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## THE ROLE OF CYTOKINES IN UBD PROMOTER REGULATION AND MALLORY-DENK BODY-LIKE AGGRESOMES

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### Abstract

Mallory-Denk bodies (MDBs) are found in chronic liver diseases. Previous studies showed that Diethyl-1, 4-dihydro-2,4,6,-trimethyl-3,5-pyridinedicarboxylate (DDC) induced formation of MDBs and the up regulation of UbD expression in mouse liver. UbD is a protein over expressed in hepatocellular carcinomas. It is a potential preneoplastic marker in the mouse. It is hypothesized that inflammatory cytokines play a critical role in UbD up regulation and MDB formation. TNF $\alpha$  and IFN $\gamma$  treatment of HCC cell line Hepa 1–6, induced the expression of UbD and the expression of genes coding for the immunoproteasome (LMP2, LMP7, and MECL-1 subunits). TNF $\alpha$  and IFN $\gamma$  induced the activity of the UbD promoter, using a luciferase assay. The co-treatment with TNF $\alpha$  and IFN $\gamma$  induced the activity of the UbD promoter through an Interferon Sequence Responsive Element (ISRE). In addition, long term treatment with TNF $\alpha$  and IFN $\gamma$  induced the formation of MDB-like aggresomes in Hepa 1–6 cells, which emphasizes the role of inflammation in the formation of MDBs leading to the formation of liver tumors, in the mouse. Identifying the mechanism that regulates gene expression of UbD supports the hypothesis that down regulation of UbD and the proinflammatory gene expression would prevent MDBs and HCC formation. Previous studies indicate that S-adenosylmethionine or betaine prevented IFN $\gamma$  induced UbD and MDB formation.

### Keywords

MDB: Mallory-Denk Bodies; IFN $\gamma$ : Interferon gamma; TNF $\alpha$ : Tumor Necrosis Factor alpha; ISRE: Interferon stimulated response element; UbD: Di-Ubiquitin (Fat10); Ub: Ubiquitin

## INTRODUCTION

Mallory-Denk Bodies (MDBs) are a morphologic feature of alcoholic steatohepatitis (ASH). They also occur in a variety of other liver diseases, such as non-alcoholic steatohepatitis (NASH), which represents a progressive form of non-alcoholic fatty liver disease called NAFLD, as well as intestinal bypass surgery, certain types of drug-induced liver diseases e.g., related to amiodarone, chronic cholestasis (in particular, primary biliary cirrhosis), copper storage related diseases (Wilson's disease, idiopathic copper toxicosis, Indian childhood cirrhosis), alpha-1-antitrypsin deficiency, and in benign and malignant hepatocellular neoplasms and tumor-like lesions e.g., focal nodular hyperplasia (French, 1981a; French,

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1981b; Nakanuma and Ohta, 1985; Zatloukal et al., 2007). MDBs are aggregates of proteins containing p62, cytokeratin (CK8 and 18), Ubb+1 and ubiquitin (Ub) (Bardag-Gorce et al., 2003; Nan et al., 2004; Ohta et al., 1988). Mice fed diethyl-1,4-dihydro-2,4,6-trimethyl-3,5-pyridine decarboxylate (DDC) added to a normal mouse diet for 10 weeks, formed MDBs in clusters of hepatocytes (Yuan et al., 1996). MDBs mostly disappear by 1 month of DDC withdrawal (Oliva et al., 2008). However, following 7 days of DDC refeeding, MDBs form again (Oliva et al., 2008; Yuan et al., 1996). Recent studies have shown that MDB formation is linked with UbD over expression (Bardag-Gorce et al., 2008; Oliva et al., 2008), the switch of proteasome population to immunoproteasome (Oliva et al., 2009a), and the activation of TLR2/4 signaling mechanism of proinflammatory gene expression.

A connection between inflammation and cancer has long been suspected. Epidemiological studies have established that many tumors occur in association with chronic infectious diseases (Schutte et al., 2009). It has also been shown that persistent inflammation in the absence of infections increases the risk of cancer and accelerates its development (Shan and Liu, 2009; Wu and Zhou, 2009). One clear example of inflammation-related cancer is hepatocellular carcinoma (HCC)(Schutte et al., 2009). HCC is a type of tumor that slowly develops on a background of chronic inflammation mainly triggered by exposure to infectious agents (hepatotropic viruses) (Heydtmann and Adams, 2009) or to toxic compounds (ethanol) (Mandrekar and Szabo, 2009). The molecular links that connect inflammation and cancer are not completely understood, but evidence gathered over the past few years are beginning to define the precise mechanisms. However, the evidence has shown the important role played by transcription factors, such as NF-kappa B and STAT3, the role of cytokines such as IL-6 and IL-1 alpha and other inflammatory mediators in cancer development, with special emphasis in the case of HCC (Aggarwal and Gehlot, 2009; Yu et al., 2009). The molecular dissection of the pathways connecting the inflammatory reaction and neoplasia could pave the way to more effective therapies for treating cancers. Studies have shown a link between the inflammatory response and the development of HCC (Berasain et al., 2009). Cytokines and downstream effectors (STAT1, STAT3, and NFkB) are activated during inflammation, which could be the mechanism of activation of genes which leads to tumor formation. STAT1 and STAT3 are downstream signals of TNFa and IFNg cytokine pathway (Adamkova et al., 2007), cytokines known to induce the expression of UbD (Lukasiak et al., 2008; Raasi et al., 1999) and the immunoproteasome protein LMP2 (Lukasiak et al., 2008).

After 8–15 months withdrawal from DDC, tumors formed in the liver of mice (Oliva et al., 2008). These tumors over expressed UbD protein, a preneoplastic marker expressed by liver cells (Oliva et al., 2008). Indeed, during the progression of tumor formation, we observed the formation of clusters of cells that over express UbD (Oliva et al., 2008). In parallel studies, UbD was found to be over expressed in HCC (Lee et al., 2003; Lukasiak et al., 2008). Lukasiak *et al.* showed a correlation between the over expression of human UbD, also called FAT10 in humans, and the protein LMP2, a specific protein of the immunoproteasome (Lukasiak et al., 2008).

UbD is a member of the ubiquitin-like modifier family of proteins, and is thought to play an important role in the immune system (Canaan et al., 2006), cytokine response (Bates et al., 1997; Raasi et al., 1999), apoptosis (Raasi et al., 2001), and mitosis (Liu et al., 1999). The expression of UbD is regulated by various transcription factors such as p53 (Ji et al., 2009; Zhang et al., 2006) and retinoid nuclear receptors (Dokmanovic et al., 2002). In the present study, we have investigated the regulation of the expression of the UbD gene and immunoproteasome specific genes in response to TNFa and IFNg cytokine treatment. Hepa 1–6 cells were used to study the regulation of the UbD promoter. In addition, an in vitro long term treatment with TNFa and IFNg was done to study the formation of MDBs to mimic sustained drug-induced chronic liver disease where MDBs are formed.

