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## THE ROLE OF INNATE IMMUNITY IN THE PATHOGENESIS OF PRENEOPLASIA IN DRUG-INDUCED CHRONIC HEPATITIS BASED ON A MOUSE MODEL

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### Abstract

Innate immunity factors such as conversion of the 26S proteasome to form the immunoproteasome and the Toll-like receptor signaling pathways are activated in chronic hepatitis induced by the carcinogenic drug DDC. Over time, preneoplastic hepatocyte phenotypes appear in the liver parenchyma. These changed hepatocytes expand in number because they have a growth advantage over normal hepatocytes when responding to chronic liver injury. The changed hepatocytes can be identified using immunofluorescent antibodies to preneoplastic cells e.g. FAT10/UbD, A2 macroglobulin, glutathione transpeptidase, alpha fetoprotein, glycipan 3, FAS, and gamma glutamyl transpeptidase. The formation of the preneoplastic cells occurs concomitant with activation of the Toll-like receptor signaling pathways and the transformation of the 26S proteasome to form the immunoproteasome. This transformation is in response to interferon stimulating response element on the promoter of the FAT10/UbD gene. NF $\kappa$ B, Erk, p38 and Jnk are also up regulated. Specific inhibitors block these responses *in vitro* in a mouse tumor cell line exposed to interferon gamma. Mallory-Denk bodies form in these preneoplastic cells, because of the depletion of the 26S proteasome due to formation of the immunoproteasome. Thus, MDB forming cells are also markers of the preneoplastic hepatocytes. The UbD positive preneoplastic cells regress when the liver injury induced chronic hepatitis subsides. When the drug DDC is re-fed to mice and chronic hepatitis is activated, the preneoplastic cell population expands and Mallory-Denk bodies rapidly reform. This response is remembered by the preneoplastic cells for at least four months indicating that an epigenetic cellular memory has formed in the preneoplastic cells. This proliferative response is prevented by feeding methyl donors such as S-adenosylmethionine or betaine. Drug feeding reduces the methylation of H<sub>3</sub> K4, 9, and 27 and this response is prevented by feeding the methyl donors. After 8 to 15 months of drug withdrawal in mice the preneoplastic liver cells persist as single or small clusters of cells in the liver lobules. Multiple liver tumors form, some of which are hepatocellular carcinomas. The tumors immunostain positively for the same preneoplastic markers that the preneoplastic cells. Similar cells are identified in human cirrhosis and hepatocellular carcinoma indicating the relevance of the drug model described here to the preneoplastic changes associated with human chronic hepatitis and hepatocellular carcinoma.

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## Keywords

Mallory-Denk bodies; ubiquitin D; 26S proteasome; immunoproteasome; Toll-like receptors

## INTRODUCTION

The review outline follows: 1) Role of the shift from 26S proteasome to immunoproteasome in the pathogenesis of preneoplasia in chronic drug hepatitis; 2) Role of the TLR signaling system in the pathogenesis of preneoplasia in the liver; 3) Role of NF $\kappa$ B p38, Src, JNK, Erk, STAT1, STAT3 and p53 in the pathogenesis of preneoplasia in the liver; 4) Role of epigenetics in the pathogenesis of preneoplasia in the liver; 5) Relevance of the preneoplastic liver cell in the mouse model to the pathogenesis of human hepatocellular carcinoma.

### 1. The role of the shift from the 26S proteasome to the immunoproteasome in the pathogenesis of preneoplasia in chronic drug hepatitis

Chronic hepatitis develops after 10 weeks of feeding diethyl 1, 4-dihydro-2, 4, 6-trimethyl-3, 5-pyridinedicarboxylate (DDC). At this time preneoplastic hepatocytes are identified, some of which have formed Mallory-Denk bodies (MDBs) (Nan et al., 2006a). After 1 to 4 months of withdrawal of the drug the liver returns largely to normal except for the persistence of scattered preneoplastic hepatocytes some of which have retained a few small MDBs (Nan et al., 2006b; Li et al., 2008). The preneoplastic liver cells forming MDBs immunostain positive for the catalytic subunits of the immunoproteasome (Bardag-Gorce et al., 2010a). There is at this time a reduction in 26S proteasome activity (Bardag-Gorce et al., 2010a). The immunoproteasome colocalizes with FAT10/UbD in the MDB forming hepatocytes (Bardag-Gorce et al., 2010a). The loss of the 26S proteasome activity in the MDB forming preneoplastic cells provides a mechanism for MDB formation. Aggregates of keratin 8 and 18 form are the result of the decreased turnover of the aggregated proteins. These proteins are normally turned over by the 26S proteasome (Bardag-Gorce et al., 2010a). Quantitation of the immunoproteasome catalytic subunits LMP2, LMP 7 and MECL-1, both by Western blot and quantitative PCR, showed that the subunits were up regulated when DDC was refed for 6 days in mice withdrawn from the drug for 1 month (Bardag-Gorce et al., 2010a). The increase in the subunit proteins of the immunoproteasome was also found after the initial 10 week feeding of DDC (Bardag-Gorce et al., 2010a).

