammation in the prostate induces lipid metabolic enzymes: Long chain acyl-CoA synthetase (ACSL1) and fatty acid synthase (FAS) – effect of CO and HO-1

Lipid metabolic enzymes such as ACSL1 regulate immunological reactions. In our study we asked a question whether expression levels of lipid metabolic enzymes were affected by pathogen-induced prostatitis. Immunoblots showed induction of ACSL1 and FAS as early as 6h after injection, in comparison to naïve animals, with a stronger increase in *E. coli* exposed mice compared to PBS group (Figure 4A-B). Similar
induction in expression of ASCL1 and FAS was observed after 24h and 3 weeks post-infection. FAS showed the strongest induction after 24h. Further, immunofluorescence staining of ACCL1 and CD45 (Figure 4C-D) showed that residential CD45+ leukocytes were positive for ACCL1 in normal prostate (Figure 4C). In inflamed tissue, ACCL1 was strongly induced in prostate epithelium 24h after injection of PBS and even more pronounced in the E.coli group (Figure 4D). The 3 week time-point yet needs to be evaluated.

Further, we tested whether inhaled CO has any effect on the lipid metabolic enzymes and therefore we performed immunoblotting on prostate lysates from CO treated mice, as described earlier, and compared them to Air control mice (Figure 5A-B). Early induction of ACCL1, observed 2h after injection of E.coli, was almost totally abolished in CO treated mice as compared to controls (Figure 5A). Inhibition of ACCL1 was also seen in CO treated mice after 24h and 3 weeks (Figure 5B), even though it was not as strong as for the earliest time-point tested. FAS was not changed in the early time point after application of CO, but was inhibited after 3 weeks, both in PBS and E.coli treated mice. The 6h time-point is still under analysis.

To evaluate the role of HO-1 in the model of prostatitis we analyzed the expression of ACCL1 and FAS in prostates from LysM-Cre:Hmox1fl/fl 24h after injection of bacteria or PBS (Figure 5C). We did not see any difference in ACCL1 protein levels between knock-out and wild type mice. FAS expression was strongly expressed in wild type PBS as compared to knock-out mice, while it was slightly lower in wild type as compared to LysM-Cre:Hmox1fl/fl in group of mice injected with E.coli. The 2h, 6h and 3 weeks-time points remain to be analyzed.
Anchorage independent growth of PC3 and PNT1A cells, respectively, is enhanced by E.coli induced macrophage derived factors, but can be suppressed in the former cell type by CO treatment.

We wanted to investigate the potential of macrophage derived factors to promote anchorage independent growth, a characteristic of transformed and malignant cells, in the prostate cell lines PC3 (metastatic lesion of PCa) and PNT1A (transformed, but pre-malignant prostate cells). Preliminary results from quantifications of PC3 colonies cultured in soft agar showed that wells with cells treated with supernatant (for 18 days) from E.coli stimulated (10 MOI for 2h) WT BMDMs had significantly higher number of colonies compared to RPMI treated control (P<0.001) and supernatant from unstimulated BMDMs (P<0.05). However, the increase in PC3 colony number due to supernatant was suppressed when the BMDMs were treated with CO (250 ppm for 1h) starting 1h after infection with E.coli P<0.001. Figure 6A-B). PNT1A cells grown as described above were generally more slow-growing and smaller in size than PC3 cells, but showed a similar pattern with increased number of colonies in the soft agar after 31 days of treatment with supernatant from E.coli activated BMDMs, compared to control P<0.001, and compared to sterile BMDMs P<0.05, but in contrast the increased potential of
anchorage-independent growth in the BMDM + *E. coli* supernatant treated group of PNT1A was not suppressed by CO treatment of the macrophages prior to harvest of supernatant (**Figure 6C-D**).
first-line treatment in the guide-line from the Prostatitis Expert Reference Group (PERG) primarily provide relief from pain and symptoms in patients with prostatitis, but often fail to cure the disease permanently (125). To target the immune system itself and redirect skewed immune responses might be a new strategy to help patients suffering from chronic prostatitis. This requires deeper understanding of the underlying mechanisms of the disease and also more attention in the literature.

