Early heme oxygenase 1 induction delays tumour initiation and enhances DNA damage repair in liver macrophages of Mdr2−/− mice

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Multi drug resistance protein 2 knockout mice (Mdr2−/−) are a mouse model of chronic liver inflammation and inflammation-induced tumour development. Here we investigated the kinetics of early heme oxygenase 1 (HO-1) induction on inflammation, tumour development, and DNA damage in Mdr2−/− mice. HO-1 was induced by intraperitoneal injection of cobalt protoporphyrin IX (CoPP) twice weekly for 9 consecutive weeks. Immediately after HO-1 induction, liver function improved and infiltration of CD4+ and CD8+ T cells was reduced. Furthermore, we observed increased p38 activation with concomitant reduction of Cyclin D1 expression in aged Mdr2−/− mice. Long-term effects of HO-1 induction included increased CD8+ T cell infiltration as well as delayed and reduced tumour growth in one-year-old animals. Unexpectedly, DNA double-strand breaks were detected predominantly in macrophages of 65-week-old Mdr2−/− mice, while DNA damage was reduced in response to early HO-1 induction in vivo and in vitro. Overall, early induction of HO-1 in Mdr2−/− mice had a beneficial short-term effect on liver function and reduced hepatic T cell accumulation. Long-term effects of early HO-1 induction were increased CD8+ T cell numbers, decreased proliferation as well as reduced DNA damage in liver macrophages of aged animals, accompanied by delayed and reduced tumour growth.

Chronic inflammation of the liver, e.g. by hepatitis B or C virus infections, initiates wound healing processes and immune responses which promote development of hepatocellular carcinoma (HCC)1,2. During the course of chronic inflammation, permanently activated regenerative processes increase the risk of mutations, as well as chromosomal aberrations and DNA damage1. Mdr2−/− mice are a widely used model of inflammation-induced HCC. These animals lack the multi drug resistance protein 2 (MDR2), a phosphatidylcholine transporter, resulting in dysfunctional phospholipid secretion and increased oxidative stress, which in turn leads to chronic liver damage, leukocyte infiltration, fibrosis, and over time to HCC development3,4.

Immune cells mediate pro- or anti-carcinogenic effects depending on cell type and environment. Myeloid cells for example, can promote carcinogenesis through regulation of senescence, angiogenesis, extracellular matrix remodelling, or metastasis formation5. Furthermore, a majority of HCC patients display defects in dendritic cell (DC) maturation, which eventually impairs T cell responses6. Alterations of the adaptive immune response in HCC patients include a reduced CD4+ T cell response and impaired CD8+ T cell function7,8.

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Cyclin D1 signalling, which may regulate marrow-derived macrophages (BMDMs) from Mdr2−/− mice. Following silencing of HO-1 reduced tumour growth in an orthotopic liver tumour mouse model. In contrast, HO-1 reduces tissue damage by suppressing the activation and cytokine production of heme sensing macrophages, polymorphonuclear cells, and DCs. HO-1 activity inhibits T cell activation in various mouse models, including promotion of T cell tolerance against transplanted organs. Whether HO-1 attenuates inflammation and subsequent tumour development, or promotes adverse effects such as suppression of tumour-specific immune responses appears to highly depend on the time point of induction.

The present study investigated short- and long-term effects of early HO-1 induction on liver function, proliferation, DNA damage, and DNA damage repair (DDR) and tumour growth in Mdr2−/− mice. Next to short-term effects of early HO-1 induction on liver damage and T cell infiltration, we observed a lasting downregulation of Cyclin D1 expression most likely via p38 activation. We also found severe DNA damage especially in hepatic macrophages of aged Mdr2−/− mice, which was significantly reduced in CoPP treated animals. In addition, bone marrow-derived macrophages (BMDMs) from Mdr2−/− mice, in contrast to WT and CoPP treated Mdr2−/− mice, failed to induce HO-1 as a stress response upon DNA damage. CoPP treatment attenuated DNA damage in vitro and in vivo as well as it reduced phagocytic activity of macrophages, which suggests reduced oxidative stress in hepatic and BM derived macrophages as a consequence of HO-1 induction. Taken together, early HO-1 induction interfered with several inflammatory and tumour promoting processes.