Interferon gamma (IFN $\gamma$ ) and tumor necrosis factor have been shown to induce the formation of the immunoproteasome at the expense of the 26S proteasome (Kloetzel, 2001). Likewise, FAT10/UbD is over expressed along with the up regulation of LMP2 in response to IFN $\gamma$  and TNF $\alpha$  (Lukasiak et al., 2008). In the mouse DDC feeding model the receptors for both IFN $\gamma$  and TNF $\alpha$  were up regulated and liver TNF $\alpha$  expression was also up regulated. IFN $\gamma$ , but not TNF $\alpha$ , increased MDB formation *in vitro* when it was added to the tissue culture media of primary cultures of hepatocytes from mice refed DDC (Bardag-Gorce et al, 2010c). When IFN $\gamma$  was added to the media of mouse tumor Hepa 1-6 cell line cultures, UbD, LMP2, LMP7 and MECL-1 were all up regulated, but TNF $\alpha$  was not effective when added to the media (Oliva et al., 2010a). However, the combination of IFN $\gamma$  and TNF $\alpha$  was synergistic. IFN $\gamma$  activated the promoter for the UbD gene *in vitro* by binding to the interferon stimulated response element (ISRE) (Oliva et al., 2010a). When different truncated promoter of the UbD were transfected in Hepa 1-6 cells, only the D1 promoter region was activated by IFN $\gamma$  and TNF $\alpha$ , using the luciferase reporter gene. The promoter activation by IFN $\gamma$  was repressed by presence of p53 consensus sequence. The ISRE was shown to be located at the D1 promoter region. When the mouse tumor cell line Hepa 1-6 was incubated with both TNF $\alpha$  and IFN $\gamma$  added to the media, MDB like

aggresomes (CK8 and UB positive) formed *in vitro* presumably by activating the immunoproteasome *in vitro* (Oliva et al., 2010a).

Human hepatocytes express immunoproteasome subunits in normal human liver in health and chronic hepatitis (Vasuri et al., 2010). Normal human hepatocytes express UbD/FAT10, which colocalizes with the subunits of the immunoproteasome in the cytoplasm of hepatocytes as well as in MDBs in mice refed DDC and in human hepatocytes and MDBs (French et al., 2011). Similarly, in humans, hepatocellular carcinomas that form MDBs also show colocalization of ubiquitin and the immunoproteasome subunits in the MDBs (French et al., 2011). Thus both mouse and human hepatocytes co express UbD/FAT10 and the immunoproteasome subunits when the markers of the preneoplastic phenotype are over expressed. These markers are also over expressed in HCCs in both human and mouse livers (French et al., 2011; Oliva et al., 2008).

INF $\gamma$  stimulates the up regulation of the expression of FAT10 and the 3 immunoproteasome subunits LMP2, LMP7 and MECL-1 at the expense of the 26S proteasome catalytic subunits. This causes a down regulation of the 26S proteasome activity, and consequently accumulation of proteins. These proteins aggregate and form Mallory Denk bodies (MDB) in the DDC refed mouse model of phenotypically switched hepatocytes (Bardag-Gorce et al., 2010a; Strehl et al., 2005; Schroder et al, 2004). At the same time, the expression of the INF $\gamma$  receptors (IFN $\gamma$ R1 and 2) were up regulated as were the TNF $\alpha$  receptors (TNFR21 and TNFRS21a). TNF $\alpha$  protein levels in the liver were also increased (Bardag-Gorce et al., 2010a). Hepatocytes from these DDC refed mice, isolated and grown in primary culture, increased the numbers of MDBs formed when INF $\gamma$ , but not TNF $\alpha$ , was added to the media (Bardag-Gorce et al., 2010a). When INF $\gamma$ , but not TNF $\alpha$ , was added to the media of Hepa 1-6 mouse liver tumor cells, MDBs were also induced (Oliva et al., 2010a).

## 2. Role of the TLR signaling system in the pathogenesis of preneoplasia in the liver

Machida (Machida, 2010; Machida et al., 2009) has shown in mice transfected with HCV NS5A and fed ethanol chronically, develop cancer-initiating stem cells (CSC) which indicates that synergism between alcohol and HCV may lead to liver tumorigenesis through TLR signaling. By analogy we found that the TLR signaling system was also involved in the induction of the proliferation of preneoplastic cell phenotype, which forms tumors long after the drug has been withdrawn in the chronic mouse DDC drug-model of carcinogenesis (French et al., 2010a; French et al., 2010b). The TLR signaling pathway is an important part of the innate immune system (Szabo et al., 2007). It is involved in the development of cirrhosis and hepatocellular carcinogenesis in chronic alcoholic liver disease and hepatitis C (Szabo et al., 2007; Gao et al., 2011) where progenitor cells and cancer stem cells are found, both at the cirrhosis and carcinoma stages (Szabo et al., 2010; Oliva et al., 2010b; French 2010c). Because of this, TLR4 has been characterized as a putative proto-oncogene (Machida 2010).