## MATERIAL AND METHODS

### Cell Culture

Hepa 1–6 (ATTC, Manassas, VA) derived cells were maintained in Dulbecco's minimum essential medium (DMEM) with phenol red supplemented with 5% Fetal Bovine Serum (FBS medium). To study the RNA and protein expression,  $10^6$  Hepa 1–6 cells were seeded in 100mm cell culture dishes, with or without FBS, without antibiotics. They were incubated for 48h. Cells, in the presence or absence of FBS, were treated for 48 h with : PS-341 0.1mM (LC Laboratories, Woburn, MA), TNFa, 0.2 U/ml (BD Pharmigen, San Jose, California), IFNg, 0.2 U/ml (Shenandoah Biotechnology, Warwick, PA), NFkB inhibitor, 40 nM (Calbiochem, Gibbstown, NJ), SP600125 10uM (Sigma-Aldrich, St. Louis, MO) or SB202190 20uM (Sigma-Aldrich, St. Louis, MO).

### Cloning of the UbD Promoter Region and cDNA of UbD

No putative promoter region in the UbD gene has been characterized so far. Upstream regions of the UbD gene were cloned from -4260 bases, including the 250 first bases in the first exon to test promoter activity after different treatments. The sequence used to design the primers is named: NT\_039649.6 in the mouse genome, in the NIH website. The different promoters were cloned in the pGL3Luc+ in the cloning site (Promega, Madison, WI): XhoI and MLUI for the IIL, and SacI for D1, D2, and D3 promoter (New England Biolabs, Ipswich, MA). PCR was performed using mouse genomic DNA from mouse liver and primers (Table 1):

Primers to clone the promoter of UbD

Name of the Amplified product	Forward Primers	Reverse Primers
IIL	CCTCACGCGTGGAGTAGGGGCTCCTGAAG (MLUI)	CAAG CTCGAG TCCAGTTTAGCAG (Xho I)
D1	CTCCGAGCTCAGACACACCAGAAGAGGACATCA (SACI)	CAAG CTCGAG TCCAGTTTAGCAG (Xho I)
D2	CTCCGAGCTCAAGCAGCATGTCAGCACCAT (SACI)	CAAG CTCGAG TCCAGTTTAGCAG (Xho I)
D3	CTCCGAGCTCCACATGTCTCTGGCCTCAGG (SACI)	CAAG CTCGAG TCCAGTTTAGCAG (Xho I)

PCR was done with Phusion® Flash High-Fidelity PCR (Finnzymes, Woburn, Massachusetts) on 10 ng of mouse genomic DNA or 2 ng of mouse cDNA, with the following conditions for PCR: 98°C for 30 s, and then 40 cycles at 98°C for 15 s, 60°C for 30 s, 72°C 30 s, and finally 5 min at 72°C.

The PCR products were purified using the NucleoSpin Extract II kit (Macherey-Nagel, Bethlehem, PA). PCR products were cloned in the pGMET vectors, according to the company's protocol (Promega, Madison WI) and sequenced. The inserts were digested with the corresponding restriction enzyme for 90 min at 37°C (New England Biolabs, Ipswich, MA). The inserts were purified using the NucleoSpin Extract II kit (Macherey-Nagel, Bethlehem, PA). The purified product was cloned in the pGL3Luc+ vector (Promega, Madison, WI).

### Transient Transfection of the Promoter of UbD

Fifty thousand Hepa 1–6 cells were seeded in 24-well plates in the absence of FBS for 48 h. On the third day, the cells were transfected with the vectors using JET PEI, according to the company's protocol (Polyplus, Illkirch, France). After 48 h. of treatment, in the absence of FBS, the cells were stopped by adding 300  $\mu$ l of cell lysis buffer (Promega, Madison, WI) to each well. 100  $\mu$ l was used to measure Beta-galactosidase activity to standardize the luciferase activity (37°C). Beta-galactosidase activity was measured spectrophotometrically at 420 nm (Promega, Madison, WI). 20  $\mu$ l of cell lysis buffer was used to quantify the luciferase activity of the reporter gene, using the luciferase assay system (Promega, Madison, WI).

### Long term treatment of Hepa 1–6 with TNF $\alpha$ and IFN $\gamma$

$5 \times 10^6$  cells were seeded in 100mm cell culture dishes. The cells were treated with the cytokines TNF $\alpha$  and IFN $\gamma$ , in the presence of 1% of FBS for 43 days. Every third day, the media and the cytokines were renewed. At confluence, the cells were trypsinized and split. Some cells were put on slides for immunohistochemistry. Cells were also seeded in 100mm cell culture plates in order to continue the long term experiment.

### Quantitative Real-time RT-PCR Assay

Total liver RNAs were extracted with Trizol Plus RNA Purification kit (Invitrogen, Carlsbad, CA). Synthesis of cDNAs was performed with 5  $\mu$ g total RNA, and 50 ng random hexamer primers using SuperSriptIII RNase H<sup>-</sup> Reverse Transcriptase (Invitrogen, Carlsbad, CA). PCR primers were designed with the Primer Express software (Applied Biosystems, Foster City, CA). The primers used for UbD, LMP7, LMP2 and MECL-1 were:

UbD	NM_023137
Forward	GATTGACAAGGAAACCACTATCCA
Reverse	ACAAGGGCAGCTCTTCATCAC
LMP7	NM_010724
Forward	GGCTCTCGGGACAGATGTTTT
Reverse	ACCACTGTCCATCACCCATA
LMP2	NM_013585
Forward	TGGAGCTACACGGGTTGGA
Reverse	GAGATGTTCTTACCACGTTTGC
MECL-1	NM_013585
Forward	TTGTGTTCCGAGATGGAGTCAT
Reverse	GCCACAACCGAATCGTTAGTG

**Sense and anti-sense**—Quantitative PCR was achieved by using the SYBR Green JumpStart™ Tag ReadyMix (Sigma, St. Louis, MO) on an ABI PRISM 7000 Sequence Detector system (Applied Biosystems, Foster City, CA). The thermal cycling consists of an initial step at 50°C for 2 min, followed by a denaturation step at 95°C for 10 min, then 40 cycles at 95°C for 15 s, and 60°C for 1 min. Single PCR product was confirmed with the heat dissociation protocol at the end of the PCR cycles. Quantitative values were obtained from the threshold PCR cycle number (Ct) at which point the increase in the signal associated with an exponential growth for PCR product begins to be detected. The target mRNA abundance in each sample was normalized to its 18S level as  $\Delta Ct = Ct_{\text{target gene}} - Ct_{18S}$ . For each target gene, the highest  $\Delta Ct$  was assigned as  $\Delta Ct_{\text{max}}$ .