Results

Long-term effects of early HO-1 induction in Mdr2−/− mice. To investigate long-term effects of early HO-1 induction, Mdr2−/− mice were treated with either PBS or CoPP for 9 weeks (week 5–14) and sacrificed for analysis at different time points throughout a period of 65 weeks (Supplementary Fig. 1A). Analysis of Ho-1 mRNA expression confirmed successful induction of HO-1 in 14-week-old Mdr2−/− mice treated with CoPP (Fig. 1A). Subsequently, the Ho-1 mRNA expression did not differ between control and CoPP treated Mdr2−/− mice. However, CoPP treated mice displayed a significantly higher body weight gain than control treated animals up to one month after termination of treatment, indicating an improved overall health (Fig. 1B). PBS treated Mdr2−/− mice presented with increasing liver damage over time, demonstrated by rising plasma levels of ALT, which was only marginally reduced in CoPP treated animals (Fig. 1C). The strongest effect of CoPP treatment was seen in 24-week-old animals. However, plasma levels of albumin were significantly elevated in CoPP treated Mdr2−/− mice until week 48, indicating improved liver function in response to HO-1 induction (Fig. 1D). Flow cytometric analysis (Fig. 1E; gating strategy in Supplementary Fig. 1B) revealed significantly decreased CD4+ and CD8+ T cell infiltration into the injured liver of 14-week-old CoPP treated mice compared to PBS treated mice. Aged PBS treated Mdr2−/− mice displayed strongly decreased numbers of CD4+ T cells compared to the 14-week-old mice. The CoPP treated mice on the other hand showed only a slight reduction of CD4+ T cells over time and the same number of cytotoxic CD8+ T cells at 65 weeks of age. Since CD8+ T cells are known to play an integral part of the anti-tumour response and were decreased in 65-week-old PBS treated Mdr2−/− mice (Fig. 1E), the significantly increased number of CD8+ T cells in aged Mdr2−/− mice treated with CoPP may indicate a less tumour susceptible microenvironment.

Early HO-1 induction delays tumour development in Mdr2−/− mice. Chronic hepatic inflammation and fibrosis in Mdr2−/− mice results in tumour development within approximately one year of age. Long-term effects of early HO-1 induction on tumour development were monitored by magnetic resonance imaging (MRI). The cumulative tumour volume was measured for each mouse at different time points, starting from the first appearance of dysplastic nodules (48 weeks) until the mice were sacrificed at 65 weeks; an exemplary image sequence of MRI scans over time is presented in Fig. 2A. CoPP treated animals showed significantly decreased mean tumour volumes from week 50 to 58 compared to control treated mice (Fig. 2B). Histological analysis of dysplastic nodules in younger mice (up to 50 weeks) as well as of tumour tissue from older mice (50+ weeks) revealed no phenotypical differences of malignancies derived from PBS and CoPP treated animals. This is probably due to the formation of numerous tumours per animal along with a great variance in histomorphology. PBS as well as CoPP treated animals displayed tumours with varying morphology, vascularization, fat content and differentiation state. Nevertheless, early HO-1 induction successfully delayed malignant growth for over 2 months.