Toll-like receptors 2 and 4 (TLR2, TLR4) were up regulated when DDC was refed for 7 days in the mouse model of preneoplasia and hepatocellular carcinogenesis (Nan et al., 2006a; Bardag-Gorce et al., 2010b). This was true for the expression of the genes measured by qRT-PCR and when TLR4 was measured by Western blot (Bardag-Gorce et al., 2010b). The up regulation of the expression of the two genes was completely blocked by feeding SAME with DDC, probably as a result of the methylation of histones H3K9 and H3K27, which silences gene expression (Oliva et al., 2008; Bardag-Gorce et al., 2008).

TLR2 and 4 are up regulated by IFN $\gamma$  and TNF $\alpha$  (Oliva et al., 2008; Bardag-Gorce et al., 2008). This may be the mechanism for the up regulation of TLR2 and 4 in the DDC refed mouse model of preneoplasia and carcinogenesis, because IFN $\gamma$  up regulates the expression

of the TLRs MyD88 and IRAK (Schroder et al., 2004; Franklin et al., 2009). CD14 is also up regulated in the DDC refeed model which supports a role for the increased sensitivity of the TLR signaling response to LPS stimulation seen in alcoholic liver disease (Szabo et al., 2007). MyD88, downstream in the TLR signaling pathway, was also up regulated in the mouse model (Bardag-Gorce et al., 2010b). TRAF-6, down stream in the TLR signaling pathway, tended to be up regulated in the TLR signaling pathway in the DDC refeeding mouse model (Bardag-Gorce et al., 2010b). Evidence for activation of NF $\kappa$ B downstream from TRAF-6 leads to the up regulation of cytokines including IL-1. IL-1 is up regulated in the DDC refeed mouse model (Bardag-Gorce et al., 2010b).

### 3. Role of NF $\kappa$ B, p38, SRC, JNK, ERK, STAT1 and 3 and p53 in the pathogenesis of preneoplasia in the liver

Evidence that NF $\kappa$ B activation plays an essential role down stream from the activation of the TLR signaling response is based on *in vitro* assays done on primary cell cultures of liver cells derived from the DDC mouse model in which DDC is fed for 10 weeks then withdrawn from DDC for 1 month (Nan et al., 2005). Using this model the preneoplastic liver cells spontaneously formed MDBs *in vitro* over a 6 day period (Nan et al., 2006a). Even 3 h after seeding the isolated hepatocytes from the one month drug withdrawn mice, the microarrays showed that IKK, ERK, JNK, MEKK1 and p38 were up regulated compared to liver cells from control mice (Nan et al., 2006b). FAT10/UbD positivity was increased beginning on the 2<sup>nd</sup> day of incubation at the same time as NF $\kappa$ B expression was up regulated and MDBs began to form *in vitro* (Nan et al., 2006b). NF $\kappa$ B was up regulated both in the livers of DDC refeed mice *in vivo* (Yuan et al., 2000) and in the primary liver cell cultures *in vitro* (Nan et al., 2006a). When a NF $\kappa$ B inhibitor was added to the medium, no MDBs formed over 6 days of culture (Nan et al., 2006b). The NF $\kappa$ B inhibitor also blocked the up regulation of SRC, NF $\kappa$ B, p105, ERK, JNK, MEKK1 and the phosphorylation of JNK and ERK $\frac{1}{2}$  (Nan et al., 2006b). Using the mouse HCC cell line Hepa 1-6 model, previously used to study the immunoproteasome up regulation by IFN $\gamma$  (Oliva et al., 2010a), it was shown that IFN $\gamma$  plus TNF $\alpha$  up regulation of FAT10 expression was blocked by inhibitors of NF $\kappa$ B, JNK and p38 as well as by the presence of two p53 consensus sequences (Oliva et al., 2010a). Using this *in vitro* assay it was shown that IFN $\gamma$  treatment *in vitro* activated the phosphorylation of ERK and STAT1 and 3 (Oliva et al., 2010a). Activation of hepatocyte gene expression by IFN $\gamma$  requires the activation of both STAT 1 and IRF-1 transcription factors (Howas et al., 2011).

To further study p38 up regulation, the DDC withdrawn MDB formation 6 day *in vitro* assay was used. It showed that p38 phosphorylation was up regulated when MDBs formed *in vitro* and both the phosphorylation of p38 and MDB formation were blocked by an inhibitor of p38 phosphorylation (SB202190) (Nan et al., 2006b). Likewise, specific inhibitors of ERK and JNK phosphorylation inhibited MDB formation in the 6 day primary liver tissue culture assay (Nan et al., 2006b; Wu et al., 2005). ERK, MEKK1 and SRC were up regulated *in vivo* in the liver, after 10 months of DDC feeding and also after 7 days of DDC refeeding (Wu et al., 2005). TGF $\beta$ 1 and 2 and PKC $\beta$  were up regulated in the livers *in vivo*, after 10 weeks of DDC feeding and also after 7 days of DDC refeeding (Nan et al., 2006b). In prior studies using Northern blots, where DDC was fed for 5 months, withdrawn for 1 month, and then refeed for 7 days, c-myc and c-jun were up regulated and PPAR $\alpha$  and RXR $\alpha$  were down regulated (Nagao et al., 1998). Gel retardation assays showed up regulation of AP-1, which correlated with the marked proliferation of the preneoplastic FAT10/MDB forming hepatocytes, as indicated by PCNA positive nuclear counts (Nagao et al., 1998; Oliva et al., 2008; Yuan et al., 1996). A similar proliferation response occurred when another liver toxin, thioacetamide was fed instead of DDC (Roomi et al., 2006), indicating that the preneoplastic cell phenotype has a growth advantage over the neighboring normal cells and is a

nonspecific response. It also indicates that any kind of toxic liver cell injury will cause the FAT10 positive cells to proliferate.