## Western Blot Analysis

Proteins (50 ug) from liquid nitrogen frozen stored livers were separated by SDS-PAGE gels and transferred to a PVDF membrane (Bio-Rad, Hercules, CA) for 1 h in 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 20% methanol. The membranes were stained using ERK/p42/44 (Cell Signaling, Danvers, MA) STAT1 (Abcam, Cambridge, MA), STAT3 (Abcam, Cambridge, MA) and beta actin (Sigma-Aldrich, Saint louis MO). Appropriate species anti polyclonal and monoclonal HRP-conjugated antibodies were used as the secondary antibodies. The membranes were subjected to chemiluminescence detection using luminal, according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ).

## Immunohistochemistry

Cells on coverslips and liver biopsy sections were double stained with the primary antibodies rabbit anti UbD (Fat10) (BioMol, Plymouth Meeting, PA), rabbit anti CK8 (Fitzgerald Industries Intl, Concord, MA) and mouse anti ubiquitin (Chemicon, Temecula CA). Secondary antibodies conjugated with FITC or Texas Red (Jackson, West Grove, PA) were used to detect binding of the primary antibodies. Dapi was used as the nuclear stain The slides were examined using a Nikon-400 fluorescent microscope with a triple color band cube to detect simultaneously FITC, Texas Red and DAPI staining. UbD positive hepatocytes were detected.

## Proteasome Chymotrypsin-Like Activity Assay

Nuclei were isolated, and 1 µg of total protein was used (Oliva et al., 2009b). The reaction mixture contained 50 mM Tris-HCl pH 8, 1 mM DTT, and 40 µM Suc-LLVY-AMC substrate for chymotrypsin-like activity. The mixture was incubated for 30 min at 37°C, and then stopped by adding 100 µM monochloroacetate and 30 mM sodium acetate pH 4.3. Fluorescence was determined by measuring the release of AMC ( $\lambda$  excitation: 355 nm,  $\lambda$  emission: 430 nm) using a Perkin Elmer LS 30 spectrofluorometer.

## Results and Discussion

Cytokines, such as TNFa, IFN $\gamma$ , and IL-6, are released by Kupffler cells and hepatocytes during inflammation. Activated intracellular pathways stimulate genes involved in antigen presentation (Wang et al., 2007) (Knolle et al., 1997). Bates *et al.* (1997) identified UbD as one of the genes induced during inflammation from human dendritic cells (Bates et al., 1997). UbD is a small protein of 17 kDa, and its functional role is not well understood, nor is the cellular localization known (Lee et al., 2003). The UbD gene is located on the mouse MHC-I locus (chromosome 17) and on the human MHC-I locus (chromosome 6) (Table 1A). Other relevant genes are present in this locus, i.e., genes involved in the presentation of antigen (HLA), and those coding for the immunoproteasome subunits: LMP2 and LMP7 (Table 1A). Homologous gene localization is found for the human locus. The activation of the inflammatory pathway also activates the other genes present in the MHC-I locus: UbD, LMP2 and LMP7.

Previous studies have shown that IFN $\gamma$  and TNFa increase the expression of UbD (Lukasiak et al., 2008; Raasi et al., 1999) and LMP2. The latter is a catalytic subunit of the immunoproteasome. However, it is important to notice that the induction by the cytokines was absolutely different depending on the cell line. Even some human cell lines do not show up regulation of UbD by treatment with cytokines. According to Raasi and Lukasiak, the cell culture conditions were not indicated as to whether the cells were cultivated in the presence of serum or starved (Lukasiak et al., 2008; Raasi et al., 1999). We thus decided to study their regulation in the presence of 5% FBS or in the absence of FBS, incubated for 48h and then treated for 48h. We observed a dramatic difference in the regulation of gene expression depending on whether there was serum present or absent. Indeed, in the presence of FBS, the cytokines TNFa and IFN $\gamma$  were not able to induce the expression of UbD, MECL-1, LMP2 and

LMP7 (Fig 1 ABCD). Also, in the absence of serum, TNF $\alpha$  was not able to induce their expression (Fig 1 ABCD). However, IFN $\gamma$  was able to induce their expression. This had been shown in different cell lines (Raasi et al., 1999). We observed a synergistic effect with the co treatment of the two cytokines in the absence of serum, including LMP2 and LMP7 (Fig 1). The different treatments did not have an effect on the expression of the control gene beta-Actin (data not shown). Raasi and Lukasiak showed that cytokines directly induced the expression of UbD without *de novo* synthesis of the protein, and that lactacystin (a proteasome inhibitor) blocked the effect of TNF $\alpha$  and IFN $\gamma$  (Raasi et al., 1999).

There was an increase in ERK/p42/44, STAT1 and STAT 3 phosphorylation induced by IFN $\gamma$  and the co treatment of the two cytokines. TNF $\alpha$  alone was only able to induce the phosphorylation of ERK/p42/44 (Fig 3). Gerber *et al* showed that IFN $\gamma$  increased the phosphorylation of STAT1 (Gerber et al., 2009). TNF $\alpha$  was able to induce the phosphorylation of STAT1, which was the same result that Wesemann and Benveniste (2003) reported, possibly because of the cell line (Fig 3) (Wesemann and Benveniste, 2003).

We also used inhibitors of NF $\kappa$ Bi, SP600125, SB202190, NF $\kappa$ B, JNK and p38 respectively to determine the pathway which activates UbD and the specific genes of the immunoproteasome. The importance of the NF- $\kappa$ B pathway in IFN $\gamma$  signaling is still emerging. IFN $\gamma$  can induce DNA binding of NF- $\kappa$ B in a STAT1-independent manner (Deb et al., 2001). The inhibitor of I $\kappa$ B kinase (IKK) maintains the NF $\kappa$ B components in an inactive state. In response to stimuli by IFN $\gamma$ , I $\kappa$ B was phosphorylated by IKK leading to I $\kappa$ B degradation, and, thus, the release of active NF- $\kappa$ B components. In our experiment, NF $\kappa$ B inhibitor blocked the induction of UbD expression (Fig2 A). However, the inhibitor did not inhibit the expression of LMP2, LMP7 and only slightly inhibited the expression of MECL-1 (Fig 2 ABCD). No previous studies have shown the promoter regulation of the genes for MECL-1, LMP2 and LMP7 by NF $\kappa$ B, indicating that the IFN $\gamma$ /TNF $\alpha$  treatment induced the expression of these genes by other pathways. Other pathways, such as the MAPK pathway, could be activated by treatment with TNF $\alpha$  and IFN $\gamma$ . IFN $\gamma$  triggers the activation of the MEK1/ERK1/2 pathway in a range of cell lines and primary cell cultures, which could occur through a number of different molecular pathways (Gough et al., 2007). It has also been reported that IFN $\gamma$  does not activate JNK/MAP kinase (Gough et al., 2007). However, it has been shown recently that IFN $\gamma$  can activate JNK in macrophages, where it appears to be required for the expression of genes associated with antigen presentation (Valledor et al., 2008).