Early induction of HO-1 in Mdr2−/− mice may interfere with hepatocellular proliferation. Tumour development is driven by cell cycle activation and tumour cell proliferation which is regulated by cyclins such as Cyclin D1. We found previously that HO-1 induction reduces Cyclin D1 mRNA expression in young Mdr2−/− mice (12 weeks) treated with CoPP. Here we show that early HO-1 induction resulted in sustained suppression of Cyclin D1 expression over months after termination of CoPP treatment (Fig. 3A). Furthermore, we found increased protein expression and phosphorylation of p38 (p-p38; Fig. 3B,C) a known negative regulator of Cyclin D1 expression in older mice (36 weeks). Interestingly, in 65-week-old animals the p-p38/p38 ratio is still elevated (Fig. 3D,E). These findings suggest that early HO-1 induction has a long-term effect on MAP-Kinase signalling, which may regulate Cyclin D1 mediated proliferation.
HO-1 induction reduces DNA damage in macrophages in vitro and in vivo. Malignant transformation is associated with accumulation of mutations, DNA damage and altered capacity of DDR over time. We therefore investigated DNA damage in Mdr2−/− mice by the assessment of γH2AX+ cells in liver tissue sections of aged animals. γH2AX+ nuclei were present in F4/80+ macrophages or HNF4-α+ hepatocytes (Fig. 4A,B) in tissue
sections of 65-week-old WT and Mdr2−/− mice. While we observed DNA damage in few hepatocytes, γH2AX+ nuclei were predominantly found in F4/80+ macrophages in Mdr2−/− mice. Interestingly, CoPP-treated Mdr2−/− mice displayed significantly reduced numbers of γH2AX+ macrophages compared to control treated animals (Fig. 4C). By contrast, HNF4α+ hepatocytes did not show a significant change in DNA damage occurrence upon CoPP treatment.

To verify that DNA damage accumulated in macrophages rather than hepatocytes, we stained tissue sections of 14- as well as 36-week-old Mdr2−/− mice (Supplementary Fig. 2A,B) and additionally performed western blot analysis of isolated hepatic macrophages and hepatocytes from these mice (Supplementary Fig. 2C). In both cases we observed little overall DNA damage in 14-week-old Mdr2−/− mice, which increased with age (36-week-old animals) in macrophages and to a much smaller extent also in the hepatocytes.

In order to investigate the functional consequences of DNA damage in macrophages, we induced DNA double-strand breaks (DBS) in BMDMs of WT and Mdr2−/− mice (PBS vs CoPP treated [10 µg/ml; 24 h]) by gamma irradiation [6 Gy]. WT macrophages were able to significantly induce Ho-1 expression upon irradiation (Fig. 4D), whereas Mdr2−/− mice derived BMDMs displayed a reduced basal expression of Ho-1 and failed to induce it as a stress response to irradiation. Mdr2−/− mice derived BMDMs pre-treated with CoPP in vitro showed increased Ho-1 expression compared to untreated Mdr2−/− BMDMs and further increased its expression upon irradiation. We also investigated the basal DNA damage in BMDMs from 14-week-old (Supplementary Fig. 2D) and 65-week-old (Fig. 4E) WT or Mdr2−/−, mice with or without CoPP treatment in vitro and in vivo (14-week-old). A frequency distribution of γH2AX+ foci per cell illustrates significant differences between WT and Mdr2−/−, as well as CoPP treated vs. control treated Mdr2−/−-derived BMDMs. BMDMs derived from 65-week-old Mdr2−/− mice showed a significantly higher number of γH2AX+ foci per cell compared to WT derived BMDMs. HO-1 induction for 24 h in vitro significantly decreased the number of γH2AX+ foci per cell in Mdr2−/− mice derived BMDMs (Fig. 4E). BMDMs isolated from 14-week-old Mdr2−/− mice confirmed a similar reduction of γH2AX+ foci per cell when mice were treated with CoPP in vivo (week 5–14) to those treated in vitro (CoPP treatment for 24 h) (Supplementary Fig. 2D).

A further indicator of altered macrophage function in response to early HO-1 induction was seen in the significantly decreased hepatic expression of Cd36 in CoPP treated animals throughout the first 48 weeks of life (Fig. 4F). As CD36 can mediate phagocytosis of macrophages24, we further investigated the phagocytic capacities
of BMDMs from WT and Mdr2−/− mice. BMDMs derived from Mdr2−/− mice, showed a significantly reduced phagocytic activity after HO-1 induction (CoPP [10 µg/ml] for 24 h) (Fig. 4G, gating strategy in Supplementary Fig. 1C, representative dot plots are presented in Supplementary Fig. 3). These findings imply that early HO-1 induction has a persistent effect on DNA integrity and phagocytic activity of macrophages in Mdr2−/− mice.