#### 4. Role of epigenetics in the pathogenesis of preneoplasia in the liver

The role of epigenetics in liver preneoplasia pathogenesis is important as indicated by the observation that SAME or betaine prevented virtually all of the changes in gene expression that resulted from DDC refeeding. This included the prevention of the formation of the preneoplastic cell phenotype UbD/FAT10, MDB formation, as well as the up regulation of TLR2, TLR4, MyD88 and TRAF6, IFN $\gamma$  and TNF $\alpha$  receptors, CD14 and IL-1. The proliferation of the FAT10 positive liver cells was also prevented by SAME and/or betaine (Bardag-Gorce et al., 2008; Oliva et al., 2009; Bardag-Gorce et al., 2010a; Oliva et al., 2008; Bardag-Gorce et al., 2010b). In addition, the expression of a large number of genes was changed in the liver after DDC refeeding and the changes were prevented by feeding SAME or betaine with DDC (Li et al., 2008; Oliva et al., 2009). The cellular epigenetic memory was retained by the UbD/FAT10 preneoplastic hepatocytes, at least for 4 months after DDC was withdrawn (Li et al., 2008). UbD/FAT10 was expressed by individual hepatocytes and small groups of hepatocytes 8 to 15 months after DDC withdrawal when hepatocellular tumors developed (Oliva et al., 2008) indicating that the epigenetic memory of the phenotype change was retained through many generations of hepatocytes.

Many changes in acetylation and methylation of histones and methylation of DNA cytosine developed in the liver after DDC refeeding and these changes were prevented by feeding SAME or betaine (Li et al., 2008; Bardag-Gorce et al., 2010a, Oliva et al., 2008; Bardag-Gorce et al., 2008). The expression of enzymes which methylate or demethylate DNA and histones; acetylate and deacetylate, or ubiquitinate histones were changed by DDC refeeding and SAME or betaine prevented these changes (Bardag-Gorce et al., 2008, Bardag-Gorce et al., 2010b). The expression of the enzymes involved in SAME, betaine and methionine metabolism were altered by DDC refeeding. Feeding SAME or betaine prevented most of these changes, including changes in intermediates such as S-adenosylhomocysteine (Bardag-Gorce et al., 2008; Oliva et al., 2009). SAME prevented MDB formation in 6 day cultures of primary liver cells (Li et al., 2008) as did the inhibition of deacetylation of histones by TSA (Oliva et al., 2008). Probably the most significant change induced in histone methylation was the decrease in H3K9me3 and H3K27 me3 (Bardag-Gorce et al., 2010b and 2008) induced by DDC refeeding and prevented by SAME. A decrease in methylation of H3K9me3 and H3K27me3 would decrease the silencing of gene expression. Prevention of the demethylation of these histones would maintain the silencing of gene expression (Blackledge and Klose, 2010).

#### 5. Relevance of the preneoplastic mouse liver cell model to the pathogenesis of human hepatocellular carcinoma

The preneoplastic cells of the DDC mouse model of chronic hepatitis stain positive for markers of progenitor cells seen in human cirrhosis and in preneoplastic foci including FAT10, MDB, AFP, OV6, CD133, A2M, GST, GGTP, HGF, CK19, H19, EGF, AIR, OV6, Nanog and FAS (Oliva et al., 2010b; Nan et al., 2006a; Li et al., 2008; French et al., 2011; French 2010c; Nan et al., 2005; Roomi et al., 2006; Nagao et al., 1999; Tazawa et al., 1983). Human cirrhosis and/or HCC progenitor/preneoplastic/cancer stem cell (CSC) markers, which stain positive immunohistochemically or by FISH, are similar to the mouse model studied here as follows: GP3, AFP, GST, MDBs, FAT10, OV6, Nanog, Oct 3, Oct4, CD49f, and CD133 (Oliva et al., 2010; Wang et al., 2008). The CSC markers which stain positive in HCC stem cells have been further extended to include CD44, ALDH and CD90 (Lingdala et al., 2010). The markers for CSC in the mouse model have been expanded to include YAP1, IMP3, (IGF2bp3) CD133 and Nanog in 29 mouse tumors and surrounding chronic hepatitis.