We investigated the role of myeloid-derived HO-1 and the effect of exogenously applied CO in pathogen-induced non-lethal prostatitis. We established a mouse model of bacterial prostatitis by inoculating male mice (7-10 weeks old) with live E.coli MG1655 (2x10⁸ CFU/animal) transurethrally. This method has been previously used with success (65, 67), causing evident prostatic inflammation with increased influx of inflammatory cells, proliferation of cells and finally transformation of prostate epithelium into pre-cancerous PIN lesions. Our results confirmed infiltration of CD45+ leukocytes, expression changes in cytokine (IL-1β, IL-10) and activation of MAPK signaling pathway, which is involved in proliferation. We also noted an increased cell division (p-HH3+ cells) in the prostates in animals injected with E.coli for 6h. Sustained inflammation was observed up to 3 weeks after injection, which indicates chronic prostatitis in these mice. Interestingly, mice injected with PBS also showed signs of inflammation after 24h, but the immune response was transient and was not significantly different from naïve controls after 3 weeks. This was earlier noted in mice by Kwon and colleagues (65), who explained the effect as a consequence of physical injury caused by the catheterization, urine reflux and increased pressure in the prostate due to injection of liquid. We noted that in order to minimize the risk of back-ground inflammation in the prostate all injections should be performed with great care and the solution should fulfill physiological conditions of the local environment in case of osmolarity and pH. Since PBS is sterile, yet induces urine reflux to the prostate upon the injection, it might represent a model of sterile prostatitis.

Despite the transient activation of inflammatory responses by injection procedure, the transurethral injection does more adequately resemble the urinary tract infection pathology in humans than does the alternative method: direct intra-prostatic injection that is also used in prostatitis models in vivo. In addition, the intra-prostatic injection increases the risk of complications due to open surgery and also causes great tissue trauma and hemorrhage in the prostate after the procedure.

HO-1 activity in macrophages has been documented to act differently on inflammatory processes depending on the cellular context, being able to trigger lytic function in immune cells but also promote immunosuppressive cells, such as T regulatory cells and inhibit antigen presenting cell maturation and function (126-129). In our study, we showed induction of HO-1 in the prostate after injection of PBS or E.coli and localization to the stroma, indicating activation of stromal cells in response to the tissue insult. Yet, this data is preliminary and needs to be confirmed by complementary analyses and further investigated in terms of different time points and additional animals. Technical issues regarding western blots and immunohistochemistry left the experiment to be completed after trouble-shooting of staining methods. Further, when applying the model in mice with HO-1
deficient myeloid cells we observed an increase in inflammatory infiltrates in these mice as compared to wild type controls indicating an impact of myeloid derived HO-1 on inflammatory responses in the prostate. However, further experiments are needed to explain the mechanism behind the role of HO-1 in this model. Early induction of IL-1β after infection and even higher levels in CO treated mice are in accordance with earlier results from our group (96), suggesting HO-1 and CO as a co-stimulatory signal for macrophage activation in response to bacteria. Our results indicate CO triggering an earlier burst of pro-IL-1β protein, shown after 6h in comparison to Air treated controls, where the cytokine is not induced until 24h after bacterial challenge. An increased influx of immune cells in HO-1 knockout mice might indicate that HO-1 activity mediate the initial phase of bacterial clearance (95, 130), inhibiting development of acute prostatitis. The rate of proliferation was not associated with lack of myeloid derived HO-1. Proliferation and differentiation in the prostate are both regulated by steroid hormones via a complex paracrine signaling between stromal cells and epithelium (63, 131). Our studies suggest that expression of HO-1 might not regulate proliferation yet influence other changes in the cells that contribute to carcinogenesis. Additional studies how the heme degradation pathway may have effect on prostate cell proliferation are required to investigate the cross-talk between androgen signaling and metabolic pathways.