The treatment with both cytokines synergistically increased the expression of UbD (Fig 2 A). SP600125 (a JNK inhibitor) blocked the effect of the IFN $\gamma$  and TNF $\alpha$  co-treatment on the expression of UbD, but not in the expression of the others genes (Fig 2 B, C, D). The activation of p38 MAP kinase by IFN $\gamma$  is somewhat controversial. IFN $\gamma$ -stimulated recruitment of MyD88 to the receptor has been shown to trigger the activation of the MKK6/p38 MAP kinase pathway (Sun and Ding, 2006). Furthermore, c-Src activation at the IFN $\gamma$ R results in the activation of the calcium-dependent kinase Pyk2, leading to the activation of the Mekk4/MKK6/p38 MAP kinase pathway (Halfter et al., 2005). However, other authors have been unable to demonstrate the phosphorylation of p38 MAP kinase in response to IFN $\gamma$  (Gough et al., 2007; Ramsauer et al., 2002). Using SB202190 (inhibitor of p38), we showed that the inhibition of p38 kinase blocks the expression of genes (Fig 2).

Using the MDB mouse model, we observed a switch of the proteasome to form the immunoproteasome during DDC feeding (Oliva et al., 2009a). The 20S proteasome activity decreased and the immunoproteasome catalytic subunits such as LMP2, LMP7 and MECL-1 were over expressed (Oliva et al., 2009a). In parallel, we found an increase in the expression of TNF $\alpha$  and IFN $\gamma$  receptors in the liver (Oliva et al., 2009a). Here, we hypothesized that a similar mechanism would occur in the Hepa 1–6 cell lines treated with IFN $\gamma$  and TNF $\alpha$ . We

used PS-341 as a positive control to show the effect of inhibition of the proteasome activity. IFN $\gamma$  induced the activity of the proteasome catalytic subunits of the immunoproteasome population (Fig 4). Osna *et al* (2003) also showed the increase in the 20S proteasome activity with IFN $\gamma$  treatment (Osna *et al.*, 2003). However, TNF $\alpha$ , surprisingly, repressed the activity of the 20S proteasome, which has not been reported. However, the combined treatment neutralized the proteasome chymotrypsin-like activity of the 20S proteasome to control levels (Fig 4).

We cloned the hypothetical UbD promoter (4260 bases upstream from the initiation point) to analyze its regulation by TNF $\alpha$  and IFN $\gamma$ . The transfection of the longer promoter, called IIL, was not up regulated by TNF $\alpha$ , IFN $\gamma$  or the co treatment. However, the promoter D1 had higher basal activity than the IIL promoter. We hypothesized that the longer promoter was down regulated by the protein p53, as shown by Zhang *et al.* (Zhang *et al.*, 2006). The mutant of p53 leads to a gastric tumor and also an over expression of FAT10 (Ji *et al.*, 2009). Indeed, the mouse UbD promoter showed the presence of two potential p53 consensus sequences of the deleted promoter that could explain the increase in the basal activity (Table 1B) (Fig 5). However, TNF $\alpha$  alone was not able to induce the expression of UbD promoter D1 which correlates with the qRT-PCR results (Fig 5a) (Fig 1A). IFN $\gamma$  alone and the co treatment were able to induce the expression of the UbD promoter, exactly as we showed with qRT-PCR (Fig 1A). By comparing the promoter sequences, we found the presence of an ISRE sequence on the D1 promoter (Table 1 B, C). This ISRE has a similar sequence to the ISRE of ISG15, another gene which was induced by the interferon gamma and consensus sequence of ISRE (Levy *et al.*, 1988; Weihua *et al.*, 1997). In the absence of this ISRE sequence, the TNF $\alpha$ +IFN $\gamma$  co-treatment was not able to induce the activity of the UbD promoters D2 and D3 (Fig 5 C).

MDB formation in mice is reversible since MDBs become smaller and less numerous after withdrawal of the drug (Yuan *et al.*, 1996). However, both “recovered” humans and animals remain highly predisposed to MDB re-formation (Jensen and Gluud, 1994a; Jensen and Gluud, 1994b),(Yuan *et al.*, 1996). Moreover, the re-formation of MDBs can be induced by a variety of non-specific stress inducing agents when introduced after withdrawal of the drug in the drug primed mouse. (French *et al.*, 2001). The purpose of this study was to maintain long term *in vitro* treatment of Hepa 1–6 cells, with TNF $\alpha$  and IFN $\gamma$  to mimic long term proinflammatory conditions in the liver caused by alcohol. The treatment of the cells was stopped at different time intervals. The cells were immunostained for ubiquitin, which is a marker of MDBs (Ohta *et al.*, 1988). After 21 days of treatment, we observed the formation of MDB-like aggresomes in the liver tumor cells (Fig 6 D, J, P). The MDB-like aggresomes were still present after 43 days of continuous treatment, indicating that the proinflammatory cytokines in the liver could induce the formation of MDB-like aggresomes *in vitro* (Fig 6 F,L,R). The MDB-like aggresomes were shown to be formed by the presence of Ub and CK-8, which colocalizes in the aggresomes (Fig 7).

DDC feeding induced the formation of tumors, after 8–15 months withdrawal from DDC (Nan *et al.*, 2006; Oliva *et al.*, 2008). DDC induced the expression of tumor markers, such as AFP, UbD, and GST $\mu$ 2, and continued to over express UbD proteins after months of withdrawal (Nan *et al.*, 2006; Oliva *et al.*, 2008). There was an increase of two hepatocellular carcinoma markers (AFP and Gankyrin) in the tumors formed after nine months of withdrawal. UbD (Fat10) was still over expressed in these tumors (Oliva *et al.*, 2008). The increase of PCNA positive nuclei in UbD positive cells indicates that UbD positive cells had a growth advantage similar to tumor cells (Qin and Tang, 2002; Roomi *et al.*, 2006). UbD over expression reduces the cell cycle time (Nan *et al.*, 2006), and regulates the cell cycle by interacting with MAD2 (Liu *et al.*, 1999). UbD induces apoptosis in renal cells during HIV infection (Ross *et al.*, 2006; Snyder *et al.*, 2009) and induces apoptosis of the lymphocyte population in knock-out mice (Canaan *et al.*, 2006). UbD can also stimulate cell proliferation in HCT116 cells (Lim *et*

al., 2006). The knowledge of the regulation of the expression of UbD could be used to understand the mechanism of the development of HCC and UbD expression could be used as a preneoplastic marker, in the liver based on the observations reported here.