Figure 1 shows some histochemical double stained hepatocytes with antibodies to two different CSC markers in each double stain (FAT10, Nanog, CD133, Yap 1 and IMP3) from human cirrhosis and HCCs. These are compared with mouse liver tumors and chronic drug hepatitis from the DDC mouse model (Fig.2). Both single small cells representative of CSC and large differentiated hepatocytes stain positive for the CSC markers. The CSC cells present in cirrhotic livers, HCC, mouse liver tumors and mouse chronic hepatitis, stain positive in the cytoplasm in these cells. The nucleus rarely stained positive in these cells. The positive stained cells often showed colocalization of two CSC markers in the same cell when double stained with two CSC antibodies. Since the markers which stained positive in liver cells were present in both the tumors and the surrounding non-tumor liver tissue they must be present in both precursor progenitor cells as well as in CSC cells.

Nanog is a marker for pluripotential stem cells (Villasante et al., 2011). Its expression is regulated by Ezh2, in pluripotent stem cells (Villasante et al, 2011). TLR4 activation up regulates Nanog down stream, which leads to CD133-Nanog positive CSC cells present in liver tumors (Machida et al., 2009). When Nanog levels are high, CSC cell self renewal is supported. When Nanog levels are low, CSC cell differentiation is supported (Villasante et al., 2011). When Nanog levels are high, Imp3 and Yap1 are activated. Up regulation of Imp3 and Yap1 develops when Nanog is increased in pluripotential stem cells, in both human and mice livers (Fig.1 and 2). Ezh2 is a histone methyltransferase which regulates H3K27me3. When both Ezh2 and H3k27me3 are low, Nanog levels are high, and self renewal is supported. When the levels of H3K27me3 and Ezh2 are high at the Nanog promoter, cell differentiation is favored. Ezh2 activity is inhibited when phosphorylated by CDK1 and 2 (Chen et al., 2010) which allows the H3K27me3 epigenetic marker to persist through cell divisions (Zeng et al., 2011). In the mouse DDC refeed model, H3K27me3 levels were significantly decreased, which would favor Nanog supported self renewal and the progenitor cells would proliferate (Bardag-Gorce et al., 2010b). Immunohistochemical staining of livers from mice refeed DDC for both the phosphorylated and unphosphorylated Ezh2 showed that pEzh2 was sequestered within the MDBs formed by the progenitor cells (Fig. 3). This would favor self renewal. When pEzh2 levels are low Nanog levels are high, which supports cell proliferation. The results support the concept that the UbD/MDB progenitors, which proliferate in response to DDC refeeding, reduce the activity of EZH2 by phosphorylating it and sequestering the phosphorylated Ezh2 in MDBs in the phenotypically changed hepatocytes. These hepatocytes have depressed levels of H3K27me3 and reduced methylation of nuclear DNA methylcytosine (Oliva et al., 2008; Li et al., 2008). These changes would epigenetically be remembered in the phenotypically changed hepatocytes through the cell cycle when they proliferate in response to DDC refeeding. This response is completely prevented by feeding SAME with DDC where the decrease in H3k27me3 is prevented by the methyl donor which methylates H3K27.

The presence of Nanog positive, Yap1 positive, Imp3 (Igf2bp) positive CSCs in both human cirrhosis and HCC as well as DDC induced liver tumors, implicates TLR4 activation as the primary driving factor in the induction and maintenance of CSCs derived from precursor progenitor cells located within the chronic hepatitis and cirrhotic livers. Preliminary studies showed that a TLR4 knockout mice refeed DDC showed a reduction in the proliferating FAT10 positive hepatocytes (Fig.4). This correlates with that SAME or betaine prevents both TLR4 up regulation by DDC and ethanol feeding (Bardag-Gorce et al, 2010b; Oliva et al,2011), prevents the TLR4 up regulation by DDC refeeding, prevents FAT10/UbD and MDB formation by DDC refeeding (li et al 2008; Oliva et al 2008; Oliva et al, 2009). There was no reduction however, in the FAT10 positive cells in the DDC refeed TLR2 mouse compared to the DDC refeed wild type mouse (Fig 4).

## Summary

TLR4 up regulation is triggered and maintained by interferon receptor IFN $\gamma$ R 1 and 2. NF $\kappa$ B, STAT 1 and 3, ERK, JNK and p38 phosphorylation are increased by INF $\gamma$ +TNF $\alpha$  up regulation (Fig 5). Inhibitors of NF $\kappa$ B prevented MDB formation, FAT10/UbD up regulation and the up regulation of SRC, NF $\kappa$ B, ERK, JNK, and MEKK1. They also prevented the phosphorylation of JNK and ERK. The increase in CD14 enhances the TLR4 up regulation by LPS. The increase in activation of NF $\kappa$ B increases the expression of cytokines such as IL1. INF $\gamma$  increases the activity of STAT1, 2 and 3. INF $\gamma$  converts the 26S proteasome to the immunoproteasome which leads to 26S proteasome deficiency and MDB formation. The reduction of gene silencing by the low levels of H3K9me3 and H3k27me3 and the prevention of this change by feeding the methyl donors SAME and betaine indicates that epigenetic mechanisms are involved. These epigenetic mechanisms include the maintenance of the oncogene Nanog self renewal of CSC in the DDC tumorigenic model of carcinogenesis, where multiple liver tumors develop (Fig 6).