Even though lack of myeloid derived HO-1 was not shown to affect the proliferation rate, inhalation of CO for 1h after induction of prostatitis was able to suppress cell division in wild type mice injected with bacteria in the 3 weeks’ time point. Earlier, CO has been shown to promote regeneration of injured tissues (89, 93), however these results originate from studies using inflammations induced by heme toxicity and trauma. HO-1 and CO work in a situation-dependent manner to favor host survival, potent of initiate different types of responses. CO might act to restore homeostasis by suppressing proliferation and prevent development of hyperplasia and sequential PIN lesions. Indeed CO suppressed growth of prostate cancer and PIN development in TRAMP prostate cancer model in mice (111).

Fatty lipid metabolism enzymes ACSL1 and FAS have been shown to be liked to macrophage activation and inflammation in diabetic mice (123) as well as to prostate cancer (116). In normal, non-inflamed tissue we found that ACSL1 was almost exclusively expressed in CD45+ leukocytes, indicating a role of this enzyme in the function of residing tissue macrophages. We also observed strong induction of ACSL1 and FAS in the epithelial cells after bacterial challenge. These results indicate that FAS and ACSL1 are regulators of the inflammatory process in the prostate following bacterial infection. Interestingly, the induction of ACSL1 was rapidly inhibited by CO treatment, which indicates that CO might regulate the inflammation, at least in part, through the lipid metabolic enzyme ACSL1. We have not established whether CO works directly on the epithelium or only on macrophages, as indicated in a preliminary figure illustrating our hypothesis (Figure 7). CO mediated suppression of proliferation might be mediated in part through lipid metabolic enzymes. If CO inhibits activation and production of fatty acids which are utilized for diverse anabolic processes in the epithelial cells, it might regulate their division directly by re-directing their metabolism. We have previously shown that CO targets glucose metabolism in cancer cells and thus blocks their proliferation (111).
Deregulation of the macrophage polarization status is known to play a role in chronic inflammation and cancer. Reprogramming of tumor associated macrophages into cancer promoting phenotype by cancer cells has been described in numerous studies. Anchorage-independent growth is a characteristic of malignant cells and preliminary data from one experiment showed that transformed prostate epithelial cells (PNT1A) and prostate adenocarcinoma cells (PC3) tended to grow larger colonies in soft agar, after treatment with supernatant from BIDMCs treated with E.coli for 2h than from non-stimulated macrophages. This indicate that factors released from bacteria-induced M1 macrophages are able to accelerate the malignant phenotype of prostate epithelial cells in different stages of transformation. Similar effects were previously noted by Helm et al. in a model of pancreatic cancer, where pro-inflammatory macrophages potently induced epithelial-mesenchymal-transition in transformed cells (77). CO treatment of bacteria-stimulated BIDMCs suppressed the growth of PC3 colonies, but not PNT1A colonies treated with this supernatant, as compared to Air treated control group, further supporting the theory of CO mediated inhibition of prostate proliferation, which we observed in our in vivo model of pathogen-induced prostatitis. This result indicates that CO effect on macrophages only, is able to cause regression of prostate adenocarcinoma cells. However, this experiment has to be repeated with more extensive analysis of supernatant content, macrophage activation and of the cellular response in prostate cells to the treatment.
5. Conclusion

In summary, our results suggest that HO-1 in myeloid cells might have a regulatory function in the established model of bacterial infection, by decreasing the number of leukocytes present in the prostate. Further studies warrant investigation on the role of HO-1 in specific cell types, signaling pathways, inflammatory factors and proliferative responses of epithelial cells in the inflamed prostate. We provide the first evidences that CO treatment might have beneficial effect on inhibiting inflammation in the prostate, yet further proof-of-concept studies are required to evaluate potential therapeutic effects of CO in prostatitis.

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