Discussion

Chronic liver inflammation is a major health issue and often associated with HCC development, which is the second most frequent cause of cancer related death worldwide25. Apart from surgical procedures and irradiation, only the multikinase inhibitor sorafenib is known to prolong survival of advanced HCC patients for about 3 months26. A better understanding of immune mediated processes and molecular changes during tumour development is therefore essential to improve treatment options for HCC patients.

We showed previously, that HO-1 induction in Mdr2−/− mice not only reduced inflammation and subsequent fibrosis, but also induced fibrolysis and attenuated the amount of dysplastic nodules in female Mdr2−/− mice up to 19 weeks of age16. Here, we monitored short- and long-term effects of HO-1 induction (week 5–14) until one year after termination of treatment. Early induction of HO-1 had broad beneficial effects on the overall health. The improved body weight gain and albumin release are strong indicators of an improved health status and liver function in response to CoPP treatment. However, serum ALT activities remained unchanged at most of the time points investigated. This might have been due to the fact that HO-1 function does not interfere with the underlying liver pathology of Mdr2−/− mice based on accumulation of toxic bile salts. Rather, HO-1 induction interferes with subsequent events, such as inflammation and fibrosis16 which are known to drive tumour progression5,27.

An immune inhibitory effect was demonstrated by significantly reduced numbers of CD4+ and CD8+ T cells in liver tissues of young Mdr2−/− mice immediately after HO-1 induction. In contrast, 65-weeks-old tumour bearing Mdr2−/− mice showed significantly increased numbers of CD8+ T cells in response to early HO-1 induction compared to PBS treated animals, which likely contributes to a more efficient anti-tumour immune response.

In line with that and our previous data16, we demonstrated beneficial long-term effects of early HO-1 induction on proliferation and tumour growth. Although CoPP-treated Mdr2−/− mice were not completely protected from tumour development, the tumour volume was significantly reduced and delayed due to early HO-1 induction.
We observed no effect of early HO-1 induction on formation of dysplastic nodules at the pre-cancerous stage at 48 weeks of age, although CoPP treatment successfully delayed the progression to the malignant state and significantly reduced tumour growth for over two months. We speculate that the equal amount of dysplastic tissue at week 48 is associated with specific features of disease progression in the Mdr2 mouse model. Katzenellenbogen et al. demonstrated, that the Mdr2−/− phenotype is dominated by a strong inflammatory response in young animals (3 months), which is less pronounced at 12 months of age. Therefore, they concluded that dysplastic nodule...
formation at 12 months of age is most likely not a direct result of overwhelming inflammatory activity, but of aberrant hepatocyte cell death resulting from either leakage of toxic bile acids or non-direct immune mediated mechanism. Therefore one could speculate that at 48 weeks of age, the anti-inflammatory effect of early HO-1 induction has little effect on the underlying cause of dysplasia. Another possibility is that small differences in dysplastic nodule formation cannot be quantified by histological analysis. Since early CoPP treatment clearly delayed tumour growth in mice older than 48-weeks, we therefore hypothesize that the immune modulatory effect of CoPP is more prominent and allows a more efficient anti-tumour immune surveillance. This is in line with the fact that while 65-week-old PBS treated animals show significantly decreased populations of hepatic CD4+ and CD8+ T cells, whereas CoPP treated Mdr2−/− mice were able to retain almost equal numbers of hepatic T cells over time.