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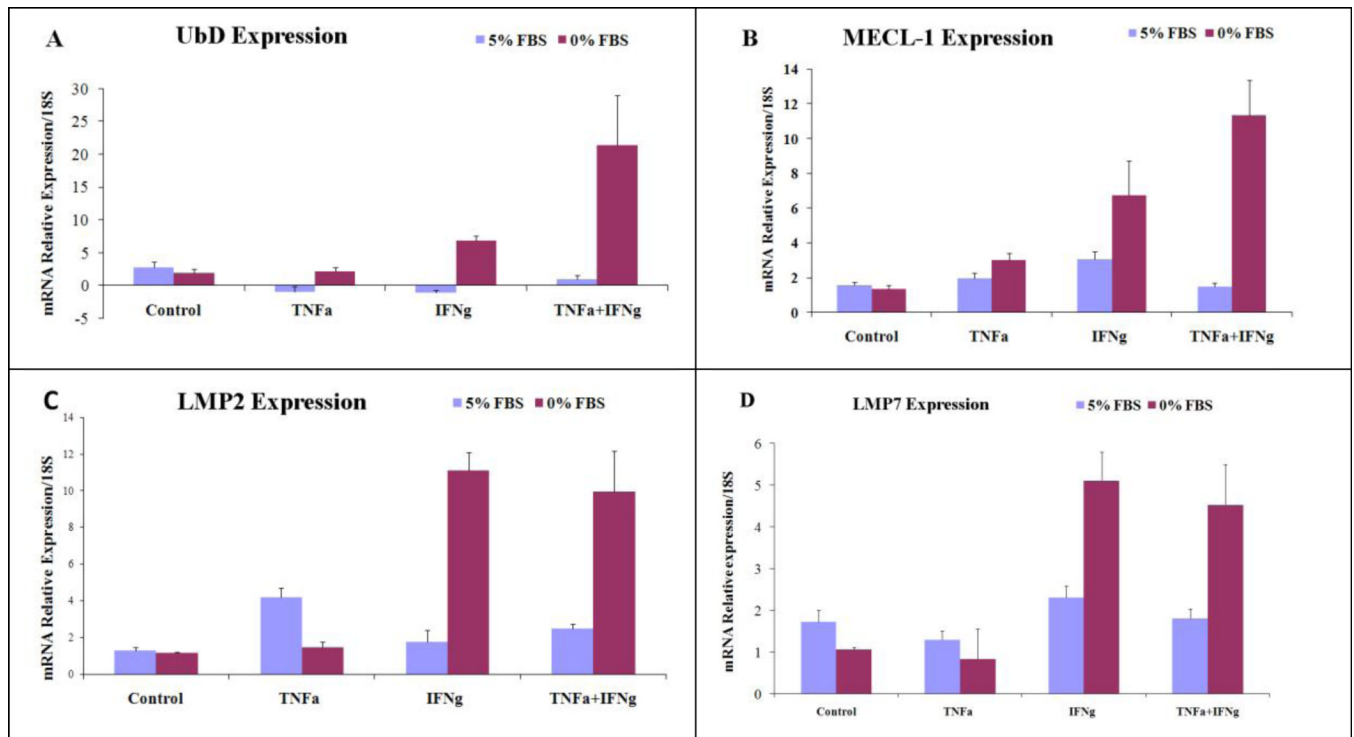
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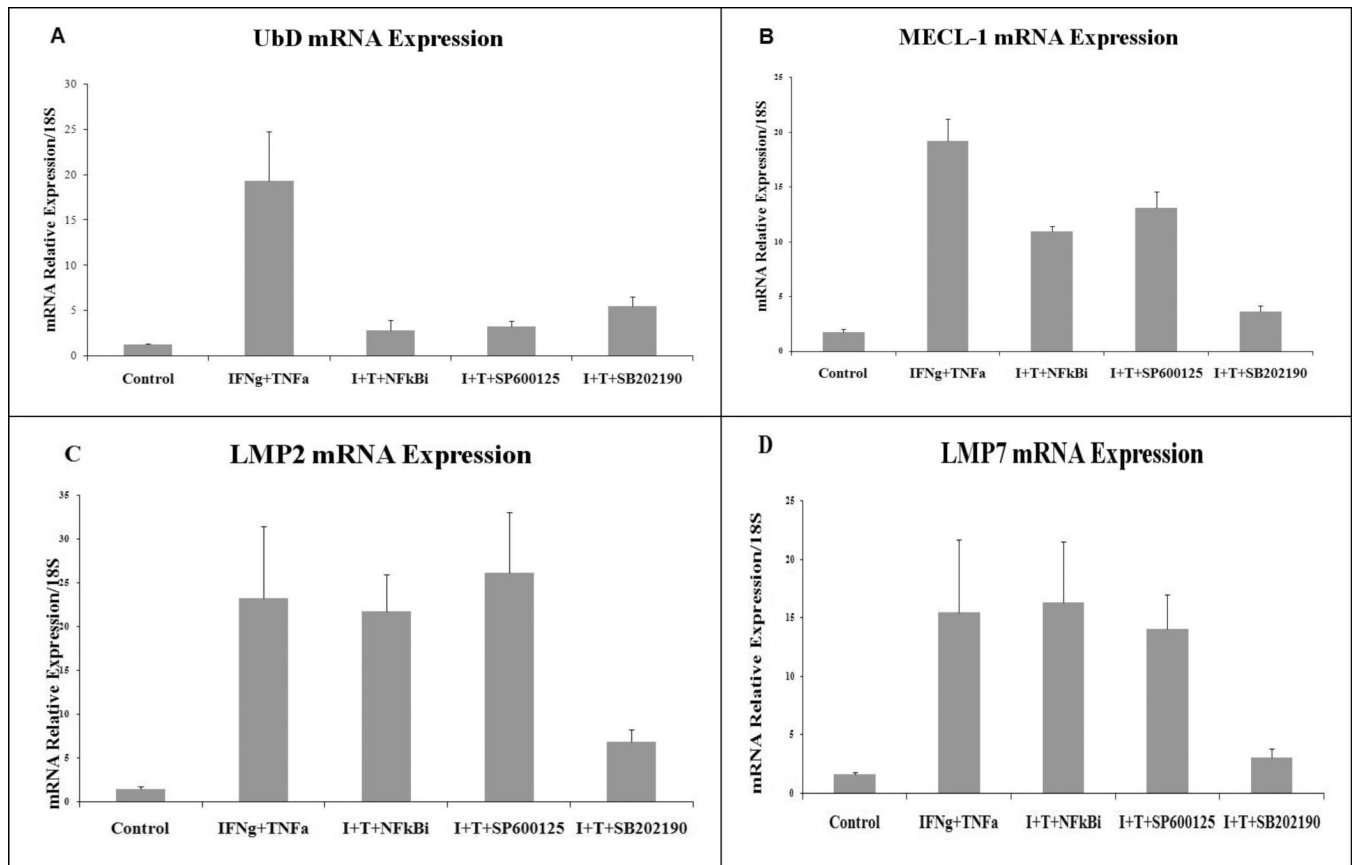