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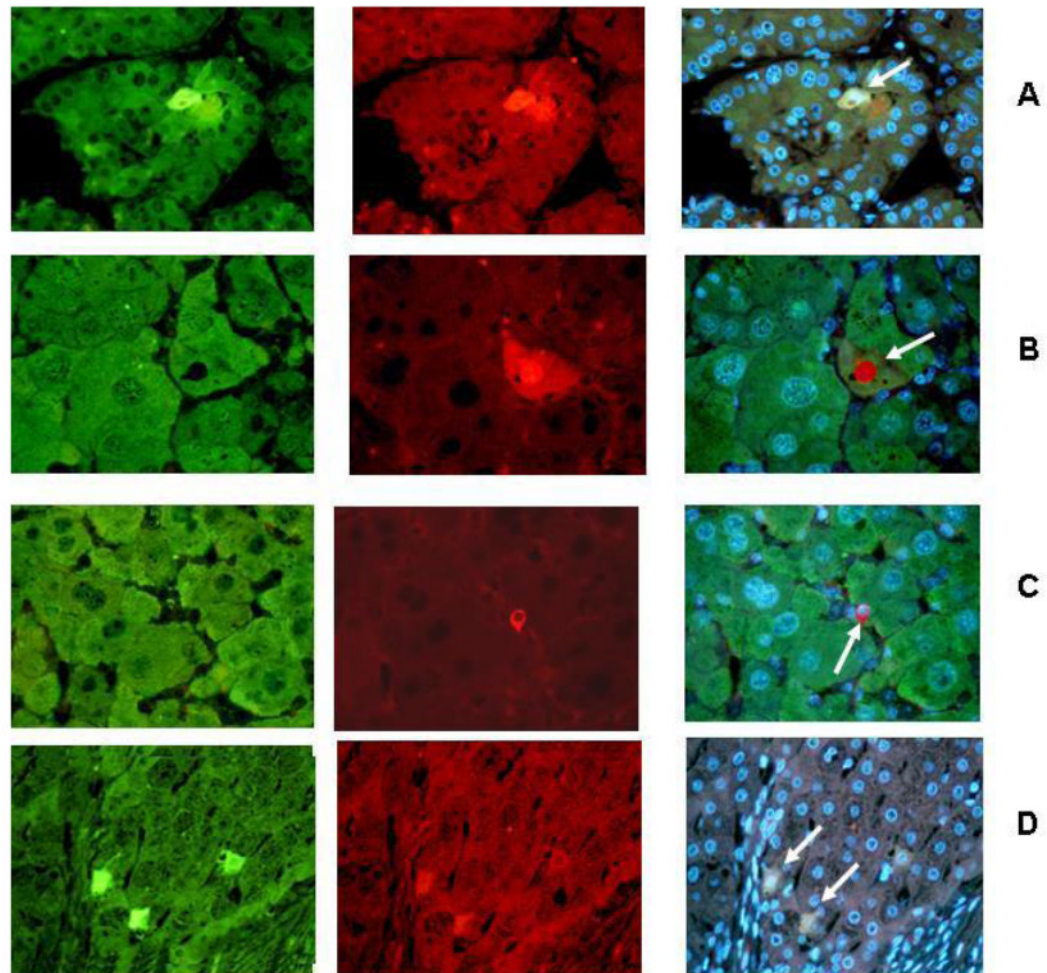
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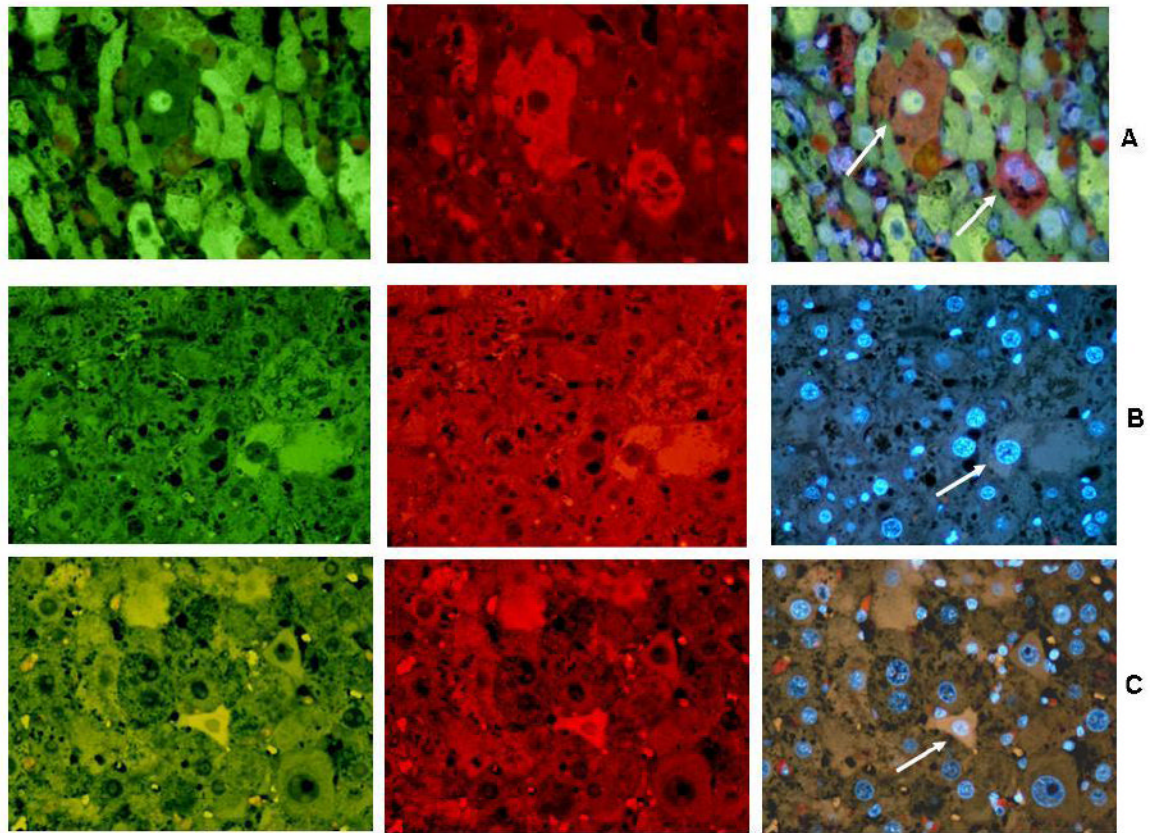
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## Abbreviations