Furthermore, during the early phase of liver disease HO-1 function may alternatively affect regenerative proliferation in Mdr2−/− mice through interfering with Cyclin D1 expression up to 5 months after termination of treatment. This is in line with previous reports of anti-proliferative functions of HO-1 in different mouse models. In addition, we observed HO-1 mediated induction of p38 protein expression and phosphorylation, which may negatively regulate Cyclin D1 expression up to eight months after CoPP treatment. Chronic regenerative proliferation and exposure to oxidative stress increases the risk of accumulating mutations and chromosomal aberrations. Once the regenerative capacity is exhausted, chromosome uncapping induces DNA damage signals, senescence, or apoptosis. To our surprise, DNA damage increased over time in F4/80+ hepatic macrophages, and much less in hepatocytes. HO-1, as well as its product CO, are known to be essential for DDR after treatment with cytostatic agents or irradiation. Either overexpression of HO-1 or application of CO has been shown to accelerate DDR through phosphorylation of ataxia-telangiectasia mutated protein and downstream repair processes, while the absence of HO-1 results in high levels of DNA damage in various tissues and decreased DDR. We observed a reduced basal Ho-1 mRNA expression in Mdr2−/− mice derived BMDMs and an inability to upregulate Ho-1 expression as a stress response. This shows that once DNA damage occurs these macrophages cannot initiate DDR via HO-1. DNA damage in hepatic macrophages has been associated with a more pro-inflammatory phenotype. Under physiological and pathophysiological conditions, macrophages are essential for the systemic response to DNA damage. Macrophages have been shown to enhance existing DDR networks and accelerate double strand break repair in neighbouring cells during tissue injury. This is an essential tumour preventing function that might be altered in macrophages with DNA damage.

The involvement of macrophages in all stages of carcinogenesis has been analysed extensively. It has been shown that depending on the microenvironment macrophages can promote malignant transformation in pre-neoplastic lesions, tumour associated neo-angiogenesis, invasion, and metastasis formation. Therefore, maintenance of accurate macrophage function is of great importance in regard of tumour development. DNA damage in macrophages is often the result aberrant ROS production. Increased ROS production in macrophages is associated with phagocytic activity, since they are essential for the efficient break down of internalized materials. Another link between DNA damage and phagocytic activity of macrophages has been demonstrated by Pinto et al., who showed that irradiated macrophages show increased DNA damage, which led to an increased phagocytic activity and modulated the cytokine production profile of these cells. Furthermore, they could show that while accumulation of DNA damage was associated with a more inflammatory phenotype, these cells retained the ability to promote cancer cell invasion and tumour associated angiogenesis. It can therefore be assumed, that the accumulation of DNA damage in hepatic macrophages contributes to the exacerbation of inflammation in PBS treated Mdr2−/− mice, whereas early HO-1 induction reduces inflammation, counteracts ROS production and overall protects genomic stability and functionality of liver macrophages.

Taken together we conclude that early HO-1 induction not only improved chronic inflammation throughout treatment, it mediated anti-inflammatory, anti-tumour effects and DDR in Mdr2−/− mice throughout life. We showed that early induction of HO-1 during chronic inflammation lasting altered the cellular composition of the adaptive immune response. MR imaging confirmed that HO-1 induction delayed tumour growth. Moreover, early induction of HO-1 reduced the expression of Cyclin D1, possibly by inducing p38 expression and phosphorylation. On the other hand, HO-1 induction reduced DNA damage in macrophages, which may inhibit their pro-inflammatory phenotype and thereby reduce their tumour promoting function, which overall led to the delayed tumour growth. Collectively these HO-1 mediated effects ameliorated chronic inflammation and delayed tumour development in Mdr2−/− mice.

One would not prescribe CoPP as a drug for patients as it contains cobalt, but a variety of approved drugs and compounds induce HO-1 in addition to their pharmacological function. The widely prescribed group of statins as well as curcumin have been shown effective in treating various diseases as well as being effective inducers of HO-1. We therefore conclude it is worth considering HO-1 inducing compounds as an additional therapeutic option in early chronic liver diseases.

Material and Methods

Animals. For all experiments female Mdr2 knockout (Mdr2−/−; FVB.129P2-Abcb4tm1Bor) mice were used, which display a more severe pathology than male mice. FVB/N (WT) mice served as controls. All mice received care according to the FELASA guidelines; the animal protocols were approved by the Hamburg Federal Authority for Health and Environment. Mice were housed in IVC cages under controlled conditions (22 °C, 55% humidity, and 12-hour day-night rhythm) and fed a standard laboratory chow (LASvendi, Soest; Altromin, Lage, Germany).