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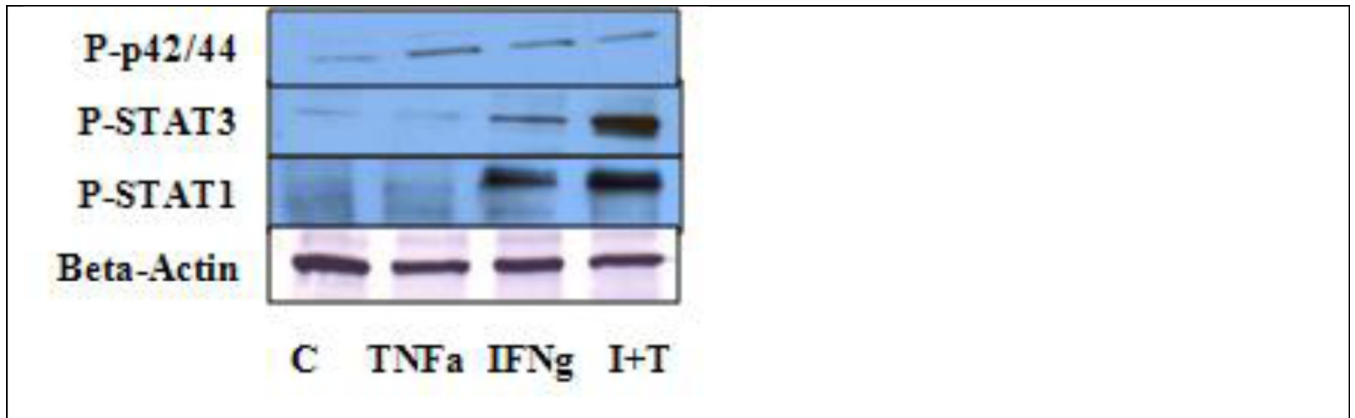
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**Fig 1.**

Changes in the expression of UbD and the catalytic subunits of the immunoproteasome were measured by qReal-Time PCR (N=4). Beta-Actin gene was used as the negative control for the effect of TNFa and IFNg cytokine transcriptional regulation. (UbD for 0%: TNFa+IFNg vs. C  $p < 0.001$ ; TNFa+IFNg vs TNFa  $p < 0.001$ ; TNFa+IFNg vs IFNg  $p = 0.002$ ; MECL-1: TNFa+IFNg vs. C  $p = 0.002$ ; TNFa+IFNg vs. IFNg  $p = 0.044$ ; TNFa+IFNg vs. IFNg  $p = 0.004$ ; IFNg vs. C  $p = 0.054$ ).

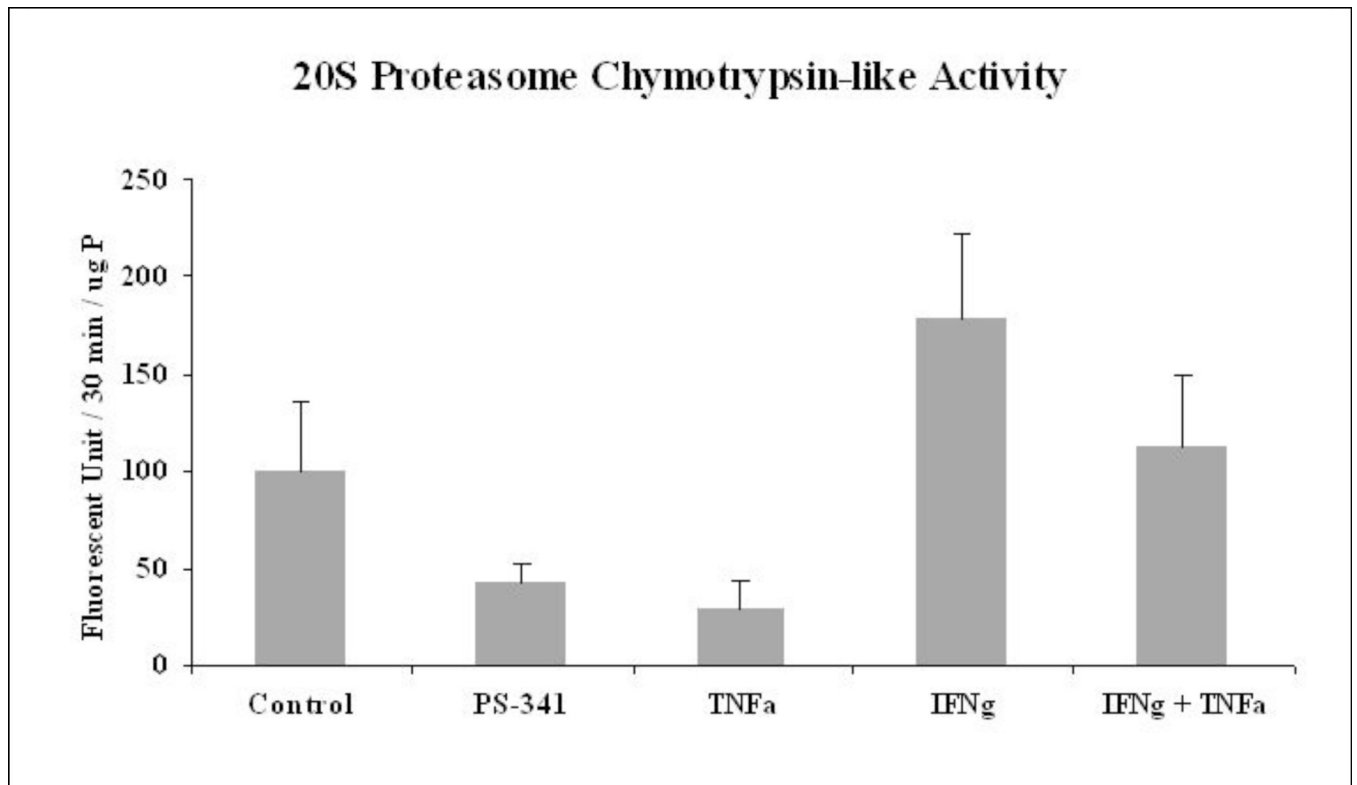
**Fig 2.**

Effects of NF $\kappa$ B, JNK and p38 inhibitors on the expression of UbD and immunoproteasome catalytic subunits induced by TNF $\alpha$  and IFN $\gamma$ . (n=4) (Statistics for **UbD**: Control vs. I+T p=0.002; I+T vs. I+T+Nf $\kappa$ Bi p=0.005; I+T vs. I+T+SP600125 p=0.002; I+T vs. I+T+SB202190 p=0.002; for **MECL-1**: I+T vs. Control p<0.001; I+T vs. I+T+SB202190 p<0.001; for **LMP2**: Control vs. I+T p=0.043; I+T vs. I+T+SB202190 p=0.096; for **LMP7**: Control vs. I+T p=0.044; I+T vs. I+T+SB202190 p=0.045. (I+T : IFN $\gamma$  + TNF $\alpha$ ).