<b>DDC</b>	diethyl-1, 4-dihydro-2, 4, 6-trimethyl-3, 5-pyridinecarboxylate
<b>MDBs</b>	Mallory-Denk bodies
<b>UbD</b>	ubiquitin D in mice, FAT10 in man
<b>ISRE</b>	interferon stimulated response element

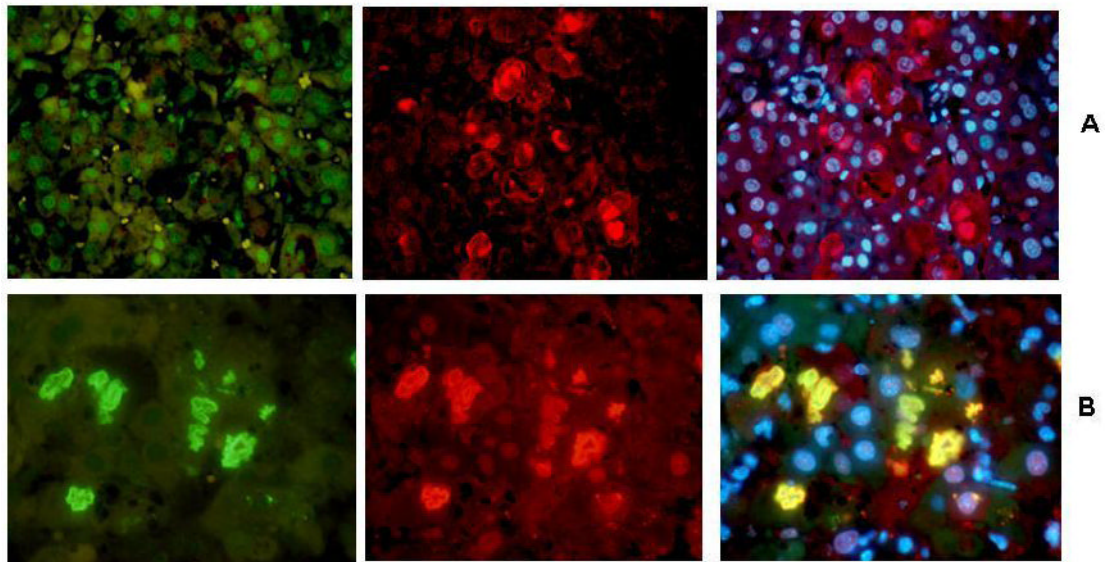


**Fig 1.** CSC markers were used (arrows) to identify progenitor /stem cells in human HCCs and cirrhosis, (1A-1D). **A.** Human HCC stained for Imp3 (green), Yap1 (red) and tricolor x 260. **B.** Human HCC stained for FAT10 (green), CD133 (red) and tricolor, x780. **C.** Human HCC stained for FAT10 (green), CD133 (red) and tricolor x520. **D.** Human cirrhosis stained for Imp3 (green), Yap1 (red) and tricolor x520.