HO-1 induction in vivo. For the induction of HO-1, Mdr2−/− mice were injected intraperitoneally with 5 mg/kg Cobalt protoporphyrin IX (CoPP; Frontier Scientific Europe, Ltd., UK) or PBS twice weekly, for nine weeks (5 to 14 weeks of age). CoPP is a well-established non-substrate inducer of HO-1, proven to be effective in vitro.
and in vivo. At the age of either 14, 24, 36, 48 or 65 weeks mice were sacrificed for analysis. Non-invasive analyses were performed throughout life.

**Determination of plasma transaminase activity and albumin levels.** Plasma enzyme activity of alanine aminotransferase (ALT) and plasma levels of albumin were determined as described previously.

**Isolation of Hepatocytes.** Hepatocytes were isolated as described previously.

**Isolation of hepatic macrophages.** Hepatic non-parenchymal cells were isolated as described previously. F4/80⁺ were enriched by magnetic activated cell sorting using a biotinylated anti-F4/80 antibody (Biolegend; San Diego, CA) and Streptavidin coated magnetic beads (Milteny Biotec, Bergisch Gladbach, Germany).

**Hepatic protein isolation and western blot analysis of p38.** Protein lysates were prepared and analysed via western blotting as described previously. Antibodies are summarized in Table 1.

**Western blot analysis of γH2AX in hepatocytes and hepatic macrophages.** Total protein was extracted from isolated hepatocytes and hepatic macrophages. 40 µg of total protein were resolved by SDS-PAGE using a 4–15% gradient gel (Bio-Rad, Hercules, CA). After transfer to a Nitrocellulose membrane (Licor, Lincoln, NE), proteins were detected by Anti-phospho-Histone H2A.X (Ser139) Antibody, (1:2000; Millipore Burlington, MA) and anti-β-actin (Sigma, 1:20000) and IRDYE 680RD conjugated anti-mouse IgG (1:7500; Licor, Lincoln, NE) was used. Antibodies are summarized in Table 1.

**Analysis of mRNA expression.** Isolation of total RNA, cDNA synthesis and real time RT-PCR were carried out as described previously. Oligonucleotides are summarized in Table 2.

**Immunohistochemistry and H&E staining.** Liver and tumour tissue was processed as described previously. Cryo sections were processed as described previously. Antibodies are summarized in Table 3. Routine hematoxylin & eosin staining of tumour tissue was performed for histological tumour typing and grading by a pathologist.

**Flow Cytometry.** Flow cytometric analysis was performed as described previously. Antibodies are summarized in Table 4.

**MR Imaging.** The MRI dataset consists of 1305 measurements for 87 subjects over 30 weeks (15 measuring points). Measurements were taken every other week, from approximately 38 weeks of age throughout life, using a T2-weighted turbo spin echo sequence, as described previously.

**Image Analysis.** Quantitative image analysis was performed using the open source software ImageJ (National Institutes of Health, Bethesda, MD) and qMapIT plugin. Regions of interest were manually placed and tumour volume of each individual tumour was measured.

**Irradiation.** Cells were irradiated at room temperature with 200 kV X-rays (Gulmay RS225, Gulmay Medical Ltd., Byfleet, UK; 15 mA, 0.8 mm Be + 0.5 mm Cu filtering; dose rate of 1.2 Gy/min).
Table 3. Antibodies for immunohistochemistry. HNF4: hepatocyte nuclear factor 4; γH2AX: phosphorylated histone H2AX; IgG: immunoglobulin.