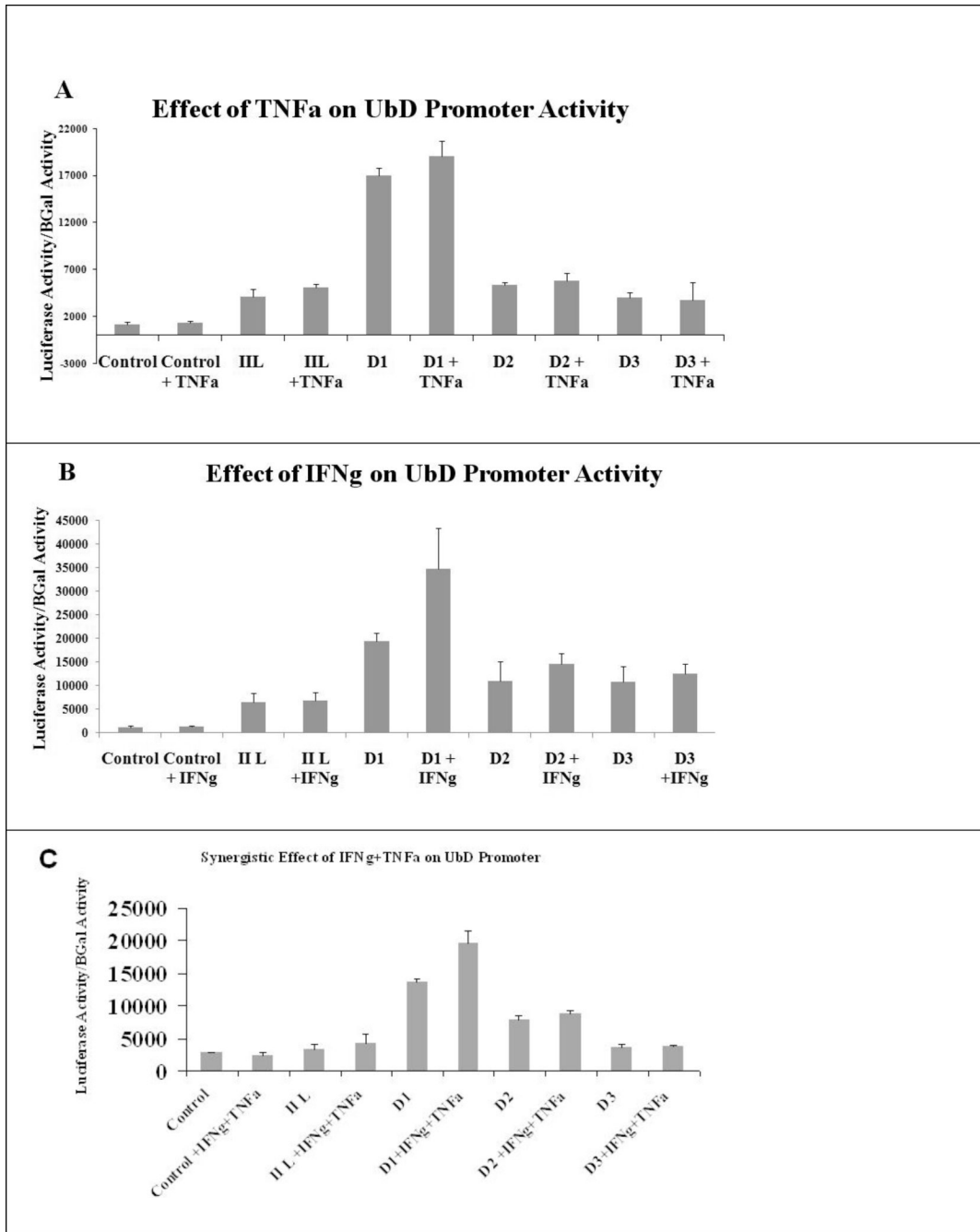
**Fig 3.**

Analysis of the phosphorylation levels of ERK/p42/44, STAT1 and STAT3. TNF $\alpha$  was able to induce the phosphorylation of p42/44. IFN $\gamma$  increased the phosphorylation of p42/44, STAT1 and STAT3. The combination of the two cytokines (I+T) had a synergistic effect on the phosphorylation of STAT1 and STAT3. (N=3)

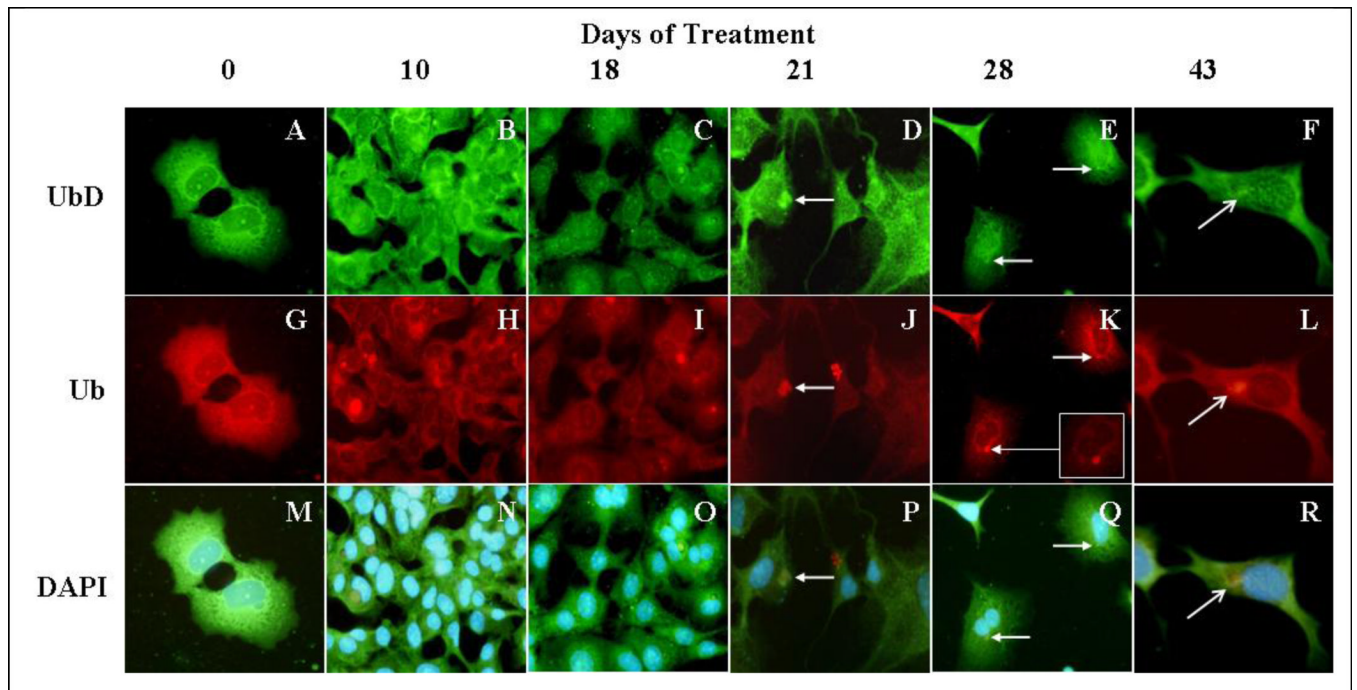


**Fig 4.**

Proteasome chymotrypsin-like activity was measured in the Hepa 1–6 cells treated with PS-341 (a proteasome inhibitor) TNF $\alpha$ , IFN $\gamma$  and IFN $\gamma$ +TNF $\alpha$ . (SEM, N=3; Differences between all the groups:  $p=0.047$ ).