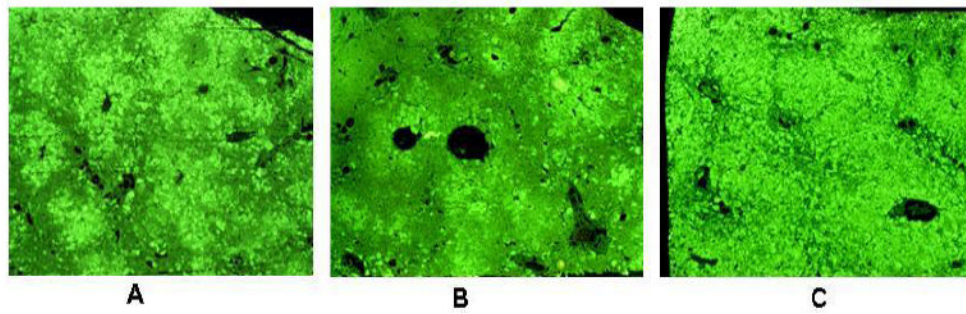


**Fig 2.** CSC markers were used to double stain mouse liver tumors and a non tumor area (arrows) (A-C). **A.** Mouse non tumor stained for FAT10 (green), CD133 (red) and tricolor x 780. **B.** Mouse liver non tumor stained for Nanog (green), FAT10 (red) and tricolor x 520. **C.** Mouse liver tumor stained for Yap1 (red), Imp3 (green) and tricolor x 520.





**Fig 3.** Livers of DDC re-fed mice double immunostained for EZH2 or phosphorylated EZH2 (pEZH2), ubiquitin and Dapi. **A.** Stained for EZH2 (green), ubiquitin (red) and tricolor x520. **B.** Stained for pEZH2 (green), ubiquitin (red) and tricolor x730.

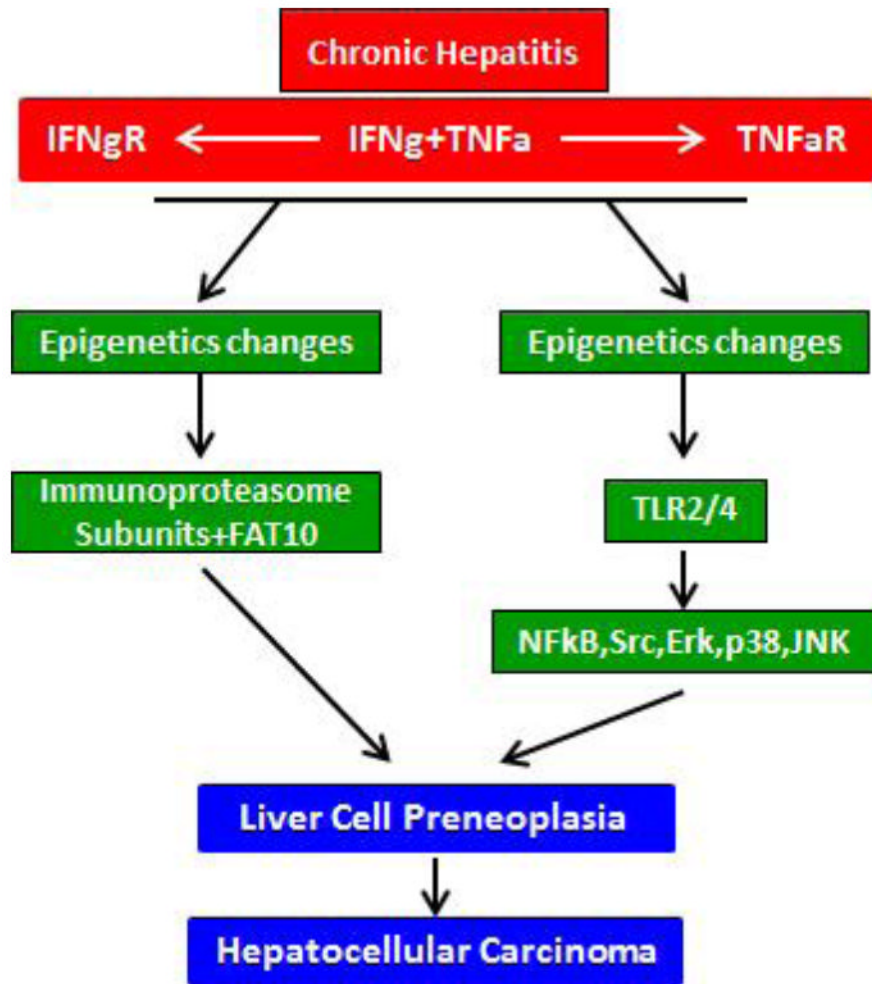


**Fig 4.**

Livers from TLR4 and 2 knock out mice re-fed DDC showing FAT10 liver cell proliferation.

**A.** Centrilobular proliferation of FAT10 positive cells is shown x 520. **B.** This shows a reduction in the proliferation of FAT10 positive liver cells in the liver of a TLR4 knock out mouse x 520. **C.** This shows an increase in the proliferation of FAT10 positive cells in the liver of a TLR2 knock out mouse. Almost all of the liver has been replaced by FAT10 positive liver cells x 520





**Fig 5.** A scheme showing two signaling pathways converging to induce liver cell preneoplasia, which presumably, eventually form liver tumors. SAME blocks both pathways by preventing epigenetic changes.



**Three Hepatocellular Carcinomas Developed 15 Months After DDC Withdrawal**

**Fig 6.** Example of multiple tumors forming in the liver of a mouse fed DDC for 10 months and then the DDC withdrawn for 15 months. Note that 3 HCCs were formed (arrows).