| Target       | Host | Dilution | Distributed by       |
|--------------|------|----------|----------------------|
| F4/80        | rat  | 1:200    | #MCA497R, Bio Rad, Hercules, CA |
| HNF4-alpha   | goat | 1:100    | sc-6556, Santa Cruz, Dallas, TX |
| γH2AX        | rabbit | 1:200 | #2577, Cell Signaling, Danvers, MA |
| anti-Rabbit IgG (H + L) Alexa Fluor 488 | goat | 1:200 | #A-11008, Life Technologies Carlsbad, CA |
| anti-Rat IgG (H + L) Alexa Fluor 594 | donkey | 1:200 | #A-21209, Life Technologies, Carlsbad, CA |
| anti-Phospho-Histone H2A.x | mouse | 1:200 | #05-636, Millipore, Burlington, MA |
| anti-mouse ALEXA fluor 594 | goat | 1:600 | #A-11005 Thermo Fisher, Waltham, MA |

Table 4. Antibodies for flow cytometry. CD: cluster of differentiation; FITC: fluorescein isothiocyanate; Foxp3: forkhead-box P3.

| Target | Conjugate | Dilution | Clone | Distributed by |
|--------|-----------|----------|-------|----------------|
| CD19   | Brilliant Violet 785 | 1:200 | 6D5 | BioLegend, San Diego, CA |
| CD3    | Brilliant Violet 650 | 1:200 | 17A2 | BioLegend, San Diego, CA |
| CD8a   | FITC      | 1:200 | 53–67 | BD Pharmingen, San Jose, CA |
| CD4    | Brilliant Violet 711 | 1:500 | RM4–5 | BioLegend, San Diego, CA |
| NK1.1  | PE        | 1:200 | PK 136 | BioLegend, San Diego, CA |
| Foxp3  | PE-Cy5    | 1:100 | FJK165 | eBioscience, San Diego, CA |

Bone marrow derived macrophages. Bone marrow derived macrophages were differentiated according to a protocol adapted from Weischenfeldt et al.46. In brief, bone marrow cells were isolated from the tibia and femur. Cells were washed with PBS and cultured on tissue culture plates in RPMI medium containing 1% penicillin/streptomycin, 20% FCS, and 30% supernatant of L929 cells. BMDMs were incubated for 4 days; medium was changed after 46 h. Cells were treated with or without CoPP [10µg/ml] for 24 h prior to further procedures.

Phagocytosis Assay. BMDMs (2 × 10^5) were cultured with 5 × 10^5 APC+ BD Calibrite beads™ (BD bioscience, Franklin Lake, NJ) for 4 h at 37°C. Cells were subsequently fixated in 2% PFA solution and analysed with a BD FACSCanto™ II system (BD bioscience, Franklin Lake, NJ).

Immunofluorescence. Immunofluorescence staining of γH2AX was performed as described previously47. Antibodies are summarized in Table 3.

Statistical analysis. Data sets were analysed using either a student's t test with post-hoc Welch's correction or a Mann-Whitney test if two groups were compared. If three or more groups were compared a one-way ANOVA with Bonferroni post-test was used. For frequency distribution analysis, a paired t test with a Wilcoxon's post-test were used. All data in this study are expressed as mean ± SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions
R.B.: obtained funding, study concept and design, study supervision, acquisition of data, analysis and interpretation of data, statistical analysis, drafting of the manuscript. L.B.: acquisition of data, analysis and interpretation of data, statistical analysis, drafting of the manuscript. J.B.: acquisition and analysis of data (immunofluorescent staining of γH2AX, HNF4α and F4/80). D.S.: interpretation of data (immunofluorescent staining of γH2AX, HNF4α and F4/80), critical revision of the manuscript. T.E., H.I. and G.A.: concept, design, acquisition of MRI data. A.P.: acquisition of irradiation data, analysis and interpretation of data (immunofluorescent staining of γH2AX in BMDMs). C.C.: statistical analysis of MRI data. T.K.: analysis and interpretation of data (H&E staining; determination of inflammation, fibrosis and HCC grading). K.K.: concept and design of FACS data. G.S., G.T.: obtained funding, study concept and design, critical revision of the manuscript for important intellectual content.

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