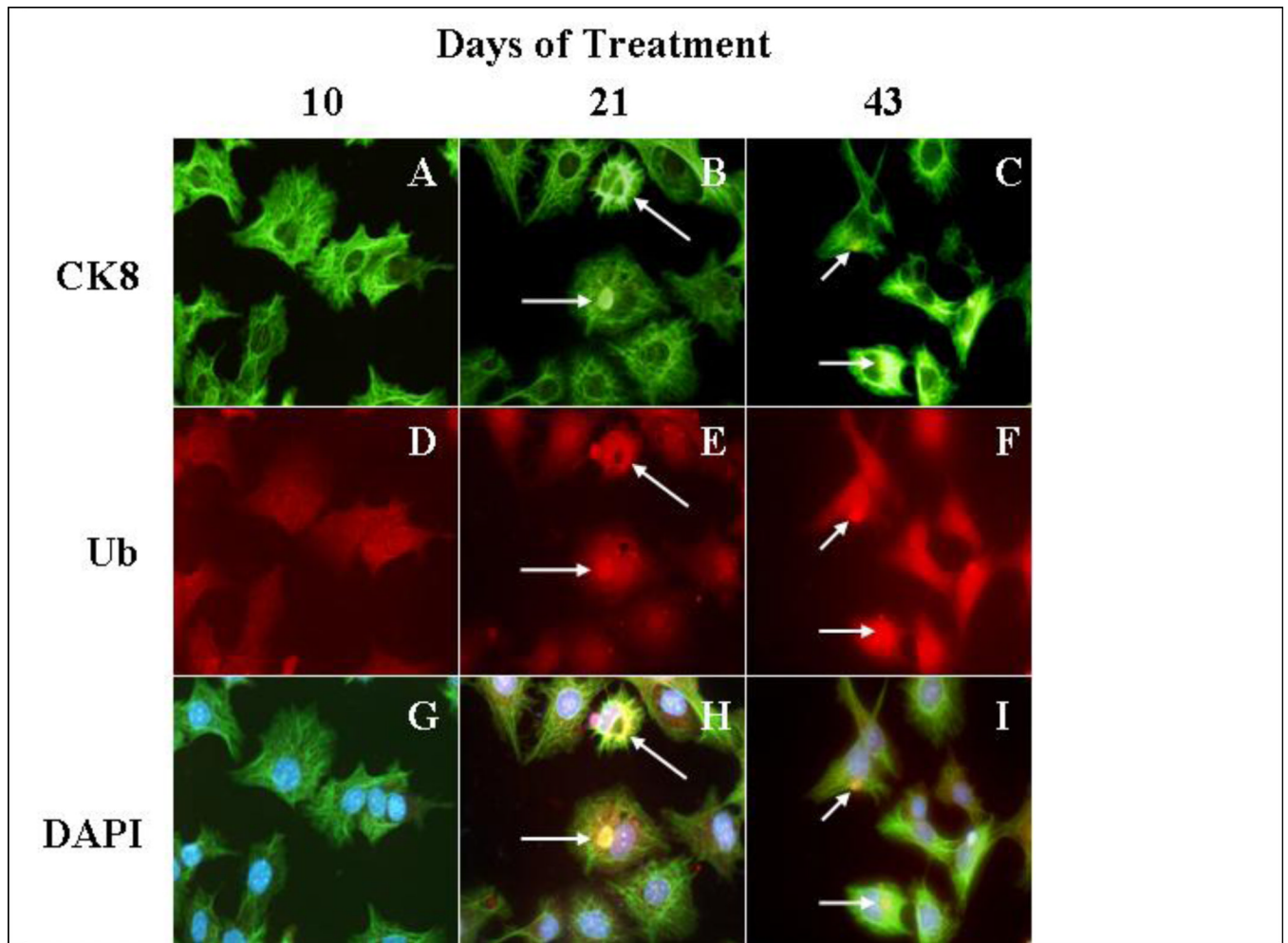


**Fig 5.** The IIL UbD promoter was repressed by p53 protein. IFNγ, but not TNFα, was able to induce the activity of the UbD promoter (D1 promoter) when the p53 consensus sequences were absent. However, IFNγ is not able to induce the activity of D2 and D3 shorter UbD promoter, indicating the presence of an ISRE sequence in the IIL UbD promoter. (**TNFα treatment:** D1 vs. IIL  $p < 0.001$ ; **IFNγ treatment:** D1 vs. IIL  $p = 0.151$ ; D1 vs. D1+IFNγ  $p = 0.005$ ; **TNFα+IFNγ treatment:** D1 vs. IIL  $p < 0.001$ ; D1 vs. D1+IFNγ+TNFα  $p < 0.001$ )

**Fig 6.**

Study of the formation of MDBs in Hepa 1–6 cells treated continuously for 43 days with a cotreatment of IFN $\gamma$  and TNF $\alpha$ . UbD localized with aggresomes (white arrows) similar to the MDB-like aggresomes seen after 21 days of cotreatment with cytokines. The immunostaining was done using antibodies against endogenous UbD (green) and ubiquitin (red). Nuclei were stained with DAPI (blue). A to F; cells stained with UbD antibody. G to L; cells stained with Ubiquitin antibody. M to R; cells stained with DAPI (Magnification  $\times 175$ ).





**Fig 7.**

Study of the formation of MDBs in Hepa 1–6 cells treated continuously for 43 days with a cotreatment of IFN $\gamma$  and TNF $\alpha$ . CK8 localized with aggresomes (white arrows) similar to MDB-like aggresomes, after 21 days of cotreatment with cytokines. The immunostaining is done with antibodies against endogenous CK8 (green), ubiquitin (red). The nuclear stain was DAPI (blue). A to C cells stained with CK8 antibody. D to F cells stained with Ubiquitin antibody. G to I cells stained with DAPI (Magnification  $\times 175$ ).

**Table 1**

A) Comparison of the histocompatibility MHC I locus between human and mouse. B) Scheme of mouse UbD Promoter. The size of the cloned UbD promoter is 4.2 Kb. Using Tfsitescan software, we identified different consensus sequences involved in the expression of UbD: p53 down regulating UbD expression 28,29, RRE (Retinoid Responsive Elements) have been found to be involved in this down regulation 30, and TAT HIV sequence has been found to be involved in the up regulation of UbD in the kidney 52. One ISRE consensus

sequence was found in the promoter at 3.5 Kb upstream from the starting point. C) Comparison of the UbD ISRE, ISG15 ISRE and the consensus ISRE is shown 43,44.

